STUDIES ON ORF VIRUS INFECTIONS IN SHEEP

by

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A thesis submitted to the University of London
for the degree of Doctor of Philosophy

1980

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Abstract

The clinical, immune and serological responses of a flock of 44 sheep which suffered an outbreak of natural orf infection was monitored over a period of one year.

Animals were shown to be almost solidly resistant to experimental reinfection of the mouth with a heterologous strain of orf virus up to one year after the outbreak. All animals tested were, however, at least partially susceptible to infection of the thigh within one month of recovery from disease. Full susceptibility appeared to be regained within a year.

Lambs born to recovered ewes one year after the outbreak were uniformly fully susceptible to experimental mouth infection, but the ewes remained resistant to udder infection while nursing the infected lambs.

Most, but not all, sheep developed precipitating antibodies in association with the outbreak of disease. An antigen of high potency was required to detect such antibodies, the presence of which fluctuated in individual animals during the monitoring period. Results also varied with the use of antigens prepared from different strains of orf or milker's nodule virus.

Using a plaque-reduction assay, some sheep were found to develop low levels of serum neutralizing antibodies, either in association with the outbreak of disease or following experimental reinfection.

There was no correlation between the severity of clinical lesions and susceptibility to subsequent challenge infection of mouth or thigh and the development of precipitating or serum neutralizing antibodies. Precipitating and serum neutralizing antibodies also appeared to develop independently of each other and were not passively transferred from ewe to lamb to any significant extent.

Sheep vaccinated by thigh scarification were, in most cases, susceptible to reinfection within one month. Precipitating and serum neutralizing antibodies were detected after one or two revaccinations.

In agar-gel diffusion tests, five strains of orf virus appeared antigenically identical. Orf and milker's nodule viruses were similarly indistinguishable and both were very closely related to bovine papular stomatitis virus.

Acknowledgements

No man is an Island, entire of itself;
every man is a piece of the continent, a part of the main.

John Donne. Meditation XVII.

I am most grateful to Dr A B Paterson, former Director of the Central Veterinary Laboratory, Weybridge, for permitting me to carry out this study and allowing me the use of laboratory staff and facilities without which the work could not have been completed.

My supervisor at the London School of Hygiene and Tropical Medicine, Dr C.J.M. Rondle, took a great personal interest in the subject of the thesis and his guidance and encouragement, particularly when progress seemed to be faltering and spirits sagging, was invaluable.

The study was supervised at the Central Veterinary Laboratory by Dr W.J. Brinley Morgan who was always ready to offer his help whenever requested. I am particularly indebted to him for his excellent advice in the writing of the thesis.

I should like to record the help given me by Mr R.A. Huck, who extended to me the benefit of his considerable experience with poxviruses. He and his colleagues also obtained and looked after all the sheep used in the study.

The bulk of the experimental work was carried out with the technical assistance of Mr M.J. Carwardine and Mr G.C. Evans who undertook such diverse activities as blood sampling sheep, propagating viruses, counting innumerable plaques and scrutinizing gel-diffusion plates with consummate skill and enthusiasm.

The excellent photographic work to be found in the thesis was undertaken by Mr R Sayer and the photographic display pages were prepared by Mr D. Kimber.

Miss Nancy Hebert kindly carried out the necessary statistical analyses of the results and provided Appendix IV.

Many of the library staff, particularly Mr R. Park, Mrs Margaret Thompson, Mrs Vera Hicks and Miss Rosemary Jones, assisted me greatly in obtaining references and translations and in compiling and checking the list of references.

The arduous task of typing both draft and final manuscripts was carried out by Mrs Joan Lawrence.

I am also indebted to other colleagues at the Central Veterinary Laboratory and elsewhere who provided materials and advice which greatly facilitated my work.

Finally, for my wife Coral, who made the original observations in sheep which initiated the study, I hope the thesis will stand as a token of recognition and appreciation of her contribution.

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INTRODUCTION

Orf vaccines licensed for use in the United Kingdom are required to meet the standards of quality specified in the relevant monograph of the British Pharmacopoeia (Veterinary) 1977. The potency requirement of the monograph is that vaccines should contain not less than 100 minimum infective doses per sheep dose and that this should be determined by vaccinating at least two healthy, susceptible sheep with serial dilutions of vaccine applied to the scarified skin. In practical terms, the inner aspect of the thigh is used for the test as this is a hairless area large enough to provide multiple scarification sites which are protected from interference by the test animal itself as well as being protected from contact with other animals or objects. A 1/100 dilution of a satisfactory vaccine should give rise to characteristic lesions of orf at the vaccination site on the fourth to eighth day after inoculation.

This test has been used at the Central Veterinary Laboratory, Weybridge for a number of years to monitor the potency of commercially manufactured vaccines but during this time a number of observations have been made which have given rise to doubts over the meaningfulness of the prescribed test. Firstly, vaccines passed the potency test even when assayed in sheep which had suffered a natural orf infection less than six months earlier. Secondly, vaccines passed the potency test when assayed in sheep which had already been vaccinated between one and six months earlier and thirdly, sheep which had been used for vaccine assay twice within the previous 12 months were still susceptible to thigh infection and could be used for assay purposes yet again, although the vaccinal lesions appeared less severe than those which had developed in the initial assay. Thus it appeared that sheep remained "susceptible" to experimental infection even though previous vaccination or natural infection should have rendered them "immune".

Examination of the published literature revealed a considerable number of conflicting, often vague or unsubstantiated accounts relating to immunity following orf infection and it was considered appropriate to undertake further studies on this aspect of the disease with a view to modifying the current potency test or developing a new type of test, should this be indicated. Before any experimental work was initiated however, a natural outbreak of the disease occurred in the flock of sheep purchased for this purpose and the opportunity was therefore taken to study some aspects of the clinical and immune response of naturally infected animals which were considered of relevance to vaccination procedures and vaccine potency testing.

The present work is an account of the study carried out on this flock of sheep together with observations on other experimentally infected animals and an investigation of the antigenic relationship between different strains of orf virus and other selected poxviruses.

HISTORICAL BACKGROUND

I. The Disease

Nomenclature

The word orf is said to be derived from the English dialect word "hurf", itself a word probably of Scandinavian origin meaning a crust or scab on a wound (Webster's Third New International Dictionary, 1961). It was first introduced into the literature by Walley (1890) as a synonym for contagious dermatitis, a disease affecting the feet and legs of sheep in the border areas of Scotland. In these areas the disease was also described as "carbuncle of the coronary band" or "hair and hoof" when lesions were confined to the feet or "mouth and foot" when the lips and face were also infected (Walley, 1890). Berry (1901) made no distinction between orf, hair and hoof or mouth and foot disease and described the condition in which either or both the mouth and feet were involved under the heading of contagious pustular dermatitis. This term was used so as to emphasize that when the mouth was affected the disease was essentially a pustular condition of the skin in contrast to foot-and-mouth disease which was a vesicular condition of the mucous membrane. Some years earlier Walley had also described a disease of sheep known as malignant aptha. This was an infectious pustular disease affecting principally the mouths of suckling lambs and the udder and teats of the nursing ewes. He considered this term a misnomer and proposed that the name "pustular fever" or "contagious ecthyma" should replace it (Walley, 1888).

Hoare (1913) considered that all these terms were used to describe different manifestations of the same disease and included the terms "lip and leg ulceration of sheep", "Crusta labialis" and "ulcerative stomatitis" as additional synonyms. Lip and leg ulceration and Crusta labialis have since been recognised as being synonomous with the disease now known as ulcerative dermatosis of sheep, a condition pathologically and immunologically distinct from orf (Tunnicliff, 1949).

Nowadays the terms orf, contagious pustular dermatitis and contagious ecthyma are the three synonyms most commonly used. Contagious pustular dermatitis tends to be favoured by British workers whereas contagious ecthyma is used more often by American and European workers, in standard text-books and in publications by international organisations such as FAO, WHO and OIE. Orf is the term often used by virologists and orf virus is currently listed as the type species of the parapoxvirus genus of the poxvirus family (Fenner, 1976).

A number of other technical terms for the disease have appeared in the literature in the past including contagious pustular stomatitis (Aynaud, 1921) and infectious labial dermatitis (Seddon and Belschner, 1929). These have now become obsolete but two early colloquial descriptions, namely sore mouth (Newsom and Cross, 1931) and scabby mouth (Seddon and Belschner, 1929), still appear in print particularly in farming journals and non-technical publications.

Natural infection

1) Clinical disease: Orf is an infectious disease of sheep and goats caused by orf virus. Clinically it takes the form of an acute eruptive dermatitis affecting principally the hairless areas of skin of the face, especially the mouth, the feet, the udder of ewes nursing infected lambs or, less commonly, the external genitalia. Following infection and an incubation period of two to three days a sequential development of papules, vesicles, pustules and scabs characteristic of a poxvirus infection occurs during the next ten days or so. The disease shows considerable variation in severity from one outbreak to another with corresponding variations in morbidity and mortality but it usually lasts for two to four weeks when the scab material drops off and affected tissues return to normal.

Numerous clinical descriptions of the disease have appeared in the literature over the years, the very early ones of which were fully summarized by Hoare (1913). In the 1920's detailed descriptions of the disease were published by Aynaud (1923) in France, Glover (1928) in England, Theiler (1928) in South Africa, Seddon and Belschner (1929) in Australia and Howarth (1929) in the USA. These accounts and those of other workers are all essentially the same and form the basis of the descriptions found in standard text-books on sheep diseases (Marsh, 1965; Jensen, 1974), veterinary medicine (Hungerford, 1967; Blood and Henderson, 1974), veterinary pathology (Jubb and Kennedy, 1963; Runnells, Monlux and Monlux, 1965) and microbiology (Buxton and Fraser, 1977).

2) Susceptible species: Orf is a naturally occurring disease of sheep and goats and the susceptibility of these species to the virus is unquestioned.

Among other species of animals the naturally occurring disease has only been seen with any degree of frequency in the chamois. Bouvier, Burgisser and Schweizer (1951) first described the disease in this species in Switzerland and further cases have been recorded by Carrara (1959) and Guarda (1959) in Italy. Daniel and Christie (1963) reported that since 1940 at least eight epidemics of orf had occurred in the chamois and thar (an Indian species of deer) populations of New Zealand. Although Grausgruber (1964) was unable to find any difference between sheep and chamois derived orf viruses in experimental animal studies and Gerstl (1964) likewise could not differentiate between virus strains from these two origins under the electron-microscope, chamois contagious ecthyma virus has been listed as a separate species in the parapoxvirus genus in the Second Report of the International Committee on Taxonomy of Viruses (Fenner, 1976).

A single instance of a natural outbreak in dogs has been recorded by Wilkinson, Prydie and Scarnell (1970). The disease occurred in a pack of hounds, with lesions developing mainly around the head. Skin biopsy material proved infective for sheep and electron-microscope examination of sheep lesion material revealed the presence of typical orf particles.

Orf is not usually classified as a naturally occurring disease of man but the virus is recognised as being infectious for man and it is considered an occupational hazard for those involved in the sheep and goat and allied industries. The early literature on human infection was summarized by Taylor and Lea (1957) who refer to ten published reports of human cases, including the first authentic report by Newsom and Cross (1934b), and describe three additional cases. A later review by Leavell et. al. (1968) also describes the clinical and pathological features of 19 human cases and an even larger series of 119 cases encountered in Norway between 1957 and 1973 has been documented by Johannessen et. al. (1975). In the United Kingdom human infection is probably more common than is generally believed. In 1977 a total of 28 human orf-paravaccinia cases were confirmed by the Public Health Laboratory Service, an incidence only slightly less than the average annual total of 37 for the period 1972-1976 (PHLS, unpublished data). It should be borne in mind however that these figures include milker's nodule as well as orf virus infections.

3) Prevalence: There would seem to be little doubt that orf is a disease with a worldwide distribution. Accounts of infection occurring in sheep or goats in many different countries are to be found in the published literature and the most recent FAO/WHO/OIE Animal Health Yearbook lists orf as occurring in no less than 84 countries from which information was obtainable (Table I).

In the United Kingdom itself the disease is probably ubiquitous. Many years ago Glover (1933) obtained strains of virus from Cambridgeshire,

Table I

Geographical distribution of orf, 1977

Cap. = Caprine
Ov. = Ovine

	Cap.	Ov.
AFRICA		
Algeria	+	+
Benin	+	
Botswana	+	+
Central African Empire	+	+
Chad	+..	+..
Congo P.R.	+	...
Egypt		+
Gabon	++	+ +
Ghana		+
Guinea	+	+
Kenya	+	+
Lesotho	+	+
Libya	+	+
Madagascar	+	+
Malawi	(+)	(+)
Mali		++
Morocco	(+)	+
Mozambique	+	+
Niger	(+)	(+)
Rhodesia	+	+
Rwanda	(+)	(+)
Sierra Leone	++	++
Somalia	+	+
South Africa	++	++
Swaziland	+	+
Togo	+	+
Tunisia	+	+
Zaire	++	
Zambia	...	+
ASIA		
Afghanistan	...	+
Bahrain	+	+
Bhutan	(+)	(+)
Burma	+	
Cyprus	++	++
India	+	++
Iran	+	+
Iraq	+	+
Israel	+	+
Jordan	+	+
Kuwait		+
Lebanon	+	+
Malaysia	+	...
Nepal		+
Sri Lanka	(+)	
United Arab Emirates	+	+
Vietnam	+	
Yemen	(+)	(+)

	Cap.	Ov.
AUSTRALASIA		
Australia	+..	++
New Zealand	++	++
EUROPE		
Albania	+	+
Bulgaria		+
Czechoslovakia		(+)
France	+	+
German Democratic Republic		+
Greece	+	+
Hungary	+	+
Iceland		(+)
Ireland		+
Italy		(+)
Netherlands		+
Poland		+
Portugal	+	+
Romania		+
Spain	(+)	(+)
Switzerland	(+)	(+)
UK (Great Britain)	(+)	+
UK (N. Ireland)		+
USSR		+
Yugoslavia		+
N. AMERICA		
Canada		+
Cuba	(+)	(+)
Dominican Republic	++	+
Guatemala	+..	+..
Jamaica	+	
Mexico	+ +	++
USA		+
S. AMERICA		
Argentina	+	+
Bolivia	+	+ +
Brazil	+	++
Chile	+	++
Colombia	+	+
Peru		++
Uruguay		++
Venezuela	+	+

Key:-

(+)	exceptional occurrence
+	low sporadic incidence
++	moderate incidence
+..	disease exists; incidence unknown
...	no information available

Extracted from FAO/WHO/OIE

Animal Health Yearbook, 1977

Dorset, Isle of Wight, Northumberland, North Wales, Rosshire, Somerset, Suffolk and Worcestershire and during 1977 the virus was positively identified in 37 samples of ovine scab material from 17 English and Welsh Veterinary Investigation Centres, ranging from Penrith in the north to Truro in the south and Bangor in the west to Norwich in the east (Central Veterinary Laboratory, unpublished data).

Immunity

1) Immunity following natural infection: It is generally recognised that an animal which has recovered from a naturally acquired infection possesses a considerable degree of immunity to reinfection but the length of time such immunity persists has been the subject of various opinions. Lanfranchi (1925) considered that it was not long lasting and might disappear from five to eight months after infection. Glover (1928) however was able to show that recovered sheep were resistant to reinfection for at least eight months and Jacotot (1926) demonstrated that goats retained complete immunity for at least two and a half years. Hardy (1964) held the view that in sheep a very strong life-long immunity developed but only against the same strain of virus. Samojlov and Aliverdiev (1968) also recognised that long-lasting resistance developed in adult sheep but were of the opinion that this arose through periodic re-immunisation in the field, since adult sheep kept in isolation after experimental infection became susceptible to reinfection after 10-12 months.

The length of time immunity persists also appears to depend to a certain extent on the age at which infection occurs. Olah and Klek (1953) noted that lambs which had sustained a natural infection when only a few days old contracted the disease again one year later whereas those a few months old at the time of the initial infection remained

immune. Samojlov and Aliverdiev (1968) state that it is not unusual to find cases where suckling lambs which succumbed to the disease have suffered a further infection following weaning.

Apart from these observations on the immune status of the very young lamb, there is no substantial evidence to show that the state of immunity is otherwise age-related. Aynaud (1923), Theiler (1928), Seddon and Belschner (1929) and Hardy (1964) have all affirmed that adult animals, not previously exposed to infection, are fully susceptible although these same authors as well as other workers generally agree that the disease is not as severe in the adult animal as in the lamb. Cases in which nursing ewes have remained free of lesions while their lambs were clinically affected have been attributed to an immunity acquired from an earlier infection and not to ageing (Aynaud, 1923; Glover, 1928).

2) Immunity following experimental infection: Although it had been established during very early studies of the disease that orf could be experimentally transmitted to susceptible animals (Walley, 1890), it was not until the 1920s that experimental infections were utilised to gain further understanding of the nature of immunity to the virus.

Initially, Aynaud (1921) reported that experimental thigh infection of sheep gave rise to an immunity lasting at least nine months. The same author subsequently published the results of further experiments with sheep which led to the application of a practical method of thigh vaccination still widely followed to the present day (Aynaud, 1923). His more significant observations were that a solid immunity to thigh reinfection developed 15-20 days after initial infection; reinfection before 15 days resulted in less severe and more rapidly forming and healing lesions; animals infected by scarification of the tail developed immunity 20 days later even when the tail had been amputated five days after infection; immunity could be induced by subcutaneous or intravenous inoculation of

infective material without the development of visible lesions; immunity could not be transferred via serum from immune animals. The overall conclusion was that in orf a tissue immunity, but not a humoral immunity, developed associated with recovery from either natural or experimental infection.

As part of a study to compare the disease as it occurred in England with that described in France, Glover (1928) repeated much of Aynaud's work and obtained similar results although immunity to reinfection was found to develop somewhat earlier, being absolute by the 15th day post-infection. He confirmed that immunity developed in lambs infected by scarification of the tail which was subsequently amputated and that immunity was induced by subcutaneous or intravenous inoculation of scab material without the development of any disease symptoms. Although these conclusions were based largely on the results of experimental infections using scarification of the skin of the thigh, it was also observed that the lips of animals which had recovered from a thigh infection had "acquired a considerable resistance against the virus." Marais (1928) found that animals infected by scarification of the neck developed complete immunity of the lips within ten days and that this immunity persisted for about six months. Seddon and Belschner (1929) also reported that experimental infection of the inguinal region of sheep induced resistance to a test infection of the muzzle and added that the reverse was also true. This development of immunity of the muzzle following thigh infection was substantiated by the results of additional experimental studies (Seddon and McGrath, 1933). Manley (1934) however, found that sheep infected by scarification of the flank developed only a partial immunity to reinfection of the flank and Nisbet (1954) observed that flank immunity persisted no longer than three months.

The observation by Aynaud (1923) that reinfection before absolute immunity developed resulted in more rapidly developing and healing lesions has also been reported by Boughton and Hardy (1934), Manley (1934), Glover (1935), Hart, Hayston and Keast (1949) and Olah and Elek (1953). This abortive type of reaction has also been seen to occur when the state of absolute immunity had presumably waned and only a partial immunity remained.

Many of the seemingly contradictory findings of the above workers were further investigated by Schmidt (1962). He was able to show that following infection by scarification of the inner thigh, partial immunity at the lip site could be detected by the eighth day post-infection and absolute immunity by 13 days. Absolute immunity at the lip site persisted for five months but contrary to expectations, 80% of animals were fully susceptible to reinfection at the thigh site within two months of the initial scarification. Furthermore, these animals could be similarly reinfected a second or third time at two-monthly intervals without any apparent change in sensitivity to the virus. Schmidt (1967c) further reported that the healed lesion area of the thigh was more resistant to reinfection than neighbouring skin sites; intravenous administration of virus induced immunity on the mouth but not the thigh; sheep infected on the feet developed immunity to challenge infection on the mouth as well as the feet and sheep infected on the mouth, udder or inner thigh developed immunity on the feet. Udder infection also induced immunity in the mouth region but 83% of the animals which had recovered from an udder infection were susceptible to udder reinfection while remaining resistant to a mouth infection. Many of these findings were confirmed by Kovalev et. al. (1971), particularly in respect of the time taken for immunity to develop, the duration of immunity and the difference in sensitivity to reinfection between the mouth and thigh regions. Kovalev et. al. (1971) also confirmed the observation of Lopatnikov (1968) that two scarification

infections of the thigh at a seven to ten day interval enhanced the resistance of all other skin sites on the body to a further test infection.

3) Immunity following vaccination:

a) Live vaccines: Vaccination as a preventative measure and also as a means of controlling an existing outbreak of orf in a flock of sheep was first investigated by Aynaud (1923). The vaccine itself consisted of scab material, collected at the 15th-20th day of disease, which had been dried over sulphuric acid, exposed to chloroform for 24 hours and again dried. When required for use a 1% w/v emulsion of the powder was made in 50% v/v glycerine in normal saline and a few drops applied to the scarified inner aspect of the thigh. Scarification was carried out with a needle and took the form of an H, each arm of the H being 8-10 cms long. Aynaud vaccinated 10,000 animals in this way without generalisation of vaccinal lesions occurring in any animal. In those flocks known to be free of infection before vaccination, the subsequent introduction of infected animals into the flock did not result in disease developing in the vaccinates. Vaccination of already infected flocks was also considered successful in that the period during which disease was present in the flock was reduced from an expected five to six weeks to three weeks. Moussu (1923) also recommended this vaccination procedure for controlling the disease in infected flocks, asserting that animals not yet affected would not develop the disease and those in the incubation stage would only develop small localized lesions. Melanidi and Stylianopoulo (1928) reported that this method of vaccination had proved very effective in sheep in Greece and Hatziolos (1930) observed that none of 5,226 vaccinated sheep became infected even though they remained in an infected environment. Bubberman and Kraneveld (1931) and Kraneveld and Djaenoedin (1933) showed that vaccination on the thigh protected sheep against an experimental challenge on the lips although immunity was not absolute in every instance.

An extensive series of field trials with sheep in Texas, USA was undertaken by Boughton and Hardy (1934). In the first trial 7,804 lambs from five ranches were vaccinated and 10,173 lambs from the same premises were left as controls, the two groups being kept apart. The final results showed that only 0.38% of vaccinated animals contracted the disease whereas 65.3% of the controls became infected. It was also noted that those vaccinated animals which contracted the disease showed only very mild lesions. The following year approximately one and a half million animals were vaccinated and with no reports being received from the participating ranchers of any vaccinated animal contracting the disease, the results were considered highly successful. The sheep vaccinated in the initial trial remained on infected pastures for the following two years during which time none of them developed orf. It was concluded from this that vaccination afforded immunity against field infection for at least two years (Boughton and Hardy, 1935). Hardy (1964), in fact, was of the opinion that vaccination conferred life-long immunity.

A field investigation was also carried out in England by Glover (1935) with approximately 1,870 vaccinated and 2,420 uninoculated animals maintained under similar conditions to act as controls. In the vaccinated group 6.8% of animals developed the natural disease compared with an incidence of 11.6% in the control group. It was emphasized, however, that the lesions in the vaccinated lambs were distinctly milder than in the control group. The vaccine was found to be unsuitable for very young lambs as it frequently caused lesions of undue severity. It was probably this latter observation which led to advice being given to flock owners in the UK not to vaccinate lambs under six weeks of age. Kerry and Powell (1971) however, have since carried out trials which demonstrated that lambs could be safely and effectively vaccinated as early as 24 hours of age and the six-week age bar is now no longer applied.

In New Zealand, vaccine prepared from scabs was considered to be highly effective in preventing orf and was prepared by the Department of Agriculture and issued free to farmers who were encouraged to avail themselves of the service (Peddie, 1947, 1950). Vaccination as a means of disease control was also used in Australia and Hart, Hayston and Keast (1949) reported that the results of vaccinating approximately 53,500 sheep during a six year period were "all that could be desired". In most cases no unvaccinated control animals were left on the premises used but in one instance 1,300 lambs were vaccinated and 400 were left as controls. Only three mild cases developed in the vaccinated lambs whilst about 40 of the controls contracted the disease, many in a severe form.

A vaccine prepared in a manner similar to that of all the previous investigators was also evaluated in Hungary by Olah and Elek (1953). These authors considered that the vaccine gave good results, particularly in reducing losses in endemically infected flocks. Immunity appeared to persist for about one year except where lambs under one week of age were vaccinated when immunity was shorter lasting. Tunkl and Aleraj (1964), in Yugoslavia, also considered this type of vaccine to be both safe and effective with immunity lasting for about one year.

Lopatnikov (1968) reported that in the USSR over two million lambs on farms where orf was a problem had been vaccinated with complete success. It should be noted, however, that a double vaccination procedure was used with these animals. A vaccine trial was also carried out in Bulgaria by Ganovski (1973) with two to three month old lambs distributed between five farms. Two months after vaccination an outbreak of disease appeared on two of the farms affecting 375 of 838 unvaccinated animals but none of the 1,050 in-contact vaccinates.

In Holland, Dijkstra (1967) used a heat-treated, live vaccine to control an outbreak of disease in two experimental flocks of sheep. He found the vaccine prevented further spread of the disease within the flocks and accelerated the recovery process in already affected animals. Richter and Jansen (1968) compared the performance of a similar heat-treated vaccine with that of an unheated product and found the unheated preparation to be greatly superior in preventing disease spread in an infected environment but it had the disadvantage of inducing much more severe vaccination reactions. Apart from these two trials using a heat-treated vaccine, all the foregoing investigations were carried out using vaccines prepared in essentially the same manner as that described by Aynaud (1923) with only minor modifications being used by the different workers. Indeed, a method of preparation for this type of vaccine is still described in the British Pharmacopoeia (Veterinary) 1977 and the two commercial vaccines currently available in the UK are both prepared from scab material.

Jacotot (1926) found that goats could also be successfully immunized in the same way as sheep but recommended the caudal fold as the vaccination site of choice for milking and suckling animals. Immunity appeared to persist for at least one year. Schmidt and Hardy (1932) vaccinated 77 kids in a field experiment in which 70 kids were left as controls. Approximately 80% of the control kids later developed the natural disease while the vaccinated animals remained free of infection.

In recent years interest has been shown in the possibility of using tissue culture derived vaccines and Khanduev et. al. (1968), Sjurin et. al. (1968) and Ramyar (1973) briefly reported such products to be safe and immunogenic. A more comprehensive study, comprising three trials on farms with an orf problem, was carried out by Kovalev et. al. (1971) in which a tissue culture derived vaccine was found to be only marginally

less effective than a scab derived product in inducing immunity. Ergin and Koklu (1977) found that a virus strain passaged 28 times in calf kidney cells immunized lambs for up to six months.

Tissue culture virus emulsified with Freund's incomplete adjuvant and inoculated subcutaneously has also been tested for vaccine potential (Johnston, 1971). Fifty-five sheep were given the preparation and challenged three to eight weeks later with virulent virus. The results were considered encouraging and further trials recommended although the severity of some local reactions which occurred was an undesirable feature of the vaccine.

b) Inactivated vaccines: Glover (1935) was unable to demonstrate that a heat-killed preparation administered by scarification or a formalin-killed preparation given subcutaneously afforded lambs any degree of immunity but two lambs given 10ml of the heat-killed preparation subcutaneously appeared to develop some degree of immunity. Olah and Elek (1953) also tested a formalin-killed vaccine but adsorbed the inactivated fluid on to aluminium hydroxide gel before subcutaneous inoculation. This product appeared to stimulate an immunity but the authors point out that one dose of the vaccine required the same quantity of virus as would make 30,000 doses of live vaccine.

4) Passive immunity: Aynaud (1923) reported that a lamb given 240ml intravenously of citrated blood from immunized animals was not protected against an experimental infection administered 24 hours later. Boughton and Hardy (1934) observed that lambs born of immune mothers, and which had suckled at least once, were fully susceptible to experimental infection administered within 72 hours of birth. This apparent absence of serum or colostral orf antibody in amounts sufficient to provide passive protection to the lamb has been supported by the studies of Manley (1934), Glover (1935), Olah and Elek (1953), Lopatnikov (1968) and Richter and Jansen (1968).

Although the weight of published evidence strongly suggests that lambs do not acquire any significant degree of protection via colostrum from immune mothers, Ganovski (1973) has produced evidence to the contrary. In a study carried out on three sheep-rearing farms, 598 (39%) of 1520 suckling lambs born of non-vaccinated ewes contracted disease whereas only 29 (2.7%) of 1080 lambs from vaccinated animals were noted as being similarly affected. Poulain, Gourreau and Dautigny (1972) have also demonstrated that lambs born of immune mothers may have significant levels of neutralizing antibody in their serum indicating that such antibodies may be secreted in the colostrum. Unfortunately, these authors only present the results obtained with the serum of a single lamb and as this same lamb was not apparently challenged with infective material it is not possible to draw any conclusion in respect of the relationship between neutralizing antibodies and protection.

Serological response to infection

1) Neutralizing antibodies: Following either natural infection or vaccination, sheep appear to develop at best only low levels of neutralizing antibody in their serum. Aynaud (1923), in fact, was unable to demonstrate any neutralizing activity in the sera obtained from animals which had recovered from natural infection or those which had been vaccinated one to two months previously. Initial experiments by Glover (1933) also failed to reveal the presence of neutralising antibodies in the sera of recovered lambs but he was subsequently able to demonstrate neutralizing activity when an autolysate of crusts was used as the source of virus. Titration of serum-virus mixtures in lambs showed that serum from a lamb which had recovered three weeks previously reduced the severity of the lesions, as compared with normal serum, but did not completely inactivate the virus. Manley (1934) obtained essentially

the same results as Glover with serum from two lambs artificially infected by scarification and Selbie (1945) detected a low level of neutralizing activity in a small sample of sera from recovered lambs but also found that some lambs gave completely negative results. Olah and Elek (1953) were unable to observe any neutralization of virus by convalescent sera. Using the intradermal inoculation of rabbits to titrate serum-virus mixtures, Abdussalam (1958) was able to demonstrate a recognisable degree of inhibitory activity in the serum of two convalescent sheep three to four weeks after infection.

All the above workers used relatively crude antigen preparations and carried out their titrations on the skin of sheep or rabbits. The results obtained were necessarily of a qualitative nature and the neutralizing activity of the sera tested could not be expressed in precise quantitative terms.

Plowright, Witcomb and Ferris (1959) used a tissue culture technique to assay the neutralizing antibody levels of sheep infected by dermal scarification or intradermal inoculation. Using the constant serum-varying virus method on sera from eight sheep, neutralization indices of between $\log_{10}0.5$ - $\log_{10}2.4$ were obtained, the lower values being considered of only marginal significance. Nagington and Whittle (1961), using a plaque-reduction test, demonstrated neutralizing antibodies in convalescent sera from sheep severely affected in an orf epidemic but Macdonald and Bell (1961), in preliminary tissue culture experiments, were unable to demonstrate unequivocally the development of neutralizing antibody after infection in either sheep or man. Liess (1962) used both the constant serum-varying virus and constant virus - varying serum methods in examining sera from four sheep, three of which had been infected by lip scarification and the other by intravenous inoculation. No neutralizing antibodies could be detected during the course of disease

or convalescence nor in the intravenously infected animal when serum was tested 33 days after inoculation. Other workers have also reported negative findings. Khanduev, Guesev and Dzhakulov (1969) could not detect neutralizing antibody in twice vaccinated sheep two weeks, three months or six months later or in naturally infected animals two, six and nine months after clinical recovery and Schmidt (1967c) was unable to find antibodies in the serum of either convalescent or hyperimmunized sheep. Khanduev et. al. (1973) also reported the absence of neutralizing antibodies in skin extracts of vaccinated or naturally infected sheep. Although Kovalev et. al. (1971) also obtained negative findings with sheep vaccinated by scarification of small areas (4 x 2cm) of skin, serum neutralizing antibodies were detected after applying a massive infection to extensively scarified areas (20 x 10 cm) or following the subcutaneous inoculation of a large quantity of virus (10^7TCID$_{50}$/ml.).

Trueblood, Chow and Griner (1963) tested sheep convalescent serum by both <u>in vivo</u> and <u>in vitro</u> methods. When serum-virus mixtures were inoculated into sheep "no demonstrable reduction of lesions" was observed but neutralization indices between $\log_{10}1.8$ - $\log_{10}2.1$ were obtained in tissue culture assay. Sawhney (1966c) also obtained neutralization index values of $\log_{10}0.6$ - $\log_{10}2.0$ in sera from sheep experimentally infected by scarification. Poulain, Gourreau and Dautigny (1972) used a plaque-reduction test to assay neutralizing antibodies and found that a 1/50 dilution of serum from two, twice-vaccinated sheep and from a convalescent animal gave a 50% reduction in plaque formation. Neutralizing activity was also found in sera from actively infected lambs and in the serum of lambs from immunised mothers. In the latter case the antibodies were presumed to be colostrum derived. Neutralizing activity was said to be enhanced by the addition of guinea-pig complement to the serum-virus mixtures.

2) Complement-fixing antibodies As part of a study into methods of diagnosing orf infection, Glover (1933) investigated the possible occurrence of complement-fixing antibodies in the sera of recovered and hyperimmunized lambs. Using an autolysate of infective scab material an antigen, clear-cut and specific fixation was obtained with hyperimmune sera but convalescent sera gave results varying from occasional complete fixation to, more commonly, no detectable fixation. The serum of an experimentally infected lamb showed complete fixation three weeks after recovery but by the ninth week these antibodies could no longer be detected. Manley (1934) obtained a similar result in that serum from one experimentally infected animal gave a positive complement-fixation reaction but subsequent tests with other sera proved unsatisfactory. Rottgardt, Arambur and Garcia Pirazzi (1949) detected complement-fixing antibodies in the sera of sheep 8 and 31 days after experimental infection whereas Nisbet (1954) found that specific fixation could not be detected in the majority of serum samples from lambs 15-60 days after experimental infection. Olah and Elek (1953) were able to detect antibodies for the first time 11 days after infection and found that 19 of 26 infected animals still had antibodies in their sera five months later. These workers also found that 23 of 83 sheep which were susceptible to infection had complement-fixing antibodies in their sera whereas a number of immune animals proved negative in the test. This latter observation was explained by the fact that immunisation had occurred more than six months previously. Abdussalam (1958) also detected complement-fixing antibodies in sheep sera three to four weeks after orf infection but the titres obtained were variable and not as high as in hyperimmunized animals.

Using a tissue culture derived antigen, Macdonald and Bell (1961) showed that serum from experimentally infected sheep fixed complement at

dilutions up to 1/64 three weeks after infection but Trueblood, Chow and Griner (1963), also using a tissue culture derived antigen, found no evidence of complement-fixing antibodies in convalescent sheep sera. Schmidt (1967d) observed an eight-fold increase in antibody titres of three sheep following repeated experimental infection at weekly intervals for 16 weeks. Titres declined to pre-infection levels by four months after final reinfection.

Romero-Mercado (1969) used 1/100 dilutions of orf scab suspensions as antigen to test convalescent and post-vaccinal serum samples from sheep. Complement-fixing antibodies were readily detected in the sera of convalescent sheep four weeks after the onset of disease and persisted for at least 20 weeks, the longest period tested. Similarly, vaccinated lambs developed antibodies within four weeks of vaccination which persisted for 20 weeks. Groups of vaccinated lambs were also challenged 4, 8, 12 and 16 weeks after vaccination and serum samples assayed one and two weeks later. Overall, challenge did not alter the complement-fixing antibody levels.

3) Precipitating antibodies: The possibility that circulating precipitating antibodies may be formed following orf infection in sheep was first investigated by Glover (1933) using the flocculation test. Despite many attempts however, he was unable to demonstrate a specific flocculation reaction with mixtures of scab extract and serum from recovered lambs. Manley (1934) also used the flocculation test to examine the serum from a sheep recovered from experimental infection by scarification and subsequently hyperimmunized by four intravenous inoculations of virus. Negative results were obtained with both convalescent and hyperimmune serum. Abdussalam (1958) confirmed these observations when using scab extract as antigen but with a partially purified and concentrated antigen he obtained a weakly positive flocculation reaction

with a convalescent sheep serum and a stronger reaction with a hyperimmune serum.

Trueblood, Chow and Griner (1963) used both the Ouchterlony double diffusion and tube precipitin tests to examine convalescent sera. Precipitating antibodies could not be demonstrated but the nature of the antigen used was unclear from the protocol. Likewise, Schmidt (1967e) was unable to obtain a precipitin line in the double diffusion test with sera from either convalescent or hyperimmunized sheep. Here again, details of the antigen used in these particular tests are not given in his report. Johnston (1966; 1967), on the other hand, was able to induce the production of detectable levels of precipitating antibody by hyperimmunizing sheep with tissue culture virus in Freund's adjuvant and Sawhney, Dubey and Malik (1973) were able to identify three distinct lines of precipitation in the double diffusion test when scab extracts from 15 infected sheep and goats were reacted against sheep hyperimmune serum but four convalescent sera from orf recovered sheep tested against the same antigen preparations gave negative results. Capurso, Traballesi and Guarino (1976) also found that serum samples from clinically affected or convalescent sheep or goats generally gave negative results.

Although all the above workers were unable to demonstrate the development of precipitating antibodies following natural orf infection, convincing evidence of their occurrence has been provided by Romero-Mercado (1969). Using the supernatent fluids from 20% w/v suspensions of orf scabs as antigen in double diffusion tests he identified antibodies in both convalescent and post-vaccinal sheep sera. In a group of 16 naturally infected animals precipitating antibodies appeared in the serum of 25% of animals within one week of the onset of disease and in 100% of animals by the fourth week. Antibodies had fallen to an undetectable level by the sixteenth week. All convalescent sera produced

one line of precipitation only. In a group of 25 six-month-old orf-susceptible lambs, precipitating antibodies were detected in five out of six animals tested four weeks after vaccination but no antibodies were detected by the eighth week post-vaccination. Vaccination within one week of birth of 12 lambs born of immune ewes resulted in the appearance of antibodies in 30% of animals three weeks later. The number of positive lambs rose to 63% at eight weeks and then fell until all animals were negative by 20 weeks post-vaccination. Like the convalescent sera, post-vaccinal sera produced only a single line of precipitation. In a further experiment however, it was observed that two lines of precipitation formed with sera from some animals which had been experimentally challenged by scarification following recovery from a natural infection or vaccination.

4) Agglutinating antibodies: In contrast to the considerable number of studies carried out with respect to the development of neutralizing, complement-fixing and precipitating antibodies following orf infection or vaccination, few workers have investigated the occurrence of agglutinating antibodies. Following the demonstration by Blakemore, Abdussalam and Goldsmith (1948) that man developed specific agglutinins in his serum after either natural or experimental infection, Olah and Elek (1953) used a similar tube agglutination method to test sera from five experimentally infected sheep. Agglutination of serum dilutions of 1/50 or greater were obtained with these animals compared with dilutions of 1/10 or less in control sheep. Abdussalam (1958) mixed dilutions of sheep serum with elementary body suspensions in a capillary tube and read the results after 18 hours at room temperature. Convalescent, and more particularly hyperimmune sera, showed a four-fold or greater rise in titre. Alexaj and Tunkl (1964) used essentially the same technique to test sera from four infected sheep. Low titres of

agglutinating antibody were detected 6-10 days after infection with four-fold rises being observed by 15 days. These workers concluded that the test was a reliable diagnostic test for orf infection.

5) Haemagglutination-inhibiting antibodies: Unlike the viruses of the orthopoxvirus genus, orf virus is not recognised as possessing a specific haemagglutinin (Fenner, 1976) and this has been supported by the observations of a number of workers. Blakemore, Abdussalam and Goldsmith (1948) reported that two strains of virus did not produce haemagglutinins but the species spectrum of red blood cells tested is not stated in their publication. Olah and Elek (1953) were unable to obtain agglutination of chicken, rabbit or sheep erythrocytes and only inconsistent and doubtful agglutination of frog, guinea-pig and horse cells. Agglutination of hamster cells was observed in about half of the tests carried out and inhibition of the reaction by convalescent serum was obtained in a single instance. Kujumgiev (1954) also occasionally observed agglutination of guinea-pig erythrocytes with high concentrations of virus but cells from chickens, rabbits, sheep, horses and pigs were not agglutinated. Similarly, Abdussalam (1958) found that erythrocytes from chickens, rabbits, sheep and a horse were non-agglutinable by the virus and, in addition, that cells from a goat, a calf, pigeons, guinea-pigs and mice were unaffected.

In contrast to the above findings, Sawhney (1966b) demonstrated agglutination of chicken, guinea-pig, mouse and human "O" erythrocytes with clarified suspensions of chorioallantoic membranes infected with orf virus. The reaction was also shown to be inhibited by anti-orf sera prepared by the hyperimmunization of chickens, rabbits and sheep. It should be noted however that haemagglutination titres increased with increasing egg-passage of virus and that no significant titres were obtained with antigen prepared from the first two egg passages. Infected

scab extracts and elementary body preparations of the two virus strains used also failed to agglutinate red blood cells. These observations, together with the additional finding that the haemagglutination obtained could be inhibited with anti-vaccinia serum, must cast some doubt on the validity of these findings.

II. The Virus

Structure and Classification

One of the earliest suggestions that the causative agent of orf might be a member of the poxvirus family came from Whalley (1888) who observed a very close similarity between the lesions of orf infection of the udder and teats and the lesions of sheep pox at the same site. Aynaud (1923) was one of the early workers to establish the filterability of the agent and, because of the pathogenesis of the disease, classified the agent as a virus in the vaccinia-variola group. Blanc, Melanidi and Caminopetros (1922), Jacotot (1926), Glover (1928) and Newsom and Cross (1934a) all confirmed the filterability of the virus, the latter two workers giving a hint as to the size of the infective particle by finding that it would only pass through the coarser grades of filter. Further filtration studies by Blanc and Martin (1941) enabled them to estimate the infective particle to be 100-260 nm in diameter.

Ishii, Kawakami and Fukuhara (1953) first described the morphology of the virus as revealed by the electron microscope. Ellipsoidal particles, 200-250nm in diameter were observed in smears of pustular material. Abdussalam and Cosslett (1957) made a more detailed study of morphology using dark-ground illumination of unstained particles, light microscopy of stained material and electron microscopy. Particles of mean length of 258nm and a mean breadth at the widest point of 158nm and with rounded ends were observed. The axial ratio (length/breadth) was 1.6 and the

virus was distinguished on this basis from the viruses of molluscum contagiosum, ectromelia, myxoma, canary-pox, pig-pox and vaccinia, all of which have lower ratios.

Downie and Dumbell (1956) and Fenner and Burnet (1957) both considered on the basis of particle morphology that orf virus should be a candidate member of the poxvirus group.

The size of the virus has since been determined by a number of other workers. Nagington and Whittle (1961) and Nagington and Horne (1962) obtained average dimensions of 263nm (range 220-300nm) by 157 nm (range 150-175nm) and an axial ratio of 1.62 which compare well with the figures of Abdussalam and Cosslett (1957). Schulze and Schmidt (1964b) also obtained almost identical values (258nm x 157.5nm) for the strain they examined and Faizulina et. al. (1973) found the majority of particles they examined to have dimensions of 270nm x 170nm. Kujumgiev and Todorov (1961) however obtained much lower values - 108nm x 143nm - and an axial ratio of 1.45 and Knocke (1962) observed oval particles in the range of 170-240nm in length by 120-170nm in breadth.

Nagington and Horne (1962) also described two forms of orf virus particle - an incomplete and a complete form. In the complete form a well-defined criss-cross or woven pattern of parallel stripes of material running diagonally across the particle was demonstrated.

Nagington, Newton and Horne (1964) confirmed the nucleic acid of orf virus to be DNA and determined the molecular weight of the nucleic acid to be 171×10^6 per particle, a value similar to that of other poxviruses.

Orf virus has been shown to possess the ability to reactivate heat-inactivated members of the poxvirus group, a property considered characteristic of all poxviruses (Fenner and Woodroofe, 1960).

A study of the morphology of bovine papular stomatitis virus revealed particles remarkably similar to orf (Nagington, Plowright and

Horne, 1962). They were found to be oval in shape with a conspicuous criss-cross pattern and with mean dimensions of 177nm x 169.5nm. Friedman-Kien, Rowe and Banfield (1963) found that the milker's nodule virus also exhibited a marked similarity to orf in overall appearance although the dimensions (280nm x 120nm) were different. Peters, Muller and Buttner (1964) considered that on the basis of virus structure, orf, bovine papular stomatitis and milker's nodule (paravaccinia) viruses belonged unequivocally to the poxvirus group but that they formed a distinct subgroup for which the name paravaccinia was proposed. Nagington (1964) also considered that these three agents formed a distinct morphological type and Nagington, Tee and Smith (1965) argued that the subgroup formed should more appropriately be termed the orf subgroup rather than the paravaccinia subgroup. Nevertheless, the term paravaccinia came to be used to categorise these agents (Huck, 1966; Nagington, 1968; Joklik, 1968) even though Melnick and McCombs (1966) formally classified them as the orf-like viruses subgroup.

In 1974 the International Committee on Taxonomy of Viruses (ICTV) approved the constitution of a family of viruses to be known as Poxviridae with six genera being established within the family. The genus parapoxvirus (para = 'by the side of' (Gr.)) comprised the viruses of orf, ulcerative dermatosis of sheep, bovine papular stomatitis and milker's nodule (Fenner et. al., 1974). The Second Report of the ICTV in 1976 omitted ulcerative dermatosis of sheep and included chamois contagious ecthyma virus in the parapoxvirus genus. Orf virus was established as the type species of the genus (Fenner, 1976).

Resistance

Orf virus has been shown to be markedly resistant to the effects of desiccation. Aynaud (1923) found that crusts from lesions, powdered and

dried over sulphuric acid, remained fully infective for at least one year and Glover (1928) considered this method of preservation to be preferable to any other. Seddon and Belschner (1929) and Cauchemez (1933) reported that dried scab material held at room temperature retained infectivity for over a year and Glover (1933) found that low temperature storage of crusts preserved infectivity for at least 4½ years. Hart, Hayston and Keast (1949) found dried scab material still contained viable virus after 15½ years storage at room temperature and Livingston and Hardy (1960) were able to induce typical lesions in lambs with sulphuric acid dried and powdered material stored in a refrigerator for over 22 years. This same material has since been shown to be infective after 27 years storage (Hardy, 1964).

The virus also appears to be comparatively resistant to heat treatment. Exposure of infective tissue culture fluids to 37°C for one week reduced infectivity approximately 100-fold (Plowright, Witcomb and Ferris, 1959; Sawhney, 1972) and a similar fall in titre was observed with fluids held at 55°C for 30 minutes (Plowright, Witcomb and Ferris, 1959). Schmidt (1967a) however, observed a 100-fold drop in the titre of a scab suspension after only 10 minutes at 56°C with a further 1000-fold fall after 20 minutes. Heating at 60°C has produced varying results. After 30 minutes at this temperature Sawhney (1972) observed a fall not dissimilar to that obtained with material held at 37°C for one week and Dijkstra (1967) also found that the virus survived these conditions whereas Richter (1969) obtained complete inactivation after 45 minutes at 60°C which supported the much earlier findings of Boughton and Hardy (1934) who noted a complete loss of infectivity after 30 minutes at 59°C.

Freezing and lyophilization appear to have no deleterious effect on the virus (Olah and Elek, 1953; Plowright, Witcomb and Ferris, 1959; Kovalev et. al., 1971; Sawhney, 1972).

Ultrasonic treatment has also been shown to have no adverse effect on virus infectivity but ultra-violet irradiation does have an inactivating effect (Sawhney, 1972).

Resistance to the action of lipid solvents has been found to be somewhat variable. Glover (1928) found that scab emulsions treated with chloroform or ether had greatly reduced infectivity titres for lambs after storage for eight days and he concluded that the virus was slowly destroyed by these agents. Plowright and Ferris (1959) demonstrated a low degree of sensitivity to ether treatment. These workers observed a similar degree of inactivation with vaccinia, a virus usually considered to be very resistant to ether (Andrewes and Pereira, 1972). Trueblood and Chow (1963) could not detect any significant loss of activity after ether treatment and Sawhney and Toschkov (1972) found only a low degree of sensitivity with the five strains they examined. Precausta and Stellman (1973) reported a "medium" degree of sensitivity with five other strains of virus. Both the latter groups of workers found however that all strains were markedly sensitive to chloroform treatment. The true position is probably as stated by Andrewes and Pereira (1972) that orf virus holds a position intermediate between the ether-sensitive and ether-resistant poxviruses but like all other members of the same family, is chloroform-sensitive.

Although orf virus has proved to be relatively resistant to inactivation by physical factors such as heat and desiccation, it appears to be fairly sensitive to chemical disinfectants. Manley (1934) demonstrated that emulsions of infective scab material could be rendered innocuous by treatment for 20 hours at room temperature with 0.5% carbolic acid, 0.05% formalin or 0.005% mercuric chloride but was resistant to 0.01% potassium permanganate. Grishaev et. al. (1971) reported that treatment of infective scab material for one to two minutes on two

to three consecutive days with 5-10% solutions of formalin or copper sulphate rendered the material non-infective for sheep and recommended copper sulphate for the treatment of lesions on infected animals as a means of preventing spread of the disease. Evans, Stuart and Roberts (1977) have shown that under laboratory conditions the virus may be inactivated by a 3% solution of an iodophor disinfectant, 2.5% lysol, 1% phenol, 2% glutaraldehyde, 2% formalin, 1% hypochlorite or 0.1% peracetic acid solutions.

Immunological relationships of orf virus

1) Relationship between strains: The balance of published evidence firmly indicates that all strains of orf, if not immunologically identical, are certainly very closely related. Glover (1928), using cross-protection tests in lambs, found three English strains to be immunologically indistinguisable from each other. Seddon and McGrath (1933), using a similar type of test, found a number of strains isolated in New South Wales to be very closely related and also showed that one of these strains and one of Glover's strains conferred a solid cross-immunity against each other. Kraneveld and Djaenoedin (1933) demonstrated complete immunological uniformity between two strains from widely separated areas of the Dutch East Indies.

Glover (1933) extended his earlier observations by examining a further 15 strains from different areas of Britain, two strains from France and one each from California, Tanganyika and Cyprus. All strains were sheep derived except those from Tanganyika and Cyprus which were goat isolates. Cross-protection tests in lambs showed that all the sheep strains fell into a single immunological group although marked differences in potency of viruses from different sources was noted. The results with the goat strains were not conclusive, principally

because the reactions of sheep to these strains were always mild but also because some doubt existed as to whether the Cyprus strain represented an orf isolate or a goat-pox isolate. Horgan and Haseeb (1947) investigated this observation further by carrying out additional cross-protection studies with English and Cyprus strains of virus including an orf isolate from a Cyprus goat. Although differences in potencies of the strains were again observed, the overall conclusion was that all strains were immunologically identical or closely related. Olah and Elek (1953) also considered all strains to be immunologically homogeneous. Mundu and Mohan (1961) came to the same conclusion with respect to two Indian strains and Sabban, El Dahaby and Hussein (1961) found an American and an Egyptian strain to be antigenically identical.

Hardy (1964) however considered that strain differences did exist and identified six variants or different immunological types on the basis of cross-immunity tests in sheep. Sawhney (1966c) also demonstrated the existence of two distinct immunogenic groups of virus based on the results of cross-protection tests in sheep with six strains of virus from England, Bulgaria, Iran, Rumania and Czechoslovakia. Although the methodology of this study indicates that adequate control animals were used, the results obtained with the controls are not presented so that a critical assessment of the conclusions is not possible. Sawhney (1966c) also claimed that his cross-protection study findings were supported by the results of serum neutralization tests in tissue culture but agar-gel diffusion tests failed to reveal any antigenic differences between the strains. A later study using the gel-diffusion technique with 12 sheep and 3 goat strains of orf isolated in India failed to reveal any antigenic differences between any of these strains (Sawhney, Dubey and Malik, 1973). An in-vitro serum neutralisation test has also been used by Precausta and Stellman (1973) in a study of four French and one Iranian strain of virus.

All five strains were found to be closely related, four of them certainly falling wthin a single group and the fifth being only marginally different.

2) Relationship with other poxviruses: Early workers were primarily interested in the possible immunological relationship between orf and sheep pox viruses but Blanc, Melanidi and Caminopetros (1922), Hudson (1931), Kraneveld and Djaenoedin (1933) and Sabban, El Dahaby and Hussein (1961) all demonstrated by means of cross-immunity tests in sheep that the two viruses were immunologically distinct. Subba Rao and Malik (1979) also failed to demonstrate any relationship using cross-neutralization tests in cell cultures. A serological relationship between the two viruses has however been shown to exist by Schmidt (1967f) using a complement-fixation test.

Manley (1934) also used cross-immunity tests to demonstrate that goat pox virus and orf were unrelated and this was supported by the additional finding that serum from a pox-immune goat failed to reduce the potency of orf virus. Other workers however have not found such a clear cut difference between these two viruses. Bennett, Horgan and Haseeb (1944) carried out fairly extensive cross-protection tests in goats which showed quite clearly that goat pox immunized against orf infection but that orf infection failed to afford even partial protection against goat pox. Sharma and Bhatia (1958) were able to repeat this finding in goats and also showed that goat pox immune serum neutralized orf virus whereas anti-orf serum failed to neutralize goat pox virus. Mundu and Mohan (1961) on the other hand obtained results rather similar to those of Manley (1934) except that they concluded nevertheless that a goat pox vaccine may afford some protection against orf infection. Evidence of a serological relationship between the two viruses has been strengthened by the observation of Sawhney, Dubey and Malik (1973) that a single line of precipitation developed in the agar-gel diffusion test

when orf virus was reacted against goat pox hyperimmune serum. More recent *in vitro* serum neutralization and *in vivo* cross-immunity studies by Renshaw and Dodd (1978) indicated quite strongly that goat pox and orf are antigenically dissimilar viruses but Subba Rao and Malik (1979) on the other hand, also using cross-neutralization tests in a cell culture system, found that goat pox hyperimmune serum neutralized orf virus. As has been observed by all other workers however, orf antiserum did not neutralize goat pox virus.

The relationship between orf and vaccinia viruses has been investigated by many workers. Blanc, Melanidi and Caminopetros (1922) and Jacotot (1926) found that vaccination with vaccinia did not afford the sheep or goat any protection against orf virus infection and this was confirmed in a more extensive cross-protection study in sheep by Horgan and Haseeb (1948). Webster (1958) could show no significant cross-neutralization between orf and vaccinia hyperimmune sera and Nagington and Whittle (1961) likewise were unable to demonstrate cross-neutralizing activity with human and sheep convalescent sera.

Macdonald (1951) was unable to demonstrate any immunological relationship between orf and vaccinia in a cross-complement-fixation test and Abdussalam (1958) found that vaccinia convalescent human serum showed only very weak complement-fixing activity in the presence of orf virus. Both Webster (1958) and Schmidt (1967f) however found that orf and vaccinia viruses possessed a common complement-fixing antigen. Blakemore, Abdussalam and Goldsmith (1948) and Abdussalam (1958) found that orf antisera did not inhibit haemagglutination of chicken red blood cells by vaccinia virus and Abdussalam (1958) was also unable to demonstrate specific agglutination of orf virus by vaccinia antiserum. The interrelationship of orf and vaccinia viruses has also been studied by agar-gel diffusion techniques. Webster (1958) demonstrated the exis-

tence of a common, soluble precipitating antigen in the two viruses and this was later confirmed by Schmidt (1967f) but Huck (1966) and Papadopoulos et. al. (1968) were unable to demonstrate any such relationship between the two agents.

Webster (1958) found that ectromelia virus was related to orf to an extent similar to that of vaccinia but fowl pox virus showed no evidence of any immunological relationship as judged by the results of complement-fixation and agar-gel diffusion tests.

Although the condition known as ulcerative dermatosis presents many clinical features similar to orf, Tunnicliff (1949), Trueblood, Chow and Griner (1963) and Hardy (1964) have all shown on the basis of cross-protection tests that the causative agents of the two diseases are immunologically separate entities.

As orf, bovine papular stomatitis and milker's nodule viruses are now classified together as members of the parapoxvirus group on the basis of virus morphology and structure it may be expected that these agents also exhibit an immunological relationship closer to each other than to the other members of the poxvirus family. Huck (1966) found that in the agar-gel diffusion test a line of identity developed between homologous and heterologous combinations of orf and milker's nodule viruses and their antisera, but no precipitation was observed with vaccinia antiserum. Papadopoulos et. al. (1968) also obtained a line of precipitation with combinations of orf and milker's nodule viruses and their antisera which were absent when both cowpox and vaccinia viruses were reacted against milker's nodule antiserum. Liebermann (1966) used the direct fluorescent-antibody technique with labelled antisera prepared against orf and bovine papular stomatitis viruses to demonstrate a close immunological relationship between orf, bovine papular stomatitis and milker's nodule viruses. No specific fluorescent occurred when the orf or bovine papular stomatitis

labelled antisera were reacted against vaccinia infected tissue cultures.

Virus propag tion

1) In vivo propagation: Orf is generally considered as a disease of sheep and goats and the susceptibility of these species to the virus is unquestioned. Other animals are certainly less susceptible and most species appear entirely refractory.

Aynaud (1923) reported the successful transmission of infection to a calf and Jacotot (1926) obtained characteristic lesions in one of 34 cattle tested. Bennett, Horgan and Haseeb (1944) found no difficulty in infecting calves and Ishii, Kawakami and Fukuhara (1953) also reported cattle as a susceptible species. Howarth (1929) and Boughton and Hardy (1934) however, were both unsuccessful in infecting cattle although both workers only attempted transmission in a single animal. More recently, Huck (1966) reported the development of lesions on the muzzle and buccal mucosa of calves following the subcutaneous inoculation of orf virus at these sites.

Horses were found to be resistant to experimental infection by Jacotot (1926) and Ishii, Kawakami and Fukuhara (1953). The same workers also found pigs and dogs to be resistant, an observation supported by Howarth(1929) and Olah and Elek (1953) and also by Boughton and Hardy (1934) in respect of the dog.

It is possible that the monkey, like man, may be susceptible to infection as both Jacotot (1926) and Bennett, Horgan and Haseeb (1944) were each able to transmit infection to two animals of this species.

The fowl and pigeon have both proved to be resistant (Jacotot, 1926; Glover, 1928; Olah and Elek, 1953).

A considerable number of workers have investigated the susceptibility of laboratory animal species to orf virus, partly to characterize this member of the poxvirus family but more importantly to obtain a laboratory animal model for immunological and pathological studies. Without exception, attempts at transmission to the guinea-pig have proved unsuccessful (Aynaud, 1923; Jacotot, 1926; Glover, 1928; Howarth, 1929; Newsom and Cross, 1934a; Boughton and Hardy, 1934; Selbie, 1944; Ishii, Kawakami and Fukuhara, 1953; Olah and Elek, 1953; Greig, 1956; Abdussalam, 1957). Although not as intensively studied as the guinea-pig, the mouse would appear to be equally resistant to infection (Glover, 1928; Selbie, 1944; Greig, 1956; Abdussalam, 1957) as are rats (Jacotot, 1926; Selbie, 1944).

The rabbit is the only laboratory animal in which successful transmission of the virus has been reported but this has not been a consistent finding among all workers concerned. Aynaud (1923) simply stated that the rabbit was not susceptible to orf. Glover (1928), Howarth (1929), Newsom and Cross (1934a) and Boughton and Hardy (1934) also found that inoculation of the virus by various routes gave negative results. Selbie (1944) observed the development of erythematous, scaly patches on the inoculated area of three rabbits 20 days after infection but he was unable to transmit this rabbit material back to lambs (Selbie, 1945). Olah and Elek (1953), Greig (1956) and Plowright, Witcomb and Ferris (1959) also observed the appearance of erythematous areas around the inoculation site one to four days later but vesico-pustules never developed. In contrast to the above findings Blanc and Martin (1933) observed the development of severe pustular lesions and Bennett, Horgan and Haseeb (1944) experienced no difficulty in effecting transmission to rabbits although Glover (1944) was unsuccessful in his attempts to infect rabbits with the same strain of virus. Ishii, Kawakami and

Fukuhara (1953) have reported successful transmission by lip inoculation. Abdussalam (1957), using the same virus strain which had previously been successfully transmitted by Bennett, Horgan and Haseeb (1944) and unsuccessfully by Glover (1944), found no difficulty in infecting rabbits by scarification or intradermal inoculation. Lesions comprised areas of erythema and papulation on the third day after inoculation. Infection was serially passaged up to the 22nd rabbit passage and material from the 5th, 9th and 18th passage was infective for lambs. More recently, Maglione and Venturoli (1976) found that the virus could not be reisolated from rabbits following intradermal inoculation.

2) In vitro propagation:

a) Propagation in embryonated eggs: Many workers have attempted to propagate the causative agent of orf on the chorioallantoic membrane of embryonated hens' eggs but with only a variable degree of success. Beveridge and Burnet (1946) found no indication that multiplication of the virus occurred and Hart, Hayston and Keast (1949) reported that a filtered scab suspension, shown to be infective for sheep, produced no significant lesions three days after inoculation of two, 12-day old developing eggs. Three blind serial passages of membrane material also failed to produce lesions. Lyell and Miles (1950) however found that vesicle fluid from a human case of orf resulted in the formation of a number of very small pock-like lesions on the chorioallantoic membrane of 10-day old eggs incubated for three days and that this material was infective for lambs. Further passage in embryonated eggs resulted in fewer lesions developing at each passage until no lesions were observed at the fourth passage. Abdussalam (1951) reported essentially the same findings with an elementary body suspension of the virus. Minute lesions were observed after two days of incubation and the material was successfully passaged for three further generations before infectivity for both

membranes and lambs was lost. Macdonald (1951) attempted to propagate both sheep and human strains in 10-15-day old eggs incubated for three days at temperatures ranging from 35-39°C and although minute lesions were sometimes seen on first passage material, histological examination showed only minor ectodermal proliferation and mesodermal infiltration and no evidence to indicate that the lesions were the result of specific virus invasion. Other unsuccessful attempts to propagate orf virus in eggs have also been reported by Ishii, Kawakami and Fukuhara (1953), Greig (1956), Valadao (1961), Liess (1962), and Precausta and Stellman (1973). On the other hand, Webster (1958) was able to repeat the observations of Lyell and Miles (1950) and Abdussalam (1951) by serially passaging a strain of virus for four generations before infectivity was lost. He was also able to detect intracytoplasmic inclusion bodies in ectoderm cells at the first passage level. Nagington and Whittle (1961) observed that a tissue-culture adapted strain of virus gave rise to discrete pocks on the chorioallantoic membrane of 12-day old embryos but no attempt was made to serially passage this material. Sawhney (1966) also succeeded in infecting membranes with two strains of virus. He found 12-day old embryos more susceptible to infection than 9, 10, 11 or 13-day-old embryos and also noted that only about 70% of embryos could be infected. Like earlier investigators he observed discrete, pin-point lesions and was able to demonstrate intracytoplasmic inclusion bodies. Serial passage resulted in the disappearance of lesions by the fourth generation but further blind passaging led to their reappearance after the sixth generation and infection was then serially transmitted through to the fifteenth generation. However, material from the tenth passage did not produce a specific lesion on the scarified skin of a young lamb. Sawhney and Spasova (1973) have also demonstrated the presence of elementary bodies in the chorioallantoic membrane of infected eggs using electron microscopy.

b) Propagation in tissue cell cultures: The possibility of using tissue cell cultures as a means of propagating orf virus was first investigated by Greig (1957). He succeeded in isolating and serially propagating three Canadian strains of the virus in monolayer cultures of embryonic sheep skin. Webster (1958) was also able to grow the virus in suspended embryonic sheep skin fragments but found that the strain, used for vaccinating sheep in New Zealand, was non-cytopathogenic for monolayer cultures. Plowright, Witcomb and Ferris (1959) were able to propagate an English strain of the virus in monolayer kidney cultures derived from embryonic sheep, calves and kid-goats and also in sheep and calf testis cultures. Macdonald and Bell (1961) confirmed the ability of sheep embryo kidney cultures to support growth of the virus and extended the range of susceptible culture substrates by obtaining good growth in monolayer cultures of human amnion, human embryonic kidney and human embryonic liver cells. They suggested that human amnion cells might be the most suitable substrate for isolating the virus from human lesions. Nagington and Whittle (1961) also showed that human amnion cells were suitable for isolating the virus from human cases and that the ovine strain used by Plowright, Witcomb and Ferris (1959) could be grown in this substrate. One of the human strains isolated in human amnion cultures also grew in secondary monkey kidney cell cultures but attempts at propagation in HeLa, MK2 and Am9 (human amnion) continuous cell lines were unsuccessful. Ramyar (1963) however reported the successful isolation and passage of an Iranian ovine strain of orf virus on a monkey kidney cell line and Scott (personal communication) recently adapted a Scottish strain, isolated in lamb kidney culture, to growth in Vero cell line cultures. Nagington (1968) finally succeeded in adapting a human isolate to growth in HeLa cells and Sawhney and Toschkov (1971) also reported the successful adaptation of a Bulgarian strain, isolated in

lamb testis, to growth in this substrate. The variety of substrates suitable for orf virus propagation has now been extended further than for most other viruses with the successful use of newborn rabbit kidney cells by Kujumgiev and Todorov (1961), a rabbit cell line by Johnston (1964), pig kidney cells by Sawhney (1966) and chick embryo fibroblast cultures by Trueblood (1966) and Rossi (1973), the latter worker also finding duck embryo fibroblasts at least as sensitive to orf virus as chick embryo cells. Goat testis cells have been used successfully by Renshaw and Dodd (1978).

Nevertheless, the majority of workers propagating orf virus in vitro have utilized cell cultures of ovine or bovine origin for this purpose. Zueva et al. (1968) used a sheep kidney cell line to study the growth characteristics of two strains of virus and Precausta and Stellman (1973) used primary lamb kidney cultures for a comparative in vitro study of five strains of the virus. Faizulina et. al. (1972), Khanduev et. al. (1973) and Vdovina et. al. (1973) used both sheep kidney and sheep skin cultures to study plaque development, tissue neutralizing antibodies in infected animals and growth of virus in cultures of tissues from immune animals respectively. A sheep fetal muscle cell culture system has been used by Poulain, Gourreau and Dautigny (1972) to assay serum neutralizing antibodies.

Bovine cell cultures have been used almost as extensively as ovine cultures. Liess (1962) isolated and passaged virus from both human and ovine orf infections in calf testis monolayers and the histopathological changes occurring in calf testis cultures following infection with orf virus were described by Schimmelpfennig and Liess (1962). Huck (1966) and Papadopoulos et. al. (1968) both used calf testis as the cell substrate for the preparation of orf virus antigen for gel diffusion precipitin tests. Trueblood and Chow (1963) utilized embryonic bovine kidney to propagate the agents of orf and ulcerative dermatosis of sheep and Schulze

and Schmidt (1964a) studied the effect of orf virus on calf kidney cells. Schmidt (1967b) also made observations on the growth in similar cell cultures of the orf strain used in earlier studies by Plowright, Witcomb and Ferris (1959).

Sawhney (1966d) used kidney and testis cultures from both sheep and calves in a comparative study of orf virus strains from six different countries and found the testis cultures to be more sensitive to infection than the kidney cultures. Nagington (1968) compared calf testis with sheep testis cultures in respect of the suitability of these substrates for primary isolation of orf virus from clinical material and concluded that sheep testis was superior for this purpose but that this was not the case with strains already adapted to tissue culture growth.

MATERIALS AND METHODS

Virus strains

Orf viruses

1) Strain EB/64/CT (referred to as EB) - obtained from Dr G R Scott, Centre for Tropical Veterinary Medicine, Edinburgh, at the 11th passage level in primary lamb testis culture. It was further propagated in secondary calf testis culture and used between the 3rd and 5th passage level in this substrate.

2) Strain ET 411 (referred to as ET) - a long established laboratory strain maintained at the Central Veterinary Laboratory, Weybridge. It was used between the 18th and 20th passage level in secondary calf testis culture.

3) Strain CVL/77 (referred to as CVL) - isolated from scab material from one of the naturally affected sheep used in this study and used between the 10th and 12th passage level in secondary calf testis culture.

Milker's nodule virus

Strain FS 198 (Huck, 1966) - obtained as freeze-dried material and propagated in secondary calf testis culture.

Bovine papular stomatitis virus

Strain 67333 - obtained as a calf kidney culture isolate from clinical material submitted to the Central Veterinary Laboratory, Weybridge and adapted to growth in secondary calf testis culture.

Other poxviruses

A vaccinia and a cowpox virus strain obtained from Dr C J Rondle, London School of Hygiene and Tropical Medicine, as suspensions of lesions from experimentally infected rabbits.

Fowl pox virus - a vaccine strain propagated on the chorioallantoic membrane of embryonated hens' eggs.

Shope fibroma virus - isolated in Vero cell culture from a commercially available myxomatosis vaccine.

Vaccines

Two commercially available live virus vaccines, licensed for use in the United Kingdom, were used. These were designated as vaccines A and B respectively.

Antisera

1) Orf antisera were obtained from naturally infected, experimentally infected and vaccinated sheep.

2) Milker's nodule antiserum was obtained from a calf experimentally infected 42 days previously with strain FC198 of milker's nodule virus.

3) Bovine papular stomatitis antiserum was obtained from a calf experimentally infected 53 days previously with strain 31099 of bovine papular stomatitis virus.

4) Vaccinia, cowpox and Shope fibroma virus antisera were raised in rabbits and fowl pox antiserum was obtained from a vaccinated chicken.

Animals

A group of 44 commercially bred Suffolk and Suffolk-cross sheep comprising 43 female and one castrated male animal was used to study immunological aspects of naturally occurring orf. The animals were four months old at the time of purchase and had been obtained from premises in South Wales on which orf was not recognised as having occurred previously and where orf vaccination was not practised. They were housed for quarantine purposes in four adjacent loose-boxes, 11 animals per box, where they were retained for the following five months when they were released to pasture as a single flock.

Six Suffolk x Hampshire lambs were obtained the following year from ewes in the above group and used to study aspects of orf immunity in the young, unweaned animal.

Dorset Horn castrated male sheep, six to nine months old, were used as control animals for experimental orf infections and for vaccination studies. These animals were laboratory bred and were known to be unvaccinated and to have remained free from any orf infection prior to use.

Animal infection

Experimental infection of sheep with orf virus was carried out by scarifying and inoculating the mouth or inner aspect of the thigh. Orf vaccination and experimental infection with milker's nodule or bovine papular stomatitis virus was carried out on the thigh site only. A hypodermic needle was used to scarify the inoculation sites.

When the mouth was used three or four scratches, sufficient to cause minor bleeding, were made on the upper lips extending forwards for approximately 3 cms from the commissures. When the inner thigh was used each inoculation site comprised two single scratches in the form of an X on the hairless area of skin. Each arm of the X was 3-4 cms long and made deep enough to cause minor capillary bleeding. Test material was applied to the scarified areas using a pair of wooden aural probes held together closely and which had been dipped into the virus preparation to a depth of about 2.5cms.

Results were recorded at seven day intervals following infection.

Assessment of susceptibility of sheep to infection

1) Mouth infection: Susceptibility to mouth infection was assessed subjectively. The absence of any specific lesion one week after infection was considered to indicate a solid immunity of the mouth region whereas the presence of specific orf scabs which extended beyond the inoculation site, and which were still evident two to three weeks after infection, indicated complete susceptibility. Lesions of intermediate severity indicated a partial immunity.

2) Thigh infection: Susceptibility to thigh infection was assessed following the application of undiluted, 1/10 and 1/100 dilutions of virus preparation or vaccine to scarified sites on one or both thighs. A 10% v/v glycerin-saline solution was used to prepare dilutions.

A positive reaction was only recorded when specific vesicles or pustules could be identified at the site of inoculation. Inoculation sites which were characterised by varying degrees of inflammation without vesicle or pustule formation were interpreted as negative reactions. In order to compare the susceptibility of different animals, a scoring system was applied to the thigh lesions. A numerical value of 1 was assigned to positive reactions occurring with undiluted material and values of 2 and 4 assigned to positive reactions with 1/10 and 1/100 dilutions respectively. If multiple focal lesions or a confluent chain of lesions developed at the inoculation site, the value for that site was doubled. Thus, for example, an animal which developed confluent lesions with the undiluted material and isolated lesions with the 1/10 dilution but no lesions with the 1/100 dilution would score (1x2) + 2 + 0 = 4. An animal with confluent lesions at the undiluted and 1/10 dilution inoculation site and isolated lesions at the 1/100 site would score (1x2) + (2x2) + 4 = 10.

Figure 1 shows examples of lesions obtained with undiluted material only, giving scores of 1 and 2.

Figure 2 shows isolated lesions obtained with undiluted and 1/10 diluted material (score 3), multiple focal lesions with undiluted and isolated lesions with 1/10 diluted material (score 4) and confluent lesions with both undiluted and 1/10 diluted material (score 6) but no lesions with 1/100 dilutions in any instance.

Figure 3 illustrates the variety of combinations obtained when lesions occurred with all three dilutions of test material, the scores ranging from 7 - 14.

a) **Score : 1**

1 - isolated lesions

b) **Score : 2**

1 - confluent lesions

Figure 1. Orf lesions on scarified thigh : positive reactions with undiluted material only.

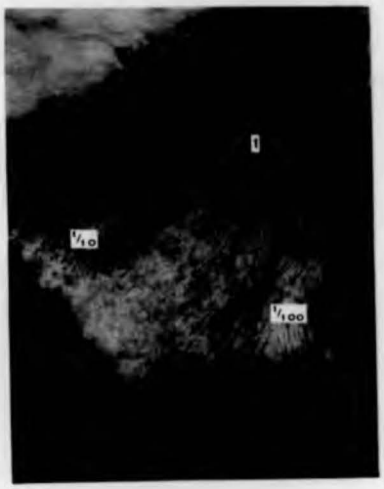
a) Score : 1

↑ - isolated lesions

b) Score : 2

↑ - confluent lesions

Figure 1. Orf lesions on scarified thigh : positive reactions with undiluted material only.



a) **Score : 1**

- isolated lesions

b) **Score : 2**

- confluent lesions

Figure 1. Orf lesions on scarified thigh : positive reactions with undiluted material only.

a) Score : 3

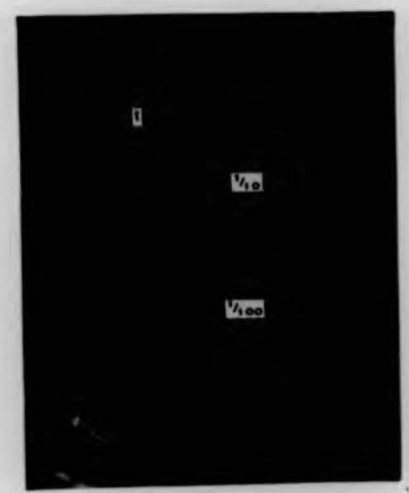
1 - isolated lesions

1/10 - isolated lesions

b) Score : 4

1 - multiple lesions

1/10 - isolated lesions



c) Score : 6

1 - confluent lesions

1/10 - confluent lesions

Figure 2. Orf lesions on scarified thigh : positive reactions with undiluted and 1/10 diluted material.

a) Score : 3

1 - isolated lesions

1/10 - isolated lesions

b) Score : 4

1 - multiple lesions

1/10 - isolated lesions

c) Score : 6

1 - confluent lesions

1/10 - confluent lesions

Figure 2. Orf lesions on scarified thigh : positive reactions with undiluted and 1/10 diluted material.

a) **Score : 3**

1 - isolated lesions

1/10 - isolated lesions

b) **Score : 4**

1 - multiple lesions

1/10 - isolated lesions

c) **Score : 6**

1 - confluent lesions

1/10 - confluent lesions

Figure 2. Orf lesions on scarified thigh : positive reactions with undiluted and 1/10 diluted material.

a) **Score : 7**

1 - isolated lesions

1/10 - isolated lesions

1/100 - isolated lesions

b) **Score : 8**

1 - confluent lesions

1/10 - isolated lesions

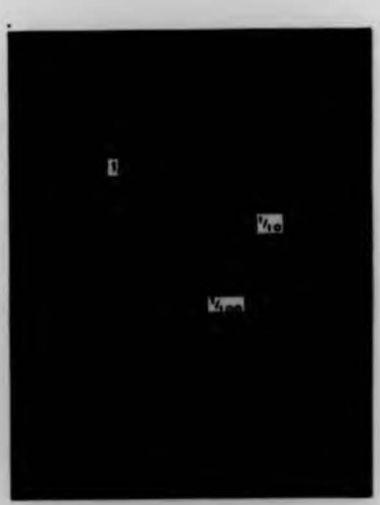
1/100 - isolated lesions

c) **Score : 10**

1 - confluent lesions

1/10 - confluent lesions

1/100 - isolated lesions



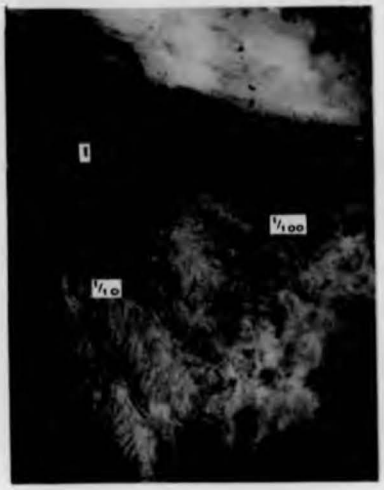
d) **Score : 14**

1 - confluent lesions

1/10 - confluent lesions

1/100 - confluent lesions

Figure 3. Orf lesions on scarified thigh : positive reactions with undiluted, 1/10 and 1/100 diluted material.



a) **Score : 7**

1 - isolated lesions

1/10 - isolated lesions

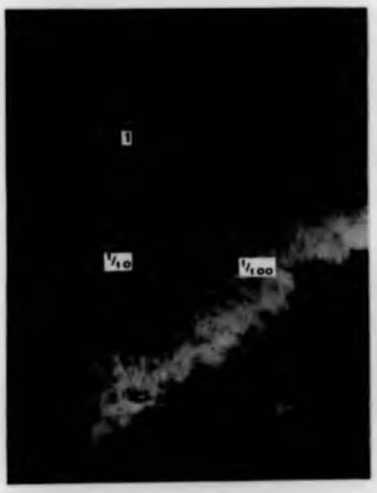
1/100 - isolated lesions

b) **Score : 8**

1 - confluent lesions

1/10 - isolated lesions

1/100 - isolated lesions

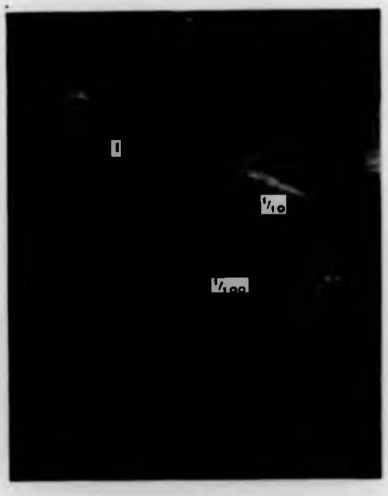


c) **Score : 10**

1 - confluent lesions

1/10 - confluent lesions

1/100 - isolated lesions



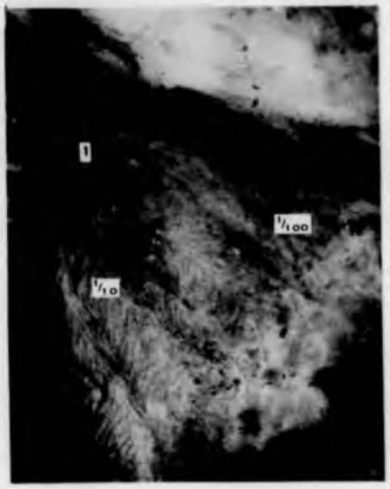
d) **Score : 14**

1 - confluent lesions

1/10 - confluent lesions

1/100 - confluent lesions

Figure 3. Orf lesions on scarified thigh : positive reactions with undiluted, 1/10 and 1/100 diluted material.

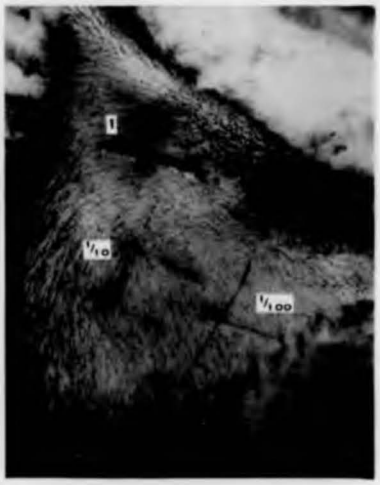


a) Score : 7

1 - isolated lesions

1/10 - isolated lesions

1/100 - isolated lesions

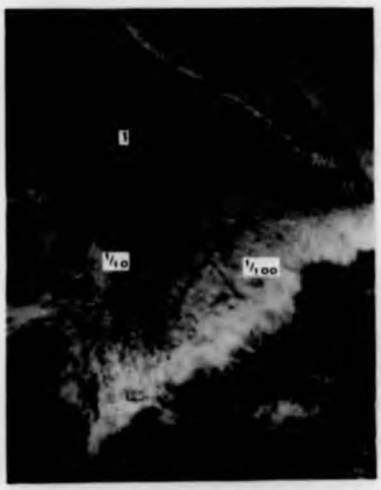


b) Score : 8

1 - confluent lesions

1/10 - isolated lesions

1/100 - isolated lesions



c) Score : 10

1 - confluent lesions

1/10 - confluent lesions

1/100 - isolated lesions

d) Score : 14

1 - confluent lesions

1/10 - confluent lesions

1/100 - confluent lesions

Figure 3. Orf lesions on scarified thigh : positive reactions with undiluted, 1/10 and 1/100 diluted material.

Electron microscopy

Suspensions of scab material mounted on carbon coated formvar grids and negatively stained with 2% phosphotungstic acid at pH 6.6 were examined for virus particles in a Philips EM300 microscope (Harkness, Scott and Hebert, 1977).

Tissue culture

Secondary calf testis monolayers were used throughout for the isolation of orf virus from scab material, orf virus assays and serum neutralization tests, and the propagation of orf, milker's nodule and bovine papular stomatitis viruses for use in agar-gel precipitin tests.

Testes were obtained from calves less than one week of age sent for slaughter at local abattoirs. Primary cell cultures were prepared according to the method of Ferris and Plowright (1958) except that cell suspensions were adjusted to contain approximately 1.8 million cells/cm^3 and the growth medium was slightly modified to contain 0.01% yeast extract and 10% fetal calf serum (Appendix I). Monolayers were prepared in Roux flasks, 20oz medical flats or 75cm^2 or 25cm^2 Falcon bottles (Becton-Dickinson and Co.) as required. Earle's saline was not used to replace the primary medium as secondary cell monolayers were prepared from the primary cell sheets as soon as these were confluent, usually after two to three days.

For the preparation of secondary calf testis cell cultures the growth medium was poured off the primary cell sheet and the monolayer rinsed with trypsin diluent (Appendix I). Sufficient trypsin/versene solution (Appendix I) to cover the monolayer was added, swirled rapidly over the culture, and discarded. The bottle was replaced in an incubator at 37°C for up to one minute when the whole monolayer was observed to have detached from the surface and the cells to have formed a

suspension in the residual fluid. Eagle's medium M.E.M. with 10% fetal calf serum (Appendix I) was used as growth medium for secondary cell cultures and 5-15ml was added to the trypsinised cells and thoroughly mixed with the cell suspension. Cell counts were made in an improved Neubauer counting chamber and the suspension diluted with growth medium so as to contain 200,000-400,000 cells/cm^3. This was distributed into Roux flasks (100ml), 20oz medical flats (40ml), 75cm^2 (30ml) or 25cm^2 (6ml) Falcon bottles or six-well, moulded polystyrene plates (3ml/well) (Dynatech Laboratories Ltd.) with or without a coverslip in the bottom, as required.

Cultures were incubated at 37°C in a conventional incubator or, in the case of the six-well plates, in an atmosphere of 5% CO_2 in a carbon dioxide incubator. Confluent monolayers were obtained in two to three days and were maintained by replacing the growth medium with Eagle's medium M.E.M. supplemented with 0.5-2% fetal calf serum (Appendix I) depending upon how well the cells had grown.

Virus propagation

a) Isolation from scab material: The method for isolating orf virus strain CVL from scab material was similar to that described by Plowright, Witcomb and Ferris (1959). A 10% w/v suspension of scab material was prepared by grinding with pestle and mortar a weighed amount of scab with an appropriate volume of tissue culture maintenance medium. The suspension was clarified by centrifuging at 2000 r.p.m. (700G) (MSE Super Minor) for 10 minutes and 0.5-1.0ml of the supernatant inoculated into bottles of newly confluent secondary calf testis monolayers. The inoculum was allowed to adsorb for one hour at 37°C before adding further maintenance medium.

After 24 hours of incubation the cell cultures showed non-specific cytopathic changes and the medium was therefore discarded, the cultures washed with medium and fresh medium added. In this study, the CVL strain did not produce macroscopic cytopathic effects in monolayer cultures after incubation for a further six days but stained coverslip cultures initiated at the same time as the bottle cultures showed the presence of characteristic intracytoplasmic inclusions (Plowright, Witcomb and Ferris, 1959). The bottle cultures were therefore subjected to two freeze/thaw treatments at -70°C for 30 minutes and the resulting cell suspensions clarified by low-speed centrifugation as before. The supernatant material was then centrifuged at 12,000 r.p.m. (18,000G) (MSE High Speed 25) at 4°C for one hour and the deposit resuspended in a small volume of maintenance medium for inoculation on to fresh cell cultures. This blind-passage procedure was repeated through five serial passages when specific macroscopic cytopathic changes started to appear after two to three days. Three further passages were carried out, omitting the high-speed centrifugation procedure, to establish the adaptation of the virus to growth in tissue culture. At this stage freeze/thawed, clarified culture fluids were distributed in bijou bottles and stored at -70°C until required for use.

b) <u>Propagation from freeze-dried state or culture fluids</u>: Freeze-dried cultures of orf, bovine papular stomatitis and milker's nodule viruses were reconstituted with a small volume of maintenance medium and inoculated on to secondary calf testis monolayers. Liquid cultures were inoculated directly on to similar cell cultures. Specific cytopathic effects, similar for all these viruses, developed after two to three days of incubation at 37°C. Virus in the form of clarified tissue culture fluid was harvested after freeze/thawing the infected monolayer cultures.

Virus titration

Orf virus preparations were titrated using the plaque assay method developed for use in the present study (Appendix II).

Newly confluent monolayers of secondary calf testis cells were propagated in six-well, polystyrene plates and 0.1ml of virus preparation inoculated onto the centre of each of the six cell cultures. After allowing adsorption for one hour at 37°C, each of the wells was overlaid with 3ml sodium carboxymethyl cellulose (SCMC) overlay medium (Appendix I) and the plate placed in a carbon dioxide incubator at 37°C.

Plates were incubated for five to seven days when the overlay medium was carefully removed by pipette, the cell sheets fixed for 10 minutes with 70% industrial methylated spirits and stained for 20 minutes with 1/10 Giemsa. After rinsing in tap water the plates were allowed to air dry and plaques identified and counted with the aid of a microfilm reader (Carl Zeiss Jena DLII).

Virus neutralization tests

Serum neutralizing antibodies were assayed using an *in vitro* plaque-reduction test.

Orf virus strain EB in the form of clarified, infective tissue culture fluid was used throughout as the test virus. Each batch of virus was assayed as described above and then adjusted to contain an estimated 100–300 plaque-forming units per 0.05ml by dilution with maintenance medium prior to use in the neutralization test.

All sera to be tested were diluted 1 in 5 in tissue culture maintenance medium and inactivated by heating at 56°C for 30 minutes. One volume of diluted, inactivated serum was mixed with an equal volume of test virus and left to neutralize for approximately 72 hrs at 4°C. This neutralisation time and temperature was adopted as it was convenient from a practical point of view and a comparative study had shown it to be

as suitable as 18 hours at 4°C or 2 hours at 37°C (Appendix III). At the end of the neutralization period, 0.1ml volumes of the serum-virus mixture were inoculated onto the centre of newly confluent monolayers of secondary calf testis cells propagated in six-well polystyrene plates. At least six wells were inoculated with each mixture, the usual procedure being to inoculate one well in each of six different plates. In this way a statistically valid plaque count could usually be obtained even when some cell cultures were lost through contamination or other causes during the assay period. The serum-virus inocula were allowed to adsorb for one hour at 37°C before adding 3ml SCMC overlay medium to each well.

Plates were incubated for five to seven days when the cell sheets were fixed and stained and plaques counted as previously described.

Precipitin tests

The double diffusion in two dimensions procedure (Ouchterlony, 1967) was used to examine sheep serum samples for the presence of precipitating antibodies and for antigenic relationship studies with selected poxviruses.

Tests were carried out in 9cm diameter plastic petri dishes containing 15ml of 1% w/v agarose A (Pharmacia Fine Chemicals) in phosphate buffered saline (pH 7.2) with 0.5% phenol added as preservative. Agarose A was preferred to Noble agar (Difco Laboratories) or Agarose for electrophoresis (British Drug Houses) as it was found to form the clearest gel layer. The 15ml volume was also found to be the most suitable as larger volumes resulted in slight distortions of any precipitin lines which developed and smaller volumes produced weaker reactions.

Wells were cut in the solidified gel using a template providing 7mm diameter holes at a distance of 5mm apart after preliminary experiments had shown this configuration to give more satisfactory results than smaller wells at either greater or lesser distances apart.

Antigen preparations for all parapoxviruses and Shope fibroma virus consisted of clarified and concentrated infective tissue culture fluids. Concentration was found to be an essential procedure as repeated tests of unconcentrated material failed to give readable precipitin reactions. The principle of ultrafiltration using an Immersible Molecular Separator (Millipore Corporation) fitted with a Pellicon type membrane with a nominal molecular weight cut-off level of 10,000 was used to concentrate material. This unit was immersed in the culture fluid and a vacuum applied by connecting it to a 20ml Vacutainer (Becton-Dickinson and Co.). Concentration was allowed to take place at room temperature or 4°C, replacing the Vacutainer when necessary, until a 30-50-fold degree of concentration had been achieved. This procedure took up to three days to complete when large volumes (± 500ml) were processed. Faster rates of filtration could have been achieved with a positive pressure method of concentration but as infective material was being handled the gentler and safer, albeit slower, method of vacuum filtration was preferred. Best results were obtained when culture fluids from cell cultures showing extensive cytopathic effect were concentrated without delay and the test carried out immediately after the concentration procedure had been completed. Almost equally good results were obtained with fluid stored at -70°C but storage at room temperature or 4°C appeared to have a marked adverse effect on antigen quality.

Vaccinia, cowpox and fowl pox antigens used were rabbit skin lesion or chorio-allantoic membrane suspensions.

Although precipitin reaction lines were often seen within 24 hours of starting the test, many weaker reactions took some days to appear. Consequently all plates were allowed to develop for seven days in a humid atmosphere at room temperature before the result was finally assessed.

RESULTS

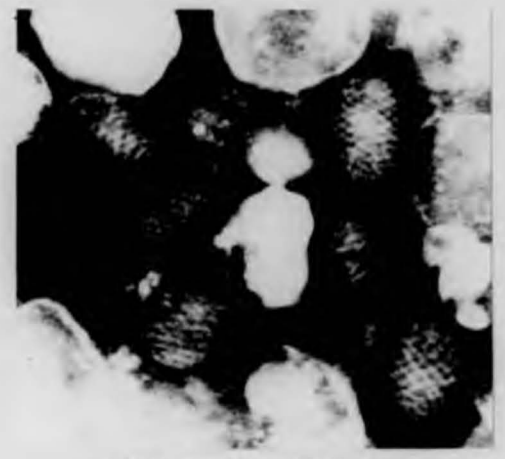
1. Clinical disease in naturally infected animals:

Lesions characteristic of orf virus infection were first observed during a routine clinical examination of the group of 44 sheep three weeks after their arrival at the laboratory. Diagnosis was confirmed by examination of scab material under the electron microscope which revealed the presence of typical orf virus particles (Fig. 4.). The sheep had been randomly divided into four groups of 11 animals each and housed in four adjacent loose-boxes but the disease was initially limited to boxes 2 and 4 only, with the animals in boxes 1 and 3 showing no obvious lesions.

The occurrence of macroscopic lesions in the sheep in boxes 2 and 4 is shown in Table II. Not all animals showed clinical evidence of disease when first examined. One animal in box 2 (no.26) developed lesions four weeks later but three animals in box 4 (nos. 34, 39 and 41) never developed macroscopic lesions during the following three months of observation.

Although the occasional animal in boxes 1 and 3 exhibited lesions suggestive of orf during the outbreak in boxes 2 and 4, typical lesions affecting the majority of sheep in boxes 1 and 3 did not develop until five weeks after the disease was first diagnosed. At this time almost all lesions in the originally infected animals had resolved completely. The occurrence of macroscopic lesions in the sheep in boxes 1 and 3 is shown in Table III. As was observed with the animals in boxes 2 and 4, not every sheep developed lesions, two animals in one box (nos. 8 and 9) and four in the other (nos. 13, 16, 20 and 22) remaining clinically unaffected during the following two months of observation.

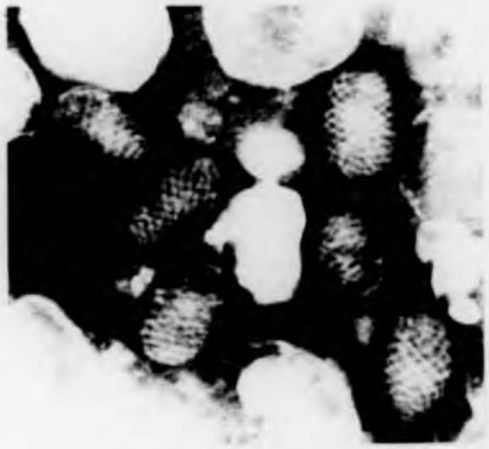
Clinically, the disease followed a very similar course in all 35 affected animals. Lesions developed at one or both commissures of the lips in almost all cases, but in some instances were so small that some



Magnification : x 62,500

Magnification : x 100,000

Figure 4. Crf virus particles



Magnification : x 62,500

Magnification : x 100,000

Figure 4. Orf virus particles

Table II

Occurrence of orf lesions in sheep in boxes 3 (sheep 33-35) and 4 (sheep 34-44).

Sheep	Weeks after arrival at laboratory						
	3	4	5	6	7	8	9
23	.	X	.	.	.	.	.
24	X	.	.	.	X	X	.
25	X	X	X	X	X	.	.
26	.	.	.	.	X	X	.
27	X	.	.	.	.	.	.
28	X	X	.	.	.	.	.
29	X	X	.	.	.	.	.
30	X	.	.	.	.	.	.
31	X	X	X	X	.	.	.
32	X	X	X	X	.	.	.
33	X	X	X	X	X	.	.
34	.	.	.	.	.	.	.
35	X	X	.	.	.	.	.
36	X	X	.	.	.	.	.
37	X	X	.	.	.	.	.
38	X	.	.	.	.	.	.
39	.	.	.	.	.	.	.
40	X	X	.	.	.	.	.
41	.	.	.	.	.	.	.
42	X	.	.	.	.	.	.
43	X	X	.	.	.	.	.
44	X	X	.	.	.	.	.

X = observable lesion

. = no visible lesion

Table III

Occurrence of orf lesions in sheep in boxes 1 (sheep 1-11) and 3 (sheep 12-22)

Sheep	Weeks after arrival at laboratory									
	3	4	5	6	7	8	9	10	11	12
1	.	X	.	.	.	.	X	.	.	.
2	.	.	.	.	.	X	X	X	X	.
3	.	.	.	.	.	X	X	X	.	.
4	.	.	.	.	.	.	X	.	.	.
5	.	.	.	.	.	.	X	X	X	.
6	.	.	.	.	X	X	.	.	.	.
7	.	X	.	.	.	.	X	X	X	.
8	.	.	.	.	.	.	.	.	.	.
9	.	.	.	.	.	.	.	.	.	.
10	.	.	.	.	.	.	.	X	X	.
11	.	.	X	.	.	X	X	.	.	.
12	.	.	.	.	.	.	.	X	.	.
13	.	.	.	.	.	.	.	.	.	.
14	.	.	.	.	.	X	X	X	.	.
15	.	.	X	.	.	X	X	.	.	.
16	.	.	.	.	.	.	.	.	.	.
17	.	.	.	.	.	X	X	X	.	.
18	.	.	.	.	.	X	X	.	.	.
19	.	.	.	.	.	X	X	.	.	.
20	.	.	.	.	.	.	.	.	.	.
21	.	.	.	.	.	X	X	.	.	.
22	.	.	.	.	.	.	.	.	.	.

X = observable lesion

. = no visible lesion

doubt existed as to whether they were indicative of specific infection. Extension of lesions along the lips from the commissures occurred in approximately 25% of cases and the occasional animal also developed lesions on the muzzle, nose, eye or ear. No feet or genital lesions were ever observed. The sites of lesions on the individual animals are shown in Table IV.

In general, the course of the disease was short lasting. Very few animals had observable lesions for more than three weeks with the maximum period being five weeks in two cases (nos. 25 and 33).

Table IV

Sites of lesions in orf infected sheep

Sheep	Site	Sheep	Site
1	Lc.	23	Lc.
2	Lc.	24	Lc, lip
3	Lc, Rc, lips, muzzle.	25	Lc, Rc.
4	lip, nose.	26	Lc, Rc.
5	Lc, Rc.	27	Rc.
6	Lc.	28	Lc, Rc, lips.
7	Lc, Rc.	29	Lc, Rc, lips, muzzle.
8	-	30	Lc, Rc.
9	-	31	Lc.
10	Lc, lip.	32	Lc, Rc.
11	Lc, Rc.	33	Lc, Rc, lips.
12	nose.	34	-
13	-	35	Lc, Rc, nose.
14	Rc, nose.	36	Lc, Rc, lips.
15	Rc.	37	Rc.
16	-	38	Rc, nose.
17	Lc, Rc.	39	-
18	Lc, Rc, lips, ear.	40	Lc, Rc.
19	Lc, eye.	41	-
20	-	42	Lc.
21	Rc.	43	Lc, Rc.
22	-	44	Lc.

Lc. = left commissure.

Rc. = right commissure.

2. Resistance to reinfection of naturally infected animals:

2.1. Resistance at predilection sites

Three months after recovery from the outbreak of orf, five animals from the infected flock together with two control sheep were challenged by scarification on both sides of the mouth with a tissue culture suspension of strain EB of orf virus. Results are shown in Table V. Three sheep, including one which had not originally developed clinical disease (no.13), appeared to be solidly resistant to mouth reinfection. A single pustule was observed to have developed in one animal and a pustule and scab in another. In the latter case, the scabby lesion developed on the same side of the mouth as was originally affected. In both animals however, the lesions were neither as severe nor as persistent as in the control sheep.

Eleven months after recovery from the outbreak, a further five sheep were similarly challenged on the mouth with the same virus preparation. In this case, five 2-3 week old lambs, born to five other ewes in the flock, were used as controls. The results are shown in Table VI. Overall, the reaction of the animals was very similar to that observed with the three-month post-recovery group. Two animals appeared solidly resistant; two animals, both of which had not originally exhibited clinical infection (nos. 8 and 22), developed only the mildest of lesions and the remaining animal developed lesions which were markedly less severe and less persistent than in the control lambs.

The lambs used as controls continued to suckle their dams with no apparent difficulty or loss of condition throughout the period of infection. Four of the five dams, all of which had developed mouth lesions during the initial outbreak of disease, never developed any udder lesions during the time the lambs were infected. The fifth animal, the mother of the oldest and most severely affected lamb

(no.533), exhibited 3–4 ruptured vesicles on the udder and a single pustule at the base of one teat two weeks after the lamb had been infected but these lesions did not progress and resolved completely within one week.

Table V

Occurrence of mouth lesions following challenge three months after clinical recovery from natural orf

Sheep		Weeks following challenge		
		1	2	3
2	R	–	–	–
	L	–	–	–
7	R	–	–	–
	L	pustule	–	–
9	R	–	–	–
	L	–	–	–
13	R	–	–	–
	L	–	–	–
19	R	pustule	–	–
	L	scab	–	–
561 (control)	R	scab	extensive scab	scab
	L	scab	extensive scab	scab
569 (control)	R	scab	scab	–
	L	scab	scab	small scab

R = right side of mouth
L = left side of mouth
– = no specific lesion

Table VI

Occurrence of mouth lesions following challenge

11 months after clinical recovery from natural orf

Sheep		Weeks following challenge 1	2	3
5	R	–	–	–
	L	–	–	–
8	R	–	–	–
	L	scab	–	–
14	R	scab	small scab	–
	L	erosion	small scab	–
21	R	–	–	–
	L	–	–	–
22	R	erosion	–	–
	L	erosion	–	–
Lambs (controls)				
533	R	scab	extensive	small scab
	L	scab	spreading scab	small scab
537	R	scab	scab	small scab
	L	scab	scab	small scab
568	R	scab	extensive scab	small scab
	L	scab	extensive scab	small scab
569	R	scab	extensive scab	small scab
	L	scab	extensive scab	small scab
570	R	scab	extensive scab	–
	L	scab	extensive scab	–

R = right side of mouth
L = left side of mouth

– = no specific lesion

2.2. Resistance to thigh infection

One month after recovery from the outbreak of orf, five sheep from the infected flock together with two control animals were challenged on the inner thighs with undiluted, 1/10 and 1/100 dilutions of a tissue culture preparation of strain EB and Vaccine A. Lesions were examined and scored seven days later. The results are shown in Table VII. The scores are not intended to give a quantitative estimate of the immune status of any individual animal but can be used to assess relative degrees of immunity. Thus, four of the five recovered animals appeared to be more resistant than a control animal to challenge with the EB virus but a similar difference was not observed when Vaccine A was used as challenge. Also, animals 31 and 41 appeared to be the most resistant of the group to EB virus infection. Interestingly, sheep 41 was the one animal in the group which had not shown earlier clinical infection. It was also found that the lesions on the control animals took approximately three weeks to resolve compared with two weeks for the animals from the infected flock.

Three months after clinical recovery, five further sheep from the infected flock and two control animals were challenged in the same way as the previous group using the same preparations of strain EB and Vaccine A. In addition, the five animals used to assess mouth resistance three months after infection were challenged on the thigh at the same time as on the mouth, again using the same preparation of EB virus. The responses of both these groups (1 and 2 respectively) to the thigh infections are shown in Table VIII. The overall impression was that susceptibility to the EB virus had increased slightly compared with the one-month post-recovery test whereas an increased resistance to Vaccine A seemed to have developed although this might possibly have been due to a loss of infectivity of the vaccine with storage. In

Table VII

Thigh lesions following challenge one month after clinical recovery from natural orf

Sheep	Strain EB				Vaccine A			
	10^{0}	10^{-1}	10^{-2}	Score	10^{0}	10^{-1}	10^{-2}	Score
23	+	+	-	3	+	+	+	7
27	+	+	-	3	+	+	+	8
31	+	-	-	1	+	+	+	7
41	+	-	-	2	+	+	+	8
43	+	+	+	8	+	+	+	8
462 (control)	Not done				+	+	+	10
565 (control)	+	+	+	8	Not done			

+ = specific orf lesion(s)
- = no specific lesion

Table VIII

Thigh lesions following challenge three months after clinical recovery from natural orf

Sheep	Strain EB				Vaccine A			
	10^{0}	10^{-1}	10^{-2}	Score	10^{0}	10^{-1}	10^{-2}	Score
Group 1								
29	+	+	-	4	+	+	-	6
32	+	+	-	3	+	+	-	4
34	+	+	+	8	+	+	+	10
35	+	+	-	6	+	+	-	4
36	+	+	-	3	+	+	-	4
518 (control)	+	+	-	6	+	+	+	14
544 (control)	+	+	+	10	+	+	+	10
Group 2								
2	+	+	+	10	Not done			
7	+	+	-	4				
9	+	+	-	3				
13	+	+	-	6				
19	+	+	+	8				
561 (control)	+	+	+	10				
569 (control)	+	+	+	10				

+ = specific orf lesion(s)
- = no specific lesion

group 1, the animal which had not developed earlier clinical lesions (no.34) appeared marginally more susceptible to infection than the other sheep in the group but this was not observed in group 2 in which sheep 9 and 13 had not developed earlier clinical disease.

As had been done in the three-month post-recovery test, the group of sheep used to study mouth resistance 11 months after recovery were challenged on the thigh at the same time as on the mouth. Again, the same preparation of EB virus was used as before. The results are shown in Table IX. As was observed with the three-month post-recovery test, susceptibility to the EB virus appeared to have increased with time and although no control animals were available for inclusion in this test, comparison with the control animal results with the same virus preparation in the two previous tests would suggest that at 11 months post-recovery, sheep may have become as susceptible to thigh infection as previously non-infected animals.

Table IX

Thigh lesions following challenge 11 months after clinical recovery from natural orf

Sheep	Strain EB			
	10^{0}	10^{-1}	10^{-2}	Score
5	+	+	+	10
8	+	+	+	10
14	+	+	+	8
21	+	+	+	10
22	+	+	+	10

+ = specific orf lesions(s)
- = no specific lesion

3. Clinical response of sheep to vaccination/experimental infection:

Vaccination/experimental infection studies were carried out using seven- to nine-months-old laboratory bred sheep known to have been free from any orf infection prior to use. Infections were limited to scarified areas of the inner thigh and as all test preparations consisted of suspensions of live orf virus, the term "vaccination" was adopted to describe experimental infection with laboratory prepared virus suspensions as well as the application of conventional vaccines.

The preparations used were two commercially produced vaccines, A and B, and clarified tissue culture suspensions of strains EB and ET of orf virus.

One sheep was used for each virus preparation and the vaccination schedule used in each case was as follows:-

Vaccination	Dose(s)	Site
First	Neat, 1/10 and 1/100 dilutions	Right thigh
Second	Neat, 1/10 and 1/100 dilutions	Left thigh
Third	Neat only	Right thigh

An interval of exactly four weeks separated each vaccination. The animals were examined weekly and lesions recorded. The results obtained seven days after vaccination are shown in Table X. The scoring system used in the reinfection studies with the naturally infected flock (Section 2.2) was not used in these vaccination experiments as comparisons were made of the response of one vaccinated animal with itself at successive points in time, whereas with the infected flock a comparison was made between different animals at a single point in time.

Two distinct patterns of response were observed. Vaccine A, gave rise to specific lesions at all dilutions when first used but no lesions

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Two distinct patterns of response were observed. Vaccine A, gave rise to specific lesions at all dilutions when first used but no lesions

developed following either the second or third vaccinations. On the other hand, Vaccine B gave rise to specific lesions on all three occasions, although not with the 1/100 dilution when used initially. The EB and ET preparations gave a response pattern similar to Vaccine B but both preparations appeared less potent than either commercial product. The absence of lesions following the second vaccination with strain ET must be of doubtful significance as only the undiluted material induced lesions initially.

Table X

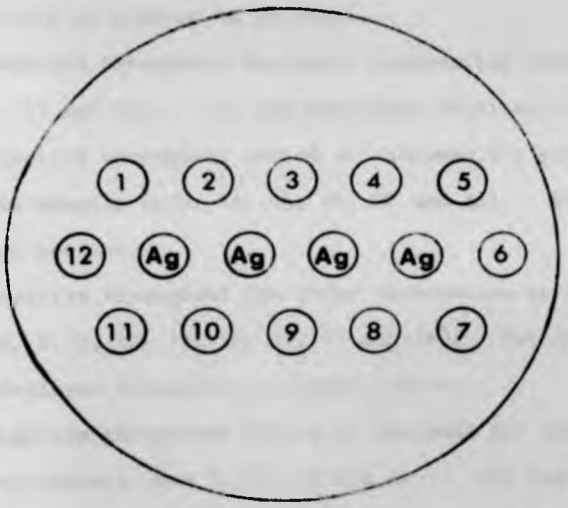
Clinical response of sheep to vaccination

Virus Preparation	Dose response of sheep						
	1st vaccination			2nd vaccination			3rd vaccination
	10^{0}	10^{-1}	10^{-2}	10^{0}	10^{-1}	10^{-2}	10^{0}
Vaccine A	+	+	+	-	-	-	-
Vaccine B	+	+	-	+	+	+	+
EB	+	+	-	+	+	-	+
ET	+	-	-	-	-	-	+

+ specific orf lesion(s) present
- no specific lesion

4. Precipitating antibodies in orf infections:

All serum samples obtained from the naturally and experimentally infected sheep used in this study were tested for the presence of precipitating antibodies. The basic pattern of wells used is shown below and was selected to permit comparisons to be made between serial serum samples from an individual animal. The inner wells contained the antigen preparation and the surrounding wells were used for the serum samples. The first serum sample obtained from each animal was placed in the top left-hand well of the plate and subsequent samples placed in sequential order in a clockwise direction from the first sample.



Key: Ag - antigen preparation

1 - 12 - serum samples

Well pattern for gel diffusion precipitin tests.

4.1. Natural orf infection

A single batch of antigen prepared from cultures infected with strain EB of orf virus was used throughout.

Positive reactions were normally seen as single, rather weak lines of precipitation (Fig 5(a)) but a few serum samples from some animals produced a second, even weaker, precipitin reaction line towards the serum well side of the plate (Fig 5(b)). The results obtained for the naturally infected flock of animals are shown in Table XI, which is also annotated to show when orf lesions were present at the time of blood sampling.

The response of the 27 animals which survived the nine month observation period, and which were not subjected to an intervening experimental infection, could be grouped as follows:-

i) Negative throughout the whole observation period - four animals (nos. 3, 25, 37 and 40). All had developed clinical lesions.

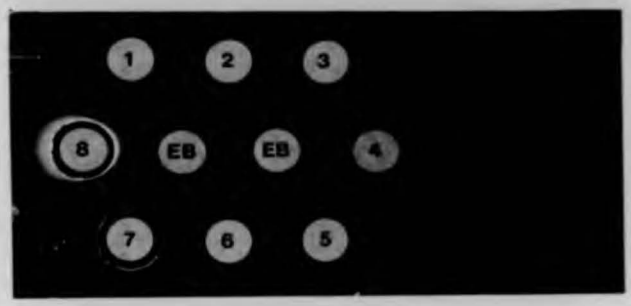
ii) Negative throughout period of outbreak but positive at nine months - five animals (nos. 14, 15, 18, 22 and 44). All except sheep 22 had developed lesions.

iii) Positive throughout the whole observation period - eight animals (nos. 6, 8, 10, 11, 16, 17, 21 and 26). Two animals (nos. 8 and 16) never developed clinically observed lesions.

iv) Positive throughout period of outbreak but negative at nine months - four animals (nos 5, 24, 28 and 30). All had developed lesions.

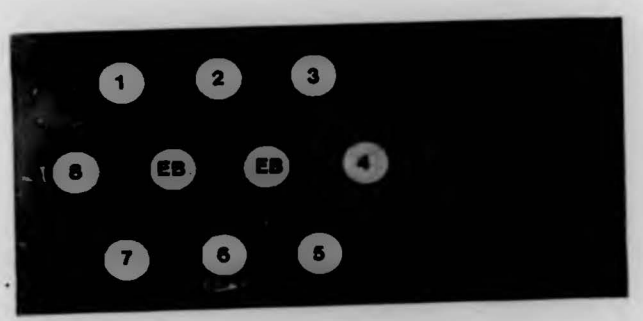
v) Variable positive and negative response - six animals (nos. 4, 12, 20, 33, 38 and 42). All except sheep 20 had developed lesions.

In the five animals in which a double precipitin line was observed (nos. 1, 5, 8, 21 and 31), the second line appeared in samples taken during the outbreak of disease in the flock but did not persist longer than one month. The animal in which the second line persisted longest (no. 8) was the one animal in the group which did not develop clinical disease.



a) (i) Sheep 6 - single precipitin lines only

(ii) Sheep 23 - single precipitin lines only



b) Sheep 8 - single and double precipitin lines

Key: EB - antigen preparation
1-10 - serial serum samples

Figure 5. Gel diffusion with orf virus strain EB

a) (i) Sheep 6 - single precipitin lines only

(ii) Sheep 23 - single precipitin lines only

b) Sheep 8 - single and double precipitin lines

Key: EB - antigen preparation
1-10 - serial serum samples

Figure 5. Gel diffusion with orf virus strain EB

Table XI

Occurrence of precipitating antibodies in naturally infected sheep

Sheep	Weeks after arrival at laboratory											
	3	4	5	6	8	9	10	11	8	20	25	30
1	-	+ (L)			+	+ (L)	+	+	+			Dead
2	-	-			- (L)	- (L)	- (L)	- (L)	-			- Chal Dengue
3	-	-			- (L)	- (L)	- (L)	-	-			-
4	-	-			-	+ (L)	+	+	-			-
5	-	-			+	+ (L)	+ (L)	+ (L)	-			-
6	-	-			+ (L)	+	+	+	+			-
7	-	+ (L)			+	+ (L)	+ (L)	+ (L)	-			- Chal Dengue
8	-	+			+	+	+	+	-			-
9	-	-			+	+	+	+	-			- Chal Dengue
10	-	+			+	+	+ (L)	+ (L)	-			+
11	-	+	(L)		+ (L)	+ (L)	+	+	-			+
12	-	-			+	+	- (L)	-	-			-
13	-	-			+	+	+	+	-			- Chal Dengue
14	-	-			- (L)	- (L)	- (L)	-	-			-
15	-	-	(L)		- (L)	- (L)	-	-	-			-
16	-	-			+	+	+	+	-			+
17	-	+			+ (L)	+ (L)	+ (L)	+	-			-
18	-	-			+ (L)	+ (L)	-	-	-			-
19	-	-			+ (L)	+ (L)	-	-	-			- Chal Dengue
20	-	-			+	+	+	+	-			+
21	-	-			+ (L)	+ (L)	+	+	-			-
22	-	-			-	-	-	-	-			+
23	+	+ (L)	+	-			- Chal Dengue					-
24	+ (L)	+	+	-	(L)		-					+
25	- (L)	- (L)	- (L)	- (L)			-					+
26	+	+	+	-	(L)		-					+
27	+ (L)	+	+	-			- Chal Dengue					-
28	+ (L)	+ (L)	+	-			+					-
29	- (L)	- (L)	-	-			+				- Chal Dengue	
30	+ (L)	+	+	-			+					+
31	+ (L)	+ (L)	+ (L)	- (L)			+					- Chal Dengue
32	- (L)	- (L)	- (L)	- (L)			+				- Chal Dengue	
33	- (L)	- (L)	- (L)	- (L)			+					+
34		+	+	-			+				- Chal Dengue	
35		- (L)	-	-			+				- Chal Dengue	
36		- (L)	-	-			+				- Chal Dengue	
37		(L)	-	-			+					-
38		- (L)	-	-			+					+
39		+ (L)	+	-			+					+
40		-	+	-			+					-
41		- (L)	- (L)	-			+					+
42		-	+	-			+ Chal Dengue					
43		+ (L)	+	-			+					+
44		- (L)	- (L)	-			+ Chal Dengue					
45		- (L)	- (L)	-			-					+

+ = double precipitation line
(L) = single precipitation line
- = no precipitation line
Chal Dengue = challenge with dengue virus

Table XI

Occurrence of precipitating antibodies in naturally infected sheep

Sheep	Weeks after arrival at laboratory											
	3	4	5	6	8	9	10	11	15	20	25	40
1	+	+ (L)			‡	‡ (L)	‡	‡	+			Dead
2	-	-			- (L)	- (L)	- (L)	- (L)	-		- Challenged	
3	-	-			- (L)	- (L)	- (L)	-	-			-
4	-	-			-	+ (L)	+	+	+			-
5	+	+			+	+ (L)	‡ (L)	‡ (L)	+			-
6	+	+			+ (L)	+	+	+	+			+
7	+	+ (L)			+	+ (L)	+ (L)	+ (L)	+		+ Challenged	
8	+	‡			‡	‡	‡	+	+			+
9	+	+			+	+	+	+	+		+ Challenged	
10	+	+			+	+	+ (L)	+ (L)	+			+
11	+	+	(L)		+ (L)	+ (L)	+	+	+			+
12	-	-			+	+	+ (L)	+	-			+
13	+	+			+	+	+	+	+		+ Challenged	
14	-	-			- (L)	- (L)	- (L)	-	-			+
15	-	-	(L)		- (L)	- (L)	-	-	-			+
16	+	+			+	+	+	+	+			+
17	+	+			+ (L)	+ (L)	+ (L)	+	+			+
18	-	-			- (L)	- (L)	-	-	-			+
19	+	+			+ (L)	+ (L)	+	+	+		+ Challenged	
20	-	+			+	+	+	+	+			-
21	+	+			‡ (L)	‡ (L)	‡	+	+			+
22	-	-			-	-	-	-	-			+
23	+	+ (L)	+	+			+ Challenged					
24	+ (L)	+	+	+		(L)	+					-
25	- (L)	- (L)	- (L)	- (L)			-					-
26	+	+	+	+		(L)	+					+
27	+ (L)	+	+	+			+ Challenged					
28	+ (L)	+ (L)	+	+			+					-
29	- (L)	- (L)	-	-			+			+ Challenged		
30	+ (L)	+	+	+			+					-
31	+ (L)	‡ (L)	‡ (L)	+ (L)			+ Challenged					
32	- (L)	- (L)	- (L)	- (L)			-			+ Challenged		
33	- (L)	- (L)	- (L)	+ (L)			-					-
34	-	+	+	+			+			+ Challenged		
35	- (L)	- (L)	-	-			-			+ Challenged		
36	- (L)	- (L)	-	-			-			- Challenged		
37	- (L)	- (L)	-	-			-					-
38	- (L)	-	+	+			+					-
39	-	-	+	+			+					+
40	- (L)	- (L)	-	-			-					-
41	-	+	+	+			+ Challenged					
42	+ (L)	+	+	-			-					+
43	- (L)	- (L)	-	-			+ Challenged					
44	- (L)	- (L)	-	-			+					+

KEY

‡ double precipitin line
+ single precipitin line
- no precipitin line
(L) orf lesion present

4.2. Experimental reinfection following natural infection

During the nine month monitoring period, 15 animals from the flock were used to study the effect of experimental reinfection with orf virus. Five animals were subjected to simultaneous thigh infections with strain EB and Vaccine A one month after recovery, a further five animals were similarly challenged three months after recovery and five animals were challenged on the mouth and one thigh with strain EB only, also three months after recovery. An account of the clinical observations following the reinfection of these animals has been given earlier (Section 2.2.) and the precipitating antibody response prior to reinfection is recorded in Table XI. The antibody response following reinfection is shown in Table XII.

The two animals with no demonstrable antibody at the time of reinfection (nos. 2 and 36) both became seropositive within two weeks of reinfection. Most of the remaining animals, all seropositive at the time of reinfection, showed no change in antibody status throughout the subsequent monitoring period although a double precipitin line was observed to develop in two instances (nos. 31 and 35). Two animals (nos. 31 and 32) also became seronegative sometime later and one animal (no.29) was atypical in that it became seronegative within two weeks of reinfection but was found to be seropositive once more when tested four months later.

4.3. Effect of different antigen preparations on precipitin test results

To study whether different orf virus preparations might affect the result of gel diffusion precipitin tests, all serum samples from nine sheep in the flock were tested against the CVL strain of orf virus in the same way as had been done for the whole flock using the EB strain.

Examples of the gel diffusion patterns obtained with the CVL strain are shown in Fig. 6 and these may be compared with the patterns obtained from the same three animals with strain EB (Fig 5).

Table XII

Occurrence of precipitating antibodies in sheep experimentally reinfected following recovery from natural orf

Sheep	Time since recovery	Site of reinfection	Weeks after reinfection						
			0	1	2	3	4	20	30
23	1 month	Thigh	+	+	+	+	+		+
27	"	"	+	+	+	+	+		-
31	"	"	+	+	‡	‡	‡		-
41*	"	"	+	+	+	+	+		+
43	"	"	+	+	+	+	+		+
29	3 months	Thigh	+	+	-	-	-	+	
32	"	"	+	+	+	+	+	-	
34*	"	"	+	+	+	+	+	+	
35	"	"	+	+	‡	‡	‡	+	
36	"	"	-	+	+	+	+	+	
2	3 months	Mouth & Thigh	-	-	+	+	+		
7	"	"	+	+	+	+	+		
9*	"	"	+	+	+	+	+		
13*	"	"	+	+	+	+	+		
19	"	"	+	+(L)	+	+	+		

*denotes animals which did not develop clinical lesions during disease outbreak

‡double precipitin line

+single precipitin line

-no precipitin line

(L) orf lesion present following mouth reinfection

(i) **Sheep 6** - single precipitin lines only

(ii) **Sheep 23** - single, double and triple precipitin lines

(iii) **Sheep 8** - single precipitin lines only

Key: CVL - antigen preparation
1-10 - serial serum samples

Figure 6. Gel diffusion with orf virus strain CVL

(i) **Sheep 6** - single precipitin lines only

(ii) **Sheep 23** - single, double and triple precipitin lines

(iii) **Sheep 8** - single precipitin lines only

Key: CVL - antigen preparation
1-10 - serial serum samples

Figure 6. Gel diffusion with orf virus strain CVL

The main identifiable differences between the two sets of patterns are as follows:-

i) Intensity of precipitin lines.

ii) Occurrence of positive and negative reactions.

iii) Absence of double precipitin line with CVL antigen in the case of sheep 8 - a double line formed with EB antigen.

iv) Presence of double and triple precipitin lines with CVL antigen in the case of sheep 23 - only a single line formed with EB antigen.

v) The second precipitin line formed against CVL antigen appears to be towards the antigen well side of the plate - the opposite occurred with EB antigen.

The results of all gel diffusion tests with both EB and CVL antigens for the nine selected sheep are shown in Table XIII. Almost every possible combination of reactions was observed among the 75 serum samples tested and the frequency of occurrence of the different combinations was as follows:-

Precipitin reaction EB	CVL	No. of tests
-	-	10
+	+	21
‡	‡	4
‡	+	7
+	‡	10
+	‡	2
+	-	8
-	+	12
‡	-	1

Table XIII

Precipitating antibody responses using two different orf antigens (EB and CVL)

Sheep	Serum sample 1		2		3		4		5		6		7		8		9		10		11	
	EB	CVL	EB	CVL	EB	CVL	EB	CVL	EB	CVL	EB	CVL	EB	CVL	EB	CVL	EB	CVL	EB	CVL	EB	CVL
2	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	+	‡	+	‡
6	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	-						
8	+	-	‡	-	‡	+	‡	+	‡	+	+	+	+	+	+	-						
10	+	+	+	+	+	+	+	‡	+	‡	+	‡	+	‡	+	-						
21	+	-	+	+	‡	‡	‡	+	‡	+	+	+	+	‡	+	+						
23	+	+	+	+	+	+	+	+	+	‡	+	‡‡	+	+	+	‡	+	‡‡	+	-		
25	-	+	-	+	-	+	-	+	-	+	-	-										
31	+	+	‡	+	‡	+	+	+	+	+	+	‡	‡	‡	‡	‡	‡	‡	-	-		
40	-	+	-	+	-	+	-	+	-	-	-	-										

‡‡ triple precipitin line + single precipitin line

‡ double precipitin line - no precipitin line

Complete matching of results (EB − CVL − ; EB + CVL + or EB ‡ CVL ‡) was attained with only 35 serum samples. In a further 19 cases the sera gave positive reactions to both antigens, although the results were inconsistent in respect of the number of precipitin lines formed, but in 21 tests completely contradictory results were recorded (EB + CVL − or EB − CVL +).

All 75 sera were also tested against the FS 198 strain of milker's nodule virus. The results obtained using this antigen are shown in Table XIV.

Table XIV

Precipitating antibody response with milker's nodule antigen

Sheep	Serum sample										
	1	2	3	4	5	6	7	8	9	10	11
2	−	−	−	−	+	+	−	−	−	‡	‡
6	−	−	+	+	+	+	+	−			
8	−	−	‡	+	+	+	+	−			
10	+	+	+	+	+	+	−	−			
21	−	−	+	+	+	‡	+	+			
23	−	+	+	+	−	−	+	+	+	−	
25	+	+	+	+	+	−					
31	+	+	+	+	−	−	+	+	+	−	
40	+	+	+	+	−	−					

‡ double precipitin line

+ single precipitin line

− no precipitin line

Forty-eight of the serum samples gave positive reactions with the milker's nodule antigen. All these sera had also given a positive reaction with at least one of the orf antigens. Of the 27 samples which gave a negative reaction with the milker's nodule antigen, 10 were also negative with both orf antigens, but the remaining 17 had given positive results with one or both antigens. The relationship between the reactions obtained with the milker's nodule antigen and the two orf antigens can also be summarized as follows:-

	EB +	EB −	CVL +	CVL −
FS 198 +	37	11	46	2
FS 198 −	16	11	9	18

From this it can be seen that the reactions obtained with the FS 198 antigen corresponded more closely with the reactions obtained with the CVL antigen than with the EB antigen.

The appearance of a second precipitin line with milker's nodule antigen was much less frequent than with either of the orf virus antigens and was only observed in four instances as follows:-

Sheep	Serum	Antigen		
		EB	CVL	FS 198
2	10	+	‡	‡
2	11	+	‡	‡
8	3	‡	+	‡
21	6	+	+	‡

4.4. Vaccination/experimental infection

During the study of the clinical response of four sheep to vaccination/experimental infection (Section 3), serum samples were taken at weekly intervals. These were examined for the presence of precipitating antibodies at the same time as the sera from the naturally infected flock of animals using the same preparation of EB virus as antigen. The results obtained for the four vaccinates are shown in Table XV.

Table XV

Occurrence of precipitating antibodies in sheep following vaccination

Virus Preparation	Weeks after primary vaccination									
	1	2	3	4	Revaccination	5	6	7	8	Revaccination 9
Vaccine A	–	–	–	–		–	–	–	–	+
Vaccine B	–	–	–	–		+	+	+	+	+
EB	–	–	–	–		–	+	+	+	+
ET	–	–	–	–		–	+	+	+	+

+ single precipitin line
– no precipitin line

None of the animals were seropositive prior to vaccination and all remained seronegative during the four weeks following primary vaccination. Three animals developed precipitating antibody after the second vaccination but the remaining animal (Vaccine A) did not seroconvert until the third vaccination had been carried out. It was this latter animal that appeared on clinical evidence to develop the most marked degree of resistance following primary vaccination, failing to develop any lesions following the second or third vaccination procedures.

5. Serum neutralizing antibodies in orf infections

5.1. Natural orf infection

Serum samples obtained at various times from 20 animals in the naturally infected flock were tested for *in vitro* neutralizing antibody activity by a plaque-reduction assay. The plaque counts obtained for each assay are shown in Table XVI. Missing values for serum-virus mixtures were estimated wherever possible, using statistical methods, by comparison with other row (plate) and column (serum-virus mixture) values. Generally, this could only be done when not more than two values were missing from a row or a column. Missing values for the virus preparations were not estimated in the same way, but were simply taken as the mean value of the other virus counts obtained in the assay. All estimated values are shown in brackets.

Each serum-virus mixture count was expressed as a percentage of the virus count for that plate and the mean percentages computed (Appendix IV). For ease of comparison, each mean percentage value was tabulated to show when the serum samples were obtained and at what point some of the animals had been experimentally reinfected (Table XVII). The results were also divided into four groups, each group comprising the set of sera which was assayed during one of four separate working sessions.

It was initially intended to analyse each result to determine which sera were associated with the development of significantly reduced numbers of plaques when compared with the corresponding virus titrations. Such an analysis however gave totally illogical results. For example, all 29 serum-virus mixtures from the first group of animals gave lower plaque counts than the corresponding virus preparations whereas 16 of the 17 mixtures from the third group gave higher counts than the virus preparations. As the principal purpose of the study was to monitor the development of neutralizing antibodies over a period of time rather than

to determine actual antibody titres, the initial serum samples were thus used as reference preparations and the neutralizing activity of subsequent samples assessed relative to them.

Significant levels of neutralizing antibody were found to develop in 5 of the 11 animals not subjected to experimental reinfection during the 40-week observation period. In addition, 2 of the 9 reinfected animals developed significant antibody levels prior to reinfection.

The time taken for neutralizing antibodies to develop varied considerably. Although they appeared in four sheep 8 to 10 weeks after arrival at the laboratory (nos. 8, 14, 20 and 41), in the remaining animals they did not appear until 15 (no.18), 25 (no.2) or 40 (no.17) weeks after arrival. In most cases however, once antibodies had appeared, they tended to persist for the remainder of the observation period.

5.2. Experimental reinfection following natural infection

Nine of the 20 animals monitored for serum neutralizing antibody activity were subjected to experimental reinfection one or three months after recovery from natural infection (Section 2.2.). Neutralization test results for these animals are included in Tables XVI and XVII.

Of the three animals reinfected by thigh scarification one month after recovery, one developed a significant level of neutralizing antibody for the first time (no.31), one showed a boosting effect (no.41) and one showed no change (no.43).

In the group of animals reinfected by thigh scarification three months after recovery, sheep 36 developed significant neutralizing antibody activity but sheep 34 showed no change. The results for sheep 29 must be of doubtful significance due to the high number of missing values obtained in the plaque-reduction assay.

In the group of animals reinfected by both mouth and thigh scarification three months following recovery, a more uniform response was observed. The two animals which had not developed significant levels of neutralizing antibody prior to reinfection (nos.9 and 19), both became seropositive within two weeks and the third animal (no.2) showed a further rise in titre.

5.3. Vaccination/experimental infection

Selected serum samples obtained from the four sheep used in the vaccination/experimental infection study (Section 3) were assayed for neutralizing antibody activity. The plaque counts for each assay are shown in Table XVIII. As with the assays of sera from the naturally infected flock of sheep, missing values were computed and are shown in brackets.

The mean counts for the virus preparation and the serum-virus mixtures are presented in Table XIX. Statistical comparison of the values obtained for the virus preparation with those for the serum-virus mixtures showed that the animal which received Vaccine A developed a significant level of neutralizing antibody four weeks after primary vaccination. This activity was not maintained following the second vaccination procedure however, but reappeared within two weeks of the third vaccination. Sera from the sheep which received Vaccine B and strain ET of orf virus also showed significant neutralizing activity within two weeks of the third vaccination. The animal infected with strain EB however, showed no evidence of seroconversion even after three vaccinations.

Table XVI

Serum neutralization test plaque counts: naturally infected sheep.

Sheep no. 2

Plate	Virus	Serum-virus mixture				
		4	8	10	25	27
1	244	204	171	141	81	9
2	[220]	[111]	[104]	35	71	6
3	251	160	153	117	59	23
4	211	178	159	114	94	19
5	168	187	226	132	90	15
6	229	156	141	171	60	16

Sheep no. 3

Plate	Virus	Serum-virus mixture				
		4	8	10	15	40
1	168	153	146	[153]	[12:]	131
2	226	152	151	163	137	115
3	251	170	177	197	139	144
4	239	155	[152]	144	119	142
5	281	218	184	153	151	144
6	269	219	216	169	162	133

Sheep no. 8

Plate	Virus	Serum-virus mixture				
		4	8	10	15	40
1	76	116	78	53	[69]	59
2	103	103	77	117	58	71
3	98	101	73	[73]	85	[71]
4	77	121	87	72	64	75
5	68	76	94	50	77	46
6	123	94	61	85	85	86

Sheep no. 9

Plate	Virus	Serum-virus mixture				
		4	8	10	25	27
1	202	133	124	195	136	30
2	239	155	153	146	140	52
3	175	164	120	119	118	16
4	209	170	173	135	147	38
5	209	209	155	163	139	35
6	242	181	140	108	34	46

Sheep no. 14

Plate	Virus	Serum-virus mixture				
		4	8	10	15	40
1	96	133	64	99	99	80
2	53	102	53	68	78	77
3	83	91	[64]	97	38	88
4	66	100	[66]	92	65	69
5	72	91	67	107	51	70
6	73	105	82	67	59	72

Sheep no. 17

Plate	Virus	Serum-virus mixture				
		4	8	10	15	40
1	65	150	53	114	82	68
2	86	82	84	77	102	51
3	101	102	100	131	119	89
4	46	80	137	135	91	62
5	72	106	101	109	83	69
6	91	61	105	90	99	77

Table XVI (cont.)

Sheep no. 18

Plate	Virus	Serum-virus Mixture				
		4	8	10	15	40
1	246	167	(154)	103	55	82
2	202	152	172	148	81	(98)
3	179	107	159	95	70	74
4	274	169	162	90	63	89
5	264	186	153	90	87	61
6	237	166	110	97	92	77

Sheep no. 19

Plate	Virus	Serum-virus Mixture				
		4	8	10	25	27
1	208	129	87	83	100	38
2	224	84	66	65	65	49
3	242	131	115	88	107	56
4	218	122	106	85	89	38
5	226	103	89	72	(82)	49
6	201	104	101	92	101	55

Sheep no. 20

Plate	Virus	Serum-virus mixture				
		4	8	10	15	40
1	272	128	(48)	67	95	-
2	205	105	37	66	65	-
3	205	111	38	64	77	-
4	220	124	51	89	81	-
5	259	104	49	48	86	-
6	-	-	-	-	-	-

Sheep no. 21

Plate	Virus	Serum-virus mixture				
		4	8	10	15	40
1	77	69	74	78	84	57
2	85	81	60	59	76	73
3	87	121	82	104	72	74
4	72	86	75	70	81	72
5	94	94	101	88	100	69
6	76	80	96	76	96	73

Sheep no. 22

Plate	Virus	Serum-virus mixture				
		4	8	10	15	40
1	99	90	118	79	89	70
2	114	91	95	114	83	(80)
3	117	102	91	106	82	80
4	94	86	128	78	84	77
5	(104)	104	84	104	89	(82)
6	98	64	69	119	87	80

Sheep no. 28

Plate	Virus	Serum-virus mixture			
		3	5	10	40
1	74	113	87	91	123
2	69	(117)	96	91	85
3	71	(130)	132	110	123
4	68	130	151	127	92
5	97	139	109	109	75
6	85	85	170	108	97

Sheep no. 29

Plate	Virus	Serum-virus mixture				
		3	5	20	22	40
1	87	-	-	50	38	70
2	-	-	-	44	28	56
3	53	31	-	83	32	-
4	-	47	58	-	33	49
5	82	-	35	48	-	50
6	-	-	-	-	-	-

Sheep no. 30

Plate	Virus	Serum-virus mixture			
		3	5	10	40
1	104	125	115	126	111
2	97	105	86	112	114
3	88	84	116	91	100
4	64	150	99	138	137
5	98	122	105	126	93
6	87	117	(94)	101	98

Table XVI (cont.)

Sheep no. 31

Plate	Virus	Serum-virus mixture 3	5	10	12	40
1	59	(77)	(72)	57	34	55
2	90	53	(63)	117	27	56
3	(88)	77	78	57	39	59
4	102	129	61	63	41	57
5	101	68	64	(69)	(35)	55
6	-	-	-	-	-	-

Sheep no. 34

Plate	Virus	Serum-virus mixture 3	5	20	22	40
1	88	62	77	86	27	(69)
2	71	59	-	-	65	-
3	52	67	73	54	73	60
4	86	88	91	53	89	76
5	100	68	70	70	70	53
6	90	66	75	54	73	68

Sheep no. 36

Plate	Virus	Serum-virus mixture 3	5	20	22	40
1	72	68	50	79	48	-
2	86	84	81	91	34	-
3	65	68	65	50	51	-
4	75	90	80	68	70	-
5	(74)	(100)	108	98	52	-
6	70	107	112	83	81	-

Sheep no. 41

Plate	Virus	Serum-virus mixture 3	5	10	12	40
1	48	79	72	40	34	42
2	109	104	77	35	24	43
3	102	75	53	43	36	33
4	87	67	(60)	51	42	42
5	81	86	62	52	27	39
6	-	-	-	-	-	-

Sheep no. 42

Plate	Virus	Serum-virus mixture 3	5	10	40
1	78	136	129	116	87
2	75	155	138	131	78
3	52	46	100	94	135
4	57	109	105	109	73
5	62	106	53	134	103
6	71	138	(110)	109	76

Sheep no. 43

Plate	Virus	Serum-virus mixture 3	5	10	12	40
1	74	91	86	60	69	55
2	95	64	(80)	75	91	72
3	62	78	84	60	72	69
4	96	(90)	106	85	68	85
5	101	128	60	128	(100)	70
6	100	65	116	96	91	92

• = no. weeks after arrival at laboratory
( ) = estimated value
- = no count

Table XVII

Serum neutralization test plaque counts (expressed as mean percentages of virus titrations) : naturally infected sheep

Gp	Sheep	Serum-virus mixture (weeks after arrival at laboratory)											
		3	4	5	8	10	12	15	20	22	25	27	40
1	2		77		75	55					36** R	7**	
	9		80		68	69					61 R	17**	
	19		51		42	37					41 R	22*	
	3		75		72	70		59					60
	18		68		67	46		33					35*
	20		50		19**	29**		35					-
2	8		117		92	82*		82*					76*
	14		144		92**	121		91**					106*
	21		108		100	97		104					87
	22		86		95	96		83					75
3	17		136		139	156		131					94*
	28	157		163		139							132
	30	136		117		133							127
	42	171		161		178							146
4	29	61		61					83 R	47			71
	34	89		97					79 R	85			81
	36	117		113					106 R	77*			-
	31	94		81		84 R	41**						67
	41	103		84		56** R	42**						51**
	43	100		104		95 R	94						85

9 = denotes animals which did not develop clinical lesions

\- = no result

R = reinfection

* = significantly different from 1st serum-virus mixture (*P<0.05; **P<0.01;)

Table XVIII

Serum neutralization test plaque counts: vaccinated sheep.

Vaccine A

Plate	Virus	Serum-virus mixture				
		*2	4	5	8	10
1	25	28	18	15	15	11
2	25	25	13	18	19	14
3	20	20	28	17	13	12
4	17	12	13	14	18	11
5	15	15	12	15	15	12
6	19	15	5	21	35	11

Vaccine B

Plate	Virus	Serum-virus mixture				
		2	4	5	8	10
1	[21]	23	23	24	27	18
2	25	16	18	17	21	13
3	21	17	16	21	24	15
4	18	23	34	18	24	15
5	20	26	20	23	[25]	16
6	20	18	21	21	19	13

Strain EB

Plate	Virus	Serum-virus mixture				
		2	4	5	8	10
1	32	18	18	25	10	17
2	20	18	15	23	14	11
3	15	7	9	27	14	11
4	25	20	16	18	32	[17]
5	18	15	14	36	17	13
6	12	20	25	13	14	13

Strain ET

Plate	Virus	Serum-virus mixture				
		2	4	5	8	10
1	19	28	25	19	15	16
2	36	18	29	22	21	13
3	29	25	24	24	13	15
4	20	24	28	16	21	15
5	14	18	36	27	21	10
6	18	29	21	30	31	10

* = no. of weeks after 1st vaccination

[ ] = estimated value

Table XIX

SN test mean plaque counts: vaccinated sheep

	Virus	Serum-virus mixture				
		2	4	5	8	10
Vaccine A	20	19	15*	17	19	12**
Vaccine B	21	21	22	21	23	15**
EB	20	16	16	24	17	14
ET	23	24	27 (V_2)	23	20 (V_3)	13*

V_2; V_3 = second and third vaccination

*; ** = significantly lower than mean virus count ($P<0.05$; $P<0.01$)

6. Transfer of passive immunity to lambs

The possibility of passive immunity to orf being transferred to lambs via colostrum was investigated in five lambs born from ewes in the infected flock. The five ewes concerned had all developed clinical lesions during the initial outbreak of disease almost a year earlier, but they had not been subjected to any experimental reinfection. The lambs were 2-4 weeks old when placed on experiment, at which time they were blood sampled for precipitating and serum neutralizing antibody investigation and then challenged by scarification and inoculation of both sides of the mouth with the preparation of EB virus used in the previous reinfection studies (Section 2).

6.1. Clinical response to challenge

All five lambs responded to challenge in a notably consistent manner. One week later, characteristic orf scabs had developed at the inoculation sites in all cases. During the following week the scabby lesions extended along the lips beyond the initial areas of scarification and in one animal, the eldest, the front of the muzzle became affected. During the third week the lesions started to heal and this progressed steadily to the point of complete resolution by the end of the fourth week (Table VI).

Throughout the period of infection, the lambs continued to suckle without apparent difficulty and remained in good condition.

6.2. Precipitating antibodies

Gel diffusion tests, using both EB and CVL strains of orf, carried out on the lamb sera taken at the time of challenge showed no evidence of any precipitating antibody in any of the sera.

Serum samples from the ewes themselves, taken approximately 2½ months before lambing, were positive for precipitating antibody in three cases (nos.17, 18 and 42) and negative in the other two (nos.25 and 28). The full record of precipitin test results for these animals is shown in Table XI.

6.3. Serum neutralizing antibodies

The results of plaque-reduction assays of the lamb sera are shown in Table XX. By comparison with the values obtained for the virus preparation alone, the serum from only one lamb (no.568) appeared to have a significant level of neutralizing antibody. Unfortunately, the neutralizing antibody status of this lamb's mother (no.25) prior to lambing was not determined through a lack of an adequate serum sample. Samples from the other four ewes, taken approximately 2½ months before lambing, were found to have significant levels of neutralizing activity in two instances (nos. 17 and 18) but not in the other two (nos.28 and 42) (Table XIX).

Table XX

Serum neutralization test plaque counts: uninfected lambs

Plate	Virus	Lamb serum				
		533	537	568	569	570
1	-	-	-	66	124	91
2	63	54	55	35	-	82
3	161	52	-	36	-	68
4	79	57	78	70	-	75
5	74	61	75	41	120	63
6	78	79	59	47	98	66
Mean	91	61	67	49*	114	74

* - significantly different from mean virus count ($P<0.05$)

7. Antigenic relationships of orf virus

7.1. Relationship between different strains of orf virus

The antigenic relationship between the EB, ET and CVL strains of orf virus was examined using the double diffusion in gel technique. A diffusion pattern comprising a central well with six peripheral wells was adopted for the study. The central well contained the antigen preparations and the peripheral wells the serum samples.

The same set of six sera were used in tests against each of the three antigens as follows:

1) Anti-ET serum - obtained from the sheep vaccinated three times with strain ET (Section 4.4.).

2) Anti-Vaccine B serum - obtained from the sheep vaccinated three times with Vaccine B (Section 4.4.).

3) Anti-EB serum - obtained from the sheep vaccinated three times with strain EB (Section 4.4.).

4) Anti-Vaccine A serum - obtained from the sheep vaccinated three times with Vaccine A (Section 4.4.).

5) Anti-CVL serum - obtained from sheep 6 in the naturally infected flock.

6) Anti-CVL serum - obtained from sheep 8 in the naturally infected flock.

A single precipitin line of identity was formed between each antigen preparation and the six antisera tested (Fig 7). The position of formation of the lines in relation to the wells in the plates varied slightly with each antigen but the overall pattern appeared to be similar, with the weakest reactions being observed with the anti-ET serum and the strongest with an anti-CVL serum.

A second precipitin line of identity also formed in the reactions between the CVL antigen and the anti-EB and anti-Vaccine A sera (Fig.7(c)).

A second precipitin line was not observed however in the reaction between the EB antigen and the serum from sheep 8 (Fig.7(a)), whereas such a line had appeared when this particular serum was previously tested against the EB strain (Fig 5(b)). Different preparations of the EB antigen were used for the two tests however.

7.2. Relationship between orf and other parapoxviruses

The antigenic relationship between orf virus strain EB, milker's nodule virus strain FS 198 and bovine papular stomatitis virus strain 67333 was studied using the same method as described for the different orf virus strains.

Antigen preparations were placed in the central wells of the gel diffusion plates and antisera in the peripheral wells as follows:

1) and 2) Anti-orf serum - obtained from sheep 6 in the naturally infected flock.

3) and 4) Anti-bovine papular stomatitis serum - calf derived.

5) and 6) Anti-milker's nodule serum - calf derived.

This distribution of serum samples was chosen to enable the greatest number of comparisons between antigen-antiserum reactions to be made within the limitations of the gel diffusion pattern selected.

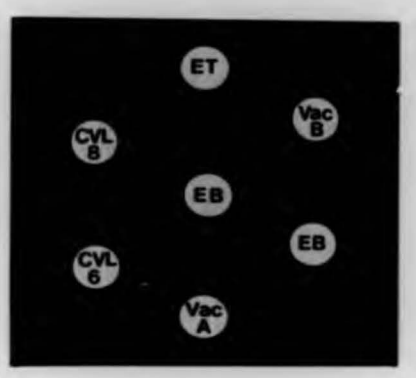
The results obtained are shown in Fig 8. Although marked differences in the clarity of the precipitin lines formed were observed with the different antigens, a single line of identity was seen to develop between each of the three antigens and the six serum samples tested. In addition, a second precipitin line could be identified in the reaction between the bovine papular stomatitis antigen and homologous antiserum (Fig 8(c)).

In a supplementary study, the milker's nodule antigen was tested against the same six anti-orf sera used to study the interrelationship of orf virus strains. The result obtained is shown in Fig 9. A single precipitin line of identity developed between the antigen and all six anti-orf sera.

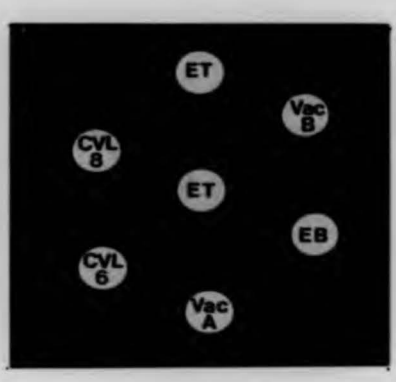
Finally, as a check on the identity of the three parapoxviruses used, a three-week old lamb was challenged by simultaneous scarification of the thigh with viable tissue culture fluid suspensions of each of the three viruses. The orf virus preparation gave rise to the development of characteristic orf lesions whereas the milker's nodule and bovine papular stomatitis viruses produced no lesions at all.

7.3. Relationship between orf and other poxviruses

The EB strain of orf was reacted against vaccinia, cowpox, Shope fibroma and fowl pox antisera in the gel diffusion test. No precipitin lines developed between these reagents. Similarly, no precipitin lines could be identified when serum from an orf infected sheep was reacted against vaccinia, cowpox, Shope fibroma or fowl pox antigens.



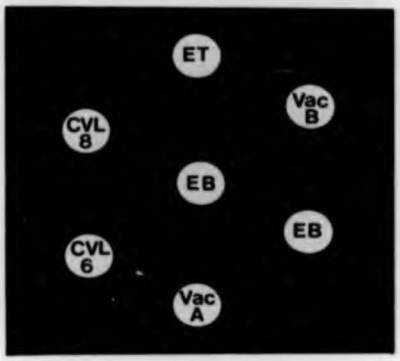
(a) EB antigen



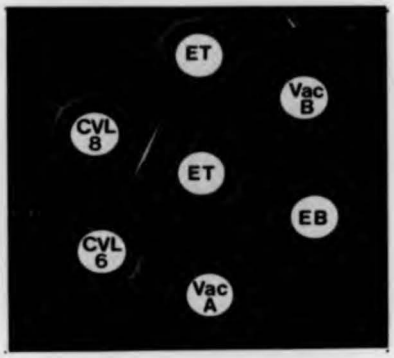
(b) ET antigen

(c) CVL antigen

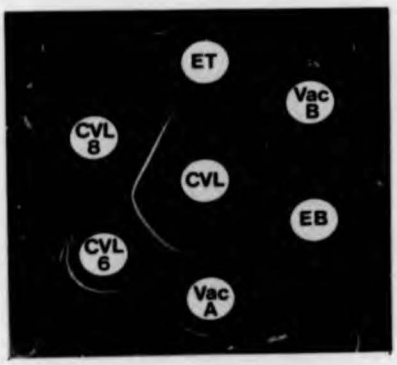
Figure 7. Gel diffusion precipitin reactions of orf virus antigens against orf antisera



(a) EB antigen



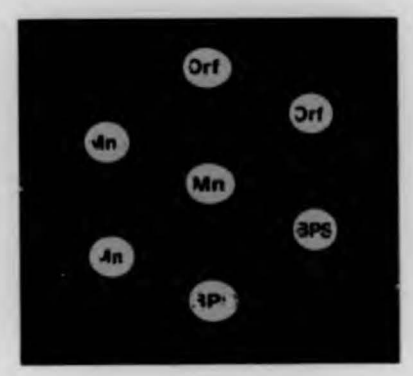
(b) ET antigen



(c) CVL antigen

Figure 7. Gel diffusion precipitin reactions of orf virus antigens against orf antisera

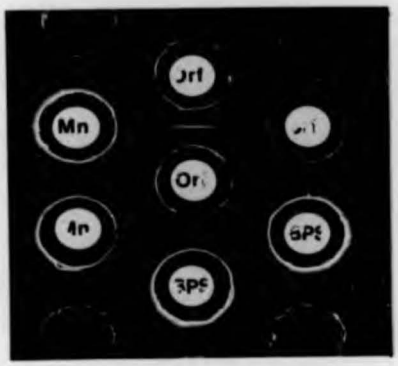
(a) Orf (strain EB) antigen



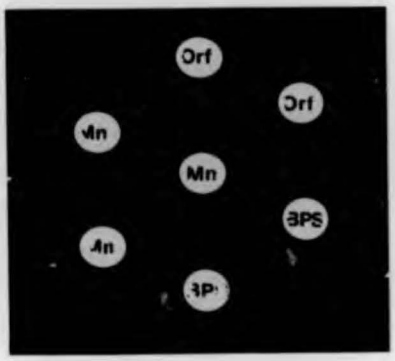
(b) Milker's nodule antigen

(c) Bovine papular stomatitis antigen

Figure 8. Gel diffusion precipitin reactions of parapoxvirus antigens against parapoxvirus antisera



(a) Orf (strain EB) antigen



(b) Milker's nodule antigen

(c) Bovine papular stomatitis antigen

Figure 8. Gel diffusion precipitin reactions of parapoxvirus antigens against parapoxvirus antisera

Milker's nodule antigen

Figure 9. Gel diffusion precipitin reaction of milker's nodule virus antigen against orf antisera

Milker's nodule antigen

Figure 9. Gel diffusion precipitin reaction of milker's nodule virus antigen against orf antisera

DISCUSSION AND CONCLUSIONS

The first problem which arose in this study related to the unexpected outbreak of orf in the flock of 44 sheep which had been obtained for experimental purposes. It was apparent from the outset that an accurate determination of the time of infection of these animals would have an important bearing on the conclusions drawn from much of the succeeding experimental work.

Clinical lesions were first observed exactly 22 days after the sheep had arrived at the laboratory at which time they had developed to form friable, proliferating scabs which, when removed, exposed a spongy, haemorrhagic tissue surface. Authoritative accounts of the pathogenesis of orf vary slightly in respect of the time taken for scabs to appear, reach a stage of maximum development and resolve. Seddon and Belschner (1929) judged that development started six to eight days after infection and reached the stage of healing at 12 to 18 days. Aynaud (1921) observed scabs developing eight days after infection and Theiler (1928) considered that they reached the stage of maximum development at about 10 days. Glover (1928) states that lesions advance to the scab stage by the fifteenth day. As these authors all studied the disease under the widely different climatic and husbandry conditions pertaining in Australia, South Africa, France and England, it would have been most surprising to find total agreement as to precisely when scabs formed and healed. Nevertheless, from all these observations it can be concluded with some certainty that scabs develop to a recognisable degree within 15 days of infection at the latest.

Thus, the sheep used in the present study must have been on the laboratory site for at least one week before becoming infected. This view is also supported by the observed distribution of infected animals when the disease was first diagnosed. If infection had occurred before

arrival at the laboratory, the disease would have been expected to appear fairly evenly distributed among the whole flock whereas, in actuality, it was initially limited to two of the four groups only.

The source of the laboratory acquired infection remains rather more speculative. Again, because of the unequal distribution of the initial infection between the four groups of sheep, it is most improbable that the virus was mechanically transferred to the laboratory when the sheep were transported from Wales as a single group of animals. The possibility of infection being introduced from the farms of origin or the transport vehicle can thus be reasonably discounted.

Within the laboratory, a number of possible sources of infection might be identified including other sheep, personnel, accommodation, food and water and vehicles and farm implements. Direct infection through contact with other sheep can be eliminated as no other sheep were present on the same laboratory premises either immediately before or during the study. Similarly, indirect infection via personnel seems unlikely as the staff responsible for the sheep were limited to work within the quarantine premises only. Likewise, farm implements were not transferred between the quarantine and other laboratory premises and vehicles were not permitted to proceed beyond the entrance to the site.

One of the more likely sources of infection would be the loose-box accommodation as this had been used to house sheep three months previously. Orf virus is known to be particularly resistant to the effects of desiccation and dried scab material has been shown to retain infectivity for over 15 years when stored at room temperature (Hart, Hayston and Keast, 1949) and 27 years when stored in a refrigerator (Hardy, 1964). Although clinical orf had never been observed in the animals which occupied the loose-boxes prior to the experimental group, they had also been purchased from outside the laboratory and might possibly have

introduced the virus which survived to infect the incoming animals. Another possible source of infection, and one which has not previously been reported in the literature, might have been the hay or straw used for feeding and bedding. Very little published information is available with respect to the longevity of the virus on pastures, but Boughton and Hardy (1934) found that during hot, dry summer conditions the virus survived for one to two months on the ground and scabs exposed to the weather over the autumn and winter months proved infective in the following spring. Hardy (1964) also reported that dry virus, protected from the sun but subjected to fluctuating temperatures, survived for over three years. On this evidence it would seem possible that, given favourable weather conditions, hay or straw obtained from contaminated pastures could transmit infective material.

Irrespective of the exact origin of the infection, it was important to determine if possible, whether these four-month-old sheep were suffering a first or second infection. Samojlov and Aliverdiev (1968) state that it is not unusual for suckling lambs which have succumbed to the disease to suffer a further infection following weaning. This is probably due to a weak active immune response generally encountered in lambs under two months of age (Halliday, 1978). All observers have agreed however, that a second infection following fairly closely on an earlier infection gives rise to an abortive-type reaction with the development of only mild and rapidly healing lesions (Boughton and Hardy, 1934; Manley, 1934; Glover, 1935; Hart, Hayston and Keast, 1949; Olah and Klek, 1953). In this study, although the lesions observed were not severe, they persisted for upwards of two weeks in almost all cases whereas, had an abortive-type of infection occurred, any scabs which formed would have resolved by this time. Thus the type of lesions observed, together with the assurance of the farmers who supplied the

sheep that no orf had occurred earlier, indicate that the outbreak encountered was a first-time infection.

Challenge experiments carried out on some of the sheep from the flock showed that animals naturally infected at four months of age developed and retained an almost solid resistance to reinfection of the mouth for at least 11 months after recovery from clinical disease. Immunity to challenge infection of the mouth was also observed in animals in which orf lesions had not been identified during the outbreak. It is not possible however to draw any firm conclusions from this latter observation as it cannot be stated with absolute certainty that lesions had not occurred at some point prior to challenge.

Although Lanfranchi (1925) considered that immunity might disappear within five to eight months of infection, the above observations support more the findings of Glover (1928), who demonstrated that resistance persisted for at least eight months after recovery and Hardy (1964), who found sheep to be immune to challenge 18 months after recovery. In fact, Hardy considered that a life-long immunity developed, but only to the same strain of virus. Samojlov and Aliverdiev (1968) found that adult sheep kept in isolation became susceptible to reinfection after 10 to 12 months and suggested that the long-lasting immunity observed in the field arose through periodic reinfection from the environment. Some support for this concept can be obtained from the present study based on the gel diffusion test results, to be discussed more fully later, in which the presence of precipitating antibodies appeared to fluctuate over the nine months following infection. This suggests that the animals experienced one or more sub-clinical infections during this time, so maintaining an immunity to clinical disease.

The present study has also confirmed the opinion of other workers (Aynaud, 1923; Glover, 1928) that sheep which have recovered from mouth

infection with orf develop an immunity to udder infection and are able to nurse infected lambs without the development of anything more than mild, transient lesions. This may be considered one of the few advantages conferred to sheep which suffer orf, as losses amongst lambs which become infected are often due to the mother preventing suckling because of the presence of painful udder lesions rather than any direct debilitating effect of the disease on the lambs themselves.

Aynaud (1921) was the first worker to utilise the inner aspect of the thigh for orf immunity studies. His interest was principally directed towards vaccine development but the site was soon adopted by other workers to study many other immunological aspects of orf infections. The inner thigh commends itself for this type of study in that it is a comparatively hairless area, large enough to provide multiple scarification sites, and is protected from interference by the animal itself as well as other animals and objects. At the present time the site is still recommended, and widely used, for the routine vaccination of sheep and for potency testing orf vaccines as this latter procedure requires the application of serial dilutions of vaccine to scarified areas of skin (British Pharmacopoeia (Veterinary) 1977; Code of Federal Regulations, 1976).

The ability of thigh infections to induce immunity to subsequent infection of the mouth was demonstrated experimentally during the early years of research on orf (Glover, 1928; Seddon and Belschner, 1929; Seddon and McGrath, 1933). Worldwide vaccination trials, sometimes involving many thousands of animals, in which the scarified thigh was inoculated with an emulsion of dried scab material in glycerine have also demonstrated that under field conditions a thigh infection induces resistance to clinical disease (Aynaud, 1923; Moussu, 1923; Melanidi and Stylianopoulo, 1928; Hatziolos, 1930; Bubberman and Kraneveld, 1931;

Kraneveld and Djaenoedin, 1933; Roughton and Hardy, 1934; Glover, 1935; Hart, Hayston and Keast, 1949; Olah and Elek, 1953; Tunkl and Alerajl, 1964; Lopatnikov, 1968; Ganovski, 1973). Of more particular relevance to the present study however, was whether mouth infection induced immunity of the thigh, as the prescribed potency tests for orf vaccine require the use of "susceptible" sheep. Although Seddon and Belschner (1929) stated that experimental infection of the mouth induced resistance at the inguinal region, Kovalev et.al. (1971) found that lambs experimentally infected on the mouth were fully susceptible to thigh infection, but resistant to mouth infection, when challenged six months later. This latter observation is consistent with results obtained at the Central Veterinary Laboratory in recent years in which 1/100 dilutions of commercial orf vaccines have induced thigh lesions in sheep which suffered a natural orf infection less than six months earlier (Frerichs, unpublished data).

This earlier observation has been confirmed as sheep from the naturally infected flock were found to be susceptible to thigh infection as early as one month after recovery from the disease. However, a comparison of the lesions obtained at this time with those obtained when other sheep were similarly challenged three and eleven months after recovery, indicates that at least a partial immunity is established at the thigh which gradually diminishes and finally disappears within one year of mouth infection. It is not possible to draw an absolutely firm conclusion on this point however, as there were many variable factors associated with the study. Firstly, each individual animal could only be challenged once so comparisons had to be made between the response of groups of similar, but by no means identical, animals. The control animals were also of a different breed. Secondly, the groups themselves were numerically small. Thirdly, each group of animals was

naturally of a different age at the time of challenge and individuals may also have experienced further sub-clinical infections at different times. There were also unavoidable differences in the amount of virus applied at challenge as the procedure of scarification and inoculation cannot be accurately standardized from sheep to sheep. In order to avoid any variation which might have arisen due to the use of different virus preparations for the different groups of sheep, the same preparations were used throughout but this introduced the possibility of loss of infectivity with storage. Although this did not seem to occur with the tissue culture derived virus preparation, it may have been a significant factor with the commercial vaccine, particularly as dried scab emulsions in glycerine have been shown to lose infectivity over a period of a few months (Glover, 1928). This would account for the apparently greater resistance of sheep to challenge with the commercial vaccine three months post-recovery compared with those challenged just one month post-recovery. The fact that the fully susceptible control sheep used on both occasions responded to the vaccine to a similar extent does not necessarily prove the stability of the product over this period of time. The results in these animals indicate that the 1/100 dilution of vaccine did not adequately approach the end-point of infectivity and a loss of infectivity with storage might have been apparent had dilutions of 1/1000 or even 1/10,000 been incorporated in the control animal tests.

No correlation could be established between the severity of clinical disease and resistance of the skin of the thigh to subsequent challenge infection. Of the five animals challenged on the thigh one month after recovery from clinical disease, one of the two sheep which appeared most resistant to reinfection had earlier developed the most severe clinical lesions whereas the other had remained free from any detectable infection. Similarly, amongst the ten animals challenged

three months after infection, the three sheep which appeared most susceptible to reinfection included one which had developed severe lesions and one which had remained clinically normal. Likewise, the three most resistant sheep included both a severely affected and a non-affected animal. At eleven months post-infection all five animals challenged, including two which had not developed clinical disease previously, responded very uniformly.

From the results of the present study and the earlier unpublished observations, it appears quite clear that orf virus infections induce varying degrees of immunity at different body sites. A mouth infection leads to a substantial and long-lasting immunity of the mouth itself and a similar degree of immunity appears to be extended to the udder. Schmidt (1967c) found the same to be true in respect of the feet and that the converse was also true in that infection of the feet or udder induced immunity of the mouth. However, the cross-relationship between infection and immunity of the skin of the thigh and the sites associated with natural infection appears to be fundamentally different. All the experimental studies and vaccination trials cited earlier indicate that infection of the thigh induces a significant degree of immunity of the mouth and Schmidt (1967c) has also shown that a thigh infection induces immunity of the feet, but the converse is not true however, and natural infection of the mouth stimulates no more than a partial and relatively short-lasting immunity of the thigh.

The early, authoritative work of Aynaud (1923) and, more particularly, Glover (1928) indicated that experimental infection of the thigh itself led to a solid immunity to reinfection of the same or opposite thigh within 15 days of inoculation and that this immunity persisted for at least eight months. These conclusions were generally accepted for decades, although Manley (1934) had observed that sheep infected by

scarification of the flank developed only a partial immunity to flank reinfection and Nisbet (1954) had found that flank immunity persisted no longer than three months. In an investigation into the development of local immunity in orf infections in sheep, Schmidt (1962) obtained results in total contrast to those of the early investigators. Almost 90% of sheep were susceptible to reinfection of the thigh within two months of the initial scarification and furthermore, they could be reinfected a second or third time at two-monthly intervals without any apparent loss in sensitivity to the virus. The small-scale vaccination/experimental infection investigation carried out as part of the present study supports to some extent the findings of all the above workers. Some animals were found to be fully susceptible to infection of the opposite thigh within a month of the initial infection and the same thigh within two months of infection but one animal developed an apparently solid resistance to reinfection of either thigh. As all the animals involved in the experiment were tested with different virus strains, the variations in response may be attributable to differences in immunogenicity or virulence of the virus preparations, or susceptibility of the individual sheep, or a combination of these and possibly other factors.

Irrespective of the reasons for the differences in response of sheep to thigh infection noted by other workers as well as the present author, the practical implication for the assessment of efficacy of orf vaccines is considerable. The present method of selecting sheep assumed to be "susceptible" to infection, applying the product to scarified areas of the thigh and observing for the development of specific lesions, leaves something to be desired.

If the present type of vaccine continues in use, and testing in sheep remains the most appropriate method of potency testing, the use of a standard vaccine preparation in all potency assays would obviate many of

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If the present type of vaccine continues in use, and testing in sheep remains the most appropriate method of potency testing, the use of a standard vaccine preparation in all potency assays would obviate many of

the reservations held over the validity of the results presently obtained. Cell culture techniques, not available when orf vaccines were first developed, should facilitate the production of a suitable standard preparation. It is envisaged that such a preparation would be used in parallel with the vaccine under test, dilutions of vaccine being applied to one thigh and dilutions of the standard preparation to the other thigh. The activity of the vaccine could then be assessed relative to the standard preparation. Variations in susceptibility of different breeds as well as individual animals would be of less significance than at present, the age of the test animals need not be so closely defined and even re-use of animals for additional tests may be possible.

An alternative, or perhaps complementary, approach to improving the validity of the potency test might be to introduce a method of determining in advance whether a sheep had experienced any prior orf infection. The application of a gel diffusion precipitin test for this purpose was considered worthy of investigation as this is often a comparatively straightforward laboratory technique.

The possibility that naturally infected sheep might develop precipitating antibodies was first investigated by Glover (1933) using scab extract as antigen in a flocculation test. He was unable to demonstrate specific antibodies in the sera from recovered lambs. Manley (1934) was similarly unsuccessful and Trueblood, Chow and Griner (1963), Schmidt (1967c), Sawhney, Dubey and Malik (1973) and Capurso, Trabellesi and Guarino (1976) were unable to demonstrate precipitating antibodies in infected or convalescent sera using the double diffusion in gel technique. Abdussalam (1958) however reported that he obtained a weakly positive flocculation reaction with one serum sample from a convalescent sheep by using a partially purified and concentrated antigen and Romero-Mercado (1969) identified antibodies in both convalescent and post-vaccinal

sheep sera using a 20% w/v suspension of scab material as antigen. Precipitating antibodies were also demonstrable in sera from the naturally infected flock of sheep used in the present study, provided that the antigen preparations used were sufficiently concentrated. In agar gel diffusion studies of paravaccinia viruses, Papadopoulos et. al. (1968) also found it necessary to concentrate antigen preparations approximately 50-fold and it therefore seems possible that some of the negative results obtained by earlier workers with orf virus may have been due to insufficiently concentrated antigen.

The actual pattern of the precipitating antibody response obtained in the present study with the naturally infected flock seemed to bear little relationship either to the disease or state of immunity to the disease. Some animals which developed lesions never developed antibodies while others which did not develop lesions were found to have precipitating antibodies throughout the nine-month observation period. Others which were seronegative during and immediately after the outbreak, were found to be seropositive many months later and vice versa, and still others fluctuated between seropositive and seronegative states.

One of the more puzzling aspects of this series of results was the finding that a number of animals were seropositive at least one month before they were affected by clinical disease. It may be argued that these animals had experienced an earlier orf infection but, although this cannot be discounted absolutely, the circumstantial evidence available strongly suggests that this was not the case. Alternatively, the results might indicate the presence of residual, passively acquired antibody. This seems even more improbable as the animals were four months old at this time and serum from lambs subsequently born to seropositive ewes in the flock were uniformly negative, indicating that no significant colostral transfer of precipitating antibodies occurs with

orf infections. The possibility that some sheep may possess non-specific antibody which was being detected in these tests also seems unlikely as, in addition to the lambs, the laboratory-bred animals used for vaccination/experimental infection studies were all found to be seronegative prior to infection.

The use of an antigen prepared from the strain of virus isolated from the infected flock (CVL) in place of the heterologous strain (EB) did little to clarify the antibody response pattern. Less than half the sera tested gave identical results with both antigens. In a further 25% of cases a positive result was obtained with both antigens although the number of precipitin lines identified differed, but in the remainder of cases completely opposite results were obtained. A further series of tests on the same group of sera using antigen prepared from milker's nodule virus produced yet another set of conflicting results, although there appeared to be less discrepancy between the reactions to the milker's nodule and CVL orf viruses than between the two orf strains.

Experimental reinfection of selected sheep from the flock generally produced unexceptional antibody responses. Animals seropositive at the time of reinfection remained seropositive, although one sheep reverted to a seronegative state two weeks later, and the two seronegative animals became seropositive within two weeks of challenge.

The significance of the double and triple precipitin lines formed with some serum samples is not known. When EB antigen was used the second line appeared in samples taken during the outbreak of disease in the flock, although not every animal giving this type of response developed clinical lesions. A second line also appeared in two animals two weeks after experimental reinfection. With the CVL and milker's nodule antigens, the appearance of multiple precipitin lines was more commonly seen in samples taken after reinfection, but it has to be

remembered that sera from only nine of the 44 animals were tested with these two preparations. Nevertheless, the observations agree to some extent with the findings of Romero-Mercado (1969) who also observed the development of two lines of precipitation in some animals which had been experimentally challenged by scarification following recovery from a natural infection or vaccination.

No satisfactory reason for the disparities in the precipitin test results can be offered at the present time. Possible differences in the antigenic structure of the three viruses used in the tests is an obvious and attractive explanation for why many sera gave a positive result with one antigen and a negative result with another. However, the study carried out into the antigenic relationships of the strains of orf and milker's nodule viruses used to test the sheep sera showed quite clearly that at least one soluble antigen is common to all these agents. Therefore, assuming the concentrations of antigen used to be approximately the same in all cases, sera giving a positive reaction with one antigen preparation would be expected to give a positive reaction with the others and sera negative to one preparation would be negative to the others. Differences in antigenic composition might, however, account for variations in the number of secondary precipitin lines formed when a positive reaction was obtained. The reasons for the many contradictory results would thus seem more likely to be attributable to the gel diffusion test itself and a more thorough examination of the technique, incorporating appropriate reference sera in all cases, is indicated for the future.

Although the gel diffusion test as applied in the present study gave rise to many spurious results the overall impression was that even an improved test would probably be of only limited value for diagnostic purposes. Individual animals appear to vary considerably in their precipitating antibody response to infection, irrespective of the test

antigen used, and the result of a gel diffusion precipitin test at any single point in time would need to be interpreted with caution. Similarly, the test would also be of little value in screening sheep intended for use in a potency test of an orf vaccine. Certainly, those animals found to be positive to such a test could be rejected for potency test purposes but, in the absence of an accurate clinical history, a seronegative animal could not be assumed to be any more susceptible than a seropositive animal.

The fluctuating response observed with some of the sheep also suggests that precipitating antibodies may only persist in the serum for a short period of time and that their reappearance is indicative of further subclinical infections arising as the virus cycles within the flock or is reacquired from the infected environment. This supposition however needs further investigation under more controlled experimental conditions before a firmer conclusion can be reached but if it was found to be true, the gel diffusion test might then find some application in screening flocks of sheep. Endemic orf could be identified by testing a reasonable sample of sera from the flock in the expectation that at any one time at least some sera would be positive for precipitating antibodies.

Examination of sera for the presence of specific neutralizing antibodies offered itself as another alternative method for determining the immune status of sheep intended for potency test use. As with precipitating antibodies, other studies have produced a number of conflicting reports regarding the occurrence of neutralizing antibodies. The early workers inoculated sheep with mixtures of scab material and serum to assess the neutralizing activity of sera and observed either no neutralization (Aynaud, 1923; Glover, 1933; Olah and Elek, 1953) or a level of activity which was sufficient only to reduce the severity of

the lesions (Glover, 1933; Manley, 1934; Selbie, 1945). Later workers used tissue culture systems to assay sera, again with variable results. Macdonald and Bell (1961), Liess (1962), Schmidt (1967c) and Khanduev, Gusev and Dzhakupov (1969) all failed to demonstrate the presence of neutralizing antibody in sera from naturally or experimentally infected sheep whereas Plowright, Witcomb and Ferris (1959), Nagington and Whittle (1961), Trueblood, Chow and Griner (1963), Sawhney (1966c) and Poulain, Gourreau and Dautigny (1972) detected varying levels of antibody in similarly infected animals.

It appeared from these earlier studies that if neutralizing antibodies were formed in response to orf infection, they would probably be present in sera only in low levels and that a sensitive assay system would be required to quantitatively estimate such levels. The use of sheep was thus rejected because of the difficulty of quantitatively assessing small differences in responses to serum-virus mixtures combined with the problem of sheep to sheep variation which could only be overcome by the use of an inordinate number of costly animals. The utilization of an *in vitro* tissue culture system was thus indicated, and a plaque-reduction test was selected as providing a more sensitive means of determining neutralizing activity than a method involving a quantal response and statistical determination of 50% endpoint dilutions of serum.

The usual procedure in plaque-reduction assays is to determine the dilution of serum which will reduce by 50% the number of plaques which form in control cell cultures. This method was not applied in the present study for the practical reason that testing a number of dilutions of one serum utilizes the same amount of materials required for testing a single dilution of the same number of different sera, and it was considered that as many sera as resources would permit should be examined. Accordingly, all sera were tested at a single 1/5 dilution as

this was judged to be sufficiently concentrated to enable low levels of specific antibody to be detected and at the same time was sparing in the amount of serum used.

As was observed with the precipitating antibody response, there appeared to be little correlation between the development of clinical lesions and the neutralizing antibody response. Three animals which became seropositive never developed clinical disease, but three others which also never exhibited lesions remained seronegative, at least until they were experimentally reinfected. Similarly, only four of the thirteen animals which had developed lesions registered a neutralizing antibody response prior to experimental reinfection.

Experimental reinfection by thigh scarification one or three months after clinical disease also gave a variable response, with two sheep showing a positive seroconversion and one an apparent increase in antibody level, but three animals showed no significant change from their previous seronegative status. Reinfection of the mouth as well as the thigh however, gave a more uniform response, with two of the three animals seroconverting and the third developing an increased antibody level.

As with the majority of virus neutralization tests, comparisons were made between the results obtained with pairs of serum samples from individual animals. Unfortunately, the first samples available in this study were not obtained until the disease was actually present in the flock so some doubt must exist as to how closely these initial samples represent a seronegative state. In many diseases this would be of no great importance as the serological response is usually sufficiently marked to leave little doubt that antibody has or has not developed. In the case of orf however, where only low levels of antibody may be expected, the absence of a serum sample obtained prior to initial

infection leads to difficulty in the interpretation of the response following infection. In the absence of preinfection sera, more meaningful results could only have been obtained by incorporating in each test a standard positive serum having a known degree of neutralizing activity. The neutralizing activity of the test sera could then have been expressed relative to the standard serum.

Notwithstanding these shortcomings in the series of tests carried out in the present study, it can be concluded that some sheep will produce circulating neutralizing antibody in response to a primary or secondary orf infection. This response appears to bear little or no relationship to the time of development of clinical lesions or immunity to reinfection but further studies with a carefully controlled test system would be worthwhile to establish this more conclusively. Although reasons for carrying out neutralization tests in tissue culture in preference to sheep were given earlier, the results obtained indicate that it might also prove worthwhile to compare *in vivo* neutralizing activity with *in vitro* results. A study by Trueblood, Chow and Griner (1963) suggests that while neutralizing activity may be detected when convalescent sera are assayed in tissue culture, similar activity is not detected when the sera are assayed in sheep. If this observation could be confirmed, it would reduce further the significance of any antibody detected in *in vitro* neutralization tests.

As with natural infection, vaccination/experimental infection alone induced the formation of neutralizing antibody, but not in all animals. Again, the significance of this remains obscure.

There was no correlation between the presence or absence of precipitating antibodies and the development of serum neutralizing antibody.

Thus, the occurrence of clinical lesions and subsequent resistance to mouth or thigh reinfection appear to be unrelated to the development

and persistence of either precipitating or serum neutralizing antibodies, and these in turn appear to be formed independently of each other. Aynaud (1923) was the first to suggest that the development of a tissue immunity, rather than a humoral immunity, was associated with orf infections. The results of the present study tend to support this view and also that of Schmidt (1962) who suggested that immunity is limited to certain areas of the skin. By a strange quirk of Nature, these areas are the hairless extremities naturally affected by orf whereas areas such as the inner thigh, which is never naturally affected, remains relatively susceptible, irrespective of the past history of infection.

Further support for the view that tissue rather than humoral antibody is the principal mediator of orf immunity is found in the results of passive immunity studies in which lambs from previously infected ewes all proved susceptible to experimental mouth infection. Although the animals appeared to be quite severely affected and, by inference, were thus fully susceptible, it remains just possible, however, that some low level of protection may have been passively transferred via colostrum and that even severer lesions would have developed had they been born to non-immune mothers. Under field conditions, Glover (1935) found that lambs born to immune mothers were susceptible to mouth infection within three weeks of birth but noted that the lesions were less severe than those occurring in lambs from non-immune mothers, suggesting that a low level of immunity may have been passively acquired. Ganovski (1973) also reported that lambs from previously infected mothers were significantly more resistant to field infection than those from non-infected mothers.

In contrast to the above reports with natural field infections, Boughton and Hardy (1934) and Richter and Jansen (1968) observed that

lambs from immune mothers were fully susceptible to experimental infection within a few days of birth but these conclusions were based on the results of thigh infections only.

Although the present study demonstrated that precipitating antibodies were not passively transferred to lambs, the serum neutralization test results were inconclusive and further work may substantiate the findings of Poulain, Gourreau and Dautigny (1972) and Le Jan et.al. (1978) that lambs do acquire such antibodies via colostrum. The role of such antibodies in mediating immunity however is unknown, as neither of the above groups of workers carried out challenge experiments on the lambs. Furthermore, Aynaud (1923), Manley (1934), Olah and Elek (1953) and Lopatnikov (1968) all found that the intravenous or subcutaneous administration of large volumes of serum from hyperimmunized sheep did not afford protection against experimental thigh infection.

Rather surprisingly there appear to be no accounts, apart from the present very limited study, of experimental mouth infection studies in lambs, and further work on the response of lambs to mouth rather than thigh infection is indicated to elucidate the role of neutralizing antibodies in orf immunity.

One aspect of orf infections which has attracted a considerable amount of attention is the immunological relationship between different strains of the virus. This is particularly relevant to the development and evaluation of vaccines, the use of which would appear at present to be the only practical method of controlling the disease. If all orf viruses fall into a single immunological group, then only a single strain of virus should be needed to prepare a vaccine, a potentially global market would exist for the product as the disease has a world-wide distribution and manufacturers might also be encouraged to develop orf vaccines in the knowledge that costly field evaluation studies

carried out in one country could be accepted as valid efficacy data by regulatory authorities in another country.

The balance of available evidence certainly indicates that all orf virus strains are immunologically very closely related. Many workers in all parts of the world where sheep are raised have been unable to identify immunologically distinct strains on the basis of cross-protection tests in sheep (Glover, 1928, 1933; Kraneveld and Djaenoedin, 1933; Seddon and McGrath, 1933; Horgan and Haseeb, 1947; Olah and Elek, 1953; Mundu and Mohan, 1961; Sabban, El Dahavy and Hussein, 1961). Only two published reports are in conflict with these findings. Hardy (1964) reported that he had been able to identify six variants of the virus on the basis of cross-immunity tests, but detailed results are not presented so it is difficult to make an independent assessment of his findings. It would appear however that lambs were inoculated on the thigh with one strain of virus and, after recovery, challenged with another strain. If the challenge strain caused lesions, even though these were less severe than in a control animal, then this was considered to be an immunological variant. Likewise, Sawhney (1966c) firmly refuted the idea that all orf viruses formed an immunologically homogeneous group, again based on results of initial thigh infection with one strain and subsequent challenge with another.

In the present study, it has been shown quite clearly that animals naturally infected with one strain of orf may be solidly resistant to mouth infection but simultaneously susceptible, or at least partially susceptible, to thigh infection with one or more different strains. It was also shown that one strain of virus inoculated on the thigh induced a solid immunity to thigh reinfection whereas other strains did not. This may be a reflection of the relative potency of the different strains or idiosynorasies in the response of individual sheep. Bearing

these observations in mind, the claims for the existence of different immunological types of virus based on thigh infection studies should not be accepted without question.

Remarkably few *in vitro* studies on the antigenic relationship of orf virus strains have been reported in the literature. This is probably due to the fact that there has been little pressure to develop a serodiagnostic test for the disease. The symptoms and lesions are highly characteristic and, apart from ulcerative dermatosis, differential diagnosis usually presents little problem to farmer and clinician alike. The disease also lends itself to rapid and unequivocal confirmation by electron-microscopic examination of scab material. Furthermore, conventional serological tests involving the assay of neutralizing, complement-fixing or precipitating antibodies have all proved unpromising and the refinement of such tests has not been pursued.

Sawhney (1966c) inferred that *in vitro* neutralization tests supported his view that more than one immunological type of orf virus existed, but his results appear unconvincing and statistical analysis would probably provide little support for his interpretation. Precausta and Stellmun (1973) also studied five isolates using an *in vitro* neutralisation test and found all strains to be very closely related.

The double diffusion in agar technique was used by Sawhney, Dubey and Malik (1973) to compare 15 strains of orf virus. All 15 strains produced three precipitin lines of identity when reacted against rabbit and sheep hyperimmune sera. In the present study, cross-precipitin tests with three orf strains and sera from sheep naturally or experimentally infected with five strains of virus produced a single line of identity in all instances. Although a second precipitin line was formed with one antigen against two of the sera, these findings provide

further evidence that all orf strains are antigenically similar. Nevertheless, it is recognised that additional *in vivo* and *in vitro* studies would be desirable before concluding that only a single immunological type of orf virus exists.

In any study of the relationship between orf viruses, too much emphasis should not be placed on the results of gel-diffusion tests as the antigen detected appears to be common to all parapoxviruses rather than orf alone. A precipitating soluble antigen common to orf and milker's nodule viruses was first reported by Huck (1966) and again by Papadopoulos et. al. (1968) and the present study has confirmed these observations. It has also demonstrated directly for the first time the presence of a soluble, precipitating antigen common to orf, milker's nodule and bovine papular stomatitis viruses. Furthermore, it has been shown that an additional antigen may be associated with bovine papular stomatitis virus. This antigen was only demonstrated in the reaction between bovine papular stomatitis virus and homologous antiserum and could not be detected when the virus was reacted against orf or milker's nodule antisera. Similarly, when orf and milker's nodule virus were reacted against bovine papular stomatitis antiserum, only one precipitating antigen could be identified. Hitherto, it has not been possible to distinguish between these three parapoxviruses except on the basis of animal susceptibility, but the present findings suggest that an *in vitro* distinction between bovine papular stomatitis virus and the other two members of the genus might be possible. This is likely to be of more relevance to any future studies with the two natural bovine infections than with orf.

The antigenic relationship of orf to other poxviruses has also received considerable attention from a number of workers. A nucleoprotein (NP) antigen is believed to be common to all viruses in the

Poxviridae family (Fenner et. al., 1974), but this antigen has only been obtained by alkaline extraction of virus particles and, in respect of the parapoxviruses, its presence in this genus has been inferred from the demonstration that NP antibody was present in convalescent serum from cattle experimentally infected with bovine papular stomatitis virus (Woodroofe and Fenner, 1962). Using intact virus and/or the soluble antigens, these same workers were unable to demonstrate any serological cross-reactions between poxviruses of different genera. Likewise, in the present study, no serological relationship could be demonstrated between orf virus and viruses of the orthopox (vaccinia, cowpox), avipox (fowl pox) or leporipox (Shope fibroma) genera in cross-precipitin tests with soluble antigens. This agrees with the findings of the majority of workers who have studied the antigenic relationships of orf virus using cross-protection, neutralization, complement-fixation, precipitation, haemagglutination-inhibition or fluorescent antibody tests. One exception to this apparent antigenic exclusiveness of the parapoxviruses may be present in the relationship between orf and goat pox virus. Some workers have observed a one-way immunological relationship between these two agents in that goat pox virus and goat pox immune serum conferred protection against or neutralized orf virus, but the reverse effect did not occur (Bennett, Horgan and Haseeb, 1947; Sharma and Bhatia, 1958; Subba Rao and Malik, 1979). Sawhney, Dubey and Malik (1973) also found that in the gel diffusion test a single line of precipitation developed between orf virus and goat pox hyperimmune serum. Goat pox immune serum was not available for inclusion in the present study so it was not possible to confirm these observations. It would be interesting to do so however, particularly as no such relationship apparently exists between orf and sheep pox virus, which is classified together with goat pox in the capripoxvirus genus.

Throughout this discussion, a number of aspects of orf infections which would merit further study have been identified, and lines of investigation have been suggested in some instances. Although a few of such investigations could be pursued independently of the others, the majority should form part of a broader study in two particular areas:

1) A re-examination of almost all aspects of immunity of sheep to orf based on the response to mouth and feet infections instead of thigh infections.

2) An investigation into the establishment and use of standard preparations for assessing the efficacy and potency of orf vaccines.

It has sometimes been said that orf is a well-known disease about which very little is known, and the review of the literature and results of the investigations carried out in the present study suggest that there is more than a grain of truth in the aphorism.

APPENDICES

APPENDIX I

Tissue Culture Media and Reagents

Primary calf testis growth medium

Hanks buffered salt solution with yeastolate (0.01%) and lactalbumin hydrolysate (0.5%). 10x concentrate.

A.	NaCl	800g
	KCl	40g
	$MgSO_4 7H_2O$	20g
	$CaCl_2$ (anhydrous)	14g
	Phenol red soln. (0.2%)	500ml
	Distilled water to	4500ml
B.	$Na_2HPO_4.2H_2O$	6g
	KH_2PO_4	6g
	Dextrose	100g
	Yeastolate (Difco)	10g
	Distilled water to	5000ml
C.	Lactalbumin hydrolysate	500g
	Distilled water to	10,000ml

Solutions A, B and C are made up separately in the order given.

All solutions are autoclaved at 10 lbs/sq.in. for 10 minutes and stored at +4°C.

For use Add aseptically to 80ml sterile distilled water:-

5 ml soln. A

5 ml soln. B

10 ml soln. C

0.4 ml $NaHCO_3$ soln. (7.5%)

0.5 ml antibiotic soln.

10 ml fetal calf serum

Secondary calf testis growth medium

Eagle's Medium M.E.M. (Wellcome Reagents Ltd., Beckenham, England).

For use Add aseptically to 100ml Eagle's medium M.E.M.:-

1ml $NaHCO_3$ soln. (7.5%) if closed bottles are used

or 2ml $NaHCO_3$ soln. (7.5%) if open plates are used

0.5ml antibiotic soln.

10ml fetal calf serum

Secondary calf testis maintenance medium

As for secondary calf testis growth medium except that 0.5 - 2.0ml of fetal calf serum is used.

Sodium bicarbonate solution (7.5%)

$NaHCO_3$	75g
Phenol red soln. (0.2%)	50ml
Distilled water	950ml

Dissolve $NaHCO_3$ in water and then add phenol red soln.

Autoclave at 10 lbs/sq.in. for 10 minutes and store at $+4^{\circ}C$.

Phenol red solution (0.2%)

Dissolve 2g phenol red in 30ml N/10 NaOH and make up volume to 1000ml with deionized water.

Antibiotic solution

Made up in deionised water to contain:-

100 iu/ml penicillin

100 mcg/ml streptomycin

25 units/ml nystatin or 2 mcg/ml amphotericin B

Trypsin diluent (10 x concentrate)

NaCl	800g
KCL	40g
Na_2HPO_4 (anhydrous)	6g
KH_2PO_4 (anhydrous)	6g
Phenol red soln. (0.2%)	500ml
Distilled water to	9,500ml

Sterilize by autoclaving at 10 lbs/sq.in. for 10 minutes and store at +4°C.

For use Add 100ml to 900ml sterile deionised water.

Add 6ml $NaHCO_3$ soln.

Trypsin solution (40 x concentrate)

Trypsin 1:250 (Difco)	100g
Dextrose	50g
Trypsin diluent	1000ml

Sterilize by membrane filtration and store at -20°C.

For use Add 25ml to 1000ml trypsin diluent.

This is now a 0.25% soln. of trypsin.

Trypsin/versene solution (10 x concentrate)

NaCl	80g
KCl	4g
Dextrose	10g
$NaHCO_3$	5.8g
Versene	2g
Phenol red soln. (0.2%)	10ml
Distilled water to	1000ml

Sterilize by membrane filtration. Add 2.5ml trypsin soln. (0.25%) and store at -20°C.

For use Add 5ml to 45ml deionised water.

Gelled overlay media

1) Sodium carboxymethyl cellulose (SCMC)

Add 2g sodium carboxymethyl cellulose (Hercules Inc., Wilmington, Delaware, U.S.A.) to 100ml deionised water and dissolve by autoclaving at 15 lbs/sq.in. for 15 minutes. Store at +4°C.

For use Add aseptically an equal volume double strength maintenance medium.

2) Difco Bacto-agar and Oxoid agar

Add 2g Difco Bacto-agar (Difco Laboratories, Detroit, Michigan, USA) or 2g Oxoid agar No.1 (Oxoid Ltd., England) to 100ml deionised water and dissolve by autoclaving at 15 lbs/sq.in. for 15 minutes.

For use Allow to cool to 37°C in a water bath and add aseptically an equal volume double strength maintenance medium previously warmed to the same temperature.

3) Difco Bacto-agar + DEAE-dextran

Add 10mg DEAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden) to 2g Difco Bacto-agar and prepare and use as for Difco Bacto-agar.

APPENDIX II

Plaque Assay of Orf Virus

The plaque assay may be regarded as a method of estimating the number of infective virus particles in a given sample by counting the number of local lesions ("plaques") produced by the virus in a cell culture, each lesion being considered to be derived from a single infective particle (Cooper, 1967). Conventional monolayer cell cultures with fluid medium overlays allow free diffusion of the infecting virus and although some viruses are self-limiting in spread and require no artificial localization, most require the addition of a gelling substance to the overlay medium to localize the lesions and prevent the development of a generalised cytopathic effect.

The addition of agar to a final concentration of 1% w/v in fluid medium is the most generally applicable method of achieving plaque formation with viruses which would otherwise induce a generalised cytopathic effect. Unfortunately, agar has an inhibitory effect on the development of plaques by certain viruses including encephalomyocarditis (Liebhaber and Takemoto, 1961) and Western equine encephalomyelitis (Ushijima et al., 1962), but this can be overcome by the inclusion of DEAE-dextran in the overlay medium which neutralizes growth-inhibiting sulphated polysaccharides present in the agar (Fenner and White, 1970).

Methyl cellulose at final concentrations between 0.75% and 2% w/v has also been used as a gelling substance in overlay media. Initially, it found application in assays in which fluorescent antibody techniques were used as, unlike agar, it was itself non-fluorescent (Hotchin, 1955; Rapp et al., 1959). Subsequently, it was also found to be non-inhibitory in certain instances where agar inhibited plaque formation (Schulze and Schlesinger, 1963) and has been favoured because it can be stored ready for use and kept at room temperature during the overlaying procedure

(Gourlay, 1970). The principal disadvantage of methyl cellulose is that it does not form a solid gel at low concentrations, so overlayed cell cultures cannot be inverted.

Orf virus propagated in monolayer cell cultures with fluid medium will produce minute plaques (< 1mm diameter) in the early stages of propagation but these soon spread and join each other resulting in a generalized cytopathic effect (Precausta and Stellmann, 1973). Gelled overlay media however, appear to have found little application in relation to orf virus studies with only Faizulina et al. (1972) and Poulain, Gourreau and Dautigny (1972) reporting the use of agar and agarose respectively to enable them to carry out plaque assays.

As part of the investigation into the development of serum neutralizing antibodies in sheep following orf virus infection, it was considered necessary to establish a suitable method of carrying out plaque assays so that sera could be assayed using a plaque-reduction test.

Method

1% w/v Difco Bacto-agar, 1% w/v Difco Bacto-agar containing 50 ug/ml DEAE-dextran, 1% w/v Oxoid agar and 1% w/v sodium carboxymethyl cellulose (SCMC) were used as gelling agents in fluid medium (Appendix I) and assessed for suitability in plaque assays of the EB and ET strains of orf virus.

Two studies were carried out. In the first, 1/500 dilutions of stock suspensions of each virus were made and 0.2ml of each dilution inoculated onto confluent monolayers of secondary calf testis cells in six-well polystyrene plates. After allowing adsorption for 30 minutes at 37°C, four of the wells were overlayed with 3ml of medium, a different medium being applied to each well. Four replicate plates were used for each virus. The plates were incubated at 37°C for five days then fixed

and stained. The SCMC overlay was aspirated off the plate before fixation but the other three overlays were removed only after fixation had been completed.

In the second study, 1/300 dilutions of the same virus stock suspensions were prepared, 0.1ml was inoculated onto each cell culture and the absorption time extended to one hour. Six replicate plates were prepared for each virus. The incubation period in this study was extended to seven days.

Results and Discussion

a) Plaque morphology: With all four overlay media both virus strains gave rise to roughly circular plaques, 1-3mm in diameter, with deeply staining, enlarged and degenerate cells round the periphery (Fig. 10). The plaques formed under Oxoid agar were less clearly delineated than with the other three media which made it more difficult to distinguish separate plaques, but the most striking difference was observed between the three agar overlays and SCMC. The central area of the plaques under agar retained considerable amounts of cell debris whereas under SCMC the plaques appeared as clear holes in the monolayer, greatly facilitating identification and enumeration (Fig.11).

b) Plaque counts: The plaque counts obtained with the four overlay media in both studies are shown in Table XXI. Only four values for the 1/300 dilution of strain EB are shown as two of the six plates were contaminated and had to be discarded. The mean counts for the values obtained with the overlay media together with a pooled estimate of the standard error of the mean for each dilution of the two virus strains are shown in Table XXII.

a) Difco agar

b) Difco agar + DEAE - dextran

c) Oxoid agar

d) Sodium carboxymethyl cellulose

Figure 10. Orf virus plaques under gelled overlay media

a) Difco agar

b) Difco agar + DEAE - dextran

c) Oxoid agar

d) Sodium carboxymethyl cellulose

Figure 10. Orf virus plaques under gelled overlay media

Figure 11. Orf virus plaques under SCMC overlay medium. Actual size.

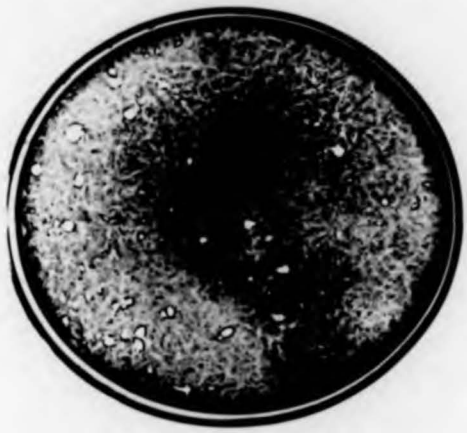

Figure 11. Orf virus plaques under SCMC overlay medium. Actual size.

Table XXI

Orf virus plaque counts with different overlay media

1/1500 virus dilution. 0.2ml inoculum

Overlay medium	Strain EB				Strain ET			
Oxoid	77	76	53	56	35	35	40	30
Difco	79	71	64	62	34	28	37	44
Difco & DEAE	55	68	60	68	37	37	38	39
SCMC	86	72	83	73	80	88	90	94

1/300 virus dilution. 0.1ml inoculum

Overlay medium	Strain EB				Strain ET					
Oxoid	168	194	115	219	192	258	258	-	299	273
Difco	215	142	162	185	257	175	177	258	272	215
Difco & DEAE	209	166	156	201	236	311	233	307	261	300
SCMC	456	352	306	359	657	657	679	770	605	783

Table XXII

Mean orf virus plaque counts with different overlay media

Overlay Medium	Strain EB		Strain ET	
	1/1500	1/300	1/1500	1/300
Oxoid	66	174	35	256
Difco	69	176	36	242
Difco & DEAE	63	183	38	275
SCMC	78	368	88	692
Pooled estimate of SEM	4.4	21.8	2.5	19.8

SEM = standard error of the mean

The highest counts were obtained with the SCMC overlay in all instances, although with the 1/500 dilution of strain EB the difference was only statistically significant in relation to the result with the Difco/DEAE-dextran medium ($P<0.05$). In all cases there was no significant difference between the counts obtained with the three agar overlay media.

It is not possible to determine from these results whether the differences observed with the different media can be attributed to an inhibiting effect by agar, an enhancing effect by SCMC or to a combination of these two effects. Further studies would be required to elucidate this.

APPENDIX III

Effect of Time/Temperature on Serum Neutralization of Orf Virus

When conducting *in vitro* assays of serum neutralizing antibodies to orf virus, different time/temperature combinations for the incubation of serum-virus mixtures have been used by different workers. Poulain, Gorreau and Dautigny (1972) incubated mixtures at 37°C for 3 hours before inoculating their cell cultures whereas Plowright, Witcomb and Ferris (1959) and Sawhney (1966c) used overnight incubation at 4°C. Khanduev et. al. (1973) allowed 23 hours incubation at 4°C.

In order to assess whether such time/temperature differences might give rise to different neutralization test results, the effects of incubation for 2 hours at 37°C, 18 hours at 4°C and 72 hours at 4°C were compared.

Method

Two sheep sera known to have neutralizing activity were reacted with a suspension of the EB strain of orf virus for the three time/temperature conditions under investigation. As control preparations, mixtures of the orf virus suspension with tissue culture maintenance medium in place of serum were simultaneously incubated under the three different conditions. At the end of the incubation periods the test and control preparations were titrated as described for the virus neutralization test, the results being read after five days of incubation.

Results and Discussion

Individual well counts and the mean counts for each set of six wells for each preparation assayed are shown in Table XXIII. The mean count obtained with the virus control preparation held at 37°C for 2 hours was significantly lower than the count obtained with the same preparation held at 4°C for 18 hours ($P<.01$). The 4°C for 72 hours preparation gave an intermediate value. The apparent reduction in infectivity titre

associated with exposure to a temperature at 37°C was not entirely unexpected as Plowright, Witcomb and Ferris (1959) and Sawhney (1972) both observed a marked fall in the titre of infective tissue culture fluids held at this temperature for one week.

The reduction in plaque counts observed after the different time/temperature neutralization periods, expressed as percentages of the corresponding virus control preparations, are shown in Table XXIV. There was no significant difference in the percentage reduction of counts after neutralization at the different time/temperature conditions with either serum.

Thus, although actual plaque count values might differ significantly with different neutralization procedures, the neutralizing activity of the serum would appear to remain constant.

Table XXIII

Orf virus plaque counts after different serum-virus incubation periods

	Incubation periods		
	2 hrs @ 37°C	18 hrs @ 4°C	72 hrs @ 4°C
Virus + serum 9	35, 38, 45, 30, 32, 49	61, 57, 59 56, 67, 61	51, 51, 71, 58, 51, 70
Mean ± SEM	38.2 ± 3.0	60.2 ± 1.6	58.7 ± 3.9
Virus + serum 19	35, 27, 36, 39, 39, 32	45, 40, 36, 41, 42, 37	37, 36, 36, 35, 32, 39
Mean ± SEM	34.7 ± 1.9	40.2 ± 1.4	35.9 ± 0.95
Virus control	71, 97, 77, 71, 122, 129	137, 158, 134, -, 114, 128	121, 97, 124, 96, 133, 78
Mean ± SEM	94.5 ± 10.6	134.2 ± 7.1	108.2 ± 8.6

SEM = standard error of the mean

Table XXIV

Percentage reduction (± SEM) in orf virus plaques after different serum-virus incubation periods

	Incubation periods		
	2 hrs @ 37°C	18 hrs @ 4°C	72 hrs @ 4°C
Virus + serum 9	59.6 ± 11.7	55.2 ± 5.5	45.8 ± 8.7
Virus + serum 19	63.3 ± 11.4	70.1 ± 5.4	66.9 ± 8.0

SEM = standard error of the mean

Table XXIII

Orf virus plaque counts after different serum-virus incubation periods

	Incubation periods		
	2 hrs @ 37°C	18 hrs @ 4°C	72 hrs @ 4°C
Virus + serum 9	35, 38, 45, 30, 32, 49	61, 57, 59 56, 67, 61	51, 51, 71, 58, 51, 70
Mean ± SEM	38.2 ± 3.0	60.2 ± 1.6	58.7 ± 3.9
Virus + serum 19	35, 27, 36, 39, 39, 32	45, 40, 36, 41, 42, 37	37, 36, 36, 35, 32, 39
Mean ± SEM	34.7 ± 1.9	40.2 ± 1.4	35.9 ± 0.95
Virus control	71, 97, 77, 71, 122, 129	137, 158, 134, -, 114, 128	121, 97, 124, 96, 133, 78
Mean ± SEM	94.5 ± 10.6	134.2 ± 7.1	108.2 ± 8.6

SEM = standard error of the mean

Table XXIV

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Virus + serum 19	63.3 ± 11.4	70.1 ± 5.4	66.9 ± 8.0

SEM = standard error of the mean

APPENDIX IV

Statistical evaluation of plaque count data in serum neutralization assays : naturally infected sheep

(Miss C N Hebert)

Missing values for serum-virus mixtures were estimated taking into account overall plate and sampling values.

Mean counts for virus alone were inserted if values for one or more plates were missing.

Each serum-virus mixture count was expressed as a percentage of the virus count for that plate and the mean percentage for each mixture then computed. Example - sheep 17:-

Plate	(serum-virus mixture ÷ virus) x 100				
	4	8	10	15	40
1	230.8	81.5	175.4	126.2	104.6
2	95.3	97.7	89.5	118.6	59.3
3	101.0	99.0	129.7	117.8	88.1
4	173.9	297.8	293.5	197.8	134.8
5	147.2	140.3	151.4	115.3	95.8
6	67.0	115.4	98.9	108.8	84.6
Mean	135.9	138.6	156.4	130.8	94.5

Since plate-to-plate variations had already been taken into account by using the virus counts in the estimation of percentages, a one-way analysis of variance of replicate percentages was carried out to assess the significance of the mean differences between the first and subsequent serum samples for each animal.

When there was a marked drop in percentage count it was found that the variance for that sampling was also considerably reduced. Pooled

estimates of error were therefore derived over all the animals tested (excluding sheep 29), separate estimates being obtained for samplings where the mean was a) greater than 60%, b) 30% to 60% and c) less than 30%.

Standard error of the mean percentage obtained for six plates:

>60% = $\pm$ 12.6; 30%-60% = $\pm$ 5.3; <30% = $\pm$ 2.5

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