I M M U N O L O G I C A L F A C T O R S A S S O C I A T E D

W I T H F O O T - R O T I N S H E E P

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

Foot-rot in sheep has troubled farmers for many years not because of any spectacular epidemics associated with high mortality but because the chronic lameness associated with foot infection, and its consequences, frequently interfere with farm husbandry. Methods of control and eradication have been known since 1940 but as they generally involve many hours of strenuous work, the development of more efficient methods of foot-rot control has been the aim of different research groups.

Two findings which suggested that foot-rot organisms might be accessible to blood-borne inhibitors helped change the attitude of researchers involved with this problem. The first was the successful use of parenterally administered antibiotics against foot-rot infection and the second was the demonstration that serum globulins were able to make contact with Fusiformis nodosus[1] in situ. The results of these experiments carried out at the McMaster Institute, Sydney led to a reappraisal of existing research projects and the formation of a new policy leading towards the development of experimental vaccines.

By 1970 when the writer's course of study began, it had been shown that bench scale vaccines conferred some protection against foot-rot infection and that vaccination was effective in stimulating resistance even after infection had become established. There were no methods available other than direct sheep challenge experiments for evaluating different types of vaccine and although the existence of anti-F. nodosus bactericidal antibody was known, there was still considerable doubt about the immune mechanisms involved in protection. There had been no investigation of the antigenic potential of F. nodosus products of growth or indeed of the bacterial cells themselves and as a consequence the influence of media components on the immunogenicity of the resulting culture had received little attention.

Before the foot-rot vaccines could be applied under New Zealand field conditions, two major problems had to be overcome. Firstly a large scale method of culture had to be developed that would produce economic yields of F. nodosus while retaining those antigens important for pro-

[1] Fusiformis nodosus was shown to be the primarily important and transmitting organism of sheep foot-rot.

tection of sheep. Secondly, an adjuvant system had to be chosen that would be effective in stimulating an adequate antigenic response but would cause minimal damage to the carcass.

To obtain the essential information upon which large scale modifications might be based, a series of experiments were designed to improve understanding of some basic aspects of foot-rot vaccine effectiveness. As a starting point for the investigation different methods of bacterial culture were used and the resulting F. nodosus cells were compared for gross morphology and ultrastructure as well as for their degree of immunogenicity after incorporation into various vaccine formulations. The serological response by the sheep to vaccination administered before and during F. nodosus infection was investigated to find a laboratory test system that would reflect the immune status of the animals. Experiments were also carried out to test the possibility of a humoral protective system, and the other contributor to specific resistance, cell mediated immunity, was investigated in normal and naturally infected sheep.

This programme of research produced results, that with the conclusions that were drawn from them, form the major part of the following thesis.

CHAPTER I

REVIEW OF THE LITERATURE

DEFINITION "Foot-rot is a contagious disease of the sheep's foot characterised by separation of a large portion of the hoof from the soft tissues due to a spreading infection immediately beneath the horn". (Beveridge, 1941).

The infection commences in the interdigital skin and horn separation is often seen first in the angle of the heels, but later moves forwards under the plantar surface and walls. In uncomplicated cases there is little suppuration: depending on under-foot conditions and the extent of the infection, the infected tissue is covered by a grey slime, or under drier conditions by dirty white caseous material. Both digits of the foot are invariably affected, each sheep often having more than one, but rarely all four feet involved. Foot-rot is a flock disease and under the environmental conditions most favourable for the associated bacteria, may spread rapidly until over 90 per cent. of the flock becomes infected.

HISTORICAL Foot-rot was well established in England during the 18th century and during the early part of the 19th century the disease was reported in France, Germany, Italy and the United States of America (Beveridge, 1959). It was first recognised as a contagious disease by Gohier (1810, quoted by Youatt, cited by Beveridge, 1959) and although this feature has been repeatedly demonstrated since (Marsh and Tunnicliff, 1934; Beveridge, 1938b, 1941; Thomas, 1962b; Roberts and Egerton, 1969) doubt persisted amongst flock masters for another 150 years (Beveridge, 1956). Uncertainty probably still does exist amongst some farmers to the

present day, principally because of important predisposing causes that largely determine the disease incidence during any one season (Beveridge, 1941; Graham and Egerton, 1968).

DIFFERENTIAL DIAGNOSIS

Digital Suppuration (Foot Abscess, Infective Bulbar Necrosis)

Murnane (1933) described two types of "foot-rot", one of which was the common subacute form which frequently passes to a chronic state, and the other was the less common acute or malignant type. Gregory (1939) advanced the view that both from the clinical and bacteriological points of view these two conditions were distinct entities. The former type was already under intensive study (Beveridge, 1935, 1938b) but for the less common acute type, Gregory (1939) proposed the name digital suppuration which was descriptive of the extensive pyogenic processes that are a marked clinical feature. In most of the cases he studied, the organism of importance was Fusiformis necrophorus, although Bacterium coli and Corynebacterium pyogenes were sometimes encountered in cultures and sometimes observed in smears. The term foot-abscess gradually took the place of digital suppuration, and this description included lesions of both the lamellar and bulbar regions of the foot (Deane, Davis, Epling and Jensen, 1955). The observations of Roberts, Graham, Egerton and Parsonson (1968) suggested that there were two distinct entities, viz. "toe abscess" affecting the lamellae, and "heel abscess" affecting the bulbar soft tissues. The most common and important form, "heel abscess", they renamed infective bulbar necrosis and found that it occurred most often in the hind feet of lambing ewes during cool, wet periods. Their experiments suggested that it was probably a true mixed infection in which the proliferation of each organism, F. necrophorus and C. pyogenes, was increased by the growth of the other. Earlier work by Roberts (1967a and b) had shown that F. necrophorus produces a leucocidal exotoxin that protects C. pyogenes from phagocytosis and thus enables it to proliferate in the tissues, while C. pyogenes produces a filterable factor that stimulates the proliferation of F. necrophorus in the tissues. The bulbar tissues become swollen and the characteristic sinus discharging into the interdigital area can be traced back to a necrotic process located in the digital cushion (Egerton and Graham, 1969). Infective bulbar necrosis can be differentiated from foot-rot by the swelling and obvious involvement of deeper tissues, the generally unilateral affliction of digits,

the suppurative changes, the failure to spread to other sheep in the flock, and the generally low incidence.

Toe-abscess (Lamellar Suppuration)

This is a far less common condition that occurs mainly at the toe in the front feet. The lesion arises from the opportunist infection of injured lamellae, originally brought about by trauma from overgrown feet, or by wall separation following laminitis (Egerton and Graham, 1969). If the original site of injury on the plantar surface becomes sealed by mud, faeces or exudate, infection can track upwards and produce a sinus above the coronet. "Shelly separation" lesions (Gregory, 1939; Littlejohn, 1967; Egerton and Graham, 1969) and horn tubule defects (Littlejohn, 1967), may become infected with F. necrophorus or other opportunist organisms. Both these conditions may be mistaken for "toe abscess", but because of the localised lesion site on one foot or digit, the presence of purulent discharge, the failure of the condition to spread to other sheep, and its sporadic occurrence, there is little chance of the disease being mistaken for foot-rot.

Foot-rot, infective bulbar necrosis and lamellar suppuration can occur simultaneously in a flock, in the same sheep, and even in the same foot (Belschner, 1939).

Characteristics of the organism F. necrophorus, and its role as a pathogen in animal diseases, have been the subject of a recent review (Simon and Stovell, 1969).

Axial Flap Lesion

This condition arises from an infective undermining of the axial wall, often traceable at the apex to a fine tract running up from the lower edge of the axial flap. The hoof is pathologically separated from the underlying tissue but remains closely applied to it (Veterinary Record, 1969). Individual animals only are affected at any one time but since conformation of the hoof tends to be hereditary, it is possible that a number of sheep of the same breeding line might be successively affected within a short period.

"Scald"[1]

Murnane (1933) referred to a condition that he had encountered in

[1] This term has been used in the past to describe any interdigital dermatitis. Interdigital dermatitis caused by F. necrophorus is now called ovine interdigital dermatitis. Interdigital dermatitis that is restricted to the interdigital integument, and caused by strains of Fusiformis nodosus is now called benign or non-progressive foot-rot.

sheep running on wet or swampy country and which closely resembled the early stage of foot-rot. It readily responded to foot-bath treatments and sometimes cleared up spontaneously, especially under drier conditions. Beveridge (1938c) recognised that "scald" might occur in foot-rot-free flocks and cause problems in diagnosis. The picture was somewhat confused by Thomas (1962a), who made a very worthy study of non-progressive foot-rot and the organism that caused it, but described the disease as "scald". Finally Parsonson, Egerton and Roberts (1967) studied "scald" in six foot-rot-free flocks. They showed the disease to be an acute necrotizing infection restricted mainly to the posterior interdigital skin and associated with an intense epidermal invasion by F. necrophorus. Experimental infection with F. necrophorus was dependent on prior skin damage and they suggested that catalase-positive diptheroids present in or on the interdigital skin would decrease the oxygen tension and Eh, much to the benefit of the anaerobe F. necrophorus. This newly defined condition of South Eastern Australia was named ovine interdigital dermatitis (Parsonson, et al., 1967). In order to avoid further confusion it has been recommended that the term "scald" should be dropped and replaced by ovine interdigital dermatitis to describe the specific F. necrophorus infection of the interdigital skin. By clinical appearance, this disease could be mistaken for foot-rot at an early stage but in ovine interdigital dermatitis, smears never show the presence of F. nodosus.

Other Conditions

The following conditions should be mentioned under Differential Diagnosis of foot-rot but in the author's opinion they all have strikingly different features that would make the chance of mistaken diagnosis highly unlikely.

In foot and mouth disease, separation of the hooves shows at the coronet and there are other systemic effects.

In suppurative cellulitis, there is patchy dermatitis of the lower limbs due to F. necrophorus and hoof involvement is not usual.

In "strawberry foot-rot", Dermatophilus congolensis infection appears in the form of multiple raised scabs between the coronet and knee, or hock. Other skin sites may be involved.

In post-dipping lameness, Erysipelothrix insidiosa infection is contracted through skin wounds and may spread to involve the sensitive laminae.

In contagious pustular dermatitis, the vesicle and pustule formation is followed by the development of indolent ulcers around the coronet. There are usually lesions present on the lips at the same time.

F O O T - R O T

Economic Importance

General

Even during the post-depression years when sheep values had made little recovery, the economic importance of foot-rot was well recognised (Marsh and Tunnicliff, 1934). Murnane (1933) referred to the loss of condition of affected animals, the depression of flock sale-value because of the existence of foot-rot in individuals, and the cost of constant treatment. Australian authorities have recognised the importance of this disease by maintaining a foot-rot research programme from 1931 until the present day, and their increased effort in the last seven years has involved a large professional staff. In New Zealand, interest has been sporadic but has been maintained steadily during the last five years.

The systemic effects of foot-rot infection appear to arise largely from pain. This causes lameness and therefore alterations in grazing behaviour. The consequences of unsatisfactory nutrition are loss in body weight, effects on wool growth, increased risk of pregnancy toxaemia, and ultimately starvation.

Calculated Losses

Many of the losses referred to above have been costed. For instance, in feeding sheep in Great Britain, Hunt (1958) found the loss of carcass value attributable to lameness, in spite of treatment for foot-rot, amounted to 9/4d per head and in another trial, Littlejohn (1964) calculated a loss of nearly 10/- per head before treatment, and an actual loss of 5/- at the time of slaughter five and a half weeks later.

To these losses must be added the cost of labour and materials associated with the treatment of individuals and flocks. In Italy, it was estimated that 1700 million lira[2], based on current prices, was the annual loss from all causes due to foot-rot, and this was in a population of

[2] 687.2 lira = $N.Z.

10 million sheep (Coppini, 1949). In New Zealand in 1955, the average cost of foot-rot treatment was about $6.00 per 100 sheep carried and of 100 sheep treated, was about ten times as much; $60, or about 63 cents per sheep (Bevin, 1955). Over 60 per cent of the farmers who replied to this questionnaire had foot-rot in their flocks every year. Ensor (1957) estimated that losses due to foot-rot in New Zealand amounted to $2 million annually. Pryor (1957) in Victoria, costed an eradication campaign in which labour plus materials charges were 2/3-3/6 per sheep at a time when labour cost 6-8/- per hour. About the same period, Stewart (1957) in Queensland, found labour charges were eight times as costly as those for medicaments. Ten years later, foot-rot in New South Wales and Victoria was estimated to cost woolgrowers $16 million annually because the loss of production was sometimes as high as $3 per sheep and once the disease was established, all cases had to be treated, which cost another 50 cents per sheep per year (Moule and Stephen Smith, 1967).

Incidence and Prevalence

From the earliest times, writers recognised that foot-rot occurred wherever sheep were farmed provided there were periods of rain and warmth. Wetness per se was not credited with causing the disease in Australia (Murnane, 1933) and in the United States of America, Marsh and Tunnicliff (1934) showed that a heavily foot-rot contaminated wet pasture, when drained, lost its infectivity within 15 days. Under conditions highly favourable for foot-rot transmission, which as defined by Beveridge (1941) were lush pasture, dampness and fairly warm weather, 90 per cent of a flock might become infected (Belschner, 1939; Thomas, 1962a). Because of the predisposing climatic influences, there may be great differences in incidence between seasons (Beveridge, 1941); and principally because of the state of pasture, between farms in one district (Ensor, 1957; Littlejohn, 1961; Whitten, 1971), Littlejohn (1966-67) noted that there was little foot-rot amongst hill sheep where soils were acid but extensive liming resulted in a higher incidence. She associated this difference with the greater activity of Fusiformis nodosus protease in an alkaline medium.

Graham and Egerton (1968) believed that moisture was the most important single factor. However, even in suitably wet conditions, foot-rot only spread when the mean ambient temperature was above 10^{o}C, and by using the two criteria of rainfall and temperature, they were able to map the likely foot-rot regions in Australia. The areas defined, closely followed those localities where foot-rot was known to occur.

Murnane (1933) and Beveridge (1941) claimed that the finer woolled breeds of sheep were less resistant to initial infection than coarser

woolled breeds and subsequent Australian experience has confirmed that Merino sheep have more feet affected, less interdigital space-only infections, and experience less spontaneous resolution than Merino/ Border Leicester cross sheep.

Whereas Murnane (1933), Gregory (1939) and Littlejohn (1966-67) found that lambs were as susceptible as adults, Beveridge (1941) found that sheep in their first year were more resistant to infection than adults, and rams, possibly because of their greater weight, more susceptible than ewes or wethers. Littlejohn and Herbert (1968) observed that ram lambs had more feet per lamb affected than ewe lambs and that in all lambs, more hind feet were infected than fore.

In some husbandry and feeding experiments, Selinanov (1955) showed that sheep kept under poor conditions and on a diet deficient in calcium, phosphorus and carotene, contracted foot-rot by natural transmission, whereas satisfactorily fed and housed sheep remained free of foot-rot.

Aetiology

Causative Agent

Murnane (1933) had shown that "pus" from foot-rot infected feet was capable of setting up typical infection by simple interdigital application and especially when the inoculated sheep were kept under wet conditions. Beveridge (1934) reproduced typical foot-rot with repeated interdigital applications of *Strongyloides* larvae together with necrotic material from a foot-rot lesion. But further understanding of the disease was severely hampered because at that time no single bacterial species had been identified in smears, isolated from infective material and subsequently shown capable of consistently reproducing typical foot-rot.

F. necrophorus was seen in smears from most infected feet, it had been isolated repeatedly from typical foot-rot cases and in tissue sections it was often the predominating organism. From the attempted infection experiment results there were some who believed *F. necrophorus* was the causative organism (Mohler and Washburn, 1904, cited by Egerton, Roberts and Parsonson, 1969; Gonharov, 1955), others who partly agreed but recognised the need for some other important unidentified predisposing agent (Marsh and Tunnicliff, 1934), and those who considered *F. necrophorus* an important secondary invader (Murnane, 1933). Beveridge (1935) was unable to produce foot-rot using *F. necrophorus* and he concluded that it was not the primary causative agent.

After repeatedly seeing a thin filamentous organism in smears, Beveridge (1938a) subsequently isolated it, but was unable to reproduce foot-rot with the culture. This was his organism "X", subsequently named Spirochaeta penortha. At this time "V-F" blood agar which consisted of a peptic digest of ox muscle and liver in blood agar, and a serum enriched agar were being used for attempted isolations from foot-rot material. On the serum enriched agar, small sunken ("etched") colonies appeared, the organisms of which were similar in appearance to those seen in small numbers in smears, and which Beveridge (1938a) had called organism "K". Initially, whole plate cultures from the serum-enriched agar produced typical foot-rot in 2 out of 9 cases, and then a culture of organism "K" together with Sp. penortha and an isolated fusiform, when used together on scarified interdigital skin produced typical foot-rot. Organism "K" by itself caused a less severe foot-rot infection. This experiment (Beveridge, 1938a) was the first time that pure cultures of organisms had been used to reproduce typical foot-rot. Beveridge (1938a) believed organism "K" probably belonged to the genus Bacteroides but as cultures did not grow readily on ordinary culture media and as the genus classification had not been widely accepted, he subsequently suggested the name Fusiformis nodosus. Beveridge (1941) believed that F. nodosus with Sp. penortha were responsible for typical field foot-rot. These observations were made originally in Australia, and confirmed by him in the United States of America.

In experimental foot-rot infections, Thomas (1962b) found that F. nodosus alone was capable of infecting a scarified site but he did not dismiss the possibility of secondary invasion by pyogenic bacteria that would add to the severity of infections caused by F. nodosus. Scarification and subsequent inoculation with F. nodosus to the horn base of one sheep caused typical foot-rot type changes and ultimately shedding of the horn.

After following the development of the disease by histological examination and carrying out experiments testing their resulting hypothesis on causation, Egerton, Roberts and Parsonson (1969) doubted whether F. nodosus could cause foot-rot without the assistance of F. necrophorus. Roberts and Egerton (1969) showed that the induction of foot-rot by F. nodosus required a factor present in faeces. When there was faecal contamination of the foot, the interdigital stratum corneum was usually colonised by F. necrophorus; an association which suggested that this organism might be the required factor in faeces. The highest incidence of infection resulted when the inoculum also included C. pyogenes. These three organisms exhibit a complex synergistic association through exotoxins or other diffusable factors

(Roberts, 1967b; Roberts and Egerton, 1969).

Roberts and Egerton (1969) concluded that foot-rot is caused by the association of F. nodosus, the transmitting agent, with F. necrophorus, a normal inhabitant of the ovine environment. The initial establishment of these two organisms is facilitated probably by the metabolism of diptheroids found at the surface of the interdigital integument (Parsonson et al., 1967).

Classification of Fusiformis nodosus

After the original isolation and cultivation of his organism "K", Beveridge (1938a) thought that it probably belonged to the genus Bacteroides. At that time the genus Bacteroides had not been widely accepted as valid by taxonomists and as he regarded the organism as a new species, Beveridge (1941) proposed the name Fusiformis nodosus (L. "nodosus" = knobbed) within the more conservative classification used by Topley and Wilson (1937). In journal publications since then, the Australian workers have, without exception, continued to use the term Fusiformis nodosus, whereas two recent New Zealand papers (Skerman, 1971; Skerman and Cairney, 1972) refer to Bacteroides nodosus (Beveridge) Mráz. et al. The latter title appears in Index Bergeyana (Buchanan, Holt and Lessel, 1966) as a legitimate name.

In those texts where the disease of ovine foot-rot is described, the authors have continued to use Beveridge's terminology, (Marsh, 1958; Beveridge, 1959; Soltys, 1963; Wilson and Miles, 1964) except for Whitten (1971) who refers to Bacteroides nodosus. Smith and Holderman (1968) also used the term Bacteroides nodosus and they included this organism amongst the other anaerobic Gram-negative non-sporing bacteria.

In the 6th edition of Bergey's Manual (Breed, Murray and Hitchens, 1948), only passing reference is made to the foot-rot causative organism. Under the second genus of the family Sphaerophoraceae Prévot, namely Fusobacterium (Knorr), they mentioned Fusiformis nodosus Beveridge, synonym Actinomyces nodosus Hagan, "because they appear to be related to the organisms in the genus Fusobacterium". In the 7th edition of Bergey's Manual reprinted in 1966, (Breed, Murray and Smith, 1957), there is no reference to F. nodosus or its synonyms. A completely new name, Ristella nodosa (Beveridge) Prévot 1948, within the genus Ristella, family Ristellaceae and Order Bacteriales was proposed by Prévot, Turpin and Kaiser (1967).

In the same edition (Breed et al., 1957) it is stated that bacteria of the genus Bacteroides are "found in the alimentary and urogenital tracts of man and other animals;". In all other respects, with the possible exception

of ability to ferment glucose, the causative organism of foot-rot complies with the requirements for the sub-genus Ristella Prévot 2 aaa. Thus habitat or source of origin is the sole criterion that stands in the way of its inclusion within this classification.

As the first name proposed by the isolator (Beveridge, 1941) is still widely accepted, the primary causative and transmitting organism of foot-rot will be referred to as Fusiformis nodosus (F. nodosus) throughout this thesis.

Isolation and Cultural Requirements

Using a 25 per cent.horse serum enriched "V-F" medium, Beveridge (1938a) isolated F. nodosus in the form of characteristic etching colonies. Anaerobic conditions were maintained by 0.1 per cent.cysteine hydrochloride incorporated into the medium, and a 5 per cent.carbon-dioxide hydrogen gas mixture was used to replace the air in the culture jar. A more detailed account of the organism's appearance and cultural requirements followed (Beveridge, 1941). Beveridge described how the horse serum additive could be replaced by gelatin but without one of these, growth on "V-F" agar or in "V-F" broth was unobtainable, or only very slight. The organism's morphology changed on subculture or when growth conditions were suboptimum, so that the knobbed ends became less prominent and the distinctive bacilli became more coccoid in form. Two types of colonies were recognised but subcultures from either gave rise to a mixture of both types.

For approximately five years from 1951, when ostensibly the same medium that had been used before was tried again in Great Britain and Australia, it failed to support growth in repeated attempts at isolation of F. nodosus although the organism was readily detectable in stained smears of the donor lesions (Beveridge, 1956). This lead to replacement of the "V-F" formulation by a simplified medium consisting of 2 per cent.ground sheep hoof and 'Lab-Lemco' meat extract in agar (Thomas, 1958). It was shown that the actual particles of horn were needed, as growth requirements were not met by the soluble fraction after filtration. For primary isolations, filter papers were placed in the petri dish lids to absorb water of condensation. Subsequent work enabled Thomas (1963) to formulate a liquid medium for the satisfactory cultivation of F. nodosus. This consisted of 2 per cent.Difco trypsin 1:250, and 1 per cent.acid hydrolysate of sheep hoof, autoclaved with 0.1 per cent.thioglycollic acid or 1 per cent.glucose or galactose. He found that an aqueous extract of pancreas could substitute for the trypsin and Difco proteose peptone could substitute for the acid hydrolysate of hoof.

A considerable advance in the technique of isolation of F. nodosus from field material was made by Merritt (1960), who used 0.25M sucrose as a diluent for the plate inoculum. He claimed that this material helped to control the growth of contaminating organisms and to prevent swarming by the motile fusiform.

Enzyme Production by F. nodosus

Amongst the biochemical properties of F. nodosus, Beveridge (1941) refers to hydrogen sulphide production, a soft clot being formed in litmus milk which is later digested, the partial digestion of meat particles in cooked heart medium and after 4 to 6 weeks culture the development of tyrosine crystals. Sucrose, maltose, galactose, lactose, dextrose and mannite were not fermented. Thomas (1958) grew F. nodosus in 'Lab-Lemco' meat extract broth containing ground hoof, and these particles were digested slowly during growth of the organism with the formation of tyrosine crystals.

The etching of agar medium by colonies of F. nodosus (Beveridge, 1938a, 1941) and the observations on digestion of meat and hoof particles, suggested that an examination of the enzymes produced by F. nodosus would be fruitful and might indicate the mechanism of pathogenesis. Thomas (1962b) suggested that the separation and liquefaction of the stratum corneum, and the detachment and disintegration of partially cornified cells, appeared to result from a digestive process and was probably caused by a keratinase elaborated by F. nodosus. Two New South Wales strains, one Victoria strain and one English strain of F. nodosus were shown by Thomas (1964a) to produce proteolytic enzymes that digested azocasein and sheep hoof powder in the presence of cysteine (Thomas, 1964b). Preparations of protease obtained at the end of the logarithmic phase of growth and again at the end of the stationary phase had similar properties, and both were inactivated at temperatures greater than 50°C.

In experiments carried out at Wallaceville Animal Research Centre (New Zealand Ministry of Agriculture and Fisheries, 1969-70) it was found that certain amino acid decarboxylases and amino acid deaminases are formed by F. nodosus and these act further on the products of proteolytic digestion.

Strains of F. nodosus Associated with Benign or Non-Progressive Foot-rot

Many pastoralists in Australia reported to Beveridge (1941) that outbreaks of foot-rot were usually preceded by the condition they termed "scald". This latter condition (Murnane, 1933) was in all probability the same that is now called ovine interdigital dermatitis and which is caused

by F. necrophorus (Parsonson, et al., 1967). But the "scald" referred to by Thomas (1957, 1962a) and Littlejohn (1966-67) is characterised by a severe interdigital dermatitis with the occasional slight separation of horn in the axial heel area. In this condition, F. nodosus-like organisms were seen in smears (Thomas, 1957) and two strains of F. nodosus were successfully isolated from the interdigital area (Thomas, 1962a). These isolates behaved like typical F. nodosus strains in sugar fermentation tests, in that they failed to produce acid or gas from a large range of sugars including glucose.

The striking difference about the new F. nodosus strains was that in culture they produced only about half as much protease as F. nodosus strains isolated from typical foot-rot cases (Thomas, 1962a). There was also a serological difference (see later in this chapter under Agglutination.).

Thomas (1962a) found that these different strains did not revert when used to reproduce the field disease experimentally i.e. "typical" strains caused typical foot-rot and interdigital dermatitis strains caused very little or no horn separation. However, Alexander (1962) concluded from his transmission experiments that a change in clinical form could occur. The work of Thomas (1962a) was confirmed by Egerton and Parsonson (1969) who distinguished two clinical forms of foot-rot. Typical foot-rot they called "virulent or progressive foot-rot", and the interdigital dermatitis associated with less proteolytic strains of F. nodosus they called "benign or non-progressive foot-rot". In their experiments, strains did not revert but "benign" cases could be converted to progressive foot-rot by superinfection with a typical strain. This last experiment showed that the clinical nature of the condition in an infected foot is due to the virulence of the infecting F. nodosus strain, and not to host or environmental factors.

Pathology

Macroscopic Changes

The following account, (after Beveridge, 1941), covers the important gross features and these have been confirmed by different workers in Australia and other countries.

The initial lesion is usually a mild inflammation of the interdigital skin which is soon followed by a break in the skin horn junction on the axial aspect and separation of the soft horn from adjacent epithelium. Over the next 5 to 15 days there is progressively more horn separation from its epithelium by the spread of infection around the heels and across the

Plantar surface. By this time there is present a little malodorious pus and necrotic detritus. The infection may extend under the abaxial walls so that all the hoof is separated from the soft tissues and remains attached only at the coronet. Both digits are invariably involved.

Beveridge (1941) went on to describe how the new growth of horn over the plantar surface usually becomes involved in the destructive process by reinfection from the interdigital area, and how granulation tissue often arises as a consequence of necrosis of the laminae. Pryor (1954) commented on the problem of excess granulation tissue, and Littlejohn (1967) blamed the failure to expose fully, chronic granulating lesions to treatment, for most of the cases of persisting infection in a flock.

Histopathology

Beveridge (1941) noted inflammatory processes in the more superficial layers of epithelium just beneath the keratinized stratum. There was cellular degeneration, infiltration with polymorphonuclear leucocytes and some pus formation that led to progressive accumulation of exudate in the intercellular spaces and pressure atrophy of surrounding cells. He regarded the changes as a superficial ulceration because new horn grew so quickly after surgical treatment that the basement membrane could not have been seriously damaged. In sections of feet from field cases of foot-rot, Deane and Jensen (1955) noted that epithelial cells of the stratum granulosum and the superficial stratum spinosum had undergone swelling, hydropic degeneration and necrosis that resulted in separation of the corneum, from the foot.

After scarification of the inoculation site, Thomas (1962b) experimentally infected sheep with F. nodosus alone. Complete separation of the horn occurred in 4 of 6 feet but as early as 9 to 12 days post-infection, F. nodosus was seen invading the stratum lucidum and the prickle and granular cells showed cytoplasmic degeneration. By eight weeks, marked changes had occurred in the stratum spinosum. Cell lysis resulted in vacuole formation, and small abscesses, caused by secondary infection with pyogenic bacteria, were commonly seen.

Bacterial Invasion

The brief history that follows is relevant to the results of later histological work, in which penetration by bacteria of various strata of the tissues was studied. Re-examination of this aspect at the McMaster Institute, Sydney led to a much better understanding of the roles played by various bacteria in the pathogenesis of foot-rot.

Murnane (1933), and Marsh and Tunnicliff (1934) constantly found F. necrophorus in even early foot-rot lesions but Beveridge (1935) failed

to reproduce the disease using pure cultures. Within three years, Beveridge (1938a) was able to report the first description of F. nodosus and at the same time he observed in foot-rot lesions a motile fusiform that resembled F. necrophorus.

Beveridge (1941) summarised his conclusions concerning the bacterial flora as follows,

"Lesions of foot-rot always contain a gross mixture of bacteria comprised largely of cocci and corynebacteria near the surface of the lesion. F. necrophorus is frequently present and there may be large numbers of a pleuropneumonia-like organism. Spirochaetes with typical morphology are sometimes, but not frequently, present. In the active areas of lesions the most prevalent organisms usually are Sp. penortha and the motile fusiform, these being constantly present. F. nodosus is constantly present but in relatively small numbers."

In sections stained with Giemsa or by the silver impregnation method, Beveridge (1941) observed that the motile fusiform, or F. necrophorus, penetrated furthest into the tissues. The organisms often appeared as a dense felted mass penetrating along fissures in the epidermis. Bacteria were not usually found beneath the epidermis and when seen in sections, F. nodosus was mostly stationed superficially. Sp. penortha was sometimes seen penetrating the epidermis in large numbers, either alone or together with the motile fusiform. Deane and Jensen (1955) saw neither bacteria nor spirochaetes at the point where the lesion met healthy tissue, but mixtures of unidentified cocci, rods and filaments, were located on the surface of the area of separation.

Thomas (1962b) thought that the dearth or apparent absence of F. nodosus in sections of naturally infected feet, seemed inconsistent with the production of the characteristic lesion of the disease by this organism. Therefore, in experimental infections he used pure cultures of F. nodosus and in order not to further complicate his study of the specific changes brought about by this organism , he reduced secondary contamination as far as possible. On the 12th day of infection, F. nodosus was seen invading the stratum lucidum beyond the point of cleavage of the epidermis. Later neutrophils cleared the ulcerated epithelium of F. nodosus but the organisms were numerous in the stratum lucidum and stratum corneum, where neutrophils could not penetrate.

When Egerton et al. (1969) re-examined sections from naturally acquired and experimentally produced foot-rot lesions, they found that cocco-bacilli and diptheroids were relatively superficial in location, but spirochaetes

and motile fusiforms penetrated more deeply and grew mainly in tissue already destroyed by the pathogenic process. A superficial invasion by F. necrophorus, probably derived from faeces, always preceded the establishment and growth of F. nodosus. The invasion of the epidermal matrix[3] by F. nodosus was followed by deeper penetration of F. necrophorus, shown by Roberts and Egerton (1969) to be due to a synergic association between these two organisms. Different stages in the recurring cycle of infection, destruction, removal, regeneration and reinfection of the epidermis were found by Egerton, et al. (1969) at any one time in interdigital skin and hoof matrix. They found that horn separation was due to the inflammatory process within the epidermal matrix and that there was no evidence of separation due to a direct bacterial attack on the horn itself, as suggested by Thomas (1962b).

Treatment

Topical Treatment

Murnane (1933) stressed the need for a thorough paring of infected feet to expose the active lesion beneath necrotic and separated horn, a principle that had been recognised by Youatt (1837; cited by Beveridge, 1941). This requirement has been stressed by nearly every observer up to the present day but because the work is exhausting and laborious, it is difficult to obtain complete co-operation from farmers working under field conditions. Detailed explanations have been given on how to trim feet and for the proper use of foot-baths containing formalin or copper sulphate by Murnane (1933), Marsh and Tunnicliff (1934), Beveridge (1935, 1941), Belschner (1939, 1953), Pryor (1954) and Littlejohn (1967).

It was found that retreatment of established foot-rot cases with any medicament must be carried out every few days to obtain the best results (Murnane, 1933; Pryor, 1956; Toop, 1957; Stewart, 1957). Formalin was found to be better than copper sulphate for two important reasons. Firstly, it did not stain wool and, secondly, Stewart (1954b) found that at concentrations greater than 2 per cent., the bactericidal effect of formalin was not diminished by even gross faecal contamination, whereas copper sulphate was affected in this way.

[3] Sisson and Grossman (1947) group the strata lucidium, granulosum, and spinosum into a single stratum germinativum, for which the term matrix is also used.

In a search for topical medicaments better than formalin, a number of antibiotics and synthetic compounds were tested - Penicillin (Coppini, 1951, cited by Littlejohn, 1966-67; Stewart, 1954a; Mutovin, 1956), Chloramphenicol (Stewart, 1954a and b; Sinclair, 1957), Chlortetracycline (Stewart, 1954a; Stewart and Thomas, 1957), "Cetavlon" (Forsyth, 1957), Oxytetracycline (Harriss, 1958) and Dichlorophen (Sinclair, 1957; Hart, Malone and Sparrow, 1962). Comparisons were made between medicaments after all affected feet had been pared. "Control" feet usually received no treatment other than the paring, and in nearly every trial, one group was treated with 5 to 10 per cent. formalin as a "positive" control. The tendency for spontaneous cure after paring varied between flocks in a single trial. Stewart (1954b) had recovery rates in untreated controls that varied from 0 to 85 per cent., whilst comparable figures for Hart et al. (1962), were 0 to 79 per cent. The newer medicaments were always as good as, or better than formalin and when a difference between them was demonstrated, chloramphenicol formulated in an alcohol base was usually shown to be the most effective.

The results of topical treatment of benign foot-rot have always been disappointing and Egerton and Parsonson (1969) suggested that the hyperkeratosis of the interdigital skin may enable F. nodosus to survive in an inpenetrable site.

Parenteral Treatment

Stewart (1954a) showed that F. nodosus was susceptible in vitro to penicillin and to a lesser extent to streptomycin. These activities were confirmed in vivo by Egerton and Parsonson (1966a) who showed encouraging cure rates of affected sheep using a mixture of 70,000 units penicillin/Kg body weight with 70 mg streptomycin/Kg body weight given once by intramuscular injection. When sheep were returned to wet pasture after treatment, only 50 per cent responded, but when sheep were returned to dry pastures or held in sheds for 24 hours after injection, the treatment was more effective and most effective if the feet were trimmed and formalin boot-bathed as well (Egerton, Parsonson and Graham, 1968). Harris (1968) produced better results with parenteral antibiotics when he severely pared rather than trimmed infected feet and this he felt rather detracted from the advantages. On two other farms his results using antibiotics were no better than conventional pare and foot-bath techniques, but Egerton (1968) suggested these poor results may have been due to wet foot conditions directly after treatment.

Control

Murnane (1933) set down guidelines for control that involved regular trimming of overgrown feet, early recognition of foot-rot infection and the use of formalin foot-baths for non-infected as well as infected sheep.

Eradication

As early as 1935, Beveridge raised the possibility of complete eradication by treating cases during periods when the disease was in abeyance. After Marsh and Tunnicliff (1934) showed that foot-rot infection rapidly disappeared from pastures that were allowed to dry out, Beveridge (1938a and b) followed up with studies on the viability of F. nodosus under field conditions. He confirmed that the organism could not remain infective outside the host for more than 14 days, and on the basis of this, devised a scheme for control and eradication (Beveridge, 1938c, 1941), that has not needed substantial modification since (Littlejohn 1966-67).

Eradication schemes based on the recommendations of Beveridge (1941) have been successfully carried out in Italy (Coppini, 1951), in Russia (Mutovin, 1956), in New Zealand (Filmer, 1948; Ensor, 1957), in Great Britain (Littlejohn, 1961), in Australia (Beveridge, 1941; Pryor, 1954; Thomas, 1957; Toop, 1957; Hayman and Triffit, 1964; Moule and Stephen Smith, 1967), and in the United States of America (United States Animal Health Association, 1968).

Prevention

The risk of reinfection from purchased replacement stock had been recognised and largely overcome by quarantine measures, careful foot examination, paring and treatment. It was realized that as goats were natural hosts for foot-rot, they had to be removed (Beveridge, 1941; Ensor, 1957) but the danger of cattle acting as carriers by naturally acquired infection was not recognised until later (Shenman, 1962; Alexander, 1962; Egerton and Parsonson, 1966b; Morgan, 1969; Wilkinson, Egerton and Dickson, 1970).

On many properties where eradication was carried out and stringent preventive measures have been maintained, foot-rot has not been re-introduced.

Immunological Aspects of Infection

Agglutination

Beveridge (1941) found that American and Australian strains of F. nodosus gave a high agglutination titre in the homologous system and a much lower but significant titre in the heterologous series. Sera from normal rabbits and sheep, and from sheep that had been infected with the homologous strain of F. nodosus gave no agglutination reaction. There was only slight, or no cross agglutination, between strains isolated from New South Wales, Victoria, and Tasmania (Gregory, cited by Beveridge, 1941).

The two strains of F. nodosus isolated from cases of benign foot-rot by Thomas (1962a), were not serologically identical with virulent field strains. Antisera prepared against the benign foot-rot strains agglutinated a typical F. nodosus strain to low titre. The one benign foot-rot strain tested, failed to absorb agglutinins from antiserum raised against the typical F. nodosus strain.

Resistance to Infection

Beveridge (1941) showed that although specific infection could persist in the sheep foot for up to three and a half years, this conferred no protection. He found that other feet of an already affected sheep could be naturally or experimentally infected, and after resolution or successful treatment, previously infected feet were immediately susceptible to re-infection. Murnane (1933) believed that because of the very nature of the disease vaccination did not hold out great possibilities, and Beveridge (1941) agreed with this view, in spite of having apparently induced some resistance in one sheep with a trial F. nodosus vaccine. In a few sheep, infection of an individual a second time, proceeded more slowly and lesions were milder. At this time, Beveridge (1941) believed eradication was the better approach to field control and he did not undertake any serious investigation with vaccination. As he put it, "from consideration of the chronic and relatively superficial nature of the lesions, foot-rot is not the type of disease in which the prospects of successful immunisation would be considered good."

Studies on diffusion of parenterally administered antibiotics (Egerton et al., 1968) stimulated renewed interest in the study of serum components found after vaccination. Originally, Beveridge (1941) had found that sheep serum was inhibitory to F. nodosus and later, workers at the McMaster Institute, Sydney, found that the injection of guinea pigs, rabbits and sheep with F. nodosus produced IgG class antibodies that were

bactericidal in the presence of complement (Commonwealth Scientific and Industrial Research Organisation, 1966-67, 1967-68, 1968-69; Egerton and Merritt, 1970). By treatment of lesion smears with fluorescin labelled anti-globulin, it was shown that serum antibodies in infected animals made contact with F. nodosus organisms in the foot (Commonwealth Scientific and Industrial Research Organisation, 1967-68).

Trial vaccines made from F. nodosus with adjuvant, and F. nodosus plus F. necrophorus with adjuvant, were used in three separate locations (Commonwealth Scientific and Industrial Research Organisation, 1967-68). In one trial, foot-rot spread less rapidly amongst vaccinated animals and in this group those lesions that did develop were less extensive than in controls. In the two other trial locations there was no protection afforded by vaccination against initiation of infection. However, in vaccinated animals, lesions of foot-rot were confined to the interdigital skin and self-cure occurred more rapidly than in control groups.

Annual Reports of the McMaster Institute gave an indication of the way their research programme was progressing but detailed reports did not appear until 1970 onwards and accordingly these published accounts of results are referred to in appropriate chapters of the main text.

CHAPTER II

LIGHT MICROSCOPE AND ELECTRON MICROSCOPE STUDIES OF FUSIFORMIS NODOSUS

Introduction It is known that cultural conditions and media constituents can influence synthesis of the cell wall and thereby may determine antigenic function (Rogers, 1970; Weinbaum, Kadis and Ajl, 1971). Accordingly the morphology of F. nodosus (J3/22) cultured by different techniques, was studied using the light and electron microscopes. F. nodosus cells contained in foot-rot necrotic material were included in the study because their morphology under conditions of natural infection provided the only standard against which cells grown on artificial media might be compared.

The opportunity was also taken to investigate the clear halo often seen around F. nodosus cells in smears from infected feet. Because the structure could be a type of capsule, indian ink stained preparations of bacteria were compared and the so-called "capsular swelling" technique was carried out. Filamentous appendages outside the cell envelope were searched for in electron microscope preparations and haemagglutinating activity of cells tested for as an indication of pili (fimbriae). Haemagglutinating activity was further defined for a number of F. nodosus strains according to their method of culture, and whether or not the activity was inhibited by D-mannose (Cruickshank, 1965).

MATERIALS AND METHODS

A. Light microscope studies

Organisms F. nodosus (J3/22) was used for all comparisons of culture

method. For each culture batch a freeze-dried vial was reconstituted on to hoof agar and four days later typical "rough" colonies were selected for subculture. "Rough" colonies were chosen because they appeared to be much the predominant type when conditions of culture had resulted in abundant growth. A second reason was that in the immediate vicinity of specific inhibitory antisera, colonies of F. nodosus on hoof agar grow in the"smooth" form (see Bacterial growth-inhibition by serum tests, Appendix XIV) and this was considered indicative of a change from the normal to an abnormal type.

Details of F. nodosus strains are described in Appendices III and IIIa.

Superficial scrapings of the interdigital integument contained F. nodosus organisms of low proteolytic activity. The donor ram was a badly affected case of benign foot-rot and from the same animal, F. nodosus (M9/4) was later isolated. (Appendix III).

Methods of culture Cells for examination were harvested from

i) a four day culture on hoof agar

ii) a 48 hr culture in biphasic medium

iii) a 24 hr culture in hoof broth

or iv) a 48 hr culture in hoof broth.

Smear preparations of the foot-rot infection were made from the interdigital detritus.

Gram stain This was carried out according to the method described in Appendix XVIII.

Methylene blue stain This was carried out according to the method described in Appendix XVII.

Capsular stain An impression smear from F. nodosus colonies grown on hoof agar, was flooded with Gunther Wagner indian ink and covered with a cover slip. Colonies of Escherichia coli growing on MacConkey agar were treated in the same manner and served as a control. The preparations were examined for evidence of capsular material according to the wet indian ink methods of Duguid (1951).

"Capsular swelling" (Neufeld) reaction Rough colonies of F. nodosus cultured for four days on hoof agar, were emulsified into two sheep sera that were undiluted or at dilutions of 1/10, 1/80 and 1/2560. One serum sample was from a normal adult sheep subsequently shown to be susceptible to foot-rot; the other was from a vaccinated sheep subsequently shown to be resistant to foot-rot infection. Colonies of E. coli were processed in

an identical manner and served as a control. The preparations were examined according to the method described by Duguid (1951) using phase contrast microscopy.

B. Electron microscope studies

Organisms and Methods of Culture The same strain of F. nodosus i.e. F. nodosus (J3/22), and methods of culture were used as for the light microscope studies.

Fixation methods Most preparations were duplicated using two of three methods of fixation as follows,

i) Double Fixation (after Karnovsky, 1965)

ii) Double Fixation (Williams and Luft, 1968)

iii) Fixation by Ryter and Kellenberger (1958).

The three methods are described in detail in Appendix V. Sections cut on the LKB "Ultrotome" were picked up on carbon coated formvar support films on 200 mesh copper grids. After staining with lead citrate (Reynolds, 1963) they were examined in a Philips EM200 electron microscope.

C. Red blood cell agglutination

Organisms F. nodosus (J3/22), F. nodosus (A8/C), F. nodosus (McM198) and F. nodosus (McM199) were compared (Appendix III). Cell suspensions in PBS were adjusted to 2 x 10^9 cells/ml.

Methods of Culture The organisms were grown for four days on hoof agar, for 48 hr on hoof broth or biphasic medium, or for 24 hr in GC broth.

Erythrocytes (RBC) Blood from adult guinea pigs was collected in acid citrate dextrose anticoagulant (Appendix XVIa) and the cells after washing were resuspended at 20 per cent v/v in PBS for storage at 4°C.

D-mannose (B.D.H., Palmerston North, N.Z.) This was held as a 2 per cent solution and used in the test at a final concentration of 0.5 per cent.

Test system Equivalent amounts in drops of RBC and F. nodosus cell suspension were mixed in cups of the standard MRC Perspex haemagglutination tray alongside an identical series to which D-mannose was also added.

A duplicate set of mixtures, again with or without D-mannose, was set out using F. nodosus cell suspensions that had been subjected to high speed treatment in an Omni-mixer homogenizer (Sowall, Norwalk, U.S.A.) in an attempt to remove the cell appendages.

The mixtures were allowed to react for up to 2 hr at 4°C. The low temperature was an attempt to prevent regrowth of pili. Haemagglutination was judged to have occurred when the RBC were prevented from settling as a discrete button and instead formed a continuous layer across the well.

RESULTS

A. Light microscope studies

i) Colony characteristics on hoof agar

The rough colonies of F. nodosus (J3/22) growing on hoof agar varied in size from 1-3 mm diameter according to the moisture present, they had a slightly crenated edge, a surface like ground glass or beaten copper, and they always left an etch mark or pit in the agar surface. With F nodosus (J3/22) in particular, the smooth, convex, butyrous colonies described by Beveridge (1941) were rarely seen, but with other strains, particularly before selection in favour of rough colonies had been applied, smooth colonies of about 1 mm diameter were commonly seen. Where heavy seeding had led to confluent growth, the agar surface appeared to be completely covered by a thin rough surfaced pellicle which exhibited the crenated or slightly fimbriate edges only at the limits of bacterial growth. An intermediate colony type was noted in all strains of F. nodosus and this consisted of a rough surfaced, crenated edge colony with the mucoid-type centre. In F. nodosus (J3/22) cultures on hoof agar, this intermediate type, although uncommon, was seen far more often than the smooth colony. In the case of F. nodosus (cbM1) (Appendix III) a consistent feature of the predominant type of colony was a mucoid centre with two or three terraces down to the crenated edge.

ii) Morphology of cells grown on hoof agar

Smears made from each of the different colony types showed an identical gross cellular morphology. When the bacteria were stained by Gram's method they appeared dark pink in colour with much bluer swollen ends and there were sometimes indistinct bluer areas in the straight body of the cell. A narrow clear zone was apparent around the cell envelope. The approximate dimensions of F. nodosus were 0.5-1 μm wide by 3-15 μm long and in this respect they were sometimes narrower and longer than the specific organism seen in smears from foot-rot infected feet. (Figures II.1, II.2, II.3, and II.4). An exception was F. nodosus (McM198) (Appendix III) in which the cells were far shorter, thicker and often had less distinct end swellings.

iii) Morphology of cells grown in hoof broth

Gram staining of cells taken during or towards the end of the log division phase showed a proportion of organisms that were poorly stained and swollen while other cells were only half the size of those grown on hoof agar.

iv) Morphology of cells grown in biphasic medium

The appearance of cells grown in this system was intermediate between

cells from solid and liquid culture. The cells were the same size as broth grown organisms but they usually stained well and held their shape provided cultural conditions had been satisfactory. Very often there were amorphous pink masses closely associated with one pole of the bacilli and occasionally groups of rod shaped structures could be seen lying in an amorphous matrix amongst the typical F. nodosus cells (Figure II.5). Cultures grown in GC broth contained organisms that looked the same as biphasic medium cultured organisms except that the additional "structures" were not seen.

v) Morphology of cells from foot-rot lesions

Smears made from the interdigital detritus of two rams with non-progressive or benign foot-rot (Thomas, 1962a; Alexander, 1962; Littlejohn, 1966-67; Egerton and Parsonson, 1969) always showed numerous F. nodosus-like organisms. These were sometimes thicker and shorter than F. nodosus (J3/22) grown on hoof agar and the cells had prominent enlargements at one or both ends (Figure II.3). Large polychromatic granules were commonly seen at the poles and often in the body of the bacillus when smears were stained with methylene blue in particular or by Giemsa's method (Figures II.6 and II.7). The characteristic "rowboat" arrangement (Beveridge 1941, 1956) caused by the close association of F. nodosus with other smaller bacilli was a consistent feature in smears from one individual (Figure II.4). Fusiformis nodosus was seen less regularly in smears prepared from necrotic epidermis exposed by the lifting horn, and these organisms appeared shorter, thicker and had less well developed terminal enlargements. Very often a clear zone was visible around the cell envelope.

vi) Capsular staining There was no evidence of a capsule or any free slime.

vii) "Capsular swelling" There was no evidence of a capsule seen by this technique.

B. Electron microscope studies

i) Morphology of cells in interdigital scrapings Sections of the scrapings showed a large amount of cell debris and a variety of bacteria. At certain cell borders there were areas where a spiral organism had been sectioned repeatedly (Figure II.8). From the general appearance and unusual wave formation this organism could be a spirochaete, possibly Spirochaeta penortha (Beveridge, 1941). There were large numbers of F. nodosus-like organisms particularly in the mixed flora on the surface or within epithelial cells (Figure II.9). Both Karnovsky and Ryter Kellenberger

methods of fixation showed the layered cell envelope typical of Gram-negative bacteria (Mahoney and Edwards, 1966; Shively, Decker and Greenawalt, 1970) and inside, prominent electron-dense granules. These were sometimes small, up to ten in number and associated with the nucleoplasm; or they occurred singly, were darker and usually larger (Figure II.9). In preparations fixed by the Karnovsky method, there was stain deposited around the borders of some cells but both fixation methods used revealed the filamentous appendages of the cell envelope (Figures II.9 and II.10). Dividing forms of bacteria were commonly seen.

ii) Morphology of cells grown on hoof agar

The Karnovsky method of fixation showed four layers of cell envelope counting the cytoplasmic membrane (Figure II.11) but no extracellular structures could be seen. However, by the Ryter Kellenberger method, filamentous appendages of varying length were obvious (Figures II.12 and II.13) and other longer filaments that appeared to be always associated with the poles (Figures II.14 and II.15). In both preparations there were small electron-dense bodies spread through the nucleoplasm. In comparison with other methods of culture, the organisms were very large and many showed terminal swellings. Both methods of fixation showed up areas that appeared to be interposed between the cytoplasmic membrane and the cell wall outer layer and that consisted of convoluted membranes (Figure II.16). These structures were not seen in cells cultured by other means.

iii) Morphology of cells grown in biphasic medium for 48 hr

In the preparation fixed by the Ryter Kellenberger method, the four layers of cell envelope were clearly separated and wrinkled in a pronounced manner (Figures II.17 and II.18). By the double fixation method of Williams and Luft (1968) the wall appeared as a simple membrane (Figure II.19).

Both methods of fixation showed distinct electron-dense bodies usually associated with very much paler areas in the centre of the cell. There were no filamentous appendages visible. Some cells were in the process of division and others showed vacuoles in the cytoplasm or complete absence of visible material inside the cell envelope.

iv) Morphology of cells grown in hoof broth for 24 hr

The different layers of the cell envelope were less distinct and were seen on fewer cells. Where the individual layers were obvious, they bore on the outer layer, very short peg-like extrusions that gave a battlement appearance. Numerous cells were in the process of, or had completely lysed, leaving a ghost shell (Figure II.20). Electron-dense bodies similar to those granules seen before in association with the paler areas were noted but less regularly. Clear vacuoles were also seen in some cells (Figure II.21)

and many of these, and other cells of more normal morphology appeared to be in various stages of division.

v) Morphology of cells grown in hoof broth for 48 hr

The Ryter Kellenberger preparation showed most of the features of cells cultured for 24 hr in hoof broth except that "folding" of the outer layer of the cell envelope was more exaggerated giving a shrunken appearance and an even more pronounced battlement effect. By the Karnovsky method of fixation the cell envelope layers and structures within the cytoplasmic membrane were less well defined.

C. Red blood cell agglutination

In the first series in which untreated F. nodosus cells were used, the cultures from hoof agar caused haemagglutination that was not inhibited by D-mannose. (Table II.I). Several other culture methods produced cells that caused slight agglutination provided D-mannose was not present.

In the second series in which the F. nodosus cells had been homogenised beforehand, the cultures from hoof agar caused a reduced degree of haemagglutination which again was not inhibited by D-mannose (Table II.I). One sample of F. nodosus (J3/22) cells cultured in biphasic medium caused slight haemagglutination that was unaffected by D-mannose.

DISCUSSION

Although F. nodosus (J3/22) was 90 per cent. rough by the working seed stage and under good growth conditions subsequent subcultures gave rise to uniformly rough colonies, the amount of moisture left on hoof agar plates at the time of inoculation did influence the appearance of colonies four days later. Surfaces left too wet or inoculation with several drops of growing culture, yielded confluent growth of colonies that had terraces like the intermediate type colony. On one occasion F. nodosus (J3/22) adapted to GC broth by 30 subcultures, produced at the first subculture on hoof agar, all three colony types amongst which the smooth variety was predominant. It therefore appears that colony type is determined largely by the cultural conditions offered and the ability to mutate remains with the strain.

Each of the three colony types contained F. nodosus organisms that appeared identical in smears stained by Gram's method. But it was claimed by Beveridge (1941) that repeated subculture, (in a medium perhaps not quite optimum for growth) could result in organisms little larger than cocci. F. nodosus (McM198) organisms were shorter, thicker and had less pronounced terminal enlargements than other F. nodosus strains examined. The passage number of this Australian strain on receipt was unknown, so it is possible that previous cultural conditions or the numbers of subcultures or both,

may have been instrumental in this morphological change.

Most F. nodosus cells cultured on hoof agar, in biphasic medium or in hoof broth, after heat fixation and Gram staining, exhibited the same narrow, clear zone around the cell envelope. This was also seen around F. nodosus organisms in preparations taken from foot-rot infected feet. The consistent occurrence of the 'structure' suggested the possibility of a capsule or slime layer but tests to demonstrate this including the wet indian ink method (Duguid, 1951) were negative. However, Egerton (1972b) has demonstrated what appears to be capsular material in indian ink preparations of F. nodosus grown on hoof agar. An Australian strain would have been used and if smooth colonies were selected for study, they could by their nature possibly include capsule bearing individuals (Rose, 1968). The narrow clear zone seen in light microscopy of bacilli can otherwise be explained as due to shrinkage retraction or else light refraction halos. After electron microscopy examination showed the various cell envelope appendages, these provided another possible explanation for the clear zone. The numerous filaments might act as a physical barrier in the immediate vicinity of the cell envelope. But in those cultures where no appendages were seen, light refraction halos would seem the most likely explanation to account for a most characteristic feature.

Smears of the broth phase of biphasic medium culture commonly showed small amorphous masses closely associated with the F. nodosus cells and also larger differentiated structures containing small rod forms and some typical F. nodosus in the matrix. Using strains of Fusiformis cultured on glucose blood agar, Morris (1953) referred to what he termed the "L cycle" that a few cells passed through and his photographs taken at certain stages show structures very similar to those associated with F. nodosus. Because of sub-optimal nutritional conditions in biphasic medium,the cell wall synthesis of some F. nodosus may be seriously interfered with and abnormal forms might appear, but like the Fusiformis strains that Morris studied there would probably be reversion to the standard bacillary form on subculture.

The observations by both light microscope and electron microscope of cultures grown in hoof broth suggested that by 24 hr autolysis is well established, many bacilli having lost their characteristic shape, stain affinity and structure. By 48 hr the rippled appearance of the outer layer of the cell envelope was pronounced and this was also true for cells cultured in biphasic medium. It was less obvious in preparations taken from hoof agar and from the interdigital space. F. nodosus possesses the layered cell envelope consisting of double-track unit membranes, which is typical of Gram-

negative bacteria (De Petris, 1965; Murray, Steed and Elson, 1965; Mahoney and Edwards, 1966; Shively, Decker and Greenawalt, 1970; Forsberg, Costerton and MacLeod, 1970). The outer layer rippled appearance is a prominent feature of most of the Thiobacillus species in which, in some instances, it appears to be an authentic characteristic of the cell rather than the effect of osmotic environment during fixation (Shively, et al., 1970). For F. nodosus the method of fixation and osmolarity do seem important because preparations double fixed by the method of Williams and Luft (1968) show a smooth outer layer to the cell envelope.

In electron microscopic examination of interdigital detritus one could not be absolutely certain of identifying F. nodosus amongst a variety of bacteria. However there was a predominant number of organisms that showed pili, a layered cell envelope, scattered volutin granules in a dispersed nucleoplasm and were in these respects identical to F. nodosus as seen in pure preparations grown on hoof agar. There was one significant difference: many of the cells grown on hoof agar showed an additional zone or areas in the cell envelope which appeared to consist of convoluted membranes interposed between the cytoplasmic membrane and the cell wall outer layers. This zone may represent a form of cytoplasmic intrusion (mesosomes) as described by Salton (1964) except that mesosomes usually emanate from the plasma membrane and are directed inwards. In view of the known enzyme producing properties of Gram-negative bacteria cell walls (Salton, 1964 p.244) it is tempting to speculate on a protease producing role for the structures. The suggestion is supported by the absence of the structures in F. nodosus of necrotic detritus, because these organisms were known to be poor protease producers having come from a case of benign foot-rot (Thomas, 1962a).

Organisms from the hoof agar and the necrotic detritus preparations bore filamentous appendages of various kinds that best fit the description of pili Type I (Brinton, 1964). This type of appendage is known to occur on other Gram-negative bacilli (Duguid and Gillies, 1957, Duguid, 1959), but for the latter, liquid culture medium as opposed to culture on agar, encourages piliation. Pili were not seen on F. nodosus cells that had been cultured in broth or biphasic medium. The presence or absence of this feature of F. nodosus cells was born out by the guinea pig erythrocyte agglutination test in which only hoof agar grown cells caused marked agglutination. The depilation by treatment in a homogenizer appears to have been only partly successful if judged by the very slight reduction in haemagglutinating activity after treatment. Although the reactions were carried out at $4^{o}C$, it is possible that the pili may have grown again as they are capable of

fast and expert regeneration (Brinton, 1964). The D-mannose resistant pili usually do not agglutinate red blood cells unless they are first tannic acid treated (Duguid, 1959; Cruickshank, 1965) but F. nodosus because of its unusual habitat and ability to survive in horn may have unique pili with a prime nutritional function.

Type I pili are also known to cause attachment of bacteria to one another and to surfaces such as glass (Duguid, 1959). Pili were not seen on F. nodosus grown in hoof broth but they may occur on cells grown in GC broth. If they do, pili might account for the plaque growth of F. nodosus seen on the sides of test tubes during bacterial growth-inhibition by serum tests. F. nodosus pili often had a terminal enlargement and in this respect they looked like a longer version of the peg-like extensions of the cell envelope that occur in Azobacter vinelandii (Lima-de-Faria, 1969) but for which no specialised function has been suggested.

The polychromatic volutin granules seen in light microscopy of F. nodosus have their counterpart in electron microscopy as the dense granules often seen dispersed in the nucleoplasm (Shively et al., 1970) or else concentrated towards one pole. In all probability these volutin granules are the granular structures containing inorganic polyphosphate as described by Friedburg and Avigad (1968). Other unidentified, less electron dense granules may be an intermediate stage in volutin synthesis as tentatively suggested by Mahoney and Edwards (1966) but disputed by Shively et al. (1970). It has been noted by Friedburg and Avigad (1968) that in most micro-organisms, the appearance of volutin granules and accumulation of inorganic polyphosphate take place when a situation of unbalanced nutrition prevails. However in Micrococcus lysodeikticus which they studied, the inorganic polyphosphate granules accumulated during the logarithmic phase of growth and disappeared during the stationary phase. In F. nodosus the change in distribution of volutin granules from a dispersed arrangement in the nucleoplasm to a much larger concentrated structure at the pole may represent a predivision tactic of the cell. Certainly the large polar structures have been noted in some cells undergoing division.

Summary and Conclusions

1. The "rough" colony of F. nodosus (J3/22) was judged to be the normal type but the ability to mutate to the "smooth" colony remains with the strain. Wetness of the surface of hoof agar at the time of inoculation influenced the appearance of colonies four days later.

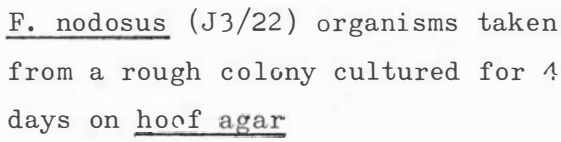
2. The morphology of F. nodosus (J3/22) grown on hoof agar was similar to that of F. nodosus-like cells seen in necrotic foot-rot material. Cells cultured on hoof agar, were longer than those cultured by other methods and end swellings were usually prominent. There were numerous pili; seen otherwise only on naturally occurring cells and there appeared to be a specialised zone within the cell envelope. A possible enzyme producing role was suggested for the latter. The piliated bacilli agglutinated guinea pig erythrocytes and the effect was not inhibited by D-mannose. The resistance to D-mannose suggested that the pili may be especially modified perhaps for some nutritional role associated with the peculiar habitat of F. nodosus.

3. F. nodosus cells grown in biphasic medium or in hoof broth showed wrinkling of the cell wall layers, vacuclation and lysis of the cells, and amorphous masses associated with the poles of some organisms. These features were suggestive of sub-optimum conditions of culture. F. nodosus (J3/22) cultured in GC broth showed a morphology largely similar to cells grown on hoof agar except that GC broth cultured cells were shorter. Their ultrastructure was not examined.

4. The Ryter Kellenberger fixation method gave excellent clarity of detail inside and outside the cell envelope. All methods of culture produced cells possessing the layered cell envelope consisting of double track membranes, and this was common to Gram-negative bacteria but the appearance was much influenced by the method of culture and the method of fixation. The nucleoplasm was dispersed and often contained volutin granules. Volutin was also seen in more concentrated form near the bacterial poles.

5. No capsule layer or slime could be demonstrated for F. nodosus (J3/22) taken from rough colonies grown on horn agar. Clear zones around the cell envelope may be due to the pili or to light refraction.

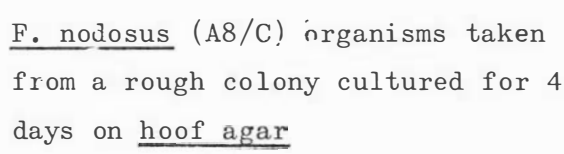
FIGURE II.1



F. nodosus (J3/22) organisms taken from a rough colony cultured for 4 days on hoof agar

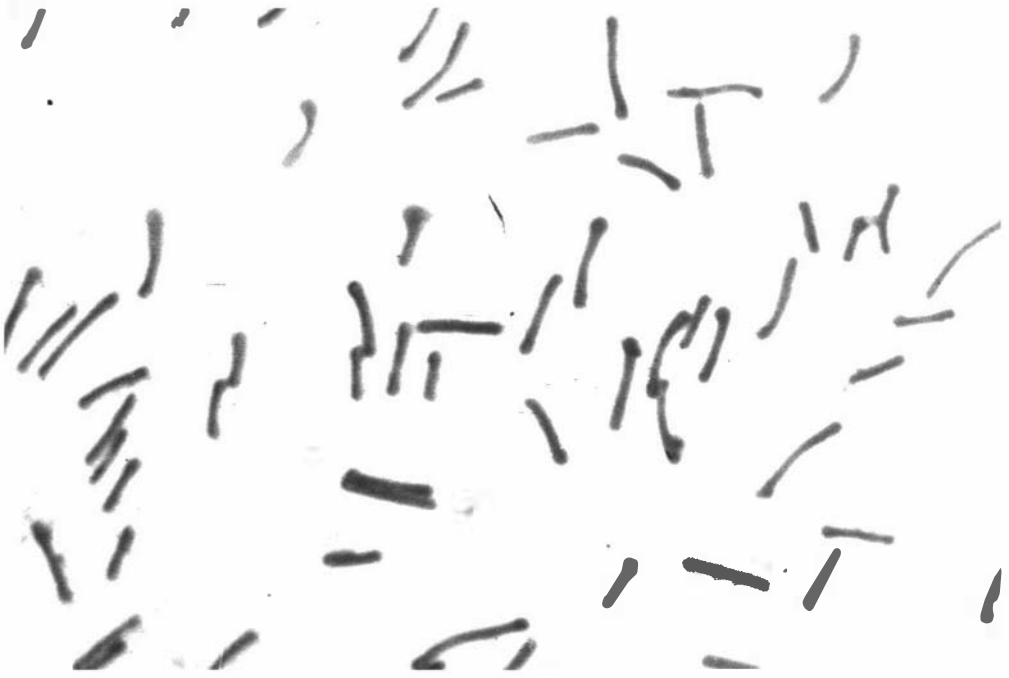
Gram, x ~2700

FIGURE II.2



F. nodosus (A8/C) organisms taken from a rough colony cultured for 4 days on hoof agar

Gram, x 2700



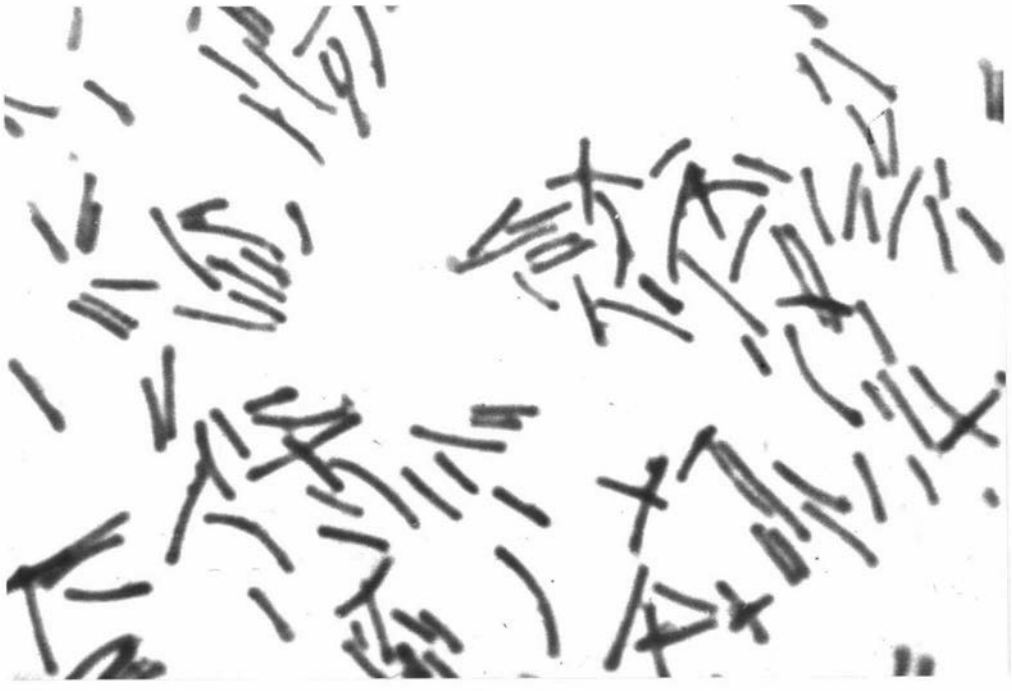


FIGURE II.3

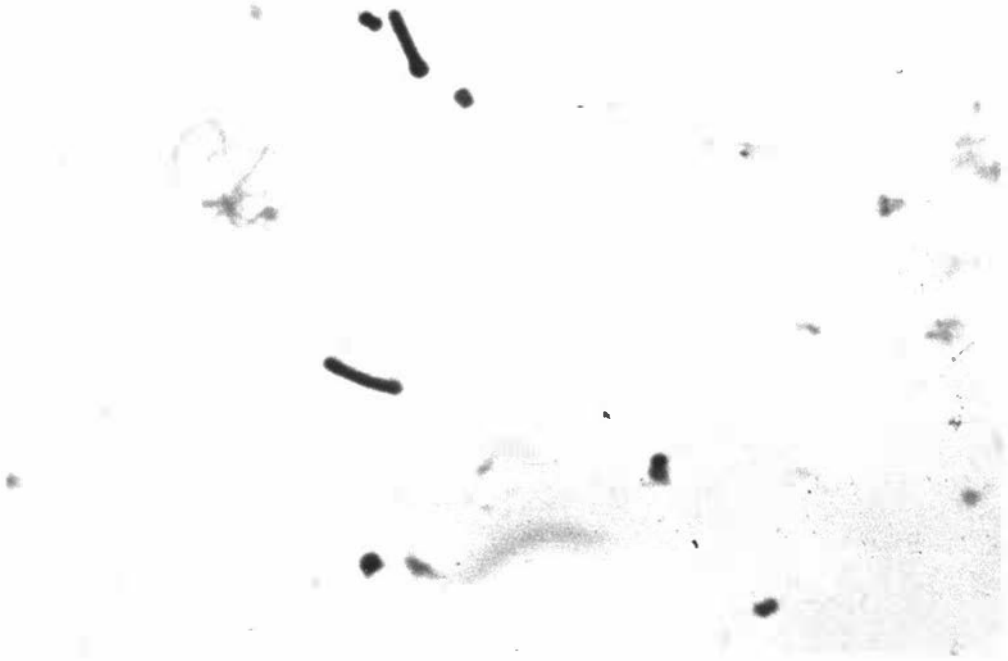
Smear of necrotic material from a foot-rot infected foot showing two F. nodosus organisms near a mass of pale stained filaments possibly of F. necrophorus

Gram, x 2700

FIGURE II.4

Smear of necrotic material from a foot-rot infected foot showing two F. nodosus organisms (arrowed) surrounded by other smaller rods in characteristic "row-boat" arrangement

Gram, x 2700



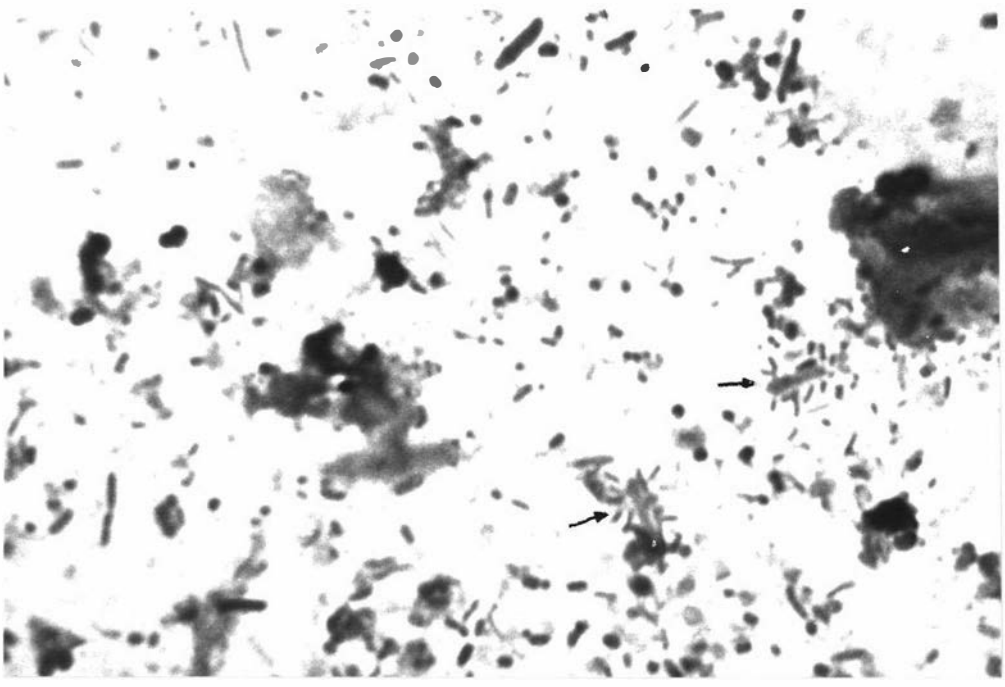
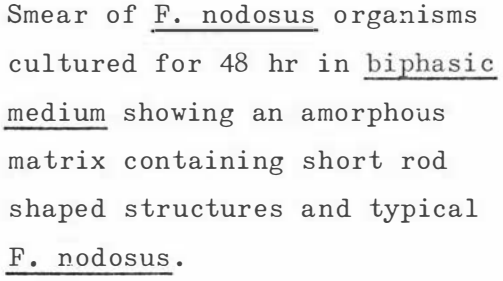


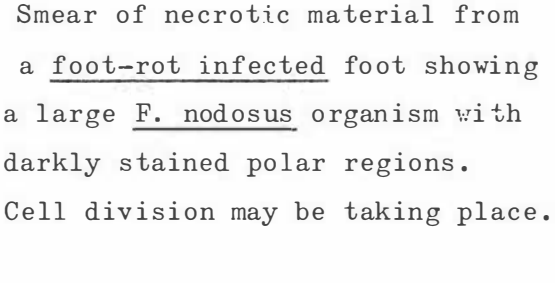
FIGURE II.5



Smear of F. nodosus organisms cultured for 48 hr in biphasic medium showing an amorphous matrix containing short rod shaped structures and typical F. nodosus.

Gram, x 2700

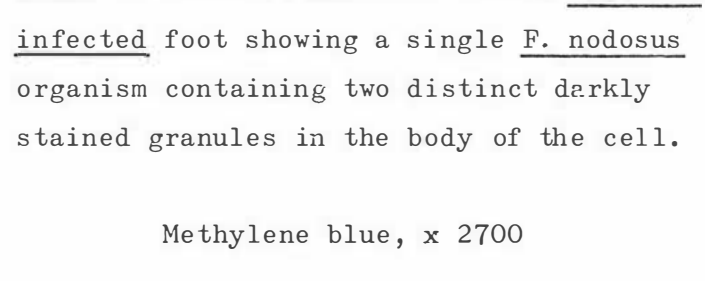
FIGURE II.6



Smear of necrotic material from a foot-rot infected foot showing a large F. nodosus organism with darkly stained polar regions. Cell division may be taking place.

Methylene blue, x 2700

FIGURE II.7



Smear of necrotic material from a foot-rot infected foot showing a single F. nodosus organism containing two distinct darkly stained granules in the body of the cell.

Methylene blue, x 2700

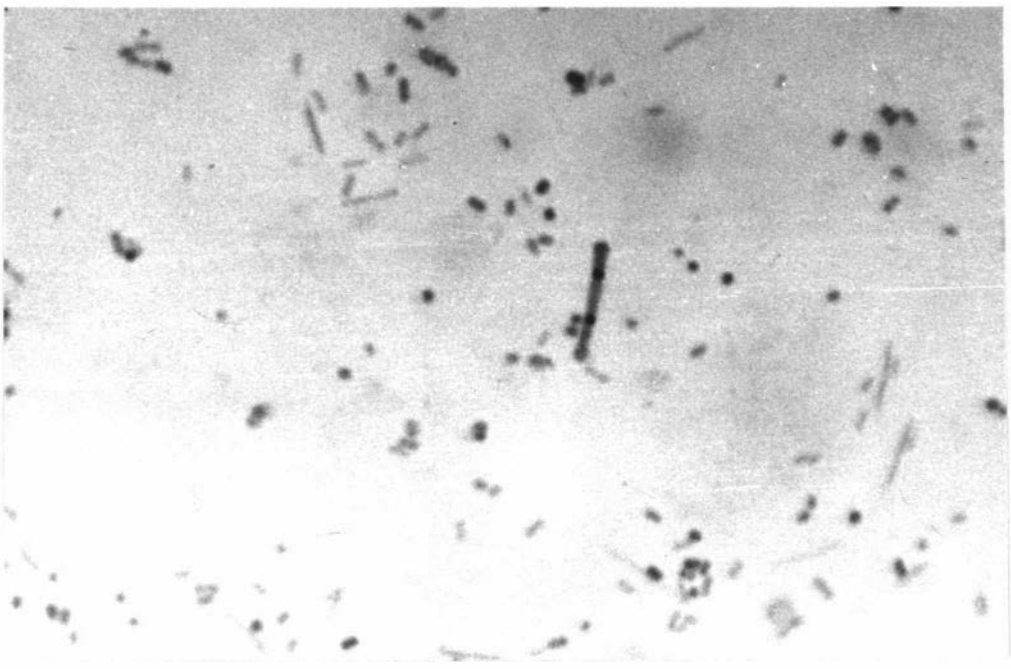


FIGURE II.8

Section of interdigital necrotic material showing cell border area with a sectioned spiral organism possibly Sp. penortha

Ryter Kellenberger, x 7,000

FIGURE II.9

Section of interdigital necrotic material with a piliated F. nodosus-like organism possibly within a cell and showing one polar granule

Karnovsky, x 18,000

FIGURE II.10

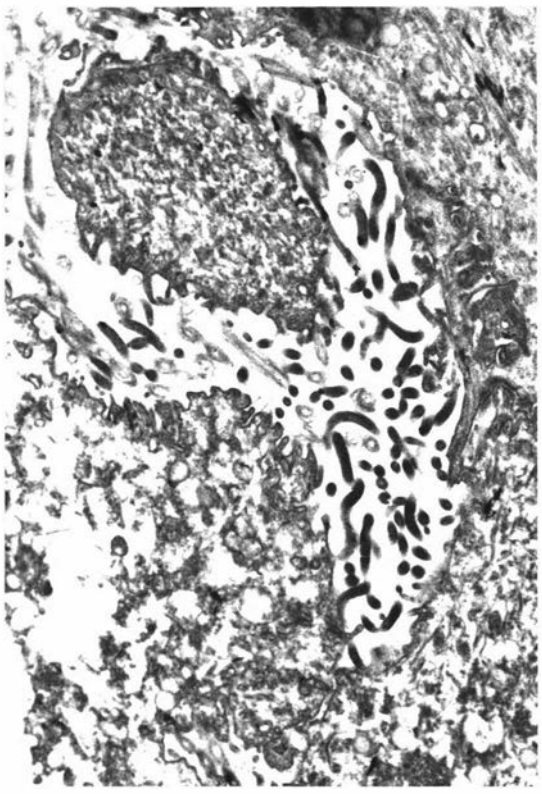
Section of interdigital necrotic material showing a piliated F. nodosus-like organism with several dense granules within the nucleoplasm

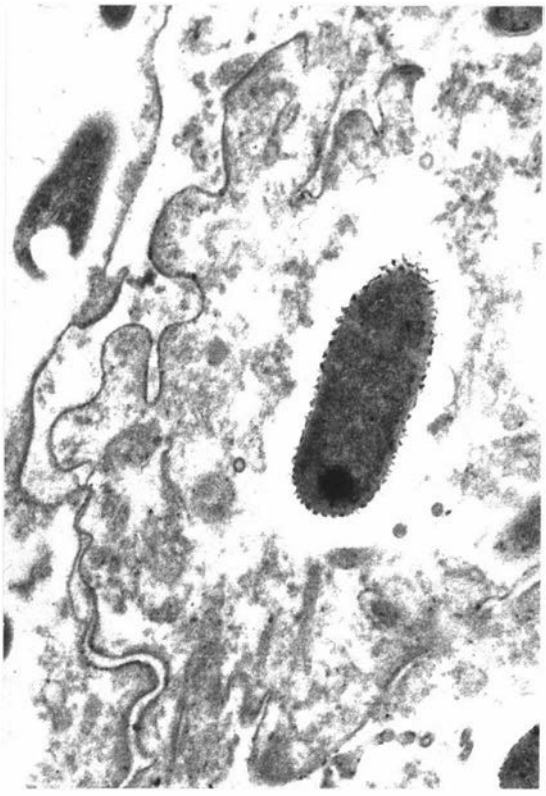
Ryter Kellenberger, x 77,000

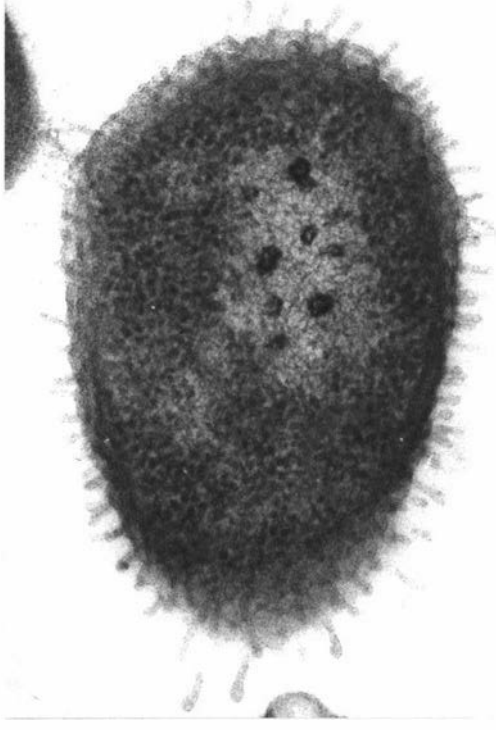
FIGURE II.11

F. nodosus cells cultured for 4 days on hoof agar, showing 4 layers of cell envelope and 2 large granules in the nucleoplasm

Karnovsky, x 35,000







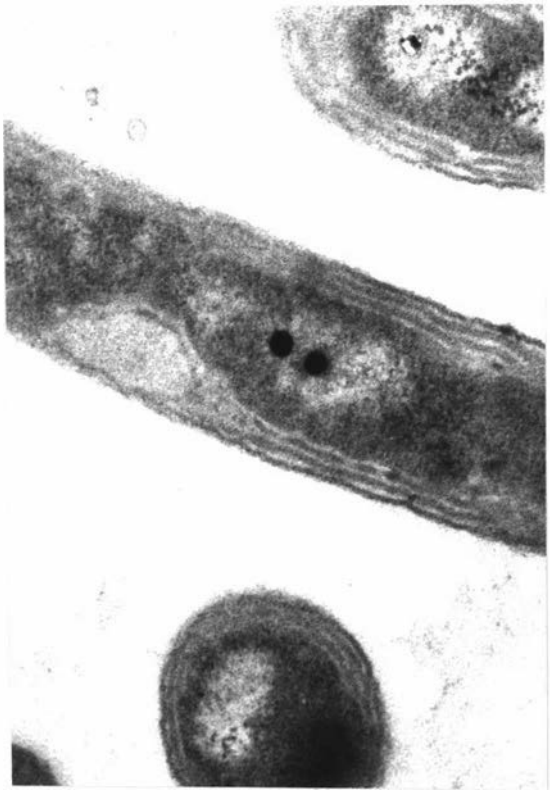
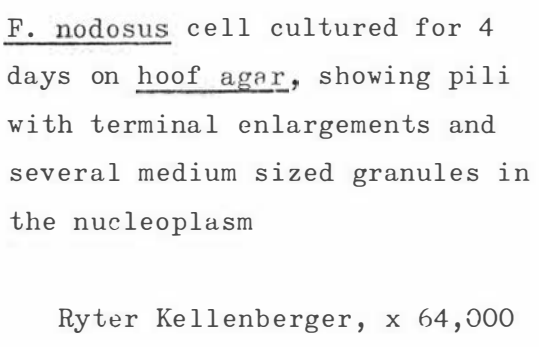


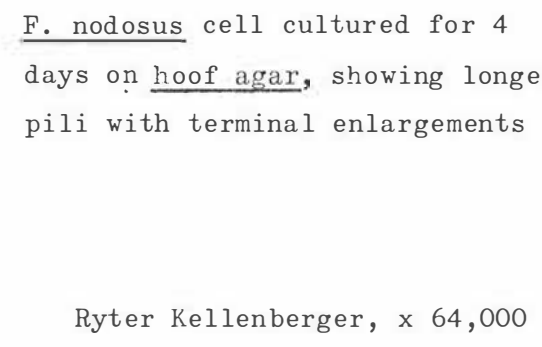
FIGURE II.12



F. nodosus cell cultured for 4 days on hoof agar, showing pili with terminal enlargements and several medium sized granules in the nucleoplasm

Ryter Kellenberger, x 64,000

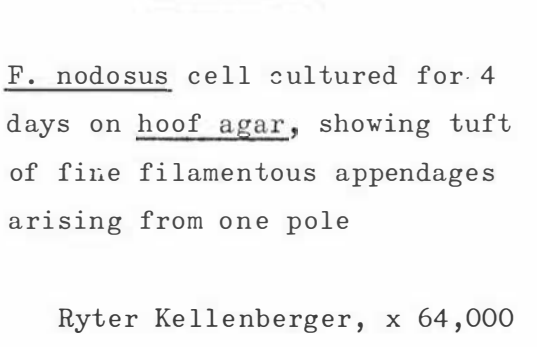
FIGURE II.13



F. nodosus cell cultured for 4 days on hoof agar, showing longer pili with terminal enlargements

Ryter Kellenberger, x 64,000

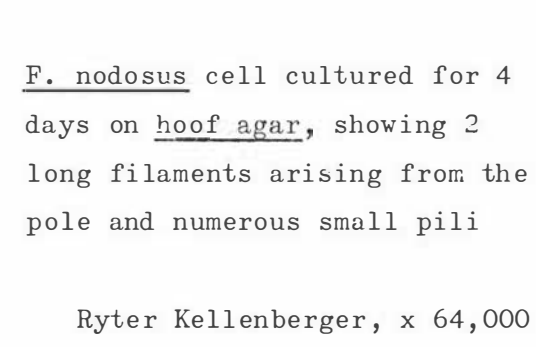
FIGURE II.14



F. nodosus cell cultured for 4 days on hoof agar, showing tuft of fine filamentous appendages arising from one pole

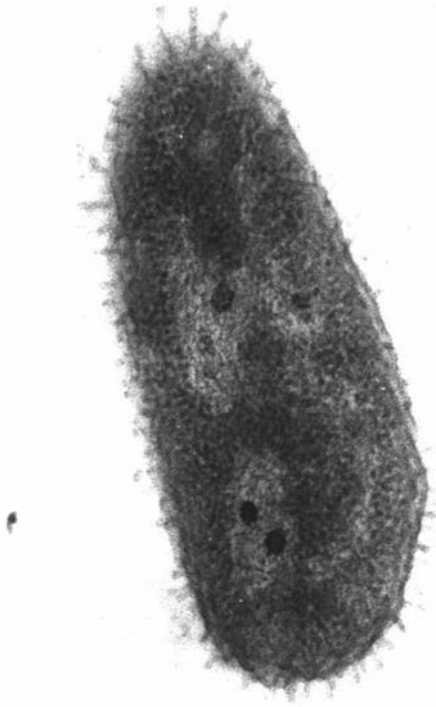
Ryter Kellenberger, x 64,000

FIGURE II.15



F. nodosus cell cultured for 4 days on hoof agar, showing 2 long filaments arising from the pole and numerous small pili

Ryter Kellenberger, x 64,000



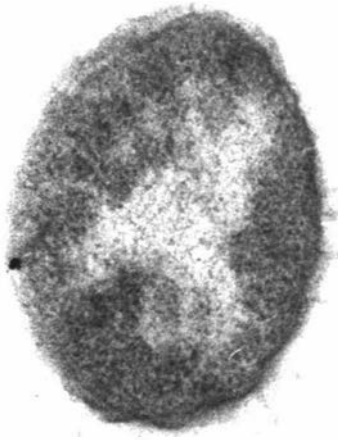
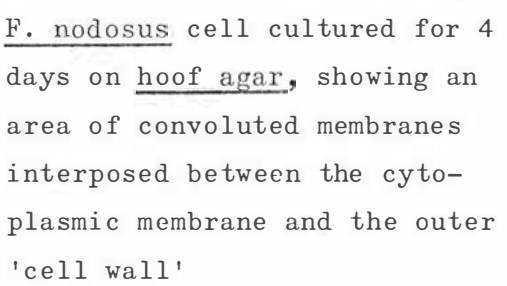


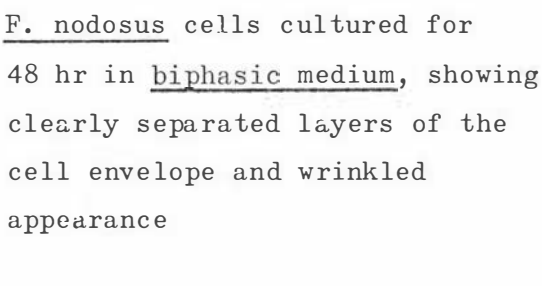
FIGURE II.16



F. nodosus cell cultured for 4 days on hoof agar, showing an area of convoluted membranes interposed between the cytoplasmic membrane and the outer 'cell wall'

Ryter Kellenberger, x 47,000

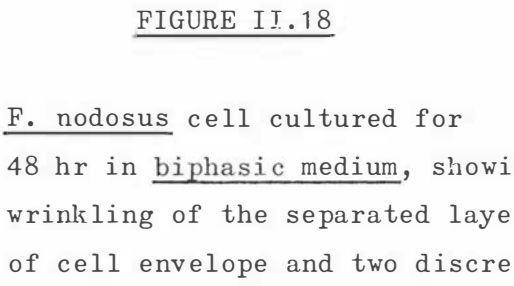
FIGURE II.17



F. nodosus cells cultured for 48 hr in biphasic medium, showing clearly separated layers of the cell envelope and wrinkled appearance

Ryter Kellenberger, x 42,000

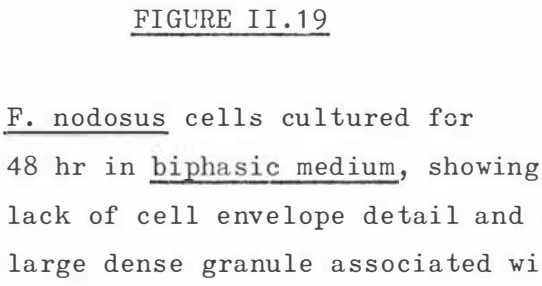
FIGURE II.18



F. nodosus cell cultured for 48 hr in biphasic medium, showing wrinkling of the separated layers of cell envelope and two discrete areas of nucleoplasm

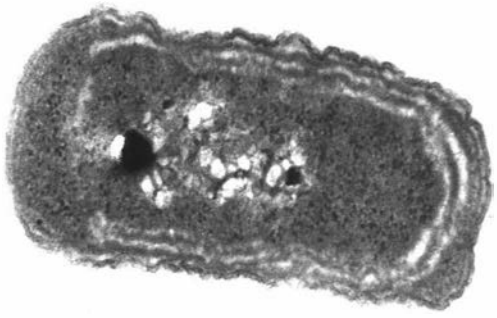
Ryter Kellenberger, x 64,000

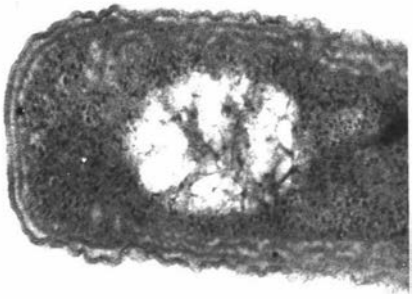
FIGURE II.19

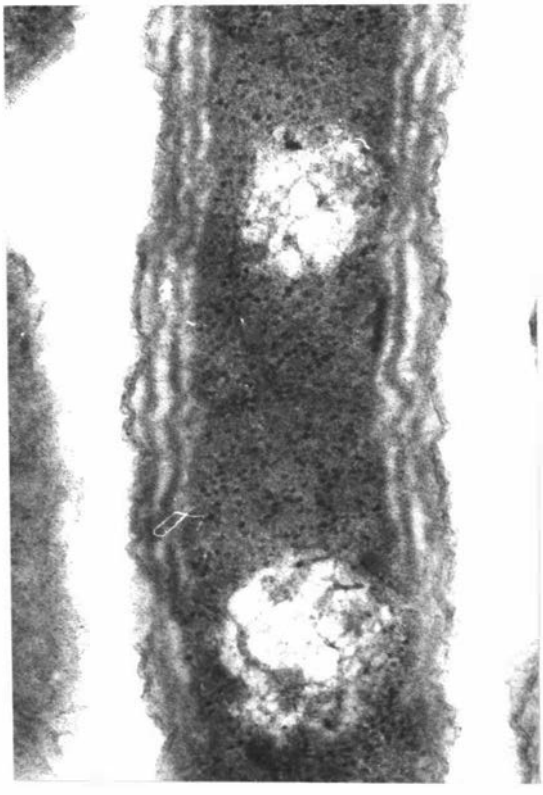


F. nodosus cells cultured for 48 hr in biphasic medium, showing lack of cell envelope detail and one large dense granule associated with the nucleoplasm

Double fixation (Williams and Luft, 1968), x 42,000







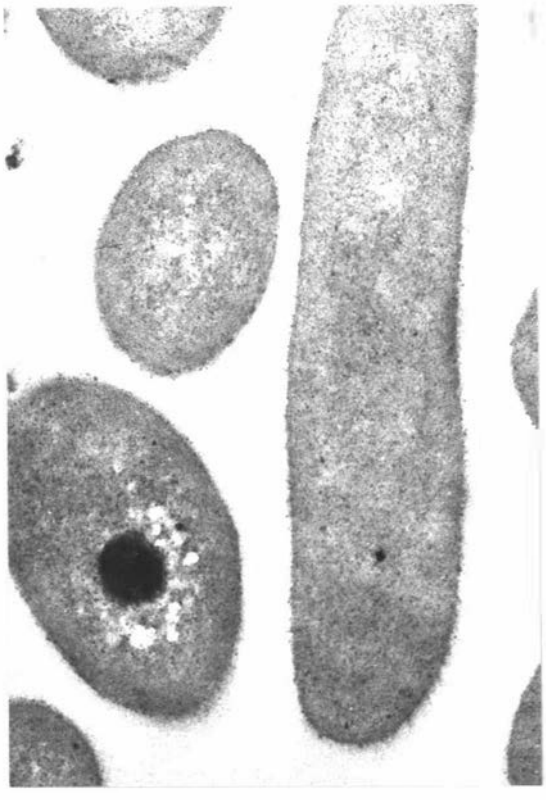
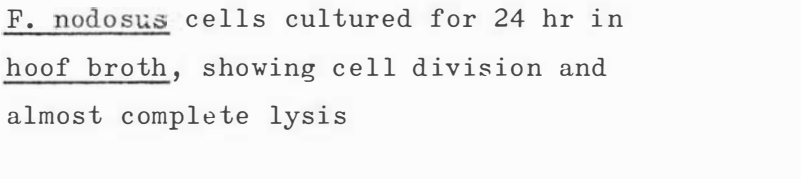


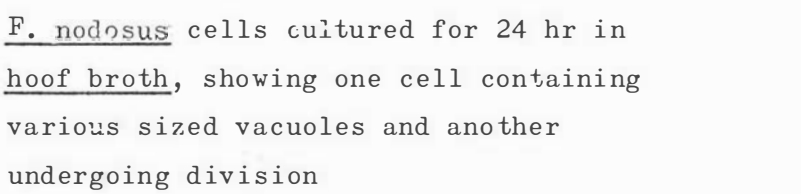
FIGURE II.20



F. nodosus cells cultured for 24 hr in hoof broth, showing cell division and almost complete lysis

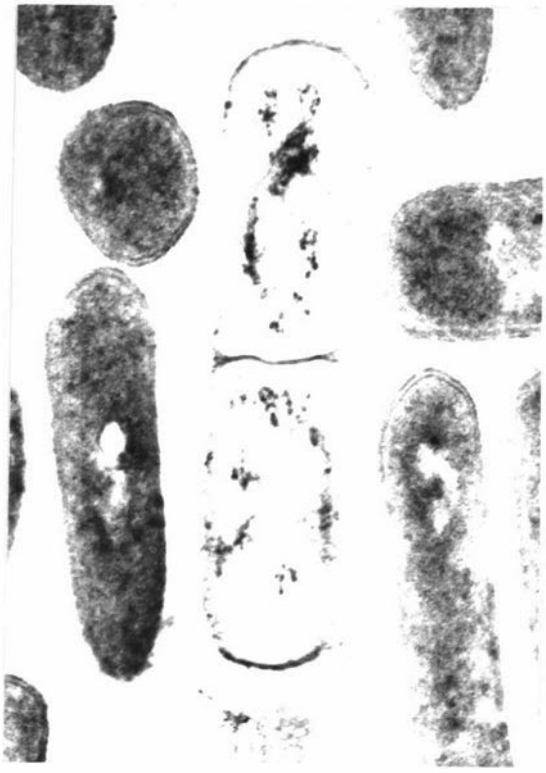
Ryter Kellenberger, x 27,700

FIGURE II.21



F. nodosus cells cultured for 24 hr in hoof broth, showing one cell containing various sized vacuoles and another undergoing division

Ryter Kellenberger, x 42,000



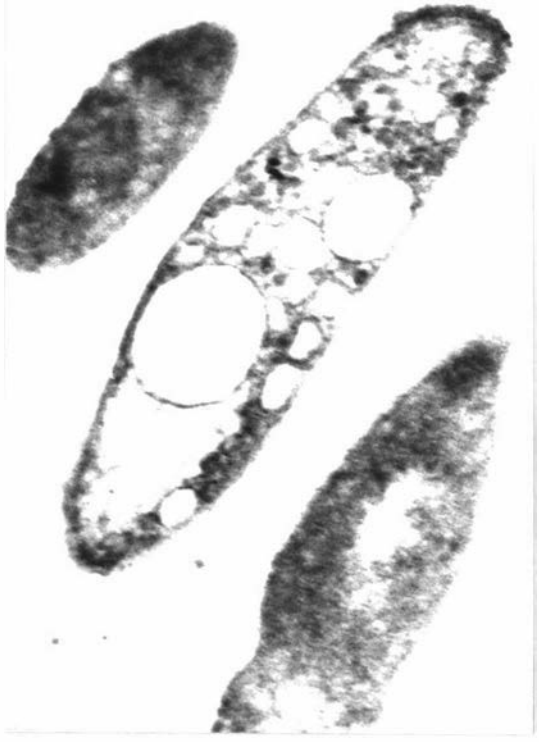


TABLE II.I

The effect of different methods of culture on the haem-agglutinating effect of four F. nodosus strains

F. nodosus strain* and culture method≠	Untreated F. nodosus		Homogenized F. nodosus	
	RBC	RBC + D-mannose	RBC	RBC + D-mannose
J3/22/8 biphasic medium 55 ml	±	-	+	+
J3/22/8 GC broth 10,000 ml	-	-	-	-
J3/22/8 hoof broth 55 ml	±	-	-	-
J3/22/8 hoof agar petri dish	++++	++++	++	++
A8/C/7 biphasic medium 55 ml	±	-	-	-
McM198 biphasic medium 55 ml	-	-	-	-
McM199 biphasic medium 55 ml	±	-	-	-

- = no haemagglutination

± to ++++ = haemagglutination

RBC = guinea pig red blood cells

* Details of strains are described in Appendix III

≠ Details of culture method are described in Appendices IIa, IIb, IIc and IId.

CHAPTER III

THE INFLUENCE OF CULTURE METHOD AND STRAIN OF ORGANISM ON THE AGGLUTINOGENS OF FUSIFORMIS NODOSUS

Introduction The first successful foot-rot vaccines contained *F. nodosus* cells that had been grown either on hoof agar or in biphasic medium (Egerton, 1970; Egerton and Burrell, 1970; Egerton and Roberts, 1971). Skerman (1971) showed that cells cultured in a liquid medium of undisclosed composition also possessed "protective" antigens. The constituents of culture media are known to influence the synthesis of cell wall components (Rogers, 1970; Weinbaum, Kadis and Ajl, 1971) and may therefore modify antigenic structure (Commonwealth Scientific and Industrial Research Organisation, 1969-70). The possibility then existed that the large scale culture of *F. nodosus* might produce cells of altered antigenicity such that after formulation into vaccines they might be ineffective in providing protection against field strains.

While Chapter II dealt with morphological features associated with different methods of culture, this chapter describes a study of the antigenicity of three *F. nodosus* strains that were cultured by four different methods. After a number of treatments the harvested cells were compared by agglutination to determine whether or not the method of culture had influenced the antigenic structure.

MATERIALS AND METHODS

F. nodosus organisms Three strains were compared.

i) *F. nodosus* (J3/22) was a virulent field strain isolated from the North Island of New Zealand.

ii) F. nodosus (A8/C)was a virulent field strain isolated from the South Island of New Zealand.

iii) F. nodosus (M9/4) was isolated from a ram with benign foot-rot at Massey University. Protease production by this strain was typically poor.

Further details of these F. nodosus strains are described in Appendices III and IIIa.

Culture methods The F. nodosus strains were cultured using either solid or liquid media: in one instance growth in liquid media was on a commercial scale. The following table shows the combinations of strains and cultural methods employed.

F. nodosus strain	Culture method	Culture volume and container
J3/22	biphasic medium	55 ml vials
J3/22	hoof agar	petri dishes
J3/22	hoof broth	55 ml vials
J3/22	GC broth	600 l fermenter tank
M9/4	biphasic medium	55 ml vials
A8/C	biphasic medium	55 ml vials

Details of the culture methods are described in Appendices II, IIa, IIb, IIc and IId.

Serology

i) Antisera Sheep serum 1876, standard anti-F. nodosus (J3/22) antiserum was used for all agglutination tests to compare the antigenic activity of different cell suspensions.

Sheep serum 2029, standard anti-F. nodosus (M9/4) antiserum, was used to provide a comparative homologous system in agglutination when testing M9/4 cells grown in biphasic medium.

Details of the methods of antisera production are described in Appendix X.

ii) Agglutination Cell suspensions were washed before or after heat treatments at 60°C for 60 min. or 100°C for 60 min. Whenever possible a formalin treated cell suspension was treated similarly for comparative tests. The formalin treated antigen was tested in parallel because this served as a reproducible standard for "K" agglutinability. Another reason was that McMaster Institute workers had experienced difficulties in maintaining stable cell suspensions (Commonwealth Scientific and Industrial Research Organisation, 1970-71) and at this stage the effective "life" of live cells was untested. Various heat treatments to test the stability of surface agglutino-

gens were based upon the methods used for the Enterobacteriaceae. The range of treatments differentiates between antigenic activity due to "K" or surface antigens (L, A and B), "H" antigens (flagella) and fimbrial (pili) antigens found in that group (Cruickshank, 1965, p. 245-246). Fifty per cent. ethyl alcohol treatment of cells that would destroy flagella but retain pili activity (Cruickshank, 1965) was also tried but all cell suspensions autoagglutinated and afterwards were not suitable for agglutination tests.

Tube agglutination tests were carried out as described in Appendix XI.

RESULTS

Pattern of agglutination The pattern of agglutination and degree of supernatant clearing was not uniform. F. nodosus cells grown on hoof agar started to agglutinate within 30 minutes as a fluffy suspended flocculation. The same material became involved in the cell precipitation and after standing overnight at 4°C the test showed "typical" agglutination i.e. a heavy deposit of cells in the bottom of the tube with a clear supernatant.

F. nodosus cells grown in biphasic medium gave typical agglutination in untreated suspensions but after heating at 60°C for 60 minutes the cell deposit became fine. It tended to settle on the slopes rather than the base of the agglutination tube and the supernatant did not clear fully.

F. nodosus cells grown in broth and afterwards left untreated or heat treated, always gave the fine type pattern of agglutination. Cell suspensions of F. nodosus (M9/4) always agglutinated in the fine type pattern without supernatant clearing.

There were marked differences in the heat stability of agglutinogens dependent on the method of culture used for the F. nodosus cells. The strain of F. nodosus also influenced the type of antigens produced. The results are presented in a form that shows the effects on antigenic structure of F. nodosus strains and methods of culture in six combinations.

Agglutination reactions of strain/culture combinations

(a) The agglutination of F. nodosus (J3/22) cells grown in biphasic medium by anti-F. nodosus (J3/22) antiserum (Table III.I)

The formalin treated cell suspension agglutinated to a higher titre than did live cells suspended in PBS. Heating of the cell suspension at 60°C for 60 minutes did not decrease the agglutination titre but 100°C for 60 minutes abolished all activity. The agglutinating activity of the cells was also removed by washing the cells before heating at 60°C for 60 minutes but washing after the heat treatment left the activity unchanged.

(bi) The agglutination of F. nodosus (M9/4) cells grown in biphasic medium by anti-F. nodosus (J3/22) antiserum (Table III.II)

Formalin treated cells agglutinated at a titre over 5000 fold greater

than did live cells in PBS. The live cell agglutinability was markedly increased by heating the cells at 60°C for 60 min. but the titre of the antiserum returned to its preheating level if the cells were washed prior to the heat treatment.

(bii) The agglutination of F. nodosus (M9/4) cells grown in biphasic medium by anti-F. nodosus (M9/4) antiserum (Table III.II)

Formalin treated cell suspensions of F. nodosus (M9/4) were agglutinated to approximately the same titres by either anti-F. nodosus (M9/4) or anti-F. nodosus (J3/22) antisera. The agglutinability of live cells was markedly increased by heating at 60°C for 60 min. However, washing the cells before heating again reduced the agglutination titre dramatically.

(c) The agglutination of F. nodosus (J3/22) cells grown on hoof agar by anti-F. nodosus (J3/22) antiserum (Table III.III)

This combination of cells and antiserum enabled a comparison to be made between agglutination of biphasic medium grown cells and hoof agar grown cells, with antisera made against F. nodosus cells grown in biphasic medium. There were insufficient cells available for a formalin treated cell suspension.

Both biphasic medium grown (Table III.I) and hoof agar grown live cells were agglutinated to a similar titre. Both cell suspensions were stable to heating at 60°C for 60 min. but marked differences in the effect of washing were seen. Whereas F. nodosus (J3/22) cells grown in biphasic medium lost agglutinability by washing combined with heating afterwards at 60°C for 60 min., cells grown on hoof agar retained all reactivity whether washing preceded or followed heat treatment.

There were marked differences between the suspensions in the stability of the antigens after heating at 100°C for 60 min. Cells grown on hoof agar that were washed and then heated at 100°C for 60 min. had a 16 fold depression in agglutinability whereas biphasic medium grown cells subjected to the same treatment lost all reactivity.

(d) The agglutination of F. nodosus (J3/22) cells grown in hoof broth by anti-F. nodosus (J3/22) antiserum (Table III.IV)

The agglutination titre of untreated live cells grown in hoof broth was the same as that for cells grown on hoof agar or in biphasic medium. Formalin treated cells agglutinated to higher titre than did live cells and this superior reactivity remained after heat treatment at 60°C for 60 min. The live cells too were stable to heating at 60°C for 60 min. but when washing was carried out before or after heating at 60°C for 60 min. all reactivity was destroyed. Heat treatment at 100°C for 60 min. destroyed all agglutinability of cells grown in hoof broth.

(e) The agglutination of F. nodosus (A8/C) cells grown in biphasic medium by anti-F. nodosus (J3/22) antiserum (Table III.V)

This combination of cells and antiserum enabled a comparison to be made between virulent F. nodosus strains. The agglutination titre using untreated live cells with a heterologous antiserum was identical to the titre obtained in the homologous system (Table III.I). Formalin treated cells were agglutinated at the same titre as live cells in the heterologous system but for formalin treated cells the titre was lower than the corresponding titre in the homologous system (Table III.I).

Heat treatment at 60°C for 60 min. or washing left the agglutination titre using untreated cells unchanged. Heat treatment at 60°C for 60 min. combined with washing before or after heating left the agglutination titre using formalin treated cells unchanged but the titre using live cells was significantly reduced or destroyed. Heat treatment at 100°C for 60 min. reduced the agglutination titre using live cells and totally destroyed reactivity of formalin treated cells.

(f) The agglutination of F. nodosus (J3/22) cells grown in GC broth by anti-F. nodosus (J3/22) antiserum (Table III.VI)

This was the only culture grown at fermenter (600 l) scale and the results are important because for commercial application vaccine production would demand culturing in volumes similar to or greater than 600 l.

There was a dramatic depression of agglutinability for either living or formalin treated cells regardless of treatments. There was still a significant advantage in heat stability of live cells over formalin treated ones, after treatment at 60°C for 60 min. or 100°C for 60 min.

The results of agglutination tests with treated cell suspensions showed that there were striking differences between agglutinogens in their stability to heat and resistance to washing. To clarify the pattern of results the more significant figures have been extracted from the preceding tables and are presented in summary form in Table III.VII. Most culture batches produced cells with agglutinability that resisted treatment at 60°C for 60 min. but which was markedly reduced or destroyed by washing prior to heat treatment. Exceptional in this regard were cells grown on hoof agar because these possessed agglutinogens stable to washing followed by heating at 60°C for 60 min. or at 100°C for 60 min. F. nodosus (J3/22) grown in GC broth at fermenter scale, produced cells that possessed very little agglutinability; all of which was resistant to heating at 100°C for 60 min.

DISCUSSION

The results of these experiments have shown significant differences in terms of antigenic structure as determined by gross and somatic agglutination tests. It can be seen from Table III.VII that the differences are brought

about largely by the method of culture but that an influence is exerted by strains with particular characteristics; for example F. nodosus (M9/4).

Apart from the GC broth/fermenter method of culture, F. nodosus cells grown by other means possessed superficial agglutinogens that were stable to heating at 60°C for 60 min., but which were dislodged by simple washing. Examples of this pattern were seen in F. nodosus (J3/22) cultured in biphasic medium or hoof broth, and in F.nodosus (A8/C) cultured in biphasic medium (Table III.VII). The other exception to this finding was in cells grown on hoof agar and in these the surface antigens appeared to be more firmly bound. Heat treatment at 60°C for 60 min. or formalin treatment of the cells before washing tended to stabilise the surface agglutinogens but not to reduce their reactivity (Tables III.I and III.V).

Heat treatment of cells at 100°C for 60 min. exposed other agglutinogens in F. nodosus (J3/22) and F. nodosus (A8/C) cells that had been cultured by three different methods (Tables III.III, III.V and III.VI). Neither washing before the heat treatment nor incubation for an extended time at a higher temperature exposed reactivity for cells from hoof broth, but on another occasion F. nodosus (J3/22) cells grown in biphasic medium agglutinated after treatment at 100°C for 60 min. This reactivity after boiling of cell suspensions is referred to as somatic or "O" agglutinability for the Enterobacteriaceae but the investigation of F. nodosus surface antigens was not continued to a stage where the envelope or "K" antigens could be further defined as "L", "A" or "B" (Kauffmann, 1947; Cruickshank, 1965). The superficial agglutinogens of F. nodosus fully resisted heat treatment at 60°C for 60 min. and therefore they are not similar to "L" antigens. (Cruickshank, 1965). In an attempt to distinguish between agglutination due to "K" antigens and that due to pili antigens, 50 per cent. ethyl alcohol treatment (Cruickshank, 1965) was tried but resulted in autoagglutination of all suspensions.

The light fluffy flocculation that was seen first in agglutination of F. nodosus cells cultured on hoof agar is typical of flagella or pili agglutination (Cruickshank, 1965). This was not seen using any other form of culture and therefore with the supporting morphological evidence of Chapter II, there seems little doubt that growth of pili on F. nodosus (J3/22) is associated with culture only on hoof agar.

Unlike cells grown in all other combinations the F. nodosus (J3/22) cells cultured in 600 litres of GC broth were exceptional in possessing no demonstrable "K" agglutinogens. This difference must have been due to influences of the culture medium as the same strain, F. nodosus (J3/22), behaved quite typically when grown by other culture methods. In terms of

immunogenicity, the absence of "K" antigens could be advantageous because when present they cause "O" innagglutinability (Chapter V). There is evidence that suggests an association between somatic agglutination titre and protection (Egerton, 1972b; Chapters V and VII) and if "O" innagglutinability occurs *in vivo*, the blocking effect could be important in lowering the response to somatic antigens and perhaps influencing subsequent resistance to infection.

F. nodosus (J3/22) and *F. nodosus* (A8/c) although isolated from different areas of New Zealand show a close serological relationship. (Tables III.I, III.V and III.VII). Information concerning the antigenic similarity between field isolates of *F. nodosus* is needed before a vaccine, developed against one strain, can confidently be predicted capable of inducing immunity against all other strains.

Summary and conclusions

1. F. nodosus (J3/22) cells grown in GC broth were lacking in certain superficial agglutinogens that were present on cells from other culture systems, were stable to heating at 60°C for 60 min. and were removed from hoof broth cultured cells by simple washing. In all other batches, heat treatment or formalin treatment before the washing tended to stabilise the agglutinogens but not to reduce their reactivity.

2. Heat treatment at 100°C for 60 min. exposed the somatic agglutinogens of F. nodosus (J3/22) and F. nodosus (A8/C) cells that had been grown by three different methods of culture.

3. When using F. nodosus (J3/22) cells that had been cultured on hoof agar, agglutination was seen first as a fluffy flocculation and this change was believed due to pili. After standing overnight at 4°C the tubes showed typical agglutination i.e. a heavy cell deposit in the tube base with a cleared supernatant.

4. F. nodosus cells autoagglutinated in 50 per cent ethyl alcohol at 37°C, so that it was not possible to distinguish further between pili and "K" antigens.

5. Standard anti-F. nodosus (J3/22) antiserum agglutinated at higher titre with formalin treated cells than with live cells suspended in phosphate buffered saline. Superficial agglutinogens appear to be easily dislodged and formalin treatment may help to stabilise the bond between antigen and cell without affecting reactivity.

6. Two virulent field strains of F. nodosus, isolated from different parts of New Zealand, were both agglutinated to the same titre by anti-F. nodosus (J3/22) antiserum.

TABLE III.I

Agglutination titres given by an anti-F. nodosus (J3/22) antiserum when reacted with F. nodosus (J3/22) cells grown in biphasic medium and subsequently treated in various ways

Cell treatment	Cells	
	F. nodosus (J3/22)/PBS	F. nodosus (J3/22) FPBS
None	20480	163840
60°C/60 min.	20480	40960
60°C/60 min. + wash	10240	ND
wash + 60°C/60 min.	40	ND
100°C/60 min.	0	0
100°C/60 min. + wash	0	ND

PBS = Live cells in phosphate buffered saline

FPBS = Dead cells in formalin phosphate buffered saline

ND = Not done.

TABLE III.II

Agglutination titres given by antisera against either F. nodosus (J3/22) or F. nodosus (M9/4) when reacted either with F. nodosus (M9/4) cells grown in biphasic medium and subsequently treated in various ways, or with F. nodosus (J3/22) cells grown in biphasic medium

Cells	Cell treatment	Antisera against: F. nodosus (J3/22)	Antisera against: F. nodosus (M9/4)
F. nodosus (M9/4)/PBS	None	40	40
F. nodosus (M9/4)/PBS	Washed	0	80
F. nodosus (M9/4)/PBS	60°C/60 min.	5120	20480
F. nodosus (M9/4)/PBS	Wash + 60°C/60 min.	40	40
F. nodosus (M9/4)/FPBS	None	20480	40960
F. nodosus (J3/22)/FPBS	None	81920	10240

PBS = Live cells in phosphate buffered saline

FPBS = Dead cells in formalin phosphate buffered saline.

TABLE III.III

Agglutination titres given by an anti-F. nodosus (J3/22) antiserum when reacted with F. nodosus (J3/22) cells grown on hoof agar medium and subsequently treated in various ways

Cell treatment	Cells F. nodosus (J3/22)/PBS
None	10240
Washed	20480
Wash + 60°C/60 min.	20480
60°C/60 min.	5120
60°C/60 min. + wash	10240
Wash + 100°C/60 min.	640

PBS = Live cells in phosphate buffered saline.

TABLE III.IV

Agglutination titres given by an anti-F. nodosus (J3/22) antiserum when reacted with F. nodosus (J3/22) cells grown in hoof broth and subsequently treated in various ways

Cell treatment	Cells	
	F. nodosus (J3/22)/PBS	F. nodosus (J3/22)/FPBS
None	10240	40960
Wash + 60°C/60 min.	0	ND
60°C/60 min.	10240	40960
60°C/60 min.+ wash	0	ND
100°C/60 min.	0	0

PBS = Live cells in phosphate buffered saline

FPBS = Dead cells in formalin phosphate buffered saline

ND = Not done.

TABLE III.V

Agglutination titres given by an anti-F. nodosus (J3/22) antiserum when reacted with F. nodosus (A8/C) cells grown in biphasic medium and subsequently treated in various ways

Cell treatment	Cells	
	F. nodosus (A8/C)/PBS	F. nodosus (A8/C)/FPBS
None	20480	20480
washed	20480	20480
wash + 60°C/60 min.	0	20480
60°C/60 min.	10240	40960
60°C/60 min. + wash	40	40960
100°C/60 min.	80	0

PBS = Live cells in phosphate buffered saline

FPBS = Dead cells in formalin phosphate buffered saline.

TABLE III.VI

Agglutination titres given by an anti-F. nodosus (J3/22) antiserum when reacted with F. nodosus (J3/22) cells grown in bulk in GC broth and subsequently treated in various ways

Cell treatment	Cells	
	F. nodosus (J3/22)/PBS	F. nodosus (J3/22)/FPBS
None	0	0
washed	40	40
wash + 60°C/60 min.	80	40
60°C/60 min.	160	0
100°C/60 min.	160	40

PBS = Live cells in phosphate buffered saline

FPBS = Dead cells in formalin phosphate buffered saline.

TABLE III.VII

Agglutination titres of anti-F. nodosus (J3/22) antiserum with cell suspensions of different F. nodosus strains grown by different methods of culture and subsequently treated in various ways

F. nodosus strain and method of culture	Cell treatment			
	None	60°C/60 min.	wash + 60°C/60 min.	100°C/60min.
J3/22 biphasic medium	20480	20480	40	0
M9/4 biphasic medium	40*	5120	40	ND
J3/22 hoof agar	10240	5120	20480	640
J3/22 hoof broth	10240	10240	0	0
A8/C biphasic medium	20480	10240	0*	80
J3/22 GC broth 600 litre	0	160	80	160

* Formalin treated cells agglutinated to 20480

ND = Not done.

CHAPTER IV

ASSESSMENT IN SHEEP OF LOCAL REACTIONS AND PROTECTION GIVEN BY VARIOUS ADJUVANT/CELL COMBINATIONS

Introduction

Egerton (1970) established that F. nodosus cells cultured either on hoof agar or in biphasic medium and then incorporated with a water-in-oil adjuvant produced a vaccine that afforded significant protection against foot-rot. The same vaccine formulation was effective in stimulating resistance amongst sheep with existing foot-rot (Egerton 1970; Egerton and Burrell, 1970; Egerton and Morgan, 1972).

Before these findings could be applied more widely two questions had to be resolved. Firstly, the local effect of the adjuvant vehicle on the animal had to be assessed as an over intense reaction at the vaccination site could severely reduce the market value of the carcass and hide. Secondly it had to be established that the large scale culturing of F. nodosus required for commercial vaccine production, would not result in the loss of "protective" antigens.

The influence of culture method on the morphology and antigenic structure of F. nodosus had been studied in experiments reported in Chapters II and III. As a result of these investigations F. nodosus (J3/22) had been selected as the vaccine strain and on the basis of the satisfactory morphology and somatic agglutinability of harvested cells, GC broth was chosen to be developed further as a large scale culture medium. It was realised that the only definitive test of the "protective" capabilities of the antigens lay in sheep challenge experiments as applied by Egerton, Roberts and Parsonson, (1969) and accordingly it was necessary to manufacture a vaccine incorporating the most appropriate adjuvant.

Eight different adjuvants were assessed for the local reactions they produced following their subcutaneous injection. Certain of these adjuvants produced severe lesions and consequently did not warrant further study. The more promising systems were evaluated further by testing their capabilities, when combined with F. nodosus grown under a variety of cultural conditions, to form a vaccine capable of protecting sheep against foot-rot.

Preliminary screening trials had indicated that both alum precipitated and aluminium hydroxide gel adsorbed vaccines produced local reactions that were largely acceptable and because of this they were considered worthy of further development. The water in mineral oil emulsion vaccine developed at the McMaster Institute, Sydney (Egerton, 1970; Egerton and Burrell, 1970), hereafter referred to as the McMaster adjuvant formulation, although known to be unacceptably irritant, was carried through into further vaccine trials both because its proven effectiveness made it a convenient positive control and also to test the hypothesis that the degree of protection afforded by a vaccine was related to the degree of local reaction it induced.

MATERIALS AND METHODS

A. Adjuvant Assessment

The following adjuvant systems which are fully described in Appendix VIII were tested,

i) Aluminium hydroxide gel, adsorbed
ii) Potash-alum, precipitated
iii) Water-in-oil emulsion
iv) McMaster water-in-oil formulation
v) Organic polymer with aluminium hydroxide gel
vi) Double emulsion
vii) Anhydrous oil with aluminium monostearate
viii) Oil-in-water emulsion with aluminium hydroxide gel.

The adjuvants were incorporated with the antigen concentrate to make a

2 ml dose vaccine containing 5 x 10^9 F. nodosus cells. Injections were made subcutaneously on the side of the upper neck in the site recommended by Cooper and Jull (1966). Local reactions were observed and palpated 7, 14 and 21 days after inoculation and were assessed using a grading system described in Appendix VIII.

B. Vaccine Trials

i) Sheep Adult Romney/Border Leicester cross ewes or wethers were selected on the basis of freedom from foot-rot or other foot blemishes.

ii) Foot conditioning All the sheep were placed in pens indoors on bedding that was kept wet continuously and upon which faecal material was allowed to accumulate (Egerton et al., 1969). This maceration of the feet was allowed to continue for 5 to 7 days before attempted infection. After challenge the bedding was kept moist for the duration of the experiment.

iii) Bacteria F. nodosus (J3/22) cells grown either in biphasic medium or in GC broth in a 10 or 600 litre fermenter , were incorporated into the vaccine as a formalin treated phosphate buffered saline suspension at 5 x 10^9 cells per dose; the concentration used by Egerton and Roberts (1971). For certain formulations formalin treated whole culture was used either instead of or in addition to the cells.

F. nodosus (J3/22) grown on hoof agar was used throughout for homologous challenge and F. nodosus (A8/C) cultured in the same way was used for the heterologous challenge used in Vaccine Trial III (see below).

iv) Assessment of lesions Each foot was examined once a week for six weeks and scored according to the assessment method described in Appendix IX using clinical observation recording sheets (Appendix IXa). Susceptibility scores were defined as the mean of assessment scores on post challenge weeks 3, 4 and 5 (Appendix IX).

In addition, where the process of infection had reached the stage of hoof separation, an attempt was made to distinguish between "active and progressive" changes and others that were in the process of resolution.

v) Statistical analysis Two by two contingency tables were drawn up for comparisons of data and analysed using the chi^2 test with Yates correction for continuity. The exact chi^2 test (Bailey, 1969) was used for calculations where expected values were less than five. Differences were considered to be non-significant when the level of probability was greater than 5 per cent.

Three vaccine trials were carried out; each with the specific objectives that are stated under the appropriate heading.

Vaccine Trial I This trial, which included four groups of sheep with seven individuals in each group had two objectives. The first of these was to confirm the protective effect of the McMaster adjuvant vaccine formulation

incorporating the New Zealand field isolate, F. nodosus (J3/22). The second objective of the trial was to compare protection afforded by a vaccine containing whole culture of F. nodosus (J3/22), with that of a similar vaccine containing only F. nodosus (J3/22) cells. Both of these vaccines incorporated aluminium hydroxide gel as the adjuvant.

(a) Vaccination The vaccines were injected subcutaneously in a 2 ml volume dose on the side of the upper neck. Two injections were given, one week apart in keeping with the method of Egerton (1970).

(b) Challenge This followed the method described by Egerton et al. (1969) and can be summarised as follows. Four days growth of F. nodosus (J3/22) on half a petri dish of hoof agar was scraped into one place and the cells on a slab of agar were applied directly to the interdigital skin. The material was held in place by a wet cottonwool swab and covered by a foot bandage. The bandages were removed five days later.

Three feet of each sheep were treated in the way outlined above and the fourth was inoculated using an infected swab taken directly from a foot-rot lesion. The swab was rubbed into the interdigital skin, after which a pad of wet cottonwool was applied under a foot bandage and left in position for five days.

Vaccine Trial II This trial involved 64 sheep that were divided randomly into eight groups. Six of these received vaccines in which the F. nodosus (J3/22) cells had been cultured in the same 10 litre fermenter and that differed one formulation from another only in the type of adjuvant used. The remaining vaccinated group received McMaster adjuvant vaccine containing F. nodosus (J3/22) cells cultured in biphasic medium to provide a basis for comparison of the immunogenicity of cells cultured in a fermenter.

(a) Vaccination Sheep were injected on alternate sides of the upper neck with two doses of 2 ml volume separated by a two week interval.

(b) Challenge The challenge procedure was carried out according to the method described by Egerton et al., (1969), as was used in the first trial. Two feet of each sheep were challenged with hoof agar grown F. nodosus (J3/22) cells.

Vaccine Trial III A further eight groups of eight sheep were involved in this trial which was largely concerned with confirming and further defining the protective potential of the Alum (one per cent.) precipitated vaccine using cells cultured in a 600 l fermenter. The trial was designed to compare protection afforded after one or two doses of vaccine against a challenge from either the homologous or a heterologous strain of F. nodosus. The opportunity was also taken to compare the immunity afforded by the alum

precipitated vaccine with that of two commercially available foot-rot vaccines. Commercial vaccine "A" was an oil emulsion vaccine presented in a 1 ml volume dose whereas Commercial vaccine "B" was an alum precipitated vaccine presented in a 4 ml volume dose. For the latter, two or more doses were recommended but the manufacturers of the first implied that one dose would provide substantial protection.

(a) Vaccination Commercial vaccines "A" and "B" were administered according to the manufacturers' instructions except that the two doses of Commercial vaccine "B" were separated by a two week interval. Other vaccines were injected subcutaneously on the side of the upper neck in a 2 ml volume dose. For the two dose schedule groups, inoculations were given on alternate sides two weeks apart: where only one dose was intended, this was given at the time of other first doses.

(b) Challenge The challenge procedure was carried out according to the method described by Egerton et al., (1969), as was used in the previous two trials. Two feet of each sheep were challenged with hoof agar grown cells: F. nodosus (J3/22) for homologous challenge and F. nodosus (A8/C) for the heterologous challenge.

RESULTS

A. Assessment of local reaction to adjuvants

In Vaccine Trial I the McMaster formulation caused severe reactions at one or both injection sites in six out of seven sheep. The reactions were characterised by abscess formation and oedema that extended down the jugular groove. All these reactions were judged unacceptable because of carcass and hide damage. Formulations containing aluminium hydroxide gel with either F. nodosus (J3/22) whole culture or with F. nodosus (J3/22) cells caused an acceptable degree of local reaction characterised by swelling and induration confined to the injection site.

In Vaccine Trial II it was possible to compare the degree of local reaction caused by the McMaster formulation with that involving other mineral oil adjuvant systems. Both the double emulsion and water-in-oil adjuvants caused fewer severe reactions than the McMaster formulation but even so they were generally unacceptable (Table IV.I). Vaccines based on an aluminium hydroxide gel adjuvant system were again entirely satisfactory while alum precipitated vaccines although causing slightly more reaction than the latter were considered largely acceptable (Table IV.I). The greater number of sheep injected with the alum precipitated vaccine in Vaccine Trial III enabled an important feature of this formulation to be observed which was that single vaccine doses caused only minimal reactions but two dose

schedules caused some lesions that were judged unacceptable (Table IV.II). The two dose schedule of Commercial vaccine "B" caused a pattern of reactions very similar to that of the Alum (one per cent) precipitated vaccine but Commercial vaccine "A", known to contain an oil adjuvant, was considerably more reactive and most of the resulting lesions were judged unacceptable (Table IV.II).

In summary, water-in-oil adjuvant vaccines caused severe reactions that were largely unacceptable. Alum precipitated vaccines caused local reactions that were usually acceptable and most of the more severe lesions were associated with a two dose schedule. Local reactions to aluminium hydroxide gel adjuvant vaccines were always acceptable.

B. Resistance of sheep to challenge

Vaccine Trial I

i) The influence of method of challenge

Although a valid comparison of the swab or horn agar culture methods of challenge could be made only within the control group, observations were made on all sheep and the results show that approximately equal numbers of feet became infected irrespective of the challenge method (Table IV.III). There was no statistically significant difference between the numbers of feet of control sheep infected by either method at 3 or 4 weeks after challenge ($p \triangleright 0.35$ and $p \triangleright 0.2$ respectively).

ii) Resistance to challenge

All controls became severely affected but all sheep vaccinated with the McMaster adjuvant/*F. nodosus* (J3/22) cells combination were strongly protected against infection ($p = 0.003$). This resistance showed in the form of group and susceptibility scores far lower than those for controls (Table IV.IV) and the fact that only one foot of one sheep in this vaccinated group showed active and progressive horn separation on post challenge week 4 (Table IV.V). In the other sheep of this group where infection became well established resolution was rapid whereas infection in control sheep progressed. In the other two vaccinated groups there were considerable between-sheep differences; for example in both groups 3 out of 7 sheep had far higher susceptibility scores than the remainder (Table IV.IV). Any extra cellular antigens present in the whole culture vaccine, as opposed to *F. nodosus* (J3/22) cells suspended in phosphate buffered saline, did not improve the protection rate. By both methods of evaluation the sheep vaccinated with the aluminium hydroxide gel/*F. nodosus* (J3/22) cells combination were as well protected as the group that received the aluminium hydroxide gel/*F. nodosus* (J3/22) whole culture combination (Tables IV.IV and IV.V).

In terms of numbers of feet infected (Table IV.V), all vaccinated groups were significantly more resistant than the control group and the McMaster formulation vaccine was superior to both the aluminium hydroxide gel adjuvant vaccines (McMaster vaccine $p = <0.001$, Aluminium hydroxide gel/whole culture $p = 0.04$, Aluminium hydroxide gel/cells $p = 0.001$; McMaster vaccine compared Aluminium hydroxide gel/cells $p = 0.023$).

Vaccine Trial II

During the period of post-challenge observations, three sheep aborted in the pens and succumbed to postparturient metritis. One other sheep developed severe foot abscess. Susceptibility scores of these animals were omitted from the final analysis.

Resistance to challenge

The susceptibility scores of all the vaccinated groups were far lower than that of the controls but in the Double emulsion, Organic polymer and Aluminium hydroxide vaccine groups, there were individual sheep that had susceptibility scores equal to or greater than the control group mean. In each of the Double emulsion and Organic polymer groups there were two uninoculated feet that developed specific foot-rot infection followed by horn separation, whereas in the control group there were four such feet.

In the control group, assessment scores on post challenge weeks 3, 4 and 5 were higher than those on week 2 whereas in all the vaccinated groups where an individual sheep was essentially protected, the assessment score reduced as from post challenge week 2.

There were two distinctly different levels of resistance to infection. The most resistant sheep were in groups vaccinated with the McMaster/biphasic medium, McMaster/fermenter or Alum/fermenter combinations. With a much lower level of resistance were all the other vaccinated groups and as these had been injected with either the least reactive or some of the most reactive adjuvant formulations, the results showed that the protection afforded by a vaccine was not necessarily related to the degree of local reaction it induced.

Susceptibility scores for individuals and groups of sheep are shown in Table IV.VI and the numbers of sheep and feet affected with active and progressive horn separation on post challenge week 4 are shown in Table IV.VII.

There was no significant difference between protection afforded by the McMaster/biphasic medium or the McMaster/fermenter combinations ($p = 0.19$). These two formulations and Alum (one per cent.) precipitated vaccine afforded significant protection ($p = 0.0001$, $p = 0.007$ and $p = 0.0003$ respectively). No significant protection was demonstrable for other groups except the water-in-oil/fermenter combination in which vaccinated sheep had fewer feet

infected than controls (p = 0.01).

Vaccine Trial III

Resistance to homologous challenge

This challenge caused a smaller number of controls to become infected and the group susceptibility score was lower than in Vaccine Trial II (Table IV.VIII). Two doses of the Alum (one per cent.) precipitated vaccine afforded no advantage over controls in susceptibility score and this group had two as against one uninoculated foot showing specific infection. However the group of sheep given two doses of the alum adjuvant formulation had fewer individuals and fewer feet showing active and progressive horn separation on post challenge week 4. The group of sheep that received one dose of alum precipitated vaccine had a lower susceptibility score than the two dose vaccinated group but the numbers showing active and progressive horn separation on post challenge week 4 were approximately the same for each schedule. The differences between the numbers of affected vaccinated and control sheep did not reach the 5 per cent. level of significance (one dose p = 0.09; two dose p = 0.06) but fewer feet of vaccinated sheep were involved (both vaccination schedules, p = <0.02).

Resistance to heterologous challenge

F. nodosus (A8/C) caused consistent infection in the control group and consistently high susceptibility scores (Table IV.IX). In the control group three uninoculated feet subsequently exhibited foot-rot infection with horn separation and every vaccinated group had one or more of these naturally acquired infections. Significant protection was evident in sheep given 1 or 2 doses of the Alum (one per cent.) precipitated vaccine (p = 0.004), which was shown by lower group susceptibility scores and fewer sheep and feet with active and progressive horn separation on post challenge week 4 than the control group. Commercial vaccine "B" showed protection at a slightly lower level (p = 0.04). The least effective protection was afforded by Commercial vaccine "A" but even in this group sheep had lower susceptibility scores than controls and fewer feet showing active and progressive horn separation on post challenge week 4 (p = 0.01).

DISCUSSION

The screening tests for local reaction in New Zealand Romney type sheep demonstrate clearly that whereas aluminium hydroxide gel adjuvants are acceptable, the McMaster and other water-in-oil adjuvants are not. Alum formulations cause generally acceptable reactions but some of these become abscesses that rupture and leave a small scar on healing. Second doses given 14 days after the first caused more local reaction (Table IV.II) and this

may be due to an Arthus-type reaction at the second site.

Skerman and Cairney (1972) state that the severity of the reaction to F. nodosus adjuvant vaccines is proportional to the amount of bacterial antigen they contain. To avoid unnecessary carcass damage it is imperative that the irritancy of any vaccine should be minimal. However, foot-rot vaccines are not so effective that one could afford to reduce the content of antigenic material. Therefore a different approach would be to concentrate upon culture purification, the identification of components responsible for evoking sheep protection and finally formulation with adjuvant using the important bacterial components only.

Of the range of adjuvants tested, aluminium hydroxide gel proved to be the best vehicle for F. nodosus because of its relatively mild reactivity, its stability, its free flowing character and the possibilities that exist for multivalent antigen formulations incorporating the clostridial range of vaccines. In an attempt to compensate for the less effective adjuvant effect of aluminium hydroxide gel, modifications were made in the antigen content but neither the inclusion of whole culture nor increasing the number of F. nodosus cells caused any improvement in protection over the standard 5×10^9 cells/dose formulation. As the local reactions caused by the Alum (one per cent.) vaccine were largely acceptable and the protection afforded approached that of the McMaster formulation, further work on the aluminium hydroxide gel formulation was stopped in favour of alum based vaccines. The results of protection in Vaccine Trial III were favourable for the Alum (one per cent.) precipitated whole culture formulation and at the present time this type of vaccine provides the best compromise between minimum degree of local damage to the carcass and hide, and maximum level of protection against foot-rot infection.

The severity of challenge was adequate as determined by the generally consistent response in control groups and by assessment scores of all sheep on post challenge weeks 1 and 2. In fully susceptible sheep, from week 2 onwards, the assessment scores continued to rise whereas in essentially resistant animals the score stopped rising by week 3 and began to fall as resolution commenced. As a measure of resistance to attempted F. nodosus infection, the susceptibility score was used to enable a more objective comparison to be made between sheep in one group. It served to do this and for individual sheep to reduce the errors arising from differences in assessment scores between weeks. These errors were believed to be caused by the activity of bacteria associated with F. nodosus because what is seen during clinical examination of foot-rot lesions is the damage caused by F. necrophorus and other bacteria aided by F. nodosus (Egerton, Roberts and Parsonson, 1969).

As a result the clinical differences observed between vaccinated groups do not necessarily or immediately reflect the effectiveness of F. nodosus vaccines. Substantial within-group variation in susceptibility scores occurred in approximately two out of eight sheep in most groups other than the McMaster/biphasic vaccine group. This variation between sheep could have arisen from either differences in response to vaccination, variable effects of the foot conditioning process, differences in the severity of challenge or may be as the result of a contribution from all three.

During the period of observations after challenge, interdigital dermatitis was seen occasionally in both vaccinated and control sheep. Without periodic and repeated smear examinations it would have been impossible to determine whether F. nodosus was invclved or not, but as protection in the field from foot-rot vaccination is rarely all or none (Egerton and Burrell, 1970) persistent interdigital infection may provide a reservoir for infection of unprotected animals.

In Vaccine Trial III the heterologous challenge by F. nodosus (A8/C) was more severe than homologous challenge by F. nodosus (J3/22) (8/8 and 6/8 control sheep infected respectively) but the Alum (one per cent) precipitated vaccine by a 1 or 2 dose schedule protected well against both strains. Commercial vaccine "A" may have been less effective because only one dose was used. Skerman (1971) suggested that single vaccination was somewhat less effective than double vaccination when using an oil adjuvant vaccine. The results of protection trials in the field against homologous and heterologous challenge (Egerton and Burrell, 1970; Skerman, 1971; Skerman and Cairney, 1972) suggest that vaccines protect better when the level of challenge is greater. For homologous challenge, this may point to a possible correlation between virulence and immunogenicity as suggested by Egerton and Burrell (1970) but the same explanation would only hold for the heterologous system if protection was related to an antigen common to all or most strains. Skerman and Cairney (1972) have suggested that antigenic components of the organism that are capable of stimulating host immunological defence mechanisms may be features of general occurrence in the natural populations of F. nodosus. At present the nature of these antigens is not known although there is evidence that they exist, and in the heat stable fraction of the bacterial cell (Egerton, 1972b; Chapter VII of this thesis).

Because of the naturally occurring form of challenge, and the natural "conditioning" of the foot, trials of a vaccine under field conditions would be much more desirable and a more realistic test of farm use potential. However indoor pen trials can be carried out at will with the certainty of infection starting on a certain day and the animals are available for repeated and detailed examination. It was found difficult to control the foot

environment for all groups so that conditions were similar and this factor of variation in pen moisture is blamed for the less severe F. nodosus (J3/22) challenge that occurred in Vaccine Trial III. Because each treatment group was kept in a separate pen,all the sheep in a trial were not exposed to the same degree of continuing F. nodosus challenge. In consequence the number of uninoculated feet subsequently exhibiting specific infection was not wholly an index of sheep protection but may also have been influenced by the magnitude of ground infection within any pen.

The underfoot conditions for the sheep are undoubtedly the worst feature of pen trials. This reached its most serious proportions during the second trial when three sheep died as a consequence of post-parturient metritis. Obviously wethers or dry sheep must be used in pen trials of this kind if the standard form of experimental infection (Egerton, et al., 1969) continues to be used. Thomas (1962b) produced clinical foot-rot in the horns of two sheep and if goats are equally susceptible in this site progressive infection could be maintained without undue discomfort to the animal. As an alternative and perhaps more suitable vaccine evaluation test the goat horn system deserves further consideration.

Only three cases of foot abscess or infective bulbar necrosis (Roberts, Graham, Egerton and Parsonson, 1968) arose amongst 132 sheep challenged in an environment presumably highly contaminated with F. necrophorus. This is similar to the ratio (3 of 121) for simple F. necrophorus interdigital infection that extended to the deeper tissues as reported by Beveridge (1941). It follows that conditions conducive to the development of infectious bulbar necrosis were not present during the pen trials. The host and environmental factors associated with F. necrophorus and C. pyogenes combined infection have been extensively studied in Australia (Roberts et al., 1968).

Summary and Conclusions

1. McMaster formulation and other water-in-oil emulsion adjuvants when incorporated into vaccines with F. nodosus, caused severe local reactions at the injection site. Alum (one per cent.) precipitated whole culture vaccine caused generally acceptable local reactions and aluminium hydroxide gel vaccines caused minimal reactions that were always acceptable.

2. The McMaster/biphasic combination was a consistently effective vaccine but other water-in-oil adjuvant vaccines although equally unacceptable because of local reactions, afforded less protection. Alum (one per cent.) precipitated whole culture vaccine produced resistance against infection that approached the degree of protection afforded by the McMaster formulation. The Alum vaccine by a one or two dose schedule was effective against homologous and heterologous challenge with F. nodosus. Aluminium hydroxide gel vaccines afforded some protection but this was not improved by including either twice the number of F. nodosus cells or F. nodosus whole culture in addition to the standard cell suspension.

3. F. nodosus (J3/22) cells grown in either biphasic medium or a 10 l fermenter were equally satisfactory for providing protective antigen. Cells grown in GC broth at the 600 l scale were used successfully as the source of protective antigen in Vaccine Trial III.

TABLE IV.I

Degree of acceptability of local reactions to two doses of vaccine formulated with different adjuvant systems

Vaccine		Number of sheep with stated lesion/No. of sheep in group		
Adjuvant	Culture	Acceptable	Marginally acceptable	Unacceptable
McMaster	Biphasic medium cells	0/8	0/8	8/8
McMaster	Fermenter* cells	0/8	1/8	7/8
Water-in-oil	Fermenter cells	0/8	4/8	4/8
Double emulsion	Fermenter cells	0/8	3/8	5/8
Organic polymer	Fermenter cells	8/8	0/8	0/8
Alum one per cent.	Fermenter whole culture	6/8	2/8	0/8
Aluminium hydroxide	Fermenter cells (10^{10}/dose)	8/8	0/8	0/8

* 10 l volume.

TABLE IV.II

Degree of acceptability of local reactions to one or two doses of vaccine formulated with alum or oil adjuvant

Vaccine		Number of sheep with stated lesion/No. of sheep in group		
Adjuvant	Culture	Acceptable	Marginally acceptable	Unacceptable
Alum one per cent.	Fermenter* whole culture 1 dose	16/16	0/16	0/16
Alum one per cent.	Fermenter whole culture 2 doses	9/16	6/16	1/16
Commercial vaccine "A" oil	1 dose 1 ml	0/8	1/8	7/8
Commercial vaccine "B" alum precipitated	2 doses each 4 ml	4/8	3/8	1/8

* 600 l volume.

TABLE IV.III

Number of feet affected with foot-rot after attempted infection by application of swab or horn agar culture (HAC)

Adjuvant	Vaccine Culture	Number of feet infected/ No. of feet challenged			
		After 3 wk		After 4 wk	
		Swab	HAC	Swab	HAC
McMaster	Biphasic medium cells	0/7	1/21	0/7	1/21
Aluminium hydroxide	Biphasic medium whole culture	2/7	8/21	4/7	8/21
Aluminium hydroxide	Biphasic medium cells	3/7	4/21	2/7	5/21
Controls		5/7	14/21	6/7	13/21

HAC = Horn agar culture.

TABLE IV.IV

Susceptibility scores for individual sheep and for groups injected with different vaccines and challenged with the homologous strain in Vaccine Trial I

Vaccine		Susceptibility scores*	
Adjuvant	Culture	Group	Sheep
McMaster	Biphasic medium cells	1	0, 0, 0, 0, 1, 2, 3.
Aluminium hydroxide	Biphasic medium whole culture	8	0, 3, 3, 4, 11, 11, 25.
Aluminium hydroxide	Biphasic medium cells	4	0, 0, 2, 3, 7, 8, 9.
Controls		14	8, 9, 11, 17, 18, 18, 20.

* Corrected to nearest whole number.

TABLE IV.V

Numbers of sheep and feet showing active and progressive horn separation on post-challenge week 4 in Vaccine Trial I

Vaccine		Number with horn separation* /No. challenged	
Adjuvant	Culture	Sheep	Feet
McMaster	Biphasic medium cells	1/7	1/28
Aluminium hydroxide	Biphasic medium whole culture	6/7	12/28
Aluminium hydroxide	Biphasic medium cells	4/7	7/28
Controls		7/7	19/28

* Active and progressive.

TABLE IV.VI

Susceptibility scores for individual sheep and for groups injected with different vaccines and challenged with the homologous strain in Vaccine Trial II

Vaccine		Susceptibility scores≠	
Adjuvant	Culture	Group	Sheep
McMaster	Biphasic medium cells	4	1, 1, 2, 4, 5, 6, 6, 6.
McMaster	Fermenter* cells	6	3, 3, 3, 5, 7, 7, 14.
Water-in-oil	Fermenter cells	10	3, 6, 7, 11, 12, 13, 14, 15.
Double emulsion	Fermenter cells	14	4, 5, 11, 14, 16, 16, (19), (24).
Organic polymer	Fermenter cells	13	7, 9, 10, 10, 11, 12, (23), (23).
Alum one per cent.	Fermenter whole culture	6	4, 5, 5, 5, 6, 9.
Aluminium hydroxide	Fermenter cells (10^{10}/dose)	13	3, 5, 8, 13, 19, 23, 23.
Controls		23	12, (17), 21, 23, 23, (27), (29), (30).

* 10 l volume

≠ Corrected to nearest whole number

(-) One uninoculated foot positive foot-rot.

TABLE IV.VII

Numbers of sheep and feet showing active and progressive horn separation on post-challenge week 4 in Vaccine Trial II

Vaccine		Number with horn separation* /No. challenged	
Adjuvant	Culture	Sheep	Feet
McMaster	Biphasic medium cells	0/8	0/16
McMaster	Fermenter≠ cells	2/7	2/14
Water-in-oil	Fermenter cells	6/8	7/16
Double emulsion	Fermenter cells	7/8	11/16
Organic polymer	Fermenter cells	7/8	10/16
Alum one per cent.	Fermenter whole culture	0/6	0/12
Aluminium hydroxide	Fermenter cells (10^{10}/dose)	6/8	10/16
Controls		8/8	14/16

* Active and progressive

≠ 10 l volume.

TABLE IV.VIII

Susceptibility scores and numbers of sheep and feet showing active and progressive horn separation on post-challenge week 4 in Vaccine Trial III

- Homologous challenge -

Vaccine		Susceptibility Scores[1]		Number with horn separation* /No. challenged	
Adjuvant	Culture	Group	Sheep	Sheep	Feet
Alum one per cent.	Fermenter≠ whole culture 1 dose	9	4, 5, 7, 7, 8, 10, 14, 16.	2/7	2/14
Alum one per cent.	Fermenter whole culture 2 doses	15	3, 11, (14), 16, (17), 18, 19, 19.	2/8	2/16
Controls		16	9, 11, 12, 12, 19, (20), 21, 22.	6/8	10/16

* Active and progressive

≠ 600 l volume

(-) One uninoculated foot positive foot-rot

[1] Corrected to nearest whole number.

TABLE IV.IX

Susceptibility scores and numbers of sheep and feet showing active and progressive horn separation on post-challenge week 4 in Vaccine Trial III

- Heterologous challenge -

Vaccine			Susceptibility Scores[1]	Number with horn separation* /No. challenged	
Adjuvant	Culture	Group	Sheep	Sheep	Feet
Alum one per cent.	Fermenter≠ whole culture 1 dose	16	6,10, 10, 15, 17, 20, (24), (26).	2/8	2/16
Alum one per cent.	Fermenter whole culture 2 doses	13	3, 9, 11, 13, 14, 14, (17), (25)	2/8	2/16
Commercial vaccine "A" oil		19	10, 16, 17, 18, 19, (20), 22, (26).	7/8	9/16
Commercial vaccine "B" alum precipitated		14	4, 8, 13, 14, (17), 18, 19, 20.	4/8	6/16
Controls		25	20, 22, 23, 24, (24), 26, (27), (30).	8/8	17/16[a]

* Active and progressive

≠ 600 l volume

[1] Corrected to nearest whole number

(-) One uninoculated foot positive foot-rot

[a] 16/16 for analysis.

CHAPTER V

SEROLOGICAL CHANGES IN SHEEP DURING FOOT-ROT INFECTION AND AFTER VACCINATION WITH VARIOUS ADJUVANT/CELL COMBINATIONS

(I) AGGLUTINATION

Introduction

By showing that the agglutination titre of sheep rose during foot-rot infection, Egerton and Roberts (1971) established that the infecting organisms make contact with the immunological system of the host. A slight rise in titre seen during natural infection was not associated with any increase in resistance to artificially induced infection whereas the very much higher titres recorded after vaccination were associated with a significant level of protection. When untreated live cells were used in the agglutination test there was no correlation between the post-vaccination titre and the degree of resistance against infection but Egerton (1972b) has suggested that the agglutination titre of serum with boiled cells might be a more reliable index of resistance in individuals. The endorsement of this hypothesis was of obvious importance in improving understanding of the mechanisms of protection and possibly predicting the immune status of sheep. Accordingly comparative agglutination tests were carried out using a single

serum sample against unwashed formalin treated cells and against washed and boiled cells. An attempt was made also to discover whether interference existed between agglutinogens *in vitro* as this might be related to the level of protection of sheep in the field.

Serum samples were available from new born and three day old lambs, normal adults and a range of mature sheep before and after vaccination. As most of these sheep had been injected with a variety of adjuvant vaccine formulations before they were challenged with *F. nodosus*, the opportunity existed to relate the agglutination titres both to the immune status of the donor and also to the effect of adjuvant employed in the vaccine. Other groups of sheep had been vaccinated during a foot-rot outbreak or had been vaccinated and then challenged with a heterologous *F. nodosus* strain. Accordingly sera from these animals was used to determine the heterologous titre. The relationship of titres between strains was studied more extensively using strain-specific antisera and specific absorption of antibody.

MATERIALS AND METHODS

Cell suspensions

i) *Unheated cells* *F. nodosus* cells were harvested from biphasic medium and, without washing were suspended in phosphate buffered saline containing 0.5 per cent. formalin. The agglutinogens of these cells that reacted with antiserum were reasoned to be situated superficially and were by definition stable to formalin treatment. As explained in Chapter III the nature of the *F. nodosus* surface antigens was not fully explored but for convenience in this chapter and the rest of the thesis they will be referred to as "K" agglutinogens and the reaction involving antiserum, as "K" agglutination.

ii) *Heated cells* *F. nodosus* cells were harvested from hoof agar, biphasic medium or GC broth cultures. They were washed three times in phosphate buffered saline, pH 7.4, boiled for 60-90 min., washed again three times and finally suspended in phosphate buffered saline. The agglutinogens of these cells were therefore reasoned to be firmly attached to the cell and by definition stable to boiling. For convenience these antigens will be referred to as somatic or "O" agglutinogens and the reaction involving antiserum as somatic or "O" agglutination[1].

All cell suspensions were adjusted to contain 5×10^8 cells/ml.

Details of the methods used for preparing cell suspensions are des-

[1] It is not the writer's intention to imply that *F. nodosus* "K" and "O" agglutinogens as defined, are identical to K and O antigens of the *Enterobacteriaceae*.

cribed in Appendix XI.

Sera Serum was collected from sheep involved in the three vaccine trials (Chapter IV) and Experiment Therapy I (Chapter XI) at various times before and after either vaccination or attempted infection with F. nodosus.

"Resistant" or "Susceptible" sheep (sera) In each treatment group of Vaccine Trials I, II and III, the most resistant and the most susceptible sheep were selected by identifying the most extreme susceptibility scores each side of the group mean score. Thereafter these sheep (sera) became entitled either"resistant" or "susceptible" and were usually paired for comparison in a number of serological tests.

Serum was collected at birth from lambs born to normal or vaccinated ewes, and further samples were taken 3 or 4 days later.

For experiments involving "K" reactions between strains, specific antisera against F. nodosus (J3/22), F. nodosus (McM198) and F. nodosus (M9/4) were used as standards. Antisera were prepared according to the techniques described in Appendix X.

Antibody absorption by F. nodosus cells

In order to study the differences in amount of unabsorbed antibody after homologous or heterologous reactions, mixtures of antiserum and cells were centrifuged after the standard agglutination reaction time and the supernatant was used again against fresh cell suspensions. Particular attention was paid to the pattern of agglutinated cells as well as to the quantitative differences.

Somatic inagglutinability

An investigation of possible interference between "K" and "O" agglutinogens, hereafter called somatic inagglutinability, was undertaken using "K" and "O" specific antisera prepared according to the method described in Appendix X.

Serology

Agglutination reactions were carried out in Dryer's tubes.

For "K" agglutination the tubes were incubated in a water bath at 37°C for 4 hr and then held at 4°C overnight.

For "O" agglutination the tubes were incubated in a water bath at 50°C for 24 hr and then held at 4°C overnight.

Details of the methods used for carrying out these agglutination tests are described in Appendix XI.

RESULTS

Antibodies reactive to both "K" and "O" antigens occurred in the sera of most normal sheep examined. Such antibodies were detectable in

sera from three days old lambs whether they had been born to normal or vaccinated ewes.

By 3 to 4 days of age, lambs born to vaccinated ewes, had "K" agglutination titres in the same range as the dam. Pre-colostral lamb serum was devoid of agglutinating antibody.

The levels of naturally acquired antibodies rose only slightly during foot-rot infections.

Agglutination titres in vaccinated sheep rose rapidly soon after the first injection and differed in level according to the adjuvant system used in the vaccine.

(a) Water-in-oil adjuvant vaccines This type of adjuvant system, of which the McMaster formulation is an example, caused the greatest rise in both the "K" and "O" agglutination titres. Seven days after the first dose of McMaster adjuvant vaccine, "K" titres of pooled sera rose from approximately 80 to 5120 and showed a further 32 fold increase following a second dose of vaccine. The high prechallenge titre in Vaccine Trial I (Table V.I) persisted at approximately the same level for the following six weeks. The Double emulsion and water-in-oil vaccine formulations caused approximately the same degree of response in both "K" and "O" agglutination titres when compared with the McMaster formulation in the same trial (Table V.II).

In the comparison between "resistant" and "susceptible" sheep, the "K" and "O" agglutination titres of"resistant" sheep were always equal to or greater than those of "susceptible" sheep but particularly in the case of "O" titres, the differences were only in the range of one or two doubling dilutions (Table V.II).

The oil based Commercial vaccine "A", by one injection produced an irregular response as measured by the "K" agglutination titre (Table V.IV). The pooled sera titre was no higher than an unstimulated control sheep whereas that of the "resistant" sheep was equal to levels produced by the water-in-oil adjuvant vaccines (Table V.II). In spite of the irregular "K" response, Commercial vaccine "A" produced a significant rise in "O" agglutination titre (Table V.IV) equal to that produced by other water-in-oil formulations.

(b) Alum precipitated adjuvant vaccines

i) Alum (one per cent.) vaccine Two doses of this vaccine produced a marginally greater rise in the "K" agglutination titre than that produced by the 1 dose schedule and this level was largely maintained for eight weeks after challenge. "Resistant" sheep usually had higher "K" agglutina-

tion titres than did "susceptible" sheep. As the rise in "O" agglutination titre did not exceed two doubling dilutions by either vaccination schedule, there were no obvious differences either between titres of "resistant" or "susceptible" sheep, or between effects of one or two doses of vaccine. (Tables V.III and V.IV).

ii) Commercial vaccine "B" The pooled sera sample had a "K" agglutination titre equal to that of unstimulated controls and the two individual serum titres were only fractionally higher (Table V.IV). Somatic or "O" agglutination titres were approximately equal to those produced by the Alum (one per cent.) vaccine and therefore little different from those of control sheep.

(c) Aluminium hydroxide gel vaccines Sheep injected with this type of vaccine responded moderately well in terms of "K" agglutination titre and the titres of "resistant" sheep were consistently equal to or higher than the titres of "susceptible" sheep in the group (Tables V.I and V.II).

The rise in "O" agglutination titre was equal to that of alum precipitated vaccines and therefore could not be considered significant.

In summary, water-in-oil adjuvant vaccines produced substantial rises in both "K" and "O" agglutination titres, whereas alum precipitated adjuvant and aluminium hydroxide adjuvant vaccines produced moderate rises in the "K" titre. Only insignificant differences were noted between pre and post-vaccination "O" agglutination titres. The immune status of a sheep could not be predicted from the post-vaccination agglutination titres except within very wide limits.

(d) Vaccination of foot-rot infected sheep The use of the Alum (one per cent.) precipitated whole culture vaccine on sheep with foot-rot produced a substantial rise in the "K" and an insignificant rise in the "O" agglutination titres (Table V.V). Both of these rises were short lived. A second dose of vaccine given three weeks after the first caused a prolongation of the titres but no anamnestic response.

(e) Reactions between F. nodosus strains For all three strains tested, "K" agglutination titres were higher in the homologous reaction than in the heterologous system (Table V. VI). In the homologous reactions, F. nodosus (McM198) cells were agglutinated to the lowest titre of the three strains. F. nodosus (J3/22) and F. nodosus (M9/4) cells were agglutinated by their homologous antisera to approximately the same titre. F. nodosus (J3/22) was the only strain in which cells were "typically" agglutinated i.e. with complete clearing of the supernatant. In reactions involving the cells of both the other strains, the supernatant remained

cloudy or was only part cleared.

(f) Antibody absorption by F.nodosus cells This experiment showed that the anti-F. nodosus (J3/22) antiserum supernatant, after mixing with F. nodosus (M9/4) cells, still contained most of the original agglutinating activity for F. nodosus (J3/22) cells whereas after the F. nodosus (M9/4) homologous reaction there was far less unabsorbed antibody for F. nodosus (J3/22) remaining (Table V.VII). The results also showed that F. nodosus (M9/4) cells had absorbed less antibody from either homologous or heterologous antiserum than had F. nodosus (J3/22) cells.

(g) Somatic inagglutinability Anti-F. nodosus (J3/22) antiserum raised against boiled cells, agglutinated washed and boiled F. nodosus cells to a higher titre than formalin treated cells (Table V.VIII). In a complementary test using the same reagents, formalin treated cells were agglutinated to a much higher titre than washed and boiled cells by the antiserum raised against formalin treated cells.

DISCUSSION

The slight rise in "K" and "O" agglutination titres in the sera of unvaccinated sheep during foot-rot infection confirmed that invasion by F.nodosus must be sufficiently deep to react with the immunological system of the host; an observation originally made by Egerton and Roberts (1971).

The response in "K" agglutinating antibody to vaccination with mineral oil adjuvant formulations was of the same order as reported earlier by Egerton and Roberts (1971) and was clearly greater than it was to less irritant vaccine formulations. Alum precipitated vaccines and aluminium hydroxide gel vaccines produced "K" titres of the same order and these were approximately equal to the geometric mean titres reported by Roberts, Foster, Kerry and Calder (1972) for another alum treated vaccine. In Vaccine Trial III (Tables V.III and V.IV), the response in "K" agglutinating antibody was of a slightly lower level in spite of the fact that serum samples were tested 14 days after the second dose of vaccine at a time when the response should be at a peak. Tests employing pools of sera and those evaluating "susceptible" and "resistant" individuals were carried out at different times using different batches of formalin treated cells and this may account for many individual serum titres being well above the pool titre. Nevertheless all end points were "read blind" and standard control systems worked well on both occasions so that comparisons between pools and comparisons between individuals may be considered valid.

Egerton (1972b) has suggested that for the McMaster water-in-oil formulation vaccine, somatic or "O" agglutination titres of 2000 or higher

may be indicative of prctection. The results of Vaccine Trial II (Table V.II) for all water-in-oil vaccine formulations would tend to support Egerton's suggestion but it is clear from the results shown in Tables V.II, V.III and V.IV that the same standard cannot be applied to other forms of adjuvant vaccine. For sheep injected with alum adjuvant vaccines the "K" agglutination titre appears to be a better indicator than the "O" titre, of response to vaccination. Although the magnitude of the agglutination titre provides no indication of subsequent level of resistance against foot-rot, individuals or groups of sheep with a pool "K" titre of not less than 10,000 usually showed significant protection.

The method of challenge and assessment of infection in sheep are imprecise techniques upon which to base an analysis of correlation between the level of protection and agglutination titre. Moreover only an experiment based on an antigen extinction design would provide all the data needed and this was considered impracticable because of the numbers of sheep required. However, the results of agglutination tests on selected sera as reported in this chapter, suggest that for alum precipitated vaccines, which at present, are the most generally accepted formulations, the "K" or "O" titre cannot be used to predict the immune status of individual sheep. In salmonellosis and leptospirosis agglutination titres are used diagnostically but without reference to protection and in brucellosis a disassociation between immunogenicity and agglutinogenicity for Brucella abortus fractions has been particularly well demonstrated (Rasooly, Boras and Gerichter, 1968). Although agglutination titres can serve as a useful index of response by the host to an introduced antigen, any association between titre and resistance to infection seems largely fortuitous.

The serological differences between strains as previously reported (Beveridge, 1941; Thomas, 1962a; Egerton, 1972a; Egerton and Morgan, 1972) were confirmed and further information was obtained. The quantitative differences in titre were within the expected range for essentially different strains but there was in addition one deposit pattern for F. nodosus (J3/22), and another for F. nodosus (M9/4) and F. nodosus (McM198 (Table V.VI). Both of the last strains were agglutinated by all three antisera as a very fine granular deposit on the slopes of the tube without full clearing of the supernatant. This pattern was similar to that obtained with F. nodosus (J3/22) cells grown in hoof broth (Chapter III). As recycling of the supernatants (Table V.VII) demonstrated typical agglutination in the homologous system but little improvement in the heterologous, the results suggest that if F. nodosus (J3/22) is grown in an appropriate medium, the cells will bear determinants not shared by the other two strains. Unlike the virulent field strain F. nodosus (J3/22),

F. nodosus (M9/4) is poorly proteolytic and was isolated from benign foot-rot. This combination of lower enzyme production and particular clinical features of the type of foot-rot suggest that the superficial antigens that appear to be missing may be associated with protease production and/or invasiveness. Although proteolytic activity is believed to be gradually lost on repeated subculture (Egerton and Parsonson, 1969), F. nodosus (McM198) produces the same amount of protease as F. nodosus (J3/22) but unlike the latter, the virulence of F. nodosus (McM198) for sheep has not been tested by the writer.

The fine type agglutination pattern produced with F. nodosus (M9/4) and F.nodosus (McM198) may indicate the absence of one or more superficial antigens, which by inference are presumably present in F. nodosus (J3/22). It is possible that the more virulent F. nodosus field strains have additional antigens associated with invasiveness or speed of adaptation to changes in nutrition and if this is the case, the selection of these "master" strains for vaccine manufacture could be an important feature in field effectiveness of the formulation.

Another unusual feature of "K" antigens has been demonstrated using F. nodosus (J3/22) grown in a 10 l fermenter. When untreated cells from this batch were mixed with dilutions of homologous antiserum there was no visible agglutination and no clearing of the supernatant. However, the same cells incorporated with a variety of adjuvant systems and injected into sheep produced "K" agglutinins to high titre so demonstrating the existence of "K" determinants which seem unable to function in antiserum. The same vaccines afforded high levels of sheep protection in Vaccine Trial II and as the cells after washing and boiling gave the best "O" reactivity of any batch (Chapter III) there is the possibility that all functional determinants were masked until exposed by washing in vitro or processing by cells of the reticuloendothelial system in vivo.

Masking of "O" agglutinogens was demonstrated by the relative somatic inagglutinability of formalin treated F. nodosus cells (Table V.VIII) which, under the conditions of the test, may be due either to the presence of superficial heat-labile agglutinogens or also to interference by formalin. Formalin fixed flagella are known to interfere with somatic agglutination (Cruickshank, 1965) but this F. nodosus cell suspension was derived from biphasic medium and electron microscope studies on cells cultured by this method have not shown filamentous appendages of any kind (Chapter II). Formalin can almost certainly be exonerated too because the antiserum against heated cells was raised using boiled, formalin treated cell suspension and therefore only heat labile determinants of the unwashed cells

would be missing. From these results, the inference is that in antiserum raised against washed and heated cells, "K" agglutinogens cause a blocking effect that prevents the full expression of "O" agglutination. Washed and boiled cells, placed in the same antiserum, are able to realise the full potential of the somatic agglutination titre. This situation for F. nodosus corresponds with that for certain other bacteria without extra cellular appendages; namely non-piliated strains of Escherichia coli that possess the surface K antigen in addition to the somatic O antigen (Kauffmann, 1947). De La Cruz and Cuadra (1969) have shown that the same relationship exists between heated and unheated cell suspensions of Bacteroides fragilis and Bacteroides ovatus.

Summary and Conclusions

1. Both "K" and "O" agglutinating antibodies against F. nodosus exist in the serum of normal sheep and both may rise slightly during foot-rot infection.

2. After vaccination, "K" agglutination titres rose higher relative to normal sheep levels than somatic agglutination titres.

3. Water-in-oil adjuvant vaccines provoked the highest "K" and "O" agglutination responses. Somatic agglutination titres of 2000 or greater may be indicative of satisfactory resistance against F. nodosus infection.

4. Alum and aluminium hydroxide adjuvant vaccines provoked far lower "K" agglutination titres and only very slight rises in somatic agglutination titres. Neither of these were directly correlated with resistance against F. nodosus infection but "K" titres of 10,000 or more were usually indicative of significant protection.

5. Injection of foot-rot infected sheep with an alum precipitated vaccine raised the "K" agglutination titre 16-fold but the somatic titre rose insignificantly.

6. Serological differences between strains were confirmed. F. nodosus (J3/22) appeared to possess additional specific determinants not shared by either F. nodosus (M9/4) or F. nodosus (McM198).

7. F. nodosus (J3/22) showed somatic inagglutinability and this was considered to be due to a blocking effect by superficial heat-labile antigens.

TABLE V.I

Post-vaccination agglutination titres of "resistant" or "susceptible" sheep and pooled sera from the same group in Vaccine Trial I

Vaccine *		Serum source	Susceptibility score	Post-vaccination Agglutination titres ≠	
Adjuvant	Culture			Formalin treated "K"	Heated "O"
McMaster	Biphasic cells	pool	1	163840	2560
		R	0	163840	ND
		S	3	20480	ND
Aluminium hydroxide	Biphasic whole culture	pool	8	10240	640
		R	0	20480	ND
		R	3	5120	ND
		S	11	5120	ND
		S	25	2560	ND
Aluminium hydroxide	Biphasic cells	pool	4	10240-20480	320
		R	0	5120	ND
		R	0	20480	ND
		S	9	5120	ND
		S	8	5120	ND
Controls		pool	15	80	320
		R	8	80	ND
		S	20	160	ND

* Two doses at one week interval. Challenged 28 days after second dose of vaccine

≠ Day before challenge

R = "Resistant"

S = "Susceptible"

ND = Not done.

TABLE V.II

Post-vaccination agglutination titres of "resistant" or "susceptible" sheep and pooled sera from the same group in Vaccine Trial II

Vaccine*		Serum source	Susceptibility score	Post-vaccination Agglutination titres≠	
Adjuvant	Culture			Formalin treated "K"	Heated "O"
McMaster	Biphasic cells	pool	4	20480	2560-5120
		R	2	16000	5120
McMaster	Fermenter[1] cells	pool	6	> 40960	2560
		R	3	8000	5120
		S	14	4000	1280-2560
Water-in-oil	Fermenter cells	pool	10	20480-40960	1280
		R	3	8000	2560-5120
		S	14	8000	2560
Double emulsion	Fermenter cells	pool	14	10240-20480	1280
		R	5	16000	1280-2560
		S	24	8000	640-1280
Alum one per cent.	Fermenter whole culture	pool	6	20480	640
		R	4	16000	640
		S	9	8000	640-1280
Organic polymer	Fermenter cells	pool	13	1280-2560	640
		R	7	8000	320-640
		S	23	8000	320-640
Aluminium hydroxide	Fermenter cells 10^{10}/dose	pool	13	5120	320
		R	3	8000	640
		S	23	ND	320-640
Controls		pool	23	160	ND
		R	12	200	320
		S	30	100	160-320

* Two doses at two week interval. Challenged 20 days after second dose of vaccine

≠ Fourteen days before challenge

[1] Volume 10 l

R = "Resistant"

S = "Susceptible"

ND = Not done.

TABLE V.III

Post-vaccination agglutination titres of "resistant" or "susceptible" sheep and pooled sera from the same group in Vaccine Trial III

Homologous challenge - F. nodosus (J3/22)

Vaccine*		Serum source	Susceptibility score	Post-vaccination Agglutination titres≠	
Adjuvant	Culture			Formalin treated "K"	Heated "O"
Alum one per cent. 1 dose	Fermenter[1] whole culture	pool	9	640	ND
		R	5	5120	320-640
		S	16	2560	320-640
Alum one per cent. 2 doses	Fermenter whole culture	pool	15	1280	640
		R	3	10240	160-320
		S	19	2560	640
Controls		pool	16	160	160-320
		R	11	80	160-320
		S	22	320	160-320

* Two doses at two week interval. Single dose given at time of other first doses. Challenged 21 days after second dose of vaccine.

≠ Seven days before challenge.

[1] Volume 600 l

R = "Resistant"

S = "Susceptible"

ND = Not done.

TABLE V.IV

Post-vaccination agglutination titres of "resistant" or "susceptible" sheep and pooled sera from the same group in Vaccine Trial III

Heterologous challenge - F. nodosus (A8/C)

Vaccine*		Serum source	Susceptibility score	Post-vaccination Agglutination titres≠	
Adjuvant	Culture			Formalin treated "K"	Heated "O"
Alum	Fermenter[1]	pool	16	640	ND
one per cent.	whole	R	6	1280	640
	culture	S	24	1280	640
1 dose					
Alum	Fermenter	pool	13	1280	ND
one per cent.	whole	R	3	10240	320
	culture	S	25	1280	640
2 doses					
Commercial vaccine "A"		pool	18	160	2560
oil		R	10	10240	1280-2560
1 dose		S	26	<640	1280
Commercial vaccine "B"		pool	14	40-80	640
alum precipitated		R	4	640	320-640
2 doses		S	20	<640	640
Controls		pool	24	40-80	ND
		R	22	160	320-640
		S	30	160	320-640

* Two doses at two week interval. Single dose given at time of other first doses. Challenged 21 days after second dose of vaccine.

≠ Seven days before challenge

[1] Volume 600 l

R = "Resistant"

S = "Susceptible"

ND = Not done.

TABLE V.V

Effect of vaccination on agglutination titres of sheep with existing foot-rot

Sheep treatment	Period after treatment	Agglutination titres	
		Formalin treated "K"	Heated "O"
none	nil	320	320
none	3 wk	320	320
none	6 wk	320	320
before treatment	nil	320	320
single vaccine* dose	3 wk	5120	640
single vaccine dose	6 wk	1280	320
two doses of vaccine separated by 3 wk	3 wk	5120	320-640

* Alum (one per cent.) precipitated whole culture F. nodosus (J3/22), fermenter volume 10 l.

TABLE V.VI

Agglutination reactions using formalin treated cells of three F. nodosus strains with specific antisera

Cells formalin treated	Anti-F. nodosus antiserum		
	J3/22	McM198	M9/4
F. nodosus (J3/22)	81920 typical	1280 part cleared	10240 typical
F. nodosus (McM198)	5120 part cleared	10240 no clearing	ND
F. nodosus (M9/4)	10240 part cleared	ND	40960 no clearing

ND = not done.

TABLE V.VII

Agglutination of F. nodosus (J3/22) cells by unabsorbed antibody present in homologous and heterologous antisera from the previous experiment (Table V.VI)

Cells formalin treated	Anti-F. nodosus antiserum	
	J3/22	M9/4
F. nodosus (J3/22)	1280 typical	640 part cleared
F. nodosus (J3/22)	40960 typical	2560 no clearing

TABLE V.VIII

Agglutination reactions using formalin treated, or washed and boiled F. nodosus (J3/22) cells with their respective and opposite specific antisera

F. nodosus (J3/22) cell treatment	Agglutination titres of anti-F. nodosus (J3/22) antisera	
	Against boiled cells	Against formalin treated cells
Washed, boiled, washed in PBS	10240	1280
Unwashed, formalin treated	1280	163840

PBS = Phosphate buffered saline pH 7.4

CHAPTER VI

SEROLOGICAL CHANGES IN SHEEP DURING FOOT-ROT INFECTION AND AFTER VACCINATION WITH VARIOUS ADJUVANT/CELL COMBINATIONS
(ii) ANTIPROTEOLYTIC

Introduction

Interest in the antiproteolytic activity of serum arose because various F. nodosus strains were known to produce proteolytic enzymes during the logarithmic and stationary phases of growth (Thomas, 1964a). Strains of F. nodosus causing benign foot-rot were known to produce approximately half as much protease as strains that caused progressive foot-rot (Thomas, 1962a; Egerton and Parsonson, 1969). As F. nodosus strains causing benign foot-rot are also non-invasive, an association between proteolytic activity and invasiveness has been assumed but the contribution that protease makes to the pathogenesis of progressive foot-rot has never been clearly defined.

Thomas, (1962, cited by Merritt, Egerton and Loi, 1971) showed that these proteases were inhibited by serum of sheep, horses, rabbits and man. The inhibitory fraction of all sheep serum, including precolostral lamb serum, was identified by Merritt, et al., (1971) as an α-macroglobulin. Further work at the McMaster Laboratory (Commonwealth Scientific and Industrial Research Organisation, 1969-70) showed that shortly after vaccination another specific F. nodosus protease inhibitor developed in the IgG_2 fraction of sera. As neither α-macroglobulin nor IgG_2 alone had any appreciable bactericidal activity against F. nodosus (Egerton and Merritt, 1970) it was felt unlikely that sheep resistance would be dependent upon antiproteolytic activity. Nevertheless the complementary interaction between IgG_2 antiproteases raised by vaccination and IgG_1 in the destruction

of F. nodosus in vitro had already been demonstrated (Merritt, et al., 1971).

The investigation described in this chapter was undertaken to determine whether or not the serum antiproteolytic activity was raised by vaccination and if so whether there were quantitative differences that could be traced back either to the type of adjuvant or to the method of culture used for the F. nodosus cells in the vaccine.

Serum samples were available from lambs before and after sucking colostrum, from normal adult sheep and from other adults either before or after vaccination or attempted F. nodosus infection.

MATERIALS AND METHODS

Sera

Pools of sera were used from treatment groups in the three vaccine trials (Chapter IV) and from Experiment Therapy I (Chapter XI). Pre-colostral and post colostral lamb sera were obtained from lambs born to either vaccinated or normal dams.

Indicator of proteolysis

Proteolytic activity in mixtures was estimated by the digestion of gelatin that occurred on 2 mm wide strips of daylight exposed x-ray film (Kodak Royal Blue RB54) placed in the mixtures. The strips of film were placed in tubes containing the reacting mixtures and where digestion had taken place, liquefaction of the gelatin exposed the transparent blue Estar-RH base beneath.

Protease

Freshly harvested culture supernatant was filtered to remove the bacterial cells and concentrated in volume 50 times by overnight dialysis at 4°C against polyethylene glycol. The concentrate was distributed in 1.5 ml amounts and stored at -20°C. Before use in the anti-proteolytic test, an aliquot was thawed and its proteolytic activity assayed as follows. Doubling dilutions of culture supernatant in phosphate buffered saline, pH 7.4, were distributed into 3 ml disposable plastic test tubes and an indicator strip added to each. The mixtures were incubated at 37°C for one hour after which the strips were examined for gelatin digestion. The end point was taken as the last tube where digestion was 50 per cent. or more completed and the reciprocal of the dilution became the proteolytic titre ascribed to the culture supernatant sample.

Serology

The antiproteolytic test was carried out in two stages.

(i) Serum dilutions in phosphate buffered saline, pH 7.4, were made up in 3 ml plastic disposable test tubes. To each tube was added 0.2 ml of serum dilution and an equivalent amount of culture supernatant at the

chosen dilution. For the antiproteolytic titration, the culture supernatant was used eight fold stronger than its proteolytic titre so that after additions of serum dilution and culture supernatant dilution, four "proteolytic doses" were available in each tube. The mixtures were placed in a water bath at 37°C for one hour.

(ii) Indicator strips of x-ray film were added to the tubes and incubation allowed to continue for another two hours after which the test was read by examining the immersed part of the indicator strips. The end point was taken as that tube where proteolytic activity had been completely or more than 50 per cent. inhibited. The antiproteolytic titre of a serum was defined as the reciprocal of the final serum dilution in the end point tube.

RESULTS

All normal sheep including precolostral and postcolostral lambs had titres of antiproteolytic activity of the same order i.e. 640 or 1280, and this did not change when the sheep became infected with progressive foot-rot. When sheep with existing foot-rot were vaccinated with one or two doses of the Alum (one per cent.) precipitated whole culture vaccine, either schedule produced up to a four fold rise in the antiproteolytic titre (Table VI.I).

Water-in-oil vaccines used on normal sheep produced up to four fold increases in the antiproteolytic titre, and two doses of alum precipitated vaccines produced a two fold increase (Table VI.II). Either aluminium hydroxide gel adjuvant vaccines by a two dose schedule, or one dose of the alum precipitated vaccines did not change the antiproteolytic titre.

DISCUSSION

The high antiproteolytic titres of normal sheep sera are in all probability due to the α-macroglobulin described by Merritt, Egerton and Loi (1971). As antiproteases raised by vaccination are of the IgG_2 class (Merritt et al., 1971) it is possible that α-macroglobulin activity in the sera of vaccinated sheep masks the response to vaccination unless the antiproteolytic gamma globulin concentration rises well above that of the α-macroglobulin.

The more reactive adjuvants such as the McMaster formulation and that used in Commercial Vaccine "A", were again responsible for producing the greatest overall rises in antiproteolytic titre. These two vaccines during Vaccine Trials I, II and III, (Chapter IV), produced some of the most and some of the least effective protection of sheep by any form of vaccine.

The disassociation between protection and antiproteolytic titre suggested by the results above is confirmed by the insignificant rise in anti-proteolytic titre produced by Alum (one per cent.) whole culture vaccine that in Vaccine Trials II and III (Chapter IV) afforded significant protection when the sheep were challenged with F. nodosus.

Summary and Conclusions

1. The sera of all normal sheep, including lambs before they had taken colostrum, had an antiproteolytic titre of the same order. This titre remained unchanged during foot-rot infection.

2. Vaccination of normal or foot-rot infected sheep caused only a slight rise in the overall antiproteolytic titre. In this respect water-in-oil adjuvant vaccines were more potent than the other vaccine formulations.

3. There was no direct relationship between the serum antiproteolytic titre of individual sheep or groups of sheep and their immunity to foot-rot.

TABLE VI.I

Effect of vaccination on serum antiproteolytic titres of sheep with existing foot-rot

Sheep treatment	Period after treatment	Antiproteolytic Titre
none	nil	1280
none	3 wk	640
none	6 wk	640
before treatment	nil	1280
single vaccine* dose	3 wk	1280
single vaccine dose	6 wk	2560
two doses of vaccine separated by 3 wk	3 wk	1280

* Alum (one per cent.) precipitated whole culture *F. nodosus* (J3/22), fermenter volume 10 l.

TABLE VI.II

Antiproteolytic titres of pooled sera from groups of sheep vaccinated with various adjuvant/cell combinations in Vaccine Trials I, II, and III

Vaccine			
Adjuvant	Culture	Vaccine Trial No.	Antiproteolytic titre
McMaster	Biphasic cells	I	5120
McMaster	Biphasic cells	II	2560
McMaster	Fermenter cells	II	2560
Water-in-oil	Fermenter cells	II	2560
Double emulsion	Fermenter cells	II	1280
Commercial Vaccine "A"		III	5120
Alum one per cent.	Fermenter whole culture 2 doses	II	1280
Alum one per cent.	Fermenter whole culture 2 doses	III	2560
Alum one per cent.	Fermenter whole culture 1 dose	III	1280
Commercial Vaccine "B"		III	2560
Organic polymer	Fermenter cells	II	1280
Aluminium hydroxide	Biphasic cells	I	1280
Aluminium hydroxide	Biphasic whole culture	I	1280
Controls		I	1280
Controls		III	1280

CHAPTER VII

SEROLOGICAL CHANGES IN SHEEP DURING FOOT-ROT INFECTION AND AFTER VACCINATION WITH VARIOUS ADJUVANT/CELL COMBINATIONS

(III) IMMUNOPRECIPITATION IN AGAR

Introduction

The influence of culture methods on both the morphology of F. nodosus and on the organism's agglutinating properties have been discussed (Chapters II and III). As double diffusion in agar gel provides a convenient system for the analysis of complex antigenic mixtures (Williams and Chase, 1971), it was reasoned that by the use of such a system the

specificity and heat stability of certain F. nodosus antigens might be further established. Furthermore, precipitation in gel would enable a more precise localisation of reacting antigens and determine their occurrence both in the bacterial cell and free in the culture medium. An additional application was seen for such a system in that agar diffusion might be used to analyse the number and intensity of antigen/antibody systems occurring between F. nodosus antigens and the sera of sheep with known histories of foot-rot infection or vaccination with different adjuvant/cell combinations.

MATERIALS AND METHODS

Bacteria F. nodosus (J3/22), F. nodosus (McM198), F. nodosus (McM199) and F. nodosus (A8/C) were grown from the working seed established for each strain.

Untreated cell extracts F. nodosus cells were harvested from biphasic medium or hoof agar. The unwashed cells were suspended in a minimal amount of phosphate buffered saline, pH 7.4, and passed twice through the French press (Appendix VI). Following centrifugation the extract was concentrated by dialysis against polyethylene glycol and stored in small aliquots at -20°C.

Culture supernatants Cells grown in biphasic medium were deposited by centrifugation and the supernatant culture fluid collected. The supernatant was sterilised by filtration through a Gamma-12 filter tube (Whatman, U.K.), concentrated by dialysis against polyethylene glycol and stored at -20°C.

Cell washings Cells were washed three times in phosphate buffered saline, pH 7.4, and the washings concentrated by dialysis against polyethylene glycol. Small aliquots were stored at -20°C.

Extracts of boiled cells F. nodosus cells cultured either on hoof agar or in 10 litre batches of GC broth, were washed three times in phosphate buffered saline, pH 7.4, before being treated at 100°C for 90 minutes. The cells were then washed a further three times before being disrupted by two cycles through the French press.

Protease Protease was obtained from the culture supernatant of a GC broth-grown F. nodosus (J3/22) culture. The protease was isolated by ammonium sulphate precipitation and column chromatography procedures and was generously donated by Mr S. Singh, Department of Biotechnology, Massey University.

Antisera Antisera were prepared in sheep against :

F. nodosus (J3/22): cells harvested from biphasic medium were incorporated with Freund's Incomplete Adjuvant.

F. nodosus (McM198): cells harvested from biphasic medium were incorporated with Freund's Incomplete Adjuvant.

F. nodosus (McM199): cells harvested from biphasic medium were incorporated with Freund's Incomplete Adjuvant.

F. nodosus (J3/22) Boiled: cells harvested from biphasic medium were boiled for 90 minutes before being incorporated with Freund's Incomplete Adjuvant.

F. nodosus (J3/22) Sonicated: cells harvested from biphasic medium were sonicated for 12 or 20 minutes and adsorbed with aluminium hydroxide gel for three days.

F. nodosus (J3/22) Culture supernatant: culture supernatant derived from a biphasic medium culture was concentrated by dialysis against polyethylene glycol and incorporated with Freund's Incomplete Adjuvant.

Control antisera were also prepared against :

Phosphate buffered saline, pH 7.4, incorporated either with Freund's Incomplete Adjuvant or McMaster formulation.

Incubated but uninoculated hoof broth from biphasic medium incorporated with Freund's Incomplete Adjuvant.

Full details of the production of these antisera are given in Appendix X.

Rabbit anti-whole sheep serum and rabbit anti-sheep IgG were obtained commercially (Miles, Kankakee, U.S.A.).

Antisera taken from sheep before, during and after naturally occurring foot-rot infections were available for study. Other sheep antisera included samples from animals involved in Vaccine Trials I, II and III (Chapter IV). For comparative purposes, sera from selected "resistant" or "susceptible" sheep were chosen using the criteria described in Chapter V.

In addition, serum samples were obtained from lambs, born to either normal or vaccinated ewes. Initial samples of sera were taken at birth before ingestion of colostrum and a second one, three to four days later.

Absorption of antisera Certain antisera were in some instances absorbed with F. nodosus (J3/22) cells grown either in biphasic medium or in GC broth. Absorptions were done at $4^{o}C$ for 1 or 24 hours: equal volumes of antisera and cell suspension at a concentration of 10^{10} cells/ml were used.

Isolation of sheep serum IgG The method used was that of Stanworth (1960). Sheep serum dialysed against phosphate buffer, 0.01M, pH 7.5 was absorbed with DEAE-cellulose (DE-32, Whatman, U.K.) that had been equilibrated with the same buffer. The mixture was held at $4^{o}C$ for five hours.

After this time the cellulose was removed by centrifugation and IgG-rich supernatant concentrated by dialysis against polyethylene glycol (IgG fraction). Further proteins were eluted from the sedimented cellulose by treatment with phosphate buffer, 0.3M, pH 7.5. This eluted material was collected by centrifugation and similarly concentrated (DEAE elution).

Double diffusion in agar Agar diffusion was carried out in one per cent. "Ionagar" No. 2 (Oxoid, London) in isotonic saline (Ouchterlony, 1968). A detailed account of the preparation and subsequent treatment of the plates is included in Appendix XIII.

Immunoelectrophoresis Immunoelectrophoresis was carried out in either one per cent. "Ionagar" No. 2 or one per cent. agarose in barbitone buffer, pH 8.6 (Appendix XIIIb).

RESULTS

1. Reactions using F. nodosus untreated cell extract

(a) General

Sera from normal sheep and those that had an existing foot-rot infection of up to three months duration, reacted against untreated cell extract with a single indistinct precipitate line (Figure VII.1 wells 3, 4 and 7). Most sera from vaccinated sheep showed in addition a more distinct band which was positioned peripherally to the original immunoprecipitate, (Figure VII.1 wells 1, 2, 5 and 8; Figure VII.2 wells 3, 5, and 7), and which differed in intensity according to the adjuvant/cell combination that had been used as vaccine. The line that arose as a result of vaccination, hereafter referred to as line "V" was far less distinct after absorption of immune sera by F. nodosus cells (Figure VII.2 wells 2 and 4). McMaster formulation vaccine from Vaccine Trial I, produced sera that regularly raised a strong "V" line and 3 or 4 other bands (Figure VII.1 well 5).

(b) F. nodosus strain relationships

In the heterologous reactions involving both anti-F. nodosus (McM198) antiserum and anti-F. nodosus (McM199) antiserum with F. nodosus (J3/22) cell extract, there were reactions of complete identity both for line "V" and other fainter lines (Figure VII.1 wells 1, 2 and 8). Less intense precipitates, not common to both strains were also demonstrated (Figure VII.3, arrowed). Boiling of the same F. nodosus (J3/22) cell extract destroyed all reactivity (Figure VII.4 well 4) whereas the boiled F. nodosus (McM198) cell extract produced a double line, blurred immunoprecipitate with its homologous antiserum, and a single line of identity when reacted against anti-F. nodosus (J3/22) antiserum (Figure VII.4 well 2).

(c) Sera from Vaccine Trial I - Comparison of "resistant" and "susceptible" sheep

Figures VII.5 and VII.6 show reactions between the homologous untreated cell extract and sera from "susceptible" or "resistant" sheep in Vaccine Trial I. The McMaster adjuvant formulation vaccine produced sera that showed 3 or 4 distinct precipitation lines (Figures VII.5 wells 2, 5 and 8; Figure VII.6 well 4) whereas sera from sheep injected with aluminium hydroxide adjuvant vaccines, showed fewer lines (Figure VII.5 wells 3, 4, 6 and 7). Precipitates against sera of "susceptible" sheep, were usually fainter (Figure VII.6) and sometimes line "V" was missing from reactions between the cell extract and sera from sheep vaccinated with aluminium hydroxide adjuvant vaccines (Figure VII.6 wells 2, 3 and 8). However, there was no single line that was consistently present in reactions involving "resistant" sheep sera and consistently absent from reactions involving "susceptible" sheep sera.

(d) Sera from Vaccine Trial II

The number and the intensity of precipitation bands between sera of vaccinated sheep and the untreated cell extract gave some indication of the type of adjuvant used in the vaccine. The McMaster adjuvant vaccine formulation gave rise to sera that reacted with several heavy lines (Figures VII.7 wells 1 and 2; VII.9 wells 2 and 3), the Double emulsion formulation to fewer but equally distinct lines (Figure VII.9 well 1), and Alum adjuvant to one distinct and sometimes another fainter line (Figures VII.8 well 1). Aluminium hydroxide adjuvant based vaccines produced sera that reacted with untreated cell extract to produce 1 or 2 faint immunoprecipitates (Figures VII.8 well 4, and VII.9 well 4). For comparison, Figure VII.10 shows reactions that were produced between the homologous boiled cell antigen and sera from sheep vaccinated with one of the three main adjuvant systems; namely aluminium hydroxide (well 1), alum (well 2), and water-in-oil emulsion (well 3). The differences in intensity of immunoprecipitates are again associated with the local irritancy of the adjuvant.

(e) Sera from Vaccine Trial III

Samples of serum taken from sheep after vaccination, reacted with untreated cell extract to produce 2 or 3 immunoprecipitates including a strong "V" line. By eight weeks after challenge, serum from sheep vaccinated with the Alum (one per cent.) vaccine had lost much of its prechallenge activity whereas activity in the sera raised by the oil based Commercial Vaccine "A" persisted over this period (Figure VII.11). A reaction of identity for line "V" was demonstrated between anti-F. nodosus (J3/22)

antiserum, IgG extracted from that antiserum and for the pool of sera from sheep vaccinated twice with the Alum (one per cent.) formulation (Figure VII.12 wells 1, 2 and 3). Sera from sheep vaccinated with Commercial Vaccine "B" showed a less intense "V" line (Figure VII.12 well 7) and this line was barely visible in the reaction involving the pooled sera of sheep vaccinated with one dose of the Alum (one per cent.) formulation (Figure VII.12 well 4). Two doses of alum precipitated vaccines were more effective than one dose in raising sera capable of producing a strong "V" line against untreated cell extract (Figure VII.13 wells 2, 3 and 4). In reactions involving boiled cell extract the same differences between the effects of one or two doses were apparent (Figure VII.14 wells 2, 3 and 4). The pool of sera from sheep injected with Commercial Vaccine "A", reacted in double diffusion against untreated cell extract with a weak double line, and against boiled cell extract with a single blurred precipitate close to the serum well (Figures VII.13 well 1 and VII.14 well 1).

(f) Transfer of maternal antibody

Precolostral lamb serum was negative in all double diffusion tests whether or not the dam had been vaccinated. Serum from a four days old lamb born to a ewe that had been vaccinated five months previously, reacted against untreated cell extract. There were more lines of precipitation and they were better defined than those between untreated cell extract and serum of the dam taken on the day of parturition (Figure VII.15).

Reaction of identity was established for one intense line, possibly line "V", between sera of the lamb and ewe. The same quantitative relationships were demonstrated when the same sera were reacted against boiled cell extract (Figure VII.16 wells 1, 3 and 4).

2. Reactions using F. nodosus culture supernatants

In tests involving sera of selected "resistant" and "susceptible" sheep of Vaccine Trial I, those from sheep injected with the McMaster formulation usually produced the most intense immunoprecipitates against concentrated culture supernatants (Figure VII.17 well 8). Amongst sera from sheep injected with aluminium hydroxide adjuvant vaccines, the immunoprecipitates were most obvious where sheep had been injected with the whole culture as opposed to F. nodosus cells alone (Figure VII.17 cf. wells 2 and 3; 5 and 7). It was expected that the serum in well 6 would have shown a similar precipitation pattern to that in well 8: similarly sera in wells 1 and 4 were expected to produce comparable precipitation lines. The obvious differences in precipitation pattern were attributed to physical changes in the gel due to dehydration. Reactions between anti-F. nodosus antisera and culture supernatant concentrates were shown to be common to three F. nodosus strains; one such cross reaction is shown in Figure VII.17.

Special antisera were used to further define the immunoprecipitates and Figure VII.18 shows that there was a single heavy line produced between culture supernatant and all sheep sera including those raised against control materials (Figure VII.18 wells 3, 4 and 5). The same line is apparent in Figures VII.1 and VII.2 and was associated with reactions in which either the untreated cell extract or culture supernatant was derived from liquid culture such as biphasic medium or hoof broth.

Serum from a three days old lamb reacted with concentrated culture supernatant in the form of a single distinct precipitation line and this activity was not removed by serum absorption with F. nodosus cells. There was no reaction between serum either from the same lamb or from a vaccinated ewe, and either hoof broth or GC broth that had been concentrated 50 times.

Reaction of identity was demonstrated for immunoprecipitate lines formed between anti-F. nodosus (J3/22) antiserum and either F. nodosus (j3/22) culture supernatant, F. nodosus (A8/C) culture supernatant or purified protease. The relationship became apparent after reactants had been adjusted to a standard proteolytic titre (Figures VII.19 and VII.20).

3. Reactions using F. nodosus cell washings

The washings (Appendix VI) of F. nodosus cells that had been cultured in either biphasic medium or GC broth or hoof broth, contained antigens that reacted against precolostral and postcolostral lamb serum, serum from foot-rot infected sheep (Figure VII.21) and serum from vaccinated sheep. One faint immunoprecipitate against homologous antiserum remained after either boiling the washings for 60 minutes or attempting absorption of antibody from the serum using F. nodosus cells (Figure VII.22).

4. Reactions using extracts of boiled F. nodosus cells

Reactions between sheep sera and the extract of washed and boiled F. nodosus cells (Appendix VI) were slower to develop and required larger amounts of reactants. The type of precipitate produced appeared to be influenced partly by the adjuvant used in the vaccine formulation that raised the serum. In general, water-in-oil adjuvants resulted in sera giving thick ill-defined lines (Figure VII.10, well 3), whereas alum and aluminium hydroxide adjuvant vaccines produced sera giving more definite immunoprecipitates (Figure VII.14 well 4). Antiserum raised by boiled F. nodosus cells (serum 2280 Appendix X) produced an intense reaction with untreated cell extract (Figure VII.23 well 4) whereas the same hoof agar derived untreated cell extract when reacted against anti-F. nodosus (J3/22) (formalised cells) antiserum, produced six moderate lines of precipitation (Figure VII.23 well 2).

Using boiled cell extract there was no reaction with precolostral lamb serum or normal sheep serum. However, serum from a sheep with severe foot-rot infection of two weeks duration produced a faintly visible reaction (Figure VII.24 well 1). Six weeks after infection a further serum sample raised a strong immunoprecipitate against this material; by three and a half months, when the hoof was being shed, the precipitate was seen as an indistinct line positioned almost on the lip of the serum well (Figure VII.24 wells 2 and 3).

Antibody against boiled cell extract appeared in the serum of a four days old lamb that had been born to a vaccinated ewe (Figure VII.16 well 4). Reactions between either F. nodosus (J3/22) or F. nodosus (McM198) boiled cell extracts and their antisera in homologous or heterologous systems were similar and appeared unaffected by serum absorptions using cells of F. nodosus (J3/22). The results were very similar irrespective of whether F.nodosus (J3/22) (Figures VII.25 and VII.26) or F. nodosus (McM198) (Figures VII.27 and VII.28) had been used to provide the boiled cell extract.

There were clearly apparent differences in the reactions between boiled cell extract and serum from either a selected "resistant" sheep or a selected "susceptible" sheep.

When sera from "susceptible" sheep were reacted, lines of precipitation were either faint or absent whereas sera from "resistant" sheep produced distinct immunoprecipitates. Two lines of reaction were sometimes seen particularly if a water-in-oil emulsion formulation vaccine had been used to raise the sera. These immunoprecipitates formed between immune sera and either homologous or heterologous boiled cell extract. Figure VII.29 (wells 1 and 2) shows immunoprecipitates produced by sera from "resistant" sheep that had been vaccinated with an aluminium hydroxide adjuvant vaccine. Sera from "susceptible" sheep of the same group produced no reaction (Figure VII.29 wells 3 and 4).

A similar distinction between the sera of "resistant" and "susceptible" sheep was apparent in different treatment groups from Vaccine Trial II. Figure VII.30 shows the difference in reactions between sera from "resistant" and "susceptible" sheep from a group vaccinated with another aluminium hydroxide adjuvant formulation (Figure VII.30 wells 1 and 2). The faint "precipitate" line between boiled cell extract and serum from a control sheep (well 4) was quite unexpected.

Sera from sheep that had been vaccinated with other adjuvant/cell combinations also reacted with boiled cell extract to produce precipitates that indicated either "resistance" or "susceptibility" (Figure VII.31).

The type of adjuvant used in the vaccine that raised the immune serum further determined the appearance of the precipitation lines (cf. wells 2 and 4).

The effect of either 1 or 2 doses of the Alum (one per cent.) precipitated whole culture vaccine on sheep already affected with foot-rot, was shown by the reactions produced by sera from sheep in Experiment Therapy I (Chapter XI). Two doses of vaccine produced slightly more precipitating antibody against boiled cell extract antigens than one dose, as judged by the intensity of the reaction line (Figure VII.32).

5. Precipitating antibody in serum fractions

When various serum fractions were compared with the whole serum in immunodiffusion, there were two quite distinct patterns of reaction according to whether either untreated cell extract or boiled cell extract were used as the source of antigen. Comparative tests showed that whereas most precipitating activity against the untreated cell extract was associated with serum proteins eluted off the DEAE slurry (Figure VII.33 cf. wells 2 and 4) the IgG fraction of serum contained most activity against the heat treated cell extract (Figure VII.34 cf. wells 2 and 4). The isolation of IgG was shown to be incomplete as the DEAE elution contained appreciable amounts of IgG (Figures VII.35b and VII.37c). The IgG fraction of anti-F. nodosus (J3/22) antiserum was shown to contain an additional component that reacted with rabbit anti-sheep serum to produce a faint precipitate line on the anode side of the well (Figure VII.35b).

In double diffusion against rabbit anti-sheep IgG, the IgG fraction produced a single intense precipitation line whereas both anti-F. nodosus (J3/22) antiserum and the DEAE elution produced two lines of reaction (Figure VII.38).

Precolostral sera of lambs born to either normal or vaccinated ewes contained no IgG as indicated by the absence of a precipitation line in the appropriate position (diagram between Figure 35 and Figure 36) when reacted against rabbit anti-sheep serum (Figure VII.36a and b). Postcolostral sera taken from the same lambs when they were 3 or 4 days old, reacted with an additional intense line in the IgG, (fast) sector (Figure VII.36a and b).

The absence of IgG from precolostral lamb sera (Figure VII.38 well 1) and its presence in postcolostral serum from the same lambs, was confirmed in a reaction system against rabbit anti-sheep IgG (Figure VII.37a and b).

Essentially the same pattern of precipitation lines was formed between rabbit anti-sheep serum and serum from either a normal ewe, a vaccinated ewe or a ewe with long-standing foot-rot (Figures VII.35a and VII.36c).

DISCUSSION

Reactions with untreated cell extract

The immunoprecipitation that occurred between all sheep sera and untreated cell extracts can be taken to be associated with the normal serum antiprotease shown by Merritt, Egerton and Loi (1971) to be an α-macroglobulin. Untreated cell washings reacted with all sera including that of precolostral lambs (Figure VII.21) but cell extracts derived from F. nodosus grown on hoof agar appeared to be less reactive. Cultures grown in biphasic medium or GC broth yielded more precipitinogens against normal serum and it is assumed that unwashed cells from these sources are coated with extracellular antigens normally found free in the supernatant. Concentrated culture supernatants derived from different F. nodosus strains were strongly reactive against all sheep sera and particularly that from vaccinated sheep. By adjusting culture supernatant and purified protease to a common proteolytic titre it was shown that the reaction is dependant upon a specific protease/antiproteolytic system which is revealed using concentrated culture supernatant. Protease is also released upon lysis or disruption of cells (Thomas, 1964a) and untreated cell extracts would be expected to react with specific antiproteolytic antibody in the form of immunoprecipitation.

The identity of line "V" is still something of an enigma. A very faint "V" line was apparent in reactions involving sera from some unvaccinated sheep that had foot-rot but it never appeared in reactions with sera of normal sheep. The antibody responsible for line "V" could be partly absorbed from serum by F. nodosus cells and therefore it appears to arise as the result of a specific immune reaction.

Line "V" intensity closely followed the reactivity of the adjuvant used in the vaccine that raised the serum. Water-in-oil vaccines were the best in this respect and although two doses of alum adjuvant vaccines raised the reacting potential of the serum more than one dose, line "V" could not be used alone to predict the immune status of the donor sheep. But because of its prominence it proved to be a good indicator of reactions of identity in reactions with complex immunoprecipitate patterns.

The reaction of identity for line "V" has shown that it is common to at least three F. nodosus strains. As the specific antiproteolytic effect of immune sera is also common to at least three F. nodosus strains, line "V" may represent either the same reaction or one involving the heat-labile surface antigens.

Figure VII.3 shows that cell extracts made from different F. nodosus

strains contain a large number of antigens, some of which are strain specific. The other important featureshown is that the anti-F. nodosus (J3/22) antiserum used in this test indicates that F. nodosus (J3/22) has many antigens in common with F. nodosus (McM198). The matter of strain specificity in regard to antigens and the implications of this in sheep protection by vaccination will be commented on further in the General Discussion (Chapter XII).

In reactions involving untreated cell extract, the situation of F. nodosus, that has antigens common to other F. nodosus strains as well as strain specific antigens in its structure, seems similar to that described for three strains of Sphaerophorus spps. by Wattre, Fievez and Beerens (1971), for Bacteroides melaningenicus by Hofstad (1969) and for certain oral Fusobacteria by Kristoffersen (1969a and b).

Transfer of maternal antibody

Immunoprecipitation in agar demonstrated a striking difference between lamb sera taken at birth and other samples from lambs that were 3 or 4 days old. In tests using untreated or heat treated cell extracts precipitating activity was absent in precolostral lamb sera, whereas after the lamb had ingested colostrum, reactivity of serum was greater than that from a vaccinated dam at parturition (Figures VII.15 and VII.16). The pattern that emerges is in keeping with the transfer of immunoglobulins raised by other classes of bacterial vaccines (Cooper, 1967) and the presence of passively acquired antibodies may help to explain why young lambs so rarely become seriously affected with foot-rot even though there may be an existing outbreak amongst the ewes.

Precipitating activity in serum fractions

The major gammaglobulin arc with its spur of partial non-identify between the slower migrating IgG_2 and the faster IgG_1 is obvious as the "gull wing" form seen in most sheep serum samples separated by electrophoresis (Figure VII.36c). The exceptions are the two specimens of postcolostral lamb sera (Figure VII.36a and b) taken when the animals were 3 or 4 days old. Jonas (1968) found that on the first day after suckling, lamb serum contained IgG_1, IgG_2 and IgM but when the lambs were 7 and 15 days of age, the IgG_2 was barely detectable. In view of these findings it seems most likely that within 3 to 4 days post suckling, all IgG activity in lamb serum has passed into the IgG_1 class and an arc associated with IgG_2 would not become apparent again until active IgG production commenced.

A yield of only 50 per cent. of the available IgG in serum is all that is claimed for the one washing batch method of Stanworth (1960), so that the presence of IgG in the DEAE elution was not unexpected. The IgG

extracted was largely uncontaminated by other immunoglobulins except for that component responsible for the faint arc shown in Figure VII.35B. An arc in that position (cf. diagram between Figures VII.35 and VII.36) could represent the sheep counterpart of IgA or IgD (Silverstein, Thorbecke, Kramer and Lukes, 1963; Aalund, Osebold and Murphy, 1965) both of which have been identified in ovine sera (Heimer, Clark and Maurer, 1969). However Jonas (1970) found a similar arc in sheep serum, provisionally named it β_1-globulin and found that the serum protein was a heat-labile component of sheep complement.

Reactions with boiled cell extract

Immunoprecipitates between boiled cell extract and immune serum consisted of up to three lines and the intensity of these was influenced by the adjuvant used in the vaccine that raised the serum. There were no confusing nonspecific lines raised between boiled cell extract and normal sheep serum but a rise and fall in serum precipitating antibody could be followed in a sheep that had had severe and progressive foot-rot in all four feet for at least three months (Figure VII.24). The immunoprecipitate between the cell extract and serum taken three and a half months after the infection commenced (well 3), appeared approximately half way between the reacting wells after 24 hours and by 48 hours was almost lost in the antiserum well. The migration of the immunoprecipitate suggests a gross inbalance between reactants (Clausen, 1969) and in this particular test implies that the antibody titre had fallen significantly. At this stage the hoof was almost shed and natural resolution of the infection may have accounted for the decline in circulating antibody.

The major immunoprecipitate involving boiled cell extract was common to all three strains of F. nodosus tested. Like line "V" in reactions against untreated cell extract, activity appeared in sera from lambs only after suckling colostrum from vaccinated dams, and reactivity was present in the IgG fraction. However, Figures VII.33 and VII.34 show that whereas most activity against untreated cell extract resided in the DEAE elution following separation of the IgG fraction, all activity against boiled cell extract lay in the IgG fraction of serum. This distribution was taken to mean that antibodies, perhaps important in protection, were located in the gammaglobulin fraction of serum.

A most significant difference was demonstrated between the precipitation lines produced by sera from "resistant" sheep, and those involving sera from "susceptible" sheep. There was a distinct quantitative difference between "pairs" of sera within each of the treatment groups involved in Vaccine Trials I, II and III. The only obvious discrepancy in an otherwise

predictable pattern arose over a control sheep. The very faint line that developed between the cell extract and well 4 (Figure VII.30) is difficult to explain as the control sheep concerned had a susceptibility score of 12. It is just conceivable that a previous natural infection may have left residual precipitating antibody: the sheep had not been vaccinated at any time.

Reference to Figure VII.14 will show that the immunoprecipitate produced by immune sera raised against one dose of Commercial Vaccine "A" (well 1) is the most dense of those visible but it is located close to the serum well. It is therefore interesting to note that sheep vaccinated with Commercial Vaccine "A" were relatively susceptible to F. nodosus infection (Chapter IV) whereas sheep vaccinated with alum precipitated vaccines (Figure VII.14 wells 2, 3 and 4) were significantly protected. The location of the precipitate line adjacent to well 1 indicates a low concentration of serum antibody and subsequent sheep challenge results suggest that as an indicator of immune status the position of precipitation lines may be as or more important than their intensity.

In general it was found that for any one treatment group, the intensity and position of immunoprecipitates between serum and boiled cell extract could be used to predict the immune status of those selected sheep.

Summary and Conclusions

1. In double diffusion in agar tests a single ill-defined immunoprecipitate appeared between either normal lambs' or adult sheep sera, and either untreated cell washings, untreated culture supernatant or F. nodosus untreated cell extract. This reaction was believed to be associated with the naturally occurring antiprotease shown by Merritt, Egerton and Loi (1971) to be an α-macroglobulin. Concentrates of liquid media did not produce reaction lines with serum of either normal or vaccinated sheep.

2. Extracts of boiled F. nodosus cells did not react with normal sheep serum.

3. The potency of the adjuvant used in the vaccine to raise a serum was a determining factor in the intensity and type of immunoprecipitate formed between that serum and either untreated or boiled cell extract.

4. Dense immunoprecipitates between sera of vaccinated sheep and either concentrated culture supernatants or F. nodosus concentrated cell washings, were shown by reactions of identity with purified protease to be associated with a specific protease/anti-protease reaction. The reaction was not strain specific.

5. When reacted with sera from sheep with existing foot-rot untreated cell extract sometimes produced an extra immunoprecipitate. Against sera from vaccinated sheep, untreated cell extract reacted with numerous lines, most of which were not strain specific. The most distinctive of these, line "V", was common to three F. nodosus strains, serum IgG and postcolostral serum from a lamb born to a vaccinated ewe. Line "V" could be partly absorbed out with F. nodosus cells and it was postulated to be associated with either the specific antiproteolytic effect of serum or certain heat labile surface antigens as these too were an activity common to all three F. nodosus strains. There was some evidence for the existence of strain specific precipitinogens.

The position or density of line "V" was not correlated with resistance against F. nodosus infection.

6. Boiled cell extract reacted with 1 or 2 lines against serum from sheep either with existing foot-rot or that had been vaccinated. The major immunoprecipitate was common to three F.nodosus strains, serum IgG and postcolostral serum from a lamb born to a vaccinated ewe. For any one vaccine treatment group, either presence, location and/or density of the immunoprecipitates was associated with resistance to F. nodosus infection by the serum donor.

7. Most precipitating activity against boiled cell extract was present in the IgG fraction of serum whereas most of the activity against

untreated cell extract was present in the DEAE elution that also contained IgG.

8. Using batch fractionation of serum with diethyl aminoethyl cellulose, an IgG fraction was obtained that was largely uncontaminated by other serum proteins. It was shown by immunoelectrophoresis that IgG was absent from precolostral lamb serum. In sera taken from 3 and 4 days old lambs, IgG activity resided solely in the IgG_1 (fast) class.

FIGURE VII.1

Reactions between untreated cell extract and sera from normal and vaccinated sheep

Serum

1. Anti-*F. nodosus* (J3/22) antiserum
2. Anti-*F. nodosus* (McM199) antiserum
3. Control, 6 wk foot-rot
4. Sheep 626, 3 mth foot-rot
5. McMaster/biphasic anti-*F.nodosus* (J3/22) antiserum
6. Control, 6 wk foot-rot
7. Freund's Incomplete Adjuvant/saline antiserum
8. Anti-*F. nodosus* (McM198) antiserum

wells diam., outer 7.5 mm, inner 4.5 mm

wells separated 20 mm

centre well *F. nodosus* (J3/22) biphasic, untreated cell extract

= 2×10^{10} cells/ml

FIGURE VII.2

Reactions between untreated cell extract and sera from vaccinated sheep - Vaccine Trial I and one special antiserum

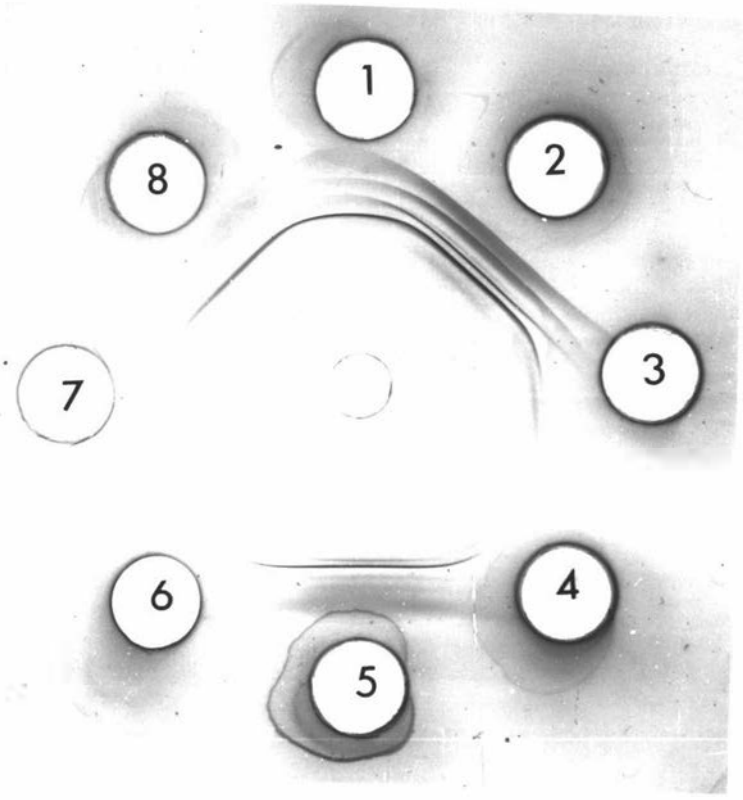
Serum

1. Controls pool, 6 wk after challenge
2. Controls pool, 6 wk after challenge absorbed
3. Aluminium hydroxide/biphasic cells prechallenge
4. Aluminium hydroxide/biphasic cells prechallenge absorbed
5. Aluminium hydroxide/biphasic whole culture prechallenge
6. Aluminium hydroxide/biphasic whole culture prechallenge absorbed
7. Alum (one per cent.)/GC fermenter whole culture (6 doses)
8. Alum (one per cent.)/GC fermenter whole culture (6 doses) absorbed

wells diam., outer 7.5 mm, inner 4.5 mm; wells separated 7 mm

centre well, *F. nodosus* (J3/22) biphasic, untreated cell extract

= 2×10^{10} cells/ml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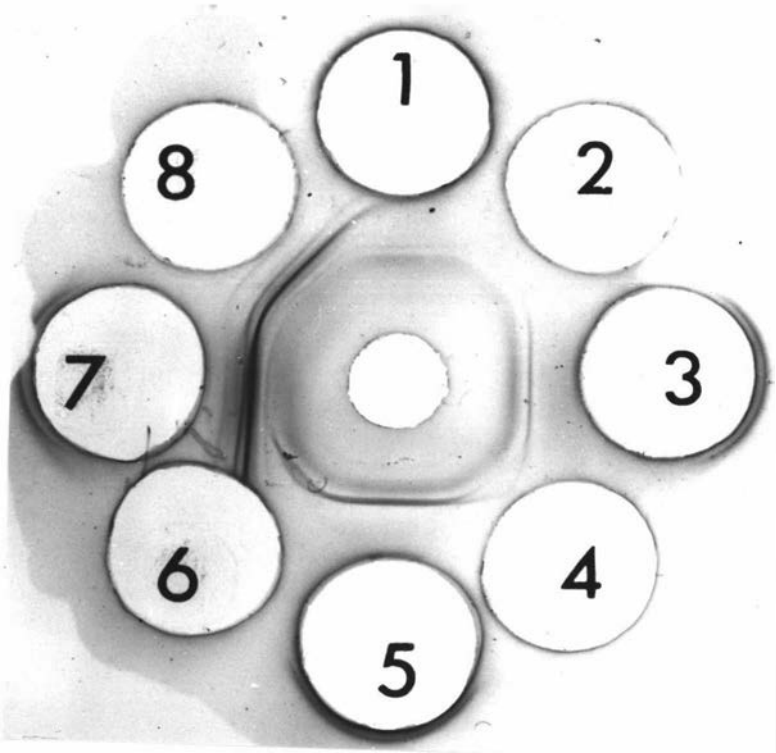
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FIGURE VII.3

Reactions of identity between F. nodosus (J3/22) and F. nodosus (McM198) untreated cell extracts, and their respective and opposite antisera

Antiserum or cell extract

1. Anti-F. nodosus (J3/22) antiserum
2. F. nodosus (McM198) biphasic, untreated cell extract
3. Anti-F. nodosus (McM198) antiserum
4. F. nodosus (J3/22) biphasic, untreated cell extract

wells diam. 7.5 mm wells separated 6 mm

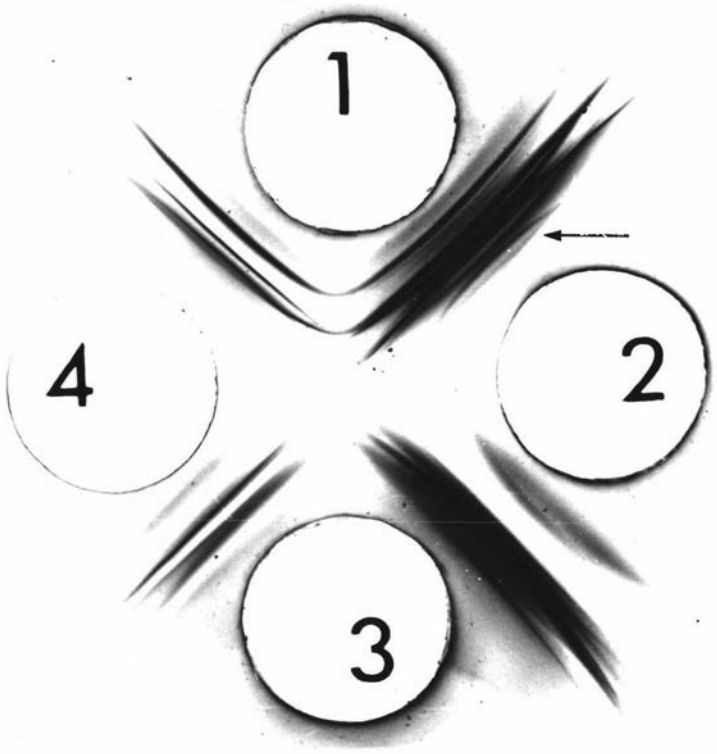
FIGURE VII.4

Reactions of identity between F. nodosus (J3/22) and F. nodosus (McM198) boiled cell extracts, and their respective and opposite antisera

Antiserum or cell extract

1. Anti-F. nodosus (J3/22) antiserum
2. F. nodosus (McM198) biphasic, boiled cell extract
3. Anti-F. nodosus (McM198) antiserum
4. F. nodosus (J3/22) biphasic, boiled cell extract

wells diam. 7.5 mm wells separated 6 mm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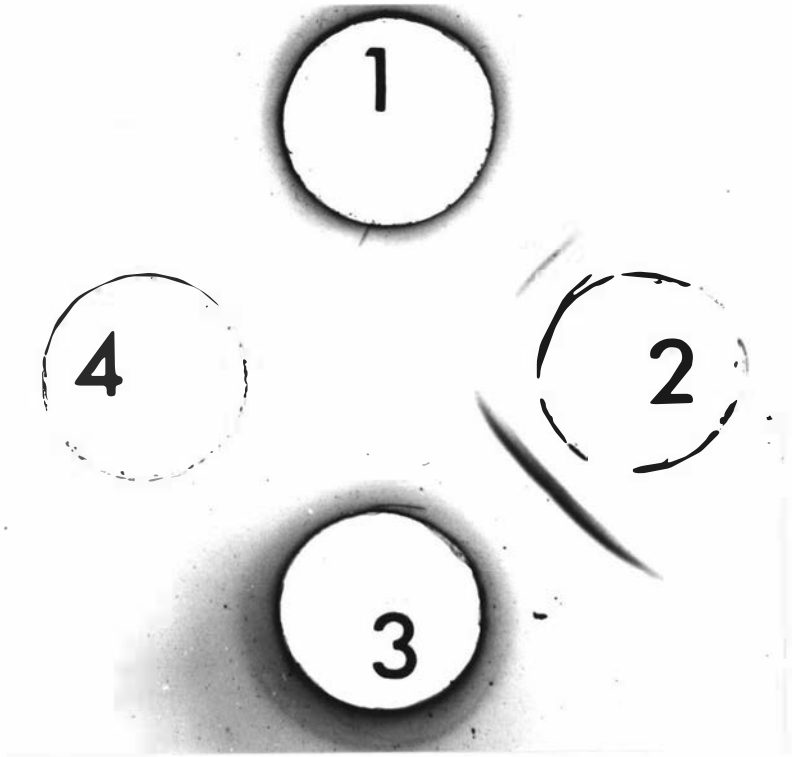
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FIGURE VII.5

Reactions between untreated cell extract and a series of "resistant" sera from Vaccine Trial I

	Serum prechallenge	Susceptibility Score
1.	Anti-*F. nodosus* (J3/22) antiserum	not done
2.	McMaster/biphasic cells	1
3.	Aluminium hydroxide/biphasic whole culture	0
4.	Aluminium hydroxide/biphasic cells	1
5.	McMaster/biphasic cells	0
6.	Aluminium hydroxide/biphasic whole culture	9
7.	Aluminium hydroxide/biphasic cells	0
8.	McMaster/biphasic cells	0

wells diam., outer 7.5 mm, inner 4.5 mm

wells separated 15 mm

centre well *F. nodosus* (J3/22) biphasic, untreated cell extract $= 1.6 \times 10^{10}$ cells/ml

FIGURE VII.6

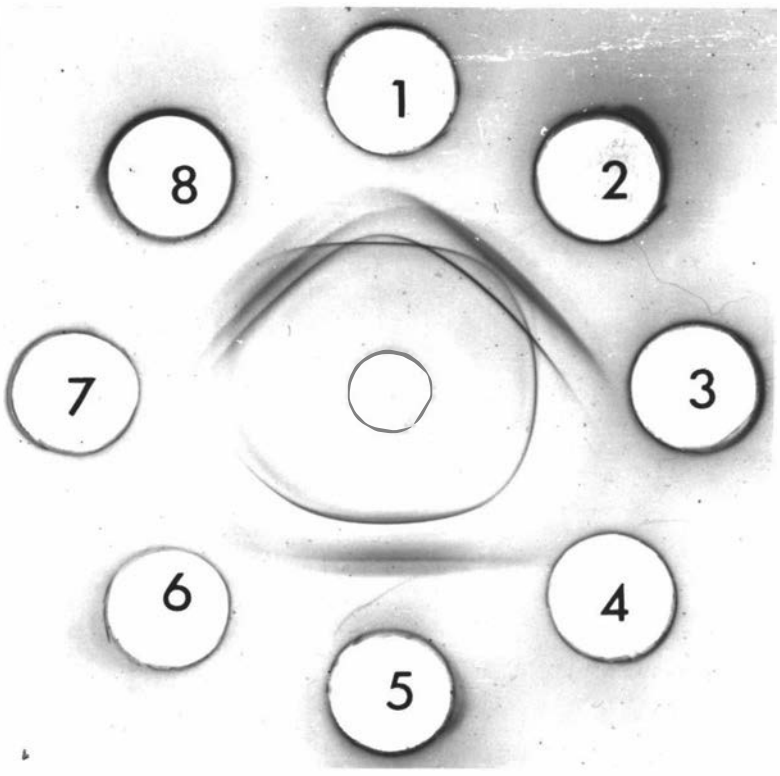
Reactions between untreated cell extract and a series of "susceptible" sera from Vaccine Trial I

	Serum prechallenge	Susceptibility score
1.	Control	18
2.	Aluminium hydroxide/biphasic cells	9
3.	Aluminium hydroxide/biphasic whole culture	25
4.	McMaster/biphasic cells (positive control serum)	3
5.	Control	20
6.	Aluminium hydroxide/biphasic cells	8
7.	Aluminium hydroxide/biphasic whole culture	11
8.	Aluminium hydroxide/biphasic whole culture	11

wells diam. outer 7.5 mm, inner 4.5 mm

wells separated 15 mm

centre well *F. nodosus* (J3/22) biphasic, untreated cell extract $= 1.6 \times 10^{10}$ cells/ml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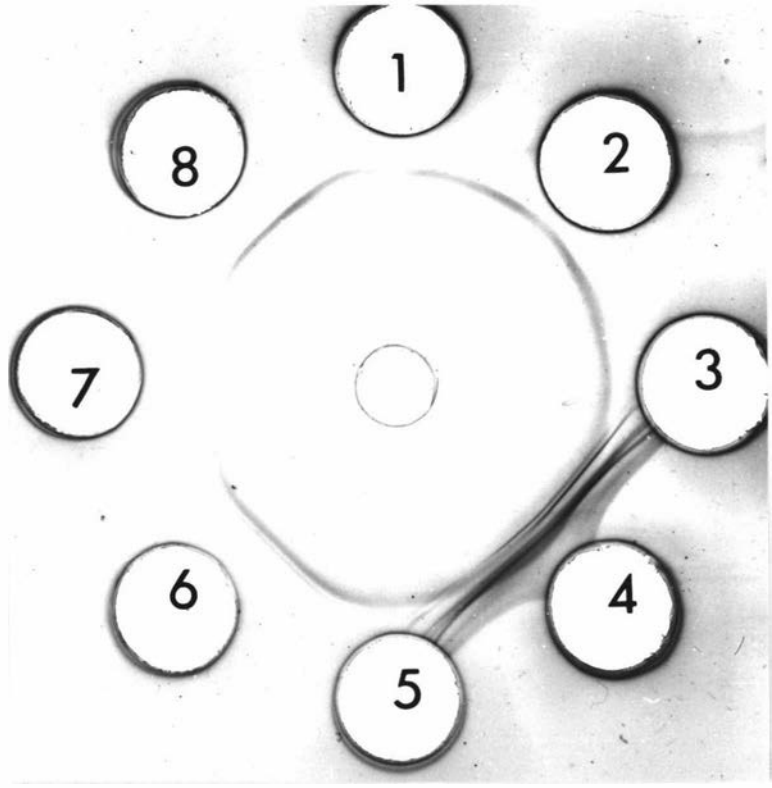
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FIGURE VII.7

Reactions between untreated cell extract and sera from sheep in Vaccine Trial II

	Serum prechallenge	Susceptibility score
1.	McMaster/fermenter cells	7
2.	McMaster/biphasic cells	6
3.	Anti-*F. nodosus* (J3/22) antiserum	not done
4.	Control	30

wells diam. 3 mm wells separated 4.5 mm

centre well *F. nodosus* (J3/22) biphasic, untreated cell extract $= 1.6 \times 10^{10}$ cells/ml

FIGURE VII.8

Reactions between untreated cell extract and sera from sheep in Vaccine Trial II

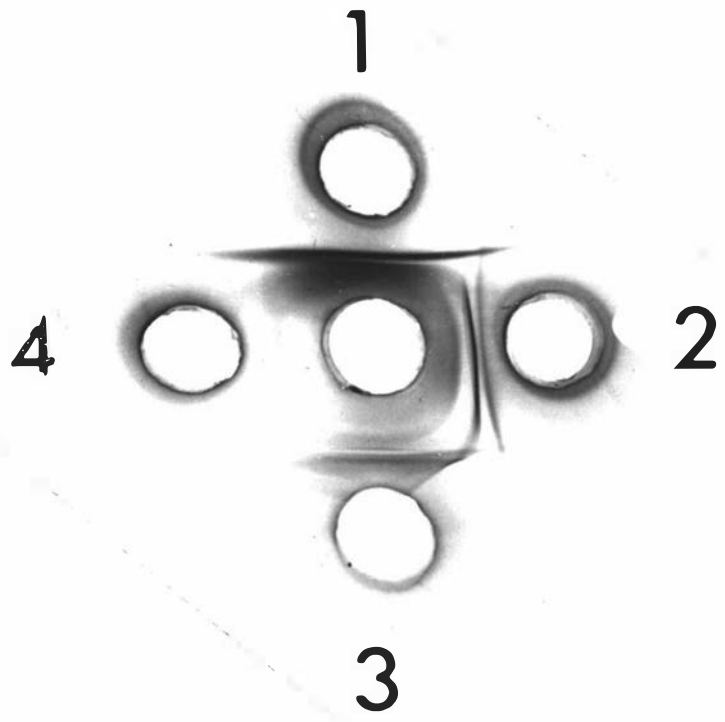
	Serum prechallenge	Susceptibility score
1.	Alum (one per cent.)/fermenter whole culture	died*
2.	Water-in-oil/fermenter cells	14
3.	McMaster/biphasic cells	0
4.	Aluminium hydroxide/fermenter cells≠	19

wells diam. 3 mm wells separated 4.5 mm

centre well *F. nodosus* (J3/22) biphasic, untreated cell extract $= 1.6 \times 10^{10}$ cells/ml

* Susceptibility score of group, 6

≠ 10^{10} cells/dose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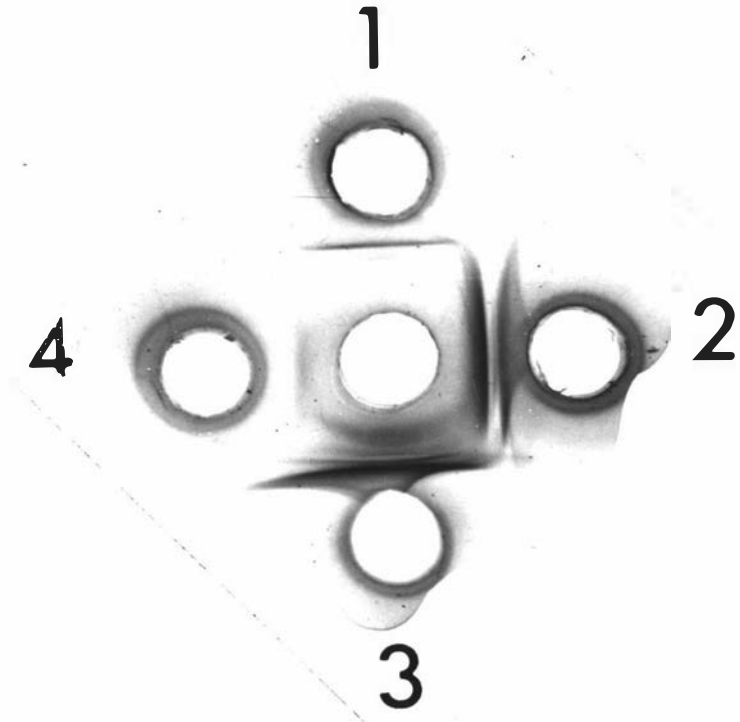
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FIGURE VII.9

Reactions between untreated cell extract and sera from sheep in Vaccine Trial II

	Serum prechallenge	Susceptibility score
1.	Double emulsion/fermenter cells	16
2.	McMaster/fermenter cells	died*
3.	McMaster/biphasic cells	0
4.	Organic polymer/fermenter cells	7

wells diam. 3 mm wells separated 4.5 mm

centre well F. nodosus (J3/22) biphasic untreated cell extract = 1.6×10^{10} cells/ml

* Susceptibility score of group, 6

FIGURE VII.10

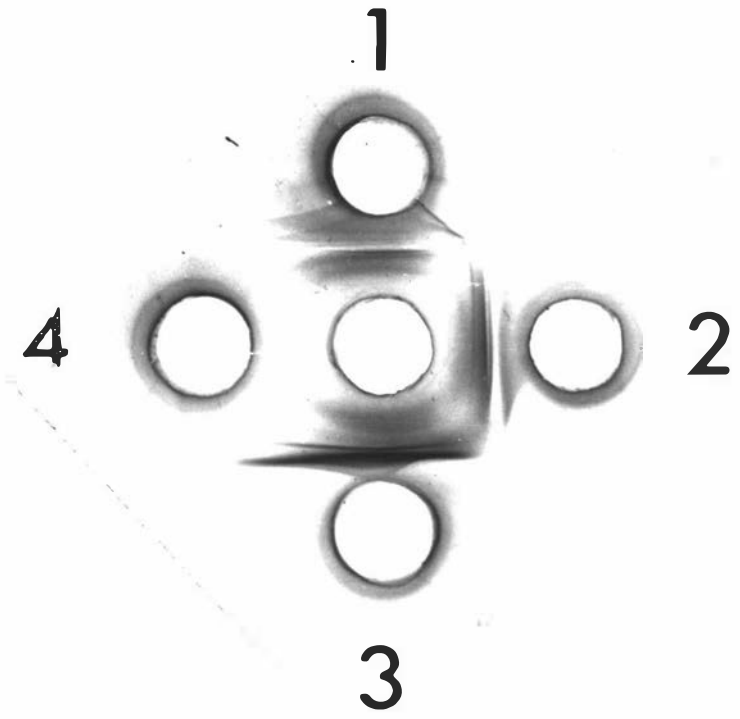
Reactions between boiled cell extract and sera from sheep in Vaccine Trial II

	Serum prechallenge	Susceptibility score
1.	pool of group Aluminium hydroxide/fermenter cells*	13
2.	pool of group Alum (one per cent.)/fermenter whole culture	6
3.	pool of group Water-in-oil/fermenter cells	10
4.	pool of group Controls	23

wells diam. 7.5 mm wells separated 5 mm

centre well F. nodosus (J3/22) hoof, agar, boiled cell extract = 8×10^{9} cells/ml

* 10^{10} cells/dose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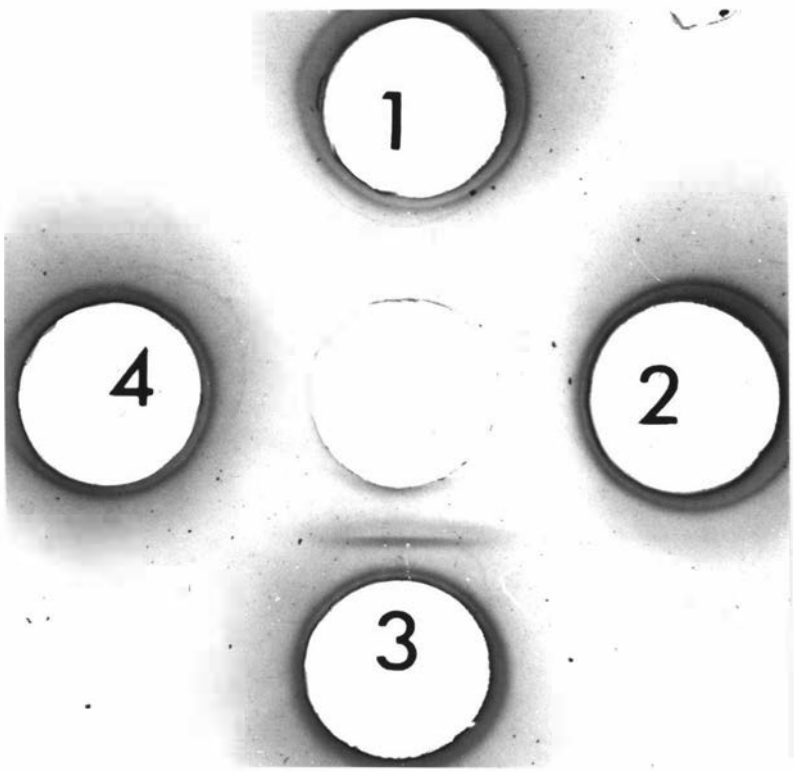
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FIGURE VII.11

Comparison of persistence of precipitating activity against untreated cell extract in pools of sera from different treatment groups in Vaccine Trial III

	Serum	Susceptibility score
1.	Commercial Vaccine "A" prechallenge	18
2.	Alum (one per cent.)/fermenter whole culture, 2 doses, postchallenge	NA
3.	Alum (one per cent.)/fermenter whole culture, 2 doses, prechallenge	14
4.	Commercial Vaccine "A" postchallenge	NA

wells diam., outer 7.5 mm, inner 4.5 mm

wells separated 5 mm

centre well F. nodosus (J3/22) hoof agar, untreated cell extract = 1.6×10^{10} cells/ml

NA = Not applicable

FIGURE VII.12

Reactions between untreated cell extract, and either specific antisera, IgG fraction, or pools of sera from different treatment groups in Vaccine Trial III

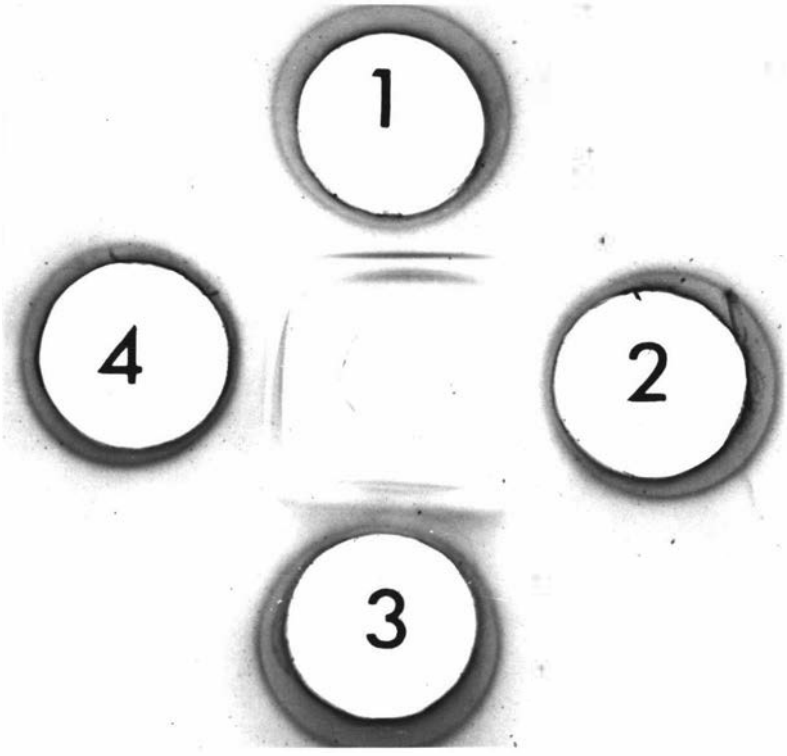
	Serum prechallenge	Susceptibility score
1.	Anti-F. nodosus (J3/22) antiserum	NA
2.	IgG fraction of above	NA
3.	Alum (one per cent.)/fermenter whole culture, 2 doses	14
4.	Alum (one per cent.)/fermenter whole culture, 1 dose	16
5.	Commercial Vaccine "A"	18
6.	Controls	24
7.	Commercial Vaccine "B"	14
8.	Normal serum (prevaccination of well 1)	NA

wells diam., outer 7.5 mm, inner 4.5 mm

wells separated 10 mm

centre well F. nodosus (J3/22) biphasic, untreated cell extract = 2.1×10^{10} cells/ml

NA = Not applicable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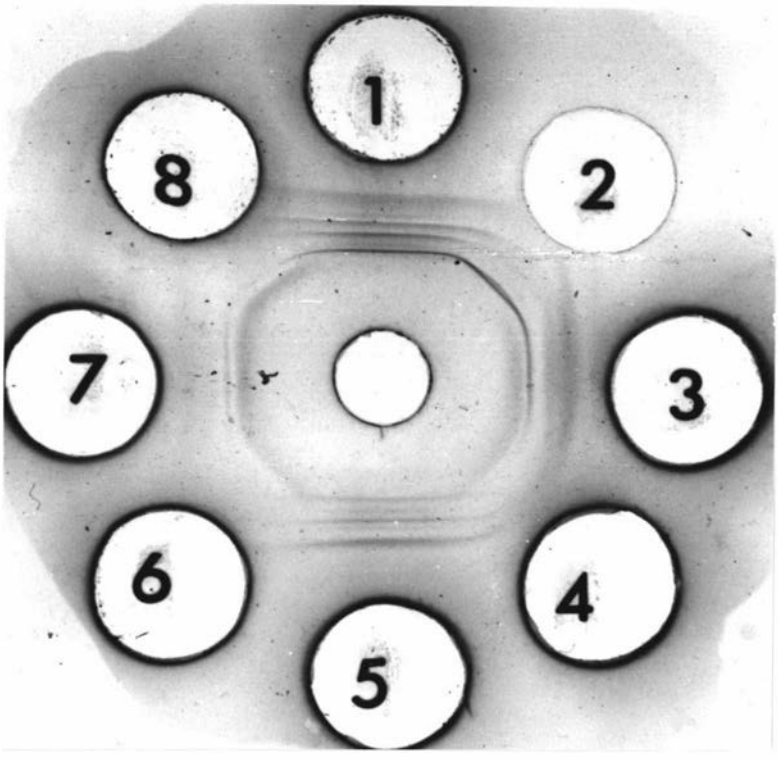
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FIGURE VII.13

Comparison of reactions between untreated cell extract and pools of sera from sheep injected with one or two doses of alum precipitated vaccines

	Serum prechallenge	Susceptibility score
1.	Commercial Vaccine "A" (oil 1 dose)	18
2.	Commercial Vaccine "B" (alum 2 doses)	14
3.	Alum (one per cent.)/fermenter whole culture, 2 doses	14
4.	Alum (one per cent.)/fermenter whole culture, 1 dose	16

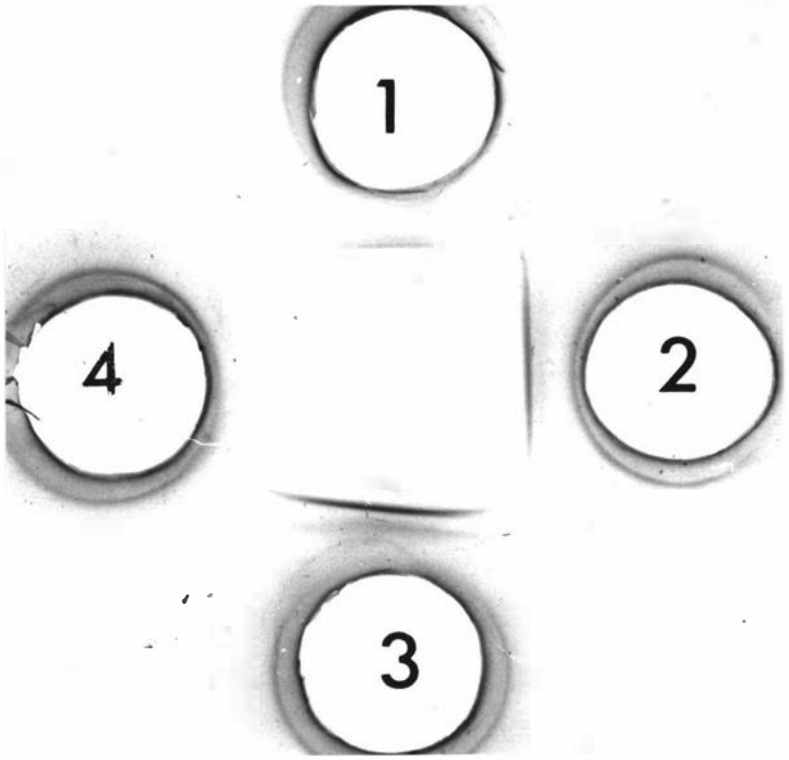
wells diam., outer 7.5 mm, inner 4.5 mm
wells separated 6 mm
centre well F. nodosus (J3/22) hoof agar, untreated cell extract $\equiv 1.6 \times 10^{10}$ cells/ml

FIGURE VII.14

Comparison of reactions between boiled cell extract and pools of sera from sheep injected with one or two doses of alum precipitated vaccines

	Serum prechallenge	Susceptibility score
1.	Commercial Vaccine "A" (oil 1 dose)	18
2.	Alum (one per cent.)/fermenter whole culture, 1 dose	16
3.	Commercial Vaccine "B" (alum 2 doses)	14
4.	Alum (one per cent.)/fermenter whole culture, 2 doses	14

wells diam. 7.5 mm wells separated 5 mm
centre well F. nodosus (J3/22) GC broth fermenter, boiled cell extract $\equiv 10^{11}$ cells/ml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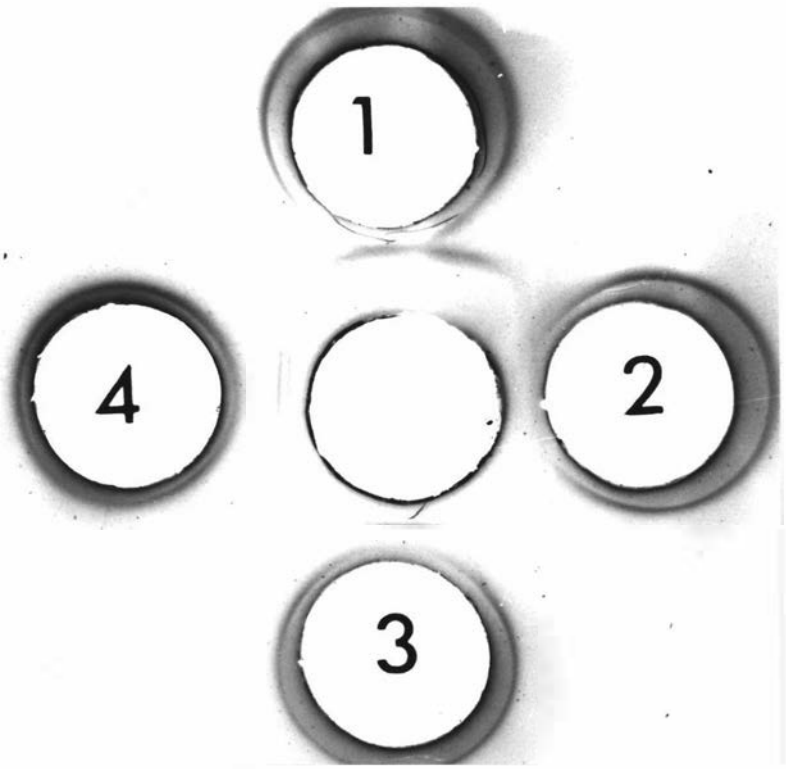
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FIGURE VII.15

Comparison of reactions between untreated cell extract and sera of either lamb or dam about the time of parturition

Serum

1. Ewe, McMaster/biphasic cells vaccine, after 2 weeks
2. Lamb precolostral
3. Ewe, McMaster/biphasic cells vaccine, after 5 months. Day of lambing.
4. Lamb 4 days old

wells diam, 7.5 mm wells separated 5 mm

centre well F. nodosus (J3/22) hoof agar, untreated cell extract $= 8 \times 10^9$ cells/ml

FIGURE VII.16

Comparison of reactions between boiled cell extract and sera of either lamb or dam about the time of parturition

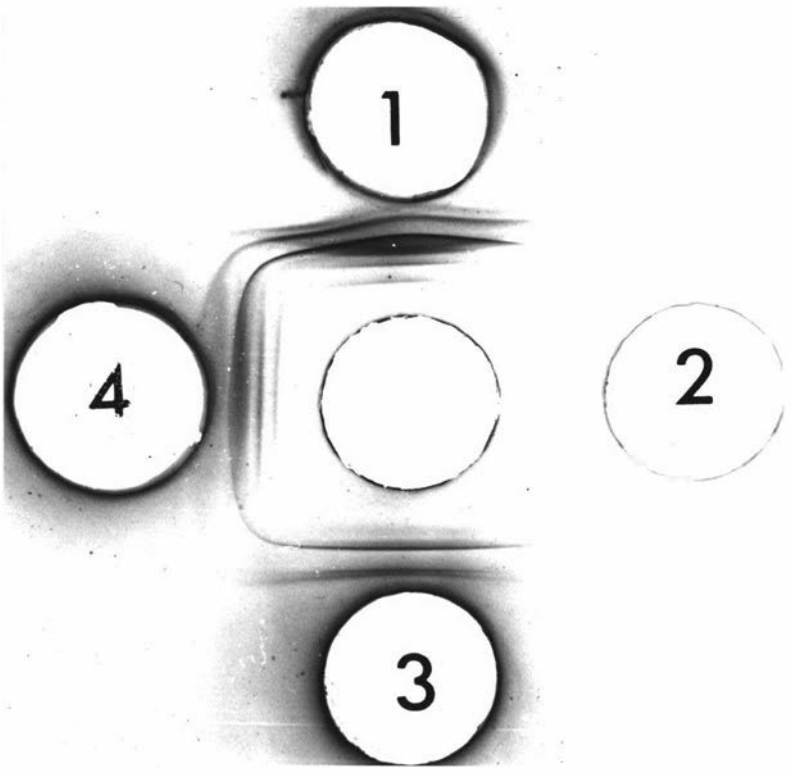
Serum

1. Ewe, McMaster/biphasic cells vaccine, after 2 weeks
2. Lamb precolostral
3. Ewe, McMaster/biphasic cells vaccine, after 5 months. Day of lambing.
4. Lamb 4 days old

wells diam., outer 7.5 mm, inner 4.5 mm

wells separated 5 mm

centre well F. nodosus (J3/22) GC broth fermenter, boiled cell extract $= 10^{11}$ cells/ml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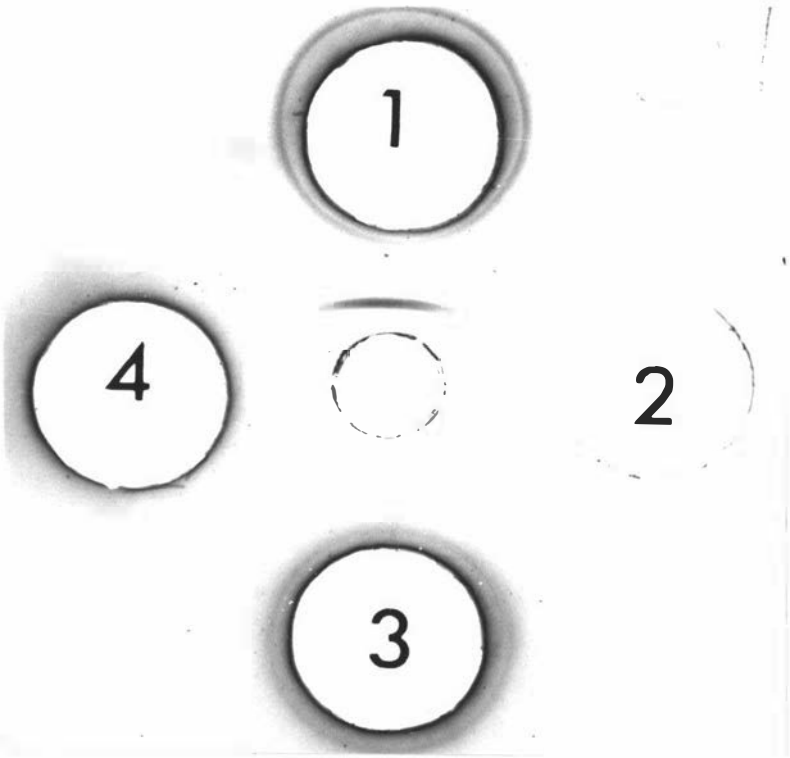
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FIGURE VII.17

Reactions between culture supernatant and sera of selected "resistant" or "susceptible" sheep in Vaccine Trial I

	Serum prechallenge	Susceptibility score
1.	Anti-*F. nodosus* (J3/22) antiersum	NA
2.	Aluminium hydroxide/biphasic whole culture	0
3.	Aluminium hydroxide/biphasic cells	26
4.	Anti-F. nodosus (J3/22) antiserum	NA
5.	Aluminium hydroxide/biphasic cells	0
6.	McMaster/biphasic cells	9
7.	Aluminium hydroxide/biphasic whole culture	25
8.	McMaster/biphasic cells	0

wells diam. 4.5 mm wells separated 10 mm

centre well *F. nodosus* (McM199) biphasic, culture supernatant

NA = Not applicable

FIGURE VII.18

Reactions between culture supernatant and special antisera* as detailed

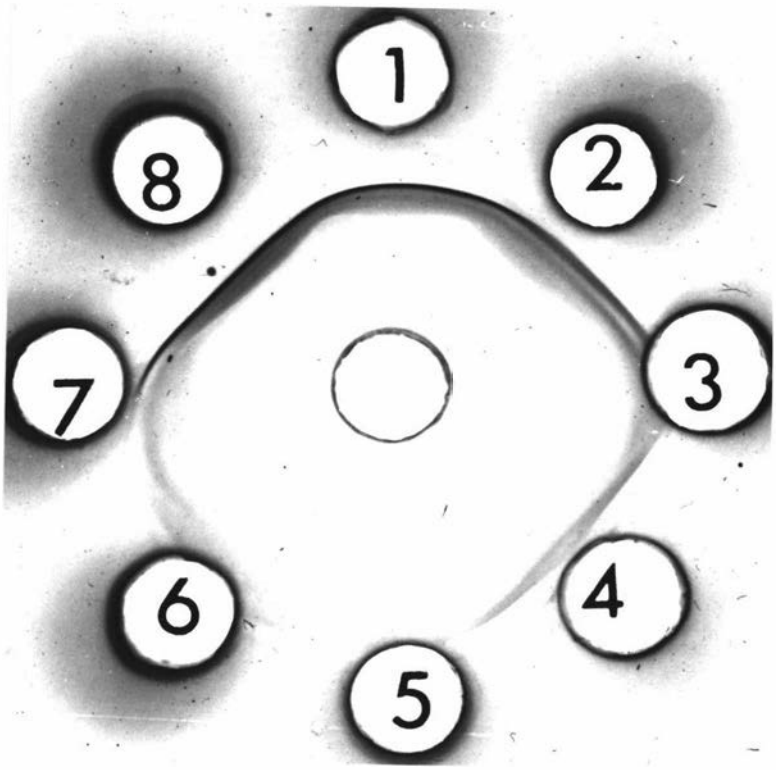
	Special antiserum raised against
1.	*F. nodosus* (J3/22) cells sonicated batch 1
2.	*F. nodosus* (J3/22) cells sonicated batch 2
3.	Freund's Incomplete Adjuvant + phosphate buffered saline
4.	McMaster adjuvant + phosphate buffered saline
5.	Freund's Incomplete Adjuvant + hoof broth
6.	*F. nodosus* (J3/22) biphasic culture supernatant
7.	*F. nodosus* (J3/22) formalin treated cells, boiled
8.	*F. nodosus* (J3/22) biphasic cells

wells diam., outer 7.5 mm, inner 4.5 mm

wells separated 7 mm

centre well *F. nodosus* (J3/22) biphasic, culture supernatant

* Detailed in Appendix X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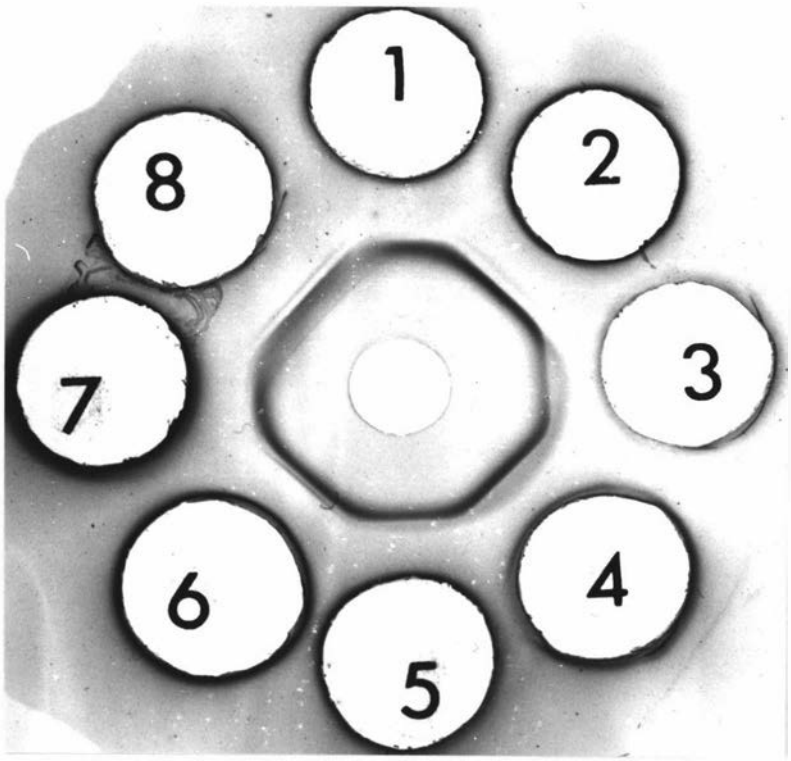
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FIGURE VII.19

Reactions between culture supernatants of different proteolytic titres with anti-F. nodosus (J3/22) antiserum

	Outer well reactants	Concentrated
1.	Incubated hoof broth	x 50
2.	F. nodosus (J3/22) biphasic culture supernatant	x 50
3.	F. nodosus (J3/22) protease (900 fold pure)	
4.	F. nodosus (A8/C) biphasic culture supernatant	x 50

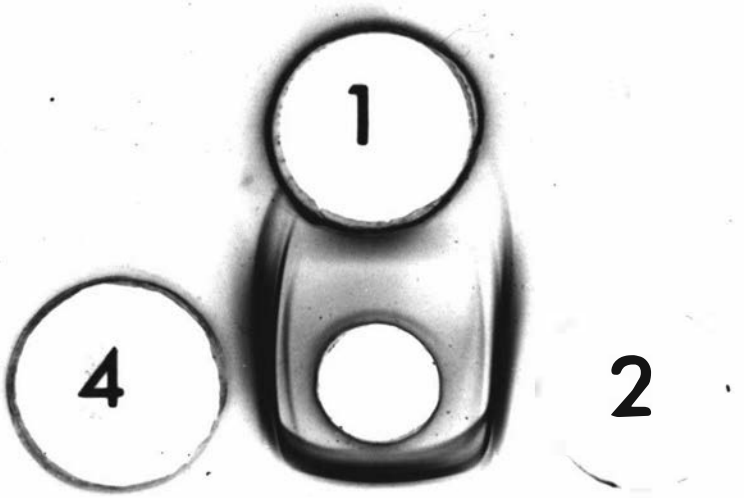
Wells diam., outer 7.5 mm, inner 4.5 mm
wells separated 5 mm
centre well anti-F. nodosus (J3/22) antiserum

FIGURE VII.20

Reactions between culture supernatants of standard proteolytic titre with anti-F. nodosus (J3/22) antiserum

	Outer well reactants	Concentrated
1.	Incubated hoof broth	x 50
2.	F. nodosus (J3/22) biphasic culture supernatant	x 50
3.	F. nodosus (J3/22) protease (900 fold pure)	x 0.5
4.	F. nodosus (A8/C) biphasic culture supernatant	x 25

wells diam., outer 7.5 mm, inner 4.5 mm
wells separated 5 mm
centre well anti-F. nodosus (J3/22) antiserum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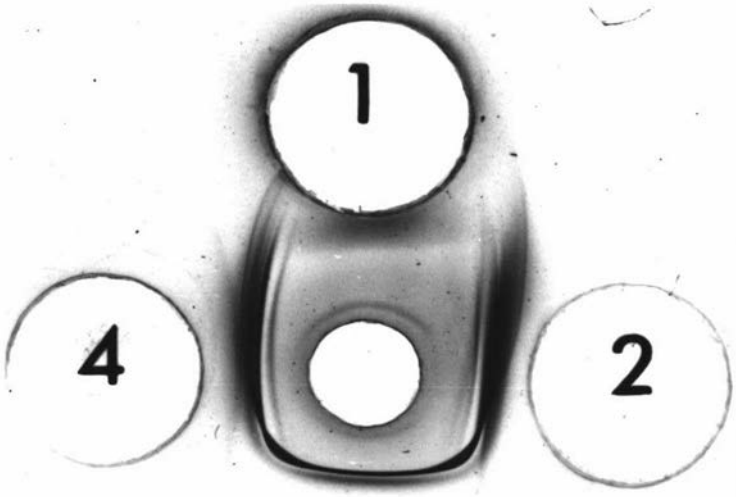
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FIGURE VII.21

Reactions between untreated cell washings and either precolostral or postcolostral lamb sera

Reactants

p Precolostral lamb serum

1. F. nodosus (A8/C) biphasic, boiled cell extract = 10^{11} cells/ml

2. Controls Vaccine Trial I, 6 wk after challenge

3. F. nodosus (A8/C) biphasic cell washings, untreated conc. x 50

4. Normal serum, lamb 3 days old

p Precolostral lamb serum

wells diam. 7.5 mm wells separated 5 mm

FIGURE VII.22

Reactions between boiled cell washings and homologous antiserum either before or after absorption

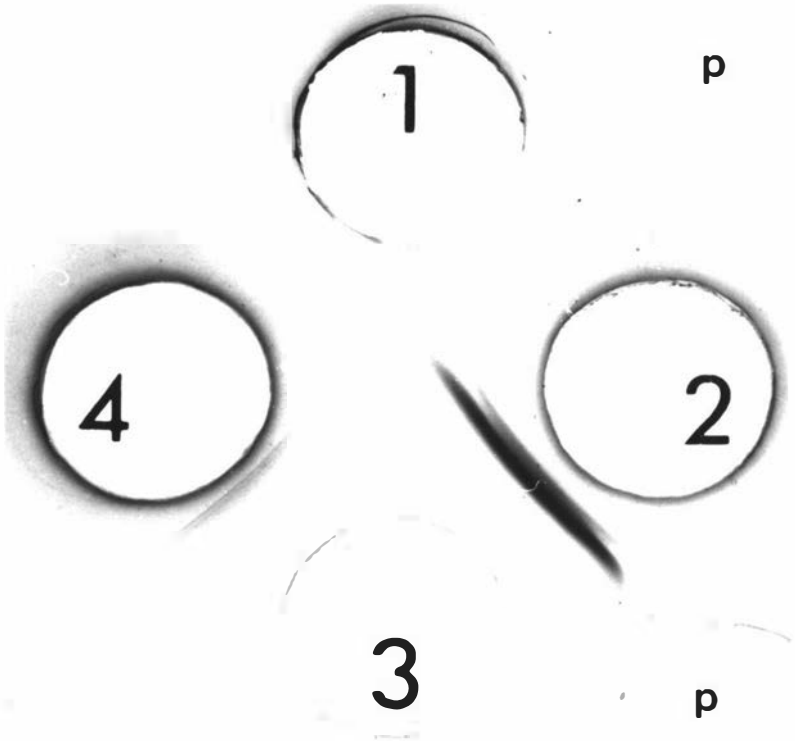
Reactants

1. Anti-F. nodosus (J3/22) antiserum, absorbed with equivalent volume homologous cells at 10^{10} cells/ml

2. Anti-F. nodosus (J3/22) antiserum

3. F. nodosus (J3/22) GC broth fermenter washings, boiled 60 min., conc. x 50

wells diam. 7.5 mm wells separated 4.5 mm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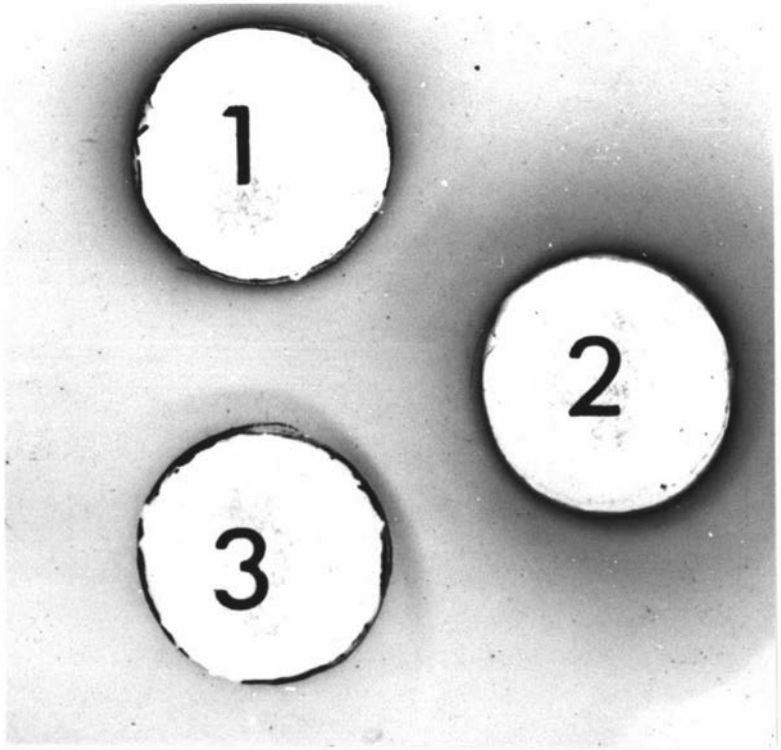
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FIGURE VII.23

Reactions between untreated cell extract and antiserum raised by either formalin treated cells or boiled cells

Serum

1. Normal sheep serum (well 2 prevaccination)
2. Anti-*F. nodosus* (J3/22) formalin treated cells antiserum
3. Normal serum, lamb 4 days old
4. Anti-*F. nodosus* (J3/22) boiled cells antiserum

wells diam. 7.5 mm wells separated 5 mm

centre well *F. nodosus* (J3/22) hoof agar, untreated cell extract = 8×10^9 cells/ml

FIGURE VII.24

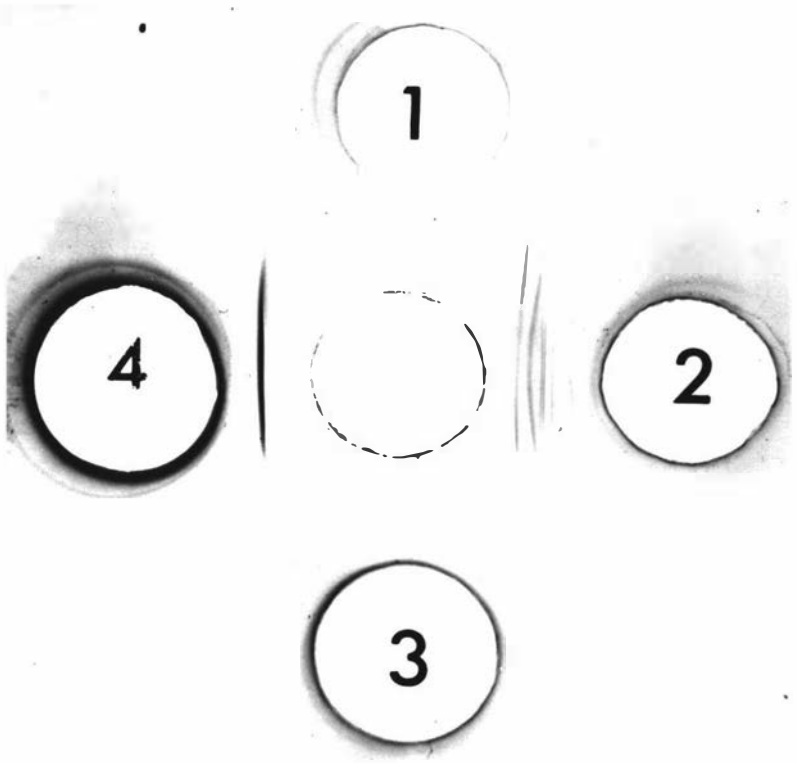
Reactions between boiled cell extract and serial serum samples taken from a sheep with foot-rot

Sheep 626 serum samples

1. Two weeks after infection
2. Six weeks after infection
3. Three and a half months after infection
4. Preinfection

wells diam. 7.5 mm wells separated 3 mm

centre well *F. nodosus* (J3/22) GC broth fermenter, boiled cell extract = 10^{11} cells/ml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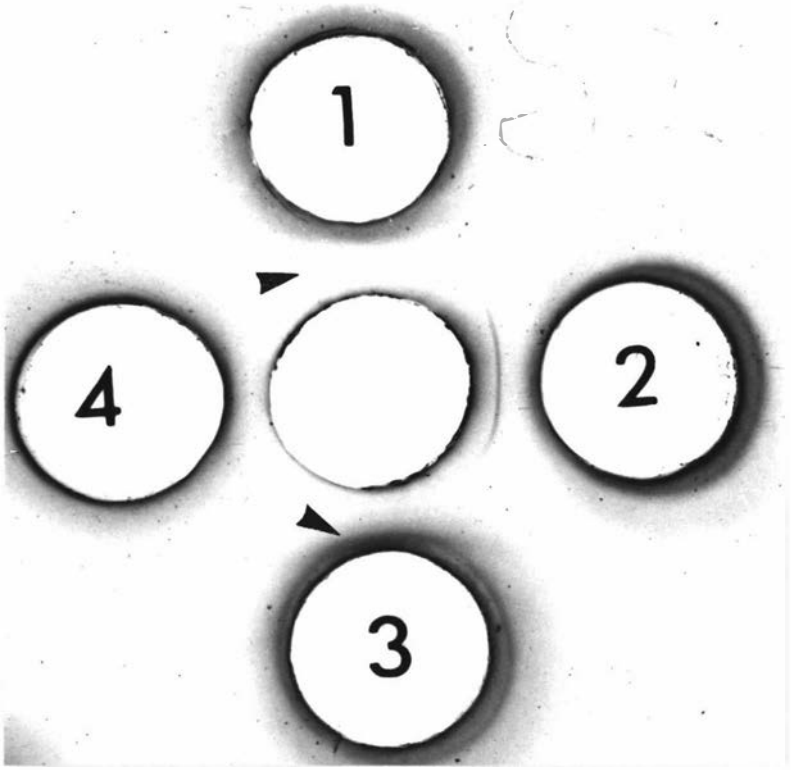
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FIGURE VII.25

Effects of absorption using F. nodosus (J3/22) cells on reactions between F. nodosus (J3/22) boiled cell extract and homologous antiserum

Anti-F. nodosus (J3/22) antiserum absorbed* with:

1. F. nodosus (J3/22) biphasic cells washed
2. F. nodosus (J3/22) biphasic cells washed and boiled
3. F. nodosus (J3/22) biphasic cells untreated
4. F. nodosus (J3/22) biphasic cells boiled

wells diam., outer 7.5 mm, inner 4.5 mm
wells separated 5 mm
centre well F. nodosus (J3/22) GC broth fermenter, boiled cell extract = 10^{11} cells/ml

* 10^{10} cells/ml, equal amounts serum, 24 hr at $4^{o}C$

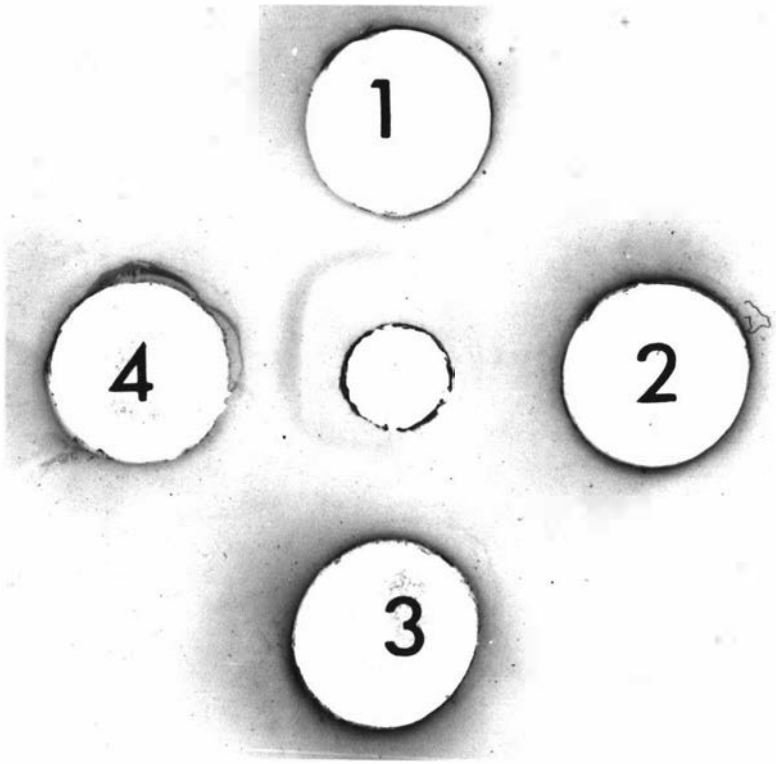
FIGURE VII.26

Effects of absorption using F. nodosus (J3/22) cells on reactions between F. nodosus (J3/22) boiled cell extract and heterologous antiserum

Anti-F. nodosus (McM198) antiserum adsorbed with:

1. F. nodosus (J3/22) biphasic cells washed
2. F. nodosus (J3/22) biphasic cells washed and boiled
3. F. nodosus (J3/22) biphasic cells untreated
4. F. nodosus (J3/22) biphasic cells boiled

wells diam., outer 7.5 mm, inner 4.5 mm
wells separated 5 mm
centre well F. nodosus (J3/22) GC broth fermenter, boiled cell extract = 10^{11} cells/ml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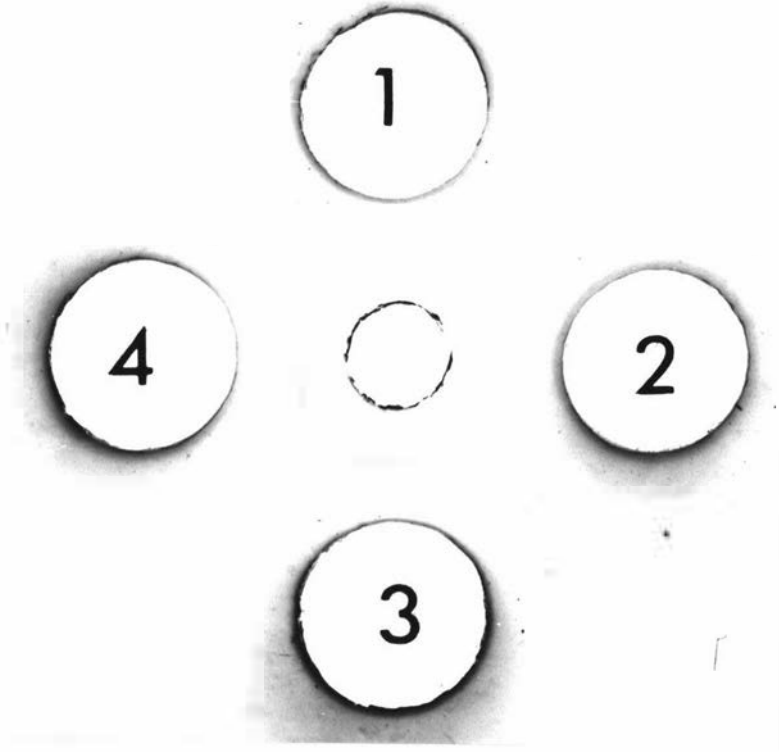
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FIGURE VII.27

Effects of absorption using F. nodosus (J3/22) cells on reactions between F. nodosus (McM198) boiled cell extract and heterologous antiserum

Anti-F. nodosus (J3/22) antiserum absorbed* with:

1. F. nodosus (J3/22) biphasic cells washed
2. F. nodosus (J3/22) biphasic cells washed and boiled
3. F. nodosus (J3/22) biphasic cells untreated
4. F. nodosus (J3/22) biphasic cells boiled

wells diam., outer 7.5 mm, inner 4.5 mm
wells separated 5 mm
centre well F. nodosus (McM198) biphasic boiled cell extract
= 5×10^{10} cells/ml

* 10^{10} cells/ml, equal amounts serum, 24 hr at 4°C

FIGURE VII.28

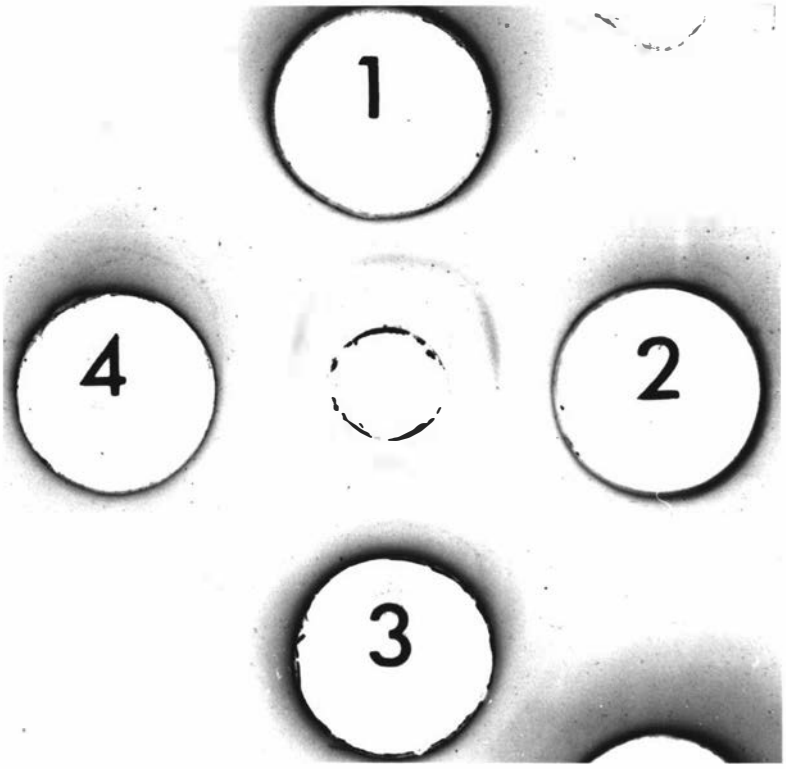
Effects of absorption using F. nodosus (J3/22) cells on reactions between F. nodosus (McM198) boiled cell extract and homologous antiserum

Anti-F. nodosus (McM198) antiserum absorbed* with:

1. F. nodosus (J3/22) biphasic cells washed
2. F. nodosus (J3/22) biphasic cells washed and boiled
3. F. nodosus (J3/22) biphasic cells untreated
4. F. nodosus (J3/22) biphasic cells boiled

wells diam., outer 7.5 mm, inner 4.5 mm
wells separated 5 mm
centre well F. nodosus (McM198) biphasic boiled cell extract
= 5×10^{10} cells/ml

* 10^{10} cells/ml, equal amounts serum, 24 hr at 4°C.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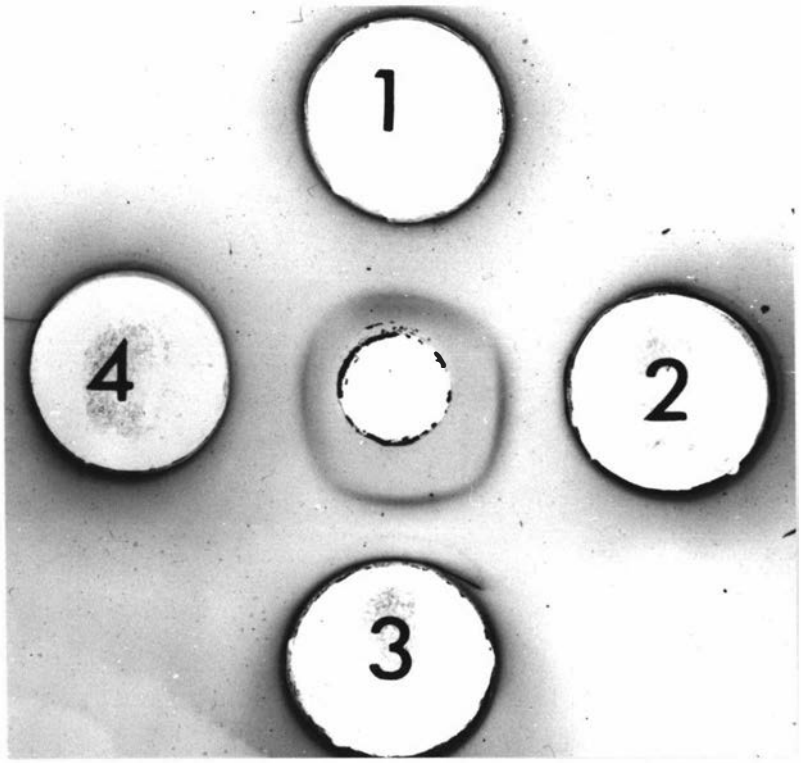
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FIGURE VII.29

Comparison of reactions between F. nodosus boiled cell extract and sera from either "resistant" or "susceptible" sheep in one group of Vaccine Trial I

	Serum prechallenge	Susceptibility score
1.	Aluminium hydroxide/biphasic cells	0
2.	Aluminium hydroxide/biphasic cells	0
3.	Aluminium hydroxide/biphasic cells	8
4.	Aluminium hydroxide/biphasic cells	9

wells diam. 7.5 mm wells separated 5 mm

centre well F. nodosus (A8/C) biphasic, boiled cell extract $\equiv 10^{11}$ cells/ml

FIGURE VII.30

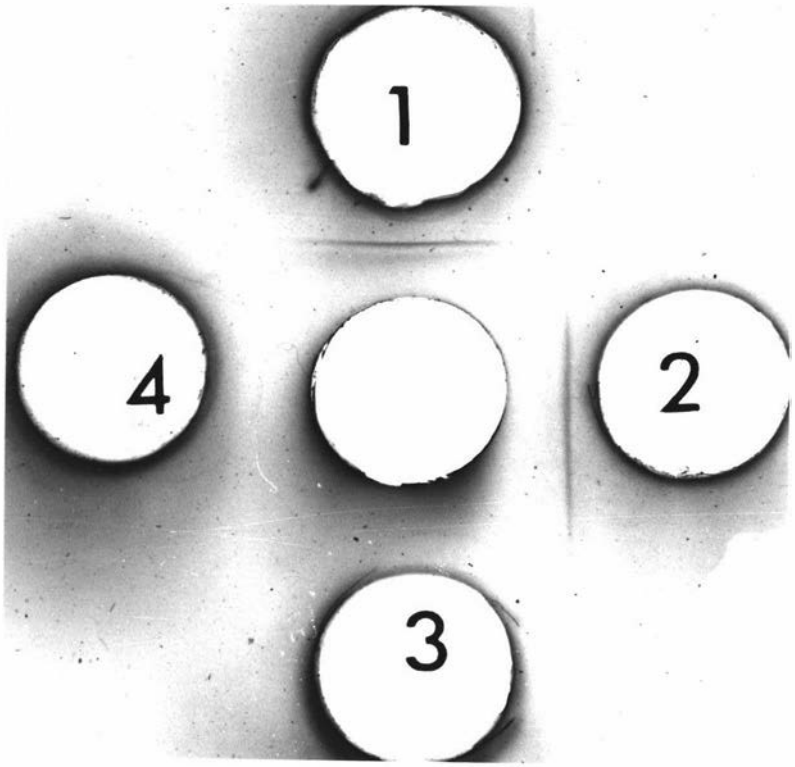
Comparison of reactions between F. nodosus boiled cell extract and sera from either "resistant" or "susceptible" sheep in Vaccine Trial II

	Serum prechallenge	Susceptibility score
1.	Aluminium hydroxide/fermenter cells*	23
2.	Aluminium hydroxide/fermenter cells	3
3.	Control	30
4.	Control	12

wells diam. 7.5 mm wells separated 3 mm

centre well F. nodosus (J3/22) GC broth fermenter, boiled cell extract $\equiv 10^{11}$ cells/ml

* 10^{10} cells/dose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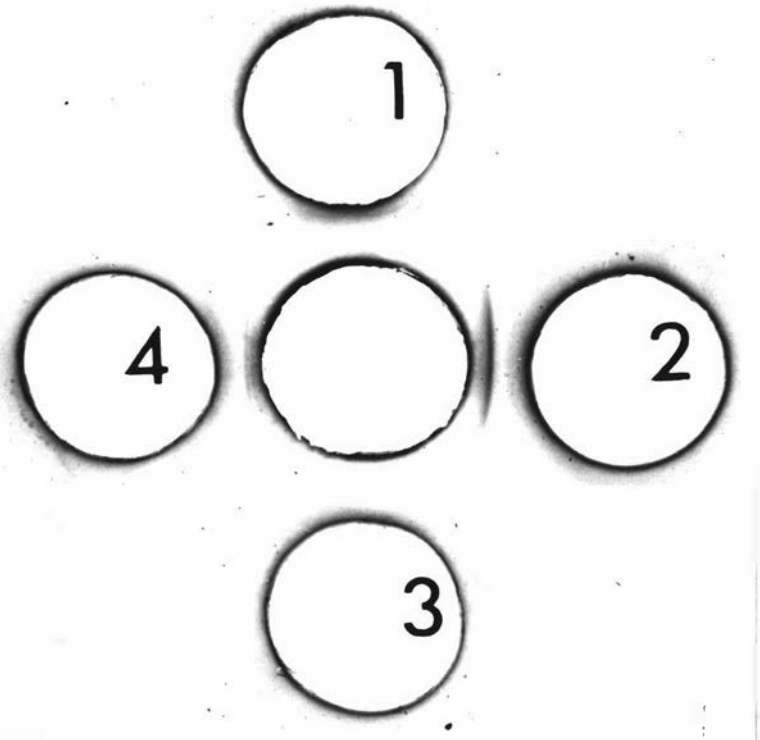
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FIGURE VII.31

Comparison of reactions between F. nodosus boiled cell extract and sera from either "resistant" or "susceptible" sheep in Vaccine Trial II

	Serum prechallenge	Susceptibility score
1.	Organic polymer/fermenter cells	23
2.	Organic polymer/fermenter cells	7
3.	Double emulsion/fermenter cells	24
4.	Double emulsion/fermenter cells	5

wells diam. 7.5 mm wells separated 3 mm

centre well F. nodosus (J3/22) GC broth fermenter, boiled cell extract = 10^{11} cells/ml

FIGURE VII.32

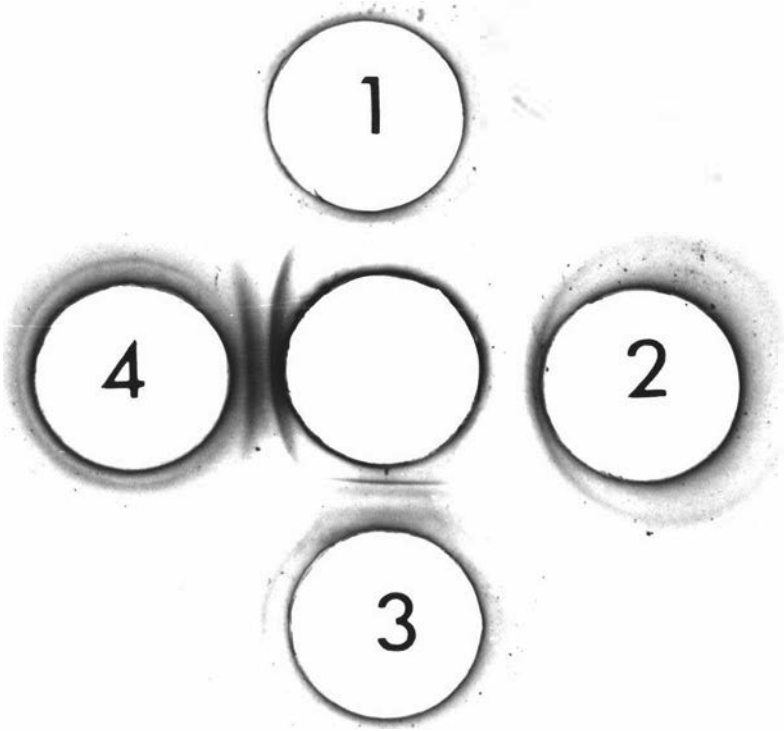
Reactions between F. nodosus boiled cell extract and pools of sera from sheep with existing foot-rot and that had been vaccinated with either one or two doses of the Alum (one per cent.) whole culture formulation

	Serum pool
1.	Before vaccination
2.	Three weeks after one dose of vaccine
3.	Three weeks after no vaccination
4.	Three weeks after two doses* of vaccine

wells diam. 7.5 mm wells separated 3 mm

centre well F. nodosus (J3/22) GC broth fermenter, boiled cell extract = 10^{11} cells/ml

* separated by three weeks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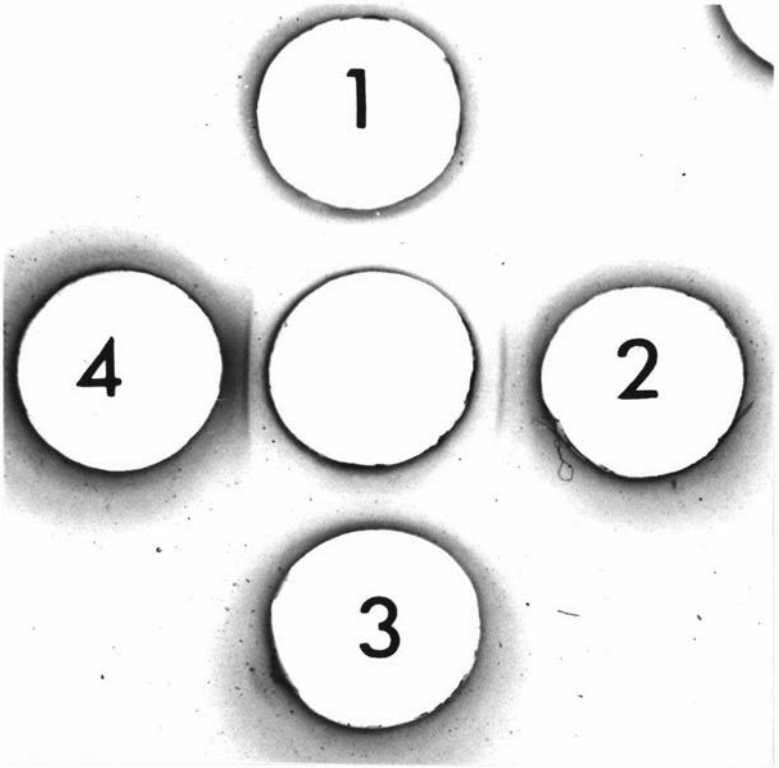
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FIGURE VII.33

Reactions between F. nodosus untreated cell extract and fractions of anti-F. nodosus antiserum

		Anti-F. nodosus (J3/22) antiserum or fraction
1.	Normal serum	prevaccination
2.	IgG fraction	post vaccination
3.	Whole serum	post vaccination
4.	DEAE* elution	post vaccination

wells diam. 7.5 mm wells separated 5 mm

centre well F. nodosus (J3/22) hoof agar, untreated cell extract $\equiv 8 \times 10^9$ cells/ml

* Diethylaminoethyl cellulose Appendix XIIIa

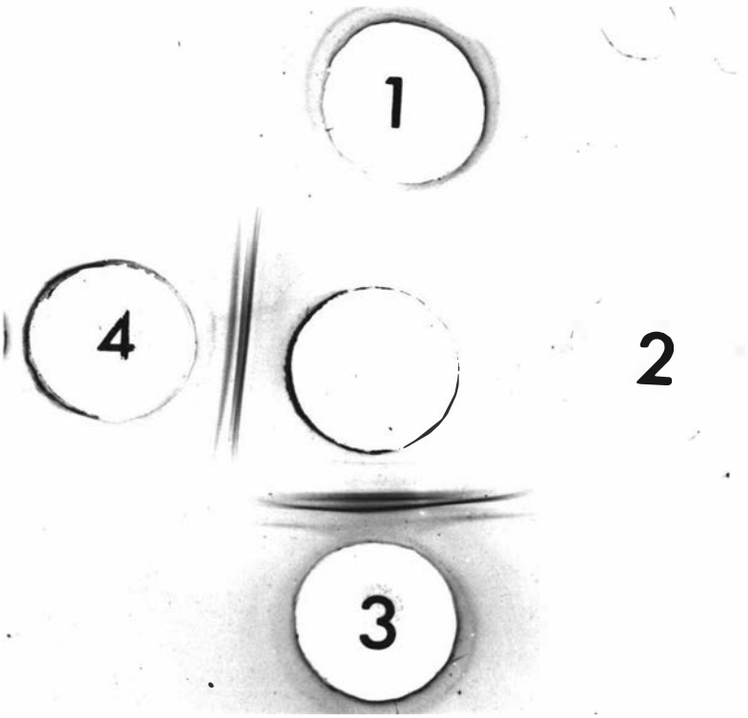
FIGURE VII.34

Reactions between F. nodosus boiled cell extract and fractions of anti-F. nodosus antiserum

		Anti-F. nodosus (J3/22) antiserum or fraction
1.	Normal serum	prevaccination
2.	IgG fraction	post vaccination
3.	Whole serum	post vaccination
4.	DEAE* elution	post vaccination

wells diam. 7.5 mm wells separated 5 mm

centre well F. nodosus (J3/22) hoof agar, boiled cell extract $\equiv 8 \times 10^9$ cells/ml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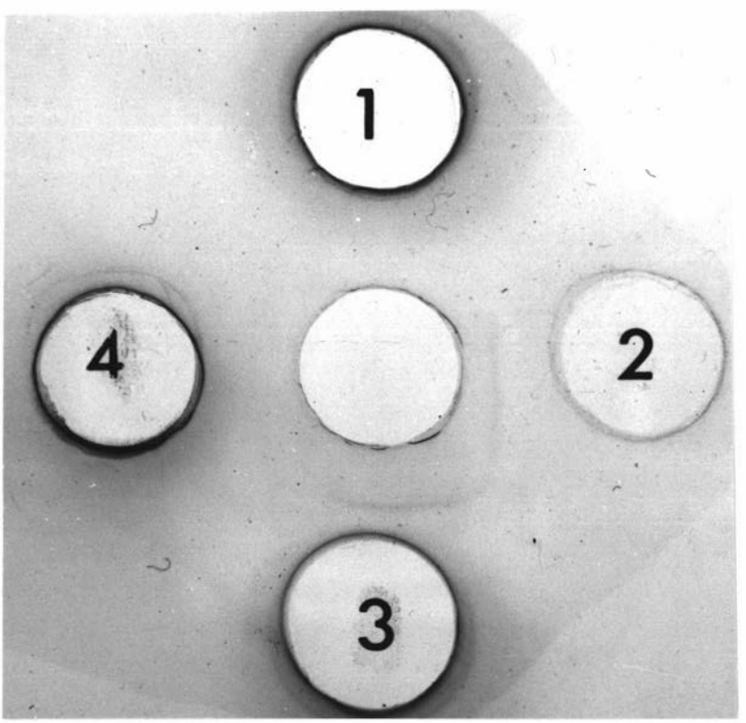
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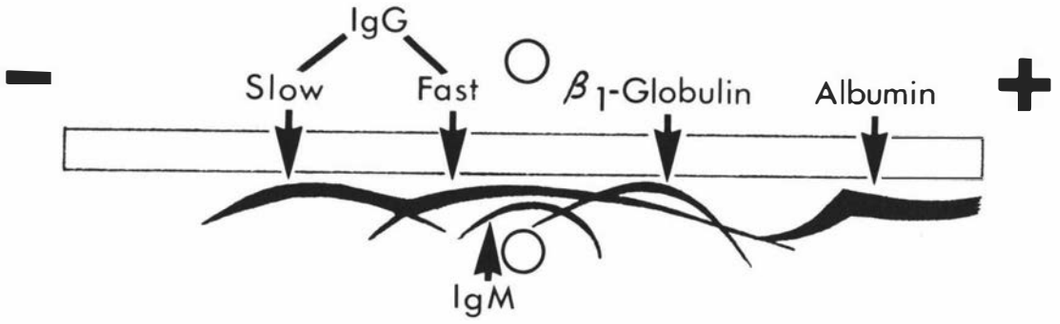
FIGURE VII.35

Reactions between rabbit anti-sheep serum and either normal serum or anti-F. nodosus (J3/22) antiserum and its fractions

a
- Normal adult sheep serum
- Anti-F. nodosus (J3/22) antiserum

b
- Anti-F. nodosus (J3/22) antiserum, IgG fraction
- Anti-F. nodosus (J3/22) antiserum, DEAE elution

All troughs rabbit anti-sheep serum



Stylised diagram showing approximate position of immunoprecipitates formed between sheep serum and rabbit anti-sheep serum

FIGURE VII.36

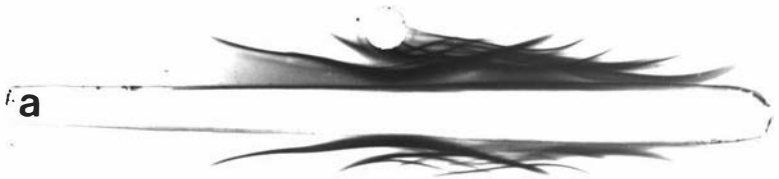
Reactions between rabbit anti-sheep serum and sera of either new born lambs, or from an adult sheep with longstanding foot-rot

a
- Precolostral lamb serum - vaccinated ewe
- Post colostral of above

b
- Precolostral lamb serum - normal ewe
- Postcolostral of above

c
- Adult sheep serum - longstanding foot-rot
- Normal sheep serum

All troughs rabbit anti-sheep serum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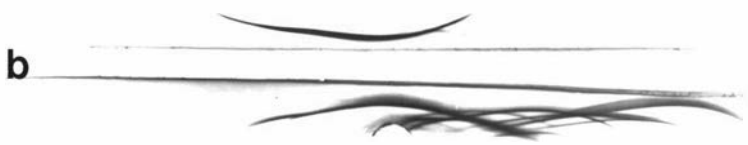
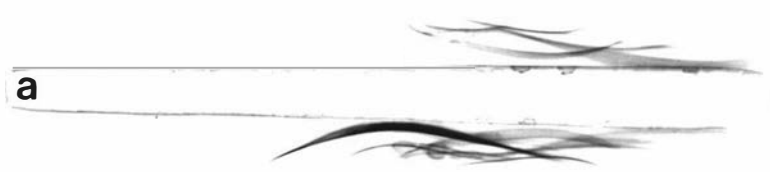
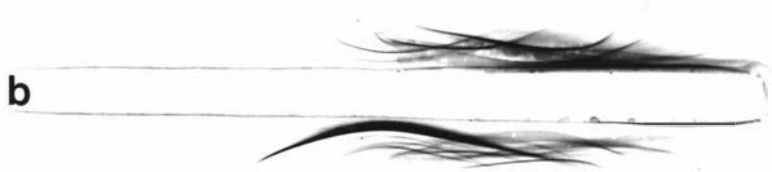

b


a


b


c

FIGURE VII.37

Reactions between rabbit anti-sheep IgG and either sera of new born lambs or anti-F. nodosus (J3/22) antiserum and its fractions

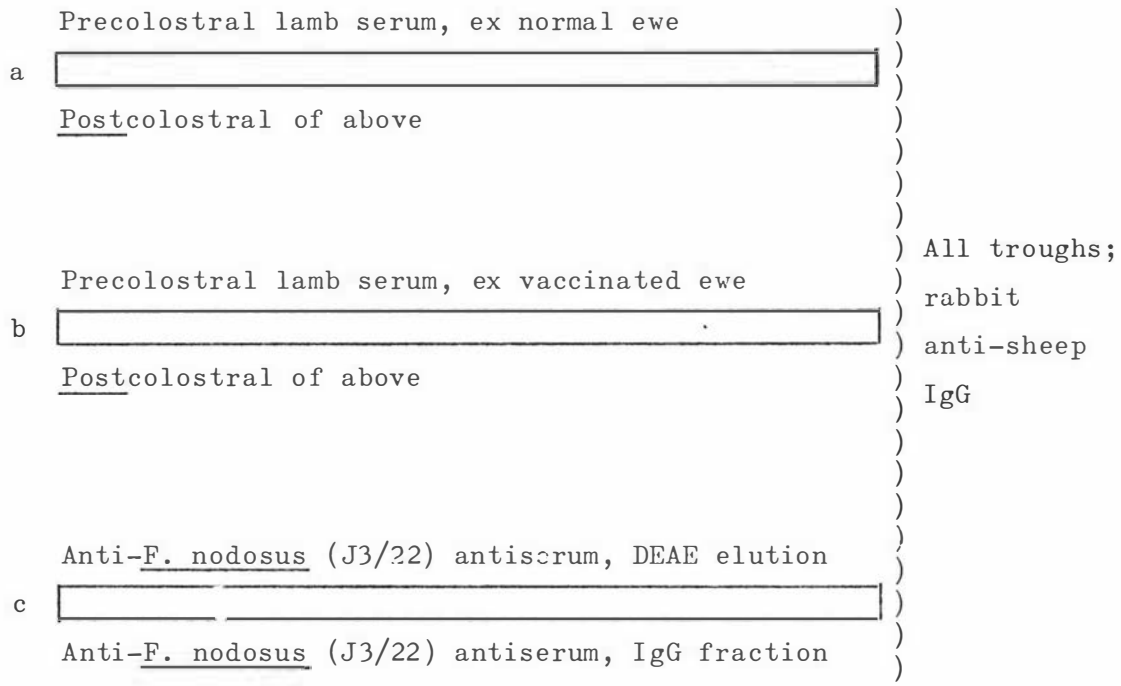


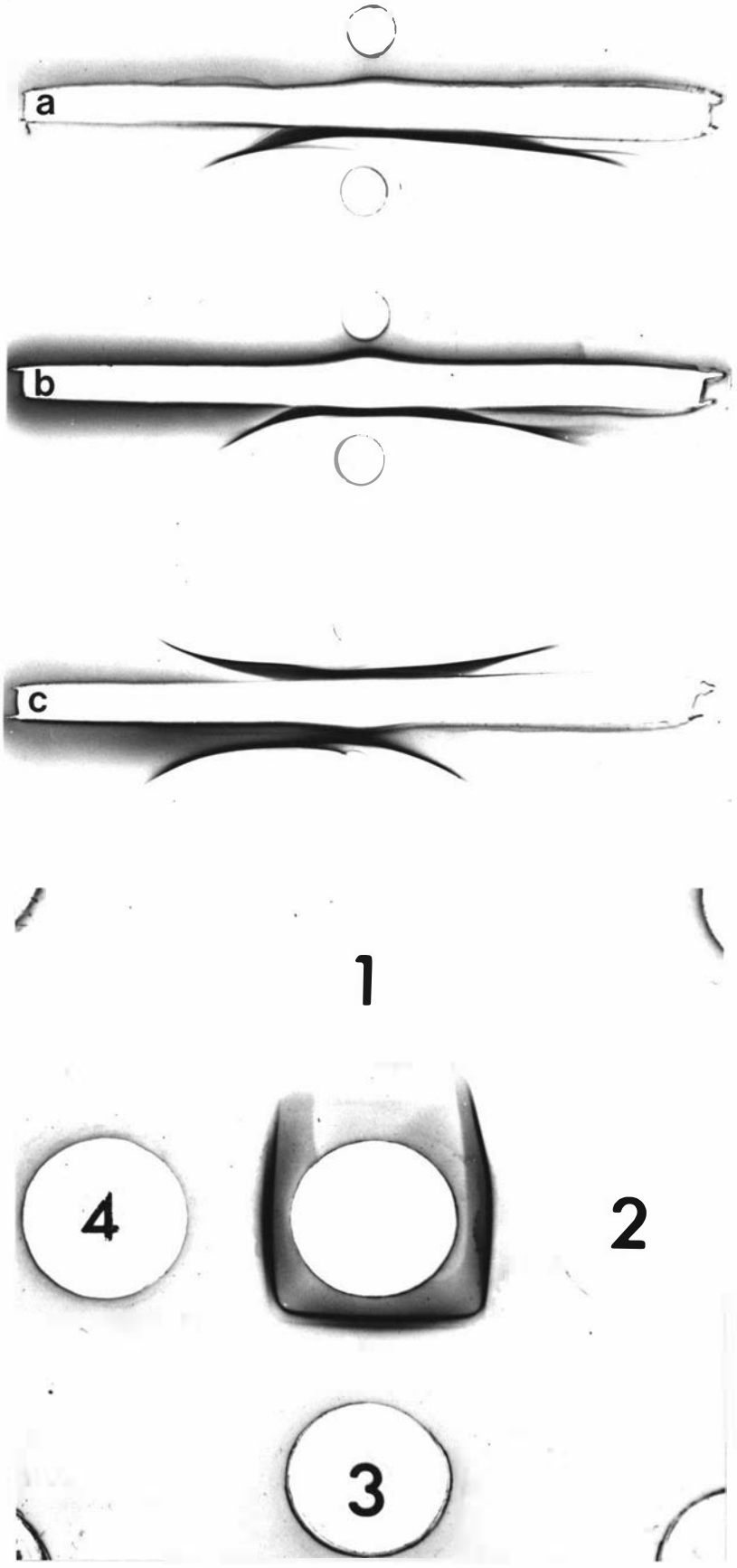
FIGURE VII.38

Reactions between rabbit anti-sheep IgG and either precolostral lamb serum or anti-F. nodosus (J3/22) antiserum and its fractions

1. Precolostral lamb serum, ex vaccinated ewe
2. Anti-F. nodosus (J3/22) antiserum, IgG fraction
3. Anti-F. nodosus (J3/22) antiserum, DEAE elution
4. Anti-F. nodosus (J3/22) antiserum, whole serum

wells diam. 7.5 mm wells separated 5 mm

centre well Rabbit anti-sheep IgG


a
b
c
1
2
3
4

CHAPTER VIII

SEROLOGICAL CHANGES IN SHEEP DURING FOOT-ROT INFECTION AND AFTER VACCINATION WITH VARIOUS ADJUVANT/CELL COMBINATIONS

(IV) BACTERIAL GROWTH-INHIBITION

Introduction

Following the observation that normal sheep serum inhibited the growth of *F. nodosus* (Beveridge, 1941), it was shown that the injection of guinea pigs, rabbits or sheep with *F. nodosus* produced bactericidal antibodies (Commonwealth Scientific and Industrial Research Organisation, 1966-67). Previous studies with fluorescin labelled antiglobulin had shown that serum antibody was capable of diffusion into foot-rot lesions and of making contact with the infecting *F. nodosus* organisms (Commonwealth Scientific and Industrial Research Organisation, 1967-68). Egerton and Merritt (1970) subsequently showed that *normal* sheep serum is bactericidal for *F. nodosus*, and that the bactericidal titre may be elevated 100 fold by vaccination. In both cases the bactericidal effect was shown to be due to the combined action of IgG antibody and heat labile components of serum. Thus there was considerable evidence from Australian work to account for the protection afforded by foot-rot vaccines (Egerton 1970; Egerton and Burrell, 1970)

and accordingly an investigation was undertaken to determine the same basic information for F. nodosus (J3/22) and its antiserum. It was reasoned that the bactericidal titre of a serum might be used as a means of predicting the immune status of an animal, particularly if the titre was shown to be positively correlated with the degree of protection afforded by a vaccine. In the event of these relationships being confirmed, a bacteriolysis test might be used to evaluate vaccines without recourse to a sheep challenge experiment.

Serum samples were available from a large range of sheep including precolostral and postcolostral lambs, normal ewes, and others infected with foot-rot and subsequently treated by vaccination. Normal sheep that were first vaccinated with a variety of adjuvant/cell combinations and afterwards challenged with F. nodosus, also provided sera before and after both vaccination and attempted infection.

MATERIALS AND METHODS

Bacteria F. nodosus (J3/22) was maintained in GC broth by subculture every 48 hr. An overnight culture was diluted on the basis of optical density so that 0.1 ml contained an estimated 20,000 organisms.

Smears of broth deposits were stained by Gram's method (Appendix XVII).

Antisera Sera were collected from sheep involved in Vaccine Trials I, II and III (Chapter IV) both after vaccination and after challenge. Additional sera were available from sheep involved in a field outbreak of foot-rot (Experiment Therapy I - Chapter XI).

Anti-F. nodosus (J3/22) antiserum was used in the growth-inhibition test as a standard antiserum.

The growth-inhibition test required serum dilutions to be first mixed with F. nodosus cells and then incubated in GC broth. Accordingly all sera were sterilised by Millipore filtration as described in Appendix XIV.

When required sera were inactivated by heating at 56°C for 30 minutes.

Complement Two pools of guinea pig serum were shown to inhibit growth of F. nodosus. Precolostral lamb serum at a dilution of 1/5 also inhibited growth. At a dilution of 1/10, no inhibition was evident and at that dilution precolostral lamb serum was used as a source of complement factors.

Antibody absorption Serum antibody absorption was carried out at 2°C for one hour using a 24 hr GC broth culture of the homologous organism at 1 x 10^9 cells/ml mixed with an equal volume of the serum dilution.

Bacterial growth-inhibition test Doubling dilutions of sera in veronal buffered saline, pH 7.2, were mixed with a standard inoculum of

bacteria, and after incubation at 37°C for one hour, were added to roller culture tubes of GC broth to determine whether or not there had been an inhibitory effect on the organisms.

Heat-inactivated sheep serum was mixed with an equal amount of diluted precolostral lamb serum before addition of bacteria.

Standard control tubes contained either veronal buffered saline plus organisms in GC broth, or veronal buffered saline plus diluted precolostral lamb serum plus organisms in GC broth.

All tubes were incubated at 37°C for three days during which visible growth was recorded twice daily. Bacterial growth took the form of a shimmering opacity throughout the broth. The end point of a titration was taken as the last tube in which failure of bacterial growth was indicated by the broth remaining clear. The titre was defined as the reciprocal of the final serum dilution in the nominated tube.

Full details of the technique are described in Appendix XIV.

RESULTS

Growth-inhibition by normal sheep serum The growth-inhibitory activity of normal sheep serum was shown by prolongation of the lag phase in tubes containing serum in high dilution and a total inhibition of growth in tubes containing less diluted serum.

Granular deposits developed in the base of tubes containing the less diluted serum particularly if the serum had been preheated. Gram stained smears of the material showed it to be a pink amorphous mass.

Sera from normal sheep had a growth-inhibition titre of 160 or 320. Absorption of these sera with F. nodosus cells caused the growth-inhibitory activity to be markedly reduced; to a titre of 10.

The growth-inhibitory activity was removed by heating normal sheep sera at 56°C for 30 minutes. The addition of diluted precolostral lamb serum restored the activity to such heat-treated sera, and control tubes confirmed that the lamb sera alone had no growth-inhibitory effect.

Growth-inhibition by sera from vaccinated sheep In high dilutions of sera from vaccinated sheep there was a prolongation of the lag phase of growth. More concentrated immune sera completely inhibited growth in the liquid medium. In those tubes containing dilutions of sera approaching the end point of the titration series, plaques of 5-10 mm diameter consisting of agglutinated F. nodosus organisms formed on the sides of the tubes. In addition to these plaques, granular deposits identical with those seen in normal serum titrations, accumulated in tubes containing less diluted serum.

The growth-inhibition titres of sera from vaccinated sheep were no higher than those recorded for the sera of non-vaccinated sheep (Tables VIII.I, VIII.II and VIII.III).

Absorption with F. nodosus cells of sera from vaccinated sheep substantially reduced the growth-inhibition titre as it had for normal sheep sera.

A striking distinction between the two classes of sera was that the activity of sera from vaccinated sheep remained unchanged after pretreatment at 56°C for 30 minutes. It was markedly reduced by heat treatment at 70°C for 10 minutes.

The results of either heat treatment or absorption by cells on the different serum samples, are compared and summarised in Table VIII.I.

Growth-inhibition by sera from sheep infected with foot-rot Sheep with existing foot-rot of at least six weeks duration had no homologous growth-inhibitory activity in their sera. The same absence of activity was confirmed in a pool of sera taken from sheep that had become infected with severe foot-rot during a field outbreak (Table VIII.II).

Growth-inhibition by sera from sheep in Vaccine Trial III Neither the adjuvant used for the vaccine nor the number of doses of vaccine administered had any demonstrable effect on the growth-inhibition titre of serum of sheep involved in Vaccine Trial III. As with the sera of other vaccinated sheep, titres of the order of 320 were realised and the activity was resistant to heating at 56°C for 30 minutes (Table VIII.III).

DISCUSSION

Although F. nodosus appeared to grow well once it was adapted to GC broth, it was noticeable that the lag phase was shortened by the addition of diluted precolostral lamb serum or heated normal sheep serum. As normal sheep serum growth-inhibitory activity was shown partly by a prolongation of the lag phase, it follows that the addition of the standard complement factor source, namely precolostral lamb serum, was providing some undefined nutrient which was absent in GC broth and which tended to enhance bacterial growth.

Precolostral lamb serum should also provide a source of antibody-free complement (Egerton and Merritt, 1970) and at the arbitrarily chosen dilution of 1/10, precolostral lamb serum provided a factor that fully restored the growth-inhibitory activity of heat inactivated serum from normal sheep.

The complement level of precolostral lamb sera was assessed by its ability to lyse sensitised (anti-erythrocyte-antibody coated) erythrocytes. Undiluted precolostral lamb serum gave some degree of haemolysis but precolostral lamb serum diluted 1/10 appeared negative in this respect. However this finding did not exclude the possibility that precolostral lamb serum at a dilution of 1/10 might contain those heat labile complement-factors necessary for the expression of growth-inhibition by normal sheep serum.

The agglutinated growth that formed on the sides of tubes was easily dislodged so freeing organisms into suspension and the resulting haziness in the broth did cause some difficulty in determination of the end point. Egerton and Merritt (1970) referred to agglutinated growth that occurred in serum from vaccinated sheep in the absence of complement. Although agglutinated F.nodosus cells from the plaques appeared morphologically typical, no form of organism, lysed or otherwise, could be recognised in the granular deposits that settled in the tubes containing less diluted serum. It was assumed that there had been complete cellular disruption with autolysis.

The term growth-inhibition rather than bacteriolysis was preferred to describe the test under consideration becaused lysed cells were not seen, and for inhibition of bacterial growth or prolongation of the lag phase, "growth-inhibition" appeared to be more appropriate.

The fact that growth-inhibition titres of all untreated sera were of the same order at 160-320, raised the doubt that differences between sera may have been masked by an imbalance in the test in which the dose of organisms was overwhelming small differences in serum antibody level. To investigate this possibility, graded doses of F. nodosus cells (calculated to contain between 1000 and 2,000,000 organisms) were added to standard dilutions of antiserum. The main effect of the titration was to extend or shorten the lag phase and as the titre remained unchanged, the sensitivity of the test was not appreciably improved. This result showed that any differences in activity between sera were not being masked by an over-large dose of organisms. It also served to confirm the statement made by Weidanz and Landy (1963) that as the percentage of bacteria killed is a function of concentration of antibody, a reduction in size of inoculum even by 1000 fold does not appreciably increase the sensitivity of the test.

The bactericidal (growth-inhibitory) activity of normal serum against Gram-negative organisms is attributable to a system comprised of specific antibody, complement and divalent cations (Osawa and Muschel, 1960; Weidanz and Landy, 1963). The normal serum activity described in this chapter fits this description and it seems reasonable to accept the view of Egerton and Merritt (1970) that antibody against F. nodosus found in normal sheep serum may have been raised by exposure to other Gram-negative organisms that share determinants with F. nodosus.

A completely unexpected finding in this series of experiments was that growth-inhibitory activity of normal sheep serum was lost when the sheep became infected with foot-rot. Egerton and Roberts (1971) had re-

ported that in their tests, infection for three weeks increased the titre of bactericidal antibody. A possible explanation for the results presented here is that levels of cross reacting antibody in normal serum are depleted by the diffusion of the antibody to the site of F. nodosus infection and this drain exceeds the rate of manufacture of such antibody. It was noticeable that the titre dropped between four and six weeks after infection (Table VIII.II) and the results of Egerton and Roberts (1971) applied only to three weeks after challenge. The challenge strain they used, F.nodosus (No. 198) of the McMaster collection, may have powers of invasiveness and of raising bactericidal antibody quite distinct to those of F. nodosus (J3/22).

The activity demonstrated in serum from vaccinated sheep was stable to heating at 56°C for 30 minutes but was wholly destroyed by heating at 70°C for 10 minutes. Such growth-inhibitory activity could be absorbed out with F. nodosus cells and therefore it appears to function specifically as an antibody system but unlike bacteriolytic antibody there appears to be no dependence upon complement factors for its full expression. Apart from the difference in heat stability of the activity associated with either normal sheep serum or that of serum from vaccinated sheep, vaccination made no difference to the growth-inhibition titre. This finding was quite unlike that of Egerton and Merritt (1970) who raised the bactericidal titre of serum from their experimental sheep at least 100 fold by vaccination. These differences can possibly be explained by different interpretations of end points particularly as these are influenced partly by the occurrence of agglutinated organisms. But there are other fundamental differences between the techniques used by Egerton and Merritt and those used for the growth-inhibition test described above that preclude an unqualified comparison of the two sets of results. Whereas Egerton and Merritt used their standard F. nodosus strains and the inoculum of 0.15 ml contained approximately 200,000 colony forming units, the technique used here employed F. nodosus (J3/22) and the inoculum of 0.1 ml contained an estimated 20,000 organisms. However, the most significant difference is probably associated with the different culture media used for each test, because as shown in Chapter III, different broth formulations have a marked influence on the resulting antigenic structure of organisms and this may determine their reactivity to antibody present in sheep serum.

To summarise, these tests have demonstrated three important features of humoral activity against metabolising F. nodosus organisms. Firstly, there is considerable growth-inhibitory activity in serum of normal sheep

and this effect is dependent on heat labile components. Secondly, the level of activity in the serum of normal sheep is no longer demonstrable when foot-rot infection has been present for at least six weeks. Thirdly, growth inhibitory activity in the serum of vaccinated sheep occurs to the same titre as that in the serum of normal sheep, is equally specific and therefore absorbable by F. nodosus organisms, but functions without the help of a factor(s) provided by diluted precolostral lamb serum.

Summary and Conclusions

1. Growth of a standard inoculum of F. nodosus cells in GC broth was inhibited to the same degree by serum either from normal sheep or from sheep that had been vaccinated with different adjuvant/cell combinations.

2. The activity of normal sheep serum was destroyed by heating at 56°C for 30 minutes but the activity could be completely restored by factor(s) present in diluted precolostral lamb serum.

3. Normal serum growth-inhibitory activity was significantly reduced by absorption with F. nodosus cells. Lower dilutions of serum were associated with amorphous deposits in the tube that developed after incubation with organisms.

4. Sera from vaccinated sheep contained growth-inhibitory activity that was unaffected by heating at 56°C for 30 minutes but was significantly reduced by heating at 70°C for 10 minutes.

5. Activity of sera of vaccinated sheep was significantly reduced by absorption with F. nodosus cells.

6. Plaques of agglutinated organisms were seen on the sides of tubes approaching the end point and amorphous deposits developed in tubes containing less diluted serum.

7. In two unrelated groups of sheep infected with severe foot-rot, pools of sera taken after several weeks infection were shown to be devoid of growth-inhibitory activity.

TABLE VIII.I

Effect of either antibody absorption or various heat treatments on the growth-inhibition titres of sera from either normal or vaccinated sheep

Serum	Serum growth-inhibition titre after stated serum treatment				
	Nil	Absorption	56°C/30min.	56°C/30min. plus PCLS	70°C/10min.
Normal serum	320	10	< 20	320	ND
Anti-*F. nodosus* (J3/22) antiserum	320	80	320	ND	< 30

PCLS = Precolostral lamb serum 1/10

ND = Not done.

TABLE VIII.II

Effect of naturally acquired or experimentally induced foot-rot on the growth-inhibition titres of sera of affected and unvaccinated sheep

Serum	Treatment	Growth-inhibition titre
Controls pre-challenge	Nil	160
	56°C/30 min.	<20
	56°C/30 min. plus PCLS	160
Controls post-challenge		
after 2 wk	Nil	160
after 4 wk	Nil	160
after 6 wk	Nil	<20
Pool Experiment Therapy I (field outbreak of foot-rot)	Nil	<20

PCLS = Precolostral lamb serum 1/10.

TABLE VIII.III

Growth-inhibition titres of heat-treated sera of groups of sheep injected with different adjuvant/cell combinations in Vaccine Trial III

Vaccine			
Adjuvant	Culture	Serum treatment	Growth-inhibition titre
Alum (one per cent.)	Fermenter whole culture 1 dose	56°C/30 min.	320
Alum (one per cent.)	Fermenter whole culture 2 doses	56°C/30 min.	320
Commercial vaccine "A" oil		56°C/30 min.	320
Commercial vaccine "B" alum		56°C/30 min.	320
Controls		56°C/30 min.	80
Controls		Nil	640

CHAPTER IX

SEROLOGICAL CHANGES IN SHEEP DURING FOOT-ROT INFECTION AND AFTER VACCINATION WITH VARIOUS ADJUVANT/CELL COMBINATIONS

(V) INDUCED CHANGE IN COLONY TYPE

Introduction

The demonstration by Egerton and Merritt (1970) of bactericidal antibodies against F. nodosus in normal sheep serum partly explained the original observation of Beveridge (1941) that sheep serum not only failed to promote F. nodosus growth on "V-F" agar but inhibited it in the presence of horse serum.

The experimental results described in the last chapter showed that there were distinctly different specific growth-inhibitory activities present in normal sheep serum as opposed to those found in serum from vaccinated animals. In view of the technical difficulties inherent in the tube test it was considered worthwhile to investigate "growth-inhibition" as exhibited on solid media by organisms that were exposed to different concentrations of sheep serum contained in filter paper discs.

MATERIALS AND METHODS

Bacteria F. nodosus (J3/22) was subcultured on hoof agar for 4-5 days and a loop of rough colonies used to inoculate the test plate of hoof agar.

Smears of colonies were stained by Gram's method and examined by the light microscope.

Sera Two sera were compared:

i) A "resistant" sheep serum sample was obtained from a sheep that had received two injections of the McMaster/biphasic cells vaccine, and that had a susceptibility score of one in Vaccine Trial I.

ii) A "susceptible" sheep serum sample was obtained from a Control group sheep that had a susceptibility score of 18 in Vaccine Trial I.

Sera were diluted in phosphate buffered saline (Appendix XVIa) and sterilised by Millipore filtration as described in Appendix XIV.

Filter paper disc test Filter paper discs (No. 740-E, Schleicher and Schnell, New York) were sterilised by autoclaving and placed four to a plate on freshly inoculated hoof agar.

Four or five drops of either serum dilution or sterile phosphate buffered saline, pH 7.4 (negative control) were delivered on to the disc by Pasteur pipette and the plates incubated agar uppermost in an anaerobic jar at 37°C for three days.

Using oblique lighting and a plate dissecting microscope, the area around the border of the discs was examined for growth inhibition or changes in colony appearance.

RESULTS

Discs saturated with either phosphate buffered saline or serum from the "susceptible" sheep, did not inhibit the growth of typical rough colonies of F. nodosus.

Where serum from the "resistant" sheep had been applied, smooth type F. nodosus colonies (Chapter II) grew in a 10 mm wide zone around the disc whereas typical rough colonies grew elsewhere on the agar. This effect of serum remained unchanged after heat treatment at 56°C for 30 minutes. No intermediate type colonies were seen.

The width of zone containing smooth colonies was approximately proportional to the dilution of serum applied to the disc (Table IX.I).

Around those discs in which the serum from the "resistant" sheep was most concentrated, there was some evidence of complete growth inhibition.

There was no apparent difference in the morphology of F. nodosus cells taken either from smooth type or rough colonies.

DISCUSSION

The ability of serum from a "resistant" vaccinated sheep to bring about the change in F. nodosus colony formation was due to a heat resistant factor not present in serum from a normal "susceptible" sheep. As a result of this it was decided to deliberately select rough colonies at the working seed plus one stage, as it was reasoned that smooth type colonies arise only as the result of some metabolic insult.

As the growth-inhibitory activity of sera (Chapter VIII) from vaccinated sheep was also resistant to heating at 56°C for 30 minutes it is possible that both of these heat stable functions are manifestations of the

same inhibitory system. The advantage of the disc test over the growth-inhibition tube test is that normal sheep serum does not produce any effect on the type of F. nodosus colony and therefore the possibility exists for comparing sera that have been raised by different adjuvant/cell combinations.

Development of the disc test was not pursued but the limited results reported here were encouraging. Lawn cultures of standard laboratory strains of either E. coli, an α haemolytic staphylococcus, a β haemolytic streptococcus or Salmonella typhimurium were not affected either in their degree of growth or colony morphology by serum from either "susceptible" or "resistant" sheep. Verification of the specificity of the activity by reacting unrelated antisera against F. nodosus cultures on hoof agar was not attempted.

A comparison of the influence on colony characteristics of F. nodosus, by antisera raised by different vaccination schedules might provide relevant data on the immune status of the serum donor.

Summary and Conclusions

1. In *F. nodosus* cultures growing on hoof agar, contact with serum from a vaccinated "resistant" sheep caused a change in colony form from the typical rough colonies to the smooth type.

2. Under identical cultural conditions, serum from a normal "susceptible" sheep had no effect on the development of typical rough colonies.

3. No intermediate-type colonies developed.

4. The activity of serum from a "resistant" sheep remained unchanged after heating at 56°C for 30 minutes.

5. The filter paper disc test results were sufficiently interesting to warrant further investigations into the specificity of the reaction.

TABLE IX.I

Induced change in F. nodosus colony type on hoof agar when exposed to serum of a "resistant" sheep

Serum	Serum treatment	Smooth type colonies when exposed to sera at stated dilution						
		1/2	1/4	1/8	1/16	1/32	1/64	1/128
"Susceptible" sheep	Nil	-	-	-	ND	ND	ND	ND
"Resistant" sheep	Nil	ND	++++	++	+	+	-	-
"Resistant" sheep	56°C/30 min.	++++	++++	++++	++	+	+	ND
Saline	Nil	-	ND	ND	ND	ND	ND	ND

- = No inhibition in growth of rough colonies

+ to ++++ = Zone of smooth type colonies

ND = Not done.

CHAPTER X

DETECTION OF DELAYED-TYPE HYPERSENSITIVITY AS AN EXPRESSION OF CELL-MEDIATED IMMUNITY IN SHEEP WITH FOOT-ROT

Introduction

F. nodosus infections are restricted to the avascular epidermis and in this site the organisms are not exposed to phagocytosis (Egerton and Roberts, 1971). Despite this relative isolation from the blood supply, foot-rot infections elicit a humoral immune response. Such a response has been demonstrated in a number of ways (Egerton and Merritt, 1970; Chapters V to IX inclusive, of this thesis) and the increased resistance of vaccinated sheep has been attributed to the destruction of *F. nodosus* by bactericidal antibody with complement, diffusing into the infected epidermis (Egerton and Roberts, 1971). However, immune responses whether they arise as a result of natural infection or as a consequence of vaccination generally show both humoral and cell mediated aspects. The mechanism of protection afforded by *F. nodosus* vaccination has not been fully explained and a complementary function of cellular defence activities and humoral antibody involvement was considered possible. The experiments that are described in this chapter were designed to see whether or not cell-mediated responses occur in animals infected with foot-rot.

In vivo tests are the most convenient and reliable techniques available for this purpose and those that are used depend upon the demonstration of delayed-type hypersensitivity. Two sites on the animal body were chosen for inoculation of antigen; intradermal and intracorneal. The intradermal site has the disadvantage that reactions of the delayed-type are often

preceded by immediate hypersensitivity. On the other hand the substance of the cornea as the site for injection of antigen has the advantage that the tissue normally has no blood vessels and therefore no contact with circulating antibody (Herbert, 1970).

Three antigens were chosen for comparison. Cell extract of F. nodosus (J3/22), the same strain used for attempted infection of sheep, was employed for homologous tests. Responses were compared with that of antigen prepared from Escherichia coli, a ubiquitous organism that would be prominent in the normal sheep environment. The third material used for injection was cell extract of a bacterium pathogenic for cauliflowers, and this provided a completely unrelated control antigen.

MATERIALS AND METHODS

Sheep Three classes of sheep were chosen for comparison. Firstly, there were four with normal feet and no history of previous foot-rot. Secondly, there were five sheep with active and progressive foot-rot and another with benign foot-rot and therefore these animals might be expected to be sensitised to F. nodosus antigens. The third class of sheep included eight individuals that had recently experienced attempted F. nodosus infection and as some had become infected with foot-rot and others remained normal, the group provided two directly comparable sub-groups.

Antigens Antigens as either untreated cell extracts, boiled cell extracts or concentrated culture supernatants, were derived from: F. nodosus (J3/22) which had been cultured in biphasic medium, Escherichia coli, strain E (Department of Microbiology and Genetics, Massey University), cultured overnight in beef heart infusion broth and Pellicularia filamentosa (Sub Department of Plant Pathology, Massey University) cultured on dextrose peptone agar at 25°C. Where possible materials were sterilised by Millipore filtration before injection.

Control materials of culture medium, phosphate buffered saline or distilled water were always tested in parallel.

Protein concentration Cell extracts were adjusted in volume to give a standard protein concentration as judged by their absorbency at 280 nm.

Intradermal skin test Approximately 0.1 ml amounts of antigen were injected intradermally either on the bare pubic area of the abdomen or else, after clipping the wool, on the side overlying the thorax. The injection site was examined daily during four days for changes in colour and palpated for swelling or induration.

Intracorneal injection Sheep were anaesthetised using thiopentone sodium or CT 1341 ("Saffan") given by intravenous injection (Appendix XV). With the eyeball immobilised by countertraction ligatures, antigen was

injected into the substance of the cornea through a 26 g (0.45 mm) x 13 mm needle. The opposite eye of each sheep was used either for comparison of another antigen or for injection of control material. Occasionally two injections were made into one cornea. Successful siting of the injection was confirmed by the appearance of an opaque bleb or "star". The injection sites were examined daily for evidence of localised or diffuse opacity.

RESULTS

The response of normal sheep or a benign foot-rot infected sheep to the intradermal injection of either F. nodosus (J3/22) cell extract or culture supernatant

Neither F. nodosus antigens nor biphasic culture medium injected as a control, caused any untoward reaction on intradermal injection into normal sheep. A transient induration that developed between 1 and 4 hours resolved overnight. A sheep with benign foot-rot showed a more marked reaction to injection with F. nodosus antigens. An ill-defined oedematous reaction of approximately 3 cm in diameter that arose one hour after injection persisted for four hours. Control injections of biphasic medium were negative in this animal (Table X.I).

The response of normal sheep to the intradermal or intracorneal injection of either F. nodosus or E. coli antigens

The intradermal injection of either F. nodosus or E. coli antigens caused a mild and transient reaction in normal sheep as described above. The intracorneal injection of either cell extract caused an immediate localised opacity that disappeared within hours. Twentyfour hours after the injection, the F. nodosus cell extract site appeared as a pinpoint white spot surrounded by a 2 mm zone of opacity. The same degree of local opacity also appeared at the E. coli cell extract site but 24 hours later.

The response of foot-rot infected sheep to the intradermal or intracorneal injection of either untreated F. nodosus antigens or untreated E. coli antigens

The intradermal injection of either untreated F. nodosus or untreated E. coli antigens into foot-rot infected sheep resulted in a similar degree of local reaction. An area of skin 1-2 cm in diameter showed swelling which reached its maximum size after 4 hours and was completely resolved within 48 hours.

The intracorneal injection of either antigen caused opacities to develop at the injection sites. Reactions to both antigens were apparent after 24 hours and persisted for four days before resolving. The opacity due to E. coli antigens was more marked than that due to F. nodosus antigens (Table X.II).

The response of normal sheep or foot-rot infected sheep to the intracorneal injection of either untreated F. nodosus cell extract or heated F. nodosus cell extract

Normal sheep responded to the intracorneal injection of either normal or heated (100°C for 60 minutes) F. nodosus cell extracts in a similar manner. The reaction had fully developed by 24 hours after injection and showed marked resolution during the next two days.

Foot-rot infected sheep also responded to both cell extracts to the same degree (Figure X.I) but in these sheep the corneal opacity was more intense than that seen in normal sheep and it persisted without fading for four days (Table X.III).

The response of foot-rot infected sheep to the intracorneal injection of either F. nodosus untreated cell extract or P. filamentosa untreated cell extract

Foot-rot infected sheep responded to the intracorneal injection of F. nodosus antigens as explained above i.e. a corneal opacity developed at the injection site after 24 hours and persisted for four days. The response to an intracorneal injection of comparable amounts of P. filamentosa antigen was minimal and did not exceed that given by saline injections used as controls.

DISCUSSION

Skin reactions to the intradermal injection of F. nodosus cell extracts were unrewarding. The extent of the reactions was difficult to follow because of the skin pigmentation. Both normal sheep and an animal with benign foot-rot showed transient responses which were maximal at four hours and resolved by 24 hours. The timing of the development of these reactions was more characteristic of antibody mediated reactions and quite unlike those expected of cell-mediated immunity. The response of normal sheep to F. nodosus antigens and the response of both normal and infected sheep to biphasic medium alone, made further evaluation of this test system appear unprofitable.

The intracorneal test, although easier to interpret than the intradermal test, was difficult to execute. Immobilisation of the eyeball and the deposition of antigens within the corneal substance were difficult to achieve. However, the differences in reactivity of normal or foot-rot infected sheep to the intracorneal injection of F. nodosus antigens as initially observed was promising. Foot-rot infected sheep responded with a corneal reaction that was both more intense and persisted longer than that of normal sheep. The absence of reaction following the intracorneal injection of P. filamentosa cell extract showed that reactions in foot-rot

infected sheep were not due simply to the deposition of foreign material in a sensitive area.

Foot-rot infected sheep also showed a similar response to the intracorneal injection of both F. nodosus untreated and heat treated cell extract. This result suggested that those antigen(s) responsible for evoking a response reside in the heat stable fraction of the cell and they may be analagous to the endotoxic lipopolysaccharide antigen of oral Fusobacteria (Kristoffersen, 1969a and b).

The specificity of the reaction shown by infected sheep to F. nodosus antigens was placed in doubt by the ability of E. coli antigens to cause similar or even more intense reactions than F. nodosus antigens in infected sheep. E. coli antigens reacted also in sheep known to have no history of clinical foot-rot infection. An explanation of these findings could be provided by the occurrence of shared antigenic determinants between the two organisms. Although agar diffusion tests failed to show any reactivity between E. coli extract and anti-F. nodosus (J3/22) antiserum, this cannot be taken as proof that such an antigenic similarity does not exist. If infection with F. nodosus causes the sensitisation of a population of lymphocytes carrying in total receptor sites for a range of antigen determinants, then exposure of the animal to any of the relevant groupings will result in cell-mediated reactions. As E. coli forms part of the normal gut flora of sheep and is widespread in the animals' environment, it is not unreasonable to expect some degree of sensitisation to the organisms, to result from such prolonged and intimate contact.

Cell-mediated immunity is associated with conditions in which the sensitising antigen remains "fixed" at its site of introduction (Bloom, 1971). In this situation the antigen rather than being carried to the lymphoid tissues and inducing antibody synthesis, makes contact predominantly with circulating small lymphocytes and elicits what is mainly a cell-mediated response. Foot-rot infections involving the prolonged occupation by microorganisms of avascular sites might act as a source of peripheral sensitisation and induce cell-mediated immunity. What role such a form of immunity might play in resistance is not known. With the current interest in cell-mediated immunity, foot-rot infection might serve as a useful model because F. nodosus is exceptional in being restricted to and adapted for long term existence in an avascular site.

Summary and Conclusions

1. As a means of testing for delayed-type hypersensitivity, the intradermal test using *F. nodosus* antigens gave inconclusive results and in its present form the test is unsatisfactory.

2. Intracorneal injection of *F. nodosus* cell extract caused an opacity that developed during the first day and that was more intense and persisted longer in sheep with foot-rot, than in normal sheep.

3. The corneal reaction provoking component in *F. nodosus* cell extract was resistant to boiling.

4. The injection of *E. coli* cell extract caused the same degree of temporary corneal opacity as *F. nodosus* cell extract. The cross-reactivity was attributed either to the occurrence of antigenic determinants common to both organisms or to the response of sheep when local environment antigens are encountered in an unusual site.

5. Cell extract of *P.filamentosa*, an agent causing "damping-off" in cauliflower seedlings, elicited no response on intracorneal injection of sheep. The absence of reaction was attributed to the complete foreigness of the antigens to sheep.

TABLE X.I

Degree of local reaction in one normal sheep and one sheep affected with benign foot-rot, in response to an intradermal injection of F. nodosus culture extract

Sheep	Inoculum/Hours after injection											
	F. nodosus (J3/22) biphasic untreated cell extract			*F. nodosus* (McM199) biphasic culture supernatant			Distilled water			Biphasic medium		
	1	4	24	1	4	24	1	4	24	1	4	24
Normal	+	+	-	+	+	-	-	-	-	+	++	-
Benign foot-rot	++ 0	+++ 0	-	++ 0	+	-	-	-	-	+	+	-

- = No reaction

+ to ++++ = Degree of local reaction

0 = Ill-defined oedema.

TABLE X.II

Extent of corneal opacity in two foot-rot infected sheep in response to intracorneal injection of either F. nodosus untreated cell extract or E. coli untreated cell extract

Foot-rot infected sheep	Inoculum/Hours after injection											
	F. nodosus (J3/22) biphasic, untreated cell extract			E. coli (E) broth, untreated cell extract			Hoof broth			E. coli cell free culture supernatant		
	24	48	96	24	48	96	24	48	96	24	48	96
Sheep A	+	+	fading	+++	+++	fading	SL	-	-	-	-	-
Sheep B	+	+	fading	++++	++++	fading	-	-	-	SL	SL	fading

- = No reaction

+ to ++++ = Extent of opacity

SL = Slight diffuse opacity.

TABLE X.III

Degree and extent of corneal opacity in either normal or foot-rot infected sheep, in response to intracorneal injection of either untreated or boiled cell extract

Sheep	*F. nodosus* (J3/22) hoof agar cell extract	Corneal opacity/ Hours after injection			
		24	48	72	96
Normal	Untreated	+ heavy	fading	faded	faded
Normal		p.p. pale	p.p. pale	fading	faded
Infected		++ pale	+ heavy	+ heavy	+ heavy
Infected		+ heavy	+ heavy	+ heavy	+ heavy
Normal	Boiled	+++ pale	+ pale	faded	faded
Normal*		++ heavy	++ pale	faded	faded
Infected		+ heavy	++ heavy	++ heavy	++ heavy
Infected		p.p. heavy	p.p. pale	p.p. fading	p.p. pale

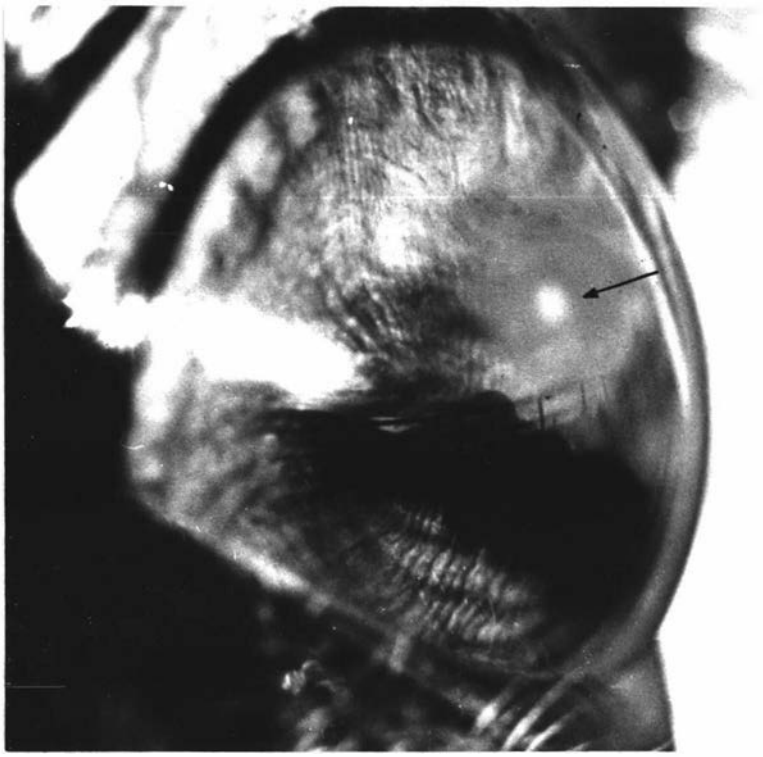
* Vaccinated 18 wk previously

\+ to ++++ = Extent of opacity

p.p. = pinpoint.

FIGURE X.1

Corneal opacity in response to intracorneal injection of F. nodosus boiled cell extract administered 48 hr previously. The sheep had severe foot-rot.



CHAPTER XI

VACCINATION AS THE SOLE MEANS OF TREATMENT IN SHEEP AFFECTED WITH PROGRESSIVE FOOT-ROT

EXPERIMENT THERAPY I

Introduction

Vaccination is rarely used as an aid in treatment of disease because protection is best afforded by prevention of infection rather than by interference with an established pathological process. In many diseases the course of infection leading to death or recovery is so rapid that even if vaccines were used in treatment, the outcome of the illness would be determined long before the host had had time to respond. But in foot-rot where infections may persist for months and sometimes years (Beveridge, 1941), the vaccinated animal has adequate time in which to respond and thus there is a logical basis for vaccine therapy.

The exact mode of action of foot-rot vaccines is still largely unexplained (Egerton and Roberts, 1971) and in prophylactic use, although vaccination prevents the majority of injected sheep from becoming infected, some sheep at risk contract interdigital skin infection and others develop a mild form of the field disease (Egerton and Burrell, 1970). However, foot-rot that occurs in vaccinated sheep is consistently less invasive, lasts a shorter time and affects fewer feet per sheep than does the disease in controls exposed to the same infections (Egerton, Morgan and Burrell, 1972).

The curative effect of foot-rot vaccines has been well documented (Egerton and Burrell, 1970; Egerton and Roberts, 1971; Skerman, 1971; Egerton and Morgan, 1972; Skerman and Cairney, 1972) and amongst vaccinated sheep, the rate of recovery is usually significantly faster than in controls.

Although much of the work presented in this thesis has been concerned both with those features of bacterial culture that might determine immunogenicity and the response of sheep to vaccination, during the course of this work an opportunity arose to investigate the therapeutic properties of vaccination.

A small flock of mixed aged Romney and Romney/Cheviot cross ewes, most of them lame because of advanced and largely untreated foot-rot, were offered for experimental investigation. Since the flock had lambed three months previously, there had been sporadic cases of foot-rot amongst the ewes and these had been topically treated at irregular intervals.

The major outbreak started after weaning when the ewes were turned onto rank pasture in river meadows during several weeks of humid weather. At the initial examination, sound-footed sheep were turned away to another paddock and the remaining 24 affected with foot-rot were put aside for a more detailed examination.

This particular group of sheep was chosen for a vaccine therapy trial because of two important features of the outbreak. Firstly, the foot-rot lesions were typically progressive and there were enough sheep affected at approximately the same stage to enable valid comparisons to be made between treated and untreated groups. Secondly, climatic conditions at that time in the early Summer were favourable for continuing infection (Graham and Egerton, 1968). It was felt that with a high level of continuing challenge, the therapeutic results of vaccination would not be obscured by natural resolution of infection amongst untreated control sheep.

MATERIALS AND METHODS

i) Clinical examination Each foot of every sheep was examined and the extent and severity of foot-rot lesions recorded (Appendix IXa) using the assessment score method (Appendix IX). Details were recorded after the initial examination and at three and six weeks after treatment by vaccination.

ii) Vaccine evaluation Treated groups were given one or two doses at three week intervals of the Alum (one per cent.) precipitated F. nodosus (J3/22) GC broth fermenter, whole culture vaccine. Assessment scores of individual sheep and groups were compared before and after vaccination.

In addition, at three weeks and six weeks after vaccination, the foot-rot lesion(s) of every sheep were evaluated as either completely active, mostly active, mostly resolved or completely resolved.

iii) Initial examination Large flaps of horn were removed by paring but no attempt was made to remove all necrotic tissue. Regardless of the foot-rot assessment score, alternate sheep after examination were given 2 ml of vaccine (treated group) or left untreated (control group). All the sheep were returned to the river pasture.

iv) Examination after three weeks All the feet were re-examined and lesions pared until active foot-rot was diagnosed or until healthy tissue was reached i.e. resolving lesions were the more likely to be extensively pared.

Six of the 12 sheep in the treated group were selected randomly for injection with a second dose of vaccine.

All the sheep were returned to the river pasture.

v) Examination after six weeks All foot-rot lesions were pared right back to expose active infection or healthy tissue. Most control sheep were vaccinated as a form of treatment.

All sheep were walked through a five per cent. formalin footbath two to three weeks later.

vi) Statistical analysis Data on the degree of resolution were contracted to produce 2 x 2 contingency tables and Fisher's exact test (Bailey, 1969) was used for direct calculation of the probabilities.

Assessment scores provide an objective means of recording the response of an individual sheep but because such scores are determined by many factors other than the state of F. nodosus infection, the data were not used for evaluation of group response.

RESULTS

i) Examination after three weeks In the vaccinated group, foot-rot lesions appeared less "active and progressive" as shown by drying of the necrotic horn matrix whereas amongst the control (untreated) sheep, foot-rot lesions remained largely unchanged. In general lameness was less apparent amongst vaccinated sheep. Of the 12 vaccinated sheep only one failed to respond as judged by assessment score whereas five of 12 untreated control sheep had the same or greater assessment scores than at the initial examination (Table XI.I). Ten out of the 12 untreated sheep had "mostly active" lesions (Table XI.II): only one out of the 12 sheep that had been vaccinated once had "mostly active" lesions. The difference in response between treated and control groups was highly significant ($p = 0.0002$).

ii) Examination after six weeks One out of six sheep vaccinated once, one out of six sheep vaccinated twice and six out of 12 untreated sheep had assessment scores greater than at the time of the pre-treatment examination.

In general the two groups of vaccinated sheep ranked equally ($p = 0.4$) and showed marked benefit from treatment (Table XI.II). Taking the two vaccinated groups together there were more sheep with "mostly resolved" lesions than amongst untreated controls ($p = 0.004$).

Three control sheep were found to have contracted cutaneous myiasis within the foot-rot lesion.

The group of sheep that had been vaccinated twice had slightly more assessment score contributed from interdigital space lesions, as opposed to hoof separation lesions, than either of the other two groups.

DISCUSSION

For the duration of the trial the 24 ewes were maintained under constantly moist or wet foot conditions and the ambient temperature remained high. This combination, coupled with considerable night humidity, was conducive to the spread and continuing activity of foot-rot infection (Graham and Egerton, 1968) thus providing a severe test for the effectiveness of the vaccine. Because extensive paring was deliberately not carried out and topical treatment was not attempted, foot conditions remained in favour of F. nodosus persisting. Even so some temporary resolution occurred in all groups including controls presumably because the superficial paring had exposed infection sites to normal drying effects and aerobic conditions. However, six weeks after the initial examination, only two of 12 control sheep had "mostly resolved" foot-rot lesions as against nine controls.

Three or four ewes that showed more of the characteristics associated with the Cheviot breed than the Romney, responded poorly to vaccination. As an example, sheep 252 (Table XI.I) was considerably worse after two doses of vaccine than at the pre-treatment assessment. It certainly seemed as though the Cheviot-type sheep either had a more persisting and severe infection or had responded to vaccination less well. No conclusions could be drawn from such a small number of sheep and because pre-treatment selection had not included breed "type" as a variable for random distribution. Other reports in the literature have drawn attention to the susceptibility of the Merino breed being greater than that of cross-bred sheep (Beveridge, 1941; Egerton, Morgan and Burrell, 1972).

Although a significant response to vaccination was demonstrated at three and six weeks after the treatment no significant difference was demonstrated between one or two doses of vaccine. This is not to say that

a difference does not exist and the use of larger groups of sheep might show this.

In large scale treatment trials, both Egerton and Burrell(1970) and Skerman (1971) found that two doses of a water-in-oil adjuvant vaccine were more effective than one dose. Egerton and Roberts (1971) using the same type of F. nodosus vaccine but incorporating Freund's incomplete adjuvant instead of the McMaster adjuvant, found that healing commenced two to four weeks after the first dose of vaccine and was complete at eight weeks. As the peak of antibody response to a water-in-oil adjuvant vaccine is not reached until about the 60th day after a single subcutaneous injection (Herbert, 1967), in order to stimulate the best response the timing of the second injection may be quite critical. Trials so far reported have employed intervals of either one week (Egerton and Burrell, 1970; Egerton and Roberts, 1971; Egerton and Morgan, 1972), two weeks (Skerman, 1971; Skerman and Cairney, 1972) or six weeks (Roberts, Foster, Kerry and Calder, 1972), and apart from the six weeks interval used for an alum precipitated formulation[1], the interval seems to have been chosen on an arbitrary basis. To obtain the maximum benefit from vaccination attention must be paid to the between-dose interval and it is likely that the optimum interval will be different for vaccines utilising either mineral oil or alum adjuvants. It is also possible that the between-dose interval for the prophylactic use of vaccine may be different to that advocated for therapeutic use because in the former a sustained response may be more useful whereas in therapy a maximum response over a short period may be more effective.

Three out of the 12 untreated sheep became affected with cutaneous myiasis in the foot during the six week observation period. Under the most desirable husbandry conditions of careful paring and topical treatment, the healing foot-rot lesion is unlikely to attract flies but the deliberate neglect method adopted in this trial has demonstrated how rapidly a necrotic foot lesion becomes attractive for blow flies and provides an ideally sheltered site for development of cutaneous myiasis.

[1] Commercial literature claims that m aximum agglutination titres were obtained when the interval between doses was extended to six weeks as opposed to shorter periods.

Summary and Conclusions

1. Vaccination administered during a foot-rot outbreak was effective in accelerating resolution of lesions noticeably within three weeks, in spite of the environmental conditions remaining favourable for continuing F. nodosus infection.

2. Vaccination should be more effective if careful paring and formalin footbathing were carried out concurrently.

3. A schedule of two doses of the Alum (one per cent.) precipitated whole culture vaccine given at a three week interval was no more effective than a single vaccine dose.

4. As in prophylactic use of the vaccine, there was an inconsistent therapeutic response to vaccination.

5. This trial highlighted the risk of cutaneous myiasis developing as a direct consequence of neglected foot-rot lesions.

TABLE XI.I

Response of foot-rot infected sheep to either one or two doses of Alum (one per cent.) precipitated whole culture vaccine

Sheep		Assessment score					
No.	Group	Pre-treatment	Mean	After 3 wk	Mean	After 6 wk	Mean
207	ReV	15		4		1	
239	ReV	32		0		0	
240	ReV	19	17	0	2	0	5
247	ReV	13		0		5	
252	ReV	8		4		19	
261	ReV	13		4		4	
268	V	17		9		6	
281	V	27		1		0	
290	V	26	23	2	7	0	10
295	V	21		21		10	
296	V	31		5		11	
299	V	18		6		31	
*209	C	33		14		35	
*218	C	23		12		4	
229	C	10		1		0	
233	C	16		38		32	
237	C	15		6		3	
243	C	15	17	3	13	5	14
248	C	15		10		21	
*254	C	27		32		16	
259	C	21		7		1	
263	C	7		16		14	
270	C	11		12		17	
284	C	7		7		17	

* Cutaneous myiasis

C = Controls

V = Vaccinated once

ReV = Vaccinated twice.

TABLE XI.II

Response of foot-rot infected sheep to either one or two doses of Alum (one per cent.) precipitated whole culture vaccine

Sheep treatment	Numbers of sheep							
	Three weeks after first treatment				Six weeks after first treatment			
	Completely resolved	Mostly resolved	Mostly active	Completely active	Completely resolved	Mostly resolved	Mostly active	Completely active
Controls untreated	1	1	2	8	1	1	nil	10
Vaccinated once	3	2	nil	1	2	3	nil	1
Vaccinated twice	3	3	nil	nil	3	1	1	1

CHAPTER XII

GENERAL DISCUSSION

As the starting point in the development of any bacterial vaccine it is standard practice to select the most virulent field strain(s) and aim to maintain their original characteristics. The same principles have been followed in the development of a foot-rot vaccine by the isolation of an appropriate strain, freeze-drying a bulk of seed to provide starting culture for each batch and by paying particular attention to the physiological requirements of the growing organism.

The virulence of lyophilized F. nodosus (J3/22) has been confirmed by the ability of cells cultured on hoof agar to infect sheep and cause severe progressive foot-rot on at least four occasions.

The morphology of such cells has been shown by electron microscopy to be strictly comparable to the naturally occurring organism in terms of intracellular structures such as volutin granules and other prominent features such as the layered cell wall. In addition, pili were seen on the cells cultured on hoof agar as well as on cells occurring in the natural state. These extracellular appendages may account for the clear zone seen around the organism in preparations made from either infected feet or the organisms in culture. Although Egerton (1972b) has demonstrated by means of indian ink extracellular slime associated with F. nodosus cells grown on hoof agar, the same material could not be confirmed by similar experiments carried out during this study.

It is known that the antigenic structure of organisms can be influenced by their conditions of culture (Commonwealth Scientific and Industrial Research Organisation, 1969-70; Weinbaum, Kadis and Ajl, 1971). The antigens of F. nodosus could be grouped into three main classes and the amounts of each demonstrated could be influenced both by the method of cell culture and by the laboratory technique used to indicate reactivity. The antigen found free in culture supernatant and associated with cells grown in liquid media, was identified as protease by reactions of identity with the purified material in double diffusion in agar tests.

Attached loosely to the bacterial cell surface were heat-labile antigens that were easily dislodged and these were designated "K" antigens. They were demonstrable by agglutination and double diffusion in agar tests.

The third class of antigens could be demonstrated after the cells had been subjected to vigorous washing, and boiling for 90 minutes. These

antigens were designated "O" and it was found that they were agglutinated to low titre in antisera raised by formalin treated F. nodosus cells.

Thus an antigenic structure is suggested for F. nodosus that closely parallels that of the Enterobacteriaceae (Cruickshank, 1965). The analogy can be taken further in consideration of the particular roles played by each of the antigen classes in virulence and possibly in the protection afforded by vaccination.

Protease production has been used by Egerton and Parsonson (1969) to classify strains of F. nodosus into two groups. Those strains producing small amounts of protease cause benign foot-rot and are relatively non-invase. The other strains produce greater amounts of protease, cause progressive foot-rot and by definition are therefore invasive. Although indicative of strain virulence, protease appears to be unnecessary as an antigen component for vaccine. This is supported by the results of Egerton and Roberts (1971) who produced a highly protective vaccine using cells harvested from hoof agar and therefore lacking in protease. Such a vaccine afforded equally as much protection as another formulation using cells grown in biphasic medium (Egerton and Burrell, 1970). F. nodosus cells grown in biphasic medium possess significant amounts of protease which can be demonstrated by double diffusion in agar. More significantly, from the results of Vaccine Trial I it was shown that a vaccine consisting of F. nodosus cells and culture supernatant, protected no better than the same formulation incorporating cells alone.

The "K" antigens were easily dislodged from most cell concentrates by simple washing but they were stabilised by either formalin or mild heat treatment. On the other hand "K" antigens present on hoof agar-grown cells were more stable to washing perhaps because the pili protected the antigen layer from dislodgement. It was clear both from the reactions of identity in double diffusion using untreated cell extract and from agglutination results after antiserum absorption, that F. nodosus (J3/22) antiserum contained antibody against the "K" antigens of other F. nodosus strains. The cross reaction included one virulent field strain isolated from another part of New Zealand. This is of particular interest since Egerton and Morgan (1972) have recently reported the partial failure of a vaccine in circumstances where a serological difference was demonstrated between the strain of F. nodosus used in the vaccine and the strain causing the field outbreak. Because anti-F. nodosus (J3/22) antiserum agglutinates other strains of organism, the strains must share antigenic determinants and it raises the possibility of "master" strains or at least the possible existence of cer-

tain virulent strains that share a common antigen. The importance of this feature in selection of strains for vaccine production is self-evident and the search for wide-spectrum antigens could be most rewarding. Neoh and Rowley (1970) have drawn attention to a protein component, common to two distinct Vibrio cholerae strains, which they suggest may be useful as a "universal" cholera vaccine to protect against all serological types. Insufficient work has been done on the antigenic structure of a significant number of F. nodosus field strains to expand further on this aspect.

One F. nodosus strain that possessed "K" antigens was "O"-inagglutinable in homologous antiserum; a feature well recognised in the "coli group" (Kauffmann, 1947). In view of the partial heat stability of F. nodosus "K" antigens (stable at 60°C for 60 minutes), by the Kauffmann scheme they would be designated B-type antigens rather than L-type which are strictly heat-labile. The great importance of "K" antigens lies in their ability to enhance bacterial virulence either by their inherent toxic properties directed at the host or by shielding the organisms from antibody directed at the bacterial membrane. Recent work in other fields has confirmed the importance of "K" antigens in relationship to virulence (Lancet, 1972). It has been suggested that not only their presence but also the actual amount of "K" antigen may be important and the possibility exists that common determinant groups may be shared between unrelated species of organisms. The widespread nature of certain determinants may partly explain the reactions that occurred in sheep injected intracorneally with E. coli antigens (Chapter X).

The "O" antigens, firmly attached to the cell well and stable to boiling, were demonstrated by agglutination and by double diffusion in agar. The latter technique showed that the same somatic antigens were common to three F. nodosus strains. Thus if the analogy with the Enterobacteriaceae is taken further, in all probability the "O" antigens represent the polysaccharide determinants of the bacterial endotoxin (Rowley, 1971).

The titres of anti-"K" or anti-"O" antibody as shown by agglutination or double diffusion have been useful in determining the role each antigen may play in stimulating response to infection or in contributing to protection afforded by vaccination. High "K" or "O" agglutination titres were not always associated with resistance against F. nodosus infection and low "K" or "O" agglutination titres were not always indicative of susceptibility. As shown in Chapter VII, the immunoprecipitation pattern produced between sera and an extract of boiled F. nodosus cells gave some indication of the immune status of the serum donor. Serological activity of this kind indicates a specific response to vaccination but because the vaccinated sheep

are subsequently found to be immune it does not necessarily follow either that protection is mediated by humoral antibody or that the antibody demonstrated plays any part in resistance against infection. Although it was valid and instructive to compare the antigenic structure of F. nodosus with that of other bacterial species such as E. coli that appear to have a similar conformation, once the aspect of protection against infection is considered, comparisons cease to be relevant because of the totally different patterns of disease.

It was shown in Chapters II and III that a virulent field strain of F. nodosus could be selected on the basis of its history and morphology: after culture by the appropriate method, the antigenic structure was retained. In Chapter IV, trials with various adjuvant systems led to the selection of a formulation that was acceptable for the degree of local reaction it caused, and its adjuvanting potency was such that incorporation with F. nodosus cells produced a protective vaccine. The foregoing evidence demonstrated the significant protection afforded by vaccination but it provided no indication of the manner in which immunity was effected.

The evidence in favour of protection being mediated by serum antibody is considerable. Egerton and Merritt (1970) showed that normal serum was bacteriolytic for F. nodosus and that the titre could be raised 100 fold by vaccination. More convincing was the fact that Egerton (pers. comm.) had protected sheep with injections of IgG derived from immune sheep sera whereas comparable controls became severely infected. The amounts of IgG transferred are unknown. Confirmatory evidence of the protective effect of immune serum was provided by Skerman (1972) who showed that when lambs were challenged at ten days of age, those born to vaccinated ewes were significantly more resistant to attempted F. nodosus infection than lambs born to unvaccinated ewes. The manner in which the parturient ruminant concentrates serum antibody into the colostrum has been recognised for some time (Brambell, 1970) and this might provide the recipient offspring with serum levels of antibody higher than those of the dam. The transfer of antibody from ewe to lamb was demonstrated by double diffusion in agar when postcolostral lamb sera, reacted against untreated cell extract, produced more prominent precipitates than did serum taken from the dam at lambing. As a result of this selective concentration, the levels of circulating antibody in young lambs may be significantly higher than in adults exposed to natural infection. Levels in lambs may be high enough to account for the low incidence of severe foot-rot amongst sheep of that age.

Egerton and Roberts (1971) have postulated that the failure of antibody induced by infection either to effect a cure or to protect against

later infection can be explained simply in terms of quantity: the amount of antibody raised by infection would be much less than the amount raised in response to vaccination. In fact bacterial growth-inhibition studies (Chapter VIII) showed that normal levels of serum activity were absent after several weeks of natural foot-rot infection. As the normal decay rate for sheep serum antibody is 16 per cent. per week (Barr, Glenny and Howie, 1953) which could not account for the reduction observed, the accelerated disappearance was attributed to a differential attraction to the inflamed area and this drain exceeded the rate of antibody synthesis.

An unsuccessful attempt, not fully reported here, was made to confirm the protective effect of passively acquired antibody. Five hundred millilitre amounts of serum from vaccinated ewes were transferred to normal recipients immediately before challenge by application of F. nodosus cells to the conditioned foot. These serum transfers were repeated weekly. The donor serum had an initial "K" agglutination titre of 1:50,000 which was sufficient to provide a final "K" agglutination titre of 1:10,000 in the recipients. The response to challenge was inconsistent and where infection did become established, progress was unusually slow. This failure of the virulent strain to infect was attributed to the low ambient temperature experienced during the trial period. Night temperatures often fell below 10^{o}C and at this level, not only does foot-rot fail to spread but there is an induced fall in temperature of the sheep extremities (Graham and Egerton, 1968). F. nodosus is slow growing at 37^{o}C and does not multiply at all below 20^{o}C (Beveridge, 1941).

The mechanism of immunity is divisable into two major classes according to the mediation of effect on the parasite: antibody-mediated or cell-mediated. Both generally occur together, in fact Rowley (1971) takes the compromise view that both humoral and cellular elements are inseparably involved.

Both antibody-mediated and cell-mediated responses occur in natural infections of foot-rot. Chapters V, VI, VII, VIII and IX dealt with responses measurable by serological means and these confirmed previous reports (Egerton and Merritt, 1970; Egerton and Roberts, 1971; Merritt, Egerton and Loi, 1971). Apart from a passing mention of the possible role of cells in foot-rot infection (Egerton and Roberts, 1971), cell-mediated immunity had not been seriously considered as a possibility before, but the results presented in Chapter X show that a cellular response does occur during natural infection and it can be confirmed by the demonstration of delayed-type hypersensitivity.

The two forms of immunity are taken to be the result of the form of

antigenic stimulus and the response to this can be explained in terms of the activities of two sub-populations of lymphocytes (Craddock, Longmire and McMillan, 1971). Antibody is formed by cells mainly resident in lymphoid tissue whereas cell-mediated immunity is a function of circulating cells that are usually exposed to antigen retained in areas remote from the main lymphoid centres.

In natural foot-rot infection there are periods of F. necrophorus invasion resulting in inflammatory changes. In such a substrate F. nodosus cells would be exposed to phagocytic and other destructive influences so that antigenic material might be carried to antibody-forming lymphoid tissue with a resulting brief and mild antibody response.

In vaccination on the other hand, antigen is largely held at the site of injection by both the adjuvant and the host reaction associated with the adjuvant/antigen combination. Under these circumstances circulating lymphocytes have ready access to antigen and may become sensitised by this exposure to a greater degree than might lymphocytes sessile in lymphoid tissue.

After vaccination the antibody level rises significantly; highest in those animals injected with water-in-oil formulations. There is no question that the rise in serum titre provides a convenient index of the degree of response to vaccination by the host. Furthermore, as circulating serum gamma globulin has been shown to diffuse to the site of F. nodosus infection (Commonwealth Scientific and Industrial Research Organisation, 1967-68), it seems likely that antibody and complement acting together would be directly damaging to F. nodosus organisms in the epidermis as suggested by Egerton and Merritt (1970).

To account for the significant protection against infection afforded by vaccination, it is postulated that injection(s) of adjuvant/antigen combinations cause a joint response that may change the relative importance of the two types of immunity. Whereas natural infection does cause both some degree of antibody production and a barely discernible sensitisation of circulating lymphocytes (Chapter X), vaccination may cause a substantial stimulation of cell-mediated immunity. Such a stimulation of both forms of immunity could provide for a formidable complementary effect.

Infection of an avascular site as occurs in foot-rot, would mitigate against contact between F. nodosus and antibody under normal circumstances, but the waves of F. necrophorus invasiveness produce inflammatory reactions and the resulting exudate offers a means of diffusion between capillaries and the epidermal matrix. Cell-mediated immunity is less dependent on passive means of access and sensitised cells would be exposed to F. nodosus

organisms during their movement through the epidermis. At times of F. necrophorus invasiveness, cell-mediated immunity would be markedly stimulated because immunologically committed lymphocytes migrate from the circulation into inflammatory exudates (Koster, McGregor and Mackaness, 1971). In connection with this suggested mode of protection, it would be interesting to examine by light and electron microscopy serial biopsy specimens of the interdigital skin of both control and vaccinated sheep for several weeks after attempted infection. Such a study would provide information on the prevalence of both normal and degenerating F. nodosus organisms, and both the degree and type of cellular response associated with the causative and other organisms.

The present work has gone some way to define the occurrence and role of cell-associated antigens that may be responsible for the undoubted effectiveness of vaccines. Although the basic mechanisms by which immunity is mediated remain unresolved, a tentative suggestion is offered to account for the beneficial effects of vaccination and ultrastructural studies are recommended to provide information on the effect of immunity at the site of infection.

SUMMARY

Foot-rot is a disease in which the primary transmitting organism Fusiformis nodosus is restricted to a superficial and avascular part of the foot. The relative isolation of the infected site from humoral influences mitigates against provocation of an immune response and in fact exposure to natural infection does not cause a sheep to be more resistant to subsequent re-infection. The announcement in 1970 that a foot-rot vaccine had been developed which was effective in both prophylactic and therapeutic roles, aroused widespread interest for two reasons. Firstly, vaccination promised a more efficient means of controlling field infection and secondly there was the academic interest in the mode of action of such a vaccine.

The work reported here had three major objectives. Firstly it was considered important to define those factors associated with the culture of F. nodosus that might influence the antigenicity of the organism. This study was undertaken in anticipation of the scaling-up process from the existing experimental techniques to the methods of culture required for the commercial manufacture of vaccine on a large scale. Secondly, an adjuvant/antigen combination was sought that would afford the maximum degree of protection without producing an unacceptable degree of carcass or hide damage at the injection site. Thirdly, the investigation aimed to select one or more in vitro tests that because of a positive correlation with sheep protection, might be used for vaccine evaluation as an alternative to sheep challenge experiments. It was hoped that study in this area would add to an understanding of the mechanism of immunity as applied to foot-rot infection.

Studies of F. nodosus preparations by light and electron microscopy showed that although colony form and bacterial morphology were inherent characteristics of a particular strain, they could be influenced either by factors in serum or by constituents of the culture medium. A conscious selection of rough colonies of the organism was always applied after it had been shown that exposure of organisms to immune serum induced a change from the rough to the smooth type colony.

Pili were observed on F. nodosus cells cultured on hoof agar as well as on naturally occurring cells but they were absent from preparations in which the cells had been grown in biphasic medium. As cells from biphasic medium had been shown to be as protective in vaccines as cells grown on hoof agar, pili were not considered a necessary antigenic component of trial vaccines. Antigenic structure, as assessed by tube agglutination, was also determined by the culture method and depended partly upon the genotypic characteristics of the seed strain.

In addition to protease found in the culture supernatant F. nodosus

cells carry both heat-labile "K" agglutinogens and heat-stable "O" agglutinogens. Although "K" antigens were easily dislodged, treatment by formalin or mild heating tended to stabilise them whereas the "O" antigens withstood vigorous washing and boiling procedures. The presence of a clear zone around the organisms and the existence of easily dislodged superficial antigens were suggestive of a capsule or slime layer, but no such structure was demonstrated by standard techniques. The clear zone seen around the organisms by light microscopy was attributed to the pili or light refraction. Culture of the organism in either hoof broth or GC broth resulted in cells that were not agglutinated by homologous antiserum but the same cells were both antigenic and immunogenic after incorporation with adjuvant. This suggested the existence of some layer that shielded the "K" antigens in antiserum but which was penetrated by elements of the defence mechanism in the sheep. One virulent field strain of F. nodosus possessed agglutinogenic determinants not shared by some other strains and this might partly account for its ability to afford protection against homologous and heterologous challenge.

Studies of the antigenic structure of cells showed that there were three classes of antigens that could be demonstrated. Firstly, protease occurred in culture supernatants and was associated with cells grown in liquid culture. The identity of the antigen was confirmed using double diffusion in agar but titrations were also made possible by utilising the antiproteolytic factors present in either normal or immune sera. Secondly, the superficial "K" antigens caused agglutination of cells in antisera and when cells grown on hoof agar were involved in this reaction there was an initial "flocculation" that may have been due to the pili. Reactions involving "K" antigens were also apparent by immunoprecipitation; in particular the transfer of maternal antibody to the lamb was demonstrated using double diffusion in agar. Thirdly the "O" antigens, firmly attached to the cell and stable to boiling, caused agglutination of F. nodosus cells in immune sera and like the "K" antigens, they caused immunoprecipitates in doubled diffusion in agar tests. Transfer of maternal antibody was also demonstrated using "O" antigens; most anti-"O" activity was present in the IgG fraction of serum.

Thus F. nodosus strains were shown to possess an antigenic structure closely resembling that of the Enterobacteriaceae. The "K" antigens of one strain caused "O" inagglutinability, a property thought to be associated with virulence. Certain "K" antigens are believed to be inherently toxic to the host and their cross-relationships between strains may be an indication of the ability to produce cross-protection; an important point for

vaccine production.

At this stage, a virulent field strain had been selected and its antigenic structure had been retained by appropriate culture techniques. The definitive test was to establish whether or not the antigens were immunogenic and accordingly F. nodosus cells were incorporated with an adjuvant for potency testing. Three different adjuvant systems were used in combinations with F. nodosus cells derived from different methods of culture. Water-in-oil adjuvant vaccines caused an unacceptably severe local reaction in New Zealand-type Romney sheep although certain formulations of this kind, namely the McMaster formulation, afforded consistent sheep protection of a high order. Other water-in-oil formulations although equally irritant locally, afforded an insignificant degree of protection. The majority of reactions caused by alum precipitated vaccines were acceptable and formulations of this kind usually provided sheep protection of the same order as that afforded by the best water-in-oil adjuvant vaccines. Aluminium hydroxide adjuvant vaccines produced a minimal local reaction but the degree of protection afforded was insufficient.

Serological tests confirmed the existence of three distinct classes of antigen associated with F. nodosus cultures. The humoral response was closely linked with the adjuvant system used in the vaccine: water-in-oil vaccines always produced the greatest response as determined by four types of serological titration. The response to protease was generally of a low order and insignificant in the case of some vaccines. Agglutination titres against the "K" antigens were of a high order and while some reactions were shown to be strain specific, most were common to all strains tested. Responses by sheep to the "O" antigens were minimal and in the case of the less irritant adjuvant formulations, insignificant.

The specific bacterial growth-inhibitory activity of normal serum was considerable and was shown to be dependant upon a heat-labile factor. The original level of activity could be restored to heated serum by the provision of a factor occurring in precolostral lamb serum. Vaccination of normal ewes did not increase the growth-inhibitory titre but there was a qualitative change in that activity after vaccination was stable to heating but still could be absorbed with F. nodosus cells.

None of the serological test results were correlated with immunity of the serum donor but certain threshold levels were usually indicative of sheep protection. It was found that extreme cases of "resistant" or "susceptible" sheep provided sera that reacted quite differently in double diffusion in agar tests. When reacted against an extract of boiled F. nodosus cells, sera from "resistant" sheep produced precipitates that were prominent

whereas sera from "susceptible" sheep produced either no lines or they were faint.

No single serological test was found that could be used to replace sheep challenge experiments as a means of vaccine evaluation but the combined results of several *in vitro* tests provide a useful indication of the vaccine potency and should be used as a means of maintaining uniform quality.

Delayed-type hypersensitivity to *F. nodosus* antigens was demonstrated in sheep with foot-rot infection and this was taken to indicate the existence of cell-mediated immunity. Although both humoral and cell-mediated immunities are stimulated in natural infections of foot-rot, the combined response seems insufficient to provide resistance to reinfection. Nevertheless vaccination of sheep with an appropriate adjuvant/antigen combination affords significant protection against both the form of *F. nodosus* infection encountered in the field and a severe experimental challenge.

Though the mechanism of protection afforded by vaccination remains unexplained, a complementary role for antibody-mediated and cell-mediated immunity has been postulated.

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APPENDIX I

MAINTENANCE OF ANAEROBIC CONDITIONS

The systems developed by the writer were based upon principles laid down by Hungate (1969) and Barnes (1969).

Two gas mixtures were used routinely for preparing media, covering inoculated media and flushing head spaces in containers during culture transfers.

(i) A mixture of 90 per cent. H_2, 10 per cent. CO_2 (N_2/CO_2) was used to replace the vacuum in anaerobic jars for culture of agar plates, and also for reducing the copper column (see below) when necessary.

(ii) A mixture of 90 per cent. oxygen-free N_2, 10 per cent CO_2 (N_2/CO_2) was used at all other times. To remove any residual oxygen from the gas lines and bladders, or traces in the gas mixture itself, the complete apparatus (Figure App. I.1) was well flushed out before use each time, as soon as the heated copper column (Moore, 1966) had reached its operating temperature of 350°C. (Figure App. I.2)

Liquid media were stored in the reduced state under N_2/CO_2 and re-autoclaved directly before use under the same gas in sealed containers. Black butyl rubber bungs Size 00 (Smith-Biolab, Auckland, N.Z.) were used for roller culture tubes No. 1801 (Bellco, New Jersey, U.S.A.) and for the 55 ml vaccine container (Glaxo, Palmerston North, N.Z.). Black butyl rubber washers were used to seal the 600 ml container rim inside the three-way distributor head. (Figure App. I.3)

To keep rubber bungs on during autoclaving, wire holders (Moore, 1966) were used on individual roller culture tubes, and screw-down clamp frames for a series of the same tubes or for the 55 ml vaccine containers (Figures App. I.4 and App. I.5).

The gas line outlets consisted of a Luer-Lok fitting, 2 ml hypodermic glass syringe plugged with sterile cotton wool (Figure App. I.1). To the end could be fitted an 18 gauge (1.1 mm) 150 mm straight hypodermic needle or any of a variety of specially shaped wider bore needles that had been designed for gassing during particular transfer manipulations. The needle was sterilised in a bunsen flame immediately before each transfer was made.

FIGURE APP. I.1

Gassing apparatus Gas entered at lower left into the heated copper column (A). This was insulated with fibreglass wadding and asbestos rope. The operating temperature of 350-370°C was maintained by an Electrothermal heat regulator (C).

Gas left the column top left, passed through the bladder (B) and by a series of glass stop cocks could be directed to outlets (D) consisting of 2 ml hypodermic syringes plugged with sterile cotton wool. A variety of hypodermic needles could be attached to the syringes for gassing during specific manipulations.

The main gas line passes out of picture top right. It was used to carry H_2/CO_2 into the open air during reduction of the copper chips.

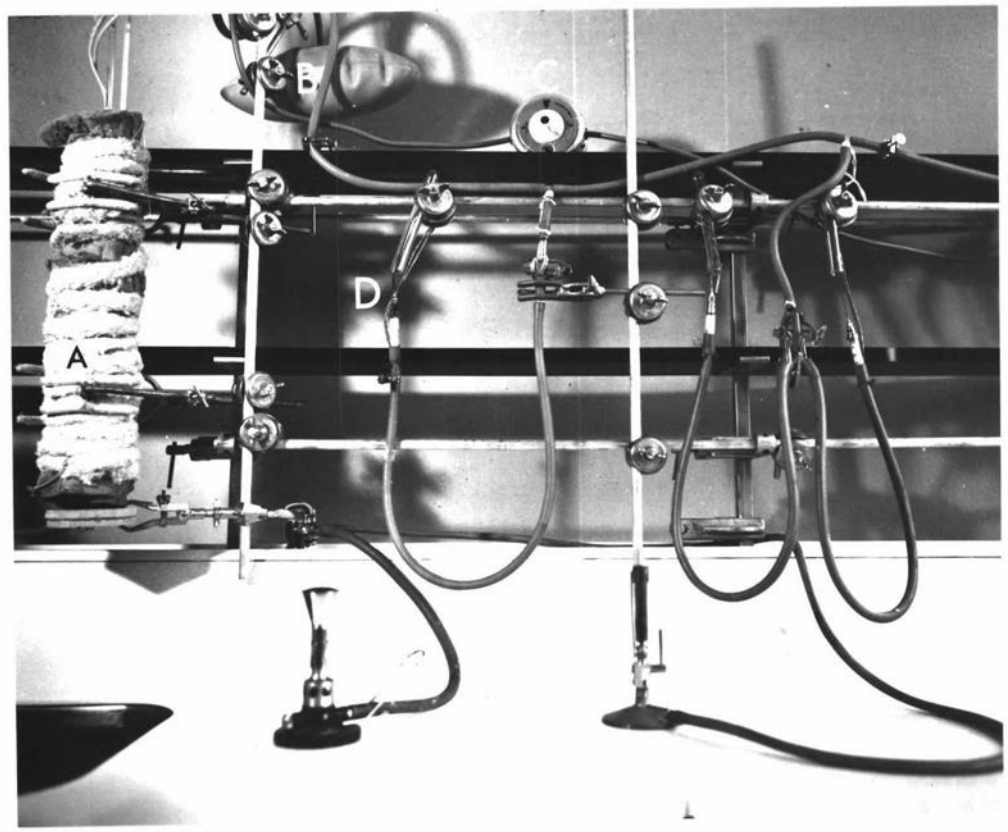

A
B
D

FIGURE APP. I.2

Part-stripped heated copper column showing inner glass column containing copper chips with thermometer alongside and surrounded by an Electrothermal heating tape.

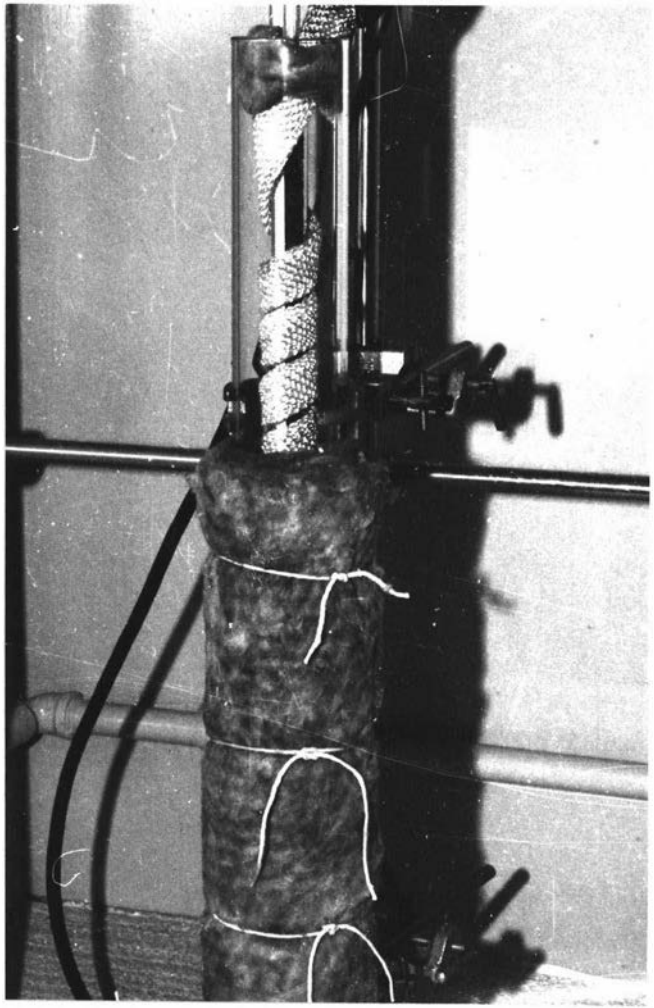
The inner and outer Pyrex glass columns were packed with fibreglass wadding.

FIGURE APP. I.3

Six hundred ml container with threaded three-way distributor head (B) sealed with butyl rubber washer.

The hooded pipette (A) connects directly with the glass tube going to the bottom of the container.

The gas filter (C) connects with the head space of the container so that N_2/CO_2 could be used to drive broth out through the pipette (A).



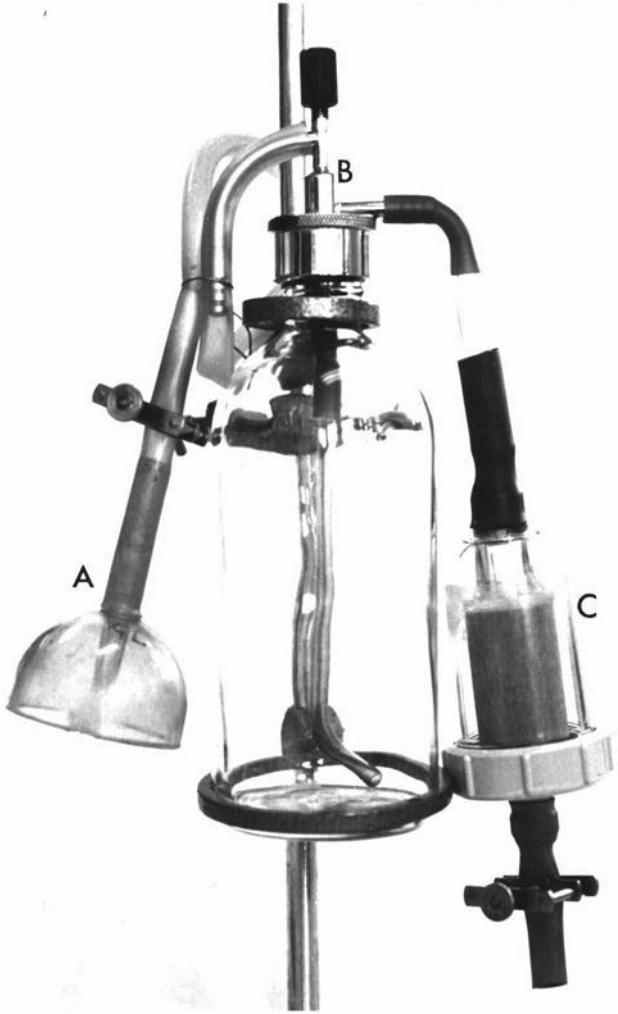

A
B
C

FIGURE APP. I.4

Screw down clamp for autoclaving roller culture tubes with black butyl bungs in place.

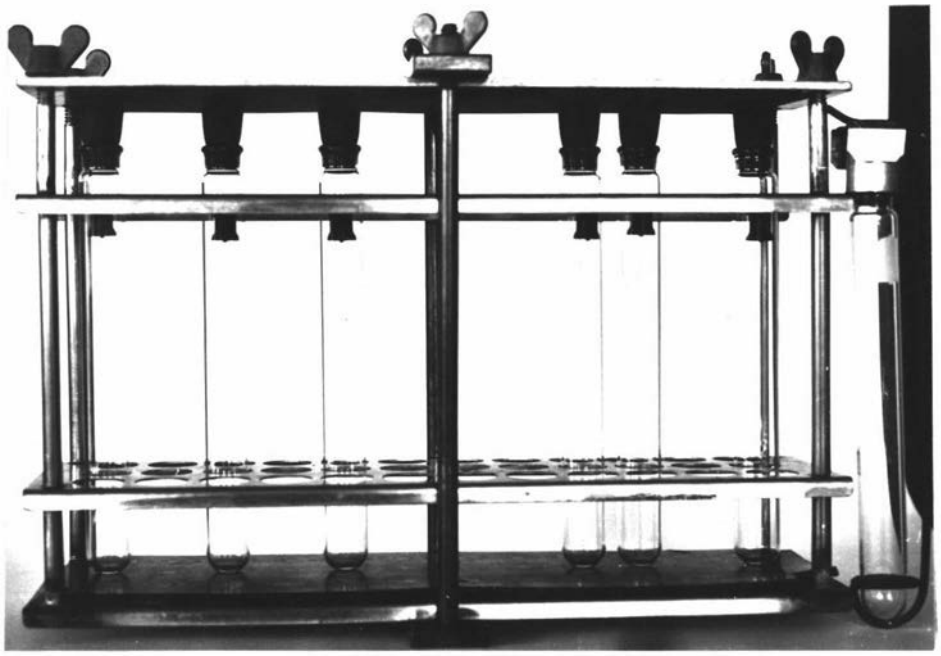
On the right a single roller culture tube is held in a wire spring with aluminium cap covering the bung.

FIGURE APP. I.5

Screw down clamp used for autoclaving up to twelve 55 ml vaccine containers.

Black butyl bungs are hidden by the aluminium caps.

Biphasic medium was routinely prepared using this apparatus for the agar slants, and the 600 ml container (Figure App. I.3) to distribute the broth.



APPENDIX II

METHOD OF CULTURE EMPLOYED FOR PRIMARY ISOLATION OF FUSIFORMIS NODOSUS

Glass petri dishes containing hoof agar were used freshly prepared, or after holding in the reduced state in an anaerobic jar for up to 48 hr. The plates were dried in a hot air oven at 70°C for 2-4 min. only and inoculated as soon as possible afterwards. The original swab or loopful of Stuart's transport medium surrounding the swab, or loopful of sucrose solution (Merritt, 1960) was plated out over four or five areas of the plate, flaming the loop between each location. Sometimes a second plate was inoculated as a continuing dilution series from the original material. Sterile filter papers were inserted in the dish lids (Thomas, 1958) before placing the plates in the anaerobic jar, agar uppermost. Approximately 20g of calcium chloride crystals in a Universal bottle were enclosed in each anaerobic jar. The filter papers and calcium chloride were included to absorb surplus moisture that might aid spreading organisms. Either Baird and Tatlock (Chadwell Heath, U.K.) cold catalyst jars, or the Gas Pak Anaerobic System (Bio Quest, Maryland, U.S.A.) were used. The jars were filled with 90 per cent H_2/10 per cent CO_2 either by three air evacuations to 650 mm Hg and replacement from a bladder for the B & T jar, or by using the gas-liberating sachets for the Gas Pak system. Incubation was carried out at 37°C for four to five days after which time the jars were opened, the calcium chloride discarded and the catalyst renewed. Plates were examined by oblique lighting at a magnification of x 10 on a Bausch and Lomb dissecting microscope with a black platform. Colonies of F. nodosus were usually in contact with miscellaneous contaminants. They were recognised by a distinctive "moat" appearance due to etching of the agar surface around the colony periphery. The centre portion was usually raised, and mucoid in appearance. Colonies of promising appearance were subcultured on to fresh plates of horn agar and incubated anaerobically for four days. Isolates in pure culture were confirmed as F. nodosus by the appearance of the colony, stained smear examination and failure to grow aerobically. The colony and individual bacteria appearances on hoof agar conformed to the description given by Beveridge (1938a, 1941) and in the Annual Report of the N.Z. Department Agriculture Research Division (New Zealand Ministry of Agriculture and Fisheries, 1968-69).

APPENDIX IIa

HOOF AGAR

This culture method was based on the modification by Roberts and Egerton (1969), of the original formulation of Thomas (1958).

Hoof powder Feet were obtained from sheep at slaughter, washed in tap water and the hoof "slipper" removed by a 15-30 second immersion in water kept near boiling. While it was still soft, the hoof was cut up into strips about 0.5 cm wide. These were thoroughly washed in distilled water and then dried on trays at 37°C for 2-3 days. The strips were run two or three times through a roller mill using a fine sieve and the resulting hoof powder was stored in tins at room temperature.

Formula	g/100 ml
Dextrose	0.15
Yeast extract ([1]Oxoid)	0.2
Proteose peptone ([2]Difco or Oxoid)	1.0
'Lab-Lemco' meat extract (Oxoid)	0.5
Sodium chloride	0.5
Distilled water	
pH adjusted with 4N NaOH to 8.1	
Hoof powder	1-2.0
Agar (Davis, Christchurch, N.Z.)	1.6
(final pH about 7.6)	

The mixture was boiled, the head space of the flask filled with a N_2/CO_2 mixture and 100 ml amounts dispensed into 'Penrose oval' bottles and sealed under the same gas.

Sterilisation Autoclaved at 121°C for 20 min.

Storage Refrigerated at 4°C.

Use The agar was liquefied in a boiling water bath and held at 56°C ready for pouring. Twenty to 25 ml agar were poured into each petri dish of 9.5 cm diameter, and hoof particles were suspended as evenly as possible during the process. After setting, the plates were dried at 70°C in a hot air oven for 2-5 minutes and either inoculated immediately or held in the reduced state in an anaerobic jar at room temperature for up to one week.

1 Oxoid, London.

2 Difco, Detroit, U.S.A.

APPENDIX IIb

HOOF BROTH

This culture method was based on the modification by Roberts and Egerton (1969), of the original formulations of Thomas (1958, 1963).

Formula	g/100 ml
Yeast extract (Difco or Oxoid)	0.2
Proteose peptone (Difco or Oxoid)	1.0
'Lab-Lemco' meat extract (Oxoid)	0.5
Sodium chloride	0.5
Distilled water	
pH adjusted with 4N NaOH to 8.1	
Hoof powder	1-2.0
Trypsin (1:250, Difco)	1.0
Autoclaved at 121°C for 20 minutes	
Hoof filtered off and pH adjusted to 7.4	

The broth was boiled, distributed and sealed under a N_2/CO_2 mixture in 100 ml amounts.

Final sterilisation was carried out by autoclaving at 110°C for 15 minutes.

Storage Refrigerated at 4°C.

Use Immediately before use the broth was boiled and the head space of the flask filled with a N_2/CO_2 mixture. Dextrose solution and sodium thioglycollate solution were added to a final concentration of 0.5 per cent. and 0.1 per cent. respectively. Distribution under gas into the final containers was preceded by a final autoclaving at 110°C for 20 minutes.

For biphasic medium (Appendix IIc), the final autoclaving was carried out in bulk and the broth in a reduced state, was distributed over the slants using an aseptic technique. (Figures App. I.1, App. I.2 and App. I.5).

APPENDIX IIc

HOOF BROTH OVER HOOF AGAR - BIPHASIC MEDIUM

This culture method was a modification of the original, described by Egerton and Burrell (1970).

Hoof agar was liquefied by boiling and distributed under a N_2/CO_2 mixture as a 10 ml slant in 55 ml round vaccine bottles. The bottles were closed with bored out black butyl bungs size 00 and placed in a screw down clamp for autoclaving at 121°C for 20 min. (Figure App. I.5). After cooling at the appropriate angle, the agar slants were removed from the clamp and used immediately or were stored for up to 14 days at 4°C. At the time of use, the bung was removed, the vaccine bottle head space flushed out with fresh N_2/CO_2 mixture, and 40 ml of pre-reduced hoof broth added under the same gas. (Figure App. I.3). The butyl bung was replaced and the biphasic medium placed at 37°C until inoculation. Two to 4 ml of culture was injected through the bung using a pregassed disposable syringe fitted with a 22 gauge (0.65 mm) x 25 mm hypodermic needle.

An account of harvesting the biphasic culture after 24-48 hr is included in Appendix XI.

APPENDIX IId

GC BROTH

This broth was developed for large scale vaccine production as an alternative to growing F. nodosus in biphasic medium (Egerton and Burrell, 1970) or on hoof agar (Thomas, 1958). Basic ingredients of the medium were the same as Thomas (1963) used for his liquid medium, but to those essentials were added enzymatic digests of other proteins and another peptone. The latter had been selected because of significant F. nodosus growth enhancement during previous screening tests for suitable nitrogen sources.

GC broth was sterilised by autoclaving at 121°C for 15 min. and was stored in 50 ml amounts under a N_2/CO_2 mixture, at room temperature.

APPENDIX III

STRAINS OF FUSIFORMIS NODOSUS

1. Strain W13, Wallaceville Animal Research Station, New Zealand, by courtesy of Dr M. Skerman, was successfully reconstituted from the freeze-dried state but was subsequently lost during subculturing. At this stage in the writer's study, the essential culturing requirements were poorly understood.

2. Strains McM198 and McM199, McMaster Institute, Sydney, by courtesy of Mr A. Webster and Mr J. Egerton. Both strains were successfully reconstituted from the freeze-dried state and a bulk of freeze-dried seed prepared from each in two passages.

3. Strain A8/C, Ashburton, New Zealand, was isolated by Mr R. Lynch at Glaxo Laboratories, Palmerston North from necrotic sheep hoof material transported in Stuart's transport medium at 4°C. One strain was isolated and a bulk of freeze-dried seed was prepared in three passages.

4. Strains R7/E, R7/N, R7/P, R7/W, and R7/Y, Riversdale, New Zealand, were isolated by the writer at Glaxo Laboratories, Palmerston North, from necrotic sheep hoof material transported in Stuart's transport medium for 24 hr at 4°C. Five strains were isolated and a bulk of freeze-dried seed was prepared from each in two passages.

5. Strains M9/4, M9/6 and M9/7, Massey University, New Zealand, were isolated by the writer in the Animal Health Department from interdigital necrotic detritus on swabs held in Stuart's transport medium at 4°C for 24 hr before plating out. Three strains were isolated and a bulk of freeze-dried seed was prepared from each in four passages. The infected animal, a five year old Romney ram, showed severe interdigital skin necrosis of all four feet and this condition persisted as such, without horn involvement, despite topical and parenteral treatment. Smears of the detritus taken over a period of 14 months always contained large numbers of typical

F. nodosus-like organisms. During a three month period during which this ram was kept on wet, faecally contaminated bedding, the clinical condition remained unchanged but a milder type of interdigital dermatitis developed in a susceptible sheep kept in the same pen. Smears of these new lesions were suggestive of foot-rot but were not fully confirmed by the appearance of typical F. nodosus-like organisms. The donor ram infection was diagnosed as non-progressive or benign foot-rot (Thomas, 1962a; Egerton and Parsonson, 1969; Morgan, Piercy and Egerton, 1972) on the basis of a persisting interdigital dermatitis that did not include horn separation, but from which F. nodosus was isolated and seen repeatedly in smears. The isolate F. nodosus (M9/4), was characteristically low in proteolytic activity.

6. Strains cbM1/B, cbM1/C, cbM1/G, and cbM1/H, Massey University, New Zealand, were isolated by the writer in the Animal Health Department from a yearling Jersey heifer that showed severe interdigital dermatitis and necrosis beneath the horn of all four feet. Swabs of the detritus and material beneath necrotic horn were transferred immediately to Stuart's transport medium and held at $4^{o}C$ for up to four hours before plating out on hoof agar containing ground sheep hoof. Four strains were isolated and a bulk of freeze-dried seed was prepared from each in four passages.

7. Strains J3/13, J3/16, J3/20, and J3/22, "Jennersmead", Bunnythorpe, New Zealand, were isolated by the writer at Glaxo Laboratories, Palmerston North from sheep artificially infected in pens by swab transfer from naturally occurring field cases of foot-rot. The resulting infection was a severe and progressive one, eventually causing shedding of the complete horn 'slipper'. Necrotic material collected from the deepest parts beneath separating horn was either used as inoculum direct, after thorough mixing with 0.25M sucrose (Merritt, 1960); or, after rubbing on a slightly moistened swab, transferred into Stuart's transport medium and held at $4^{o}C$ for up to four hours. Four strains were isolated and a bulk of freeze-dried seed was prepared from each in two passages. These vials of J3/22 were designated "grand master seeds", from which "master seeds" were produced in two more passages. A large bulk of "working seeds" was produced after a further two passages and held in the freeze-dried state at $-20^{o}C$, viz. "working seeds" were coded J3/22/6, signifying six passages. (Appendix IIIa).

APPENDIX IIIa

CODING OF FUSIFORMIS NODOSUS STRAINS

An abbreviated code was designed for security and brevity purposes and so that for each F. nodosus strain at any passage, the following details were identifiable,

(i) Geographical area of isolation.

e.g. M = Massey, R = Riversdale, J = 'Jennersmead',
cbM = cattle beast, Massey.

(ii) Number of attempted isolation experiment. Detailed records of the techniques used for each attempt were kept and from these accounts, the most successful methods were retained for future use.

e.g. J3 = third attempted isolation experiment,
M9 = ninth attempted isolation experiment.

(iii) By number or letter, the particular strain(s) cultured from any one particular isolation attempt. Each strain was derived from a different colony picked off the primary isolation plate.

e.g. A8/C = only one isolation made from attempt A8 and that strain designated "C".

J3/13)
J3/16) four strains were isolated from attempt J3.
J3/20)
J3/22)

(iv) The last number of the code always referred to the number of passages the culture had undergone since isolation.

e.g. J3/22/6 = J3/22 at working seed stage, R7/p/2 = R7/p at master seed stage.

APPENDIX IV

FREEZE-DRYING

To prepare a bank of uniform working seed material, pure cultures of F. nodosus were heavily seeded on to briefly dried hoof agar plates, and incubated at 37°C for four days in anaerobic jars containing a H_2/CO_2 mixture. The cultures were harvested by flooding each plate with 0.5-1 ml of freeze-drying medium[1] and drawing across the surface of the agar a stainless steel blunt scraper that dislodged the colonies into the fluid.

The concentrated bacterial cell suspension was distributed in amounts of 0.1 ml - 0.25 ml into 2 ml vials and immediately placed at -20°C. Freeze-drying was carried out in an Edwards PIT machine using the following schedule,

(1) The shelves were pre-cooled to -20°C, loaded, and the product held at this temperature overnight (approx. 18 hr).

(2) Full vacuum was applied for 24 hr and then the freezing unit was turned off.

(3) The shelf temperature was allowed to rise to room temperature over the next 24 hr.

(4) The vacuum was turned off, West seals applied and the vacuum replaced to a pressure of approximately 0.1 mm Hg.

(5) The vials were permanently sealed with a crimped aluminium ring.

(6) All the vials were checked for vacuum using an Edwards High Frequency Tester, before storage at -20°C or at 4°C, and before reconstitution.

Reconstitution. One ml of distilled water was added to the vial with a disposable syringe and the remaining vacuum released. With gentle agitation re-suspension of cell material occurred almost immediately. Hoof agar plates were inoculated by spreading out 1-3 drops of the cell suspension over four areas, the loop being flamed between each. The plates were incubated in an anaerobic jar for four days and the appropriate type of F. nodosus colony selected for further culture.

[1] Freeze-drying medium

Glaxo "1010" Virus F.D. Medium	80%
Horse serum (56°C/30 min.)	20%

APPENDIX V

PREPARATION OF CELLS FOR ELECTRON MICROSCOPY

Three different fixation methods were used:

METHOD I Double Fixation (after Karnovsky, 1965)

(i) Cells deposited as a pellet by centrifugation.

(ii) Pellet fixed in half strength Karnovsky fixative consisting of

2% formaldehyde
3% glutaraldehyde
0.1M phosphate buffer pH 7.2

for 2 hr at 4°C.

(iii) Washed twice in fresh buffer 15 min. each, at 4°C.

(iv) Post-fixation in 1 per cent. osmium tetroxide in phosphate buffer pH 7.2 for 1 hr at 4°C.

(v) Washed twice in fresh buffer 15 min. each, at 4°C.

Further steps carried out at room temperature.

(vi) Dehydration in graded alcohol series namely,

25% ethanol 30 min.
50% ethanol 30 min.
75% ethanol overnight
95% ethanol 30 min.
100% ethanol 30 min.
100% ethanol 30 min.

(vii) Infiltration of dehydrated pellet using

propylene oxide 100% 10 min., repeated once.

(viii) Introduction of epoxy resin "Durcupan-ACM" (Fluka AG, Buchs SG, Switzerland) in

propylene oxide	25%	1 hr
	50%	1 hr
	75%	overnight
	100%	6 hr.

(ix) Pellet embedded in fresh 100 per cent. epoxy resin in size 4 gelatin capsules for 48 hr. at 60°C.

METHOD II Double Fixation (Williams and Luft, 1968), - (TAPO)

(i) Cells deposited as a pellet by centrifugation.

(ii) Pellet fixed in following, made up 2 hr. previously,

1.2% glutaraldehyde
1% Tris. (1 - aziridinyl) phosphine oxide
0.1M phosphate buffer pH 7.0

for 20 min. at room temperature.

Proceeded as from step (iii) in METHOD I.

METHOD III Ryter and Kellenberger Fixation (Ryter and Kellenberger, 1958)

(i) Cells in culture were fixed in situ, or surface growth was scraped direct into the Kellenberger fixative. The method described by Glauert (1965) using Kellenberger's fixative, Kellenberger's buffer, tryptone medium and the uranyl acetate washing fluid was followed, except that agar embedding was found unnecessary because the pellet stayed intact.

(ii) Proceeded from the uranyl acetate wash to step (vi), in METHOD I.

Thereafter sections were cut on the L.K.B. "Ultrotome" and picked up on carbon coated formvar support films on 200 mesh copper grids. The preparations were stained for 10 min. with lead citrate (Reynolds, 1963) and examined in a Philips EM200 electron microscope.

APPENDIX VI

PREPARATION OF BACTERIAL CELL EXTRACTS

1. Crude cell extract Unwashed F. nodosus cells suspended in PBS at a known concentration were subjected to 2 runs through a precooled French press (Wabash Hydraulic Press, Indiana, U.S.A.) at a pressure of 5,000-7,000 lb/sq.in. ($\simeq$18,000 kg/sq.cm). This is equivalent to 422 technical atmospheres (at) where at = 736 mm Hg. The suspension cleared considerably and Gram stained preparations showed largely a pink lacey network containing few intact F. nodosus cells. The extract was centrifuged in a Spinco Ultra centrifuge (Beckman, Palo Alto, U.S.A.) at 51,000 x g. for 30 min. to leave a clear supernatant above a small grey-green plug. Protein estimation was based on the absorbancy of the cell extract at 280 nm in a Unicam sp500 spectrophotometer. As a positive control, sheep gamma-globulin 0.5 mg/ml (Fraction II 71-879 Run ≠ OVI Hyland, Los Angeles, U.S.A.) was run in parallel. Before use in double diffusion in agar tests, the cell extract was concentrated in volume tenfold by dialysis against polyethylene glycol. Cell extracts were stored frozen at -20°C.

2. Boiled crude cell extract The unwashed bacterial cell suspension was placed in a boiling water bath for 90-120 min. before French press treatment.

3. Washed boiled cell extract The bacterial cell suspension was washed three times in PBS and the final suspension of cells placed in a boiling water bath as above. The suspension was washed again three times before French press treatment.

4. Washed cell extract The bacterial cell suspension was washed three times in PBS before French press treatment.

5. Washings The washings from the washed cell extract first step were reduced back to the original volume by dialysis against polyethylene glycol. This material was centrifuged for 30 min. at 51,000 x g. before an estimation of protein concentration was carried out.

APPENDIX VII

ULTRA-SONIC DISRUPTION OF CELLS

F. nodosus cell suspensions in distilled water or PBS were transferred to a special glass container and capped. This container surrounded by an ice bath was fixed into a 100 watt Ultrasonic Disintegrator (Measuring and Scientific Equipment Ltd., London) bearing a 10 mm probe. The machine was set on auto and gave peak pitch at 8 microns amplitude. Samples of the cell suspension were examined at 5, 10 and 20 min. after starting sonication. There was little or no change in the appearance of cells. After 20 min. there was heating of the suspension to about 60°C in spite of the ice bath.

The failure of this technique to disintegrate F. nodosus cells in spite of lengthy sonication, and the undesirable heating of the cell suspension, caused an investigation of French press treatment. This proved successful as a means of cellular disruption (Appendix VI) and became the standard method used thereafter.

APPENDIX VIII

ADJUVANT SYSTEMS

1. Aluminium hydroxide gel "Vaxogel" (Evans, Speke, U.K.) Thirty per cent. in PBS left to adsorb at room temperature for 72 hr.

2. Anhydrous oil with aluminium monostearate (Muggleton and Hilton, 1966) The cell suspension was freeze-dried and powdered by shaking with glass beads before adding the gel.

3. Oil in water/aluminium hydroxide gel (Muggleton and Hilton, 1966) Ninety per cent. oil-in-water emulsion with cell suspension alone, or 90 per cent. oil-in-water emulsion with cell suspension adsorbed onto 30 per cent. aluminium hydroxide gel.

4. Potash-alum precipitated Six preparations were made; as 20 per cent., 10 per cent., 2 per cent. and 1 per cent. w/v alum with cell suspension, or 1 per cent. and 2 per cent. alum precipitated whole culture.

5. Water-in-oil (Muggleton and Hilton, 1966) Light paraffin oil was mixed with 3 per cent. "Arlacel 80" and 3 per cent. "Arlacel 83" and heat sterilised. Sterile distilled water 30 per cent. was emulsified with 70 per cent. of the oil/"Arlacels" mixture. The resulting water-in-oil emulsion was added to a wet cake of F. nodosus cells.

6. Organic polymer with aluminium hydroxide F. nodosus cells were incorporated with the organic polymer and the resulting mixture adsorbed with aluminium hydroxide gel.

7. McMaster formulation (Egerton and Burrell, 1970) F. nodosus cells were emulsified with Shell Ondina oil six parts, wool wax one part.

8. Double emulsion (Herbert, 1967) The saline continuous phase contained a water-in-oil emulsion containing the antigen.

ASSESSMENT OF LOCAL REACTIONS

Local reactions were assessed according to four main criteria and subgraded for recording as follows:-

1. Size
   (a) Length and width measured in cm.
   (b) Discrete, diffuse or loculated.
2. Consistency
   Soft, tense, firm, hard or very hard.
3. Depth
   Flat, slightly raised, raised, or markedly raised.

4. Serious changes

(a) Inflamed skin

(b) Necrosis (coldness, pallor, etc)

(c) Abscessation

(d) Extensive oedematous swelling.

On the basis of these recordings each site was graded as

(a) Acceptable) No reaction
) Slight reaction

(b) Marginally acceptable

or (c) Unacceptable

Judgment of Adjuvant Systems for Local Reaction

1.	Aluminium hydroxide gel alone	Acceptable
	Aluminium hydroxide gel with cells	Acceptable
2.	Anhydrous oil with aluminium monostearate alone	Acceptable
	Anhydrous oil with aluminium monostearate with cells	Unacceptable
3.	Oil-in-water emulsion alone	Acceptable
	Oil-in-water emulsion with cells	Marginally acceptable
	Oil-in-water emulsion with aluminium hydroxide gel with cells	Marginally acceptable
4.	Potash-alum 10 per cent. with cells)	
	Potash-alum 20 per cent. with cells)	Unacceptable
	Potash-alum 1 per cent. precipitated whole culture	Acceptable
	Potash-alum 2 per cent. precipitated whole culture	Marginally acceptable
5.	Water-in-oil with cells	Marginally acceptable
6.	Organic polymer with aluminium hydroxide with cells	Acceptable
7.	McMaster formulation, alone	Acceptable
	McMaster formulation, with cells	Unacceptable
8.	Double emulsion with cells	Unacceptable

APPENDIX IX

ASSESSMENT OF FOOT-ROT LESIONS

			Score
Interdigital Space		Mild inflammation	1
	or	severe inflammation	2
		Mild ulceration	1
	or	severe ulceration	2
Skin/horn Junction for each digit		Swelling	1
Hoof Separation for each digit	and	Heel	1
	and	Sole	1
	and	Toe	1
	and	extending to abaxial wall	0.25
		Possible score per foot	12.5
		Possible score per sheep	50

"Susceptibility score" was defined as the mean of assessment scores on post-challenge weeks 3, 4 and 5. This figure could be applied to an individual sheep or to a treatment group.

APPENDIX IXa

ASSESSMENT SCORE RECORDING SHEET

R.D.13 Experiment

Sheep No. Vaccine

Date of Challenge Challenge Material

Stage of Feet at Challenge Feet Challenged

Date:	Weeks Post Challenge:									
Site :		LF		RF		LH		RH		Total	Comments
Inter Digital Space :	Inflammation										
	Ulceration										
		L	M	L	M	L	M	L	M		
Skin Horn Junction:	Swelling										
Horn:	Separation: Heel										
	Sole										
	Toe										
	Extending to Wall										
	Totals:										

Date:	Weeks Post Challenge:									
Site:		LF		RF		LH		RH		Total	Comments
Inter Digital Space:	Inflammation										
	Ulceration										
		L	M	L	M	L	M	L	M		
Skin Horn Junction:	Swelling										
Horn:	Separation: Heel										
	Sole										
	Toe										
	Extending to Wall										
	Totals:										

APPENDIX X

ANTISERUM PRODUCTION

Sheep - Romney/Border Leicester cross, mixed-aged ewes.

Antisera in sheep were produced against the following F. nodosus strains,

(a) J3/22/10 cells harvested from biphasic medium — Sheep 1876

(b) McM198/6 cells harvested from biphasic medium — Sheep 583

(c) McM199/6 cells harvested from biphasic medium — Sheep 29

(d) M9/4/8 cells harvested from biphasic medium — Sheep 2029

(e) J3/22/10 cells harvested from biphasic medium, boiled for 90 min. — Sheep 2280

Method for Series (a) to (e)

A volume of cell suspension in 0.5 per cent formalin PBS (FPBS) containing 1.4×10^{10} to 3×10^{10} cells was emulsified with an equal volume of Freund's Incomplete Adjuvant (FIA-Difco, Detroit, U.S.A.) by repeatedly forcing the mixture through a 19 gauge (1 mm) needle adapted to fit a hypodermic syringe either end. Depending on the final volume, up to 7.5 ml was injected intramuscularly deep into the thigh muscles each side. The injection was repeated after one week. After a further two weeks a total dose of 0.15×10^{10} to 2.5×10^{10} cells suspended in PBS was injected subcutaneously. Blood for serum was collected ten days later.

The additional special antisera were made as follows;

Strain F. nodosus	Method of culture	Cell treatment	Adjuvant	Sheep No.
(f) J3/22/10 batch 1	biphasic medium	sonication 12 min.	Aluminium hydroxide adsorbed 3 days	2113
(g) J3/22/10 batch 10	biphasic medium	sonication	Aluminium hydroxide adsorbed 3 days	872
(h) J3/22/10 batch 1	biphasic medium	concentrated CSN	FIA	2016
(i) Incubated but uninoculated hoof broth	biphasic medium	concentrated as for CSN	FIA	888
(j) Formalin 0.5% in phosphate buffered saline pH 7.4			FIA	894
(k) Formalin 0.5% in phosphate buffered saline pH 7.4			McMaster formulation	2152

Method for series (f) to (k)

A volume of 2 ml-9.8 ml was injected subcutaneously in the mid neck region. Each dose contained an estimated 5×10^9 cells or an equivalent amount of supernatant based on a culture yield of 1×10^8 cells/ml. The same dose was repeated on the other side of the neck one week later. After a further three weeks, blood was collected for serum.

APPENDIX XI

AGGLUTINATION TESTS

Serum Sheep were bled from the jugular vein into Vacutainers (Becton-Dickinson, New Jersey, U.S.A.); after which the tubes were held at 37°C for up to 2 hr, and after freezing the clot from the glass, at 4°C overnight. The clot was removed and the serum cleared by centrifugation. The serum was used fresh or after storage at -20°C. Doubling dilutions of serum in PBS or in 0.85 per cent. normal saline were made using an automatic syringe, six mixings at each step, and the same disposable pipette throughout the series.

i) Preparation of gross antigen

Hoof agar culture F. nodosus cells were scraped and washed off hoof agar plates that had been incubated for four days. The suspending medium was PBS or PBS with the addition of 0.5 per cent. formalin (Analar, 40 per cent. formaldehyde), coded FPBS.

Biphasic medium culture F. nodosus cells used as antigen in the agglutination test were more usually obtained from biphasic medium harvested between 24 and 48 hr after inoculation. After checking each 55 ml vial for purity, the broth portions were pooled through a P1 glass sintered filter to remove gross particles. The F. nodosus cells were deposited at 4°C in a centrifuge at 2300 x g. for 40 min. The culture supernatant was removed from the deposited cells and this liquid phase was tested for proteolytic activity (see Appendix XII). The F. nodosus cells were resuspended without washing in a minimal amount of PBS or FPBS. A cell count using the Thoma slide (Hawksley, England), or else the improved Neubauer counting chamber ("Lumicyte"), was carried out and a value ascribed to the cell concentrate. The antigen was used immediately or stored at 4°C for up to ten months. Before use the concentrated cell suspension was diluted in PBS or FPBS to a calculated 5×10^8 cells/ml.

Test The gross agglutination test was carried out in Dryer's agglutination tubes using equal amounts (usually 0.3 to 0.5 ml) of serum dilution, and cell suspension at 5×10^8 F. nodosus/ml. The tubes were individually mixed by inversion twice, briskly shaken and then placed in a water bath at 37°C for 4 hr. One reading was made then, and another after holding the tubes for a further 24 hr at 4°C. The end point was taken as that tube where there was agglutination of cells as a visible deposit at the base and/or on the slopes of the tube, with some distinct clearing of the liquid phase above. Serum dilution titres quoted are the reciprocal of the end point tube final dilution i.e. after addition of antigen. A negative control tube of antigen and PBS, and a positive control series containing antigen with dilutions of standard

antiserum of known titre, were included in each test.

ii) Preparation of heat-treated antigen

F. nodosus cells from hoof agar or GC broth, harvested in PBS, were washed three times and placed in a boiling water bath for one hr. The cells were washed again three times, resuspended in PBS and one sample taken for a direct cell count. Another sample was used for a dilution series in FPBS and these latter suspensions were tested for optical density using opacity tubes (Wellcome, Beckenham, U.K.) and a modified photoelectric colorimeter (Gallenkamp). A value was ascribed to the cell concentrate which was stored at $4^{o}C$. Immediately before use the suspension was diluted with PBS to an estimated 5×10^{8} cells/ml, or approximately equal to the optical density of tube 1. For incubation, the tubes were placed in a water bath at $50^{o}C$ for 24 hr, and then held overnight at $4^{o}C$, but in all other respects the "somatic test" was carried out in the same manner described for the gross agglutination test.

APPENDIX XII

PROTEOLYTIC ACTIVITY TEST

Culture supernatants (CSN) of hoof broth, GC broth, or biphasic medium were drawn by vacuum (Ventura water-pump) through a Whatman Gamma-12 grade 03 sterilising filter. Doubling dilutions of 1 ml CSN in PBS pH 7.3-7.4 were made in disposable 3 ml plastic tubes. The indicator of proteolytic activity consisted of strips of daylight exposed x-ray film (Kodak Royal Blue, RB54) which were approximately 2 mm wide by 90 mm long and normally a flat olive green colour. One strip was placed in each tube and the rack placed in a water bath at 37°C. Readings of proteolytic activity were made after 30 min. and 1 hr. In those tubes where proteolysis occurred, the gelatin coating was digested on one or both sides of the film, exposing the transparent blue base material. The end point was taken as the last tube where digestion of the immersed portion of strip was 50 per cent or more completed, and the proteolytic titre was defined as the reciprocal of that CSN dilution.

APPENDIX XIIa

ANTIPROTEOLYTIC ACTIVITY TEST

The three components of the test were culture supernatant of a known proteolytic activity (Appendix XII), sheep serum of unknown antiproteolytic activity and x-ray film to act as indicator.

Culture Supernatant (CSN) This was material harvested from biphasic medium, filtered to remove the bacterial cells and concentrated in volume 50 times by overnight dialysis at 4°C against polyethylene glycol. The concentrate was divided into 1.5 ml amounts and held at -20°C. On the morning of a test, an aliquot was thawed and its proteolytic activity reassayed. The dilution chosen for use in the test was three doubling dilutions, i.e. eight fold more concentrated than the proteolytic titre. Therefore after adding equal parts of CSN and serum dilution, four "proteolytic doses" were available in each tube.

Sheep serum Doubling dilutions of sheep serum were made in PBS and to 0.2 ml amounts of each, an equivalent amount of the chosen dilution of CSN was added. The tubes were placed in a water bath at 37°C for one hour to allow for mixing and neutralisation.

Test Indicator strips of x-ray film were placed in the same tubes and incubation allowed to continue at 37°C for another two hours after which period the test was read. The end point was taken as that tube where proteolytic activity had been completely or more than 50 per cent prevented. Positive controls (antiserum of known antiproteolytic activity + CSN), and negative controls (re-run of CSN assay), were included in each test. The antiproteolytic titre was defined as the reciprocal of the final serum dilution[2] in the end point tube.

[2] Serum dilution after addition of CSN.

APPENDIX XIII

DOUBLE DIFFUSION IN AGAR TEST

The techniques used were based on those of Ouchterlony (1968) as described by Clausen (1969).

Double diffusion agar[3] was reliquified in a boiling water bath and a 10 ml glass pipette heated in the water alongside.

Glass plates that measured 90 x 110 x 2 mm were washed thoroughly clean and stored dry in packets away from dust. Directly before use they were flooded with 95 per cent. ethyl alcohol, rinsed with absolute alcohol and afterwards anaesthetic ether (May & Baker, Dagenham, U.K.), then drained dry and heated in a hot air oven to 70°C.

While the plate was still hot, it was placed on a level table and 7-15 ml (11 ml was found best) of double diffusion agar was evenly spread freehand with the pipette. The poured plate was covered to avoid loss of moisture and when the agar had set, was removed to a damp closed box at 4°C. After 2-48 hr, wells were cut freehand over a drawn template, using a variety of well spacings and dies of various diameters. The wells were filled to the brim with cell extracts or sera and diffusion was allowed to proceed at 25°C for up to four days.

The completed preparations were washed in a magnetically stirred 0.85 per cent. saline bath for three days, in distilled water for one hour and then covered with filter paper (Whatman grade I) to dry out at room temperature over 30 hr. Dried agar plates were stained with 0.5 per cent. Amido Black using the method of Wieme, (Clausen, 1969 - Appendix 18).

[3] Double diffusion agar

Formula	g/100 ml
"Ionagar" No. 2 (Oxoid L12)	1.0
Sodium chloride	0.85
Thiomersal B.P., 2% aqueous	final 0.01 (0.5 ml/100 ml)

Distributed in 20-25 ml amounts

Sterilisation, Autoclaved at 121°C for 15 min.

Storage, Refrigerated at 4°C

APPENDIX XIIIa

IgG FRACTIONATION

The batch method described by Stanworth (1960) was carried out using diethylaminoethyl cellulose (DEAE), (microgranular DE32, Whatman, England) and 0.01M phosphate buffer pH 7.5 (Williams and Chase, 1968).

The supernatant containing gamma-globulin, and an elution of the DEAE obtained by washing the slurry with 0.3M phosphate buffer pH 7.5, were dialysed against polyethylene glycol to a twofold concentration in terms of the original serum volume.

APPENDIX XIIIb

IMMUNOELECTROPHORESIS

Glass plates that measured 125 x 95 x 2 ml were cleaned as described (Appendix XIII) and covered with 6-12 ml of one per cent. "Ionagar" No. 2 (Oxoid, London) or one per cent. Agarose ("Seakem", Marine Colloid Inc. - Bausch & Lomb) in veronal buffer[4], pH 8.6.

The wells and troughs were cut freehand over a drawn template and the wells filled with cell extract, or serum acting as the antigen component. The plate was placed over reservoirs of the same sodium barbitone buffer pH 8.6, wicks of filter paper were attached between reservoirs and agar and the apparatus was connected to a Watson Victor Power Pack delivering a constant direct current of 15 m. amp at 100 V for 2 hr. After this period of electrophoresis, the leads were removed, the troughs filled with specific antisera, and diffusion allowed to proceed at room temperature in a closed humid container for 24-48 hr. Washing, drying and staining of the preparation was carried out in the manner described for double diffusion in agar plates (Appendix XIII).

Antisera

i)	Against *F. nodosus*	- Prepared in sheep by various schedules (Appendix X)
ii)	Against whole sheep serum	- Rabbit anti-sheep serum
iii)	Against sheep serum-IgG	- Rabbit anti-sheep IgG, lyophilized (Miles, Kankakee, U.S.A.).

[4] Formula

Sodium barbitone	20.62 g
Distilled water to	1000 ml
Adjust pH to 8.6 using 0.5M-HCl	
Distilled water to	2000 ml.

APPENDIX XIV

BACTERIAL GROWTH-INHIBITION BY SERUM

(1) TUBE TEST The technique was based on the method described by Egerton and Merritt (1970).

(a) Bacterial culture: F. nodosus (J3/22) was maintained by sub-culture every 48 hr in GC medium. The bacterial cell concentration was estimated from optical density readings using a Spectronic 20 spectrophotometer set at 480 nm. An overnight culture was diluted 1/1000-1/2000 so that the 0.1 ml dose per tube contained a calculated 20,000 organisms.

(b) Serum was centrifuged at 10,000 x g. for 10 min. at 4°C and then sterilised by passing through a Millipore filter 0.22 µm a.p.d. Dilutions were made in veronal buffered saline (Appendix XVIa).

(c) Complement source: Two batches of guinea pig serum (Baltimore Biologicals, Maryland, U.S.A. and Fort Worth, Texas, U.S.A.) at both 1/5 and 1/10 dilution, were shown to inhibit F. nodosus growth in GC medium. Pre-colostrum lamb serum (PCLS) also inhibited growth at 1/5 but not at 1/10, and at the latter dilution it was used as a complement source.

(d) The Test: This was carried out by adding the culture inoculum of 0.1 ml to serum dilutions of 0.5 ml (or 0.25 ml double strength serum, plus 0.25 ml complement source) in 6 ml bijou bottles which were then placed in a water bath at 37°C for one hour. The 0.6 ml of mixture was transferred aseptically under a N_2/CO_2 gas mixture to 4.4 ml GC medium in a roller culture tube, the head space was regassed and the butyl bung replaced. The tubes were incubated at 37°C for 2-3 days. Positive control tubes containing (i) culture inoculum, veronal buffered saline and GC broth, and/or (ii) culture inoculum, veronal buffered saline, complement source and GC broth, were always included in the test.

Visible growth in the broth was recorded daily. The end point of a titration was decided at ≏72 hr incubation, as the last tube where F. nodosus growth appearing as cells in suspension was just inhibited. The titre was defined as the reciprocal of the final serum dilution in the nominated tube.

(2) FILTER PAPER DISC TEST Filter paper discs, 12.7 mm diameter, (No. 740-E Schleicher and Schnell, New York) were sterilised by autoclaving at 121°C for 15 min. Freshly poured and quickly dried hoof agar plates were heavily seeded with F. nodosus culture from another hoof agar plate, and up to four discs firmly pressed down on the surface in an aseptic manner. Four or five drops of sterile serum dilution in phosphate buffered saline (Appendix XVIa) were delivered on to the disc by Pasteur pipette. The plates were closed and incubated agar uppermost in an anaerobic jar, at 37°C for three days. Using

oblique light and a plate dissecting microscope the area around the border of the disc was examined for growth inhibition or changes in colony appearance.

APPENDIX XV

TESTS FOR DETECTION OF SPECIFIC CELL-MEDIATED IMMUNITY

i) Antigens

Antigens were used in the form of cell extracts (Appendix VI) or culture supernatants (CSN).

Homologous antigen F. nodosus (J3/22) was cultured in biphasic medium and used as the crude cell extract. The CSN from biphasic medium culture was also used.

F. nodosus (J3/22/8) cultured on hoof agar was used either as the crude cell extract or as a washed boiled cell extract. The washings from this process were also used.

Non-related antigen

The "E" strain of Escherichia coli (Microbiology Department, Massey University) was grown up overnight in beef heart infusion broth. The bacterial cells were centrifuged at 2800 x g. for 30 min. and, without washing, subjected to two French press treatments to produce a crude cell extract.

Foreign antigen

The causative agent of "damping-off" in cauliflower seedlings, Pellicularia filamentosa was grown up over four days on dextrose peptone agar (Appendix XVI). The growth was scraped off into distilled water and a crude cell extract made by passing the mixture twice through the French press.

(ii) Intradermal Skin Test

Normal sheep, or those infected with foot-rot, were injected intradermally in the groin with 0.1 ml F. nodosus cell extract or culture supernatant. An area of skin had been prepared 24 hr previously by clipping to remove any hair and swabbing with absolute alcohol to clean away the grease.

Over the next four days the injection site was observed for changes in colour and palpated for swelling or induration.

(iii) Intracorneal injection

Sheep were anaesthetised using 5 per cent thiopentone sodium solution (May & Baker, Dagenham, U.K.) given intravenously to effect, or the steroid compound, CT1341 "Saffan" (Glaxo, Greenford, U.K.) at 12 mg total steroid per ml given intravenously as a calculated dose of 0.14 ml per Kg bodyweight.

An eyelid retractor was positioned and a single loop of nylon thread inserted into the dorsal sclera. When firmly held, this functioned as a counter force to the needle pressure on the cornea, which otherwise would have rotated the eyeball.

Cell extract, CSN, or control materials such as culture medium were injected into the substance of the cornea through a 26 g (0.45 mm) x 13 mm needle. Successful inoculation was confirmed by the appearance of an opaque bleb or star. One or two injections were made into each eye. Whenever possible the inoculum was sterilised by passing through a Millipore filter 0.22 μm a.p.d.

The injection sites were examined daily for evidence of localised or diffuse opacity.

APPENDIX XVI

CULTURE MEDIA

STUART'S TRANSPORT MEDIUM

Formula

Anaerobic Salt Solution

Thioglycollic acid (Difco)	2 ml
Sodium hydroxide 1N	12-15 ml
Sodium glycerophosphate, 20% aqueous	100 ml
Calcium chloride, 1% aqueous	20 ml
Distilled water	900 ml

Adjustment of pH to 7.2

Agar Solution

Agar	6 g.
Distilled water	1 litre

Dissolved by steaming.

Preparation of Medium

Nine hundred ml of anaerobic salt solution was added to the litre of melted agar and the pH adjusted to 7.3-7.4. Four ml of 0.1 per cent. of methylene blue in water was added and the mixture distributed into six ml bijou bottles, filling almost to the top.

Sterilisation, Autoclaved at 121°C for 20 min. and caps tightened down immediately.

Storage, Room temperature.

Nutrient agar was made up from Oxoid granules CM3

Formula	g/100 ml
'Lab-Lemco' beef extract	0.1
Yeast extract (Oxoid L20)	0.2
Peptone (Oxoid L37)	0.5
Sodium chloride	0.5
Agar	1.5

pH 7.4

MacConkey agar was made up from Oxoid granules CM7

Formula	g/100 ml
Peptone (Oxoid L37)	2.0
Lactose	1.0
Bile salts (Oxoid L55)	0.5
Sodium chloride	0.5
Neutral red	0.0075
Agar	1.2

pH 7.4

Blood agar consisted of 5-10% whole sheep blood (citrated) added to the following sterilised agar base made from Oxoid granules CM55.

Formula	g/100 ml
'Lab-Lemco' beef extract	1.0
Peptone (Oxoid L37)	1.0
Sodium chloride	0.5
Agar	1.5
pH 7.5	

Dextrose peptone agar was made up from Oxoid granules CM13.

Formula	g/100 ml
Peptone (Oxoid L37)	2.0
Dextrose	1.0
Sodium chloride	0.5
Agar	1.5
pH 7.2	

APPENDIX XVIa

BUFFERED SALINE SOLUTIONS

Phosphate buffered saline (PBS)

Formula	g/100 ml
Sodium chloride	0.8
Di-potassium hydrogen phosphate	0.121
Potassium di-hydrogen phosphate	0.034

Distilled water

Confirmation of pH 7.4

Distributed, 100 ml amounts

Sterilisation, Autoclaved at 121°C for 20 min.

Storage, Room temperature.

Formalin treated PBS had 0.5% formalin (Analar, 40% formaldehyde) added (FPBS).

Veronal buffered saline (VBS)

Made up from Complement Fixation Test Diluent Tablets (Oxoid, Code BR16)

Formula	g/100 ml
Barbitone	0.0575
Sodium chloride	0.85
Magnesium chloride	0.0168
Calcium chloride	0.0028
Barbitone soluble	0.0185

pH 7.2

Distributed, 100 ml amounts

Sterilisation, Autoclaved at 121°C for 20 min.

Storage, Room temperature.

ACD solution (Acid citrate dextrose)

Formula (for 430 ml blood)

Dextrose	1.7 g
Sodium hydrogen citrate	2.0 g
Distilled water to	70 ml

Sterilisation, by filtration using a Gradocol membrane, grade 0 3 (Whatman).

APPENDIX XVII

STAINING METHODS

Gram's method

Air dried smears were fixed by heat

- Methyl violet 0.5% — 45 sec.
- Iodine solution 2%, to wash off methyl violet, — 1 min.
- Acetone to decolourise, 2 or 3 times and immediately washed off with tap water
- Dilute carbol fuchsin, 5% of 1% strong, — 45 sec.

Washed and blotted dry

Giemsa's method

Fixation by methyl alcohol — 3-5 min.

- Giemsa stain 10% in phosphate buffer, pH 7.3, — 1 hr
- Washed in phosphate buffer pH 7.3

Blotted dry

Methylene blue

Air dried smears were fixed by heat

- Löfflers methylene blue 30% v/v of saturated alcoholic solution, — 3 min.

Washed and blotted dry.