Studies On The Vaccination Of Sheep Against *Brucella ovis* Infection

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ABSTRACT

A study was made of the efficacy and adverse effects of an inactivated Brucella ovis saline-in-oil vaccine administered either once or twice by either the subcutaneous or the intraperitoneal route.

Seven Brucella ovis isolates from various sources, including the two used in the manufacture of the Brucella ovis vaccine, were subjected to Bacterial Restriction Endonuclease Analysis and no genetic differences were found. It was concluded that there is probably only one strain of the organism.

It was found that rams vaccinated by the subcutaneous route in the neck invariably developed a palpable inflammatory lesion at the site of injection. The lesions had a mean diameter of approximately 3cm, about one-third of them discharged, and the majority persisted for at least one year after vaccination. These lesions were chronic granulomatous inflammatory reactions arranged around droplets of the oily Brucella ovis vaccine.

The intraperitoneal route of vaccination has been advocated in the past as a way of avoiding visible lesions. Necropsy of vaccinated animals revealed that in over 50% of cases, at least some of the vaccine administered by this technique failed to reach the cavity and was deposited either beneath the parietal peritoneum or between the muscles of the abdominal wall. Regardless of the site of deposition, however, the vaccine always provoked a chronic granulomatous inflammation of the tissues with which it came into contact. Changing from a subcutaneous to an intraperitoneal vaccination technique merely moved the reaction to a site where it was less visible.

Serological studies using the Brucella ovis complement fixation test, gel diffusion test and enzyme linked immunosorbent assay demonstrated a consistent difference in the antibody response of rams vaccinated by the subcutaneous technique in comparison with those vaccinated by the intraperitoneal technique. Those vaccinated by the

subcutaneous route generally developed antibody titres more rapidly and often had higher peak titres.

In the same way, animals vaccinated twice by either route generally had greater and more persistent antibody titres than those vaccinated once by the same method.

The differences in the serological response of rams to different vaccination techniques were reflected by similar differences in resistance to experimental infection. The administration of an inactivated Brucella ovis saline-in-oil vaccine by any of the techniques studied significantly increased resistance to challenge by the intravenous route. However, two spaced doses of vaccine appeared to be more effective than a single dose, and the subcutaneous technique appeared to be more effective than the intraperitoneal method.

Using intravenous inoculation, the number of bacteria required to infect 50% of unvaccinated animals was estimated to be 9.5×10^4 organisms. The administration of a single dose of vaccine by the intraperitoneal technique raised that figure to approximately 6.7×10^6, and the administration of two doses of vaccine by the subcutaneous route raised it to approximately 6.8×10^7.

A viable count of the number of Brucella ovis bacteria present in the semen of an infected ram showed that at least 3×10^9 organisms could be excreted in a single ejaculate. This was over 31,000 times the number required to infect 50% of unvaccinated rams after intravenous inoculation and 44 times that required to infect 50% of animals vaccinated twice by the subcutaneous route. There is therefore a real possibility that natural challenge through homosexual activity may result in the infection of even vaccinated rams.

It was concluded that if vaccination is to be used as a means of controlling the spread of ovine brucellosis, a programme of two doses of vaccine administered at an interval of at least four weeks should be employed. The second dose of vaccine should be administered at least four weeks before the anticipated period of risk. If this method of control is adopted, a palpable lesion at the site of injection which is likely to persist for over a year should be expected. It should also be understood that rams vaccinated in this way may not be totally resistant to Brucella ovis and may still become infected.

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CHAPTER 1

LITERATURE REVIEW

During the decade 1940-1950, it became clear that epididymitis in rams was an important cause of reproductive wastage in Australian and New Zealand sheep flocks. In 1942 it was reported by Gunn, Sanders and Granger that approximately 5.5% of 9,000 rams examined in Queensland and New South Wales had a clinical abnormality which these workers called "epididymitis with spermatocoele". Although no bacteria were isolated during this investigation, a bacterial cause was suspected from the epidemiological evidence. It is highly likely that most of these lesions were caused by Brucella ovis.

In New Zealand, concern about the prevalence of epididymitis in rams was expressed by Crawford in 1949, and in 1952 McFarlane, Salisbury, Osborne and Jebson reported finding an organism described as "Rickettsia-like" during an investigation of abortions in a flock near Masterton. This organism, which was isolated from infected placentas, was acid-fast when stained by the Modified Ziehl-Neelsen method (Stamp, McEwen, Watt and Nisbet, 1950). It was thought to be an organism similar to that reported by Stamp et.al. (1950) as causing abortions in Scotland, although preliminary tests demonstrated some basic differences. Because of the similarity to the enzootic abortion agent, a chlamydia, reported by Stamp, the New Zealand isolate was cultured in chick embryos until it was realised that it could be grown on blood agar under microaerophilic conditions (Lawrence, 1961).

An addendum to the report of McFarlane et.al. (1952) noted that the same gram-negative coccobacillus had been recovered from rams with epididymitis, and that typical cases of both the abortion and the epididymitis syndromes could be produced by inoculation of the organism into susceptible ewes and rams.

During 1953, the physical and metabolic characteristics of the organism were described both in New Zealand (Buddle and Boyes, 1953) and in Australia (Simmons and Hall, 1953). It was described as a small gram-negative bacillus, non-sporing and non-motile. Greyish white colonies 0.5mm in diameter could be seen after 48 hours incubation on blood agar in 10% CO_2 at 37°C, and the organism was acid-fast when stained by the Modified Ziehl-Neelsen technique. (Buddle and Boyes, 1953). The New Zealand workers concluded from the various physical and biochemical properties of the organism that it was a member of the genus Brucella, and further, that it was possibly a variety of Brucella melitensis. In 1956, Buddle reported the results of further biochemical and serological investigations in which the ovine organism was shown to have different characteristics to the three "classical" Brucella species: Brucella abortus, Brucella melitensis and Brucella suis. He suggested that this new variant be given the name Brucella ovis.

During the 1950s, reports of what appeared to be the same agent causing epididymitis in rams came from workers in Europe (Gdovin, Hrudka, Chladecky and Koppel, 1955), the U.S.A. (McGowan and Shultz, 1956), and South Africa (Van Rensburg, Van Heerden, Le Roux, Snyders and Van Heerden, 1958).

EPIDEMIOLOGY

The prevalence of Brucella ovis infection has been found to differ widely between individual flocks of rams. An eradication programme in Tasmania using serological testing was begun in 1959. At the initial test 18% of all ram flocks were found to be affected, with the prevalence of infection within them ranging between 10% and 74% (Ryan, 1964). The prevalence of clinical epididymitis is no less variable. Although Gunn et.al. (1942) reported a 5.5% prevalence in 9,000 rams, and this figure has been largely substantiated by several other workers (Moule, 1950; Osborne, 1955), it was found that a minority of farms had a prevalence close to the average, and that the figure varied widely between properties, from nil to over 50% (Osborne, 1955).

The prevalence of Brucella ovis infection appears to have declined under the impact of control measures. In the decade 1954-1964, 39% of specific pathogens isolated from ram testicles submitted to New Zealand diagnostic stations were Brucella ovis, but in 1965-1966 only 7% were Brucella ovis (Ekdahl, Money and Martin, 1968). It is possible, though, that this decline reflected the availability of other methods of diagnosing the disease (e.g. serology).

Reports in recent times have indicated that the level of infection in affected ram flocks can still be very high. Bruere (1982) reported the prevalence of infection at first testing to be between nil and 75% in 49 New Zealand flocks under a Brucella ovis accreditation scheme. Hughes (1972a) reported that within infected flocks, the infection level was commonly between 10% and 40% but ranged from 5%-100%. A recent survey carried out in New Zealand estimated the overall prevalence of infection in rams in New Zealand to be approximately 3%, with the average prevalence on infected properties being 5% for stud farms and 11% for commercial properties (Bruere, 1982).

Natural infection by Brucella ovis has only been recorded in sheep. Infected rams excrete Brucella ovis in their semen, and this is an important factor in the epidemiology of the disease. Rams can excrete the organisms for at least two years (Hartley, Jebson and McFarlane, 1955) and probably indefinitely. Ewes mated to infected rams can become infected, develop serological titres and produce dead lambs with grossly diseased placentas (Buddle, 1955). The syndrome caused by Brucella ovis in ewes seems to be much less commonly observed than the disease in rams. Ewes appear to be relatively resistant to infection, even those from flocks having a high proportion of actively infected rams (Hartley et.al., 1955). Infection in the ewe seems rarely to persist from one breeding season to the next (Hartley et.al., 1955; Buddle, 1955; Muhammed, Lauerman, Mesfin and Otim, 1975). Transmission from ewe to ewe could not be demonstrated by Buddle (1955) when infected and uninfected ewes were grazed together during pregnancy or when uninfected ewes grazed pasture previously contaminated by infected ewes. The evidence

suggests that the disease in ewes is contracted by the venereal route during mating with infected rams.

Rams can become infected by mating with a ewe previously served by an infected ram during the same oestrus period (Buddle, 1955) and also by direct contact with infected rams (Brown, Pietz and Price, 1973). Rams allowed to graze pasture heavily contaminated with Brucella ovis from the diseased placentas and discharges of infected ewes fail to develop infection (Hartley et.al., 1955). It therefore appears that direct contact with infected animals is required for the transmission of the disease to rams, and that the route is venereal.

The observation of Hartley et.al. (1955) that faecal material is sometimes present in the prepuce of rams, and the fact that ram to ram transmission of the disease occurs in the absence of ewes (Brown et.al., 1973) implies that homosexual activity plays an important part in the epidemiology of this disease, especially outside the mating period. Such homosexual activity is commonly seen in the field, particularly when rams are confined in small areas such as yards and shearing sheds. It has been shown, also, that active infection can develop in rams exposed to Brucella ovis by rectal inoculation (Gorrie, 1957; J.W. McDonald and J.H. Grainger, personal communication, 1984), and it is now widely accepted that this is an important route of transmission. Animals have also been infected experimentally by the intravenous, subcutaneous, conjunctival, preputial, oral, nasal, intratesticular and intraepididymal routes (Simmons and Hall, 1953).

The possibility has been raised that this disease could be transmitted through the mucous membranes of the mouth, nose and eye as animals investigate each other by licking and sniffing (Buddle, 1955). If this is the case, the potential exists for transmission through fencing barriers. However this rarely seems to happen. A flock of uninfected rams can be grazed on the same farm as a flock of infected rams with only a fence between them, and yet remain uninfected. (D.M. West, personal communication). These observations indicate that close, intimate contact is required for the transmission of the disease.

PATHOLOGY

Brucella ovis infection in the ram is characterised clinically by the presence of palpable abnormalities of the epididymis (McFarlane et.al., 1952; McGowan and Shultz, 1956; Kennedy, Frazier and McGowan, 1956; Van Rensburg et.al., 1958). The most common lesion is enlargement of the tail of the epididymis, which is usually unilateral in the early stages, and the head of the epididymis is the next most frequently affected site (Van Rensburg et.al., 1958).

The gross and microscopic pathology of this condition was described by Jebson, Hartley, McClure and McFarlane (1955). These investigators noticed that there seemed to be two distinct types of reaction in the naturally infected rams used for this study, one being an "acute" reaction and the other "chronic" in nature. The "acute" reaction was characterised in the live animal by a palpable hot doughy swelling of the scrotal contents on the affected side. Post-mortem examination revealed inflammatory oedema of the loose scrotal fascia with fibrinous exudate, hyperaemia and early granulation tissue formation in the epididymis and tunica vaginalis. The severity of the reaction ranged from slight oedema to extensive inflammation and granulation.

The more commonly encountered "chronic" reaction was characterised by a palpably enlarged, hardened epididymis on either one or both sides, which most commonly involved the tail of the organ. At necropsy, there were varying degrees of thickening and fibrosis of the visceral and parietal tunics, with the development of fibrous adhesions. These reduced the motility of the testis within the scrotum. The epididymis often contained cavities filled with partially inspissated spermatic fluid. Extensive calcification was also seen on occasion.

The acute reaction is characterised microscopically by lymphocyte and plasma cell infiltration, and is followed by the formation of granulation tissue and ultimately by fibrosis. The ducts of the epididymis may show epithelial hyperplasia, and the formation of intraepithelial cysts (Kennedy et.al., 1956). When extravasation

of sperm occurs as a result of tubular rupture, the degenerating spermatozoa are surrounded by giant cells which are bounded by peripheral zones of granulation tissue and fibrosis (Jebson et.al., 1955). The testis on the affected side sometimes becomes atrophic or fibrosed, and according to Jebson et.al. (1955) this is associated with the complete blockage of the epididymis.

In 1964, Biberstein, McGowan, Olander and Kennedy studied the pathogenesis of an experimental infection in 48 rams inoculated by the conjunctival route. The bacteria were confined to the local site at the conjunctiva for the first week, and could then be isolated from regional lymph nodes. Bacteraemia began at two weeks and continued for two months. The organism gradually disappeared from most body organs apart from the genital tract although it was occasionally isolated from the spleen, kidney and liver, and from urine.

Although epididymitis is characteristic of the disease in rams, it is by no means diagnostic, as similar lesions may be produced by other agents (Ekdahl et.al., 1968). Conversely, the absence of genital lesions does not imply that Brucella ovis infection is not present. Hughes and Claxton (1968) found in one trial that only 36% of 88 rams with bacteriological evidence of infection had palpable lesions, and Jebson, Hartley and McFarlane (1954) discovered that Brucella organisms were often excreted in the semen both before lesions developed and after they had subsided.

Reports on the effects of Brucella ovis infection in ewes have been rare in comparison with the large volume of information concerning the syndrome in rams, but there is little doubt that this organism is quite capable of causing occasional abortions (McFarlane et.al., 1952). Hughes (1972a) refuted the misconception that outbreaks of abortion were common, and reported that the most frequent consequences of infection were sporadic lamb deaths at full term, and that many lambs with diseased placentas survived to maturity. The most consistent lesions are in the placenta, and were described by McFarlane et.al. (1952) as oedema, thickening, and plaque-like yellow lesions in the intercotyledonary areas which tended to coalesce to form areas resembling chamois leather. Mollelo,

Jensen, Flint and Collier (1963) described the most obvious feature as the abundant yellow tenacious exudate in the interplacentomal areas. Hartley (1961) reported that approximately 50% of the lambs born with diseased membranes had calcified plaques on the hooves and accessory digits.

From histological studies, Hartley (1961) concluded that the organisms multiplied in the chorionic epithelial cells, eventually causing cell rupture to release large numbers of bacteria which reinfected other cells, this process resulting in severe inflammation of the mesenchyme, vasculitis and subsequent ischaemic necrosis. It was also thought that at a later stage, Brucella ovis organisms multiplied in the trophoblastic epithelium at the edge of the foetal cotyledons, again causing inflammation, necrosis and sometimes eventual calcification. Brucella ovis organisms were sometimes found in the tissues of the lamb itself. Hartley (1961) believed that if cotyledonary necrosis occurred the lamb would probably die in utero, but that full-term lambs could be born even when severe intercotyledonary lesions were present.

Mollelo et.al. (1963) speculated that abortions might result from the accumulation of exudate causing separation of the placenta from the uterus. Hughes (1972b) thought it likely that the placentitis interfered with foetal nutrition, resulting in lambs of low birth-weight.

When an uninfected ewe in oestrus is inseminated by an infected ram, vaginitis and cervicitis may develop (Van Rensburg et.al., 1958). This is a short term reaction (24-48 hours) but may recur at the following oestrus, and the possibility exists that this has some effect on reproductive efficiency.

ECONOMIC SIGNIFICANCE

The economic importance of Brucella ovis infection results from the reduction in fertility, the culling of affected rams (especially in stud flocks), and the cost of control programmes. Ryan (1964), in a paper dealing with the eradication of ovine brucellosis, noted that although ram breeders usually favoured eradication, some veterinarians questioned the importance of the disease in commercial ("grade") flocks. Ryan commented also that estimates of ram fertility based on the evaluation of semen samples might fail to assess the ability of the ram to deliver adequate numbers of spermatozoa under the conditions of frequently repeated ejaculation which prevail during flock mating. This method might therefore minimise the importance of Brucella ovis infection under field conditions.

There is now considerable evidence that Brucella ovis can significantly reduce reproductive performance in an infected flock. Several workers have demonstrated that there is a reduction in the fertility of infected rams. This involves both a reduction in semen quality (Gunn et.al., 1942; Jebson et.al., 1954; McGowan and Devine, 1960) and a reduction in the conception rate of ewes mated to affected rams (Buddle, 1955; McGowan and Devine, 1960; Plant, 1977). A reduction in the breeding performance of infected ewes also contributes to the reduced reproductive efficiency of the flock. Hughes (1972b) reported that ewes inoculated intravaginally with Brucella ovis on the day they were mated had a lower non-return rate, lower lambing percentage and lower percentage of lambs reared than unexposed ewes. It was also reported that the birth weights of lambs born to ewes showing a four-fold increase in Brucella ovis complement fixation titre after experimental exposure were significantly lower than those of lambs born to ewes negative to the complement fixation test. There was no significant difference in the mean gestation lengths of these two groups (Hughes, 1972b).

Bruere (1982) reported the findings of Quinlivan in New Zealand which reaffirmed the poor breeding performance of ewes mated to infected rams. There was an average reduction of 13% in the number of lambs docked when compared with ewes mated to uninfected rams, and in

one flock the difference was as large as 44%.

The work of Hughes (1972b) using experimentally infected ewes demonstrated that Brucella ovis increased losses from conception failure, embryonic death, abortion, and the birth of weak or under-weight lambs. All these findings clearly indicate that significant benefits could accrue from attempts to control the disease. In fact, as early as 1949, Crawford reported that in the opinion of sheep farmers, his work of examining rams and rejecting those with "permanent lesions" had resulted in a more even lamb drop and fewer dry ewes. Flocks which had had their rams examined had a lambing percentage 6.7% and 4.8% above the average for the area in the years 1947 and 1948 respectively.

DIAGNOSIS

At first, diagnosis of Brucella ovis infection in rams relied almost entirely on the detection of epididymitis by palpation of the scrotal contents. This is known to be an unreliable guide to infection in individual rams, as infected animals often excrete Brucella ovis in their semen without having any palpable abnormality of the scrotal contents (Jebson et.al., 1954; Edgar, 1959; Hughes and Claxton, 1968). However, the technique is still useful on a flock basis, and particularly as a method of routine surveillance in flocks with no recent history of the disease.

Semen samples collected from suspect rams can be a very useful source of diagnostic information. Smears of semen can be stained by the modified Ziehl-Neelsen method (Stamp et.al., 1950) and examined for the presence of acid-fast coccobacilli. Edgar (1959) and Hughes and Claxton (1968) found this quite a reliable guide to infection. However other workers, such as Biberstein and McGowan (1958) concluded that because many smears produced doubtful results, only an unequivocally positive finding had any meaning. They also found that one ejaculate might contain large numbers of bacteria while the next sample from the same ram might not contain any. The culture of the Brucella ovis organism from a semen sample is definite proof of

infection, but a negative result is more difficult to interpret. Edgar (1959) thought it necessary to have negative results from three semen samples to give a reasonable assurance that the ram was uninfected, and Biberstein and McGowan (1958) concluded that negative cultures were inadequate as proof of the absence of infection. Intermittent excretion of the organism was also reported by Hughes and Claxton (1968).

Another problem associated with using semen as a source of diagnostic information is that contamination of the sample by more rapidly growing bacteria and subsequent failure to isolate Brucella ovis from infected samples may occur (Biberstein and McGowan, 1958; Edgar, 1959; Hughes and Claxton, 1968). In addition there are the instances of failure to obtain a semen sample, or the collection of accessory gland fluid only, and these are not uncommon (Hughes and Claxton, 1968). The value of the procedure is heavily dependent on the quality of the sample, which to some extent is determined by the skill and experience of the veterinarian involved. Geographical factors such as distance from the laboratory and therefore the delay before the samples are processed also have an effect.

Because the collection of semen samples is so labour intensive and time consuming, it is unsuitable as a screening test for large numbers of animals. Edgar (1959) commented that a more accurate and practical diagnostic method was desirable, but noted that the vaccination programme which had commenced in New Zealand would make the application of any serological test more difficult.

A delayed-type hypersensitivity intradermal skin test, similar to the bovine tuberculin test but using formalin-killed Brucella ovis in suspension, was described by Hall (1955). There was a positive correlation between the test results and the presence of epididymitis in experimentally infected animals. Cedro (1963) described a similar test and confirmed its usefulness by culturing the organism from animals which had no lesions but which did have a positive reaction to the test. This method of diagnosis has not been used to any great extent.

There was a recognised need for a simple blood test to determine the presence or absence of infection in large numbers of animals (Clapp, 1955). The removal of rams with palpable lesions was not successful in ridding flocks of infection and the detection of asymptomatic carriers was considered essential to any successful eradication programme (Biberstein and McGowan, 1958).

Early experiments using serum agglutination tests were hindered by the inability to prepare an antigen suspension of satisfactory stability, and researchers began to investigate the possibility of using a complement fixation test (Clapp, 1955). The development of this test was in turn hindered by the anticomplementary activity of the antigen, but the problem was partially overcome by incubating the bacterial cells with complement and guinea pig serum for 30 minutes (Clapp, 1955). Biberstein and McGowan (1958) described an antigen of low anticomplementary activity which had been obtained by sonic disintegration of bacterial cells. It was demonstrated by Clapp (1961) that the anticomplementary activity of an antigen was related to the presence of cell debris. Because there were some difficulties associated with the complement fixation test, Ris and Te Punga (1963) decided to investigate alternatives, and described an indirect haemagglutination test which appeared to be reasonably specific, although there was some cross-reactivity with antibodies to Brucella abortus Strain 19.

Work continued on the complement fixation test, and in 1968 Hughes and Claxton reported it to have good specificity and sensitivity, although flocks where vaccination was carried out produced confusing results.

The complement fixation test became the most widely used serological diagnostic test for Brucella ovis infection, and in 1974 the development of an automated version of the "warm" complement fixation test, similar to the autoanalyser test for Brucella abortus in cattle was described (Weddell, 1974).

Ris (1974) demonstrated that a "cold" complement fixation test, in which sera were incubated with antigen and complement for 18 hours at 4°C was more sensitive than the then standard test used in New Zealand, a "warm" process in which sera were incubated for 30 minutes at 37°C. This "cold" method was later adopted as the standard test in New Zealand.

The complement fixation test is able to detect infection in some cases as early as two weeks, and in most rams by five weeks post-inoculation (Webb, Quinn, Cockram and Husband, 1980)

Worthington (1982) clearly demonstrated the effect that the use of a different antigen could have on the sensitivity and specificity of a complement fixation test. One heat-extracted Brucella ovis antigen produced a sensitivity of 85% in the "cold" complement fixation test, whereas another antigen, produced in a different way, resulted in a 97% sensitivity. An ultrasonicated antigen was found to be 10% less specific than the best heat-extracted antigen. When the most satisfactory antigen was used (a product made by the laboratory itself), the cold complement fixation test performed very well, with an expected false negative rate of 3% and false positive rate of 1%.

In recent times there has been some criticism of the complement fixation test as it is applied in eradication programmes. Hicks, Burr, Marshall and Vidler (1978a and 1978b) pointed to the inconsistent results they had experienced when using the test, such as certain animals which were positive at one test being negative on a subsequent occasion. Wagner (1982) found that animals negative to the test could be excreting Brucella ovis in their semen, and conversely, that animals not excreting Brucella ovis could be positive to the test.

The suggestion was made that some spurious results may occur as a result of vaccination or the haemolysis of blood samples (O'Hara, Anderson and Weddell, 1978). Bruere and West (1978), in defence of the test, pointed out that several flocks were now Brucella ovis free as a result of the judicious use of the complement fixation test, which was proving to be a very valuable tool in the control of the

disease. The problems in test interpretation associated with vaccination were reemphasised by McDiarmid (1978) who reported results from two farms where it appeared that complement fixation titres had persisted for three years after vaccination had taken place.

An investigation of a number of chronically infected rams revealed that a small number of individuals may react inconsistently to the complement fixation test, causing spurious results and consequent problems of interpretation (Worthington, Stevenson and de Lisle, 1985). An Animal Health Division report (1983) pointed out that animals could be positive to the complement fixation test before the organism was excreted in the semen, and that this could account for some of the so-called "false positive" results reported from the field (Wagner, 1982). The report also emphasised that the predictive value of a test depends upon the prevalence of the disease in a population as well as the accuracy of the test per se. West and Bruere (1983) presented data from 35 Brucella-free flocks which showed that of 1653 non-vaccinated and uninfected rams, only five had positive or suspicious titres in the Brucella ovis complement fixation test: this indicated a specificity of 99.7%. They emphasised the importance of knowing the history of the flock when interpreting the serological results.

The conclusion to be drawn from these reports is that in spite of some difficulties in interpretation, the complement fixation test is a remarkably good diagnostic test (Worthington, 1982), ranking amongst the most accurate of serological methods available.

Interest in the use of an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of Brucella ovis infection developed during the 1980s (Rahaley, Dennis and Smeltzer, 1983; Ris, Hamel and Long, 1984). This test does not require multiple serum dilutions to be carried out as does the complement fixation test, and it also accomodates anticomplementary sera which cannot be evaluated by the complement fixation method.

In 1984, Worthington, Weddell and Penrose compared the sensitivity and specificity of three serological tests for ovine brucellosis that were used at Wallaceville Animal Research Centre, Upper Hutt, New Zealand. These three tests were the "cold" complement fixation test, the newly developed ELISA test, and a gel diffusion test normally used for anticomplementary sera as a back-up to the complement fixation test. These workers found minimal differences between the standard complement fixation test and the ELISA, with the ELISA being slightly more sensitive and less specific, while the gel diffusion test, although highly specific, was a good deal less sensitive than the other two tests.

From these studies, it seems that in spite of occasional problems, the complement fixation test is highly reliable, and it remains the standard test for the serological diagnosis of Brucella ovis infection in New Zealand rams.

CONTROL

Attempts to control ovine brucellosis have been made from two different approaches: eradication and vaccination.

Eradication

Early attempts at eradication, based on the culling of rams with palpable lesions, were unsuccessful due to the presence of infected rams which had no clinical abnormalities (Clapp, 1955; Biberstein and McGowan, 1958). However, eradication was successful when based on a reliable serological test, as demonstrated by the elimination of the disease on the Bass Straight Islands (Tasmania) using a test and slaughter programme based on the complement fixation test (Ryan, 1964). This type of control programme has been the preferred method in Australia, where voluntary accreditation schemes are operated. Such schemes have been operating successfully in South Australia since 1966 (R.W. Newlands, personal communication, 1984), in Victoria since 1974 (M.A. Harrison, personal communication, 1984), in New South Wales since 1980 (D.R. Thompson, personal communication,

1984), and in Queensland since 1983 (K.F. Trueman and W.H. Ward, personal communication, 1984).

A voluntary ovine brucellosis accreditation scheme has been promoted by the Sheep Society of the New Zealand Veterinary Association since 1973 (Bruere, 1973). A voluntary programme in 23 New Zealand flocks was described by West and Bruere in 1979. These authors reported a keen interest in accreditation from major sheep breed societies in New Zealand, such as the Coopworth, Perendale and Romney societies, and suggested that the low prevalence of the disease, and export requirements for freedom from ovine brucellosis, should encourage and justify the cessation of vaccination in stud flocks and the official acceptance of accreditation schemes. Three years later, Bruere (1982) reported that of an estimated 1.2 million rams and ram hoggets on 26,000 properties in New Zealand, approximately 69% of rams from stud flocks and 33% of those from commercial flocks were now under veterinary inspection. He described the results of an accreditation scheme carried out on 27 commercial flocks, most of which needed fewer than three tests before all unvaccinated rams were negative to the Brucella ovis complement fixation test. However, he pointed out that in occasional "problem" flocks, up to eight tests had been required before accreditation was achieved. One of these "problem" flocks had a 12-year history of the disease, and approximately 20% of the rams had been culled each year because of the presence of lesions in the epididymis.

The problem posed by commercial breeders continuing to buy vaccinated rams was highlighted as a major obstacle to accreditation in these flocks, and concern was expressed that several vaccinated rams had that year been found to have epididymitis or to be excreting Brucella ovis in their semen. In one flock, three out of 18 vaccinated rams were found to be excretors of Brucella ovis, and in another four out of ten. In a further example, ten out of 160 vaccinated rams had lesions of epididymitis (Bruere, 1982).

Bruere (1982) also reported that in stud flocks infection was less common than in commercial flocks. Of 22 stud flocks involved in an accreditation scheme, only two were found to be infected at the initial test. One, with a low prevalence initially, became clear of infection after two examinations, while the other, with a high initial prevalence, took seven examinations before it became clear of infection. It was again emphasised that most stud groups strongly supported the control of Brucella ovis infection and the production of disease-free rams, and vaccination was a less common complicating factor on stud properties than on commercial farms.

These reports show that accreditation programmes can be successfully implemented, and in stud flocks these schemes have been very widely accepted. However, replacing large numbers of rams culled as part of an eradication programme can be expensive, and control of the disease by vaccination has been a widely used alternative to accreditation in commercial flocks, particularly those in which for various reasons, such as poor fencing, the chance of reinfection is high.

Vaccination

In 1954, Buddle reported that when working on methods of protecting animals against Brucella melitensis infection, previous authors had found killed cultures of Brucella melitensis or extracts of this organism to be ineffective as vaccines unless used in conjunction with a live, avirulent strain of Brucella abortus (Lisbonne, Roman and Quatrefages, 1947). Brucella abortus Strain 19 was used for this purpose, but was ineffective when used alone (Renoux, 1952). Experiments had also shown that the resistance produced by killed Brucella abortus or Brucella melitensis vaccines was enhanced by the addition of oily vehicles as adjuvants (Gilbert, 1943; Live, 1949; Gwatkin and Dzenis, 1952).

Acting on the basis of this information, Buddle (1954) investigated the use of a vaccine against Brucella ovis infection consisting of killed Brucella ovis cells suspended in a saline-in-oil emulsion, administered simultaneously with but at a different site to

Brucella abortus Strain 19. He found that the combined effect provided significant protection against an experimental intravenous challenge with Brucella ovis organisms. Neither of the two vaccines used alone provided significant protection for the ram lambs in this experiment.

Although Buddle was later able to demonstrate that a single dose of the inactivated Brucella ovis vaccine used alone could provide some protection (1958), he still found that the technique using both Strain 19 and the Brucella ovis vaccine was significantly better. This regime's effectiveness was confirmed by an extensive field trial involving 636 two-tooth rams challenged during the mating period by natural exposure to heavily infected sheep populations in which the average incidence of epididymitis was 18%. At the end of mating, 43% of the unvaccinated rams were excreting Brucella ovis in their semen. The corresponding figures were 16% for those vaccinated with a single dose of the Brucella ovis saline-in-oil vaccine, and only 6% for those vaccinated with the Brucella ovis vaccine as well as Strain 19 (Buddle, 1958). It was notable that neither technique was 100% effective in the face of natural challenge.

The combined Brucella ovis and Strain 19 technique subsequently became widely used (Lawrence, 1961; Claxton, 1968). However, it soon became apparent that there were several problems associated with this technique. The most obvious was a side effect of the Brucella ovis saline-in-oil vaccine. The oil adjuvant used in this vaccine caused conspicuous and persistent local reactions (Buddle, 1962; Claxton, 1968). The use of an aluminium hydroxide adjuvant as an alternative to oil was investigated by Buddle (1962). It was found that both adjuvants were similarly effective when the Brucella ovis vaccine was used in conjunction with Strain 19, but if Strain 19 was not used, a single dose of the oil adjuvanted Brucella ovis vaccine was significantly superior to either one or two doses of the Brucella ovis vaccine containing the aluminium hydroxide adjuvant.

In California, Biberstein, McGowan, Robinson and Harrold (1962) evaluated an inactivated Brucella ovis vaccine containing aluminium hydroxide adjuvant. It was found that if used in conjunction with Strain 19, none of the experimentally challenged animals became infected, whereas all the unvaccinated animals did become infected. When used alone, the Brucella ovis vaccine protected only 50% of the animals.

So, it appeared that as long as Strain 19 was used simultaneously, an aluminium hydroxide adjuvant would suffice. However, the other problems associated with vaccination against Brucella ovis infection involved the live Brucella abortus Strain 19 vaccine itself. Some rams vaccinated with Strain 19 had later been found to be excreting this organism in their semen (Buddle, 1962; Buddle, Calverly and Boyes, 1963). There was also the suggestion that vaccination might occasionally result in a temporary deterioration in semen quality for up to nine weeks (Lawrence, 1961; Claxton, 1968). Another problem was the occurrence of outbreaks of lameness following the use of the Strain 19 vaccine in conjunction with the Brucella ovis vaccine. Between 1957 and 1962 there had been a number of reports of this phenomenon, and an investigation of the problem in 12 flocks was made by Kater and Hartley (1963). It was found that lesions mainly involved the metaphyseal ends of long bones, and commonly the epiphysis of the distal radius or tibia. The lameness usually had a sudden onset, 10-20 days after vaccination, involved shifting lameness or weakness in one to four limbs, and in several outbreaks rams became prostrate for up to two weeks. There was usually a severe systemic upset and a check in growth rate, and as many as 75% of the ram flock could be affected. Strain 19 was cultured from one of the lesions. A similar outbreak was reported in which Brucella abortus Strain 19 was cultured from three out of four epiphyseal lesions examined (West, Johnstone, Bruere and Chapman, 1978).

A further disadvantage of the use of Strain 19 was the development of persistent Brucella abortus titres which precluded the export of live animals to countries which required a negative blood test (Buddle et.al., 1963; Claxton, 1968), and another was the hazard to humans handling this vaccine.

Because of the disadvantages associated with the use of live Brucella abortus Strain 19, alternative methods of vaccination were sought. In South Africa, experiments with a live, attenuated strain of Brucella melitensis (Elberg Rev 1 vaccine) had shown it to be very effective in young rams, conferring at least two years of solid immunity, although the same success was not achieved in older rams which had already been exposed to infection (Van Heerden and Van Rensburg, 1962). In South Africa, where sheep were naturally infected with Brucella melitensis as well as with Brucella abortus and Brucella ovis, the Elberg Rev 1 vaccine was a viable alternative, but in countries such as Australia and New Zealand where Brucella melitensis was unknown, it was not a valid option (Buddle et.al., 1963).

The possibility of using a killed strain of Brucella abortus was considered, but Buddle (1962) had found that the addition of killed Strain 19 to a Brucella ovis vaccine containing an aluminium hydroxide adjuvant had no significant effect on its immunological efficiency. Similarly, the addition of killed Brucella abortus Strain 45/20 did not significantly enhance the effectiveness of the Brucella ovis saline-in-oil vaccine.

Investigations were now concentrated on ways in which the Brucella ovis saline-in-oil vaccine might be used on its own, and yet still induce reasonable immunity. It was found that a single dose of Brucella ovis saline-in-oil vaccine was significantly inferior to the combined Brucella ovis/Strain 19 technique, and that two doses of the Brucella ovis vaccine, given simultaneously at different sites, were not significantly better than a single dose. However, two doses of the Brucella ovis saline-in-oil vaccine given at an interval of eight weeks was both superior to one dose ($P<0.05$) and not significantly inferior to the combined Brucella ovis/Strain 19 technique (Buddle, 1962). This experiments result was based on the proportion of animals that resisted an intravenous challenge consisting of approximately 9×10^6 Brucella ovis organisms. Out of 35 animals vaccinated with two 1ml doses of the Brucella ovis saline-in-oil vaccine, given eight weeks apart, eight became infected (23%), as compared with eight out of 33 vaccinated with 5ml of Strain 19 and 1ml

of the Brucella ovis saline-in-oil vaccine (24%), and 19 out of 35 vaccinated with a single dose of the Brucella ovis vaccine (54%). Again, it is notable that even with the most protective method of vaccination, nearly 25% of rams still became infected.

When the interval between the two doses of Brucella ovis saline-in-oil vaccine was increased from eight to 24 weeks, the resistance produced was still not significantly inferior to that from the combined Brucella ovis/Strain 19 technique (Buddle et.al., 1963). In this latter experiment, four out of 41 rams (10%) vaccinated twice with the Brucella ovis vaccine became infected after intravenous inoculation using approximately 4.7×10^7 Brucella ovis organisms, as compared with four out of 43 (9%) vaccinated by the combined Brucella ovis/Strain 19 technique, and 18 out of 42 (43%) of those given a single dose of the Brucella ovis vaccine. Once again, a significant proportion of vaccinated animals became infected.

Because of the problems associated with the method using Strain 19, and the comparable immunity provided by the regime of two doses of the Brucella ovis saline-in-oil vaccine given eight to 24 weeks apart, this two-dose technique became the most common practice for vaccinating rams against Brucella ovis (Quinlivan and Wallace, 1975).

Unfortunately, commercially manufactured Brucella ovis vaccines were not all equally effective. Claxton (1968) investigated two Brucella ovis saline-in-oil vaccines in use in Australia and found one to be very effective, while the other was not effective at all.

The local reactions that had been observed earlier by Buddle (1962) and Claxton (1968) were still causing concern in 1975, when Quinlivan and Wallace highlighted the problem in a letter to the New Zealand Veterinary Journal. These two veterinarians suggested that the unsightly subcutaneous lesions that followed vaccination (particularly arising from the double Brucella ovis method) were a reason why vaccination of rams against the disease had fallen out of favour with both stud breeders and commercial flockmen. They argued that following the recognition that the disease was once again

emerging as a production-limiting problem, interest in controlling the disease had revived. They then went on to describe a technique of intraperitoneal vaccination that had been used as an alternative to the subcutaneous route in order to avoid the development of these unsightly lesions.

The technique involved the use of a short bevelled needle inserted through the right flank with the sheep in a standing position. They reported that approximately 6,500 rams had been vaccinated by this method during 1974 and 1975 and that no lesions at the site of injection or any other undesirable side effects had been recorded in any of them. It was not stated whether these rams had been vaccinated once or twice. They also reported the results of a pilot trial involving 40 rams, half of which were vaccinated once intraperitoneally and half of which received a second injection eight weeks after the first. It was reported that all the trial animals displayed a titre to the Brucella ovis complement fixation test during an eight month period in which they were tested at monthly intervals, and that there had been no difference in titre between those vaccinated once and those vaccinated twice. It was also reported that post-mortem examination of a proportion of trial rams showed only a small number which had localised lesions in the musculature of the right flank. It was not stated how many post-mortem examinations had been carried out, or how long after vaccination they had been performed.

In 1978, an issue of the Animal Health Division's publication "Surveillance" reported that of a line of 40 surplus two-year old stud rams sent for slaughter, almost all had been found to have "abscesses" in the muscle of what was described as the "perirenal area" (probably meaning the dorsal flank region). These "abscesses" were seen under microscopic examination to contain large numbers of Brucella-like organisms, but no bacteria were grown from the lesions. The rams had been vaccinated once intraperitoneally using the Brucella ovis vaccine. The report concluded that due to the risk of abscess formation following this technique, and as its efficacy had not been proven, the double subcutaneous method would seem preferable (Surveillance, 1978).

Quinlivan and Wallace (1979) responded by stating that work "over a period of years" had demonstrated indistinguishable titre patterns in rams vaccinated by the intraperitoneal technique and the double subcutaneous method. They reported that 60,000 rams had been vaccinated by the Central Hawkes Bay group using the intraperitoneal method in the last five years, and that no problems with abscess formation had been encountered. They repeated their opinion that the unsightly lumps caused by the subcutaneous method had been a factor in the discontinuance of vaccination by ram breeders and the consequent upsurge in the incidence of the disease. They concluded that, in the incident reported in Surveillance, an error in administration must have occurred, and described their technique in full. This was based on the method described in an addendum to a paper by Thomson, Batty, Thomson, Kerry, Epps and Foster (1969) and involved an injection on the right side, at a point midway on a line between the stifle joint and the vertebral articulation of the last rib, in the lower extremity of the sublumbar fossa, using a short bevel 16-18 gauge 3/4" (19mm) needle.

In reply to the letter of Quinlivan and Wallace, Brooks (1979) expressed his concern that the efficacy of the intraperitoneal vaccination technique had not been established. He suggested that as Thomson et.al. (1969) had demonstrated rapid clearance of antigen from the peritoneal cavity, the resulting immune response might be inadequate. He also recalled that Buddle et.al. (1963) had shown that two doses of the vaccine were required to provide protection equivalent to the original combined Brucella ovis/Strain 19 vaccination technique, and questioned the evidence that a single intraperitoneal dose provided adequate protection.

Another incident involving lesions in rams sent for slaughter was reported in "Surveillance" in 1980. Ten to twenty percent of a line of approximately 100 rams slaughtered at the Tomoana Freezing works at Hastings were affected. The lesions were 5-10cm in diameter and were described as "raised, flat and fibrous with firm yellow centres". They were situated on the right flank, just beneath the peritoneum. Histological examination showed them to be granulomatous, with giant cells, macrophages and lymphocytes surrounding necrotic

tissue. The author noted that Meat Division veterinarians in the Hastings works had periodically in the last few years been called to examine lines of culled rams which at post-mortem inspection had lesions in the area of the right flank, and that it seemed likely that these lesions resulted from Brucella ovis vaccination using the intraperitoneal route.

The major unanswered questions in this debate about the use of the one-dose intraperitoneal Brucella ovis vaccination technique were summarised by Ris (1979) as follows:

1) "Is intraperitoneal administration of saline-in-oil vaccines acceptable in terms of safety and lack of adverse effects?"

2) "Does a single intraperitoneal inoculation of Brucella ovis saline-in-oil vaccine give satisfactory protection?"

The importance of resolving these questions lies in the need to understand the uses and limitations of Brucella ovis vaccines, so that their place in control programmes can be more accurately assessed. Large numbers of rams in New Zealand are currently involved in Brucella ovis control programmes. In stud flocks, where this disease is of the greatest concern, most rams are inspected by veterinarians for this disease (Bruere, 1982). Ram breeders have a responsibility to their clients, and their livelihood depends upon a reputation for producing good quality, disease-free rams. Such a reputation would be destroyed if it was found that these rams had a venereal disease. The possibility of exporting breeding stock would also be precluded by the presence of Brucella ovis in a flock.

The presence of vaccinated animals can cause special problems in eradication schemes based on serological testing. Vaccinated animals will react to the test whether infected or not. Furthermore, it is sometimes difficult to identify positively which animals have or have not been vaccinated because of poor record-keeping and inadequate animal identification. Of even more concern is the observation that some vaccinated rams may be excreting the organism, and acting as a source of infection for other rams (Bruere, 1982).

On the other hand, vaccination could be a very useful aid in controlling ovine brucellosis in situations where eradication would be difficult, for instance, where stock control is poor, fencing inadequate, and neighbouring flocks are infected. It is therefore important that the unanswered questions concerning the efficacy and side effects of the vaccination techniques currently in use be adequately resolved.

CHAPTER 2

INVESTIGATIONS ON THE CHARACTERISTICS OF BRUCELLA OVIS ISOLATES FROM VARIOUS SOURCES, INCLUDING THOSE USED FOR VACCINE MANUFACTURE AND THOSE USED FOR EXPERIMENTAL INFECTION IN THIS PROJECT.

INTRODUCTION

Brucella ovis was described by Buddle and Boyes (1953) as a small gram-negative bacillus, 0.7 - 1.2 µm long and 0.5 - 0.7 µm wide. The bacterium is non-motile, non-sporing, and arranged singly or in pairs. It has a negative reaction in the oxidase, urease, and citrate tests, does not produce indole, does not reduce nitrates, and does not produce hydrogen sulphide (H_2S). No change in the reaction of litmus milk is observed after seven days. No acid or gas is produced after 14 days from arabinose, dulcite, glycerol, inosite, lactose, levulose, maltose, mannite, raffinose, rhamnose, salicin, sorbite, sucrose, trehalose, xylose, and several other carbohydrates. The bacteria are acid-fast when stained by the modified Ziehl-Neelsen technique (Stamp et.al., 1950). The colonies formed after 48 hours incubation on blood agar in 10% CO_2 at 37°C are 0.5mm in diameter, circular, convex, smooth, greyish white and glistening, with an entire edge. After five days incubation the colonies are up to 2mm in diameter and greyish yellow. (Buddle and Boyes, 1953; Alton, Jones and Pietz, 1975; Bergeys Manual, 1984).

In New Zealand, the most important organism from which Brucella ovis must be distinguished is Brucella abortus, which has positive oxidase, urease and nitrate reactions and usually produces hydrogen sulphide (Bergeys Manual, 1984).

IDENTIFICATION OF ISOLATES

During this project, isolates of Brucella ovis were routinely subjected to the following tests:

Citrate Utilisation
H_2S Production
Nitrate Reduction
Motility
Oxidase Reaction
Urease Reaction

(See Appendix 1 for details).

In order to test further the identity of the organism, the isolate used as the challenge organism in experiments involving artificial exposure by intravenous inoculation was also subjected to additional biochemical tests using the carbohydrates arabinose, dulcite, glycerol, inosite, lactose, levulose, maltose, mannitol, raffinose, rhamnose, sorbite, salicin, sucrose, trehalose, and xylose. No significant production of acid or gas was observed over a 21 day period, confirming the findings of Buddle and Boyes (1953).

TEMPERATURE TOLERANCE

Buddle and Boyes (1953) reported the optimum temperature for the growth of Brucella ovis to be 37°C with limits for growth between 20°C and 40°C. When isolates from several sources were tested during this project, it was found that at 25°C and 42°C, growth did occur, but only extremely slowly. Colonies just visible to the naked eye appeared between 14 and 21 days. There did not seem to be any obvious differences between the isolates in their tolerance of unfavourable temperatures.

BRENDA ANALYSIS

In an attempt to discover whether different strains existed among the Brucella ovis isolates that were encountered in this project, seven selected isolates were subjected to analysis by the BRENDA technique (Bacterial Restriction Endonuclease DNA Analysis).

Method

This method involves the extraction of DNA from a homogeneous population of organisms (in this case a cloned isolate of Brucella ovis), digestion of the resultant DNA with a restriction endonuclease, and electrophoresis of the digested DNA on agarose gel. The endonuclease cleaves the DNA at specific base-pair sequences, resulting in a set of DNA fragments. These fragments migrate in the gel according to their molecular weight, resulting in a pattern of bands that, when stained with ethidium bromide, can be seen in ultraviolet light. These patterns constitute a characteristic "fingerprint" for that specific DNA, and can be used to identify subspecies of bacteria (Marshall, Wilton and Robinson, 1981).

For the treatment of the Brucella ovis isolates in this investigation, the restriction endonuclease $EcoR_1$ was used, and the method was essentially the same as that described by Marshall et.al. (1981) and Marshall, Winter, Cooper and Robinson (1985). The Brucella ovis bacteria were harvested in phosphate buffered saline from two blood agar plates.

The seven isolates tested are described in Table 2.1

Table 2.1

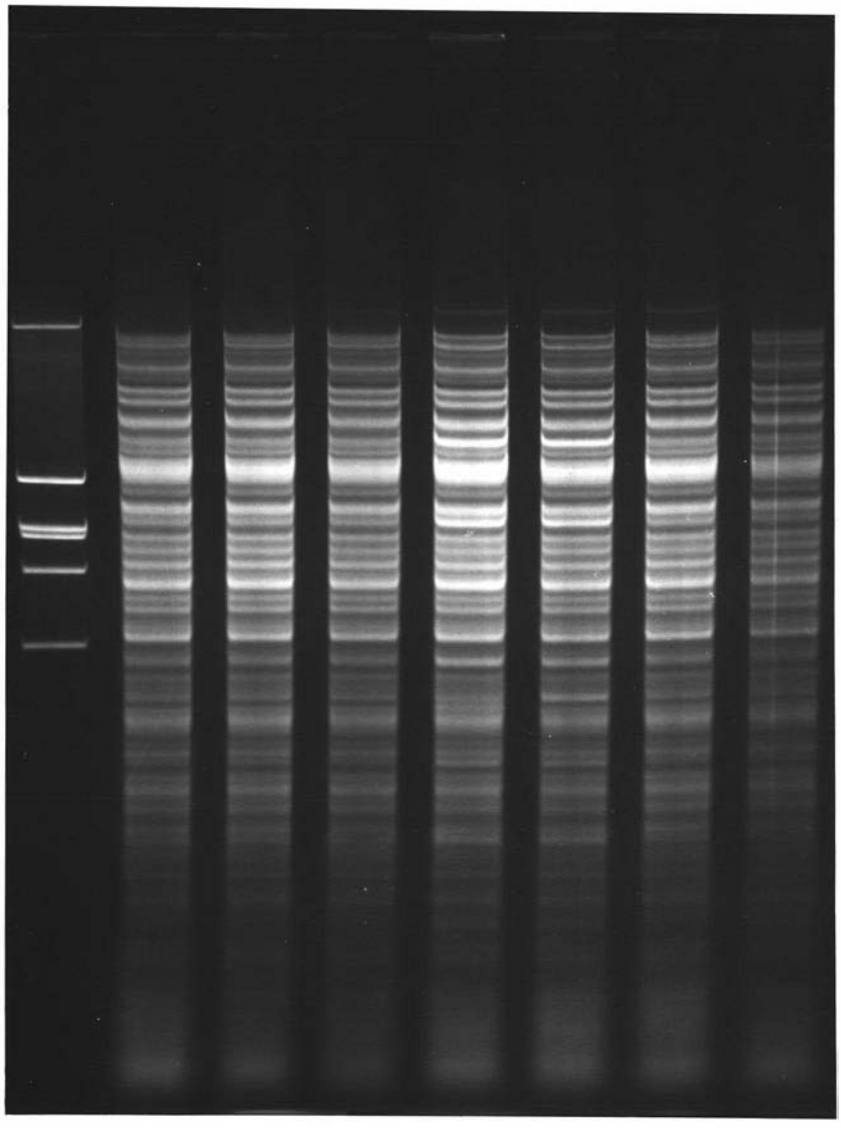
Brucella ovis Isolates Tested By Bacterial Restriction Endonuclease DNA Analysis (BRENDA)

NUMBER	IDENTITY OF ISOLATE	DESCRIPTION
1	Challenge	Isolated from the semen of a naturally infected ram and used as the challenge organism throughout this project.
2	Vaccine A	Freeze-dried culture used in the manufacture of the Brucella ovis saline-in-oil vaccine "Ramovexin" (Coopers Animal Health N.Z. Ltd), and provided by the manufacturers.
3	Vaccine B	Freeze-dried culture of a second isolate used in the manufacture of the vaccine "Ramovexin", and provided by the manufacturers.
4	NCTC 10037	Freeze-dried culture obtained from the National Type Collection, London.
5	Field 1	Isolated from the semen of a chronically infected ram having an inconsistent reaction to the Brucella ovis complement fixation test.
6	Field 2	Isolated from the semen of a ram in a naturally infected flock; Manawatu, New Zealand.
7	Field 3	Isolated from the semen of a ram in a second naturally infected flock; Manawatu, New Zealand.

Plate 2.1

Band patterns resulting from Bacterial Restriction Endonuclease DNA Analysis (BRENDA) carried out on the seven Brucella ovis isolates described in Table 2.1. The number allocated to the isolate in Table 2.1 is the lane number in the gel as shown in this plate. The isolates in each lane, as described in Table 2.1, are as follows:,

Lane: 1 – Challenge
2 – Vaccine A
3 – Vaccine B
4 – NCTC 10037
5 – Field 1
6 – Field 2
7 – Field 3



standard 1 2 3 4 5 6 7

Results

No obvious differences between patterns could be seen when this endonuclease was used (Plate 2.1).

There is the possibility that the first high molecular weight band (at the top of the pattern) which is quite clear in the NCTC 10037, Field 1 and Field 2 isolates, and less obvious in Vaccine B and Field 3, may actually be absent in the Challenge and Vaccine A isolates, and it could be argued that this indicates a strain difference. However, it is quite likely that this band, being at the high molecular weight end, may have resulted from incomplete digestion of the DNA, and could therefore be an artifact (R.B. Marshall, personal communication, 1986). If this is the case, it indicates that the isolates tested are all essentially the same strain. This conclusion is supported by the fact that no biovars are recognised (Bergeys Manual, 1984); that the Challenge and Field 1 isolates were from rams of the same age run together in the same flock; and that Australian workers using an isoenzyme electrophoresis technique could not detect any genetic variation in an experiment on 12 isolates of Brucella ovis from five veterinary laboratories in New South Wales, South Australia and Victoria (A.J.M. Belfield and M.L. Adams, personal communication, 1984).

These studies indicate that the isolate used as the challenge strain in this project was in all probability the same strain as the two isolates used in the manufacture of the vaccine "Ramovexin" (Coopers Animal Health N.Z. Ltd). Therefore, this vaccine should have the capability of stimulating a specific immunity against the challenge organism.

THE BRUCELLA OVIS VACCINE

"Ramovexin" is the only *Brucella ovis* vaccine currently available in New Zealand. The New Zealand manufacturers (Coopers Animal Health N.Z. Ltd) estimate the annual usage to be 72,000 doses (W.G. Orbell, personal communication, 1984). It is a saline-in-oil emulsion vaccine containing inactivated *Brucella ovis* organisms. Two New Zealand isolates of *Brucella ovis* are used in the production of the vaccine. After microaerophilic culture on an agar medium, the organisms are harvested in saline and killed by the addition of formalin. After inactivation the bacterial concentration is standardised, and the suspension is filtered and emulsified with the adjuvant. This adjuvant consists of 95% mineral oil (a light grade paraffin oil) and 5% of the emulsifying agent Arlacel A. It is sterilised by filtration before use. The vaccine consists of 40% bacterial suspension and 60% adjuvant. The final bacterial concentration is 6×10^{10} *Brucella ovis* bacteria/ml. Phenol is added as a preservative, and the shelf life is six months.

"Ramovexin" is essentially the same vaccine as that described by Buddle (1957) as his "Adjuvant Vaccine C". Buddle's vaccine contained the same final concentration of formalin-killed *Brucella ovis* bacteria (6×10^{10}), and the adjuvant was similar. Minor differences in the original formulation were that only 3% Arlacel A was included in the adjuvant (5% in "Ramovexin"), the mineral oil had a different name, and the proportion of adjuvant to bacterial suspension was 50:50 (60:40 in "Ramovexin"). This "Adjuvant Vaccine C" was used in Buddle's 1957 and 1958 experiments which proved its effectiveness when used in conjunction with *Brucella abortus* Strain 19. A vaccine essentially the same but containing half the bacterial concentration of "Adjuvant Vaccine C" (i.e. 3×10^{10} bacteria/ml) was used in the experiments of Buddle (1962) and Buddle *et.al.* (1963) when the use of two successive doses of the *Brucella ovis* vaccine without Strain 19 was investigated.

A single dose of the contemporary vaccine "Ramovexin" is 2ml. This would contain approximately 12×10^{10} bacteria, which is twice the number in the 1ml dose of "Adjuvant vaccine C" used in Buddle's 1957 and 1958 experiments, and four times that in the 1ml dose of the vaccine in the experiments of 1962 and 1963. It would be expected, therefore, that if any differences in efficacy existed between the modern vaccine and the preparations used by Buddle, the contemporary vaccine would be the one more likely to provide greater protection against infection.

In accordance with standard vaccine manufacturing procedures, the following tests are undertaken routinely on batches of "Ramovexin":

1) Sterility - Fourteen days incubation at 22°C and 30°C.
2) Safety - Two sheep injected subcutaneously and observed for seven days.
3) Toxicity - Five mice and two guinea pigs injected and observed for seven to ten days
4) Stability - at 37°C for seven days (observed for emulsion breakdown) and at 22°C for six months (shelf life)
5) Chemistry - to ensure concentrations of phenol and free formalin are within acceptable limits.

The vaccine manufacturers have no record of any formal complaint against the vaccine's performance. (W.G. Orbell, personal communication, 1984).

CONCLUSION

The apparent lack of diversity among Brucella ovis isolates and the reported nature of the vaccine indicate that "Ramovexin" should stimulate immunity at least equal to that demonstrated by the formulation used in Buddle's experiments. The following chapters describe investigations into various aspects of its use in the protection of rams against Brucella ovis infection.

CHAPTER 3

THE SEROLOGICAL RESPONSE OF RAMS TO VACCINATION AGAINST BRUCELLA OVIS INFECTION

INTRODUCTION

The measurement of serological titres is a well established method of assessing the response of the immune system to vaccination or infection (Herbert, 1970; Stites, 1976a).

The majority of serological tests measure circulating antibody levels by testing the serum for the presence of specific immunoglobulins. Some tests are simply qualitative (e.g. the gel immunodiffusion test). The results of these tests are reported as either positive (antibody present) or negative (antibody absent). Other tests provide quantitative information about the concentration of antibody present in the sample. Some, like the complement fixation test (CFT), are performed on serial dilutions of the serum. The number of dilutions which can be carried out before antibody is no longer detectable is an indication of the concentration of antibody in the original sample. Other tests, such as the enzyme linked immunosorbent assay (ELISA), measure the intensity of the response to the test, a more intense reaction indicating a greater concentration of antibody.

Serological tests such as these measure immunoglobulins produced by transformed B-lymphocytes (plasma cells) which multiply in response to an antigenic stimulus (Marchalonis, 1976). It is widely accepted that although resistance to Brucella species involves both humoral (B-cell) and cellular (T-cell) responses, antibody alone is not protective, and immunity depends on the presence of cell-mediated mechanisms (Collins and Campbell, 1982).

It would therefore be more indicative of the status of resistance to also measure the level of cell-mediated immunity which develops in response to an antigenic stimulus. However, in-vitro laboratory tests of cell-mediated immunity are technically demanding, and results are sometimes inconsistent and difficult to interpret (Herbert, 1970; Stites, 1976b). Serological tests are much more practical to perform, especially when large numbers of samples are involved, and when a well controlled, established test is available. The Brucella ovis complement fixation test used as a standard diagnostic technique in New Zealand is such a test. It is also known that humoral and cell-mediated immunity are not separate and independent reactions, but are both aspects of the total response to an antigenic stimulus (Marchalonis, 1976). Therefore by measuring levels of circulating antibody using a serological test, we may obtain an indirect estimate of the resistance to the disease.

Accordingly, it was decided to carry out a study of the serological response to vaccination against Brucella ovis using a commercially available inactivated Brucella ovis saline-in-oil vaccine[1].

This vaccine is based on the preparations used by Buddle in his experiments on the vaccination of rams against Brucella ovis infection (Buddle, 1957, 1958, 1962; Buddle et.al., 1963). (See Chapter 2). In his earlier experiments, the Brucella ovis saline-in-oil vaccine was used as a single dose in conjunction with a simultaneous dose of Brucella abortus Strain 19. (Buddle, 1957, 1958). Buddle's later experiments (Buddle, 1962, Buddle et.al., 1963) showed that two doses of the inactivated Brucella ovis saline-in-oil vaccine given a few weeks apart produced a degree of immunity comparable with that produced by the combined Brucella ovis/Strain 19 technique. These latter experiments formed the basis of the recommendations made by the manufacturers of the commercial Brucella ovis vaccine "Ramovexin" (Coopers Animal Health N.Z. Ltd), that the vaccine be given twice subcutaneously 8-24 weeks apart as an alternative to its use in conjunction with Strain 19.

This two-dose procedure subsequently became the common practice in New Zealand (Quinlivan and Wallace, 1975). However, this method of vaccination later appeared to fall out of favour with some farmers. In the opinion of some veterinarians this was due to the unsightly subcutaneous lesions produced by the vaccine at the site of injection (Quinlivan and Wallace, 1975). These authors suggested that the vaccine be administered by the intraperitoneal route in order to avoid these visible lesions, and reported that a pilot trial on 40 rams had shown that all the rams vaccinated this way developed serological titres, there being no difference between those vaccinated once and those vaccinated twice (Quinlivan and Wallace, 1975). Thus they were advocating not only a change in the route of administration from subcutaneous to intraperitoneal, but also a reduction in the number of injections, changing the vaccination process from a two-dose to a one-dose regime. This was undoubtedly more convenient for the farmer, but the only evidence of the method's efficacy was a later statement by these authors that work "over a period of years" had demonstrated indistinguishable titre patterns in rams vaccinated by the intraperitoneal technique and those vaccinated by the double subcutaneous method (Quinlivan and Wallace, 1979).

A large number of rams in New Zealand have subsequently been vaccinated against Brucella ovis infection using a single intraperitoneal vaccination as an alternative to the double subcutaneous regime, and these two techniques are currently the most widely used methods of vaccination against Brucella ovis (D.M. West, personal communication, 1986).

No detailed information was available on the comparative effectiveness of these two methods, and it was hoped that the examination of the serological response to the vaccination techniques would provide a preliminary basis for comparison.

The serological responses to a single intraperitoneal and a double subcutaneous vaccination regime using the commercial Brucella ovis vaccine[1] were compared in the following trial. A single subcutaneous and a double intraperitoneal regime were also included for comparative purposes.

In an attempt to provide some quantitative information about the size and persistence of the subcutaneous lesions which had prompted the introduction of the intraperitoneal technique, it was decided in addition to measure any lesions which might result from the subcutaneous vaccinations carried out in this trial.

NOTES

1) "Ramovexin" - Coopers Animal Health N.Z. Ltd. Inactivated *Brucella ovis* saline-in-oil vaccine preparation.

MATERIALS AND METHODS

Sheep

One hundred and fifteen flock rams from three commercial sheep farms in the Manawatu area were used in this trial. The three farms were all accredited Brucella ovis-free properties. The rams were of various breeds including Romney, Coopworth, Perendale, Booroola and Suffolk. Their ages ranged from two to five years, apart from one ram in the control group which was nine years old. All the rams had palpably normal external genitalia when examined at the start of the trial.

Allocation to Experimental Groups

On each property the rams were sorted by age, and then rams from each age group were randomly allocated to the four treatment groups, so that the groups were of equal sizes and contained a similar age distribution.

Three to five rams from each property were allocated as controls. These were left unvaccinated as sentinel animals to detect any entry of Brucella ovis infection into the flock. As this was considered an unlikely possibility on these farms due to their history of freedom from the disease and good control of stock movement, a total of only 11 rams were used as controls.

There were 26 rams in each of the four treatment groups and a total of 48 rams from Farm A, 34 from Farm B and 33 from Farm C were used.

The treatments allocated to the five experimental groups were as follows:

Group 1	single subcutaneous vaccination (1 s/c)
Group 2	single intraperitoneal vaccination (1 i/p)
Group 3	double subcutaneous vaccination (2 s/c)
Group 4	double intraperitoneal vaccination (2 i/p)
Group 5	unvaccinated (control)

The rams in the four treated groups were vaccinated according to the schedule shown in Table 3.1

Vaccination

A commercial, killed, Brucella ovis saline-in-oil adjuvant emulsion vaccine[1] was used. The vaccine was from a single batch (series 2935), and the dose was 2ml.

After the rams had been individually identified, allocated to experimental groups, and had preliminary blood samples taken, the individuals allocated to the double subcutaneous (2 s/c) and double intraperitoneal (2 i/p) groups received their first "sensitising" dose of vaccine. Rams in the 2 s/c group were vaccinated subcutaneously on the right side of the neck using a new 18 gauge 9mm needle and sterile syringe for each individual. The greatest care was taken to ensure that the vaccinations were carried out in a clean manner and that the correct technique was used. Rams in the 2 i/p group were vaccinated intraperitoneally using the method of Thomson et.al. (1969) as follows:

An assistant holds the sheep so the right flank is presented. The injection site is the lower extremity of the paralumbar fossa, at a point midway on a line joining the stifle joint and the vertebral articulation of the last rib. If the heel of the hand is placed on the transverse processes of the lumbar vertebrae, the fingers fall into the lower part of the flaccid abdominal wall above the point

where resistance from the small intestine is felt. This is the site. An 18 gauge 19mm needle with a short bevelled point is inserted to its full length at right angles to the body surface, and the vaccine injected. This is the same technique recommended by Quinlivan and Wallace (1979).

Ten weeks after this first vaccination was given, rams in the 2 s/c and 2 i/p groups were again vaccinated. The procedure was the same except that the left side of the neck was used for the rams in the 2 s/c group rather than the right. On the same day, rams in the single subcutaneous (1 s/c) and single intraperitoneal (1 i/p) groups were vaccinated; the 1 s/c group subcutaneously on the left side of the neck, and the 1 i/p group intraperitoneally.

Blood Collection

Blood samples were collected from all the rams in the experiment according to the experimental schedule shown in Table 3.1.

Blood was collected by jugular venepuncture using 10 ml glass vacuum tubes containing no anticoagulant, tube holders and 20 gauge 25mm needles[2]. Two full tubes were collected from each ram. After labelling, the tubes were immediately placed on sloping trays, and these were left to clot overnight in a cool place. They were not refrigerated as haemolysis seemed to occur more frequently if this was done.

The next day, the blood was centrifuged in a Sorvall GLC 1 centrifuge at 822 x g (2,000 rpm) for ten minutes. The serum was drawn off using a Pasteur pipette and dispensed into 2ml plastic autoanalyser cups[3]. It was usually possible to fill six cups with the serum from the two tubes from each ram. One vial of serum from each ram was sent for analysis immediately and the surplus stored at -20°C.

Serology

The serum samples were sent to the Central Brucellosis Laboratory of the Wallaceville Animal Research Centre, Upper Hutt, New Zealand. There they were tested for the presence of antibody to *Brucella ovis* by three methods:

1) Complement Fixation Test (CFT)
2) Enzyme Linked Immunosorbent Assay (ELISA)
3) Gel Diffusion Test (GD)

These tests are described in Appendix 2.

The results of the complement fixation test are reported according to the degree of haemolysis observed at the different dilutions of serum used in the test. The dilutions used are: 1/4, 1/8, 1/16, 1/32, 1/64 and 1/128.

100% haemolysis is reported as 0 (no antibody detected)
75% " " " " 1
50% " " " " 2
25% " " " " 3
0% " " " " 4

The results are therefore reported according to the format illustrated in Table 3.2:

Table 3.2

Examples of the Format used in Reporting Complement Fixation Test Results

SERUM DILUTION	HAEMOLYTIC REACTION			
1/4	4	4	4	0
1/8	4	4	1	0
1/16	4	4	0	4
1/32	4	2	0	0
1/64	4	0	0	0
1/128	4	0	0	0
Reported result	4^{6}	$4^{3}2$	4.1	$-^{2}4$

The last result is an example of the "prozone" phenomenon thought to be caused by excess antibody (R.W. Worthington, personal communication, 1983).

In the analysis of results, the sum of the reactions at each dilution was taken to give a test "score". Although the scoring system was not strictly mathematically correct for use in statistical comparisons, this system enabled the data to be far more easily managed. In the case of prozones, the reaction at each dilution affected was given the value 4. Examples of the scoring system as applied to the values in Table 3.2 are as follows:

$4^6 = 24$
$4^3 2 = 14$
$4.1 = 5$
$-^2 4 = 12$

The results of the ELISA test were reported as units of colour intensity as read by a microtitre plate reader (see Appendix 2), and so were already in an easily usable format.

The results of the gel diffusion test were reported as positive, negative or suspicious (see Appendix 2).

The interpretation parameters used for the other two tests are shown in Table 3.3

Table 3.3

Interpretation Parameters used for the **Brucella ovis** Complement Fixation and Enzyme Linked Immunosorbent Assay Tests

	CFT (score)	ELISA (units)
NEGATIVE	0-4	0-69
SUSPICIOUS	5-7	70-89
POSITIVE	8-24	$\geq$90

All batches of samples were sent to Wallaceville Animal Research Centre the day after they were collected and subjected to all three serological tests. At the conclusion of the trial, all the samples were retested by the ELISA method, using the stored frozen serum samples. This was done to minimise the effects of between-test variation and alterations in interpretation which had been occurring as the test was being developed, to check some anomalous results, and to evaluate the repeatability of the test.

When the results of this second test were compared with the original results, there were a number of cases in which the two figures were in different interpretation categories. e.g. one "positive" and one "negative" or "suspicious". Such samples were subjected to further retesting, when each was tested four times, giving a total of six results for each sample from which a final result could be estimated. It was decided that the mean titre of these results should be used as the final result and in all subsequent analyses. If one result was markedly different from the other five, it was omitted, otherwise, all six figures were used in the calculation of the mean. These obviously anomalous results were almost invariably from the original test, probably reflecting the fact that the test was still being developed when these original readings were made.

For those sera which did not have to be subjected to the second checking procedure, the first retest result was used in the final analysis rather than the original. This was done because these tests were carried out as a single batch and so the results should be more comparable, one with another.

As the complement fixation test was a standard procedure employing well designed quality control measures using standard sera to detect changes in sensitivity, it was not considered necessary to repeat the test on all the samples at the end of the experiment. However, samples which seemed to have given unexpected results were

retested e.g. a control animal having one positive result but negative throughout the rest of the trial, or a ram with a consistently high titre having a sudden, temporary drop in titre on a single occasion.

Samples which had been anticomplementary and thus given no result were also retested.

When the retest results were compared with the original figures, many were identical, and these were not investigated further. Those that were different were subjected to a second retesting, so that three results were available. If two of the three results were the same, that titre was taken to be the final result. If they were all different, the middle titre was taken as the result. If there were only two titres available (in the case of anticomplementary samples), the mean of the two results was used. Samples which were consistently anticomplementary were left out of the subsequent analysis.

The gel diffusion tests were not repeated.

On various occasions throughout the trial, rams would be missing from the mob either temporarily (e.g. missed the muster or being used for mating) or permanently (e.g. sold or died). This meant that the number of rams in a treatment group was as low as 18 in one group on one occasion, but usually the number ranged between 22 and 26. This variation was carefully calculated for in the subsequent analysis of the results.

Measurement of Lesions caused by Subcutaneous Vaccination

At each visit, the subcutaneous vaccination sites were palpated. If no lesion could be identified, this fact was recorded. If a lesion was palpated, it was measured across its width using callipers of the type used for measuring tuberculin reactions in cattle. Asymmetrical lesions were measured on a cranio-caudal (horizontal) axis. This was usually the smaller dimension.

In addition, the number of lesions discharging necrotic material through a sinus was recorded.

Statistical Methods

To evaluate the significance of differences in group means in this experiment, 95% confidence limits were used. (See Appendix 6)

For qualitative data, the chi-squared method was used to test the significance of any differences between groups. Yate's correction was always employed in these calculations, as there was only one degree of freedom. A difference at the 5% level of significance ($P<0.05$) was chosen as that which was to be considered statistically significant for the purposes of this study. (See Appendix 6)

Sensitivity, Specificity and Repeatability Measurements

A) The sensitivity estimates made for the serological tests were calculated as follows:

Sensitivity (%) = 100 - percentage of false negatives

Results were classified as "false negative" if they were obtained from the serum of vaccinated rams which had a positive result to the test on a prior and subsequent occasion.

B) The specificity estimates made for the serological tests were calculated as follows:

Specificity (%) = 100 - percentage of false positives

Samples giving a positive result were classified as "false positive" when they were obtained from unvaccinated rams, as these animals were free of natural infection.

C) The repeatability estimates made for the serological tests were calculated as follows:

$$\frac{\text{number of samples producing results in same interpretation category (positive, negative or suspicious) when retested}}{\text{total number of samples retested}} \times \frac{100\%}{1}$$

NOTES

1) "Ramovexin" - Coopers Animal Health N.Z. Ltd. Inactivated Brucella ovis saline-in-oil vaccine preparation.

2) "Venoject" blood collection equipment - Terumo Corporation.

3) Elkay Laboratory Products U.K. Ltd.

Table 3.1

Schedule of Vaccination and Blood Collection Procedures In a Study Of The Serological Response of Rams to Vaccination Against Brucella ovis Infection

VISIT NUMBER	DATE	WEEK NUMBER	VACCINATE 1 s/c group & 1 i/p group	VACCINATE 2 s/c group & 2 i/p group	COLLECT BLOOD SAMPLES
1	9.8.83	0		+	+
2	24.8.83	2			+
3	20.9.83	6			+
4	18.10.83	10	+	+	+
5	1.11.83	12			+
6	29.11.83	16			+
7	20.12.83	19			+
8	24.1.84	24			+
9	13.3.84	31			+
10	30.5.84	42			+
11	10.7.84	48			+
12	10.9.84	57			+

RESULTS

Freedom From Brucella ovis infection

There was no evidence of the introduction of Brucella ovis infection into any of the three flocks during this experiment. None of the rams developed palpable lesions of epididymitis, and the unvaccinated control animals remained serologically negative to the Brucella ovis complement fixation test throughout the trial.

Complement Fixation Test

There were no positive or suspicious reactions to the complement fixation test at any time from animals in the control group. Among the vaccinated groups, there appeared to be some obvious differences in titre pattern following vaccination. (See Figure 3.1). In order to test the significance of these apparent differences, the 95% confidence limits of the means were calculated and the results plotted as shown in Figures 3.2 - 3.7. Where there is no overlap between the illustrated confidence limits for the groups, there is a 95% certainty that there is a real difference between them, not due to chance.

After the completion of the vaccination programme, the mean titre of the double subcutaneous group (2 s/c) was always the highest and that of the single intraperitoneal group (1 i/p) always the lowest.

The rise in mean titre after initial vaccination appeared to take place more slowly in the intraperitoneal groups (1 i/p and 2 i/p) than in the corresponding subcutaneous groups (1 s/c and 2 s/c). The mean titre of the intraperitoneal group in each case remained significantly lower than that of the corresponding subcutaneous group until at least 12 weeks after vaccination (Figures 3.2 and 3.3). The mean titre of rams vaccinated intraperitoneally also remained lower and declined more rapidly than that of rams vaccinated by a corresponding subcutaneous technique, although on only one occasion after 20 weeks was this difference significant statistically for the

2 s/c and 2 i/p groups, and on just two occasions for the 1 s/c and 1 i/p groups.

Figures 3.4 and 3.5 illustrate the difference in the titre which was produced by one dose of vaccine in comparison with two doses when the same route of administration was used. In the case of the subcutaneous route (Figure 3.4), once the mean titre of the single-dose group (1 s/c) had peaked, decline began almost immediately, whereas the mean titre of the double-dose group (2 s/c) remained relatively stable at peak for some time before significant decline occurred. The difference between the two means was quite marked from 24 weeks onward, but did not become significant statistically until the end of the experiment at 57 weeks. The difference between single and double vaccination was far more pronounced in the groups vaccinated by the intraperitoneal route (Figure 3.5). In this case, the mean titre of the single dose group (1 i/p) peaked at a low level and began a rapid decline. On only one occasion (at 24 weeks) was the difference between the groups not statistically significant, and this was when the mean titre of the 1 i/p group was at its peak while the mean titre of the 2 i/p group had started to decline.

Among the 1 s/c, 2 s/c and 2 i/p groups there were no great differences in pattern. (See Figures 3.3, 3.4 and 3.6). The greatest differences were seen when the 1 i/p group was compared to the other three, as shown in Figures 3.2, 3.5 and 3.7. The mean titre of this group was always lower than the means of the other three groups. It was significantly lower than that of the 1 s/c group on five out of eight occasions after vaccination (Figure 3.2), significantly lower than that of the 2 i/p group on seven out of eight (Figure 3.5), and significantly lower than that of the 2 s/c group on all eight occasions after vaccination (Figure 3.7).

At two weeks after initial vaccination, a greater proportion of rams that had been vaccinated subcutaneously were positive to the complement fixation test in comparison with those vaccinated intraperitoneally, but by six weeks after the initial vaccination, all vaccinated animals were positive regardless of the technique which had

been used. (See Figure 3.8).

All the rams vaccinated twice (i.e in the 2 s/c or 2 i/p group) remained positive to the complement fixation test throughout the experiment, except for one ram in the 2 i/p group which had reverted to a suspicious titre at the last test at 57 weeks.

The rams vaccinated only once did not fare so well. One ram in each of the two groups (1 s/c and 1 i/p) had reverted to a suspicious titre at 19 weeks, only nine weeks after vaccination. By 21 weeks, two animals were negative in the 1 s/c group and three in the 1 i/p group. At the end of the experiment, three of the remaining 23 animals in the 1 s/c group were negative (13%) and six out of the 24 in the 1 i/p group (25%). When suspicious titres were also considered, nearly 30% of the rams in this group (1 i/p) were found to have lost their positive titre status within a year of vaccination.

Figure 3.1

Serological response of rams to an inactivated Brucella ovis saline-in-oil vaccine administered either once or twice by either the subcutaneous or the intraperitoneal route. Mean complement fixation titre scores.

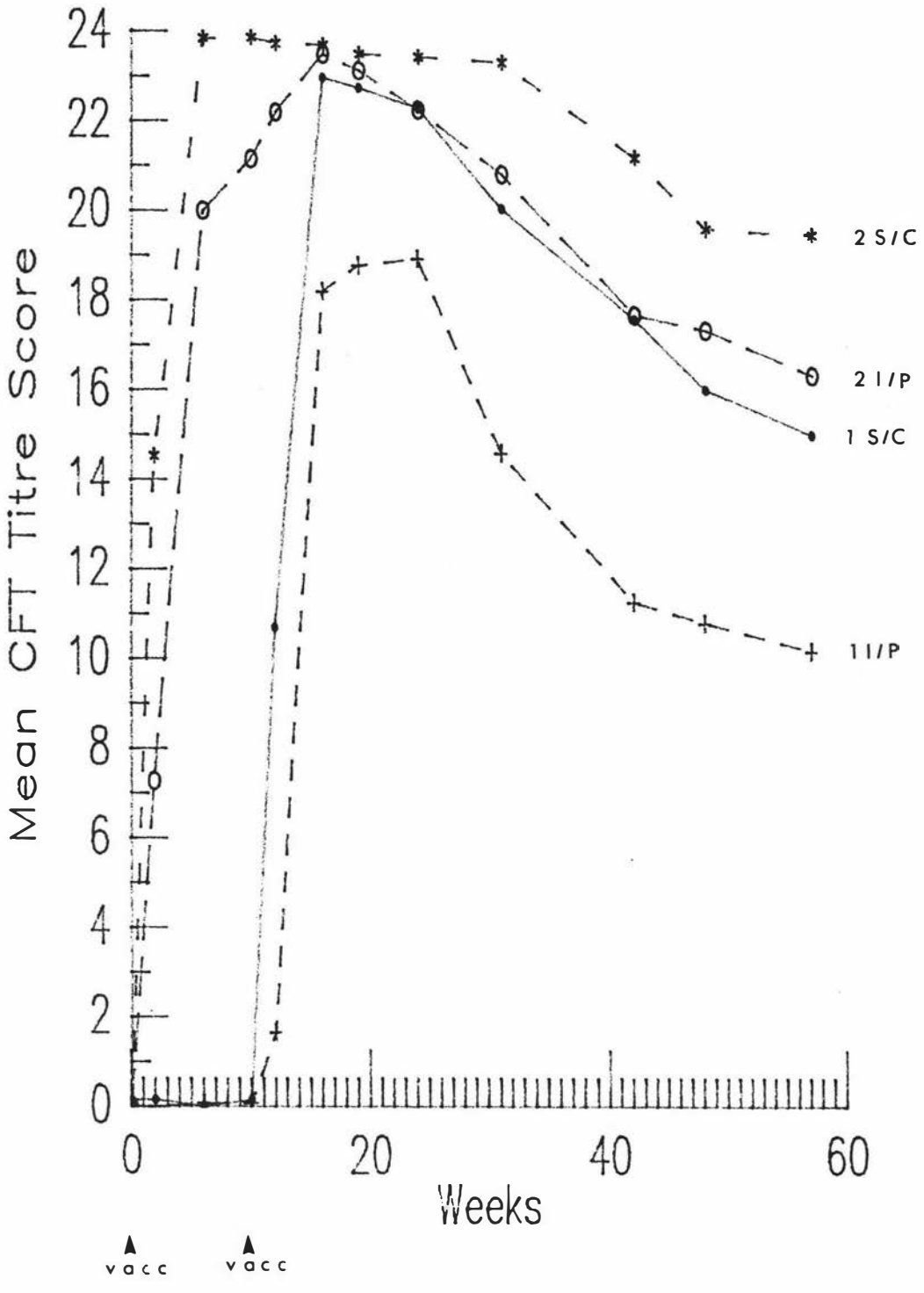
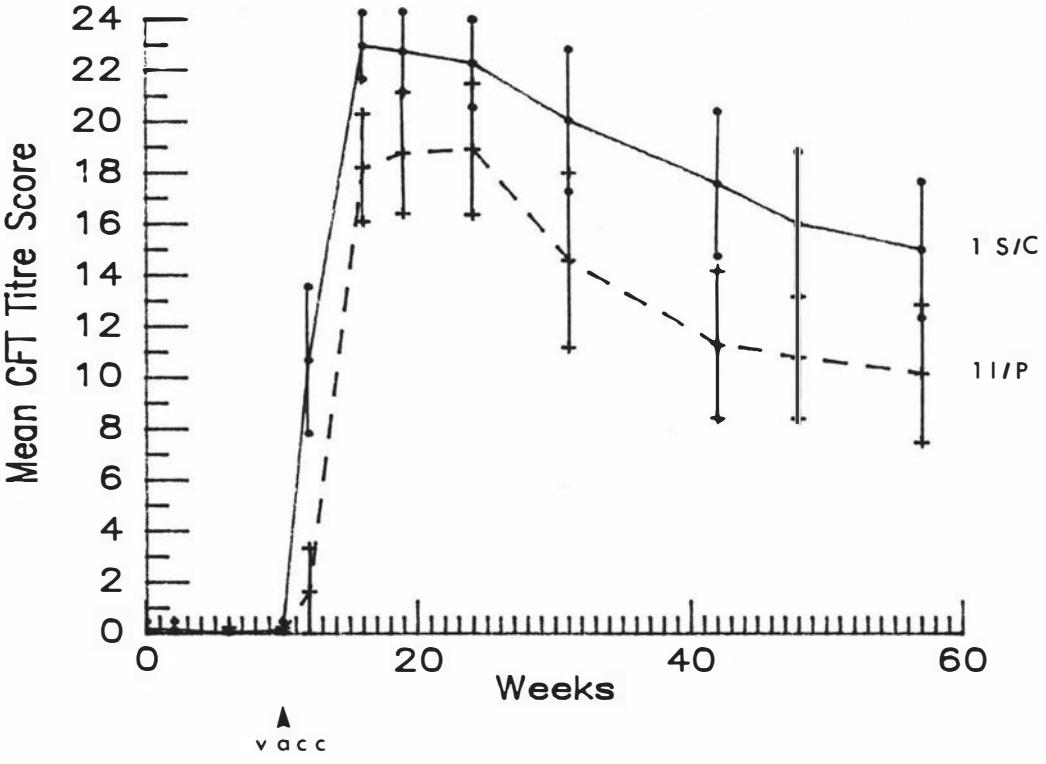

Mean CFT Titre Score
24
22
20
18
16
14
12
10
8
6
4
2
0
0
20
40
60
Weeks
vacc
vacc
2 S/C
2 I/P
1 S/C
1 I/P

Figure 3.2

Serological response of rams to an inactivated Brucella ovis vaccine administered once by either the subcutaneous or the intraperitoneal route. Mean complement fixation titre scores and 95% confidence limits.

Figure 3.3

Serological response of rams to an inactivated Brucella ovis vaccine administered twice by either the subcutaneous or the intraperitoneal route. Mean complement fixation titre scores and 95% confidence limits.


Mean CFT Titre Score
24
22
20
18
16
14
12
10
8
6
4
2
0
0
20
40
60
Weeks
vacc
1 S/C
1 I/P

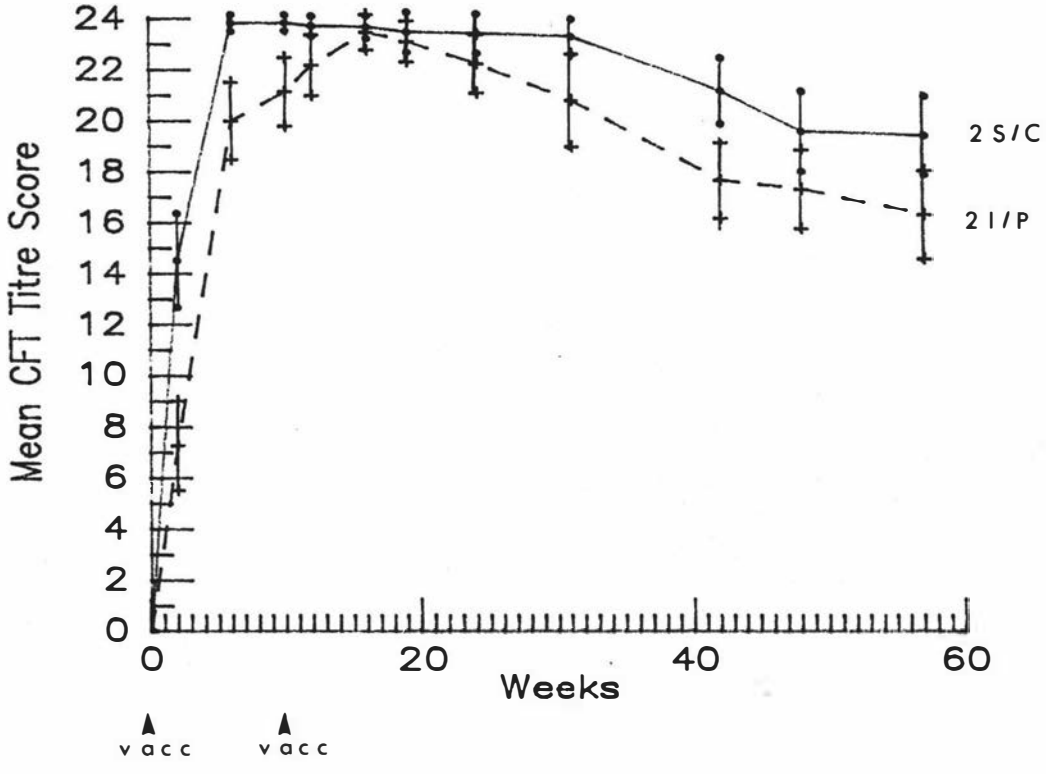
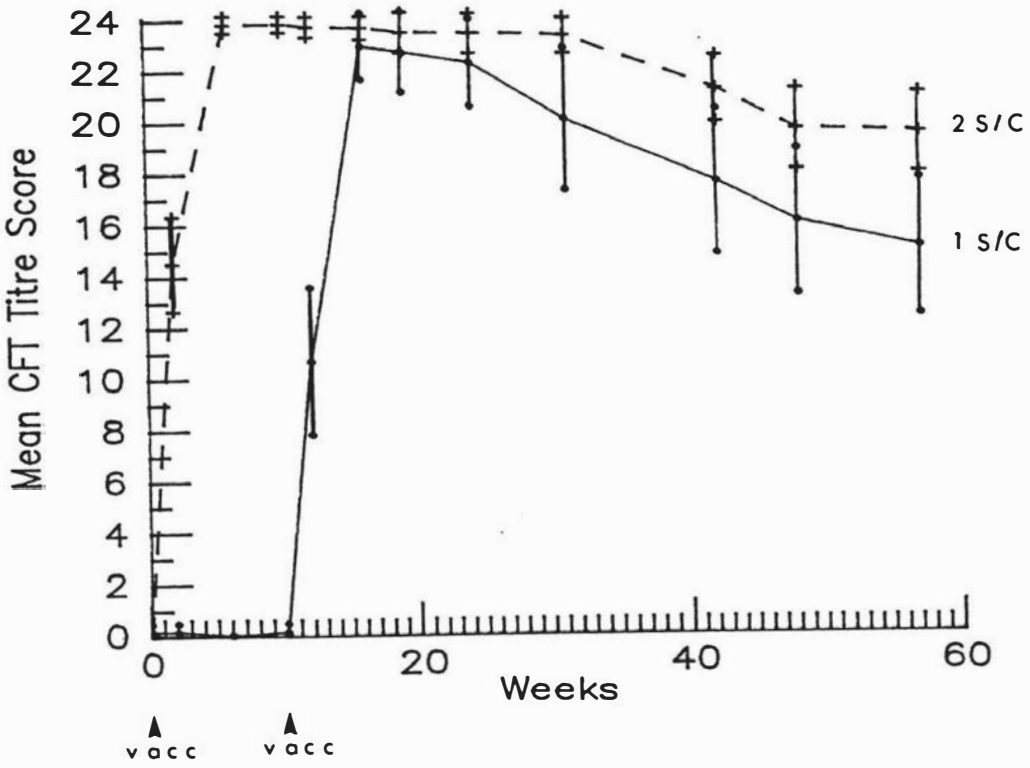

Mean CFT Titre Score
24
22
20
18
16
14
12
10
8
6
4
2
0
0
20
40
60
Weeks
vacc
vacc
2 S/C
2 I/P

Figure 3.4

Serological response of rams to an inactivated Brucella ovis vaccine administered either once or twice by the subcutaneous route. Mean complement fixation titre scores and 95% confidence limits.

Figure 3.5

Serological response of rams to an inactivated Brucella ovis vaccine administered either once or twice by the intraperitoneal route. Mean complement fixation titre scores and 95% confidence limits.


Mean CFT Titre Score
24
22
20
18
16
14
12
10
8
6
4
2
0
0
20
40
60
Weeks
vacc
vacc
2 S/C
1 S/C

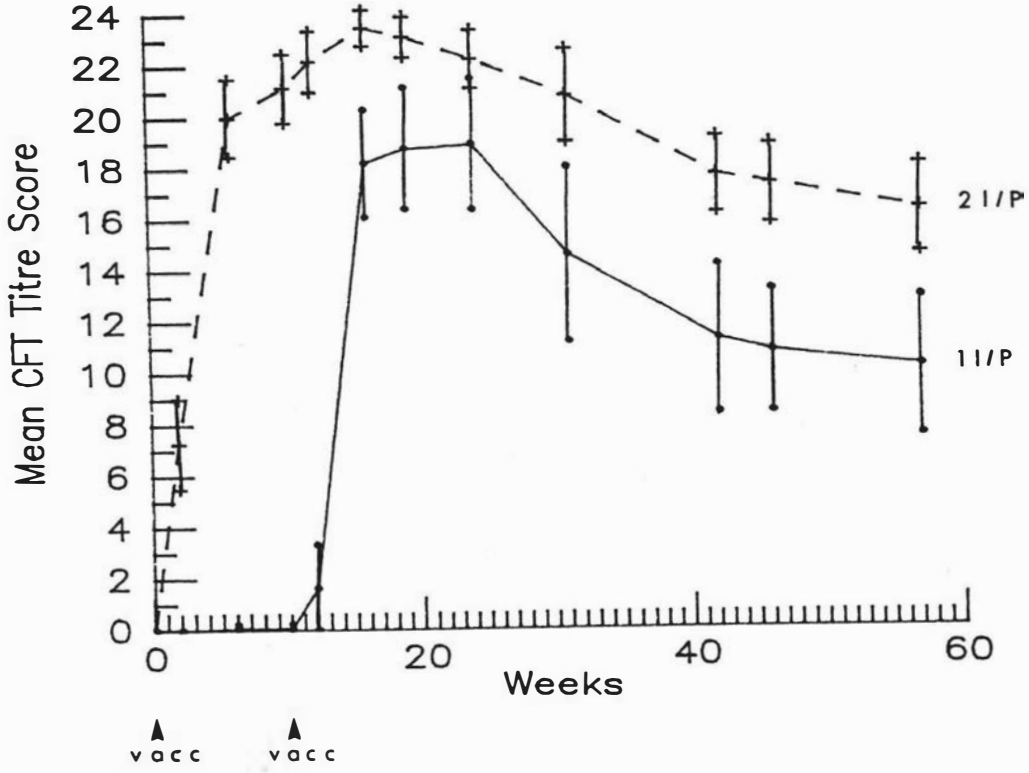
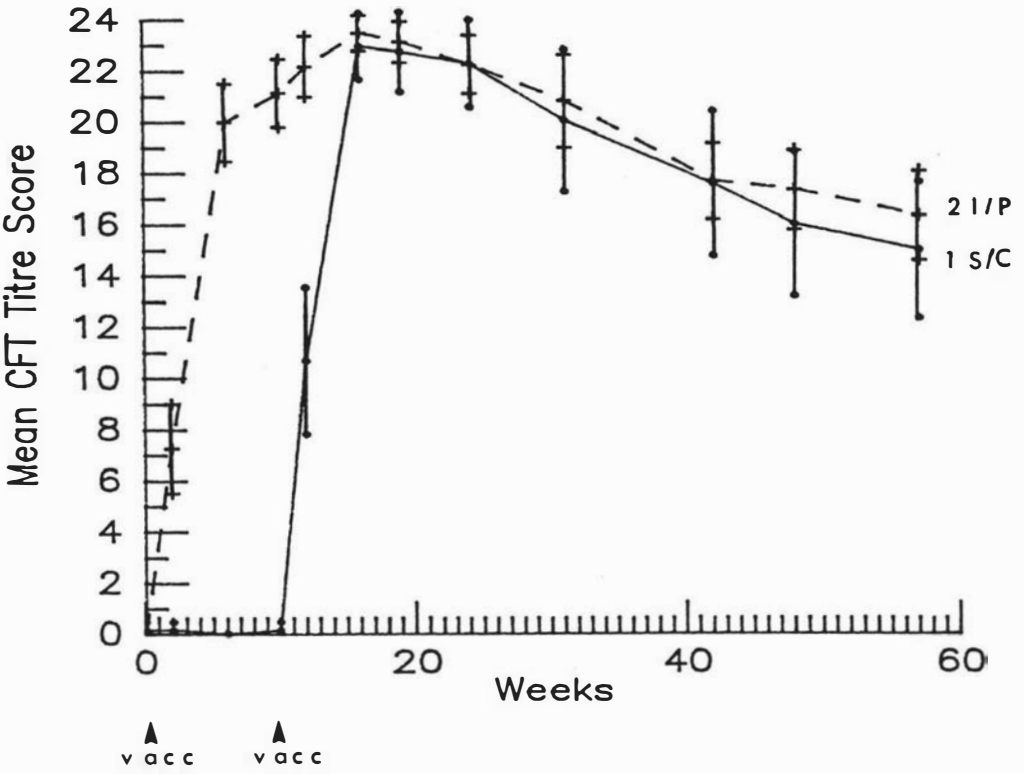

Mean CFT Titre Score
24
22
20
18
16
14
12
10
8
6
4
2
0
0
20
40
60
Weeks
vacc
vacc
2 I/P
1 I/P

Figure 3.6

Serological response of rams to an inactivated Brucella ovis vaccine administered either once by the subcutaneous route or twice by the intraperitoneal route. Mean complement fixation titre scores and 95% confidence limits.

Figure 3.7

Serological response of rams to an inactivated Brucella ovis vaccine administered either once by the intraperitoneal route or twice by the subcutaneous route. Mean complement fixation titre scores and 95% confidence limits.


Mean CFT Titre Score
24
22
20
18
16
14
12
10
8
6
4
2
0
0
20
40
60
Weeks
vacc
vacc
2 I/P
1 S/C

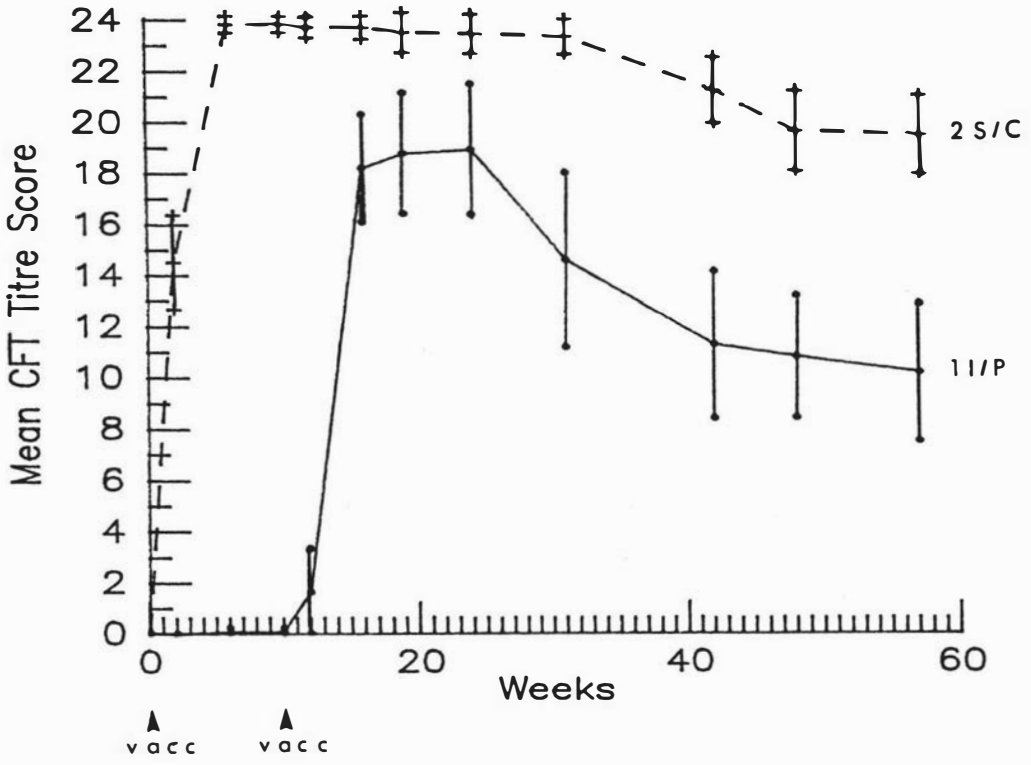
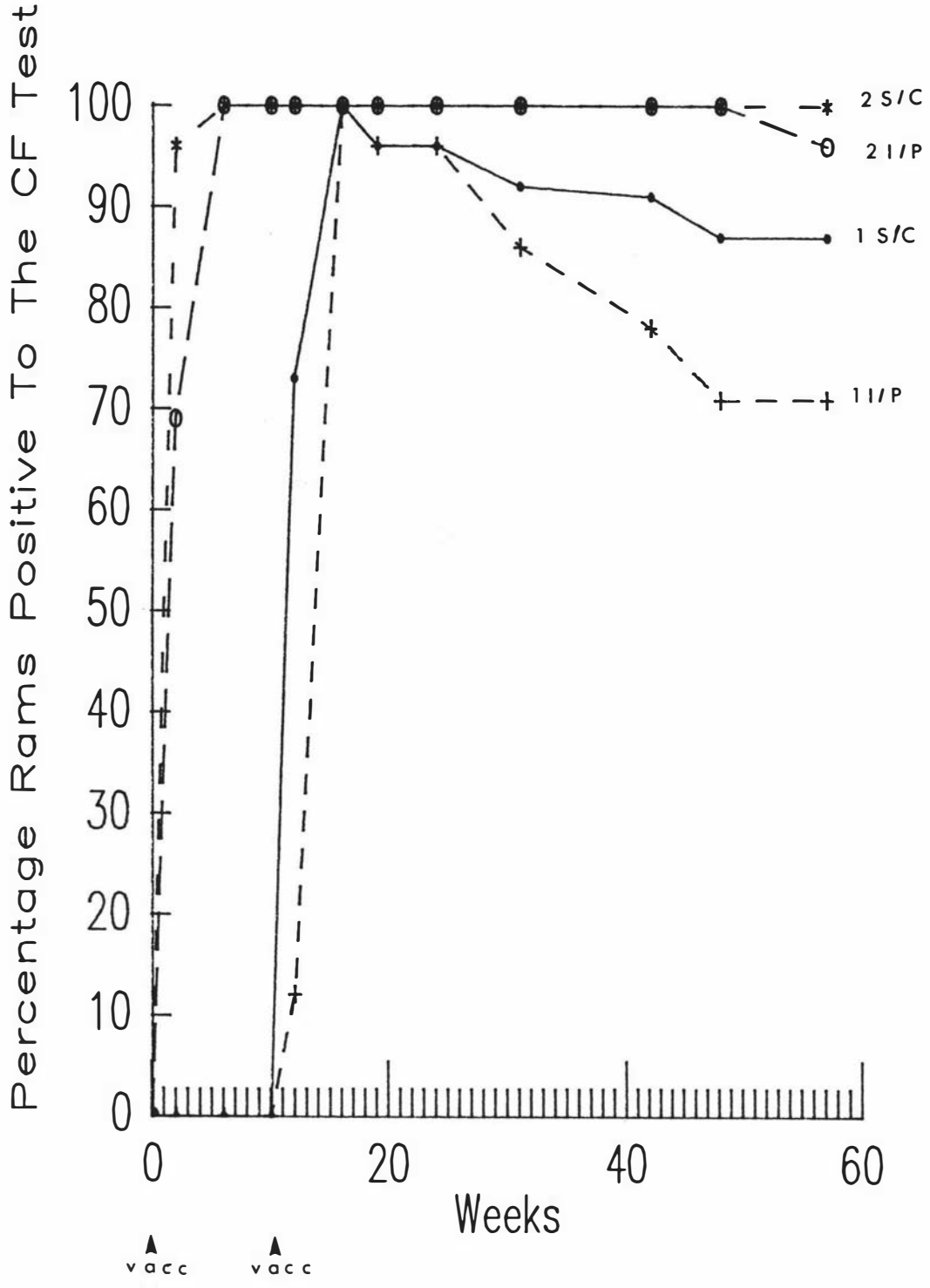

Mean CFT Titre Score
24
22
20
18
16
14
12
10
8
6
4
2
0
0
20
40
60
Weeks
vacc
vacc
2 S/C
1 I/P

Figure 3.8

Percentage of rams having a positive reaction to the complement fixation test (i.e. a titre score of 8-24) after having been vaccinated either once or twice by either the subcutaneous or the intraperitoneal route using an inactivated Brucella ovis vaccine.


Percentage Rams Positive To The CF Test
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
Weeks
vacc
vacc
2 S/C
2 I/P
1 S/C
1 I/P

Enzyme Linked Immunosorbent Assay

The mean ELISA titre of the control group always remained in the negative range. Several positive and suspicious results from control group animals in the original results were all found to be negative when the sera were subsequently retested.

The regular pattern of titres rising to a peak and then gradually declining, seen in the complement fixation test results (Figure 3.1) was not so clear for the ELISA test (Figure 3.9).

As was noted from the complement fixation results, the mean titre of rams vaccinated intraperitoneally rose more slowly, had a lower peak, and remained lower than that of rams vaccinated by a corresponding subcutaneous method. (See Figures 3.10 and 3.11).

Rams vaccinated once had a lower mean titre than those vaccinated twice by the same route. (See Figures 3.12 and 3.13). Because of the great variation in individual titres and consequently wide 95% confidence intervals, the statistical significance of any observed difference was difficult to prove.

Again, the mean titre of the 2 s/c group was always the highest, and the mean titre of the 1 i/p group always the lowest. Statistically significant differences in mean titre to the 1 i/p group could be demonstrated on four out of eight occasions after vaccination for the 1 s/c group, on five out of eight for the 2 i/p group, and on all eight occasions for the 2 s/c group. (See Figures 3.10, 3.13 and 3.14).

All the rams in the 2 s/c group were positive to the test by six weeks after vaccination and remained so throughout the trial (Figure 3.15).

One ram in the 2 i/p group failed to become positive until after the second vaccination, and by the end of the experiment this ram had the lowest titre in the group. However, once positive all the animals in this group remained so throughout the experiment.

One ram in the 1 s/c group never became positive to the ELISA test despite being positive to the complement fixation test for a two month period. However, all the other rams in that group became positive and stayed that way for the duration of the experiment.

In the 1 i/p group, two rams never became positive. Another ram was positive on only one occasion, and another positive only twice. Some of these rams were positive to the complement fixation test on occasions when they remained negative to the ELISA test. By the end of the experiment, within a year of vaccination, one third (33%) of the rams vaccinated once intraperitoneally had reverted to a negative or suspicious ELISA titre.

Figure 3.9

Serological response of rams to an inactivated Brucella ovis saline-in-oil vaccine administered either once or twice by either the subcutaneous or the intraperitoneal route. Mean ELISA titres.

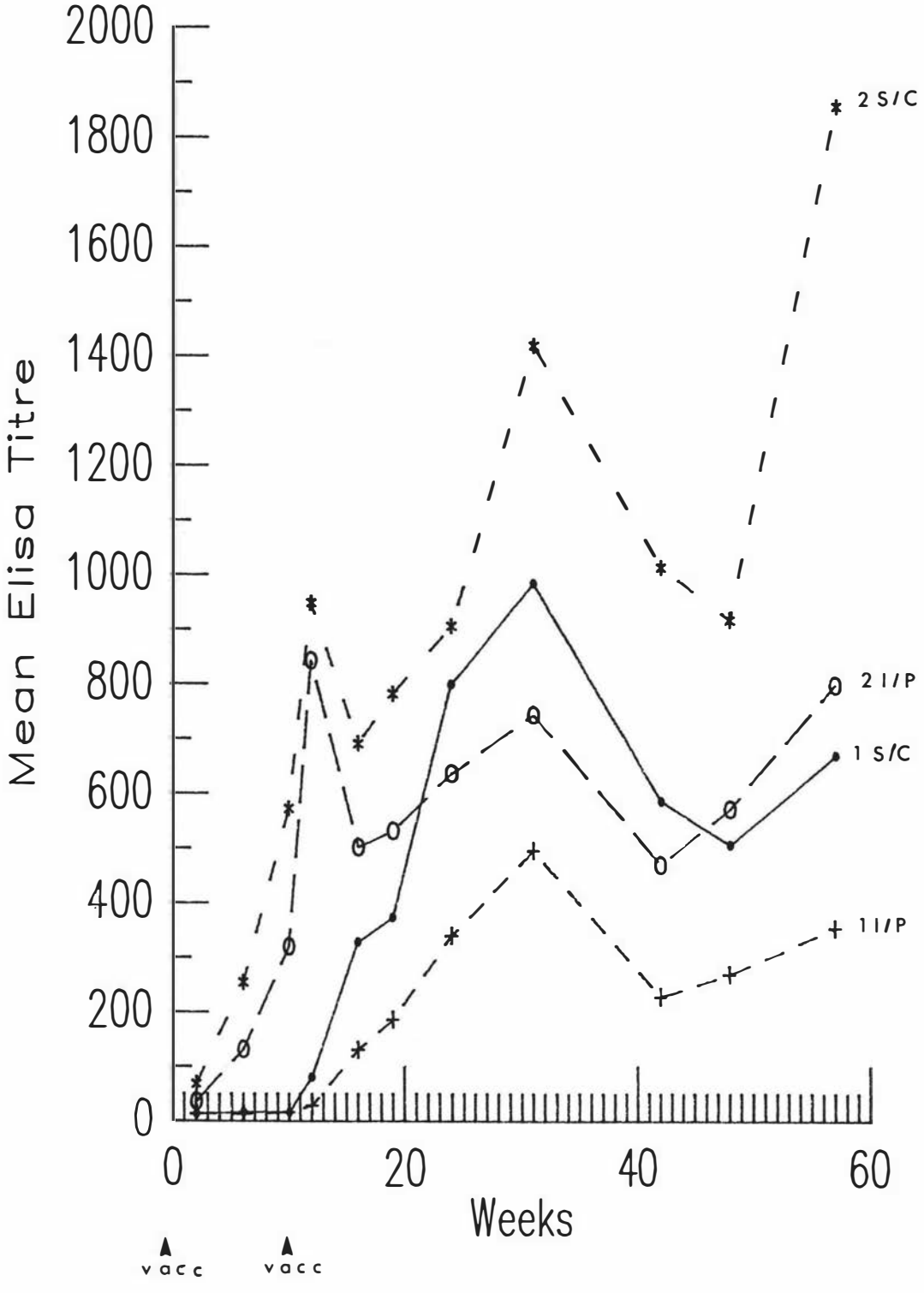
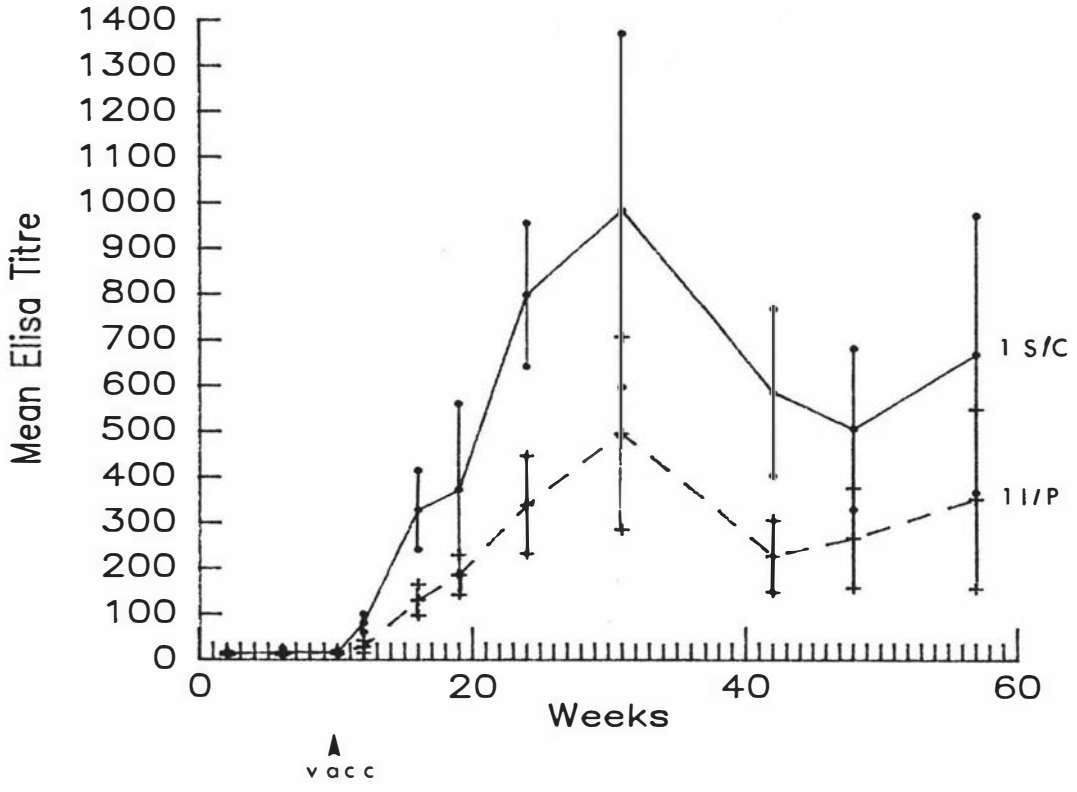

2000
1800
1600
1400
1200
1000
800
600
400
200
0
Mean Elisa Titre
0
20
40
60
Weeks
vacc
vacc
2 S/C
2 I/P
1 S/C
1 I/P

Figure 3.10

Serological response of rams to an inactivated Brucella ovis vaccine administered once by either the subcutaneous or the intraperitoneal route. Mean ELISA titres and 95% confidence limits.

Figure 3.11

Serological response of rams to an inactivated Brucella ovis vaccine administered twice by either the subcutaneous or the intraperitoneal route. Mean ELISA titres and 95% confidence limits.


Mean Elisa Titre
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
0
20
40
60
Weeks
vacc
1 S/C
1 I/P

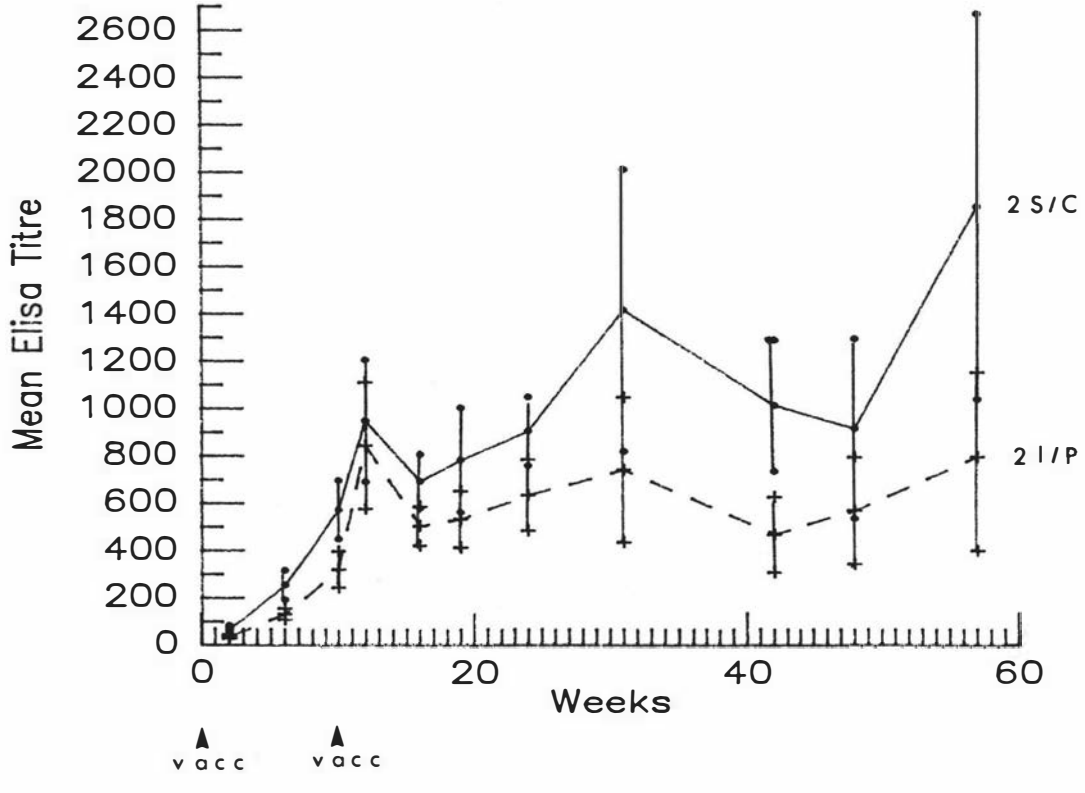
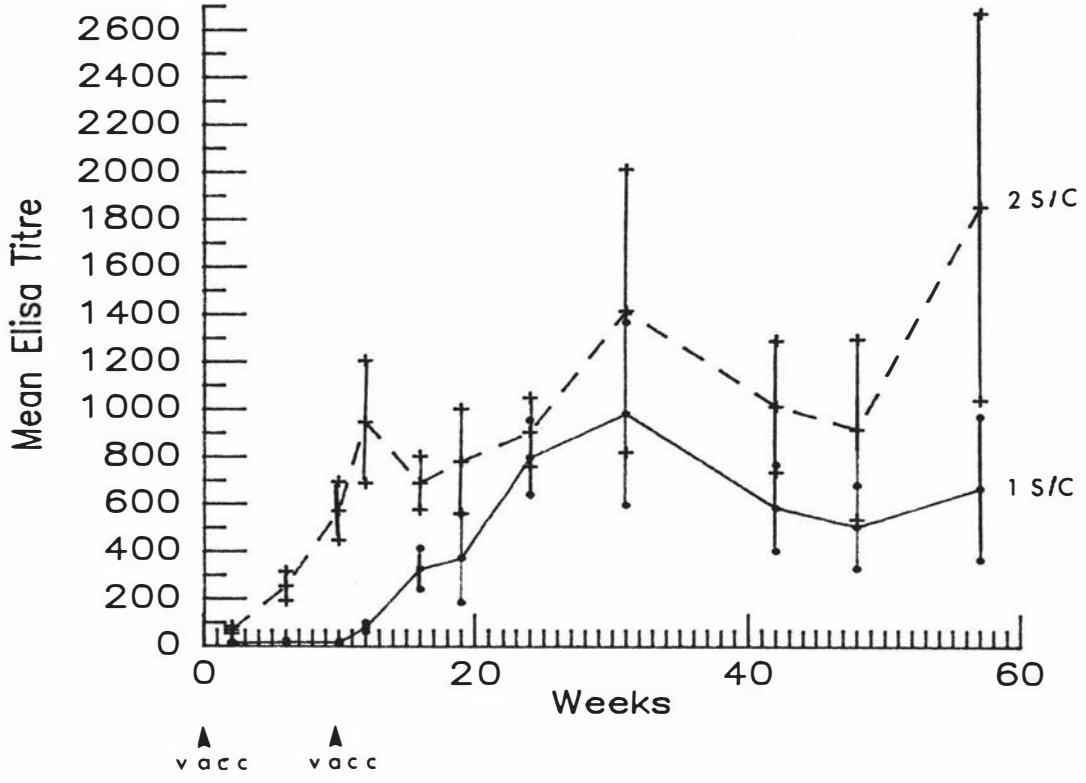

Mean Elisa Titre
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
20
40
60
Weeks
vacc
vacc
2 S/C
2 I/P

Figure 3.12

Serological response of rams to an inactivated Brucella ovis vaccine administered either once or twice by the subcutaneous route. Mean ELISA titres and 95% confidence limits.

Figure 3.13

Serological response of rams to an inactivated Brucella ovis vaccine administered either once or twice by the intraperitoneal route. Mean Elisa titres and 95% confidence limits.


Mean Elisa Titre
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
20
40
60
Weeks
vacc
vacc
2 S/C
1 S/C

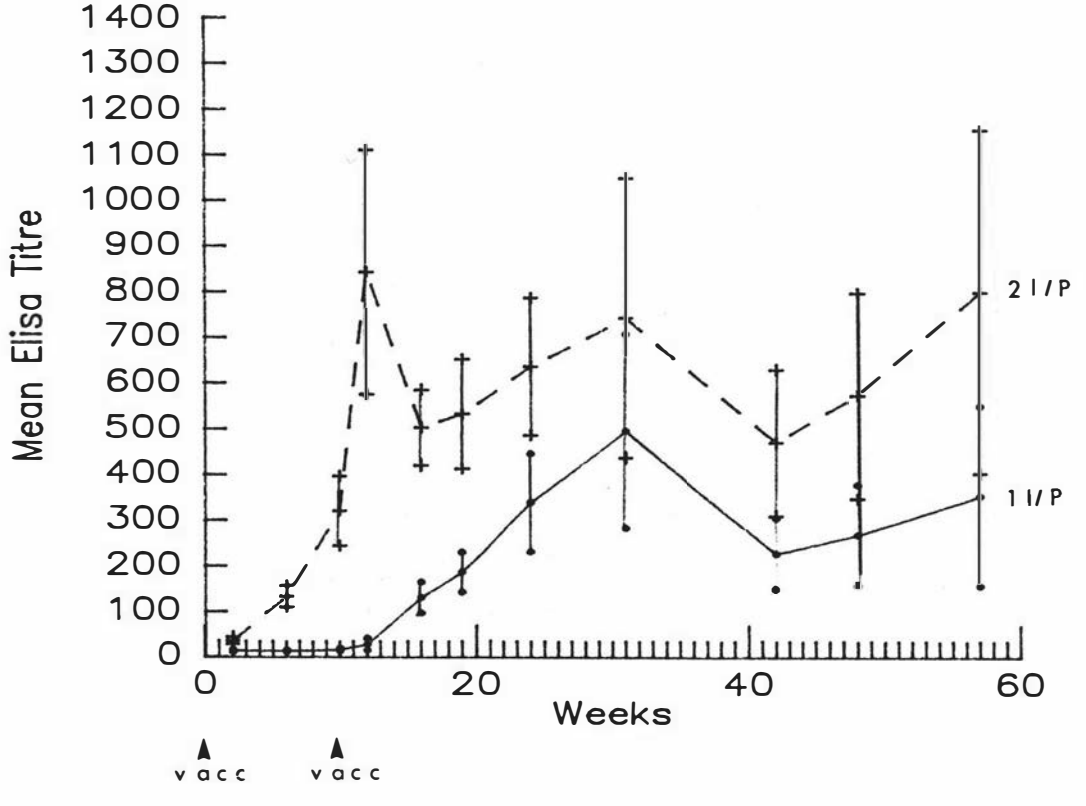

Mean Elisa Titre
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
0
20
40
60
Weeks
vacc
vacc
2 I/P
1 I/P

Figure 3.14

Serological response of rams to an inactivated *Brucella ovis* vaccine administered either once by the intraperitoneal route or twice by the subcutaneous route. Mean ELISA titres and 95% confidence limits.

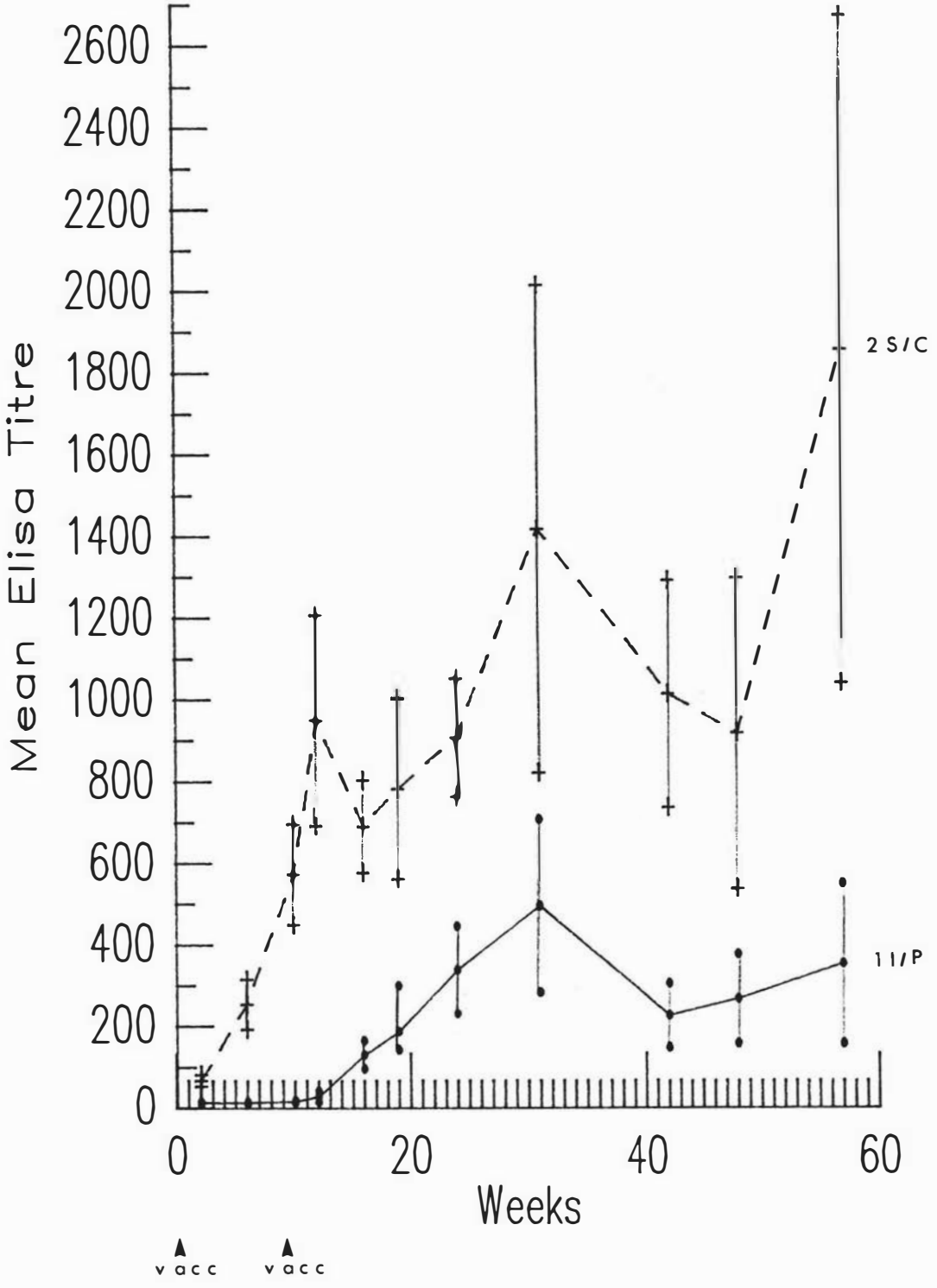
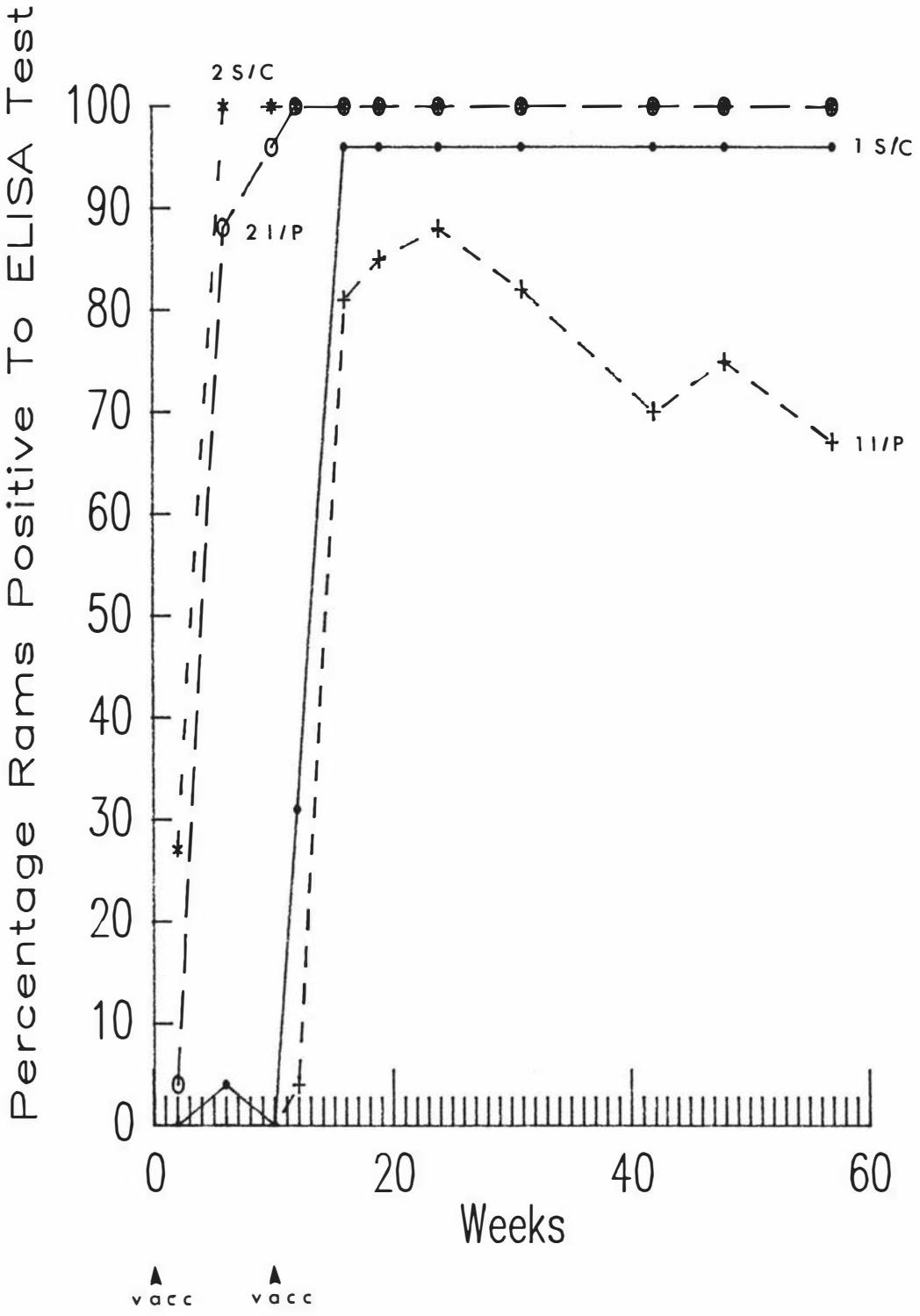

Mean Elisa Titre
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
20
40
60
Weeks
2 S/C
1 I/P
vacc
vacc

Figure 3.15

Percentage of rams having a positive reaction to the ELISA test (i.e. a titre of 90 or more) after having been vaccinated either once or twice by either the subcutaneous or the intraperitoneal route using an inactivated *Brucella ovis* vaccine.


Percentage Rams Positive To ELISA Test
100
90
80
70
60
50
40
30
20
10
0
2 S/C
1 S/C
2 I/P
1 I/P
0
20
40
60
Weeks
vacc
vacc

Gel Diffusion Test

For the purposes of these comparisons, sera giving a suspicious result in the gel diffusion test were classed as positive, as the sensitivity of this test is low (Worthington et.al., 1984), and these suspicious sera were usually positive when tested by the complement fixation test or the ELISA.

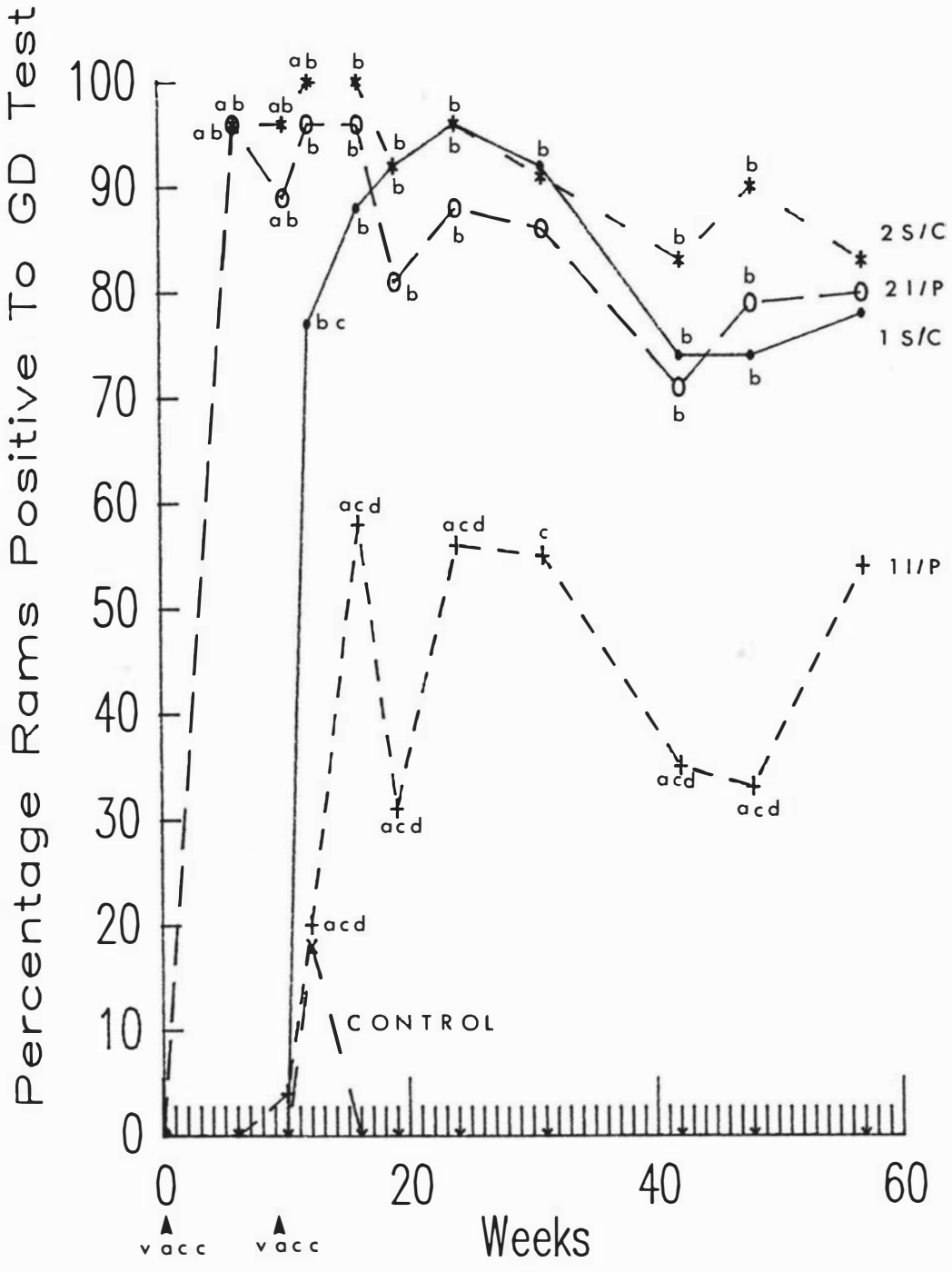
There were only two sera from rams in the control group that were not negative. At 12 weeks, one ram had a positive titre and another a suspicious reaction in the test. These two sera were both negative to the complement fixation test and the ELISA.

The 1 s/c, 2 s/c, and 2 i/p groups behaved in a similar manner to each other in their reactions to the gel diffusion test from six weeks after final vaccination (i.e. once titres had been allowed sufficient time to develop). The 1 i/p group, however, displayed a different pattern in comparison with the other vaccinated groups. After vaccination, this group had a significantly lower proportion of positive rams than any of the other three groups on all but two occasions. Only on the last test at 57 weeks was the difference between the 1 i/p group and the 2 s/c group not statistically significant.

At the end of the experiment, less than 55% of the rams in the 1 i/p group had positive reactions to the gel diffusion test, whereas over 75% of the rams in each of the other three groups were positive.

Figure 3.16

Percentage of rams having a positive reaction to the gel diffusion test after having been vaccinated either once or twice by either the subcutaneous or the intraperitoneal route using an inactivated *Brucella ovis* vaccine. The points at which between-group differences were significant at the 5% level to a chi-squared test are indicated on the graph in a manner explained by the key.



KEY- significant difference to-

1s/c group - a

1i/p group - b

2 s/c group - c

2 i/p group - d

Subcutaneous Reactions

The lesions that resulted from subcutaneous vaccination were frequently over 5cm in diameter. Two weeks after vaccination, the average size of the lesion from the second injection given to the 2 s/c group was greater than that of the lesion from the first injection given to the 2 s/c group or that of the lesion from the single injection given to the 1 s/c group when these latter two were at the same stage. (See Figure 3.17). The average size of this lesion (second vaccination site, 2 s/c group) then reduced rapidly before stabilising at an average size which was smaller than that of the other two sites. The difference between the size of the lesion at this second vaccination site of the 2 s/c group and that of the lesion at the first vaccination site of the 2 s/c group was statistically significant (when tested by 95% confidence limits) throughout the experiment, and on three occasions the difference in lesion size between the second vaccination site of the 2 s/c group and the vaccination site of the 1 s/c group was also statistically significant.

The average lesion size of the first vaccination site of the 2 s/c group was larger than that of the other two sites throughout the trial except for one occasion at 12 weeks, and on some occasions this difference was statistically significant. The lesions of the rams vaccinated only once (1 s/c group) usually had an average lesion size between that of the other two sites.

The administration of the second vaccination in the 2 s/c group appeared to cause an increase in the proportion of lesions over 30mm in diameter at the site of the first injection. (See Figure 3.18). A lesion this size would be easily felt when passing a hand over the animal's neck. Lesions of these proportions were in the majority at this first injection site throughout the remainder of the trial, while at the site of the second vaccination the proportion of lesions as large as this rapidly reduced.

Of the 78 subcutaneous vaccination sites studied, 23 (30%) were seen to discharge necrotic material. When this occurred, the size of the lesion often reduced rapidly and frequently resolved completely. The site at which the greatest proportion of lesions discharged (54%) was that of the second vaccination of the 2 s/c group. This site also had the greatest proportion of rams without detectable lesions at the end of the experiment (See Figure 3.19).

Of three rams in the 1 s/c group which had reverted to a negative complement fixation titre by the end of the experiment, the two which reverted to negative at the earliest stage had no detectable lesion at the site of injection by the end of the experiment. The lesion of one of these rams was palpable on only one occasion, two weeks after vaccination, and the lesion of the other discharged two weeks after vaccination and 12 weeks later was no longer detectable.

The most important observations were that these subcutaneous lesions can and do persist for long periods of time, at least 12 months and probably much longer; that a detectable lesion of some sort invariably developed at the vaccination site at some stage during the experiment; and that by the end of the experiment at 57 weeks, 64% of vaccination sites still had palpable lesions, and 36% of these were 30mm or more in size, and therefore quite easily felt when passing a hand over the animal's neck.

Figure 3.17

Mean size of local lesion at the site of either the first, second or sole subcutaneous injection of inactivated Brucella ovis vaccine.

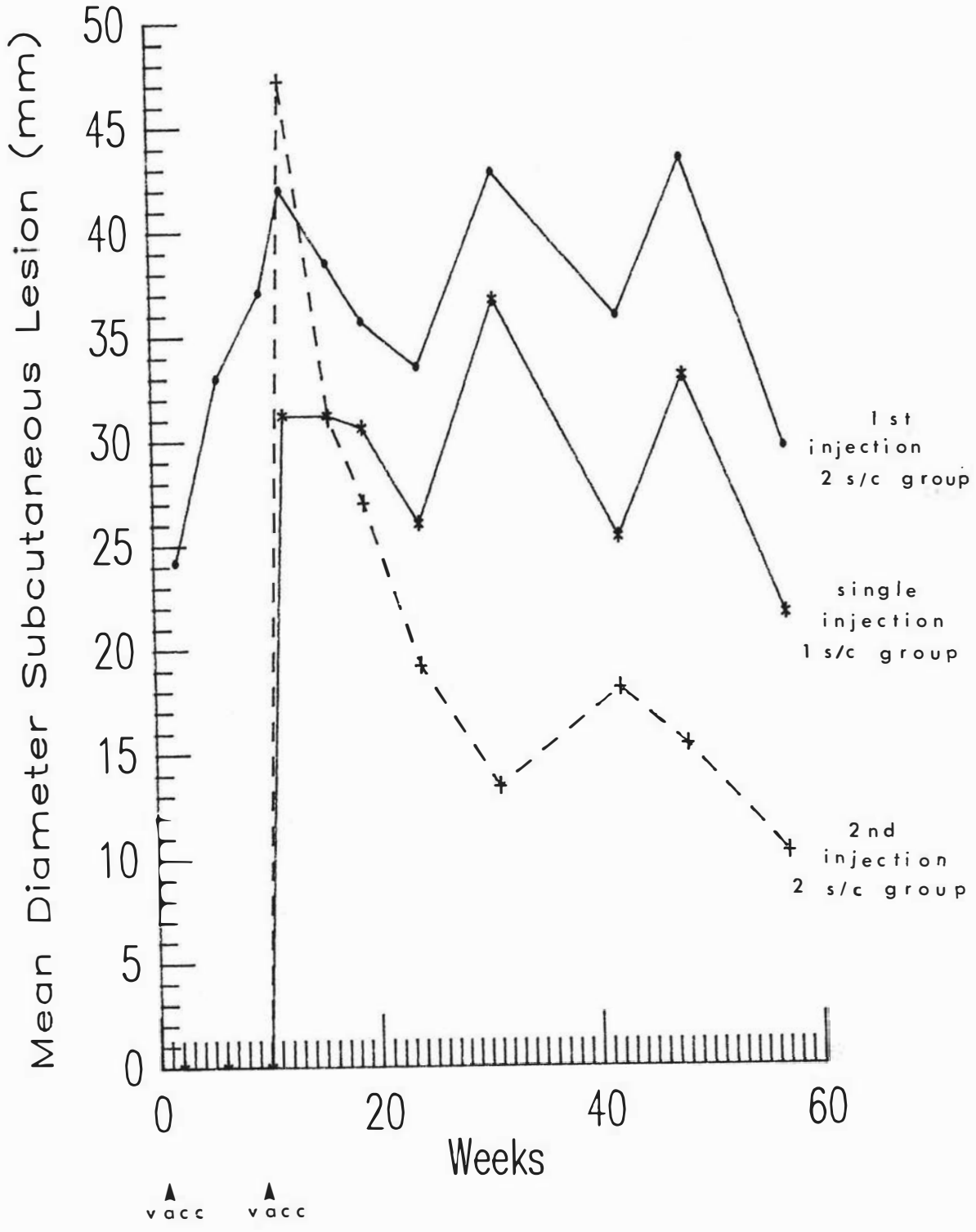

Mean Diameter Subcutaneous Lesion (mm)
50
45
40
35
30
25
20
15
10
5
0
0
20
40
60
Weeks
vacc
vacc
1st injection 2 s/c group
single injection 1 s/c group
2nd injection 2 s/c group

Figure 3.18

Proportion of local lesions resulting from either the first, second or sole subcutaneous injection of inactivated Brucella ovis vaccine which were 30mm or more in diameter.

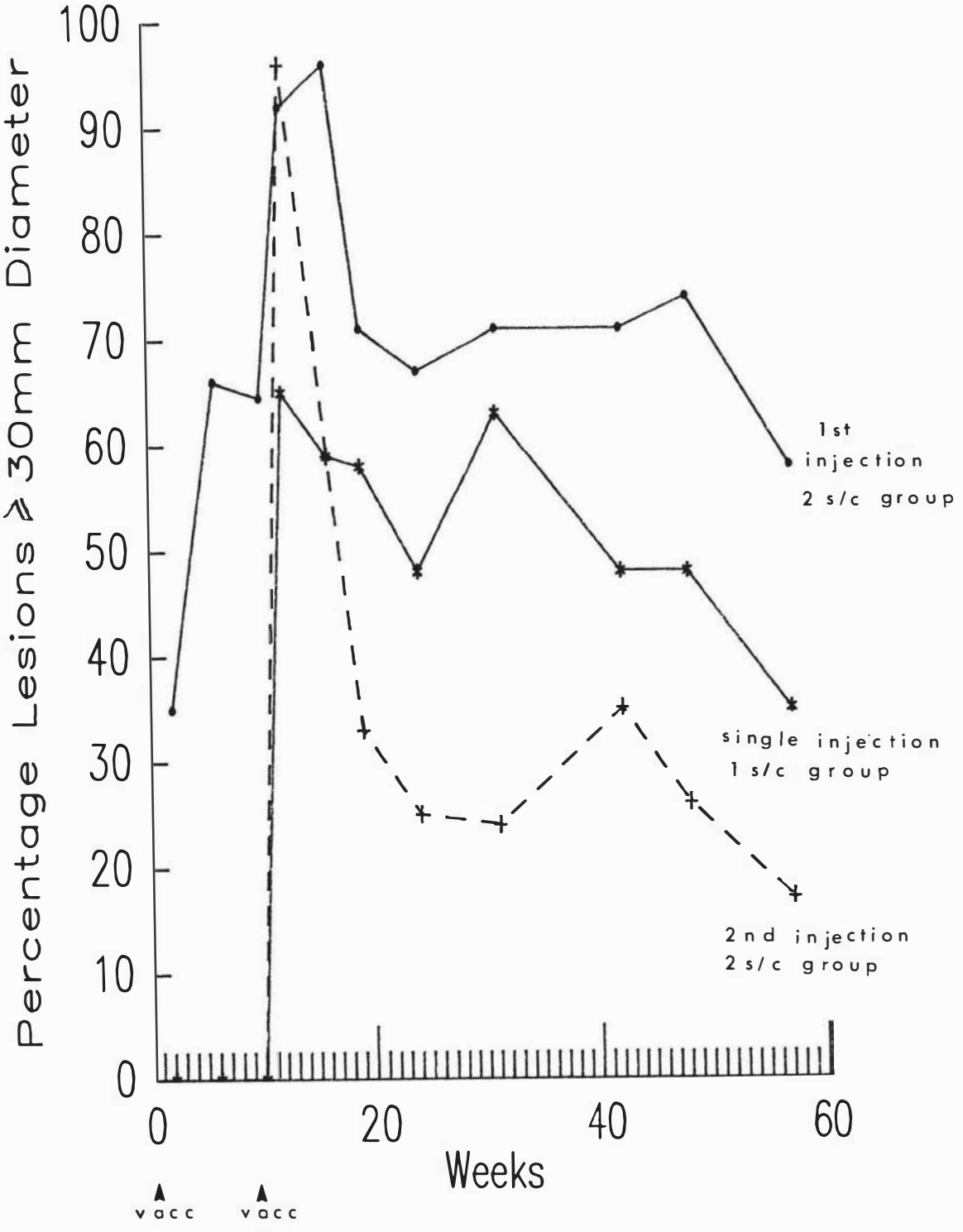
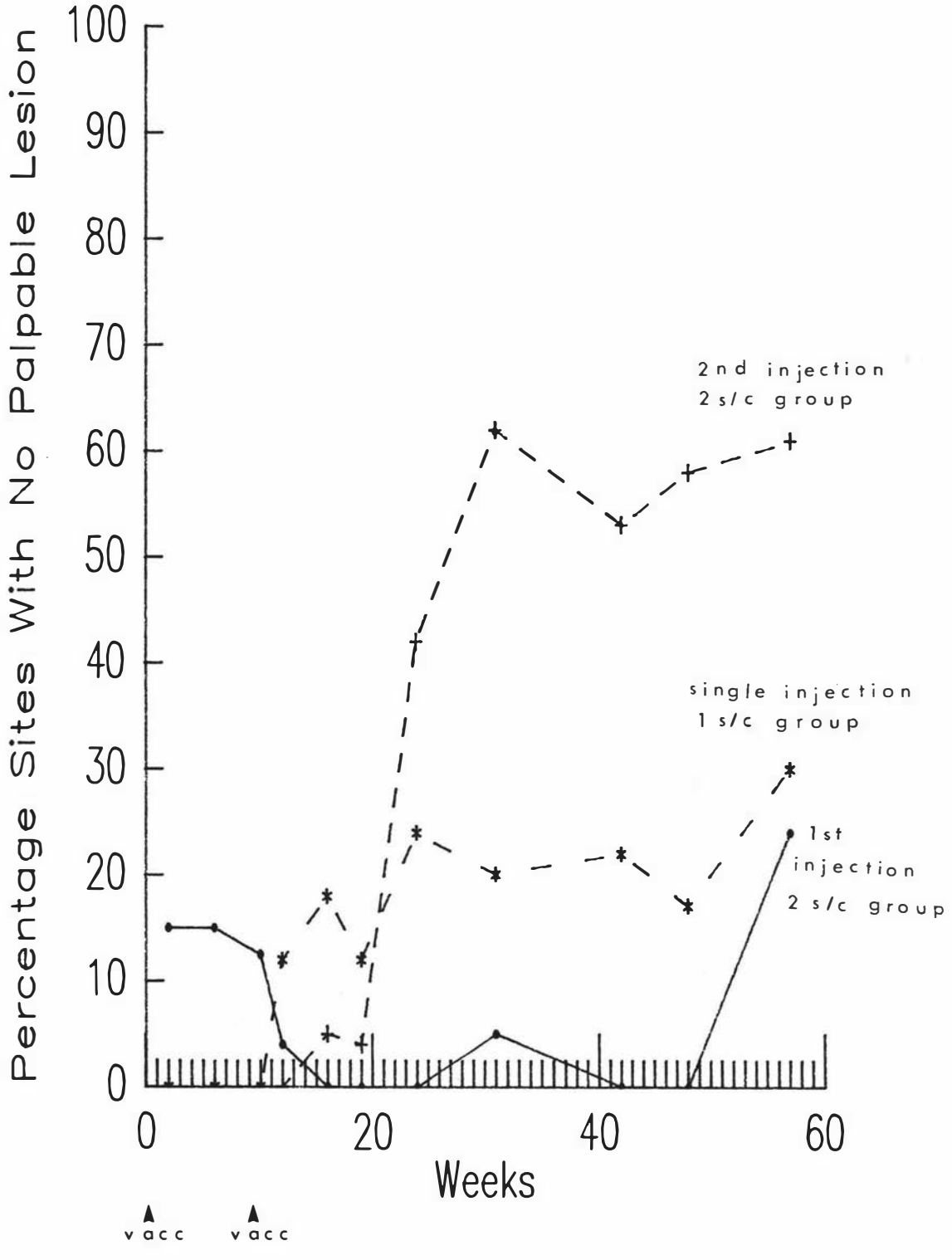

Percentage Lesions ⩾ 30mm Diameter
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
Weeks
vacc
vacc
1st injection 2 s/c group
single injection 1 s/c group
2nd injection 2 s/c group

Figure 3.19

Proportion of first, second or sole subcutaneous Brucella ovis vaccination sites which had no lesion palpable.


Percentage Sites With No Palpable Lesion
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
Weeks
2nd injection
2 s/c group
single injection
1 s/c group
1st
injection
2 s/c group
vacc
vacc

Test repeatability

Out of the 1293 samples originally tested using the complement fixation method, 39 (0.03%) were anticomplementary. These 39 samples originated from 16 animals:
9 rams had 1 anticomplementary (AC) result,
2 rams had 2 AC results,
1 ram had 3 AC results,
2 rams had 4 AC results,
1 ram had 7 AC results, and
1 ram had 8 AC results out of the 12 tests.

Of the 39 AC results, 23 (approximately 60%) originated from just four of the 16 rams (25%).

Of a total of 42 anticomplementary sera which were retested, only seven (17%) remained anticomplementary at the second test. (See Table 3.4). Of these seven, five were from just one ram.

The sera selected to be retested by the complement fixation method were not a random sample, but rather were those sera which had either given an unexpected result or been anticomplementary. Even so, the repeatability of the test for interpretation category was remarkably good. Of the sera which did not produce exactly the same titre at both tests, the vast majority (86%) were still within one serial dilution value of each other (e.g. a titre of 4^5 might give a result between 4^4 and 4^6 when retested).

For the ELISA test, the majority of sera which had changed interpretation category upon retesting were either positive or suspicious at the initial test but became negative when retested (56%). This suggests poor specificity in the original test. (It must be remembered that, at the time of this experiment, the ELISA test was still under development). A further 16% went from suspicious to positive, 10% each from negative to positive or positive to suspicious, and 8% from negative to suspicious. When subjected to further retesting (four times) to provide a final estimate based on mean titre as described in the methods, most sera had a final result

that agreed with the second test (first retest) value.

The repeatability of the complement fixation test was 96%, and for the ELISA it was 90%, (see Table 3.4), and this difference was highly significant to the chi-squared test ($P<0.001$, significant at the 0.1% level).

Test Accuracy

The estimated sensitivity of the complement fixation test was 99.9%, for the ELISA it was 99.2%, and for the gel diffusion test it was 91.3%. The estimated specificity of the complement fixation test was 100%, for the ELISA it was 99.6%, and for the gel diffusion test it was 99.1%. (See Table 3.5). The differences in the sensitivity and specificity of the complement fixation and ELISA tests were not statistically significant when tested by the chi-squared method.

Sixty-seven (approximately 50%) of the 135 negative and suspicious ELISA results from vaccinated animals had a positive complement fixation test result for the same sample, and were positive to the ELISA on a subsequent occasion. Most of these were from sera collected in the first few weeks after vaccination.

For the gel diffusion test, 187 (approximately 77%) of the 244 negative and suspicious results from vaccinated animals had a positive complement fixation test result at the same test and were positive to the gel diffusion test on a subsequent occasion.

Table 3.4

Repeatability of the Complement Fixation Test and the Enzyme Linked Immunosorbent Assay in the Detection of Antibody Response to Vaccination Against Brucella ovis Infection

	CFT	ELISA
Number of sera retested	430	1785
Number of sera from vaccination serology trial	401	1184
Number of sera from other experiments	29	601
Number anticomplementary at original test	42	-
Number of above sera anticomplementary at retesting	7	-
Number retested sera not anticomplementary originally	388	1785
Number in same interpretation category at both tests	372	1600
Estimated repeatability of test for interpretation category	96%	90%

Table 3.5

Efficiency of the Complement Fixation Test, Enzyme Linked Immunosorbent Assay and Gel Diffusion Test in Detecting Antibody Response to Vaccination Against Brucella ovis Infection

	CFT	ELISA	GD
Total number of sera	1293	1185	1185
Sera from unvaccinated animals	383	269	321
Sera from vaccinated animals	910	916	864
Proportion of sera from vaccinated animals that were a) positive	92%	85%	72%
b) suspicious	1%	3%	6%
c) negative	7%	12%	22%
% false positive results from unvaccinated animals	0%	0.4%	0.9%
% false negative results from vaccinated animals	0.1%	0.8%	8.7%
Estimated specificity	100.0%	99.6%	99.1%
Estimated sensitivity	99.9%	99.2%	91.3%

DISCUSSION

Antibody Response To Vaccination

The serological results of this trial have demonstrated a consistently different titre pattern in groups of rams vaccinated twice subcutaneously in comparison with those vaccinated once intraperitoneally. Complement fixation titres of rams vaccinated twice subcutaneously tend to rise rapidly, remain high for approximately six months and then decline slowly. In rams vaccinated once intraperitoneally, the rise in titre tends to take place more slowly, peak at a lower level, remain at peak for only approximately two months, and decline rapidly. This distinction is clearly shown in Figure 3.7. The difference in response between the two groups was clearly demonstrated not only in the complement fixation test results, but also in those of the gel diffusion test (Figure 3.16) and the ELISA (Figure 3.14 and 3.15). It was a consistent result with statistically proven significance in all three of the serological tests used.

The inferiority of the single intraperitoneal technique appears to comprise two elements:

1) The unreliability of a single vaccine dose in comparison with a double vaccination technique.

2) The route of administration.

It is clear that Buddle considered the protection conferred by a single dose of the Brucella ovis vaccine to be unsatisfactory. This was demonstrated by Buddle et.al. (1963) using the inactivated Brucella ovis saline-in-oil vaccine which became the basis of the commercial vaccine "Ramovexin" used in this serology experiment. In this trial (Buddle et.al., 1963), 18 out of 42 rams given a single dose of the Brucella ovis vaccine became infected after intravenous challenge (43%), but only 4 out of 41 which had been vaccinated twice with the same vaccine (10%). This conforms to well established

immunological principles. It is usually considered necessary to administer more than one dose of a killed bacterial preparation to confer adequate resistance, as it is not as antigenic as, and confers a less persistent resistance than a live attenuated vaccine (Herbert, 1970).

The fact that the Brucella ovis vaccine requires an oil adjuvant to enhance its immunogenicity (Buddle, 1954) also suggests that it would be unwise to rely on a single vaccine dose to induce adequate immunity, and this was borne out in the experiment of Buddle et.al. (1963), described above.

Although all the vaccinated rams in the experiment became positive to the complement fixation test, four rams failed to react to the ELISA after initial vaccination. Three of these were in treatment groups vaccinated only once, (1 s/c and 1 i/p groups) and these rams never became positive to the ELISA test. The other was in the 2 i/p group, and became positive after the second vaccination, illustrating one of the advantages of using two vaccinations instead of relying upon one.

Another characteristic of the groups vaccinated only once (1 s/c and 1 i/p) was that a much greater proportion of those rams returned to negative serological status than did those in groups vaccinated twice (2 s/c and 2 i/p), and they also did so much sooner after vaccination (see Figure 3.8). These animals having negative titres were responsible for much of the reduction in average titre in the single vaccination groups. By the end of the experiment at 57 weeks, 19% of the animals in the groups vaccinated once (1 s/c and 1 i/p) were negative to the complement fixation test, 17% negative to the ELISA, and 34% negative to the gel diffusion test. In comparison, none of the animals in the groups vaccinated twice (2 s/c and 2 i/p) were negative to either the complement fixation test or the ELISA by the end of the experiment, and only 19% were negative to the gel diffusion test.

It appears from the observations just described, that a single dose of the Brucella ovis vaccine "Ramovexin", although producing a rise in titre for a short period of time, is not sufficient to stimulate consistently a long-lived antibody response in all rams. That would be true for either the subcutaneous or the intraperitoneal route of vaccination.

The antibody response of rams vaccinated intraperitoneally appears to develop more slowly than that of rams vaccinated subcutaneously, and the mean titre remains consistently lower (Figures 3.2, 3.3, 3.10 and 3.11). Thomson et.al. (1969) claimed that following the intraperitoneal vaccination of sheep with a multicomponent clostridial oil emulsion vaccine, the vaccine was present in the local lymph nodes within 20 minutes. When the vaccine was used subcutaneously, an obvious lesion developed, but when injected intraperitoneally, no local reaction at the site and no systemic effect was noted (Thomson et.al., 1969).

It is thought that oil adjuvants such as those used in Thomson's clostridial vaccine and in the Brucella ovis vaccine "Ramovexin" may work partially by causing the vaccine to remain in-situ as a long-lived depot, so prolonging the duration of the response. ("Immunostimulation", 1980). The local lesion produced by the subcutaneous administration of the vaccine is the visible evidence of such a depot. As no local lesion was reported by Thomson et.al. (1969) at the site of intraperitoneal vaccination, it appears that the opportunity to form such a depot may be denied to vaccine deposited in the large peritoneal cavity, and this may impair its ability to stimulate the immune system, both in the long and in the short term, explaining the lower titres in the rams vaccinated by the intraperitoneal route.

The amount of inflammation itself may be important in determining the strength of the immune response. Tissue damage may cause the release of certain factors, such as nucleic acids, which amplify the reaction (Merritt and Johnson, 1965). There was some evidence from this experiment to suggest that a persistent titre may be dependent on a persistent inflammatory response at the vaccination

site. Of three rams in the 1 s/c group which had reverted to a negative complement fixation titre by the end of the experiment, the two which reverted to negative at the earliest stage had no detectable lesion at the site of injection by the end of the experiment. The lesion of one of these rams was palpable on only one occasion, two weeks after vaccination, and the lesion of the other discharged two weeks after vaccination and 12 weeks later was no longer detectable.

The way in which mineral oil enhances the immunogenicity of an antigen associated with it is still not completely understood despite extensive investigations by such workers as Freund (1957), Macher and Chase (1969), Wilkinson and White (1966) and many others.

With the combined advantages of both a subcutaneous rather than an intraperitoneal route and a double rather than a single vaccination technique, it is not surprising that the double subcutaneous technique induces what appears to be the greatest and most consistent antibody response. Equally, one would expect the finding that the single intraperitoneal technique induces the poorest, most inconsistent response. These findings have great practical significance in the field, for by changing the vaccination process from a double subcutaneous to a single intraperitoneal technique, as advocated by Quinlivan and Wallace (1975; 1979), veterinarians are not only reducing the number of vaccinations from two to one, thereby reducing the efficacy, but also changing from the subcutaneous route to the intraperitoneal one, which produces an inferior serological response. Thus, the single intraperitoneal technique which has become a common practice is, on two counts at least, inferior to the established two-dose subcutaneous method. It must be stressed that these serological differences do not necessarily imply a difference in immunity, even though this possibility is strongly suggested. Challenge trials would be required for adequate investigation of this hypothesis.

Local Lesions

Examinations of the subcutaneous injection site showed that the local reaction produced by the administration of the inactivated Brucella ovis saline-in-oil vaccine could be quite severe and long lasting. A lesion invariably developed at the subcutaneous injection site at some stage during the trial, in spite of the careful technique employed, which involved using a new, sterile needle and syringe for each animal. By the end of the experiment at 57 weeks, over 60% of the vaccination sites still had a palpable lesion and in 23% of sites the lesion was still large (over 30mm in diameter) and easy to detect.

The administration of the second vaccination in the 2 s/c group appeared to cause an increase in the proportion of lesions over 30mm in diameter at the site of the first injection. Lesions of these proportions were in the majority at this first injection site throughout the remainder of the trial, while at the site of the second vaccination the proportion of lesions as large as this rapidly reduced, indicating the possibility that the cellular response to vaccination may differ between the primary and secondary vaccination sites.

This type of local reaction following the subcutaneous administration of an oil-based vaccine is not exclusive to Brucella ovis preparations. Thomson et.al. (1969) reported a reaction of this type following the use of a multicomponent clostridial oil emulsion vaccine. Ross and Titterington (1984) made a study assessing the size and frequency of lesions induced by a commercially produced, oil-based, Bacteroides nodosus footrot vaccine. These two workers reported that following a 1ml dose of this oil-based footrot vaccine, lesions whose individual maximum dimensions ranged from 5-200mm developed, and that the size of the lesion was not reduced by swabbing the skin with methanol prior to vaccination. A proportion of the lesions burst and discharged necrotic material, which was sterile. They also reported that the lesions had been criticised by farmers, were unsightly, and caused discomfort to the animal. They suggested that these lesions may necessitate trimming and subsequent downgrading of the carcass at slaughter.

The type of water-in-oil emulsion used in preparations such as the Brucella ovis and Bacteroides nodosus vaccines is also known loosely as Freund's incomplete adjuvant. Because it is widely recognised that mineral oil often causes extensive and persistent reactions, this adjuvant is not viewed favourably in human medicine, although it has been extensively used in animals (Jollès and Paraf, 1973). This side-effect is apparently unacceptable to some animal owners also (Quinlivan and Wallace, 1975, 1979; Ross and Titterington, 1984). It was just such an unwillingness on the part of ram owners to accept this side effect which prompted the introduction of the intraperitoneal route for the administration of the Brucella ovis saline-in-oil vaccine (Quinlivan and Wallace, 1975).

Comparison of Serological Tests

The most reliable serological test for the detection of antibody response to vaccination was the complement fixation test, which had an estimated specificity of 100%, an estimated sensitivity of 99.9%, and a repeatability of 96%. The high accuracy of the test was probably largely due to the extensive experience the laboratory had had using this method. The techniques were well defined, and extensive quality control measures were routinely carried out using standard reagents and standard sera of known titre.

One of the disadvantages of the complement fixation test was the uncommon occurrence of anticomplementary activity in serum samples. Out of the 1293 samples originally tested in this experiment, 39 (0.03%), originating from 16 rams, were anticomplementary. Twenty three of these (approximately 60%) originated from just four of the 16 rams (25%). It was also notable that one of the rams had an anticomplementary result on eight out of the 12 occasions it was tested, and that of seven sera that remained anticomplementary when subjected to retesting, five came from just one ram.

These observations all suggest that this phenomenon may be due to an intrinsic characteristic of the individual ram rather than to the way the serum is handled and tested.

The majority (83%) of sera which were anticomplementary when originally tested had lost their anticomplementary activity - and indeed gave a result that appeared consistent with the serological history of the ram - when the test was repeated on the surplus serum which had been stored at -20°C. This may have been due to a change in the nature of the test itself (e.g. a different batch of red blood cells) or it may have been due to some factor associated with storage.

The ELISA was similar to the complement fixation test in its reliability as a method of detecting vaccinal titres, having an estimated specificity of 99.6%, an estimated sensitivity of 99.2%, and a repeatability of 90%. Only the difference in repeatability was statistically significant.

Sixty-seven (approximately 50%) of the 135 negative and suspicious ELISA results from vaccinated animals had a positive complement fixation test result for the same sample, and were positive to the ELISA on a subsequent occasion. Most of these were from sera collected in the first few weeks after vaccination, indicating that the complement fixation test may detect antibody response to vaccination at an earlier stage than the ELISA. In fact 56 sera from rams which later became positive to the ELISA were negative at an early stage after vaccination when at the same time the complement fixation test was already detecting them as positive.

The classical pattern of titres rising to a peak and then slowly declining, as seen in the complement fixation test, was not clearly defined in the ELISA test. This was probably partly because of the enormous variability of the individual readings on what was a much larger numerical scale in the ELISA test, and partly because there was no set upper limit to the titre readings as there was in the complement fixation test due to the predetermination of the number of serum dilutions tested. It is also known that the ELISA test measures different classes of immunoglobulin in comparison with the complement

fixation test. The complement fixation test measures IgG_1, and to a lesser extent IgM (Spencer and Burgess, 1984), although there is the suggestion that the IgM in the serum may be largely denatured during the preliminary heating process prior to the complement fixation test (Rahaley et.al., 1983). Ovine IgG_2 does not fix complement, and is therefore not detected by the complement fixation test (Rahaley et.al., 1983). The ELISA test, however, measures both IgG_1 and IgG_2, and, less efficiently, IgM (Worthington et.al., 1984). The large variations in ELISA units sometimes observed for a given complement fixation titre may be explained by the different classes of antibody that these two tests measure (Spencer and Burgess, 1984). There also seemed to be some degree of variation in the batches of sera tested by the ELISA method at each occasion (or this may have been a difference in the actual test). For example, at 31 and 57 weeks all the titres appeared to be unusually high. However, all the groups seemed to be affected in a consistent way, so that comparisons between groups at the same test were considered to be valid. Some of the apparent inconsistencies of the ELISA test may be due to difficulties encountered in developing this new technique.

The gel diffusion test was the least reliable of the three methods, with an estimated specificity of 99.1%, and an estimated sensitivity of only 91.3%.

CONCLUSION

1) The serological response of rams to vaccination using inactivated *Brucella ovis* saline-in-oil vaccine was greater, more consistent, and more persistent when the schedule involved two vaccine doses given a few weeks apart than when only a single vaccine dose was used.

2) The rise in titre was more rapid and the final titre level of greater magnitude when the subcutaneous route of administration was used than when the intraperitoneal route was employed.

3) The most satisfactory vaccination technique used in this trial, in terms of both magnitude and duration of antibody response, was a regime of two subcutaneous injections of vaccine given 10 weeks apart. The least satisfactory technique was a single intraperitoneal injection.

4) The single subcutaneous and double intraperitoneal techniques were intermediate in performance between the double subcutaneous and single intraperitoneal techniques.

5) There was evidence of the validity of the serological results in the consistency shown by the three serological tests used in this trial: the complement fixation test, the enzyme linked immunosorbent assay and the gel diffusion test. All three of these tests demonstrated the same pattern of differences in the antibody responses evoked by the various vaccination techniques employed.

6) Rams vaccinated subcutaneously invariably developed a lesion in the connective tissue at the site of injection. These were often large, and the majority persisted for over 12 months. During this period approximately one-third discharged necrotic material.

7) When two spaced doses of vaccine were given to the one animal, the tissue reaction at the two sites differed. The administration of the second dose of vaccine, 10 weeks after the first was associated with an increase in the size of the lesion at the site of the first dose. The lesion at the site of the second dose of vaccine initially developed more rapidly and then resolved more quickly than that at the first site.

8) The complement fixation test was the most satisfactory method of detecting antibody response to vaccination. It detected antibody response at an earlier stage after vaccination than did the enzyme linked immunosorbent assay and also had a higher level of repeatability. The ELISA in turn was more satisfactory than the gel diffusion test.

Because it was found in this experiment that the inactivated Brucella ovis saline-in-oil vaccine causes a persistent inflammatory reaction when administered by the subcutaneous route, it was decided to investigate what local effects the vaccine might have when administered by the intraperitoneal route. Similarly, it was decided that since a difference in the serological response to vaccination had been demonstrated depending on the schedule of administration employed, investigation should be carried out into whether this finding was reflected by a similar difference in the resistance to infection of animals vaccinated in different ways. For these purposes, pathological studies of vaccination sites, and experimental challenge trials on vaccinated rams were initiated.

CHAPTER 4

LOCAL TISSUE REACTION TO THE ADMINISTRATION OF AN INACTIVATED BRUCELLA OVIS SALINE-IN-OIL VACCINE

INTRODUCTION

Ever since Buddle's experiments in the 1950s and 1960s with an inactivated Brucella ovis saline-in-oil vaccine, similarly formulated vaccines have been recognised as a cause of lesions at the subcutaneous injection site (Buddle, 1962; Claxton, 1968; Quinlivan and Wallace, 1975). It was shown previously (Chapter 3) that these lesions can be large and persistent, and that up to 60% of injection sites may still have a lesion palpable one year after vaccination.

This type of local reaction in the subcutaneous tissue is not peculiar to the Brucella ovis vaccine but is also seen following the use of other vaccines which contain an oil-based adjuvant, such as a multicomponent clostridial oil-emulsion vaccine (Thomson et.al., 1969), and an oil-based commercial footrot (Bacteroides nodosus) vaccine (Ross and Titterington, 1984).

Because it is widely recognised that mineral oil causes this type of reaction, adjuvants based on this material are avoided in human medicine (Jollès and Paraf, 1973). This unpleasant side-effect is apparently unacceptable to some animal owners too (Quinlivan and Wallace, 1975, 1979; Ross and Titterington, 1984). Quinlivan and Wallace (1975) considered these unsightly lesions to be a major reason why vaccination against Brucella ovis had fallen out of favour with sheep farmers. A consequent upsurge in the incidence of the disease reasserted the need for control measures, and farmers again began to look at vaccination as an attractive option (Quinlivan and Wallace, 1975). It was at this time that the intraperitoneal route of administration was suggested as a way of avoiding the visible external lesions produced by the subcutaneous route of vaccination, and many rams have subsequently been vaccinated in this manner

(Quinlivan and Wallace, 1975, 1979). In 1975 Quinlivan and Wallace reported the results of a pilot trial conducted on 40 rams, half of which were given a single dose of vaccine by the intraperitoneal route and half of which received a second vaccination by the same route eight weeks after the first. Only a small number of these rams were found to have localised lesions in the musculature of the right flank when a proportion of them were necropsied. It was not clear from the report how many necropsies had been performed, or how long after vaccination they had been carried out.

Following the introduction of the intraperitoneal method of vaccination, it was reported that "abscess"-like lesions and granulomas in the flank region had been seen at post mortem inspection at the freezing works in groups of culled rams vaccinated by this technique (Surveillance, 1978, 1980). At least one of these groups consisted of surplus two-year-old rams. These were presumably a group of animals from a ram-breeding flock which had remained unsold after the ram sales. It was suggested that an incorrect technique might have caused some of these lesions (Quinlivan and Wallace, 1979), but it was difficult to assess how common it was for intraperitoneal vaccination to result in lesions of this type, mainly because so few adult breeding rams are actually sent for slaughter. In New Zealand, most rams either die of natural causes or are killed on the farm, so the carcass is rarely subjected to post-mortem examination.

It was decided, therefore, to vaccinate a small number of sheep with the Brucella ovis vaccine, both by the intraperitoneal technique described by Quinlivan and Wallace (1979) and by the standard subcutaneous method. Such an arrangement would enable a chronological study to be made of the local reaction to the vaccine at the two sites, by means of serial necropsies carried out on pairs of animals.

MATERIALS AND METHODS

Sheep

Fourteen adult ewes of various ages from farms accredited as being free from Brucella ovis infection were used in this experiment. These animals were clinically normal and had no detectable antibody against Brucella ovis when tested prior to the experiment by the complement fixation test (CFT), gel diffusion test (GD) and enzyme linked immunosorbent assay (ELISA).

Management

The sheep were kept outside in a small paddock of mixed pasture for the duration of the experiment and no supplementary feeding was undertaken.

Vaccination

The vaccine used was from a single batch (series 2935) of the commercially produced Brucella ovis saline-in-oil adjuvant vaccine[1]. It was administered to each animal by both the intraperitoneal and the subcutaneous routes. The dose was 2ml in each site, and the two doses were given one immediately after the other.

The site of the subcutaneous injection was in the anterior half of the neck on the right side (Plate 4.1). The vaccine was administered through an 18 gauge, 9mm needle attached to a sterile disposable syringe. The site was marked using animal marking spray.

The intraperitoneal injection was administered according to the method described by Thomson et.al. (1969) and later used by Quinlivan and Wallace (1979). A 17 gauge, 19mm needle attached to a sterile disposable syringe was introduced at a point halfway between the stifle joint and the vertebral articulation of the last rib on the

right side (Plate 4.1). This is usually the most hollow part of the flank, and on a shorn animal is easily identified. The site was again marked with animal marking spray.

Necropsy

Randomly selected pairs of animals were sacrificed for necropsy at 24 hours, three days, one, two, four, six, and ten weeks after vaccination. (See Table 4.1). They were killed by intravenous injection of pentobarbitone sodium solution[2]. Post-mortem examinations were carried out and gross pathological changes noted; particular attention being paid to the two vaccination sites, the local lymph nodes in the region of these two sites, the peritoneum and the abdominal viscera.

Histology

Tissue from each vaccination site was collected and fixed in 10% formol saline (see Appendix 2). For the subcutaneous site in the neck region, skin, muscle and connective tissue to the depth of the brachiocephalicus muscle was removed over an area of dimensions approximately 5cm x 5cm, with the point of needle entry at the centre. For the intraperitoneal site, a 5cm x 5cm area of tissue was again removed around the injection point, and this included all layers of the abdominal wall.

Lymph nodes in the area of each injection site were also collected and placed in fixative for subsequent histological examination. For the subcutaneous site, the right prescapular, right parotid and right submandibular lymph nodes were examined. For the peritoneal site, the right prefemoral, lumbar aortic and right inguinal nodes were collected. In addition to the routine collection of these tissues, any other tissues showing abnormality on gross examination were also collected and fixed for subsequent histological examination.

Selected trimmed sections of the tissues were embedded in paraffin wax, then cut and processed by routine histological methods.

All the tissue samples were stained with Haematoxylin and Eosin (H & E). Selected sections were also stained by the Van Gieson and Sudan Black methods. (See Appendix 2 for histological methods).

Serology

To detect the serological response to vaccination, blood samples were collected from the ewes just before they were killed, as shown in Table 4.1. Three serological tests were used, the complement fixation test (CFT), gel diffusion test (GD) and enzyme linked immunosorbent assay (ELISA). These tests were carried out by the Central Brucellosis Laboratory of Wallaceville Animal Research Centre, Upper Hutt, New Zealand. The complement fixation titres were allocated scores as described in Chapter 3.

Prescapular lymph nodes

During the initial necropsies, it was noticed that there was sometimes a discrepancy in the size of the left and right prescapular lymph nodes. In an attempt to quantify this difference, the lymph nodes were trimmed of the majority of their surrounding fat and their weights recorded after they had been fixed in formalin for at least one week. Since this difference was only noted and investigated after several necropsies had already been completed, only seven out of the fourteen animals had both the left and the right prescapular lymph nodes weighed.

Chronic Reaction

In addition to the 14 ewes used in this experiment, post-mortem examinations were also carried out on 30 rams which had been vaccinated by the intraperitoneal technique for other experiments (Chapter 6, Chapter 7). This was done in order to provide further information about the local reaction at the abdominal site. These latter necropsies were carried out six months after vaccination.

NOTES

1) Ramovexin - inactivated Brucella ovis saline-in-oil vaccine. Coopers Animal Health N.Z. Ltd.

2) Pentobarb 300 - pentobarbitone sodium solution. South Island Chemicals Ltd, Christchurch, New Zealand.

Plate 4.1

The sites used for the subcutaneous (neck) and intraperitoneal (flank) administration of the Brucella ovis vaccine are shown on this sheep by the purple dye marks.

RESULTS

No obvious signs of distress were observed in any of the ewes following vaccination.

Serology

The first positive reaction to both the complement fixation test and to the ELISA was recorded two weeks after vaccination. All the animals tested after this (four, six and ten weeks after vaccination) had positive reactions to these two tests (Table 4.1). Only five of the animals had positive reactions to the gel diffusion test at any time.

TABLE 4.1

Serological Response of Sheep to Simultaneous Subcutaneous and Intraperitoneal Vaccination Using A Brucella ovis Vaccine

Time After Vaccination	24 hrs	3 days	7 days	2 wks	4 wks	6 wks	10 wks
Animals Necropsied	1 & 2	3 & 4	5 & 6	7 & 8	9 & 10	11 & 12	13 & 14
CFT Titre (score)							
First animal	0 (-)	0 (-)	0 (-)	12 (+)	24 (+)	24 (+)	24 (+)
Second animal	0 (-)	0 (-)	4 (-)	12 (+)	20 (+)	24 (+)	24 (+)
Mean	0	0	2	12	22	24	24
ELISA Titre							
First animal	13 (-)	16 (-)	42 (-)	108 (+)	482 (+)	1452 (+)	598 (+)
Second animal	24 (-)	15 (-)	39 (-)	88(sus)	225 (+)	492 (+)	189 (+)
Mean	18.5	15.5	40.5	98	355	972	393.5
GD Result							
First animal	-	-	-	+	+	+	-
Second animal	-	-	-	+	-	+	-

KEY: + = positive reaction
- = negative reaction
sus = suspicious reaction

Gross Pathology

1) Subcutaneous Site

Twenty-four hours after vaccination there was no palpable reaction at the injection site in either of the two sheep necropsied, and no visible abnormality was noted. One of the sheep necropsied three days after vaccination, however, did have a palpable reaction at the injection site. This was a discrete, round, firm swelling in the subcutaneous tissue approximately 2cm in diameter, which contained a thin white liquid material similar to the Brucella ovis vaccine. Seven days after vaccination one of the animals examined had no palpable reaction at the injection site, but when the area was incised, white liquid similar to the Brucella ovis vaccine oozed from the tissues. The other sheep had a palpable hard swelling 3cm (dorso-ventrally) x 1cm (crano-caudally) which was firm and fibrous in texture.

From two weeks onward every sheep had a palpable reaction at the injection site (Plate 4.2) apart from one of the last pair examined at ten weeks. These lesions were variable in size and shape (Table 4.2), varying from a spherical nodule 2cm in diameter to a flattish, rectangular swelling 10cm x 4cm. The rectangular lesions always had a vertical (dorso-ventral) dimension greater than the horizontal (crano-caudal). None of the lesions were seen to discharge material to the exterior.

On incision, the centre of these lesions consisted of necrotic, semisolid, dark yellow caseous material which was encapsulated by fibrous granulation tissue. The lesions were sometimes single discrete nodules, and sometimes multilocular in structure. They were usually firm or quite hard when palpated, but were occasionally soft and easily compressed.

Table 4.2

Gross Findings At The Subcutaneous Injection Site
In 14 Sheep Vaccinated Using A **Brucella ovis** Vaccine

Time After Vaccination And Sheep Number	Lesion	Shape	Size	Consistency
24 hours				
1	no	-	-	-
2	no	-	-	-
3 days				
3	no	-	-	-
4	yes	round	2 cm diameter	hard
7 days				
5	no	-	-	-
6	yes	rectangular	3 cm x 1 cm	hard
2 weeks				
7	yes	oval	6 cm x 3 cm	hard
8	yes	round	2 cm diameter	hard
4 weeks				
9	yes	rectangular	10 cm x 4 cm	hard
10	yes	round	3 cm diameter	soft
6 weeks				
11	yes	oval	5 cm x 2 cm	soft
12	yes	round	2 cm diameter	hard
10 weeks				
13	yes	oval	5 cm x 3 cm	hard
14	no	-	-	-

Plate 4.2

Typical reaction in the neck of an animal following the administration of Brucella ovis vaccine by the subcutaneous route.

2) Intraperitoneal site

During the first week, the reaction at the site of the intraperitoneal injection consisted of the appearance of tags of fibrin on serosal surfaces and the formation of aggregations of fibrino-purulent material, both floating freely in the peritoneal cavity and attached to serosal surfaces. (See Table 4.3). Both animals necropsied at 24 hours were found to have a moderate excess of serous fluid in the peritoneal cavity. Both had clots of creamy white material floating freely in this fluid. Similar small, white, soft clots were found adherent to the large intestine in one animal (Plate 4.3) and the other had tags of fibrin-like material adherent to the greater omentum on the right side of the rumen. (Plate 4.4). A smear was made from one of the floating clots and it was found to contain a large number of leucocytes, many of them degenerate.

Three days after vaccination a soft, abscess-like structure was seen on a serosal surface adjacent to the right ovary in one animal (Plate 4.5). Some fibrino-purulent material was found adherent to the parietal peritoneum and mesentery, and the peritoneal fluid again contained clots of creamy fibrino-purulent exudate (Plate 4.6). The omentum was inflamed over most of its surface and had a roughened texture. (Plate 4.7). In the other sheep, however, the vaccine had not reached its intended site of deposition in the abdominal cavity, but had been trapped just beneath the parietal peritoneum. (Plate 4.8). The inflammatory reaction was confined to the area immediately adjacent to this deposit, and no other abnormalities were found.

One week after vaccination, one of the sheep examined had fibrinous tags adherent to the gall bladder (Plate 4.9) and omentum. An accumulation of fibrino-purulent material was seen on the peritoneum near the rectum, and there was slight congestion of the lumbar aortic lymph nodes. The other animal had a localised area of peritonitis on the parietal peritoneum at the site of injection (Plate 4.10) and mild fibrinous inflammation of parts of the mesentery and greater omentum.

From two to four weeks after vaccination, the inflammatory reaction became more extensive, and adhesions between the viscera and the parietal peritoneum covering the abdominal wall were seen. A second case of vaccine not having been deposited in the peritoneal cavity was seen in one of the animals necropsied at two weeks. In this animal a hard nodule could be palpated in the abdominal wall which was more discrete and firm than that seen in the previous case. There was only slight inflammation of the surface of the omentum in this animal, but in the other, in which the vaccine had been deposited in the peritoneal cavity, the omentum was severely affected, especially on its ventral aspect beneath the rumen (Plate 4.11). In this animal, the first instance of fibrous adhesion formation was observed. The adhesions were found attaching the large intestine to the dorsocranial abdominal wall. More adhesions were seen at four weeks, when both the animals necropsied had adhesions present between the caudo-ventral part of the omentum and the parietal peritoneum (Plate 4.12). In addition, the omentum covering the rumen in both animals had a roughened, irregular surface, and the affected tissue appeared slightly gelatinous. One animal had diffuse white, raised streaks and nodules on the caudal (abdominal) aspect of the diaphragm (Plate 4.13), while the other had a localised reaction on the parietal peritoneum at the site of vaccination.

At six weeks after vaccination, one animal had only a slight inflammatory reaction on the omentum, and the vaccine had accumulated beneath the parietal peritoneum. In the other animal the vaccine seemed to have been deposited partially beneath the peritoneum. A flat, rectangular, fibrous plaque 5cm x 3cm x 0.3cm was present on the abdominal wall at the point of injection. The most spectacular finding in this animal, however was an extensive and severe inflammation of the omentum covering the ventral surface of the rumen. This area was firm and rough in texture. (Plate 4.14). A similar but less extensive reaction was seen near the jejunum. Examination of one of the animals killed at ten weeks revealed no gross abnormalities, but in the other animal there was a plaque-like area of fibrosis on the peritoneal surface of the abdominal wall at the site of injection (Plate 4.15) and a moderate roughening of the surface of the ventral omentum.

Table 4.3

Necropsy Findings At The Abdominal Injection Site
In 14 Sheep Vaccinated By The Intraperitoneal Technique
Using **Brucella ovis** Vaccine

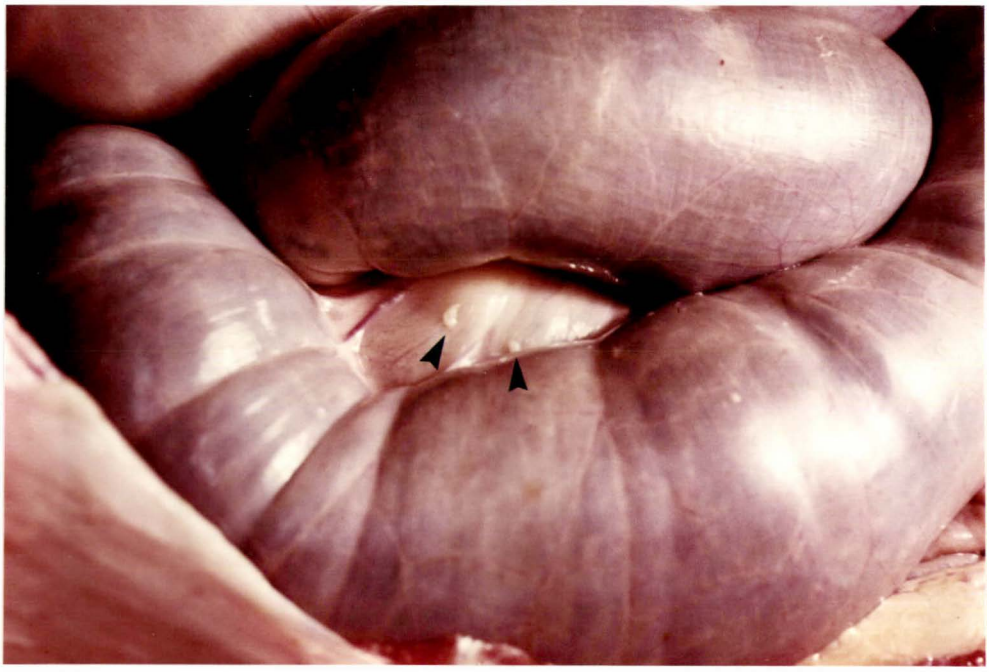
Time Of Necropsy And Animal Number	Vaccine Deposited Outside Abdominal Cavity	Parietal Peritonitis At Vaccination Site	Visceral Peritonitis	Purulent Material	Serosal Adhesions
24 hrs					
1	-	-	-	floating in cavity	-
2	-	-	mild (fibrin tags)	floating in cavity	-
3 days					
3	-	-	mild (fibrin tags)	floating in cavity/ parietal peritoneum	-
4	yes	yes	-	-	-
7 days					
5	-	yes	mild	-	-
6	-	-	mild (fibrin tags)	adherent to rectum	-
2 weeks					
7	-	-	severe	-	yes
8	yes	yes	mild	-	-
4 weeks					
9	-	-	moderate	-	yes
10	-	yes	moderate	-	yes
6 weeks					
11	partially	yes	severe	-	-
12	yes	yes	mild	-	-
10 weeks					
13	-	yes	moderate	-	-
14	-	-	-	-	-

Plate 4.3

Fibrino-purulent clots (arrowed) on serosal surfaces seen 24 hours after the administration of Brucella ovis vaccine by the intraperitoneal route.

Plate 4.4

Fibrinous inflammation of the omentum over the rumen, seen 24 hours after the administration of Brucella ovis vaccine by the intraperitoneal route.



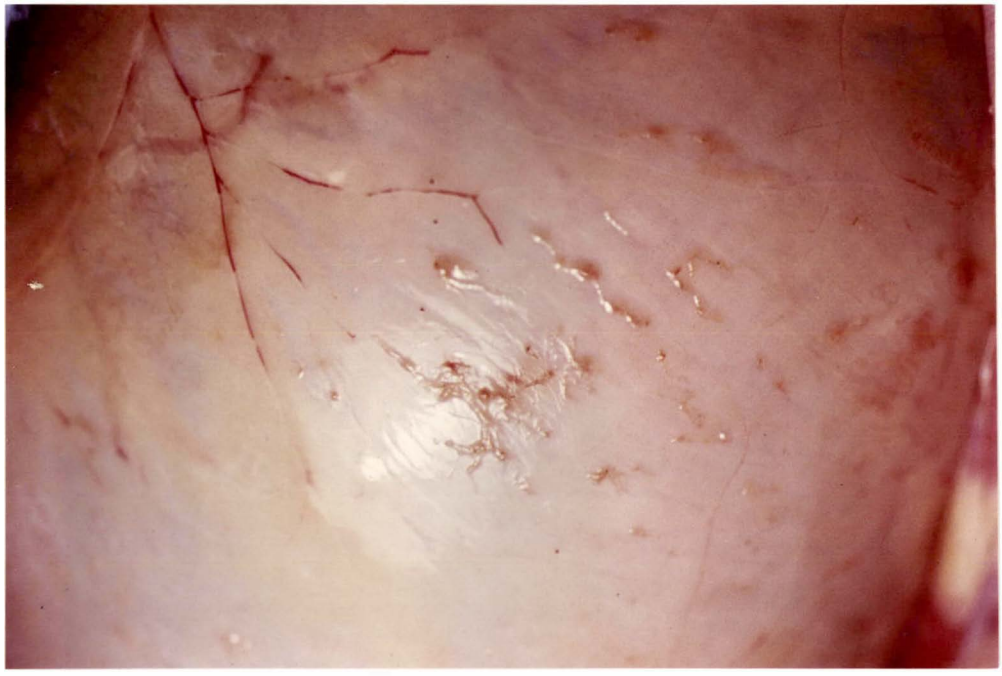
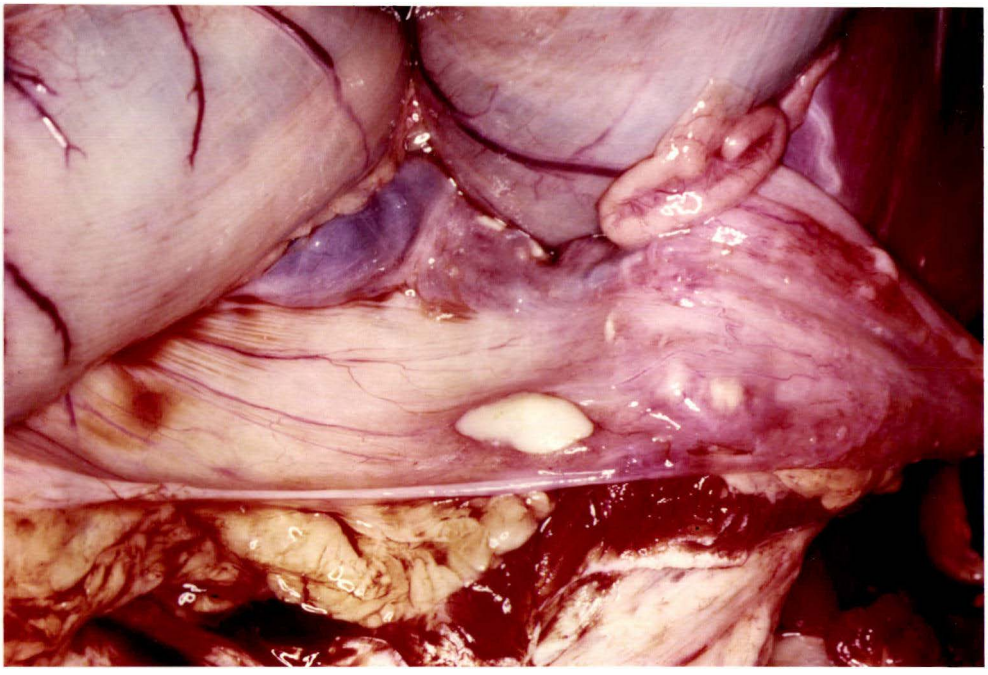


Plate 4.5

An abscess-like structure near the right ovary. Brucella ovis vaccine had been administered by the intraperitoneal route three days earlier.

Plate 4.6

Fibrino-purulent exudate (arrowed) floating in the peritoneal fluid. Brucella ovis vaccine had been administered by the intraperitoneal route three days earlier.



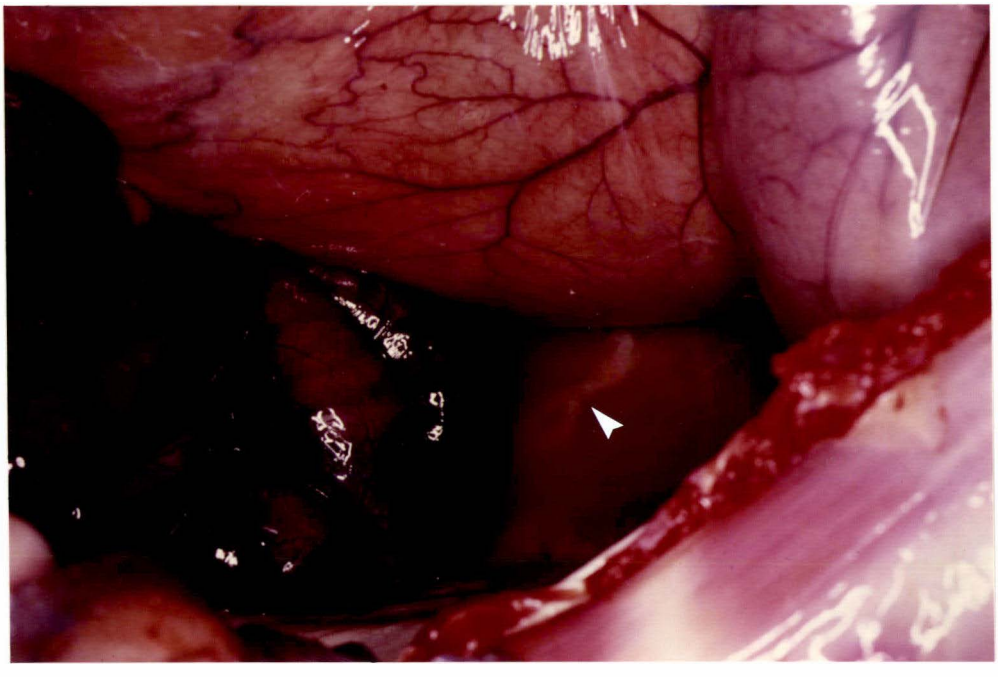
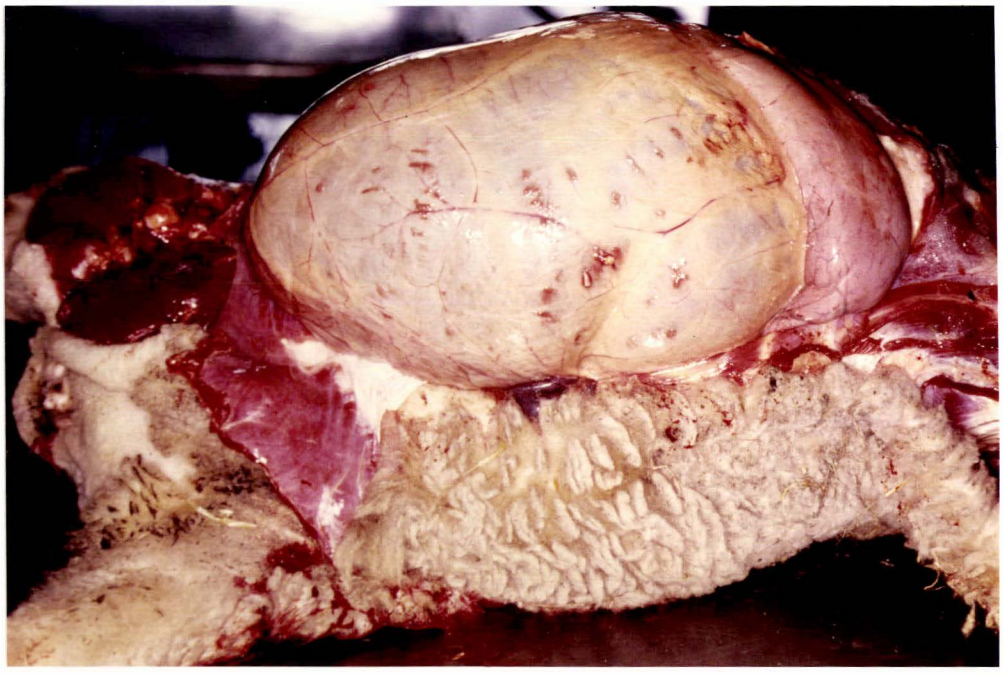


Plate 4.7

Inflammation of the omentum seen three days after the administration of Brucella ovis vaccine by the intraperitoneal route.

Plate 4.8

Brucella ovis vaccine, administered by the intraperitoneal method three days previously, trapped behind the parietal peritoneum in the flank region.



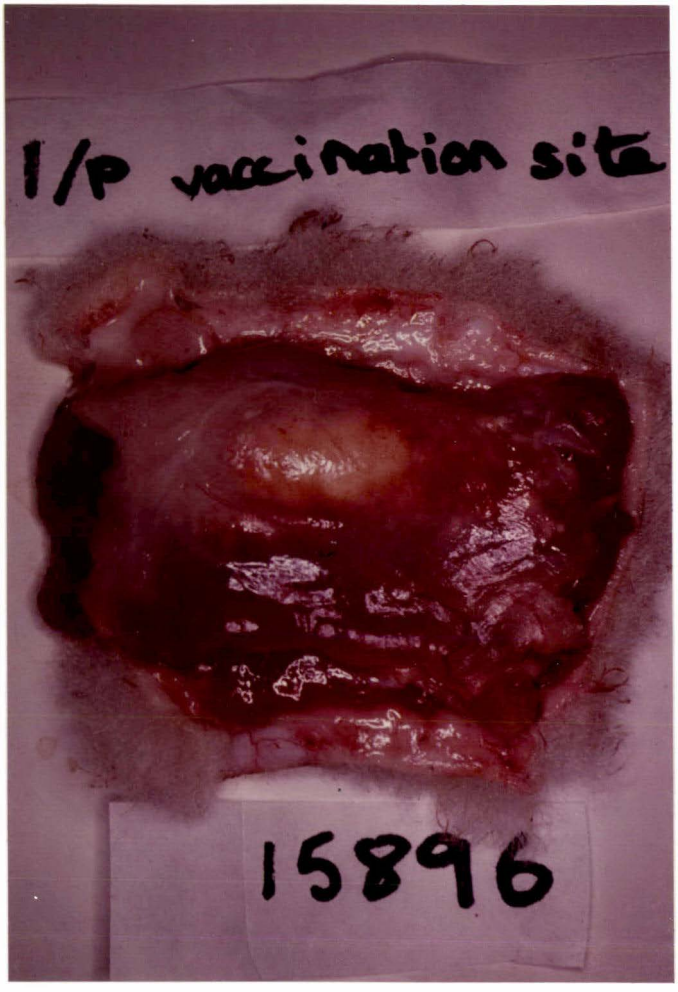
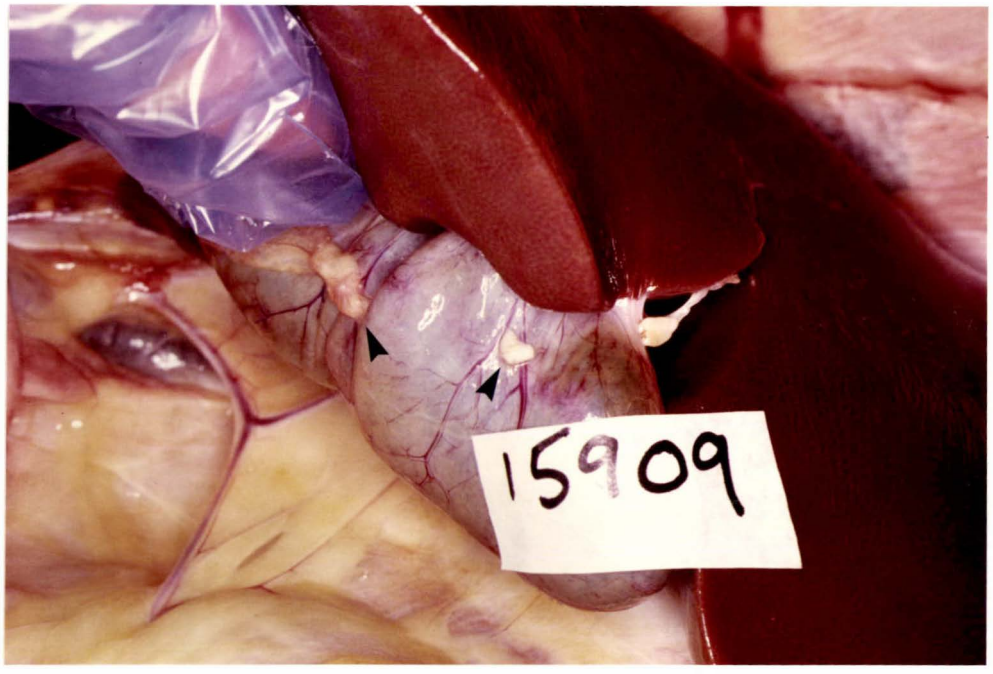

I/P vaccination site
15896

Plate 4.9

Fibrinous tags (arrowed) adherent to the gall bladder. Brucella ovis vaccine had been administered by the intraperitoneal route seven days previously.

Plate 4.10

Mild inflammation of the parietal peritoneum observed seven days after the administration of Brucella ovis vaccine by the intraperitoneal route.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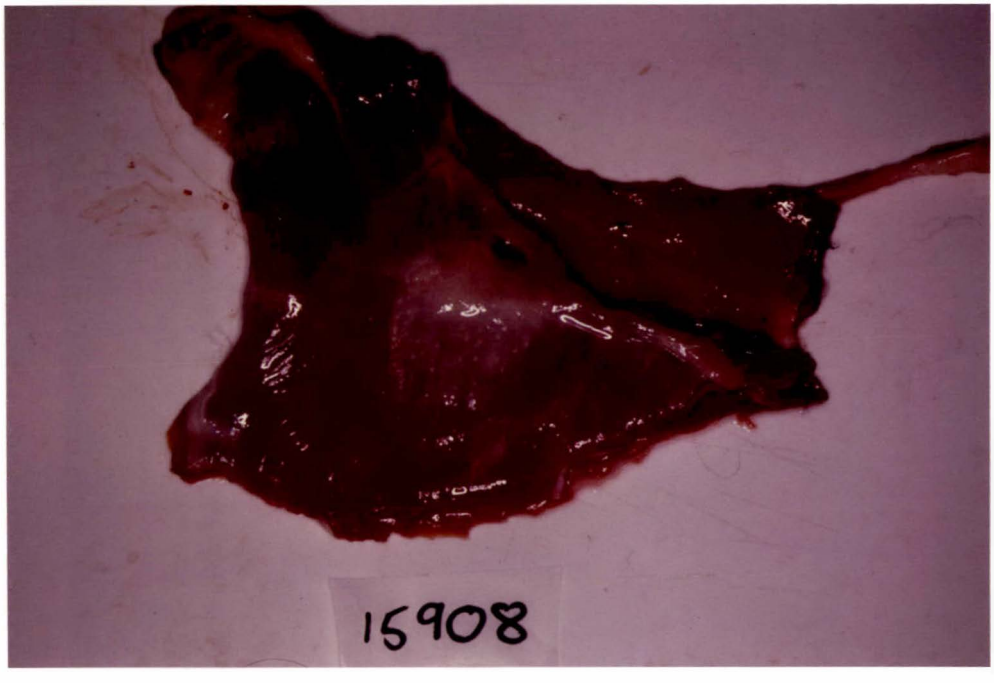
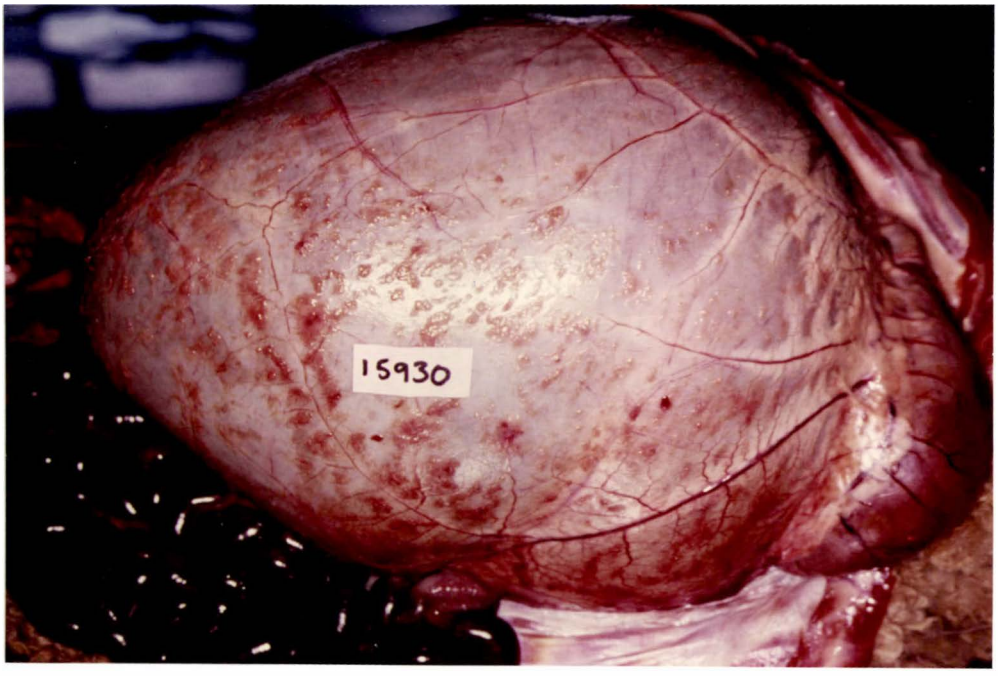

15908

Plate 4.11

Severe inflammation of the omentum. Brucella ovis vaccine had been administered by the intraperitoneal route two weeks previously.

Plate 4.12

Fibrous adhesions between the omentum and the abdominal wall seen four weeks after the administration of Brucella ovis vaccine by the intraperitoneal route.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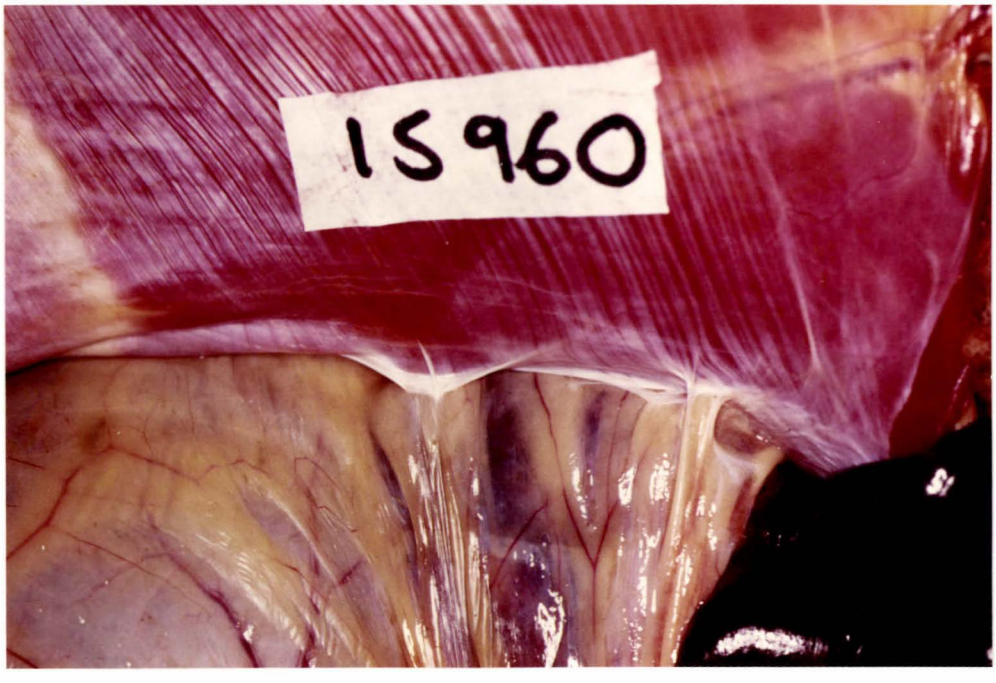
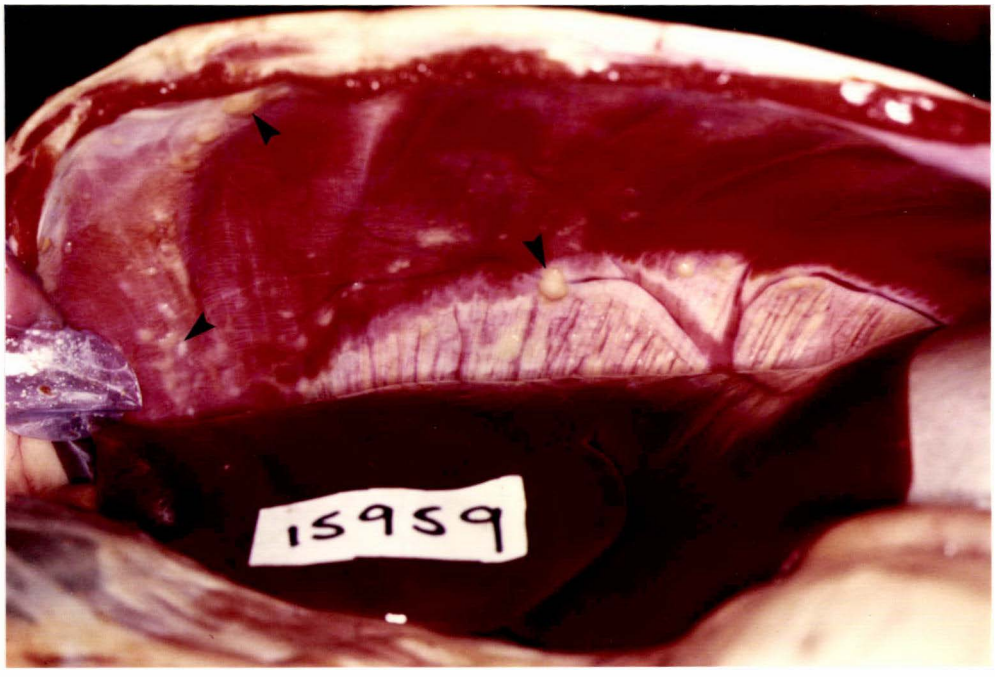

15960

Plate 4.13

Nodular reaction (arrowed) on the surface of the diaphragm seen four weeks after the administration of Brucella ovis vaccine by the intraperitoneal route.

Plate 4.14

Severe and widespread inflammation of the omentum seen six weeks after the administration of Brucella ovis vaccine by the intraperitoneal route.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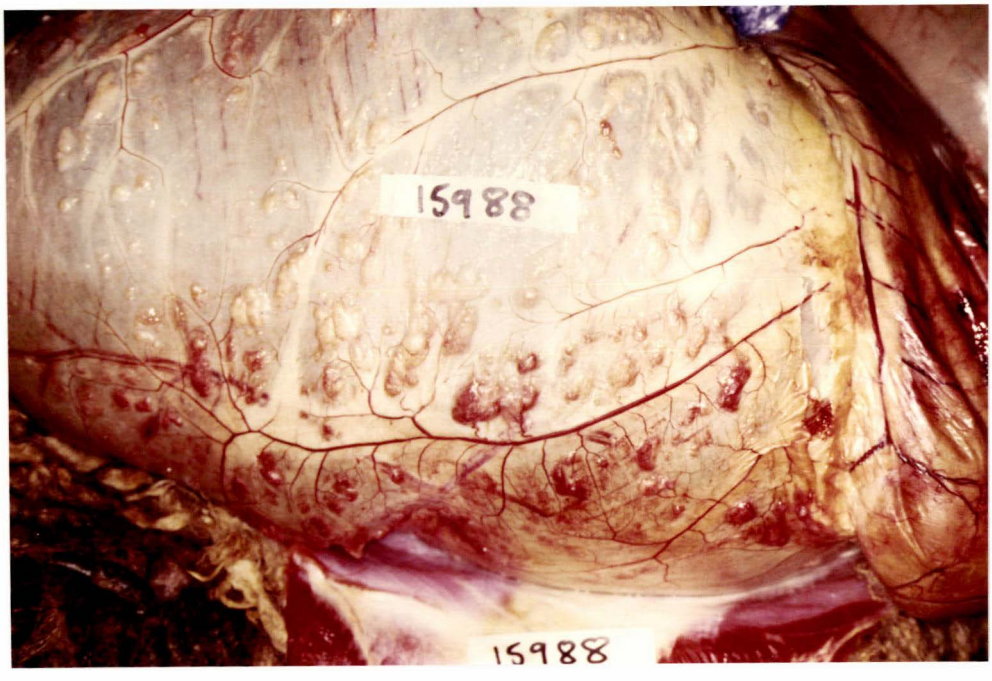
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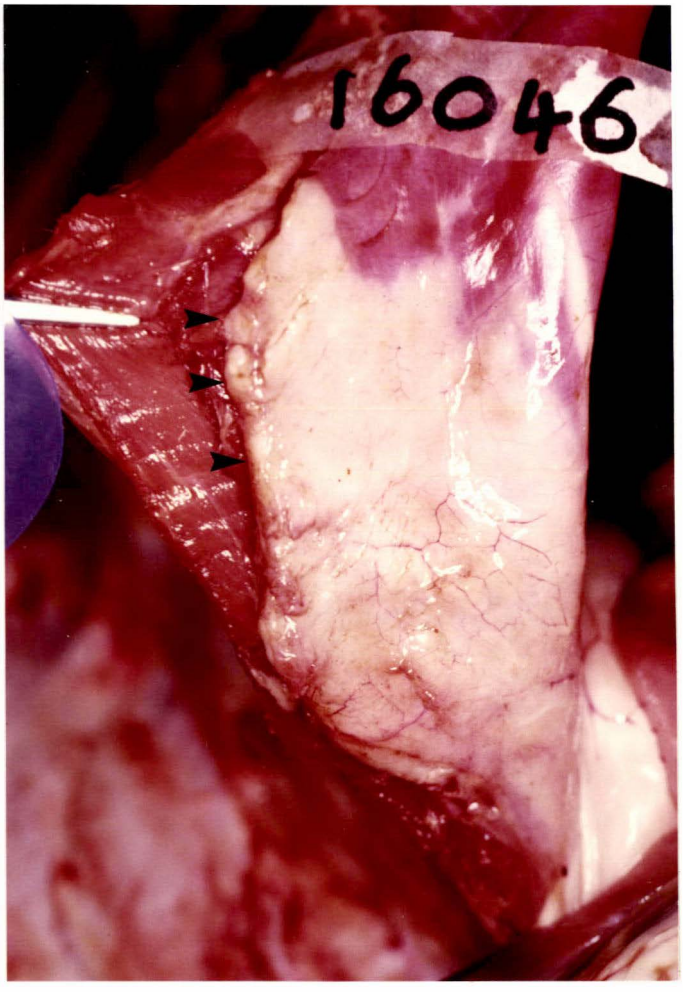
15988

Plate 4.15

Fibrosis in the abdominal wall seen on the cut surface (arrowed) of the peritoneum at the site of injection. Brucella ovis vaccine had been administered by the intraperitoneal method ten weeks previously.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Microscopic Pathology

A) Subcutaneous Vaccination

a) Injection Site

Twenty-four hours after vaccination, the reaction at the subcutaneous site was characterised by marked oedema of muscle and connective tissue, and apparent dilation of the lymphatic vessels. Round spaces, presumably occupied previously by vaccine oil which had been removed by the solvents used in processing, were present in the subcutaneous and intermuscular connective tissue (Plate 4.16). There was some acute myonecrosis. Neutrophils were the predominant cell type in the mild cellular reaction around the oil spaces, and there were also a few macrophages present.

Three days after vaccination, the vaccine lesion consisted mainly of fibrin infiltrated by leucocytes, and early proliferation of capillaries and fibroblasts was observed. The cellular reaction was now more extensive, with some of the oil spaces delineated by a dense inner margin of neutrophils (Plate 4.17), which were still the predominant cell type. Macrophages were more commonly seen than at 24 hours, and lymphocytes and plasma cells were now in evidence.

Fibroblasts were the predominant cell type seen in the vaccination site seven days after the injection (Plate 4.18). There was also marked capillary proliferation. Smaller numbers of neutrophils were seen, and lymphocytes and plasma cells were more common. Necrotic foci were present on the edges of some of the oil spaces, and multinucleate giant cells were seen for the first time.

By two weeks after vaccination, collagen formation had begun and numerous capillaries were proliferating. Macrophages were the predominant type of inflammatory cell present, and multinucleate giant cells were common (Plate 4.19). Foci of caseous necrosis were seen, and some of these were undergoing dystrophic calcification.

A granulomatous structure, similar to that which develops in tuberculosis, had begun to develop by four weeks. The necrotic central areas were becoming lined by macrophages which were in turn encircled by lymphocytes (Plate 4.20). Dense bands of fibrous connective tissue formed capsules around these inflammatory zones. Plasma cells were present in large numbers, and some giant cells were observed in the macrophage zones. Neutrophils were present only in small numbers. Examination of the subcutaneous site at six weeks revealed the granuloma structure to have developed further, becoming more discrete and having a minimal diffuse reaction. At the end of the experiment at ten weeks, the lesions displayed a well organised, chronic granulomatous structure and the reaction was largely isolated from surrounding tissue by a thick capsule of fibrous tissue. (See Table 4.4).

b) Local Lymph Nodes

1) Prescapular

Twenty-four hours after vaccination, both right prescapular lymph nodes examined contained many oil spaces (Plate 4.21), with focal accumulations of polymorphonuclear cells (especially neutrophils) around them. There were also focal aggregations of large foamy macrophages, mainly at the paracortical margin. Multinucleate giant cells in small numbers were also seen. Similar features were seen three days after vaccination, together with paracortical hyperplasia suggesting T-cell proliferation. Only one of the two right prescapular lymph nodes examined at seven days contained oil spaces, but both nodes contained large accumulations of macrophages and multinucleate giant cells. Two weeks after vaccination, both nodes examined contained oil spaces (Plate 4.22), and in one they were mainly in the subcapsular region with little tissue reaction around them, suggesting that vaccine might be continuing to drain into the node. One node had greater than normal numbers of plasma cells and lymphocytes in the medulla.

Both right prescapular lymph nodes examined at four weeks contained oil spaces, aggregations of multinucleate giant cells and large macrophages with abundant cytoplasm, and a hypercellular medulla packed with macrophages. In addition, one of the nodes contained two calcifying, necrotic foci in the subcapsular region, surrounded by giant cells and encapsulated by fibrous tissue. One focus was 2.5mm long x 2mm wide, and the other 1.5mm long x 1.25mm wide. In one of the right prescapular lymph nodes examined at six weeks, a necrotic focus was seen (Plate 4.23), and both nodes contained oil spaces. Prolific numbers of oil spaces (Plate 4.24) were seen in the medullary cords in the left prescapular lymph node of the animal which had the necrotic focus in the right.

Both right prescapular lymph nodes examined at ten weeks had reactive germinal centres and numerous follicles, indicating active transformation of stimulated B-lymphocytes. Both had haemorrhages in areas of the cortex, and aggregations of multinucleate giant cells. Oil spaces were also seen in both nodes, and in one they were prominent in the subcapsular area, the lack of cellular reaction around them suggesting continuing drainage into the node. Aggregations of giant cells were seen in both of the left prescapular lymph nodes of these animals (Plate 4.25).

2) Submandibular

Although the submandibular lymph nodes of the fourteen experimental sheep appeared normal grossly, when examined histologically, most of the nodes contained large numbers of macrophages, some having a foamy cytoplasm. Some of the nodes contained small aggregations of giant cells. Oil spaces were seen in the first two samples, taken at 24 hours and three days, and they occured mainly in the medullary cords. The cortices of the nodes appeared somewhat hyperplastic for the first two weeks after vaccination, and haemorrhages were seen in nodes collected at two, six and ten weeks.

3) Parotid

The changes in the parotid lymph nodes were minor. Most had diffuse infiltrations of numerous macrophages, together with occasional aggregations of active cells of this type which had a foamy cytoplasm. Some nodes had numerous very active germinal centres, while others had variable degrees of paracortical hyperplasia. Oil spaces were seen in the nodes of two animals; one at two weeks and one at six weeks.

Table 4.4

Histological Findings at the Subcutaneous Site of Administration of an Inactivated Brucella ovis Saline-in-Oil Vaccine

	Time Since Vaccination							
	24 hours		3 days		7 days		2 weeks	
	Animal Number							
	1	2	3	4	5	6	7	8
Diameter of oil spaces (mm)	1	0.75	1.25	1	2	0.75	2.9	1
Oedema	+++	++	+	+	+	+	+	+
Myodegeneration	++	-	+	-	+	+	+	+
Necrosis	-	+	-	+	+	-	++	+
Calcification	-	-	-	-	-	-	+	-
Neutrophils	++	++	+++	++	+	+	+	+
Macrophages	+	+	++	++	+	++	+++	+++
Lymphocytes	-	-	++	++	+	+	+++	++
Plasma cells	-	-	+	+	+	+	++	++
Giant cells	-	-	-	-	-	-	+	++
Fibroblasts	+	+	+	++	+++	+++	++	++
Collagen formation	-	-	-	-	-	-	+	+
Capillary proliferation	-	-	+	+	++	+	+++	+++
Type of Reaction	a	a	a	b	b	b	c	c

KEY:
- = absent
+ = present
++ = common
+++ = abundant

a = diffuse
b = loose circular pattern
c = granulation tissue
d = early granuloma formation
e = classic granulomatous reaction

Table 4.4 (continued)

Histological Findings at the Subcutaneous Site of Administration of an Inactivated **Brucella ovis** Saline-in-Oil Vaccine

	Time Since Vaccination					
	4 weeks		6 weeks		10 weeks	
	Animal Number					
	9	10	11	12	13	14
Diameter of oil spaces (mm)	0.6	1.5	0.75	1.5	2.9	1.625
Oedema	+	+	+	+	-	-
Myodegeneration	+	+++	+	++	-	+
Necrosis	++	+	+++	+++	+	-
Calcification	-	+	++	+	-	-
Neutrophils	+	-	++	+	+	+
Macrophages	+++	++	++	+++	+++	++
Lymphocytes	++	+++	++	++	++	+++
Plasma cells	+++	+++	+++	++	++	++
Giant cells	+	+	+	+	++	+
Fibroblasts	++	+++	++	++	++	++
Collagen formation	++	+++	+++	+++	+++	++
Capillary proliferation	++	+	+++	+++	++	+++
Type of Reaction	d	d	d	e	e	e

KEY:
- \- = absent
- \+ = present
- ++ = common
- +++ = abundant

- a = diffuse
- b = loose circular pattern
- c = granulation tissue
- d = early granuloma formation
- e = classic granulomatous reaction

Plate 4.16

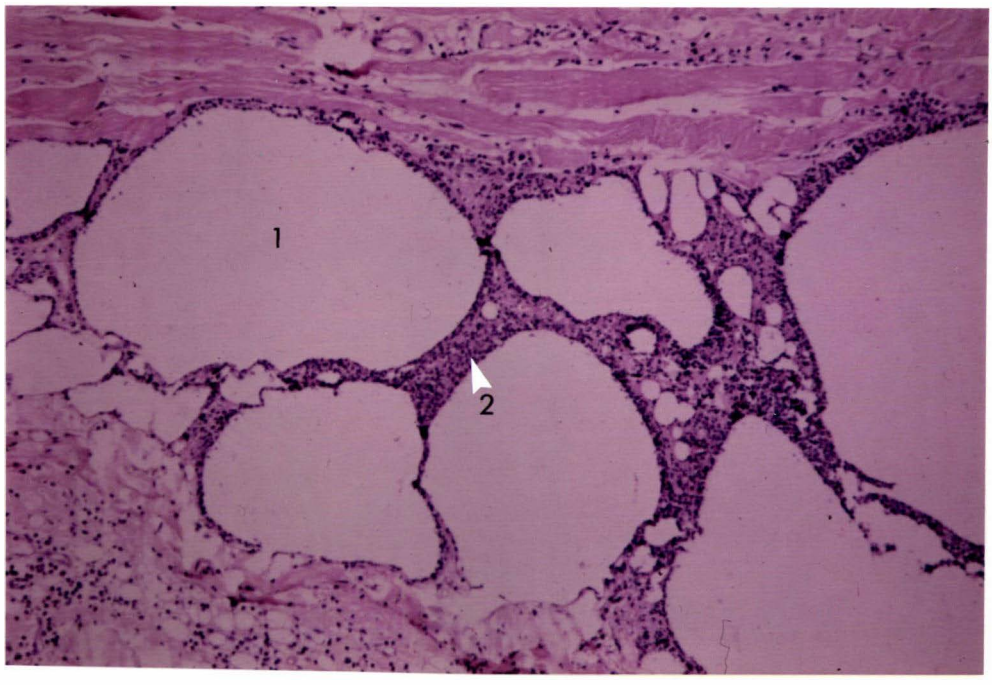
Subcutaneous injection site in the neck, 24 hours after the administration of Brucella ovis vaccine. Large oil spaces (1) are present, and the cellular reaction (2) consists mainly of neutrophils.

60 x magnification. H & E stain.

Plate 4.17

Subcutaneous injection site three days after the administration of Brucella ovis vaccine, showing oil spaces (1) lined by a dense margin of neutrophils (2).

60 x magnification. H & E stain.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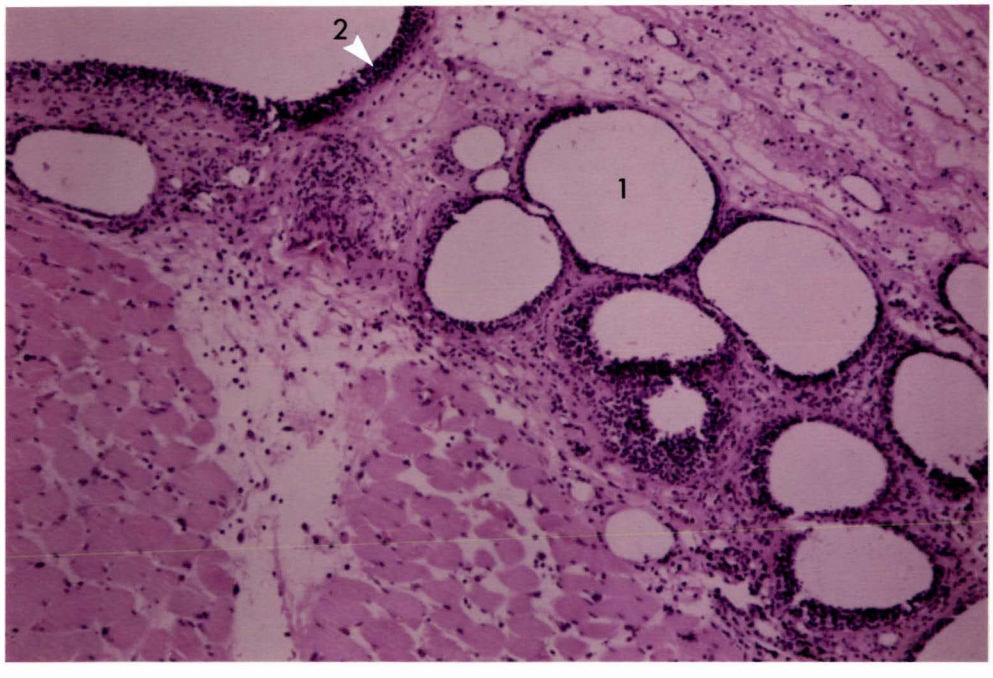
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Plate 4.18

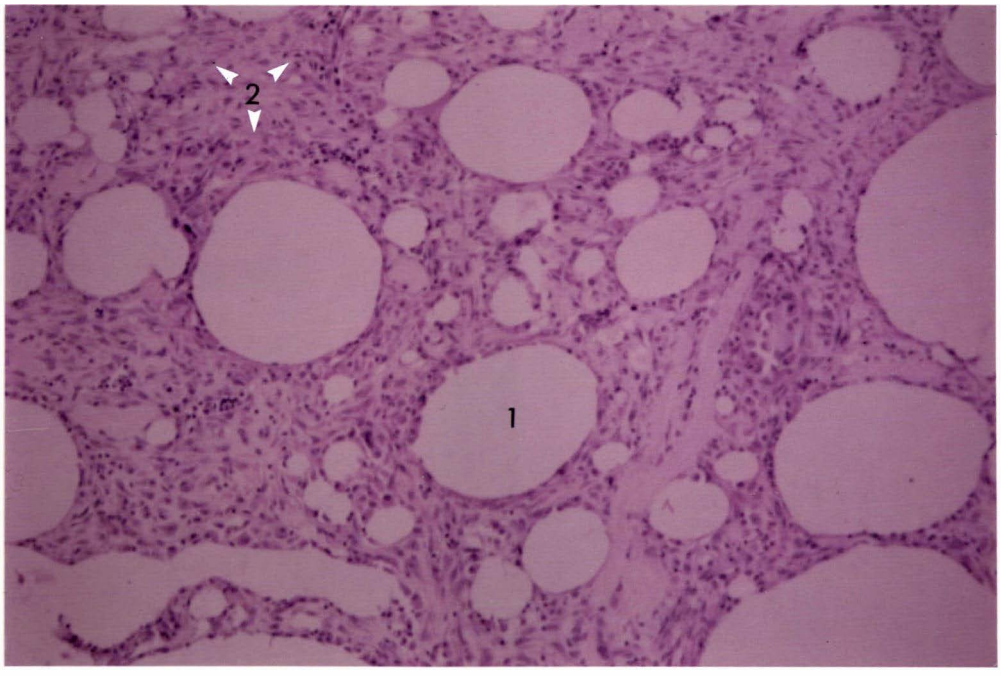
Subcutaneous injection site seven days after the administration of Brucella ovis vaccine. The oil spaces (1) are surrounded by a cellular reaction (2) consisting mainly of fibroblasts.

60 x magnification. H & E stain.

Plate 4.19

Multinucleate giant cells (arrowed) seen at the subcutaneous site two weeks after the administration of Brucella ovis vaccine.

200 x magnification. H & E stain.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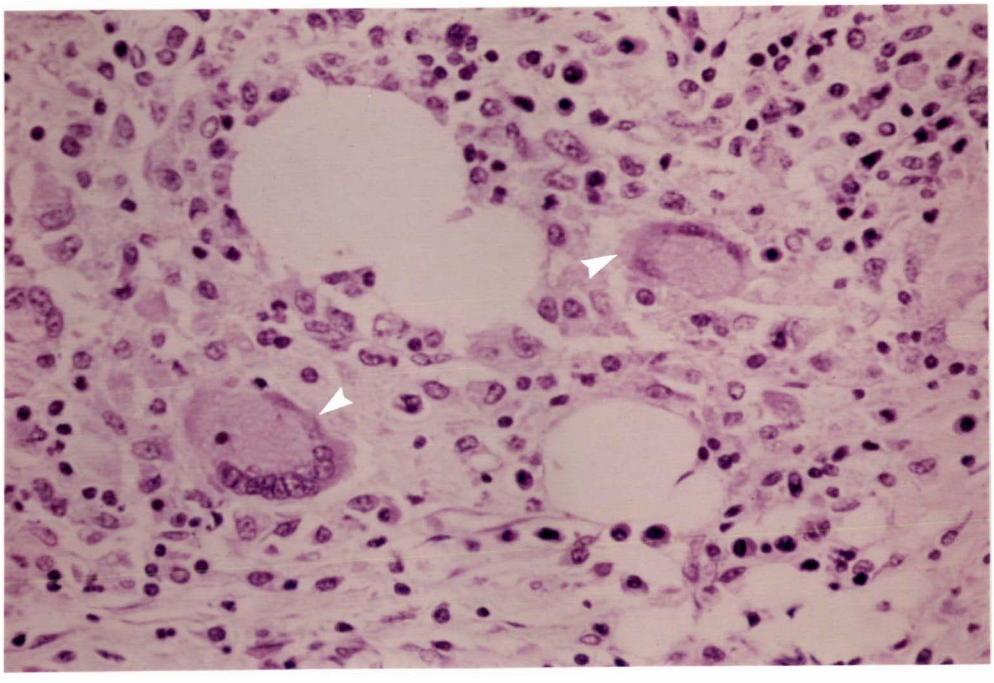


Plate 4.20

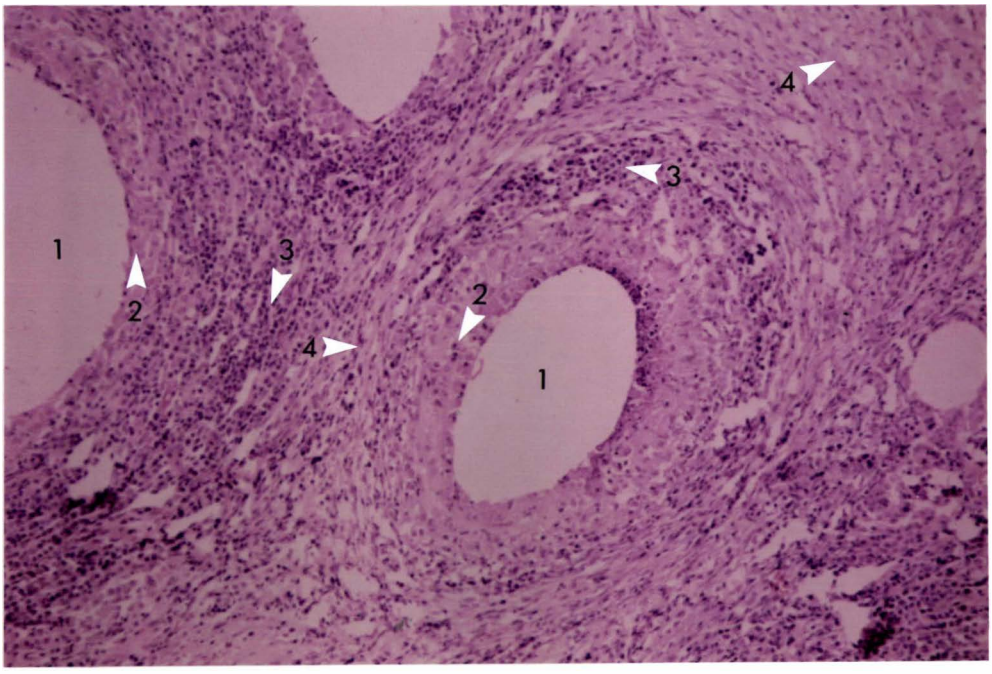
Subcutaneous injection site four weeks after the administration of Brucella ovis vaccine. A typical granulomatous structure is developing in which oil spaces (1) lined by macrophages (2) are encircled by lymphocytes (3) and surrounded by fibrous tissue (4).

60 x magnification. H & E stain.

Plate 4.21

Oil spaces (arrowed) in the right prescapular lymph node of an animal 24 hours after the administration of Brucella ovis vaccine by both the subcutaneous and intraperitoneal routes.

50 x magnification. H & E stain.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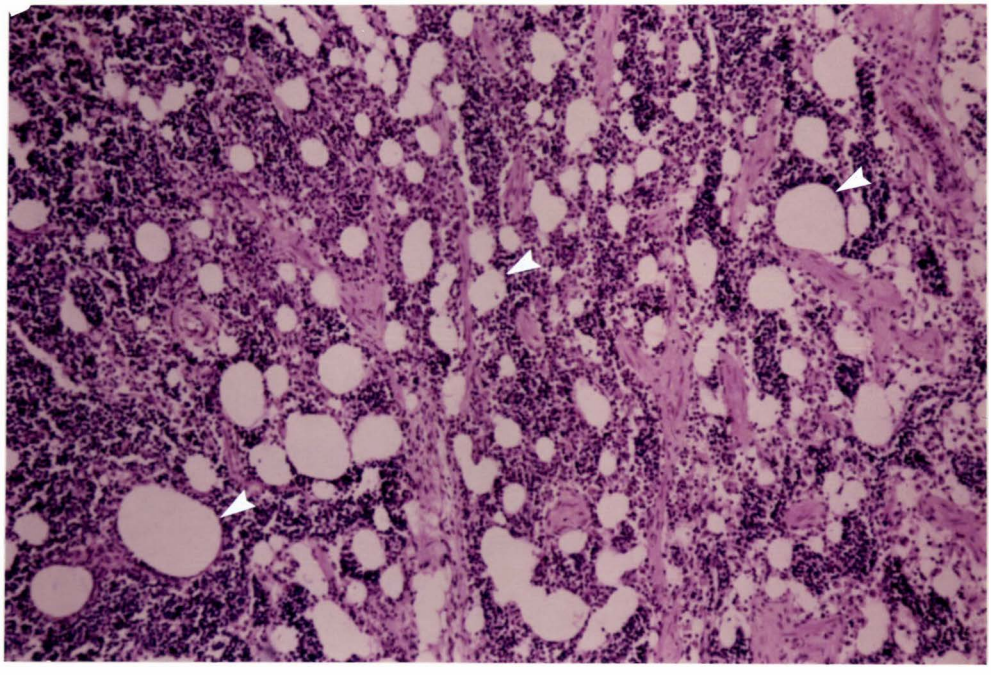


Plate 4.22

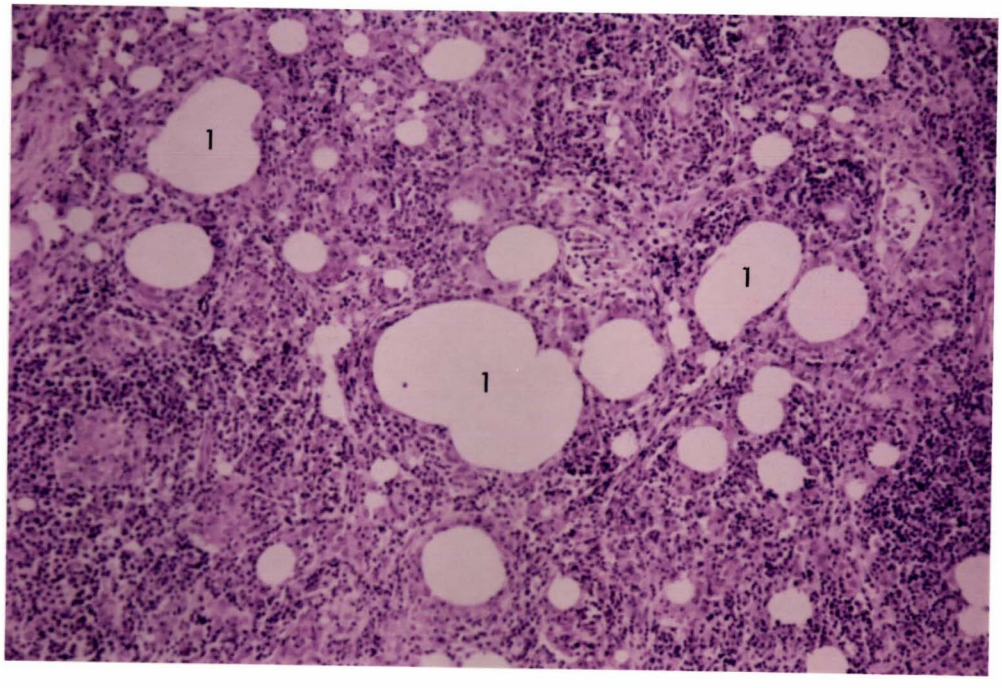
Oil spaces (1) seen in the prescapular lymph node of an animal two weeks after receiving Brucella ovis vaccine administered by both the subcutaneous and the intraperitoneal routes.

60 x magnification. H & E stain.

Plate 4.23

Focal areas of necrosis (arrowed) in a prescapular lymph node six weeks after the administration of Brucella ovis vaccine by both the subcutaneous and intraperitoneal routes.

50 x magnification. H & E stain.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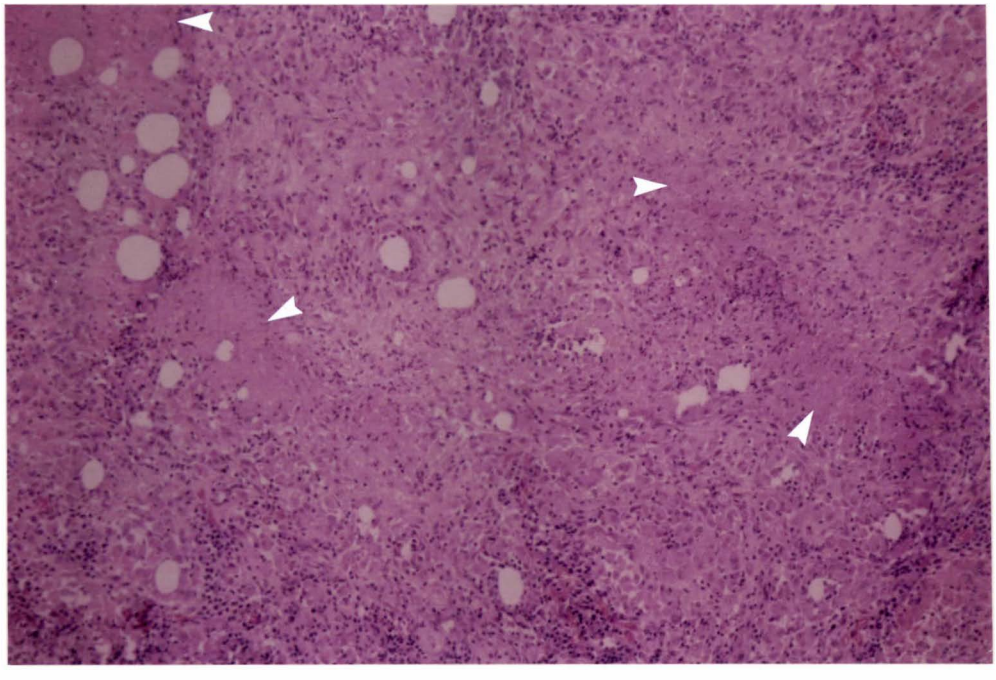


Plate 4.24

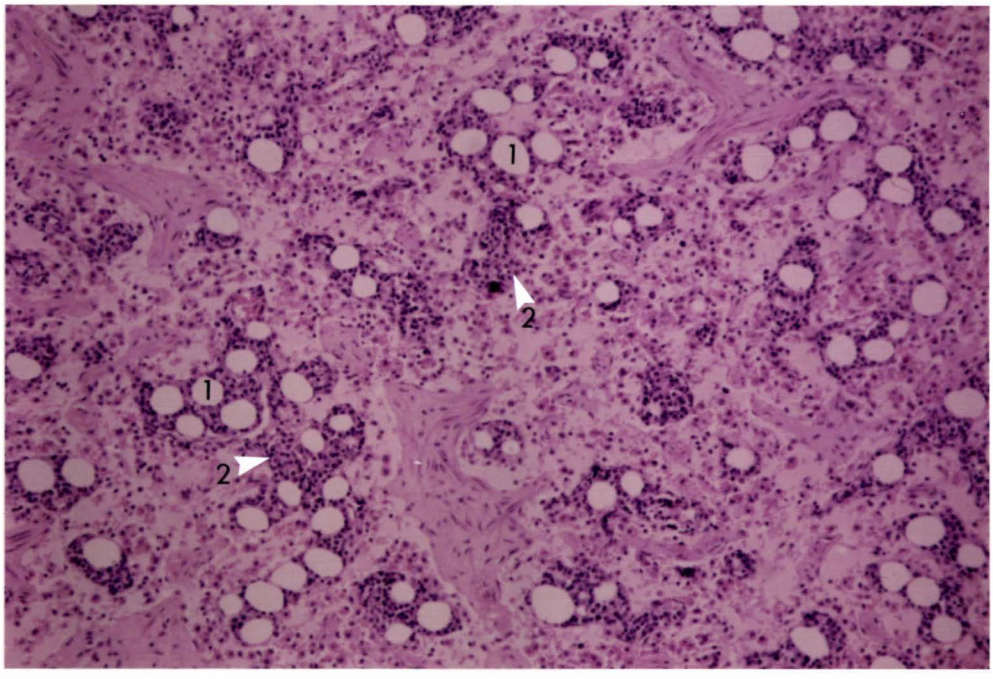
Oil spaces (1) seen in the medullary cords (2) of a left prescapular node six weeks after the administration of Brucella ovis vaccine by both the subcutaneous and the intraperitoneal routes.

60 x magnification. H & E stain.

Plate 4.25

Multinucleate giant cells (arrowed) in a prescapular lymph node ten weeks after the administration of Brucella ovis vaccine by both the subcutaneous and intraperitoneal routes.

150 x magnification. H & E stain.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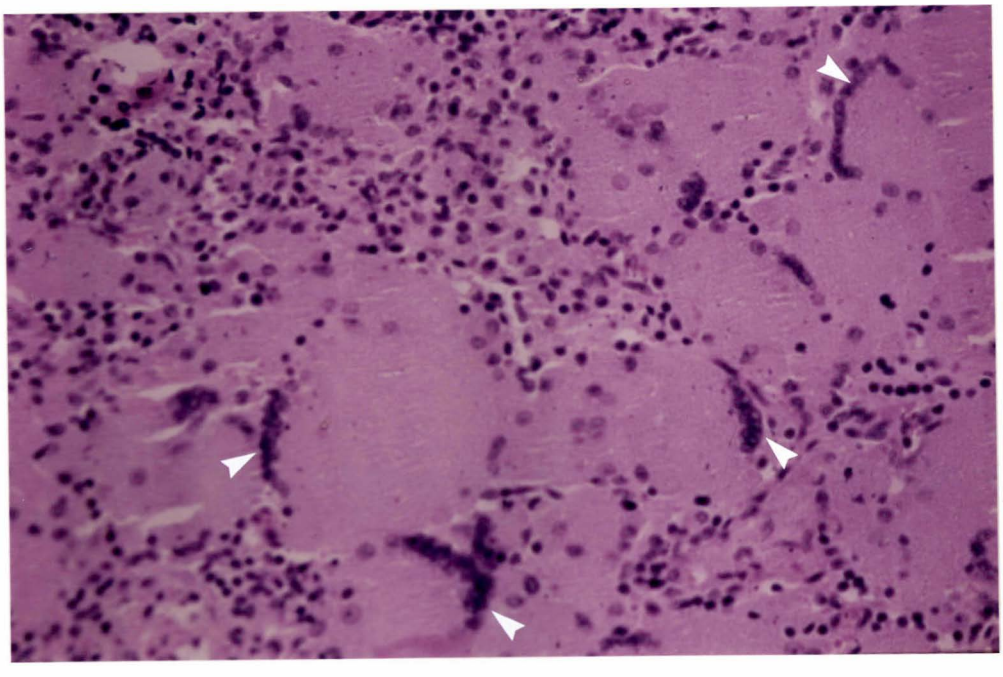


B) Intraperitoneal Vaccination

a) Injection Site

Twenty-four hours after vaccination there was a mild diffuse mixed leucocytic infiltration of the deep dermal and underlying muscle tissue at the injection site in the flank of both animals (Plate 4.26). The visceral peritoneum of one of the animals had a mild diffuse infiltration by neutrophils, and the omentum of the other was slightly oedematous and showed an infiltration of neutrophils and macrophages.

In both of the animals examined at three days there was myodegeneration in the abdominal muscles at the injection site. In one the parietal peritoneum was slightly thickened, with a mild neutrophil infiltration. In the other, the peritoneum contained several oil spaces, which were lined with neutrophils (Plate 4.27). There were also moderate numbers of macrophages and some inflammatory cell necrosis was seen.

The only abnormalities seen in the flank of one of the animals examined at seven days consisted of extensive areas of muscle degeneration and oedema. There was also a mild inflammation of the visceral peritoneum. In the other animal however, the parietal peritoneum was affected as well as the visceral peritoneum. In the centre of this parietal peritoneal reaction were several oil spaces up to 1.2mm in diameter. Macrophages were the predominant cell type, while neutrophils were less common, and giant cells were seen for the first time. (Plate 4.28). An inflammatory reaction of this type was also seen between muscle layers.

At two weeks after vaccination one animal had a severe visceral peritonitis, but only muscle degeneration in the abdominal wall. The other had oil spaces in the parietal peritoneum up to 1mm in diameter. These were surrounded by a reaction consisting mainly of fibrous tissue infiltrated by an inflammatory cell population of mostly lymphocytes and plasma cells, together with a few neutrophils.

Four weeks after vaccination, there were no significant findings in the abdominal wall of one animal, although a moderately severe visceral peritonitis was seen, but in the other, the parietal peritoneum, diaphragm (Plate 4.29) and omentum (Plate 4.30) were all affected by a granulomatous reaction containing oil spaces. This reaction was dominated by lymphocytes, macrophages and plasma cells, with occasional giant cells and focal aggregations of neutrophils. In some areas there was extensive fibrosis and early caseation.

One animal examined at six weeks had a large granuloma in the muscle adjacent to the parietal peritoneum, involving one large (12mm x 2mm) central oil space surrounded by several smaller ones. There was caseation and calcification at the centre of the granuloma. The cellular reaction consisted of macrophages and lymphocytes with a small population of neutrophils. Occasional giant cells were seen. There was a similar, but milder, inflammation of the omentum. The other animal had a severe granulomatous reaction in both the parietal peritoneum and the omentum. The lesion contained necrotic foci and oil spaces delineated by a zone of macrophages encircled by lymphocytes and plasma cells, which were in turn surrounded by fibrous tissue (Plate 4.31).

By the tenth week after vaccination, the inflammatory reaction in both animals was of a typical, chronic but active granulomatous type involving progressive fibrosis. In one animal the reaction was confined to the peritoneum, (both visceral and parietal) and in the other it was within the muscle tissue of the abdominal wall. The lesion was loosely divided into lobules by a fibrous tissue network. The cellular reaction, arranged around the oil spaces, consisted mainly of mononuclear cells (macrophages, lymphocytes and plasma cells) and occasional giant cells.

b) Local Lymph Nodes

1) Prefemoral

Oil spaces were observed in the prefemoral lymph nodes of five animals; two at 24 hours, one at three days, one at six weeks and one at ten weeks after vaccine administration. Aggregations of large macrophages or giant cells (Plate 4.32) were seen in all but two of the nodes, and both of the exceptions (one at two weeks and one at six weeks) had occasional single cells of this type. Cortical or paracortical hyperplasia was observed in the prefemoral lymph node of one animal at two weeks and another at four weeks, and focal coagulative necrosis was observed in the node of one animal at six weeks.

2) Superficial Inguinal

Of the 14 animals, three had superficial inguinal lymph nodes that appeared normal: one at 24 hours, one at two weeks and one at ten weeks after vaccination. Three others had inactive fibrous nodes: one at 24 hours, one at three days and one at seven days. All but two of the remaining eight nodes contained numerous large macrophages and/or giant cells. The other two, one at two weeks and one at six weeks, were haemorrhagic. There were four nodes that contained oil spaces, one at three days, one at two weeks, and two at six weeks.

3) Lumbar Aortic

Of the 14 nodes, four were normal and one fibrous and inactive. Five nodes contained oil spaces, one at three days, one at four weeks (Plate 4.33), one at six weeks and two at ten weeks after vaccination. Two of these, one at four and one at ten weeks, and one other (at four weeks) had an increased number of macrophages and/or giant cells. The other three nodes, from animals at 24 hours, seven days and two weeks, had a reactive hyperplasia of the cortex and/or paracortex.

Plate 4.26

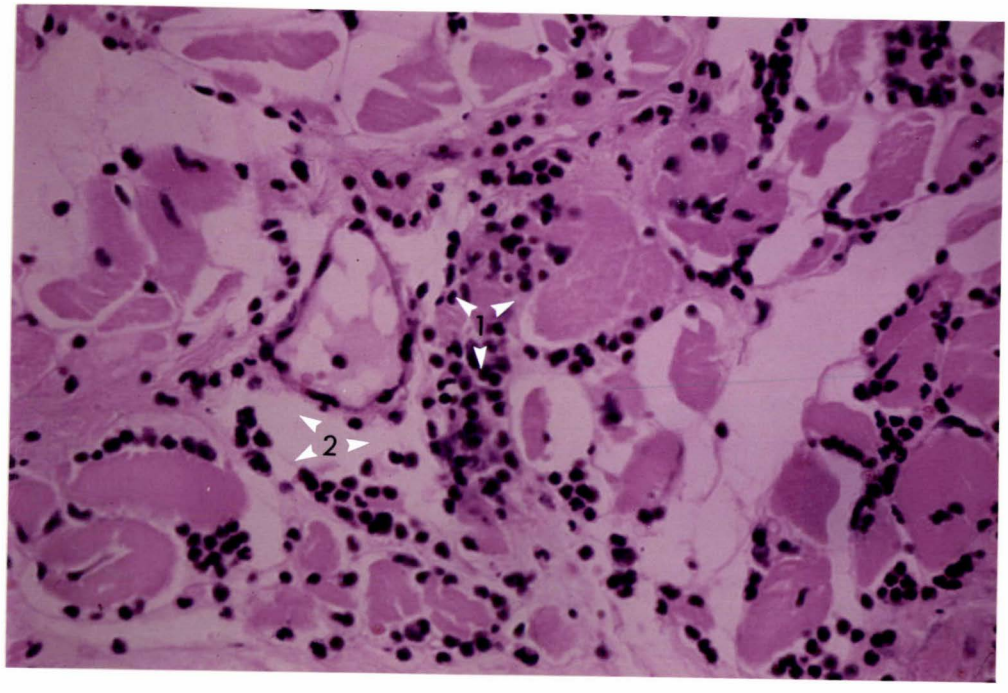
Diffuse leucocyte infiltration (1) and oedema (2) seen in the muscle tissue of the abdominal wall 24 hours after the administration of Brucella ovis vaccine using the intraperitoneal technique.

200 x magnification. H & E stain.

Plate 4.27

Large oil spaces (1) lined with neutrophils (2) in the tissue of the parietal peritoneum three days after the administration of Brucella ovis vaccine using the intraperitoneal technique.

40 x magnification. H & E stain.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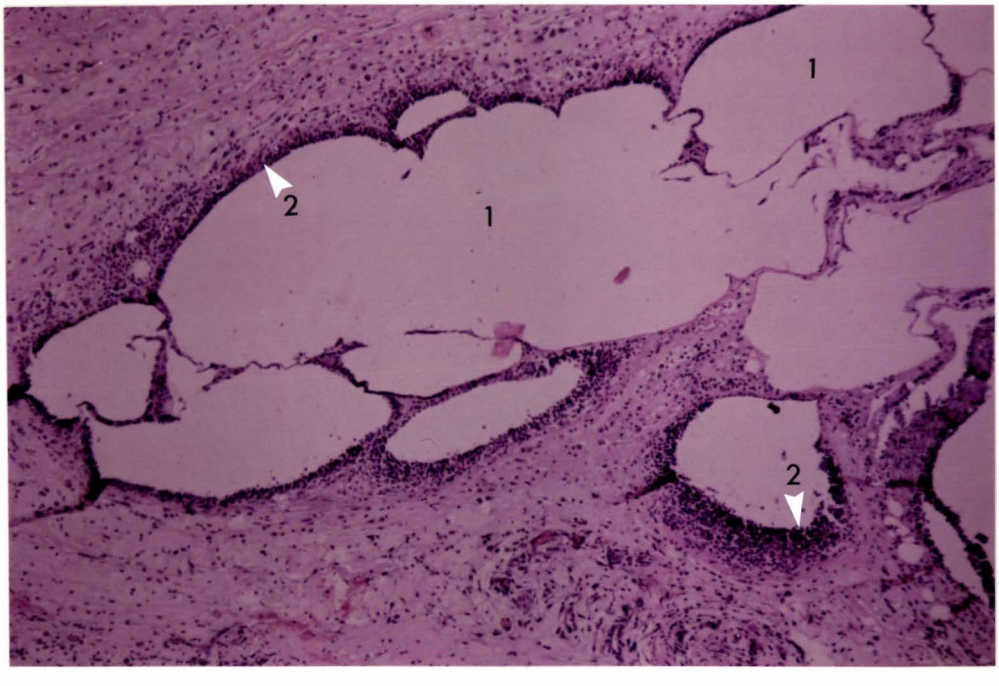
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Plate 4.28

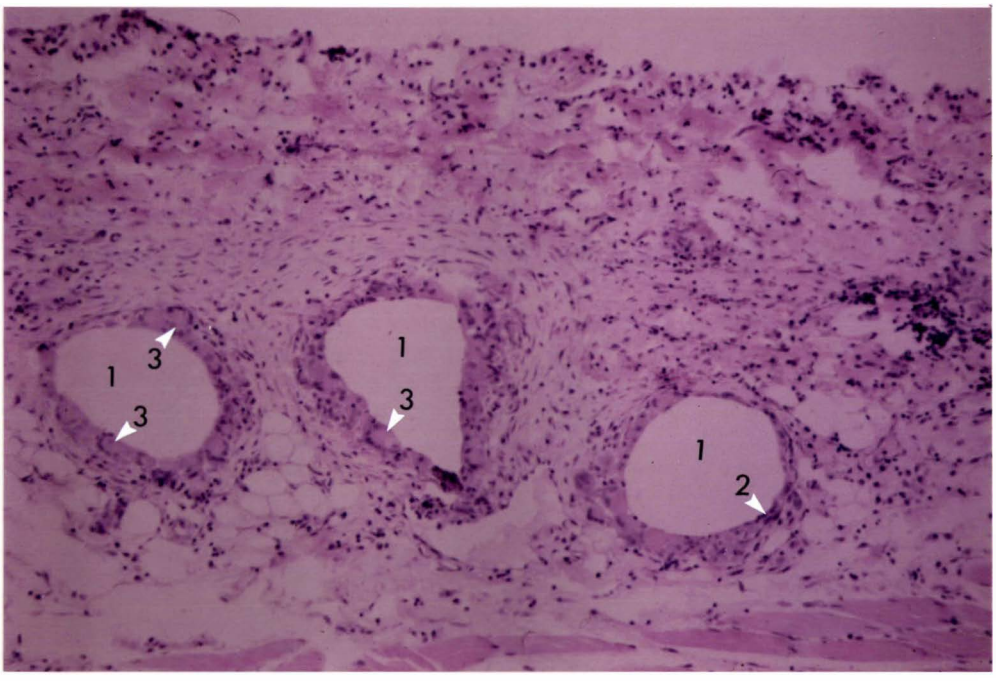
Oil spaces (1) lined by macrophages (2) and giant cells (3) in the tissue of the parietal peritoneum seven days after the administration of Brucella ovis vaccine by the intraperitoneal technique.

60 x magnification. H & E stain.

Plate 4.29

Granulomatous inflammation containing oil spaces (arrowed) seen on the surface of the diaphragm four weeks after the administration of Brucella ovis vaccine by the intraperitoneal technique.

40 x magnification. H & E stain.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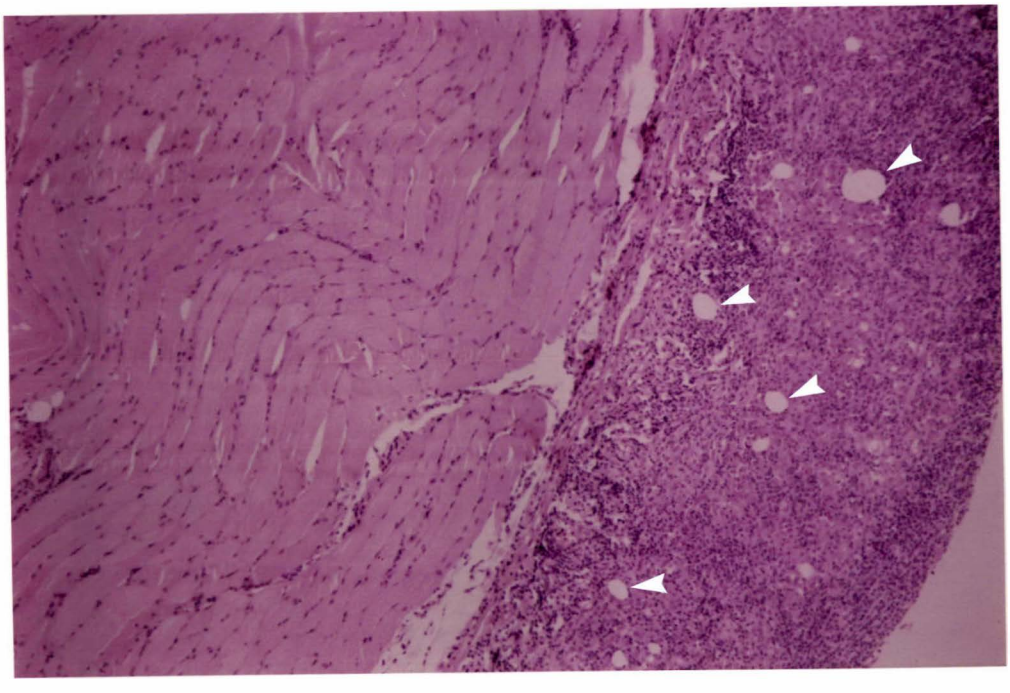


Plate 4.30

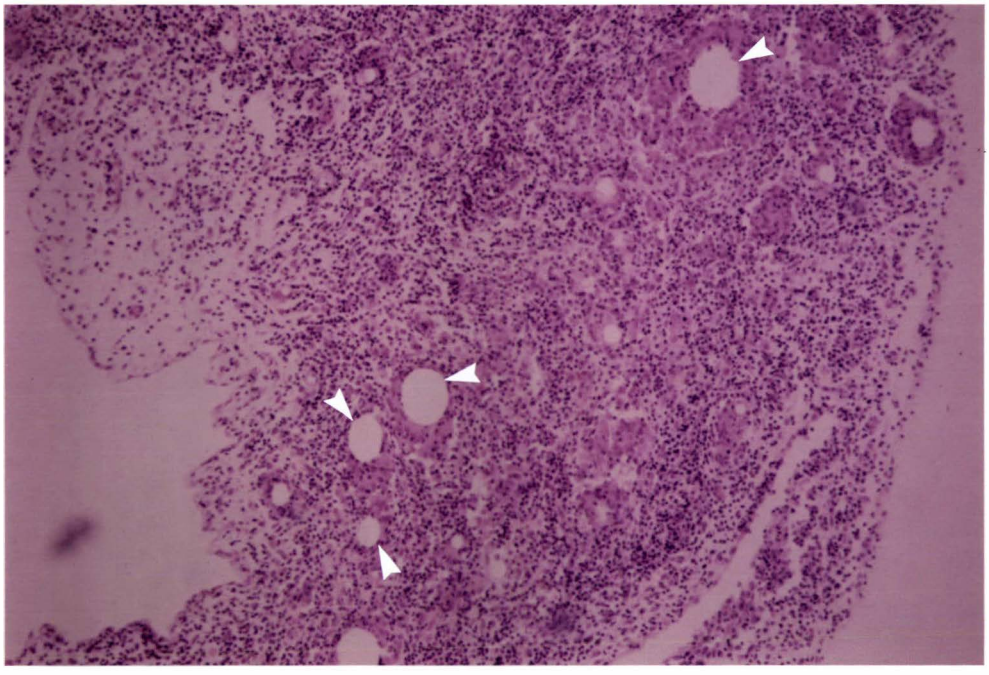
Granulomatous inflammation containing oil spaces (arrowed), seen in the omentum four weeks after the administration of Brucella ovis vaccine by the intraperitoneal technique.

50 x magnification. H & E stain.

Plate 4.31

Oil spaces (1) and necrosis (2) surrounded by granulomatous inflammation, seen in the peritoneum six weeks after the administration of Brucella ovis vaccine by the intraperitoneal technique.

40 x magnification. H & E stain.



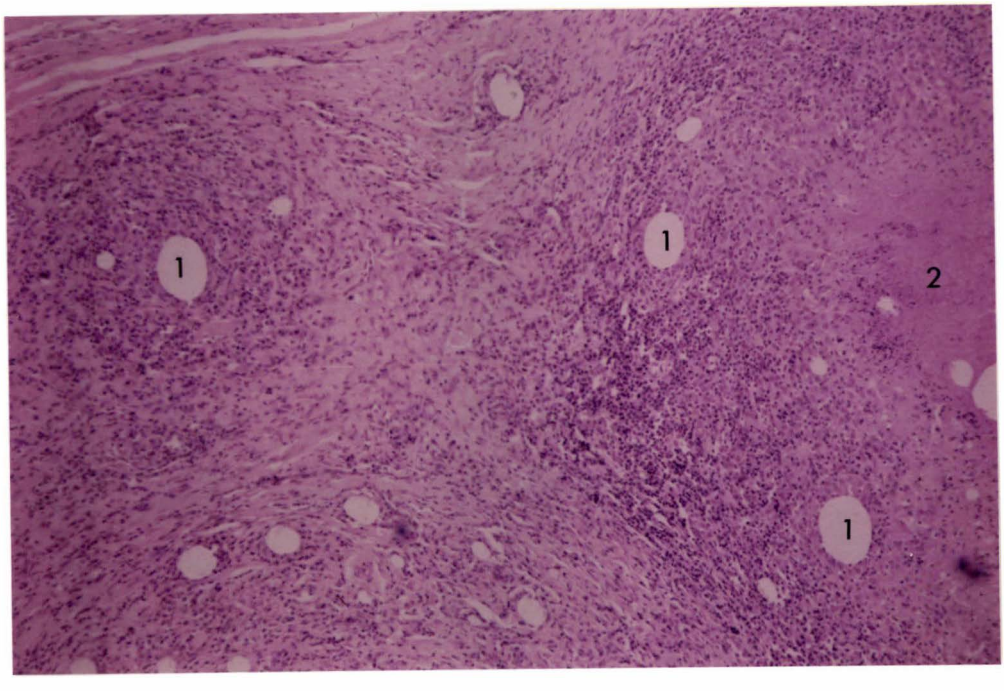

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Plate 4.32

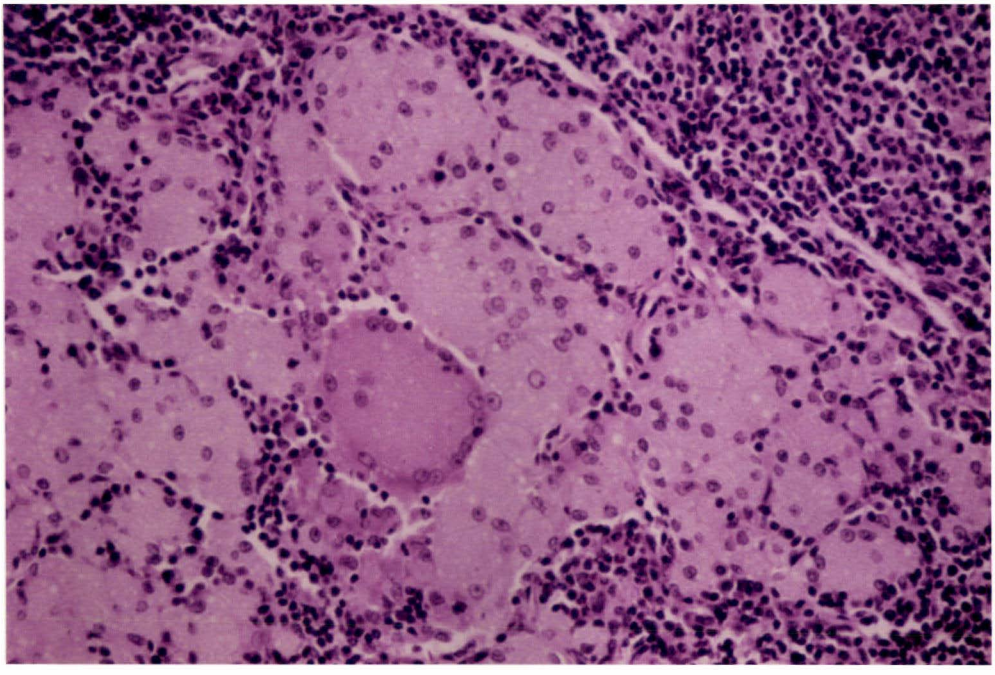
An aggregation of multinucleate giant cells in a prefemoral lymph node after the administration of Brucella ovis vaccine by both the subcutaneous and the intraperitoneal routes.

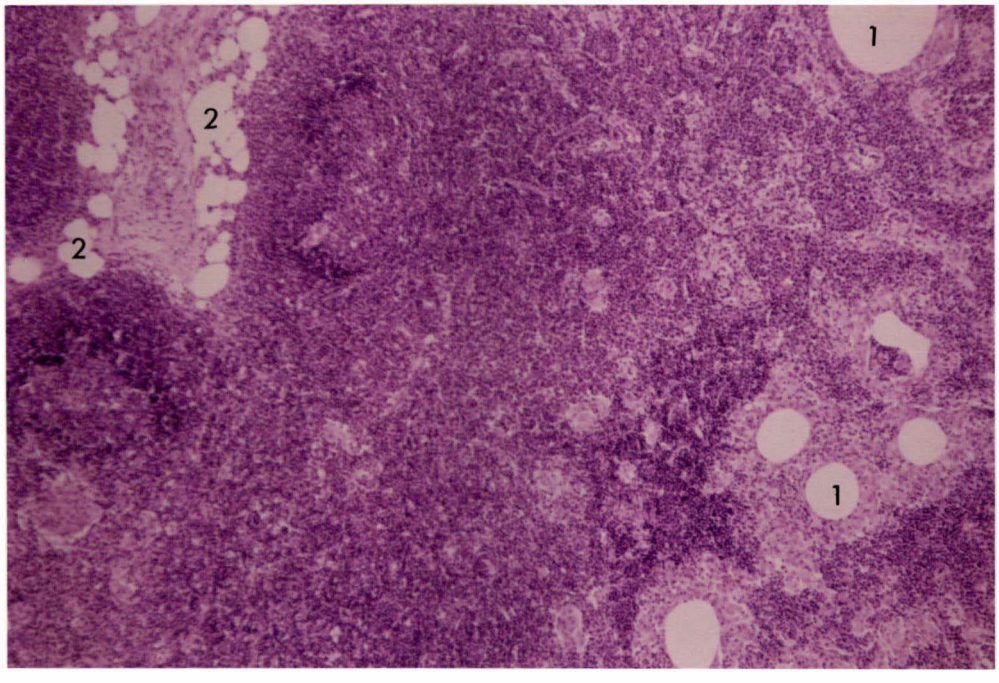
150 x magnification. H & E stain.

Plate 4.33

Oil spaces surrounded by macrophages (1), and, without an associated reaction, in the subcapsular region (2); in a lumbar aortic lymph node. Brucella ovis vaccine had been administered by both the subcutaneous and intraperitoneal routes four weeks previously.

40 x magnification. H & E stain.

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Prescapular Lymph Nodes

The fixed prescapular lymph nodes of four normal, unvaccinated adult sheep were found to range in weight from 3.34g to 5.47g, with an average weight of 4.4g. The differences in weight between right and left nodes from the same animal ranged from 0.02g to 1.87g, with an average of 0.355g, and either the right or the left could be the heavier node.

There was a noticeable difference in the average weight of the left and right prescapular lymph nodes in the experimental animals which had been vaccinated subcutaneously with an inactivated Brucella ovis saline-in-oil vaccine on the right hand side of the neck (Plate 4.34). The average weight of the node on the same side as the vaccination site was greater than that of the node on the other side. The lymph node weights are listed in Table 4.5. For the seven sheep which had both the right and the left node weighed, the mean weight of the right node was 4.04g greater than that of the left, and this difference was statistically significant at the 5% level ($P<0.05$).

There was a trend towards increasing weight difference between right and left nodes with increasing size of the lesion at the injection site. The greatest difference in lymph node weights (6.2g) was recorded for the animal with the largest vaccination site lesion ($40cm^2$), and the animal with the smallest difference in lymph node weights (1.1g) had no palpable lesion at the injection site.

Table 4.5

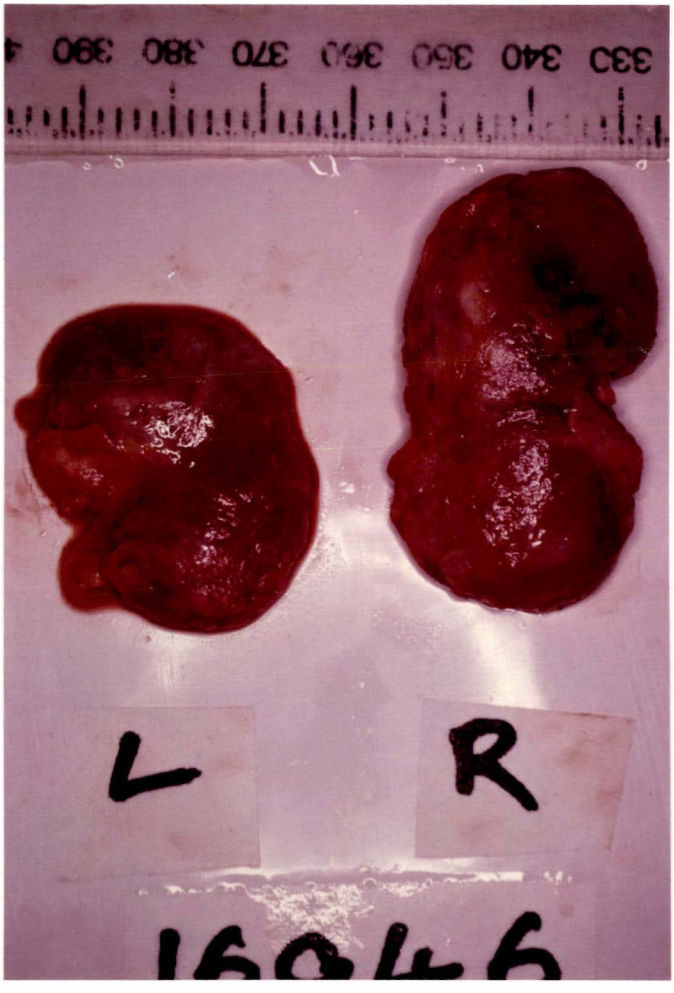
Weights Of Prescapular Lymph Nodes
And Sizes Of Subcutaneous Lesions
In Animals Vaccinated Using **Brucella ovis** Vaccine
Administered By Both The Subcutaneous And The Intraperitoneal Routes

Sheep Number	Dimensions of Subcutaneous Lesion	Weight of Right Node	Weight of Left Node	Weight Difference
1	no lesion	10.4g	NA	NA
2	no lesion	4.9g	NA	NA
3	no lesion	4.0g	NA	NA
4	2x2cm ($4cm^2$)	5.0g	2.1g	2.9g
5	no lesion	3.0g	NA	NA
6	3x1cm ($3cm^2$)	3.8g	NA	NA
7	6x3cm ($18cm^2$)	6.3g	2.0g	4.3g
8	2x2cm ($4cm^2$)	4.9g	NA	NA
9	10x4cm ($40cm^2$)	9.7g	3.5g	6.2g
10	3x3cm ($9cm^2$)	6.6g	NA	NA
11	5x2cm ($10cm^2$)	10.6g	4.5g	6.1g
12	2x2cm ($4cm^2$)	5.3g	2.2g	3.1g
13	5x3cm ($15cm^2$)	10.0g	5.4g	4.6g
14	no lesion	6.6g	5.5g	1.1g
Mean	$7.6cm^2$	6.51g	3.6g	4.04g

NB NA = Not Available

Plate 4.34

The difference in size between the left and right prescapular lymph nodes of an animal vaccinated subcutaneously using Brucella ovis vaccine on the right side of the neck.


330 340 350 360 370 380 390
L
R

Chronic Reaction To Vaccination

Thirty rams which had been vaccinated by the intraperitoneal technique were available for necropsy.

Of 16 rams that had been vaccinated once using the intraperitoneal technique six months before necropsy, four had no gross lesions when examined. Nine rams (56%) had granulomatous lesions in the abdominal wall (i.e. the musculature of the flank or the parietal peritoneum), and three (19%) had visceral peritonitis.

Of 14 rams that had been vaccinated twice, eight weeks apart, using the intraperitoneal technique, 11 (79%) had granulomatous lesions in the abdominal wall (Plates 4.35, 4.36 and 4.37), three (21%) had serosal adhesions between abdominal organs (Plate 4.38), and five (36%) had visceral peritonitis. Of the five having visceral peritonitis, three also had "extraperitoneal" granulomatous lesions in the abdominal wall. Two of the three rams which had adhesions between abdominal organs also had extraperitoneal lesions in the abdominal wall.

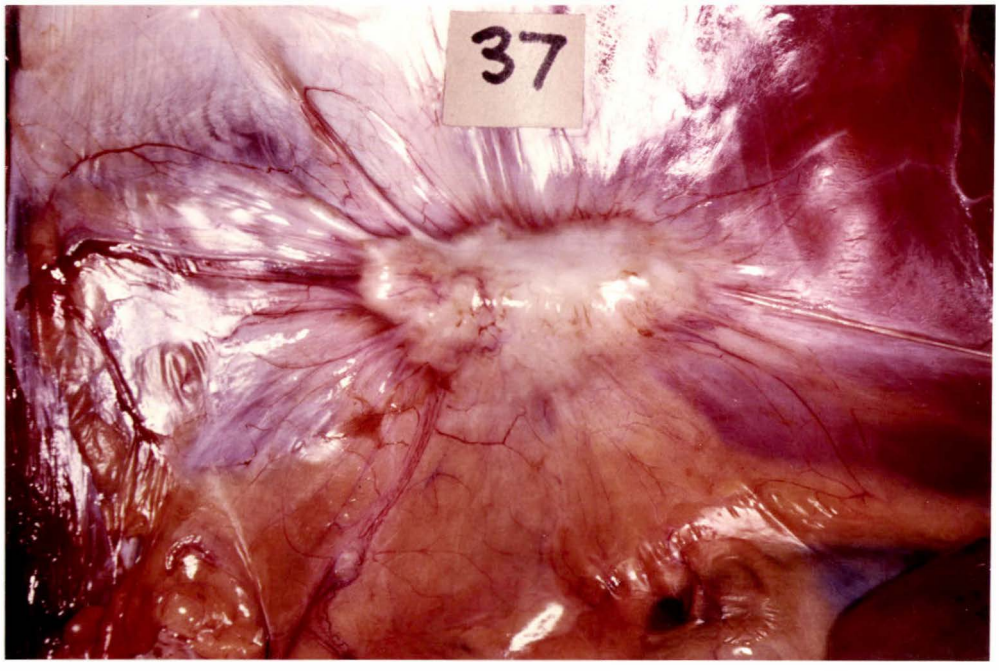
These examinations were carried out six months after vaccination, yet 27% of the 30 rams still had chronic but active visceral peritonitis visible grossly. In 66% of animals vaccinated either once or twice intraperitoneally, at least some of the vaccine had been deposited extraperitoneally.

Plate 4.35

Parietal peritoneal surface of the right flank, showing a lesion present six months after the administration of Brucella ovis vaccine using the intraperitoneal technique.

Plate 4.36

Same lesion as above, incised to show semisolid yellow contents (arrowed).


37

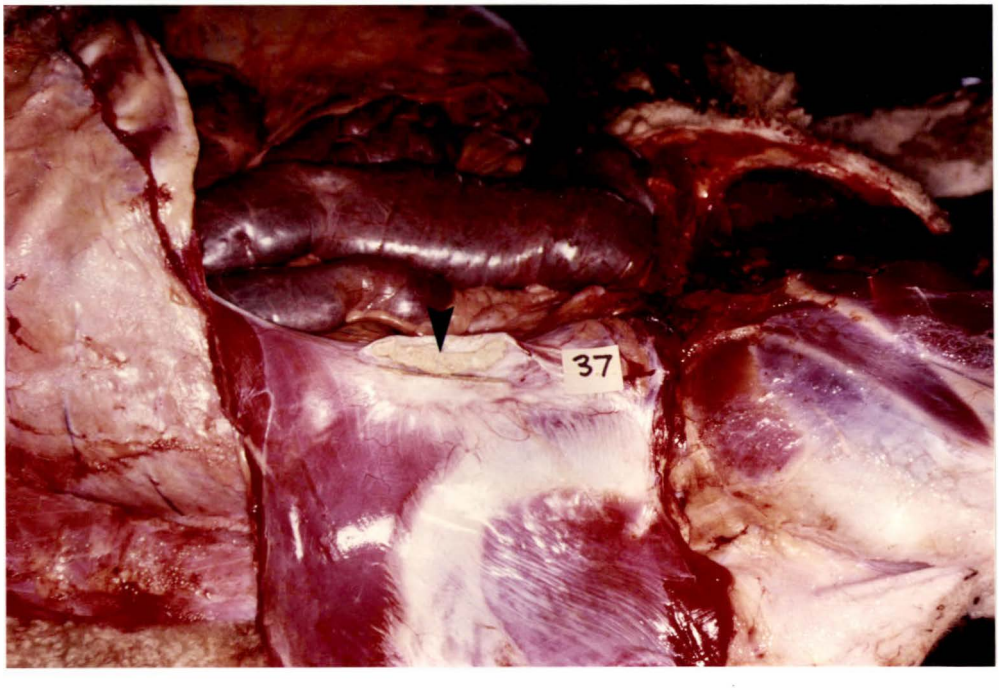
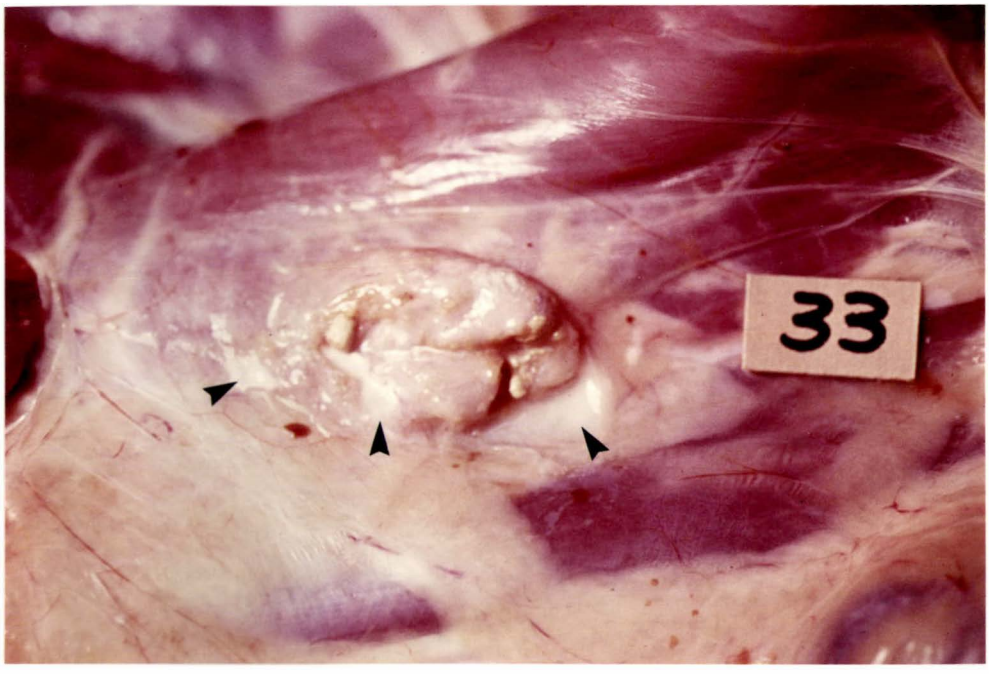

37

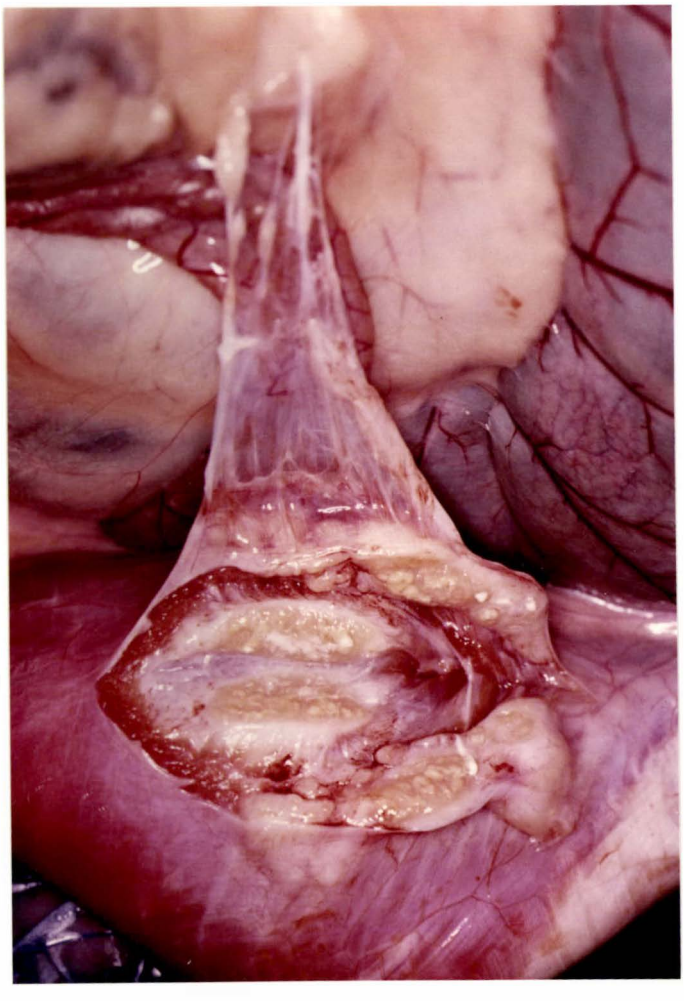
Plate 4.37

Incised lesion on the parietal peritoneal surface of the right flank, showing the white vaccine (arrowed), six months after the administration of Brucella ovis vaccine using the intraperitoneal technique.

Plate 4.38

Two distinct lesions in the musculature of the right flank, and serosal adhesions to the viscera. The ram had been vaccinated twice with Brucella ovis vaccine using the intraperitoneal technique, six months earlier.


33



DISCUSSION

The administration of an inactivated Brucella ovis saline-in-oil vaccine using the subcutaneous route in the neck caused the development of a palpable lesion in the tissues at the site of injection in nine out of the 14 sheep in this trial (approximately 64%). It is likely that even more would have eventually developed lesions if they had not been killed soon after vaccination. All but one of the animals killed at or later than two weeks after vaccination had developed a palpable lesion, and in the serology trial described in Chapter 3 a lesion developed at all of the 78 subcutaneous vaccination sites at some stage during the experiment. The use of this vaccine by the subcutaneous route must therefore be expected inevitably to result in the development of palpable lesions.

The lesions found in animals in this trial varied in size and shape from a 2cm diameter spherical nodule to a 10cm x 4cm flat rectangular reaction. The rectangular lesions always had a vertical (dorso-ventral) dimension greater than the horizontal (crano-caudal), possibly due to gravitational forces, as is seen in the ventral accumulation of many fluids when they are administered by the subcutaneous route.

These lesions were slow-developing chronic granulomatous reactions arranged in circular patterns around droplets of oily vaccine. This type of inflammation is often a reaction to the continual presence of a foreign body, in this case oil, which cannot easily be removed. It is a protracted form of response, and is similar to that seen in such diseases as tuberculosis in which the mycobacteria act as the foreign body (Robb-Smith and Taylor, 1981).

The examinations made of the lymph nodes in the region of the vaccination site showed that oily vaccine drains into these nodes within 24 hours. The presence of oil droplets in the subcapsular space that were not surrounded by an inflammatory reaction suggests that oil continues to arrive at the node for at least several weeks after vaccination. The reaction of the node to this stimulus was quite spectacular, up to a threefold increase in weight was observed

in some of the prescapular lymph nodes on the same side as the injection site in comparison with the node on the opposite side.

Although the number of normal animals available for examination was limited, the data agreed with that obtained from the carefully dissected, fresh unfixed prescapular lymph nodes of six normal adult ewes in which the average weight difference between left and right nodes was found to be minimal (0.31g) (S.T. Yao, personal communication, 1986).

Histologically, there was an increase in the number of lymphocytes in the affected lymph nodes. The paracortices, which are known to contain mainly T-lymphocytes, were particularly hypercellular. The presence of numerous very active germinal centres in some nodes was associated with the presence, within these same nodes, of large numbers of plasma cells. This is consistent with the hypothesis that germinal centres are the site of B-lymphocyte transformation. There was also the suggestion of a possible relationship between the presence of greater than normal numbers of plasma cells and the development of an antibody titre. This was indicated by the fact that the first animal observed to have a noticeable increase in plasma cell numbers in the prescapular lymph node, was also the first to have developed a positive reaction to the complement fixation test and the ELISA, at two weeks after vaccination.

There was a proliferation of large numbers of macrophages within many of the nodes, and the formation of giant cells was often observed. The large numbers of these phagocytic cell types were probably needed for the phagocytosis of the large volumes of oily material arriving at the node. This was suggested by the abundant, foamy cytoplasm of these cells. The duration of this response supports the theory that the reliability of oil-adjuvant vaccines is due in part to the continued stimulation from the long-lived vaccine depot which remains in the tissues. ("Immunostimulation", 1980).

The most unexpected outcome of this trial was the frequent failure, when using the intraperitoneal technique, to deposit the total volume of vaccine within the peritoneal cavity. From necropsy results on all the animals examined, it was demonstrated that over 50% of "intraperitoneal" inoculations resulted in at least partial extraperitoneal deposition of vaccine. It should be stressed that the vaccine was administered with extreme care, every effort being made to use new, clean equipment and to follow the recommended technique as described by Thomson et.al. (1969) and by Quinlivan and Wallace (1979). Thus under field conditions, or when injecting larger or fatter sheep, it is likely that the percentage of such failures could be even greater. This likelihood was supported by the greater proportion (66%) of "extraperitoneal" deposition observed in young rams in comparison with that in older, thinner ewes (approximately 30%). The young rams, being in better condition, had thicker abdominal walls than the ewes.

When making comparisons between the local tissue reactions resulting from the use of the subcutaneous and the intraperitoneal routes, these "extraperitoneal" reactions should not, strictly speaking, be included in the assessment, as in these cases the site of deposition of the vaccine was not intraperitoneal. However, when comparing the subcutaneous method and the intraperitoneal technique described by other workers (Thomson et.al., 1969; Quinlivan and Wallace, 1979), then the extraperitoneal lesions are of considerable importance, since it is clear from the present results that they are an inherent, unavoidable consequence of the use of this technique.

Such extraperitoneal deposits in the musculature of the flank resulted in local lesions similar to those described in "Surveillance" (1978, 1980) as having been found in some lines of rams sent for slaughter at meat works. In one of these reports the lesions were described as "raised flat and fibrous with firm yellow necrotic centres." (Surveillance, 1980). If these were the result of the vaccine having been deposited extraperitoneally it would indicate that inaccurate placement of the vaccine may also be a problem which occurs in the field. Quinlivan and Wallace (1975), also, reported finding lesions in the musculature of the flank in some of the 40 rams

involved in a pilot trial investigating the intraperitoneal vaccination technique. This implies that extraperitoneal deposition of vaccine also occurred in their trial. The extraperitoneal lesions seen in this present trial were very similar in gross appearance to those seen at the subcutaneous injection site.

Despite the care taken in using a clean, correct technique, nearly 80% of the animals inoculated by the intraperitoneal method developed visceral peritonitis of some type. Necropsies of animals from other trials that had been vaccinated intraperitoneally six months beforehand provided further evidence that the majority of animals given a single intraperitoneal injection of the Brucella ovis vaccine develop peritonitis. The reaction varied in severity and duration, but approximately 20% of animals still had grossly visible visceral peritonitis six months after vaccination. The likelihood of a more severe, chronic reaction developing appeared to increase when two vaccinations were given, as in the group of animals vaccinated twice, 36% had peritonitis six months after vaccination. This finding is contrary to the opinion of Quinlivan and Wallace (1979), which was that such local reactions were uncommon if the correct technique was used.

Examinations made of the local lymph nodes in the region of the peritoneal injection site revealed that they reacted to the vaccine in the same way as those in the region of the subcutaneous injection site. Oil arrived at the node within 24 hours, and there was some evidence that it continued to do so throughout the experiment. The node became more active, the number of lymphocytes, plasma cells and macrophages increased, and aggregations of giant cells were seen.

No matter where the vaccine was deposited in the present experiment, be it subcutaneous, intermuscular or intraperitoneal, the same type of tissue reaction was observed microscopically. This was a slowly progressing granulomatous inflammation arranged around the droplets of oily vaccine. Even on serosal surfaces in the peritoneal cavity, such as the omentum and diaphragm, the peritonitis was of the same granulomatous type, and was always sterile. This is consistent with the microscopic description of the lesions seen at slaughter in

the freezing works (Surveillance, 1980) which were described as "granulomatous with many macrophages, giant cells and lymphocytes surrounding necrotic tissue."

In Chapter 3 it was shown that following intraperitoneal vaccination the serological titre rose more slowly than it did following subcutaneous vaccination. This difference cannot be explained by a difference in the type of inflammation at these two sites, as it has been already shown that the reactions are very similar. It is possible that the intraperitoneal reaction develops slightly more slowly than the subcutaneous one, especially in the first few days. At 24 hours and three days, the peritoneal reaction appeared histologically to be more diffuse than that at the subcutaneous site. This difference in response time may have meant that immunologically active cells accumulated more slowly, and that consequently the antibody response was slower to develop. It may have been that the blood supply at the intraperitoneal site was poorer, resulting in slower migration of the immunologically active cells into the tissues at the vaccination site. A more likely possibility may have been that the lymphatic drainage was less efficient at the peritoneal site. This latter possibility gains credence when it is considered that only a local lymph node from the subcutaneous site (the prescapular) showed any gross reaction to vaccination (an increase in size), while the nodes in the region of the intraperitoneal injection appeared grossly to be normal.

In some animals the peritonitis observed was very severe and extensive adhesions were sometimes present attached to the viscera. Although no obvious signs of distress were observed in animals that were subsequently found to have peritonitis, it is possible that in some cases the discomfort of the inflammation and adhesions may make the animal disinclined to move, thus interfering with feeding or reproduction. No effort was made to measure the effects of pain (e.g. weight loss), and so this remains a subject of speculation.

This trial has shown that irrespective of whether it is administered subcutaneously or intraperitoneally, the Brucella ovis vaccine usually produces a severe local inflammatory response. This response is long-lived and the lesion essentially consists of a dense granulomatous reaction arranged around droplets of oily vaccine. Material from the site of vaccine deposition drains into local lymph nodes and stimulates them to become more active. The reaction is essentially the same at both the intraperitoneal and subcutaneous sites. The intraperitoneal route of administration merely removed this reaction to a place where it was no longer visible in the live animal.

CONCLUSION

1) Subcutaneous deposition of inactivated Brucella ovis saline-in-oil vaccine usually resulted in the development of a chronic granulomatous reaction at the site of injection. The lesion produced consisted of macrophages, lymphoctyes and fibrous tissue, and to a lesser extent plasma cells and giant cells, arranged around droplets of oily vaccine.

2) Peritonitis of both the visceral and parietal peritoneum and the serosal surfaces of abdominal organs was a common sequel to the administration of Brucella ovis vaccine by the intraperitoneal technique. Peritonitis occurred in approximately 80% of cases in this trial, and adhesions between organs were also seen. The inflammation was again of a chronic granulomatous type.

3) Oil from the vaccine reached local lymph nodes very quickly and continued to do so over many weeks. The lymph nodes became hyperplastic and in some cases obviously enlarged.

4) In over 50% of cases careful attempts to deposit the vaccine solely in the peritoneal cavity were unsuccessful, resulting in extraperitoneal (usually intermuscular) deposition of at least part of the dose. It was concluded that in the field, where conditions are less ideal, this result would occur even more frequently. The reaction around these extraperitoneal deposits was similar to that seen at the subcutaneous site.

5) Wherever the vaccine was deposited it resulted in a chronic granulomatous inflammatory reaction. Using the intraperitoneal method of vaccination did not reduce the likelihood of this reaction occurring in comparison with the subcutaneous technique, and lesions in this site could be hazardous. The use of the intraperitoneal technique merely removed the damage to a site where it was no longer visible in the live animal.

CHAPTER 5

THE ARTIFICIAL INFECTION OF SHEEP WITH BRUCELLLA OVIS (PILOT TRIAL)

INTRODUCTION

In order to to compare relative resistances to Brucella ovis infection in different groups of animals, it is first necessary to establish a reliable method of artificial challenge which consistently produces infection in susceptible individuals. Although a natural source of infection would be preferred when conducting experiments into disease resistance, the low rates of infection achieved and the inability to guarantee that each animal receives the same challenge dose makes it impractical. In order to keep animal numbers to a minimum it is best if the infection rate produced in the susceptible group is as close as possible to 100% .

Many different routes of infection have been used experimentally in attempts to infect animals with Brucella ovis. These have included the intravenous, subcutaneous, intraconjunctival, intrapreputial, intratesticular, intraepididymal, oral and nasal. (Simmons and Hall, 1953). Most workers have chosen the intravenous method as being the most convenient and consistent, and it was decided that this would be the route used in this study. By this method, infection rates of up to 90% have been achieved in susceptible sheep. (Buddle and Boyes, 1953). However, results have been extremely variable, with infection rates usually between 60% and 80%, but occasionally as low as 45% (Claxton, 1968) and sometimes less.

The magnitude of the challenge dose has varied from 1×10^6 to 1×10^9 Brucella ovis organisms. Most commonly a dose of approximately 1×10^7 bacterial "colony forming units" has been used.

It was decided to carry out a pilot trial on five rams to determine the rate of infection that could be achieved by the intravenous inoculation of approximately 1×10^8 Brucella ovis organisms to each animal.

MATERIALS AND METHODS

Sheep

The sheep used were five clinically normal, 12 month old rams from farms accredited as being free from Brucella ovis infection.

Prior to the experiment, semen samples were collected and cultured for evidence of any pathogenic bacteria including Brucella ovis. Blood samples were also collected and tested for Brucella ovis antibody by the complement fixation test (CFT).

Management

The rams were kept in a small paddock of mixed pasture species and their diet was supplemented with some hay during the winter. They were drenched regularly using 225mg of the anthelmintic Fenbendazole[1]

Production of **Brucella ovis** Challenge Suspension

A field strain of Brucella ovis was isolated from the semen of a four-year-old naturally infected ram which had lesions of epididymitis. This ram was part of a flock of chronically infected animals used in another study of ovine brucellosis (Worthington et.al., 1985). The ram had been consistently shedding Brucella ovis bacteria in its semen for over a year.

The semen sample, obtained by electro-ejaculation (see Appendix 5) was used to inoculate four sheep blood agar plates, four modified Thayer-Martin agar plates (a selective Brucella ovis medium), and two McConkey agar plates (to detect possible faecal contamination of the sample). One plate of each type of medium was incubated aerobically at 37°C, while the rest were incubated in an atmosphere of approximately 10% CO_2 at 37°C (microaerophilic conditions).

After five days, typical Brucella ovis colonies were observed growing on the blood agar plates which had been incubated in microaerophilic conditions. Two typical colonies were chosen and each was subcultured onto two sheep blood agar plates, one of which was to be cultured aerobically and the other in 10% CO_2.

The isolate was subjected to the following bacteriological tests:

1) Motility
2) Urease activity
3) Citrate utilisation
4) Hydrogen sulphide production
5) Dextrose oxidation and fermentation
6) Nitrate reduction
7) Oxidase activity

(See Appendix 1 for bacteriological methods).

These tests were all negative, further evidence of the identity of the supposed Brucella ovis isolate. Air-dried smears of the colonies, when stained by the Modified Ziehl-Neelsen technique (Stamp et.al., 1950), showed only small, red (acid-fast) cocco-bacilli. The bacteria were also gram-negative.

At 96 hours the plates cultured in aerobic conditions showed no visible growth and were discarded. The microaerophilic cultures showed growth typical of Brucella ovis. This 96 hour culture was harvested from one of the blood agar plates using a sterile cotton wool swab and used to inoculate 6ml of sterile heart infusion broth. This suspension was immediately used to inoculate 20 Trypticase Soy Agar (TSA) slopes to which had been added 5% bovine serum. The method of inoculation was to add four drops of the heart infusion broth suspension to each slope using a Pasteur pipette. The TSA slopes were incubated with loose lids in 10% CO_2 at 37°C.

After 72 hours incubation, the slopes were examined. Two were discarded as having growth atypical of Brucella ovis. the remaining slopes were harvested by adding 2ml sterile peptone saline to each, flushing the growth from the slope with a sterile Pasteur pipette, and

transferring the resultant suspension to a sterile Universal bottle. The bottles were numbered, and a smear from each was stained by the Gram and Modified Ziehl-Neelsen methods, and examined under a microscope to ensure that the cultures were typical and uncontaminated. One smear was atypical and that bottle was discarded. The remaining suspensions were pooled into two Universal bottles, one to be used in making the challenge suspension and one to be held in reserve in case of mishap.

Estimation of Bacterial Concentration of Challenge Suspension

The pooled suspension was used to make serial tenfold dilutions from 1/10 to $1/10^{10}$ to be used in a viable counting procedure (Appendix 4). The bacterial concentration of the $1/10^{2}$ dilution was estimated by filling a Petroff-Hausser bacteria counting chamber and examining it under high power by dark-field microscopy (see Appendix 4).

The dark-field count showed that the original suspension contained an estimated 1.2×10^{10} bacteria per ml.

It had been decided that the challenge dose should contain approximately 10^{8} bacteria in a volume of 5ml. Some of the original suspension was thus diluted to 1/100th to give an estimated concentration of 1.2×10^{8} bacteria per ml, and then diluted again, one in five, so that the final concentration was estimated to be 1.2×10^{8} per 5ml.

In order to establish the actual concentration of viable bacteria in the suspension, a viable count method as described in Appendix 4 was used. Duplicate plates were made of serial tenfold dilutions of the original suspension from a dilution of $1/10^{5}$ to one of $1/10^{10}$. A 1 in 5×10^{6} (one in five million) dilution was also used. Each plate was inoculated with 0.1ml of suspension spread with a glass spreader. These were then incubated in an atmosphere of 10% CO_2 at 37°C for four days before the colonies were counted. A similar count was carried out on the actual challenge suspension.

Swabs from the bottles of challenge suspension were taken for culture in aerobic and microaerophilic conditions as a test for contaminant organisms.

Experimental Infection

Using a sterile syringe and 20 gauge 25mm needle, a 5ml dose of the challenge suspension, containing approximately 1.2×10^8 Brucella ovis organisms, was injected into the jugular vein of each ram.

Detection of Infection

Semen samples (see Appendix 5) and blood samples were collected at one, two, four, five, seven, eight, nine, ten, 28 and 31 weeks after inoculation and occasionally thereafter for over a year. On each occasion the following diagnostic procedures were carried out:

1) Palpation of the external genitalia.
2) Examination of a Modified Ziehl-Neelsen stained smear of semen for the presence of inflammatory cells and bacteria. (See Appendix 1)
3) Culture of semen on two blood agar plates incubated at 37°C in an atmosphere of 10% CO_2 for four days to detect the presence of Brucella ovis.
4) Complement fixation test (CFT), gel diffusion test (GD) and enzyme linked immunosorbent assay (ELISA)to detect any antibody to Brucella ovis. The complement fixation test results were allocated "scores" as described previously (Chapter 3).

Necropsy

Two years after these five rams had been challenged they were killed and subjected to a complete post-mortem examination; particular attention being paid to the genital organs.

NOTES

1) "Panacur" - Coopers Animal Health N.Z. Ltd. Active constituent 25g/litre Fenbendazole.

RESULTS

Initial Disease Status of the Rams

Semen samples collected from the rams prior to the experiment showed no sign of Brucella ovis infection after microscopic examination of the semen and culture on blood agar. All five rams had a negative reaction to the complement fixation test at the start of the experiment.

Viable Count

Plates made from the tenfold dilutions of the original suspension concentrate just before challenge (see Plate 5.1) gave the following bacterial counts when examined after four days incubation:

Dilution	Average count from two plates	Estimated bacterial concentration of original suspension
$1/10^5$	TNTC	-
$1/10^6$	TNTC	-
$1/10^7$	121	1.21×10^{10} *
$0.5/10^7$	66.5	1.31×10^{10} *
$1/10^8$	13	1.3×10^{10} *
$1/10^9$	2	2.0×10^{10}
$1/10^{10}$	0	-

NB TNTC = too numerous to count

The estimates made from the three dilutions marked "*" were used to calculate an average to be used as the final estimate of the bacterial concentration of the original suspension:

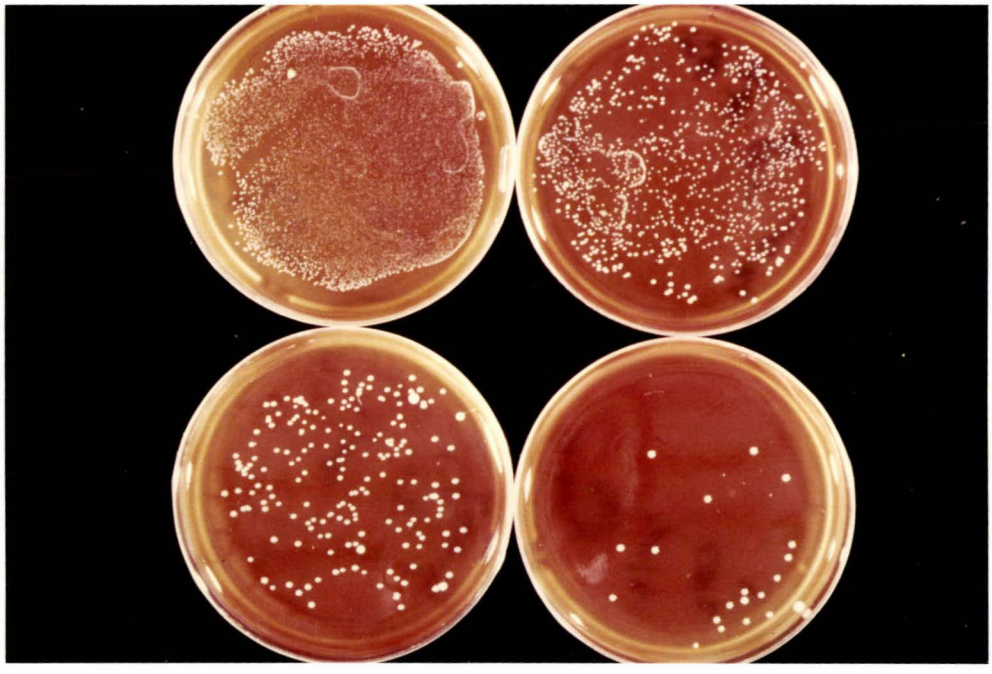
$$\frac{(1.21 + 1.31 + 1.3)}{3} \times 10^{10} = 1.27 \times 10^{10} \text{ bacteria/ml}$$

The actual number of organisms given to each animal by the intravenous route after the original suspension had been diluted 1 in 100 and then 1 in 5 was therefore estimated to have been 1.27×10^8 bacteria, slightly higher than the 1.2×10^8 bacteria estimated from the results of the dark field count.

Viable counts were also prepared from one of the bottles of suspension used for the challenge, just after the five rams had been inoculated. The number of colonies counted on these plates indicated the bacterial concentration in this bottle to have been 2.65×10^7/ml. As each ram received 5ml of the suspension, the actual dose received was therefore estimated as 1.325×10^8 bacterial "colony forming units", slightly higher than the original estimate of 1.27×10^8. As the actual injection of the inoculum took place at a time approximately halfway between the setting up of these two viable counts, the best estimate of the dose received by each animal was deemed to be an average of the two counts, which was 1.3×10^8 bacteria approximately.

Plate 5.1

Blood agar plates which have been inoculated using four serial tenfold dilutions of a suspension of Brucella ovis bacteria, so that a viable count for the estimation of the bacterial concentration of the original suspension can be made. Photographed after four days incubation in an atmosphere of 10% CO_2 at 37°C. Only the two plates at the bottom of the photograph have been diluted sufficiently to allow a count to be made.



Palpation

Palpable abnormalities of the epididymis first became apparent in two rams at six weeks after challenge. These early lesions were hot, soft swellings typical of an acute inflammation. Two other rams had palpable lesions by seven weeks, and one ram never developed any palpable lesions. (Table 5.1).

By 28 weeks, one ram which had previously had lesions had no lesions detected when the scrotal contents were manually examined, and the lesions in the other three rams were regressing. By 31 weeks none of the rams had easily palpable lesions.

In all the rams which developed epididymitis, lesions were palpated in both the right and the left epididymis at some stage during the course of the disease. The tail of the organ was the most frequently affected part, and the position and extent of the lesions was highly variable with time. (Table 5.2).

Table 5.1

The Presence of Lesions Palpated in the Epididymis of Five Rams Inoculated Intravenously Using Approximately 1.3×10^8 Brucella ovis Organisms

	Weeks Post-inoculation										
	1	2	4	5	6	7	8	9	10	28	31
Ram number											
1	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	+	+	+	+	+	+	-
3	-	-	-	-	-	+	+	+	+	+	-
4	-	-	-	-	-	+	+	+	+	+	-
5	-	-	-	-	+	+	+	+	+	-	-

Table 5.2

Site of the Lesions of the Epididymis Palpated in Rams Inoculated Intravenously using Approximately 1.3×10^8 Brucella ovis Organisms

			Weeks Post-inoculation						
			6	7	8	9	10	28	31
Ram	Side	Site							
2	Left	Head	-	-	-	-	-	-	-
		Body	-	-	-	-	-	-	-
		Tail	-	+	+	-	+	-	-
	Right	Head	-	-	-	-	-	-	-
		Body	-	-	-	-	-	-	-
		Tail	+	-	+	+	+	+	-
3	Left	Head	-	-	-	-	-	-	-
		Body	-	-	-	-	-	-	-
		Tail	-	+	+	+	+	-	-
	Right	Head	-	-	-	-	+	+	-
		Body	-	-	-	-	-	-	-
		Tail	-	+	+	+	+	+	-
4	Left	Head	-	-	-	-	-	-	-
		Body	-	-	+	-	-	-	-
		Tail	-	+	+	+	+	+	-
	Right	Head	-	-	-	-	-	-	-
		Body	-	-	-	-	-	-	-
		Tail	-	+	+	+	-	-	-
5	Left	Head	-	-	-	-	-	-	-
		Body	-	-	-	-	-	-	-
		Tail	+	+	+	+	-	+	-
	Right	Head	-	-	-	-	-	-	-
		Body	-	-	-	-	-	-	-
		Tail	+	+	+	+	+	-	-

Semen Smears

Acid-fast organisms were first seen in a Modified Ziehl-Neelsen stained smear of the semen from one ram five weeks after challenge. By six weeks four rams had positive semen smears. Smears made from the semen of these four rams contained acid-fast organisms on every subsequent occasion except at seven weeks when none were found in the smear made from the semen of one of the four rams sampled, even though the sample contained large numbers of neutrophils.

When acid-fast organisms were present in the smear, they were most commonly seen inside phagocytic cells (Plate 5.2). The presence of the bacteria was always associated with the presence of inflammatory cells. In the early stages of the disease their numbers were particularly high and polymorphonuclear cells predominated. From nine weeks onward, mononuclear cells, mainly lymphocytes but also macrophages, became more prevalent. Bacteria were sometimes seen in small aggregations outside inflammatory cells (Plate 5.3), suggesting that rupture of the host cell may have occurred, possibly during the smearing and staining process.

Culture of **Brucella ovis** from Semen

Brucella ovis was first cultured from the semen of two rams five weeks after challenge. At six weeks four of the rams had Brucella ovis cultured from their semen. Brucella ovis was recovered from every subsequent semen sample collected from these four rams.

Ram 1 never had lesions of epididymitis detected on physical examination, no acid-fast organisms were ever seen in its semen, and Brucella ovis was never cultured from the ejaculate.

Brucella ovis was isolated from 30 semen samples collected up to 31 weeks post-inoculation, of which 28 (93%) had acid-fast organisms detected in a smear. (Table 5.3)

Table 5.3

The Detection of Acid-Fast Bacteria in Smears of Semen Stained by the Modified Ziehl-Neelsen Technique, and the Culture of **Brucella ovis** from Semen in Rams Inoculated Intravenously using Approximately 1.3×10^8 **Brucella ovis** Bacteria

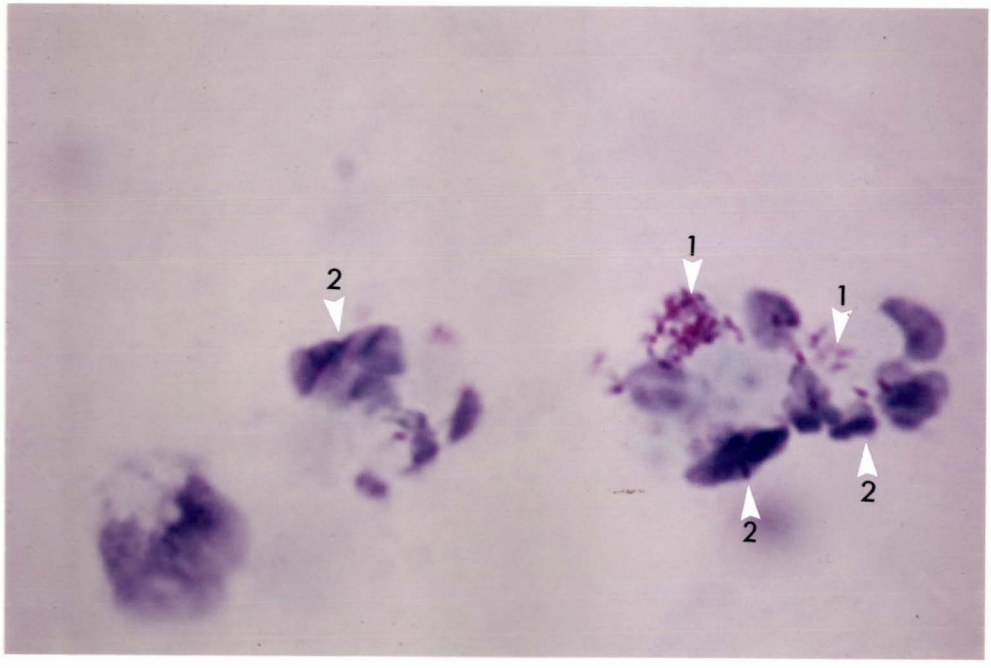
		Weeks Post-inoculation											
		0	1	2	4	5	6	7	8	9	10	28	31
Ram													
1	Smear	-	-	-	-	-	-	-	-	-	-	-	-
	Culture	-	-	-	-	-	-	-	-	-	-	-	-
2	Smear	-	-	-	-	-	+	-	+	+	+	+	+
	Culture	-	-	-	-	-	+	+	+	+	+	+	+
3	Smear	-	-	-	-	+	+	+	+	+	+	+	+
	Culture	-	-	-	-	+	+	+	+	+	+	+	+
4	Smear	-	-	-	-	-	+	+	+	+	+	+	+
	Culture	-	-	-	-	+	+	+	+	+	+	+	+
5	Smear	-	-	-	-	-	+	+	+	+	+	+	+
	Culture	-	-	-	-	-	+	+	+	+	+	+	+
Total Rams Having Positive:													
a)	Smears	0	0	0	0	1	4	3	4	4	4	4	4
b)	Cultures	0	0	0	0	2	4	4	4	4	4	4	4

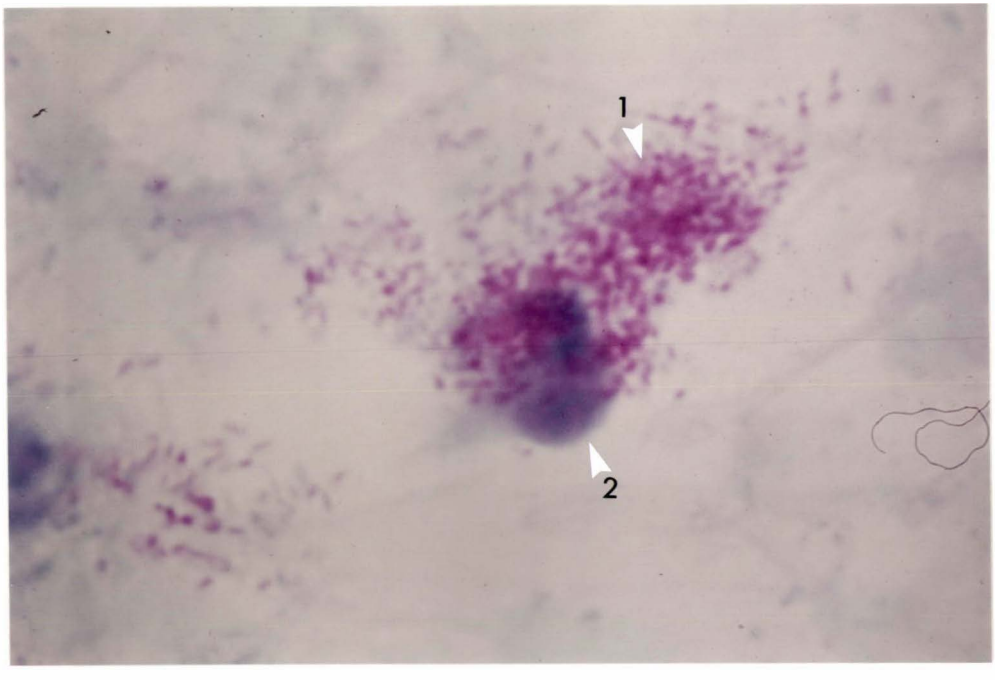
Plate 5.2

Brucella ovis bacteria (1) inside neutrophils (2) in a smear of semen stained by the Modified Ziehl-Neelsen method.

Plate 5.3

An accumulation of Brucella ovis bacteria (1) outside a neutrophil (2) in a semen smear stained by the Modified Ziehl-Neelsen method.


2
1
1
2
2


1
2

Complement Fixation Test

Two rams had a positive reaction to the complement fixation test only one week after challenge. At two weeks, all five rams had positive titres, including Ram 1 which never shed Brucella ovis in its semen. However, by six weeks this ram had lost its reaction to the test and never regained its positive titre. The other four rams had positive titres to the complement fixation test on every occasion they were tested from two weeks on (Table 5.4). From six weeks onward, all the serum samples of rams excreting Brucella ovis in their semen were positive to the complement fixation test.

Enzyme-Linked Immunosorbent Assay

One ram had a positive reaction to the ELISA test only one week after challenge. All four rams which became infected had positive reactions by two weeks, at which time Ram 1, which never excreted Brucella ovis, had a "suspicious" titre. By four weeks Ram 1 had reverted to a negative titre while the others remained positive, and this was the result at all subsequent tests (Table 5.5). From six weeks onward, all the serum samples of rams excreting Brucella ovis in their semen were positive to the ELISA test.

Gel Diffusion Test

The first "suspicious" reaction to the gel diffusion test was seen in one ram two weeks after challenge. By six weeks one ram had a positive reaction to the test, but it was ten weeks before all four rams that excreted Brucella ovis in their semen had positive reactions (Table 5.6). The gel diffusion test was less consistent than the others in detecting antibody in rams excreting Brucella ovis; detecting only 78.5% of these from six weeks after inoculation.

Of a total of 30 semen samples from which Brucella ovis was cultured, all 30 (100%) came from rams which had a positive reaction to both the complement fixation test and the ELISA test, 28 (93%) had acid-fast organisms detected in a Modified Ziehl-Neelsen stained smear, 22 (73%) came from rams which had a positive reaction to the gel diffusion test, and 21 (70%) came from rams with palpable abnormalities of the epididymis.

Table 5.4

Complement Fixation Titre Scores of Rams Inoculated Intravenously Using Approximately 1.3×10^8 **Brucella ovis** Organisms

	Ram Number				
	1	2	3	4	5
Weeks Post-inoculation					
1	0	0	16	8	1
2	12	21	21	16	17
4	9	18	17	12	20
6	0	16	20	12	16
7	0	16	20	14	20
8	2	16	20	20	24
9	0	17	24	24	24
10	0	20	24	20	24
28	0	12	20	20	24
31	0	16	24	23	22
56	0	9	17	13	18

KEY : Complement Fixation Titre Score 0 - 4 = negative
5 - 7 = suspicious
8 - 24 = positive

Table 5.5

ELISA Titres of Rams Inoculated Intravenously
Using Approximately 1.3×10^8 **Brucella ovis** Organisms

	Ram Number				
	1	2	3	4	5
Weeks Post -inoculation					
1	15	69	151	60	61
2	82	205	180	328	103
4	27	234	157	278	203
6	18	112	168	145	164
7	30	120	207	226	131
8	18	120	218	351	174
9	13	109	198	380	100
10	28	207	182	293	255
28	11	168	553	903	467
31	25	216	947	1056	724
56	12	299	1057	799	854

KEY: 0 - 69 = negative
70 - 89 = suspicious
$\geq$ 90 = positive

Table 5.6

Gel Diffusion Test Results of Rams Inoculated Intravenously Using Approximately 1.3×10^8 *Brucella ovis* Organisms

Weeks Post-inoculation	Ram Number 1	2	3	4	5
1	-	-	-	-	-
2	-	-	-	sus	-
4	-	sus	-	-	sus
6	-	-	+	-	-
7	-	+	+	-	+
8	-	+	+	+	sus
9	-	+	+	+	-
10	-	+	+	+	+
28	-	+	+	+	+
31	-	+	+	+	+

KEY: - = negative
sus = suspicious
+ = positive

Necropsy

In only two of the five rams necropsied two years after challenge were gross abnormalities of the genital organs seen. In Ram 3 the tail of the epididymis on the right side had a 2cm long fibrotic area on its caudal surface, which when incised was found to surround a 1cm diameter abscess-like structure containing dark yellow semisolid necrotic material. There were also adhesions present between the tail of the epididymis and the parietal tunica vaginalis. In Ram 5 a very small abscess-like structure 2mm in diameter containing yellow necrotic material was found in a small area of fibrosis in the tail of the right epididymis.

When these lesions were examined microscopically they were found to be chronic sperm granulomas surrounded by a thick fibrous capsule (Plate 5.4). There was perivascular cuffing of the blood vessels in the area by numerous lymphocytes (Plate 5.5).

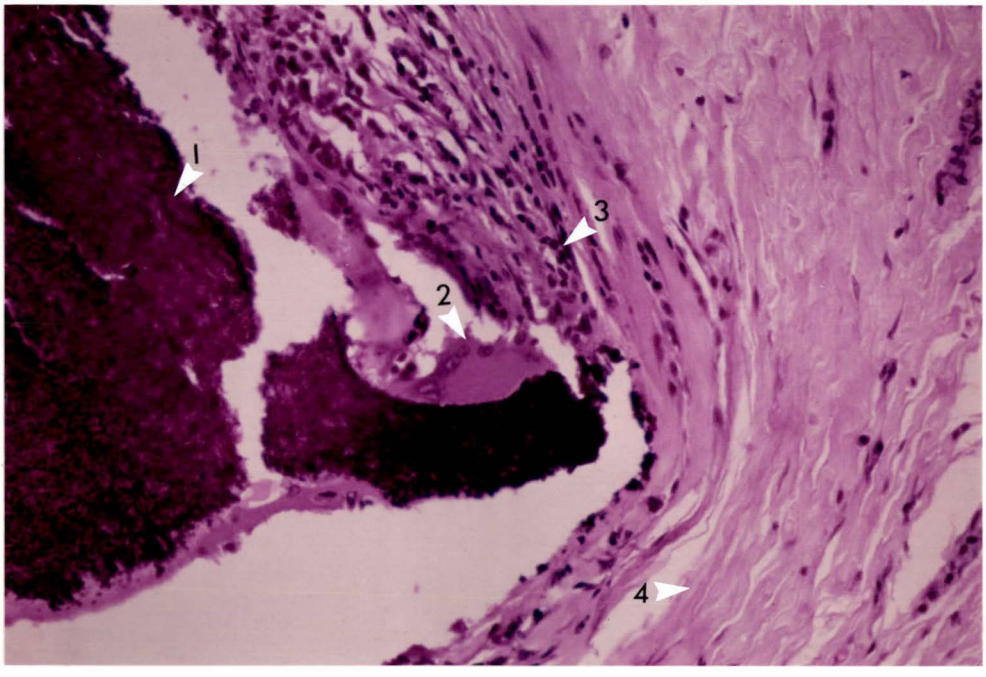
Table 5.7 summarises the chronological sequence of events that occurred following challenge.

Plate 5.4

Necrotic material (1), giant cells (2), mononuclear cells (3) and fibrosis (4) at the edge of a chronic sperm granuloma found in the tail of the right epididymis in a ram inoculated intravenously using approximately 1.3×10^8 Brucella ovis bacteria two years before necropsy.

Plate 5.5

Lymphocytes (1) densely packed around blood vessels (2) in an area of chronic fibrosis (3) seen surrounding a chronic sperm granuloma in the tail of the right epididymis of a ram inoculated intravenously using approximately 1.3×10^8 Brucella ovis bacteria two years before necropsy.


1
2
3
4

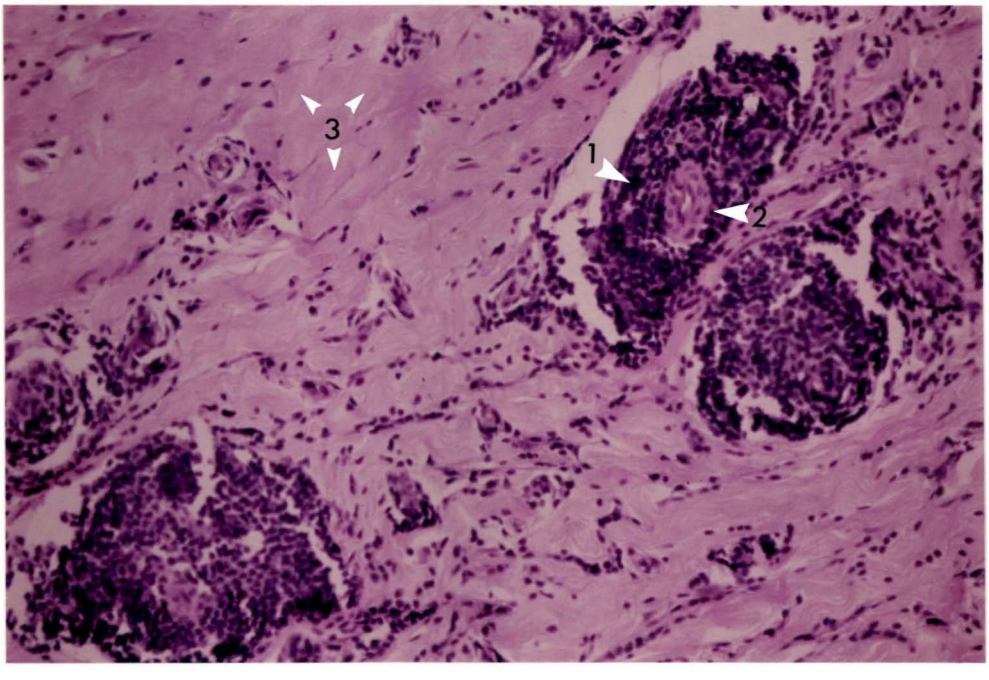

3
1
2

Table 5.7

Chronological Sequence of Events Following
The Intravenous Inoculation of Five Rams
Using Approximately 1.3 x 10^8 Brucella ovis Organisms

Weeks After Challenge	Diagnostic Procedure					
	CFT	ELISA	GD	Palpation	MZN Smear	Culture of semen
1	first positive	first positive				
2	five positive	four positive, Ram 1 "sus"	first "sus"			
4		Ram 1 lost titre				
5					first positive	first positive
6	Ram 1 lost titre		first positive	first positive	four positive	four positive
7				four positive		
10			four positive			
28				One had lost detectable lesions		
31				All four had lost detectable lesions		

DISCUSSION

Viable Count

The viable count plates prepared after the five rams had been inoculated with the Brucella ovis suspension gave a slightly higher estimate of the bacterial concentration in the inoculum than did those made before challenge. This difference may be partially explained by the fact that during the few hours between the counts, the suspension was at room temperature, and as the bacteria were in a stage of active growth when harvested from the TSA slopes, the bacterial numbers may have continued to increase during this period. Other possible sources of error in the counting procedure were: dilution errors, errors in the measurement of the 0.1ml of suspension used to inoculate the viable count plates, errors made in the physical counting of the colonies, and non-uniformity of the suspension used to make the dilutions and the viable counts, i.e. mixing error.

Detection of Infection

The complement fixation test and the ELISA were the methods which gave the earliest indication of a reaction to challenge. All the rams which subsequently excreted Brucella ovis in their semen had positive reactions to both these tests at only two weeks after challenge.

The examination of semen smears stained by the Modified Ziehl-Neelsen technique, and the culture of semen samples were effective in detecting all the infected rams at six weeks after challenge. Even though the blood agar used for the culture of the semen samples was not a selective Brucella ovis medium, overgrowth of the plates with contaminant organisms was a very minor problem as long as a good clean technique for the collection and handling of the samples was adhered to.

It was seven weeks before all four rams that eventually became infected had palpable lesions of the epididymis, and after 31 weeks this was no longer a reliable method for the detection of infection. The variability of the lesions and their gradual regression with time suggested that the majority of the obvious and extensive swelling that was detected by palpation in these rams was due to an acute inflammatory reaction rather than to the development of a chronic and persistent sperm granuloma. This was supported by the results of the post-mortem examinations, which revealed small chronic sperm granulomas in only two of the rams.

Transient Infection

The transient serological response which occured in Ram 1 in the absence of the development of epididymitis or the excretion of Brucella ovis requires some explanation. This phenomenon of serologically positive rams reverting to negative status was noted by Burgess, McDonald and Norris (1982) in flocks of naturally infected rams in Australia. These workers assumed that the rams had been infected and had subsequently recovered. However, as no bacteriological examinations were carried out, it is possible that these rams never had an established infection, and were only positive serologically. This may have been an antibody response to a brief exposure or transient bacteraemia which did not result in the establishment of infection. This was probably also the case in Ram 1 in this trial.

An established Brucella ovis infection from which the animal subsequently recovered would have been expected to produce a much more persistent immunological reaction than a single suspicious reaction to the ELISA test and a reaction to the complement fixation test of less than one month's duration, as was displayed by Ram 1 in this trial. The reasons for this phenomenon can only be speculated upon here. Perhaps the intravenous inoculation stimulated a brief response from the body defence mechanisms which completely overwhelmed the infection, not allowing it to establish in organs, or more importantly, lymph nodes. Maybe the bacteria became "trapped" in

lymph nodes remote from the urogenital system and were ultimately removed without causing infection. Alternatively, perhaps the ram may have been immunologically deficient in some way so that the serological response did not persist. The antibody produced by B-lymphocytes was measured by the serological tests, but no information about the T-lymphocyte dependent cell-mediated immune response was recorded. Investigation of this unusual immunological phenomenon was unfortunately beyond the scope of this study.

As the method of challenge used in this pilot trial successfully caused infection in all but one of the rams, it was decided to use a similar technique on rams vaccinated against Brucella ovis in various ways in order to investigate any differences in their resistance to subsequent challenge by this organism.

CONCLUSION

1) An intravenous inoculation of approximately 1.3×10^8 Brucella ovis organisms successfully caused infection in four out of five rams.

2) The earliest indication of infection was the serological reaction detected by the complement fixation and ELISA tests, between one and two weeks after challenge.

3) Excretion of Brucella ovis in the semen began five to six weeks after challenge and was consistent from that time on.

4) Epididymitis was first detectable at six to seven weeks after challenge, but became almost impossible to detect in any of the rams after 31 weeks.

CHAPTER 6

THE RESPONSE OF VACCINATED RAMS TO AN EXPERIMENTAL CHALLENGE USING BRUCELLA OVIS

INTRODUCTION

The differences in the antibody response which resulted from different Brucella ovis vaccination regimes (Chapter 3) suggested that those regimes might also induce different levels of resistance to Brucella ovis infection. In this respect, the advantage of two spaced doses of vaccine over a single dose, when the subcutaneous route of administration was used, was demonstrated by Buddle in 1962 and Buddle et.al. in 1963. In these experiments it was shown that amongst those rams which had received a single dose of Brucella ovis vaccine, two to four times as many became infected after challenge as amongst those which had received two spaced doses. There was, however, no information about the efficacy of the vaccine when it was administered by the intraperitoneal route. The differences in antibody response between animals which had received the vaccine by either the intraperitoneal or the subcutaneous technique (Chapter 3) suggested that an associated difference in effectiveness might also exist, but a challenge trial was needed to test this hypothesis.

It was shown in the pilot trial (Chapter 5) that an intravenous injection of approximately 1×10^8 Brucella ovis organisms could be expected to cause infection in approximately four out of five unvaccinated animals. It had to be assumed that vaccination would evoke an effective protection against infection, and therefore that a challenge dose of high magnitude would be required in order to demonstrate any small differences in the efficiency of different vaccination regimes. Because it would be impractical to use very large numbers of animals, the challenge dose would also need to be accurately pitched to show such small differences in protection. It was also necessary to use a challenge dose which would cause infection in a high proportion of the unvaccinated control animals to allow

valid statistical comparisons to be made.

It was decided to use a challenge dose similar to that used in the pilot trial (Chapter 5), i.e. approximately 1×10^8 Brucella ovis organisms. The trial was set up to test the resistance of rams which had been vaccinated using either one or two doses of Brucella ovis vaccine, administered by either the subcutaneous or the intraperitoneal technique.

MATERIALS AND METHODS

Sheep

The sheep used in this experiment were 50 two-year-old Romney and Romney-cross rams. They all came from farms accredited as being free from Brucella ovis infection. Prior to the experiment, they all had negative reactions to the Brucella ovis complement fixation test, all had palpably normal external genitalia, and all were free of any genital bacterial infection as determined by the culture and microscopic examination of semen samples.

Allocation to Experimental Groups

The rams were ranked in order of weight and divided into ten groups, the five heaviest in one group, the next five heaviest in another and so on. The five rams in each of these ten groups were randomly allocated to one of five treatments, so that there were ten rams in each treatment group and each group had a comparable distribution of light and heavy animals. A coloured wire indicating the treatment group was attached to the brass eartag of each ram. Plastic eartags (Allflex) numbered 1 to 50 were used to identify individual animals.

Management

For ease of management, the rams were kept in one mob throughout the experiment. They were grazed on an area subdivided into several paddocks and were regularly rotated around these paddocks. No supplementary feeding was undertaken. They were drenched each time they were mustered for experimental procedures (at least once a month) using 225mg of the anthelmintic Fenbendazole[1].

The rams were shorn before challenge to make both the inoculation of challenge suspension and the subsequent collection of semen samples easier and cleaner procedures.

Vaccine

A commercially produced, inactivated, Brucella ovis saline-in-oil emulsion vaccine[2] was used. It was from a single batch (series 2935) and was used well before the expiry date. The dose on each occasion was 2 ml.

Treatments

The following treatments were allocated to the five experimental groups:

1)	single subcutaneous vaccination	(1 s/c)
2)	single intraperitoneal vaccination	(1 i/p)
3)	two subcutaneous vaccinations, eight weeks apart	(2 s/c)
4)	two intraperitoneal vaccinations, eight weeks apart	(2 i/p)
5)	no vaccination	(control)

The subcutaneous injections were administered in the anterior half of the neck, using a new sterile syringe and 18 gauge 9mm needle for each animal. The vaccine was administered on the right side of the neck for the first dose, and on the left for the second dose.

The intraperitoneal vaccine was injected at a point midway between the stifle joint and the vertebral articulation of the last rib, in the paralumbar fossa on the right side. A new, 17 gauge 19mm needle and sterile syringe was used for each animal.

All the rams were challenged ten weeks after their vaccination programme had been completed (Table 6.1).

Production of Challenge Suspension

A Brucella ovis isolate cultured from the semen of one of the rams in the pilot trial (Chapter 5) was used in the production of the challenge suspension. A single four-day-old Brucella ovis colony from the semen culture was subcultured onto two blood agar plates. One was incubated aerobically and the other in an atmosphere of 10% CO_2. A swab of the growth that resulted from the microaerophilic culture was used to inoculate heart infusion broth, and this was immediately used to inoculate 20 TSA + serum slopes in the manner described in the pilot trial (Chapter 5).

After three days incubation at 37°C in 10% CO_2, the slopes were harvested with sterile peptone saline as previously described (Chapter 5) and a dark field count carried out on the suspension.

This technique estimated the bacterial concentration to be 1.8×10^{10} bacteria/ml. The suspension was diluted to 3.6×10^{7} bacteria/ml using peptone saline, so that each 5ml dose was estimated to contain approximately 1.8×10^{8} Brucella ovis organisms, and it was then dispensed into sterile bottles ready for use.

Duplicate viable count plates were inoculated using serial tenfold dilutions, from $1/10^{10}$ to $1/10^{5}$, of the original suspension concentrate; and also of serial tenfold dilutions from $1/10^{6}$ to $1/10^{2}$ of the resultant diluted challenge inoculum.

Inoculation Of Challenge Suspension

Using sterile syringes and 20 gauge 25mm needles, 5ml of the challenge suspension was injected into the jugular vein of each ram ten weeks after the last vaccination had been administered.

Serology

In order to detect changes in antibody titre after both vaccination and challenge, blood samples were collected from all the rams, according to the schedule set down in Table 6.1. Blood was collected by jugular venepuncture and the serum was processed and stored in the manner described previously (Chapter 3). The serological tests carried out on the serum samples at Wallaceville Animal Research Centre were the Brucella ovis complement fixation test (CFT), gel diffusion test (GD) and enzyme linked immunosorbent assay (ELISA). The complement fixation test results were allocated a "score" as described previously (Chapter 3).

Bacteriology

Semen samples were collected from all the rams according to the schedule in Table 6.1. Testing was discontinued 13 weeks after challenge, as it was considered possible that new cases of infection after this period might have resulted from the natural transmission of the organism between rams.

Semen was obtained by electroejaculation (Appendix 5).

As soon as possible after collection, two blood agar plates for each ram were inoculated using semen from the sample. These were incubated at 37°C in 10% CO_2 for four days, and then examined for the presence of Brucella ovis colonies.

In cases where there was doubt about the identity of the isolate, a colony was subcultured and the subsequent growth of organisms subjected to microscopic examination and biochemical tests (Appendix 1) until the identity was established.

Semen Smears

A smear was made from a drop of each of the semen samples. These were allowed to dry, and then stained by the Modified Ziehl Neelsen method (Stamp et.al., 1950, see Appendix 1). The smears were then examined at a magnification of x 1,000 for the presence of inflammatory cells and bacteria.

Palpation

Whenever semen samples were collected, the scrotal contents of each ram were palpated and the presence of any abnormality was recorded.

Criterion For Judging Infection

A ram was judged to be "infected" when Brucella ovis bacteria could be isolated from its semen.

Statistical Methods

To evaluate the significance of differences between group mean serological titres, 95% confidence limits of the means were calculated and compared. For qualitative data, the chi-squared test, using Yate's correction factor, was used to determine the significance of any differences between groups. Both these methods are described in Appendix 6.

NOTES

1) "Panacur" - Coopers Animal Health N.Z. Ltd. Active constituent 25g/litre Fenbendazole.

2) "Ramovexin" - Coopers Animal Health N.Z. Ltd. Inactivated Brucella ovis saline-in-oil vaccine preparation.

Table 6.1

Schedule Of Experimental Procedures In A Trial Examining The Response Of Vaccinated Rams To Intravenous Challenge Using **Brucella ovis** Organisms

Week	Vaccinate 1 s/c and 1 i/p groups	Vaccinate 2 s/c and 2 i/p groups	Collect Blood	Collect Semen
0		+	+	
2			+	
5			+	
8	+	+	+	
10			+	+
14			+	
18	DAY OF CHALLENGE		+	
22			+	+
26			+	+
31			+	+

RESULTS

Viable Count

The final estimate of the live bacterial concentration of the suspension used for intravenous challenge was 3.26×10^7 Brucella ovis bacteria per ml of suspension. Since each ram received 5ml of suspension, the actual challenge dose was therefore calculated to be approximately 1.63×10^8 Brucella ovis bacteria. (N.B. intended dose 1.8×10^8).

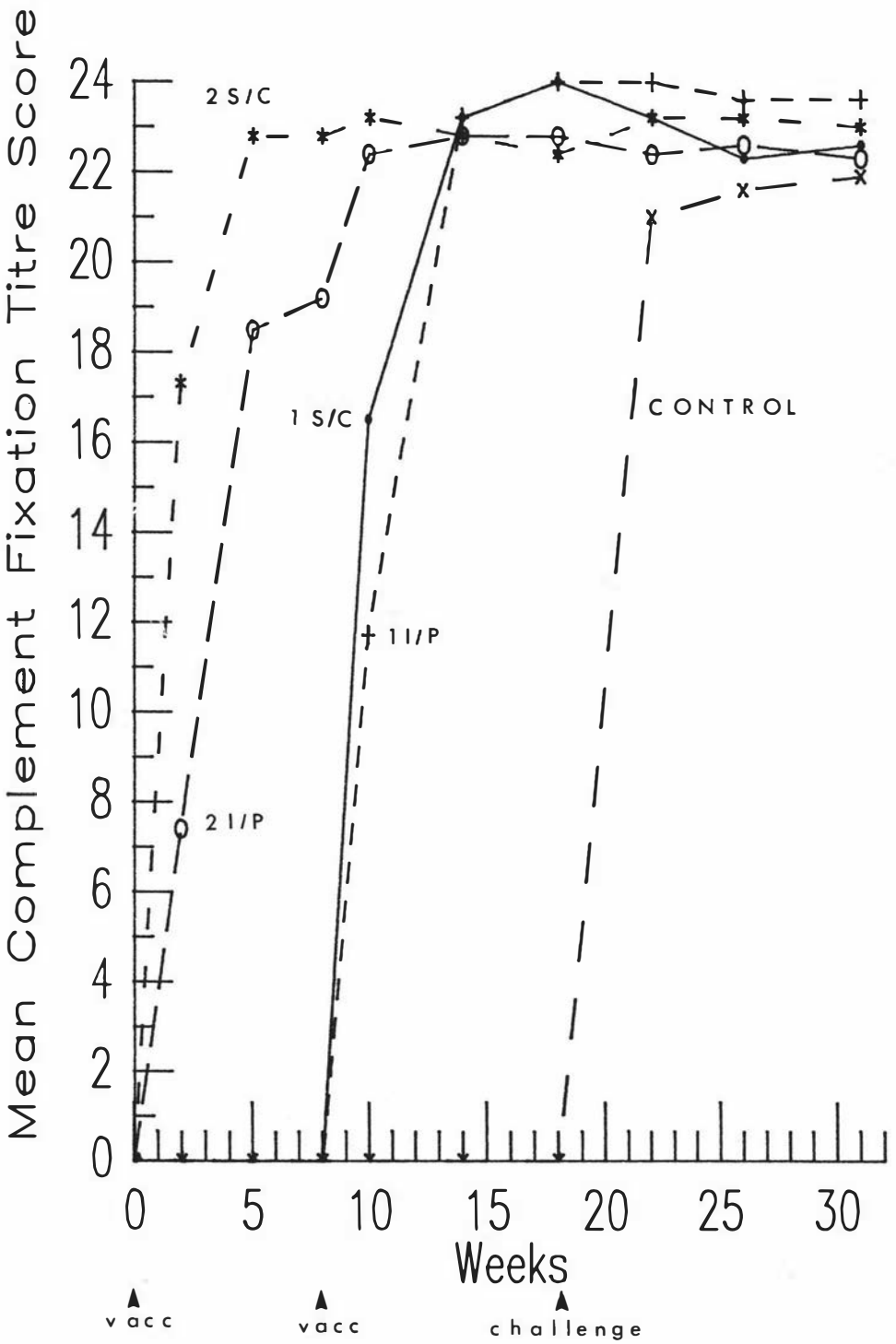
Complement Fixation Test

Following vaccination, the mean complement fixation titre scores of the various treatment groups developed in the same way as had been demonstrated previously (Chapter 3). The rams vaccinated by the intraperitoneal method tended to develop titres more slowly than those vaccinated by the subcutaneous technique, and a significant difference in mean titre could be demonstrated between the 2 s/c group and the 2 i/p group two weeks after initial vaccination. By the time that the rams were challenged, however, there were no significant differences between the mean complement fixation titre scores of any of the vaccinated groups, and this remained the case for the rest of the experiment (Figure 6.1).

The unvaccinated rams in the control group had all developed a positive reaction to the complement fixation test by four weeks after challenge. These rams, like the vaccinated animals, remained positive to the complement fixation test for the rest of the trial.

Figure 6.1

Mean complement fixation titre scores of rams challenged by the intravenous inoculation of approximately 1.63 x 10^8 Brucella ovis organisms, after receiving either one or two doses of Brucella ovis vaccine administered by either the subcutaneous or the intraperitoneal method. The reaction of a group of unvaccinated (control) rams to challenge is also shown.


Mean Complement Fixation Titre Score
24
22
20
18
16
14
12
10
8
6
4
2
0
2 S/C
1 S/C
2 I/P
1 I/P
CONTROL
0
5
10
15
20
25
30
Weeks
vacc
vacc
challenge

Enzyme Linked Immunosorbent Assay

After vaccination, the 2 s/c and 2 i/p groups followed the pattern of rising titres described previously (Chapter 3). The rams vaccinated by the intraperitoneal method exhibited a slower rise in average titre than those vaccinated by the subcutaneous route (Figure 6.2). Following the administration of the second dose of vaccine, both of these groups had a substantial rise in average titre.

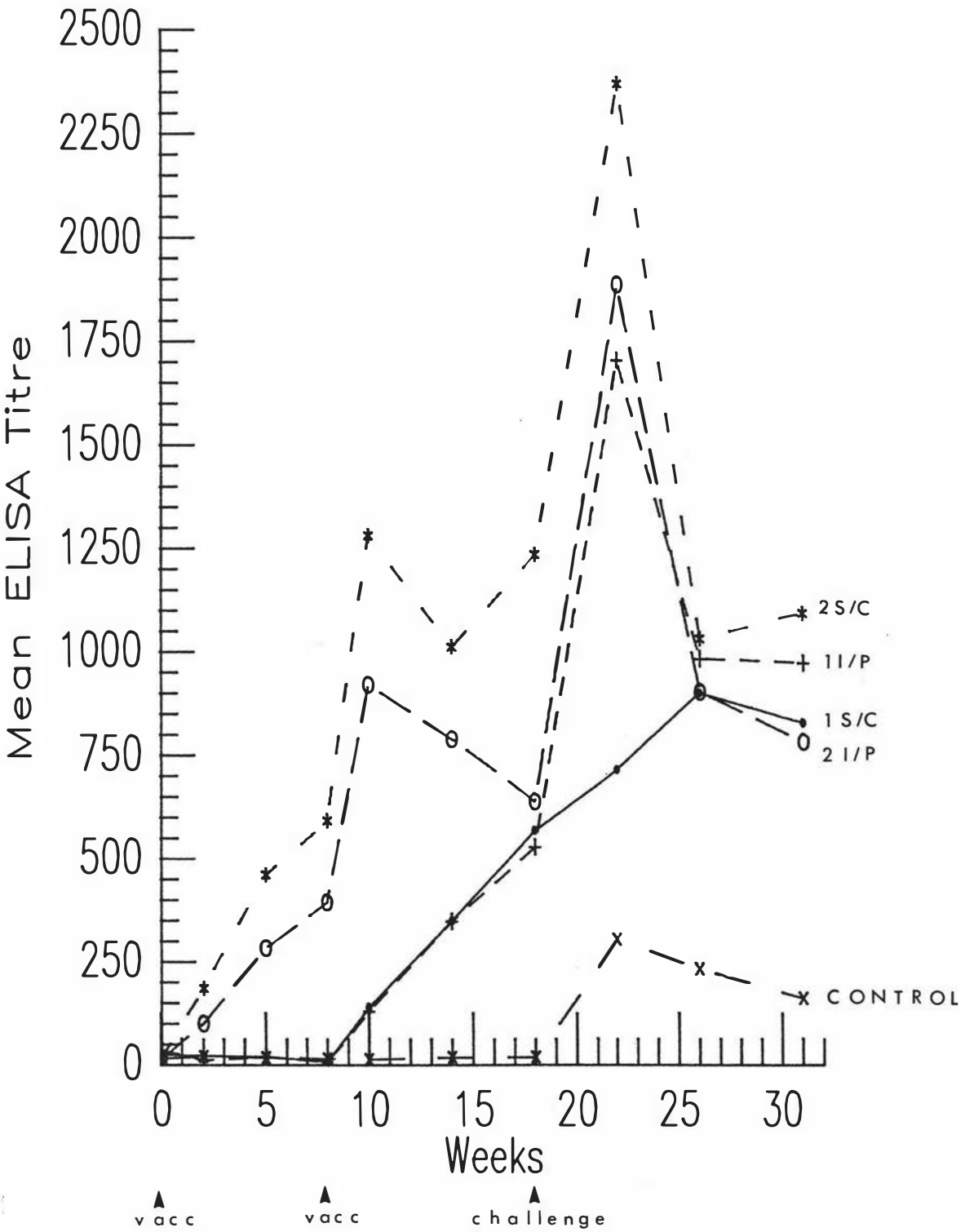
By the time the rams were challenged at 18 weeks, all the vaccinated animals had a positive reaction to the ELISA test. At this time, there were no significant differences in mean titre between the 1 s/c, 1 i/p and 2 i/p groups, but 95% confidence limits showed that the mean titre of the 2 s/c group was significantly greater than that of any of the other three vaccinated groups.

After challenge, there was a sudden rise in the mean titre of the 2 s/c, 2 i/p and 1 i/p groups, but the 1 s/c group did not have as dramatic an increase (Figure 6.2). This group had a mean titre significantly lower than that of any of the other three groups at four weeks after challenge, but this difference had disappeared by the next test, four weeks later.

All the unvaccinated rams in the control group had a positive reaction to the ELISA test by four weeks after challenge, but although these rams remained positive for the rest of the experiment, the mean titre of this group never reached the magnitude of that of any of the four vaccinated groups.

Figure 6.2

Mean ELISA titres of rams challenged by the intravenous inoculation of approximately 1.63×10^8 Brucella ovis organisms, after receiving either one or two doses of Brucella ovis vaccine administered by either the subcutaneous or the intraperitoneal method. The reaction of a group of unvaccinated (control) rams to challenge is also shown.


Mean ELISA Titre
2500
2250
2000
1750
1500
1250
1000
750
500
250
0
0
5
10
15
20
25
30
Weeks
vacc
vacc
challenge
2 S/C
1 I/P
1 S/C
2 I/P
CONTROL

Gel Diffusion Test

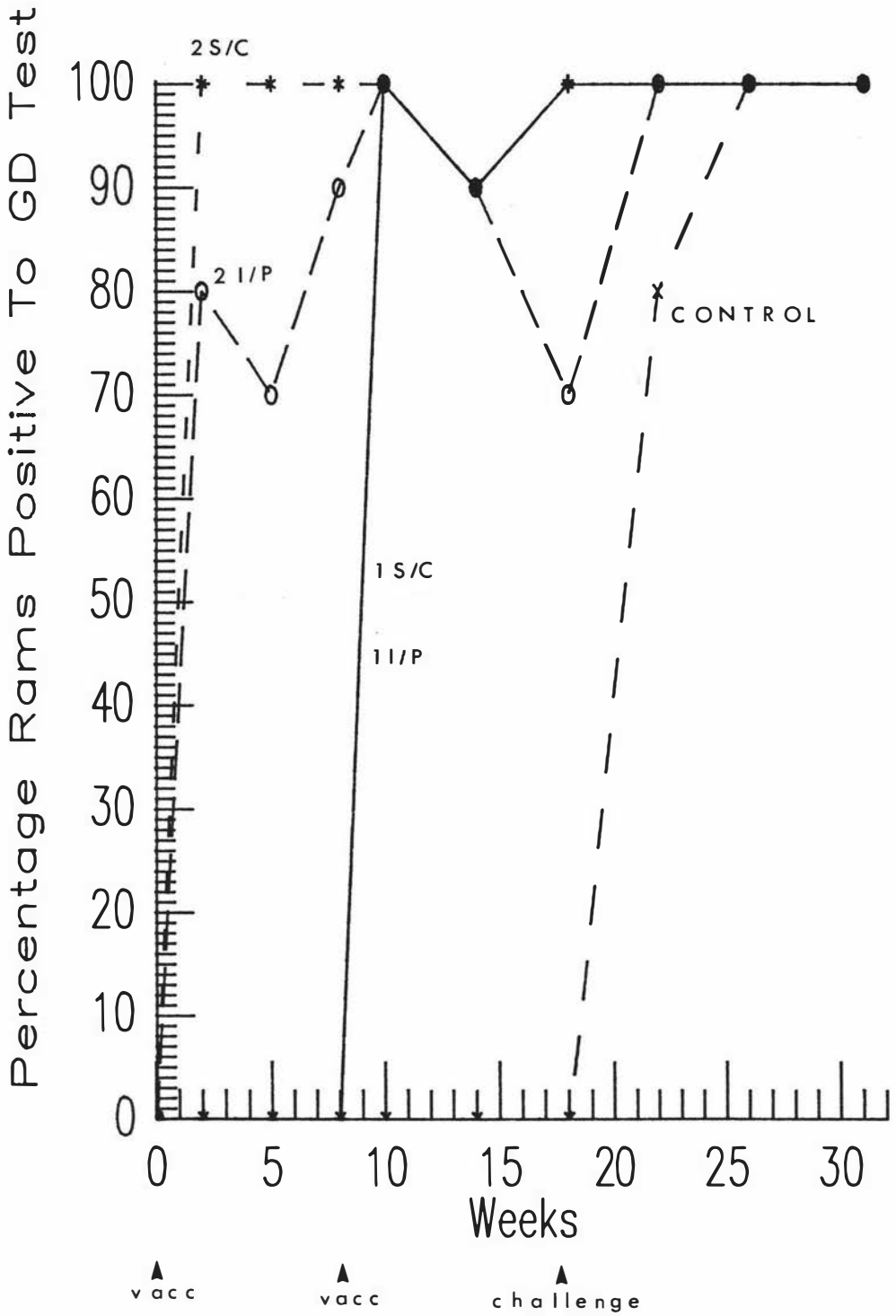
For the purposes of this trial, samples with a "suspicious" reaction to the gel diffusion test were deemed to be positive.

The serological response of the rams to vaccination was similar to that seen in the gel diffusion test in the previous study (Chapter 3).

After challenge, all the vaccinated rams had a positive reaction to the gel diffusion test on each of the three occasions they were tested. All the unvaccinated rams in the control group had a positive reaction to the test by eight weeks after challenge. (Figure 6.3).

Figure 6.3

The percentage of rams having a positive reaction to the gel diffusion test after being vaccinated either once or twice using Brucella ovis vaccine administered by either the subcutaneous or the intraperitoneal method, and then challenged by the intravenous inoculation of approximately 1.63×10^8 Brucella ovis organisms. The reaction of a group of unvaccinated (control) rams to challenge is also shown.


Percentage Rams Positive To GD Test
100
90
80
70
60
50
40
30
20
10
0
2 S/C
2 I/P
1 S/C
1 I/P
CONTROL
0
5
10
15
20
25
30
Weeks
vacc
vacc
challenge

Bacteriology

A greater proportion (60%)of unvaccinated rams had *Brucella ovis* isolated from the semen four weeks after challenge than did vaccinated rams (7.5%). (Table 6.2). This difference was significant at the 0.1% level.

By eight weeks, however, there were no longer any significant differences between the treatment groups. At the end of the experiment, 13 weeks after challenge, 75% of the vaccinated animals and 100% of the controls were infected. This difference was not statistically significant and there were also no statistically significant differences between individual treatment groups in the number of animals becoming infected after challenge.

Semen Smears

Of 87 semen samples from which *Brucella ovis* was isolated, 69 (79%) had acid-fast bacteria detected by the microscopic examination of a semen smear stained by the Modified Ziehl-Neelsen method. This result confirms the superiority of the cultural technique for diagnosis over the direct microscopic examination of a semen smear. All samples which had acid-fast bacteria detected in the smear yielded *Brucella ovis* on culture. One ram which was culturally positive never had acid-fast organisms detected in a smear on any of the three occasions on which semen samples were collected and tested.

Palpation

Thirty-six (90%) of the 40 rams which excreted Brucella ovis in their semen developed lesions of epididymitis. Of 87 semen samples from which Brucella ovis was isolated, 75 (86.2%) came from rams which had palpable lesions of epididymitis at the time the sample was collected.

There were some problems of interpretation. Minor deviations from normal were difficult to detect. In the early stages of infection, the reaction was often just a slight soft swelling, and in some cases it took a considerable period of time for the reaction to develop into a discrete, palpable lesion. The decision was made that only when an unmistakable abnormality was present would the ram be recorded as having a "palpable lesion".

Four weeks after challenge, ten rams had palpable abnormalities of the scrotal contents (Table 6.2). Of these, six had no Brucella ovis bacteria isolated from their semen at that time.

By eight weeks, all but two of the 33 rams with lesions also had Brucella ovis isolated from their semen, but seven rams which had the organism in their semen had no lesions of epididymitis.

By the end of the experiment 13 weeks after challenge, only one of the 36 rams with lesions did not have Brucella ovis isolated from its semen, and five rams excreting the organism in their semen had no lesions of epididymitis. The numbers of rams in each group which had epididymitis were not significantly different at any stage of the experiment.

Table 6.2

Number of rams either unvaccinated, or vaccinated either once or twice by either the subcutaneous or the intraperitoneal technique using Brucella ovis vaccine, which had epididymitis, or Brucella ovis isolated from the semen, after the intravenous inoculation of approximately 1.63×10^8 Brucella ovis organisms

	Weeks After Challenge					
	4		8		13	
	Diagnostic Procedure					
VACCINE GROUP	Palpation	Culture	Palpation	Culture	Palpation	Culture (FINAL NUMBER INFECTED/ NUMBER CHALLENGED)
1 s/c	0	0	6	6	6	6/10
1 i/p	3	0	7	8	8	9/10
2 s/c	1	1	6	6	6	7/10
2 i/p	3	2	6	8	8	8/10
control	3	6	8	10	8	10/10
Totals	10/50	9/50	33/50	38/50	36/50	40/50

DISCUSSION

Serology

The serological response to vaccination of the rams in this trial was similar to that shown in the results of the trial investigating vaccination serology (Chapter 3), in that the intraperitoneal method of vaccination resulted in a slower rise in titre than the subcutaneous technique.

The ELISA titres of the unvaccinated rams which developed as a result of intravenous challenge were lower and less persistent than those induced by even a single dose of *Brucella ovis* vaccine. Four weeks after challenge, when the ELISA titres of the control rams were at a peak, the mean titre was similar to that of rams in the 2 s/c group five weeks after initial vaccination. However, whereas the mean titre of the 2 s/c group had increased further by eight weeks after initial vaccination, the titres of rams in the control group had reduced when they were tested eight weeks after challenge. This difference was statistically significant, suggesting that infection may not be as effective as vaccination at stimulating antibody response. Alternatively, it could simply mean that there is less antibody in circulation in unvaccinated infected animals, because it localises into those areas which the bacteria colonise.

There was a marked serological response to challenge in the form of an anamnestic rise in titre as shown by the ELISA results of the 2 s/c, 2 i/p and 1 i/p groups. This rise in titre was not nearly as marked in the 1 s/c group. This may, however, have been an aberration in that particular run of the ELISA test, as this difference between the groups had completely disappeared at the following test.

When the complement fixation and ELISA titres of individual animals were examined, there were no obvious differences in pattern between those vaccinated animals that became infected and those that did not. Some animals with a relatively high antibody titre at the time of challenge succumbed to infection, and some with a low titre resisted the challenge. This finding indicated that antibody levels

as measured by serological tests may not be a very reliable estimation of resistance to infection in individual animals. It has been mentioned previously (Chapter 3) that resistance to intracellular bacteria such as Brucella ovis is heavily dependent on cell-mediated immune responses, which are not measured by these serological methods. Perhaps some animals, despite having high levels of circulating antibody, have a poor cell-mediated immunity. Similarly, some animals with low circulating antibody levels might have a strong cell-mediated immunity. Another possible explanation is the wide individual variation between animals in their ability to resist infection, an ability which may not necessarily be reflected in their circulating antibody levels.

In spite of these doubts cast upon the value of serology as a method of predicting resistance in individual animals, the consistent pattern of antibody titres shown by groups of animals vaccinated against Brucella ovis infection in different ways strongly suggests that a real difference in resistance exists between these groups.

Vaccine Efficacy

The results of this experiment were somewhat surprising. The use of the inactivated Brucella ovis vaccine did not appear to have had any significant effect on the resistance of rams to a single intravenous challenge using 1.63×10^8 Brucella ovis bacteria, no matter which method of vaccination was used.

Although there were no significant differences in the final numbers of rams infected in each group, there was a significant difference in the proportion of vaccinated and unvaccinated rams excreting Brucella ovis one month after challenge. This suggests that vaccination may somehow delay the establishment of an infection in the genital organs. This may be because it takes longer for the infection to overcome the enhanced immune response of the vaccinated animal sufficiently to cause detectable numbers of bacteria to be excreted in the semen.

The fact remains, however, that no significant enhancement of resistance to challenge as a result of vaccination could be demonstrated, casting doubts on the efficacy of this vaccine which has been used for over 20 years.

It could be argued that the intravenous route of challenge is highly artificial, and that under conditions of natural transmission the body defences would have had a greater chance of resisting the challenge. One of the important considerations in this respect is that of the local immune response (e.g. secretory IgA) at the first barrier to entry which would be met in natural transmission, a mucous membrane. The use of the intravenous route circumvents this initial barrier and produces an "instant bacteraemia" before the immune system has a chance to react. However, when Buddle did the initial investigations into Brucella ovis vaccines (Buddle, 1954, 1957, 1958, 1962, Buddle et.al., 1963), he used the intravenous route for all his artificial challenges, and still the vaccine appeared to perform satisfactorily.

It was noteworthy that in Buddle's 1962 experiment involving intravenous challenge, approximately four times as many vaccinated rams became infected than did so after natural challenge in an experiment carried out by Buddle in 1958. This applied both to rams vaccinated with a single dose of the Brucella ovis saline-in-oil vaccine and to those vaccinated using a combination of Brucella ovis vaccine and Brucella abortus Strain 19. This appears to support the hypothesis that the intravenous method of challenge was more severe than natural challenge. However, considering the inconsistencies of the natural challenge situation, in which many rams may never be challenged at all, a four-fold difference in the proportions of rams becoming infected could be considered to be of small significance. It could be that in the natural challenge experiment, only one-quarter of the animals were adequately challenged, and this is quite a likely possibility.

In the experiment being reported here, a challenge dose was used that was approximately 18 times greater than that used by Buddle in his 1962 experiment. This may have accounted for the difference in the proportion of rams succumbing to infection in these two trials. Of those rams vaccinated twice by the subcutaneous route, 70% became infected in this experiment, in comparison with 23% in Buddle's 1962 experiment. However, this may not be a complete explanation, as the challenge dose in this current trial was only 3.5 times greater than that used by Buddle et.al. in 1963, when only 10% of rams vaccinated twice by the subcutaneous route became infected after an intravenous challenge.

It appears, then, that the magnitude of the challenge dose may not be the only factor involved in the discrepancy between the results of this trial and those conducted by Buddle. It has to be considered that the vaccine itself might be of low efficacy.

Commercial vaccines are frequently expected to produce resistance to quite severe challenge. A challenge dose of several "ID50"s is often used to test the efficacy of vaccines (i.e. several times that dose which causes infection in 50% of unvaccinated, susceptible animals). Unfortunately, no reliable information was available concerning the intravenous ID50 of Brucella ovis, or about the numbers of bacteria that might be involved in the natural transmission processes. It was decided to make an investigation of these factors.

CONCLUSION

The use of Brucella ovis vaccine, administered either once or twice by either the subcutaneous or the intraperitoneal technique, had no significant effect on the resistance of rams to an intravenous challenge consisting of approximately 1.63×10^8 live Brucella ovis bacteria, other than to delay the onset of excretion of the organism in the semen.

CHAPTER 7

THE RESPONSE OF VACCINATED RAMS TO A RANGE OF EXPERIMENTAL CHALLENGE USING BRUCELLA OVIS (ID50 TRIAL)

INTRODUCTION

In an experiment investigating the efficacy of various vaccination techniques (Chapter 6), no significant difference in resistance to challenge with Brucella ovis could be demonstrated between vaccinated and unvaccinated animals. The challenge dose used was relatively high (1.63×10^8 Brucella ovis bacteria administered intravenously), and this inoculation caused infection in 100% of unvaccinated animals. However, this dose was similar to that used by other workers investigating Brucella ovis, such as Jebson et.al. (1954) who used 10^9 or 10^8 bacteria as an intravenous challenge dose, and Buddle and Boyes (1953) who used intravenous doses of 10^7 or 10^9 Brucella ovis bacteria.

It was considered possible that the magnitude of the challenge dose used in the previous experiment (Chapter 6) may have contributed to the apparent failure of the vaccine to produce significant resistance, and accordingly it was decided to test the effect of vaccination at a lower challenge dose. For a decision to be made concerning the specific challenge dose that should be used, some information on the likely effect of that dose on susceptible animals would be a prerequisite. Unfortunately, information such as the minimum infective dose for sheep using the local Brucella ovis strain was unavailable. It was decided that an estimate of the dose which caused 50% of susceptible rams to become infected (ID50) should be determined by experiment.

Unfortunately, most of the classical methods of making an estimate such as the ID50 involve very large numbers of animals which would be physically impractical to handle and beyond the resources of this project. However, the method of Reed and Muench (1938) offered a simple and practical alternative. The method is described in Appendix 6, but briefly, it uses a number of serial dilutions of the test material, in this case Brucella ovis challenge suspension, and at each dilution several animals are tested. By the simple expedient of assuming that an animal becoming infected at one dose would have become infected at any higher dose, and that one resisting challenge at a particular dose would have resisted it at any lower dose, cumulative results can be calculated. Thus larger numbers of animals can be used in the estimate, and the precision of the 50% end point is greatly improved over that which would otherwise have been possible using a small number of animals.

By performing a similar graded challenge procedure on groups of animals vaccinated by various techniques, it was hoped that comparisons could be made between their relative resistances to various challenge doses, as well as between the resultant ID50 estimates for these different groups. It had already been shown that there were differences in the serological responses of rams vaccinated by different techniques (Chapter 3). It had also been shown by Buddle (1962) and Buddle et.al. (1963) that rams vaccinated twice by the subcutaneous method had a greater resistance to experimental challenge than those vaccinated only once. It was hoped that by using a range of challenge doses in this experiment, information could be gained concerning the relative protective value of Brucella ovis vaccine when it is administered once or twice by the intraperitoneal technique, or twice by the subcutaneous technique.

MATERIALS AND METHODS

Sheep

One hundred and forty-four clinically normal rams were used in this experiment. They were mainly one to two year old Romneys and Drysdales, but some older animals and some rams of other breeds were also used. All the rams came from properties accredited as being *Brucella ovis* free. Prior to the experiment all rams had palpably normal scrotal contents and were negative to the *Brucella ovis* complement fixation test.

Allocation to Experimental Groups

The rams were randomly allocated to one of four experimental groups: thirty-six to each. One animal in each group was included as a "spare" in case one of the other 35 was unusable for any reason. These four rams were given blank plastic eartags, and all the others were individually identified with tags numbered 1-140.

Management

As a single grazing area large enough to maintain the whole flock throughout the experiment was not available, the animals were grazed together initially, but split into two mobs of approximately equal size immediately after challenge and thereafter grazed on two separate blocks of land. Mob A consisted of rams challenged at the higher doses, and Mob B those subjected to lower challenge doses. It was hoped that this would help to reduce any natural challenge effect from those rams most likely to become infected (high challenge) on those less likely to do so (low challenge).

As rams became infected (as shown by semen culture) they were removed from the mob, in a further effort to reduce natural transmission.

Towards the end of the experiment, dry conditions resulted in poor grass growth, and the diet was supplemented with good quality hay.

The rams were drenched regularly (about once a month) using 250mg of the anthelmintic Fenbendazole[1] and all rams were vaccinated soon after selection against salmonellosis[2], and against the five major clostridial diseases[3].

Just before challenge, all the rams were shorn to facilitate the intravenous injection of the challenge suspension and subsequent collection of clean semen samples.

Vaccine

A commercially produced, inactivated, Brucella ovis saline-in-oil emulsion vaccine[4] was used. The vaccine was made up entirely from one batch (series 2995) and was used well before the expiry date. The dose was 2ml.

Treatments

The following treatments were allocated to the experimental groups:

1) Rams 1 - 35 two vaccinations, 8 weeks apart, administered by the subcutaneous route, in the neck. (2 s/c)
2) Rams 36 - 70 two vaccinations, 8 weeks apart, administered by the intraperitoneal technique. (2 i/p)
3) Rams 70 - 105 one vaccination, administered by the intraperitoneal technique. (1 i/p)
4) Rams 106 - 140 unvaccinated. (controls)

All 36 animals in each of the three treated groups were vaccinated, including the "spares". The techniques used for administration of the vaccine were the same as those described in Chapter 3, except that the needle used for intraperitoneal vaccination was a 25mm 18 gauge needle rather than the 19mm 17 gauge needle used previously. This change was made in an effort to reduce the problem of "extraperitoneal" vaccine deposition noticed in the post-mortem studies (Chapter 4).

Animals in the 1 i/p group were vaccinated on the same day that those in the 2 i/p and 2 s/c groups received their second vaccination. All the animals were therefore challenged ten weeks after the completion of their vaccination programme. (Table 7.1)

Production of the Challenge Suspension

It was decided to use challenge doses corresponding to serial tenfold dilutions of the challenge suspension used in the previous experiment (Chapter 6); from a dose of approximately 10^8 Brucella ovis bacteria down to one of approximately 10^2 organisms.

Brucella ovis was isolated from the semen of one of the rams infected in the pilot trial (Chapter 5). This isolate was grown in pure culture, propagated on TSA slopes and harvested in sterile peptone saline in the same manner as described for the previous challenge trial (Chapter 6). The purity of the suspension was checked by examining Gram stained and Modified Ziehl-Neelsen stained smears of the preparation.

A dark field count carried out on the resultant pooled suspension gave an estimated bacterial concentration of 2.7×10^{10} bacteria/ml. A tenfold dilution of this suspension was made and 0.5ml placed in each of six sterile Universal bottles containing 13ml of sterile peptone saline, making 13.5ml of a suspension having an estimated concentration of 1×10^8 bacteria/ml in each bottle.

The suspension was then used to make a series of tenfold dilutions from 10^8 to 10^2 bacteria/ml, which in turn were used to make several 1/5 dilutions, as the volume of the challenge dose was to be 5ml. Thus, a series of seven suspensions were made for which the respective bacterial concentrations were estimated to be 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 Brucella ovis bacteria per 5ml dose.

Fifteen millilitre aliquots of the final suspension were dispensed into sterile Universal bottles, one bottle for every two animals which were to receive that dose. This arrangement allowed a 5ml margin of excess and permitted easy and accurate filling of the syringes.

As in previous experiments, duplicate viable counts were made using the challenge suspensions as a more accurate estimate of the actual concentration of viable bacteria. The plates were read after four days incubation at 37°C in an atmosphere of 10% CO_2.

Inoculation of Challenge Suspension

Ten weeks after the last vaccination procedure, 5ml of the appropriate challenge suspension was injected into the jugular vein of each ram, using a new sterile syringe and 20 gauge 25mm needle for each animal. The "spare" rams were not challenged.

Serology

Blood samples were collected from all the rams according to the schedule in Table 7.1. After challenge, only the unvaccinated control rams continued to be tested serologically. Samples were collected, processed and stored in the same manner as described previously (Chapter 3). The serum samples were sent to Wallaceville Animal Research Centre to be tested for antibody to Brucella ovis by the complement fixation method (CFT).

Bacteriology

Semen samples were collected from the rams according to the schedule in Table 7.1. Three sets of samples were collected from each mob, by which time ram numbers had significantly reduced because infected animals had been removed. It was decided to keep on testing the unvaccinated rams, as well as the vaccinated rams which had been challenged with the higher doses of Brucella ovis organisms, for a further two weeks. At ten weeks after challenge, therefore, all the unvaccinated rams and all the vaccinated rams that had received a challenge dose of 10^4 or higher were retested. At 11 weeks after challenge, all vaccinated rams which had received a challenge dose of 10^5 or higher were again retested along with the unvaccinated animals.

Semen was obtained by electroejaculation (Appendix 5). As soon as possible after collection, two blood agar plates for each ram were inoculated using a swab of the semen sample, and these plates were incubated at 37°C in 10% CO_2 for six days. They were examined daily, and any Brucella ovis colonies present were usually identifiable within three to four days. In cases where there were doubts as to the identity of the isolate, it was subcultured, and the pure growth subjected to microscopic examination until the identity was confirmed.

Semen Smears

Smears were made from a drop of each semen sample and stained by the Modified Ziehl-Neelsen method (Appendix 1). They were allowed to dry and then examined at high power using oil immersion microscopy (1,000 x magnification) for the presence of inflammatory cells and bacteria.

Palpation

At the same time as semen samples were collected, the scrotal contents of each ram were palpated and the presence of any abnormality recorded.

Ram Losses

Three weeks before challenge, two rams in the 2 i/p group were found dead. Post-mortem examination failed to reveal the cause of death. One animal was replaced by the "spare" ram for this group but the other could not be replaced, so there were only 34 rams in this group. It was decided to use only four rams in the group which was to receive the dose of approximately 10^8 bacteria, as a dose of approximately this magnitude had been used in a previous experiment (Chapter 6), so there was already some information about it.

After challenge, an outbreak of poisoning caused by the plant "Goats Rue" (Galega officinalis) claimed the lives of three more rams. Two of the rams were replaced by the "spare" animals in their respective groups, but the third, as it was from the 2 i/p group, could not be replaced. This meant that there were only four rams in the 2 i/p group that were challenged using a dose of approximately 10^3 bacteria, and that there were only 33 rams in the 2 i/p group in comparison with the 35 in each of the other three groups.

The two rams that were used as replacements for their poisoned counterparts were given the appropriate intravenous challenge dose of Brucella ovis organisms 48 hours after the others had received their challenge. A viable plate count of the suspension used to challenge these two rams revealed almost identical numbers of Brucella ovis organisms to the original plate count, and so these two individuals were not treated any differently in the subsequent analysis of results.

Statistical Methods

Chi-squared tests and 95% confidence limits were used for group comparisons. The Reed and Muench (1938) method of estimating 50% end-points and a "LOGIT" transformation were both used in the calculation of ID50 estimates. All these methods are described in Appendix 6. The analyses were performed with the help of the "MINITAB"[5] and "GENSTAT"[6] statistical packages on Massey University's "PRIME" computer.

Criterion for Judging Infection

A ram was judged to be "infected" when Brucella ovis bacteria could be cultured from the semen.

NOTES

1) "Panacur" - Coopers Animal Health N.Z. Ltd. Active constituent 25g/l Fenbendazole.

2) "Salvexin" - Coopers Animal Health N.Z. Ltd. Salmonella typhimurium and Salmonella bovismorbificans vaccine.

3) "Covax 5" - Tasvax Covax 5, Coopers Animal Health N.Z. Ltd. A combined clostridial vaccine consisting of purified toxoids of Clostridium perfringens (welchii) Type D, Clostridium septicum, Clostridium tetani, Clostridium novyi (oedomatiens), and a formol culture of Clostridium chauvoei.

4) "Ramovexin" - Coopers Animal Health N.Z. Ltd. An inactivated Brucella ovis saline-in-oil vaccine.

5) "MINITAB" - Release 82.1, copyright Pennsylvania State University 1982.

6) "GENSTAT" - Release 4.0413, copyright 1984 Lawes Agricultural Trust, Rothamsted Experimental Station.

Table 7.1

Schedule Of Experimental Procedures In A Trial Investigating The Response Of Vaccinated And Unvaccinated Rams To A Range Of Brucella ovis Challenge Doses

Week	Vaccinate	Collect Blood	Collect Semen
0	2 s/c and 2 i/p	2 s/c and 2 i/p	-
4	-	2 s/c and 2 i/p	-
8	2 s/c, 2 i/p and 1 i/p	1 i/p and control	-
15	-	All groups	-
18	DAY OF CHALLENGE	All groups	-
21	-	control, mob A	mob A
22	-	control, mob B	mob B
23	-	control, mob A	mob A
24	-	control, mob B	mob B
25	-	control, mob A	mob A
27	-	control, mob B	mob B
28	-	All controls	All
29	-	All controls	All

NB Mob A consisted of high challenge dose groups, Mob B of low challenge dose groups.

RESULTS

Viable Count

The final estimate of bacterial concentration of the challenge suspension, made on the basis of the viable count plates, was 1.23×10^8/5ml for the lowest dilution. Therefore each animal received approximately 1.23×10^2, 10^3, 10^4, 10^5, 10^6, 10^7 or 10^8 *Brucella ovis* bacteria, depending on the challenge dose group to which it had been allocated. This meant that the lowest challenge dose contained approximately 123 bacteria and the highest 123 million bacteria.

Serological Response to Vaccination

The serological results from rams vaccinated in different ways were consistent with the pattern seen in earlier experiments (Chapter 3, Chapter 6).

The rams vaccinated twice by the intraperitoneal technique had a slower rise in mean titre than those vaccinated twice subcutaneously (Figure 7.1).

The rams vaccinated once by the intraperitoneal technique had a significantly lower average titre than the other two vaccinated groups when samples were tested at 15 weeks and at the time of challenge at 18 weeks.

It took longer for all the rams in the 2 i/p group to become positive to the complement fixation test than it did for those in the 2 s/c group (Figure 7.2). By the time that the rams were challenged, at 18 weeks, all the rams in the 2 s/c and the 2 i/p groups had a positive reaction to the complement fixation test, but one of the rams in the 1 i/p group was still negative (Figure 7.2).

Figure 7.1

Mean complement fixation titre scores of rams following the administration of Brucella ovis vaccine either twice by the subcutaneous route, or once or twice by the intraperitoneal technique.

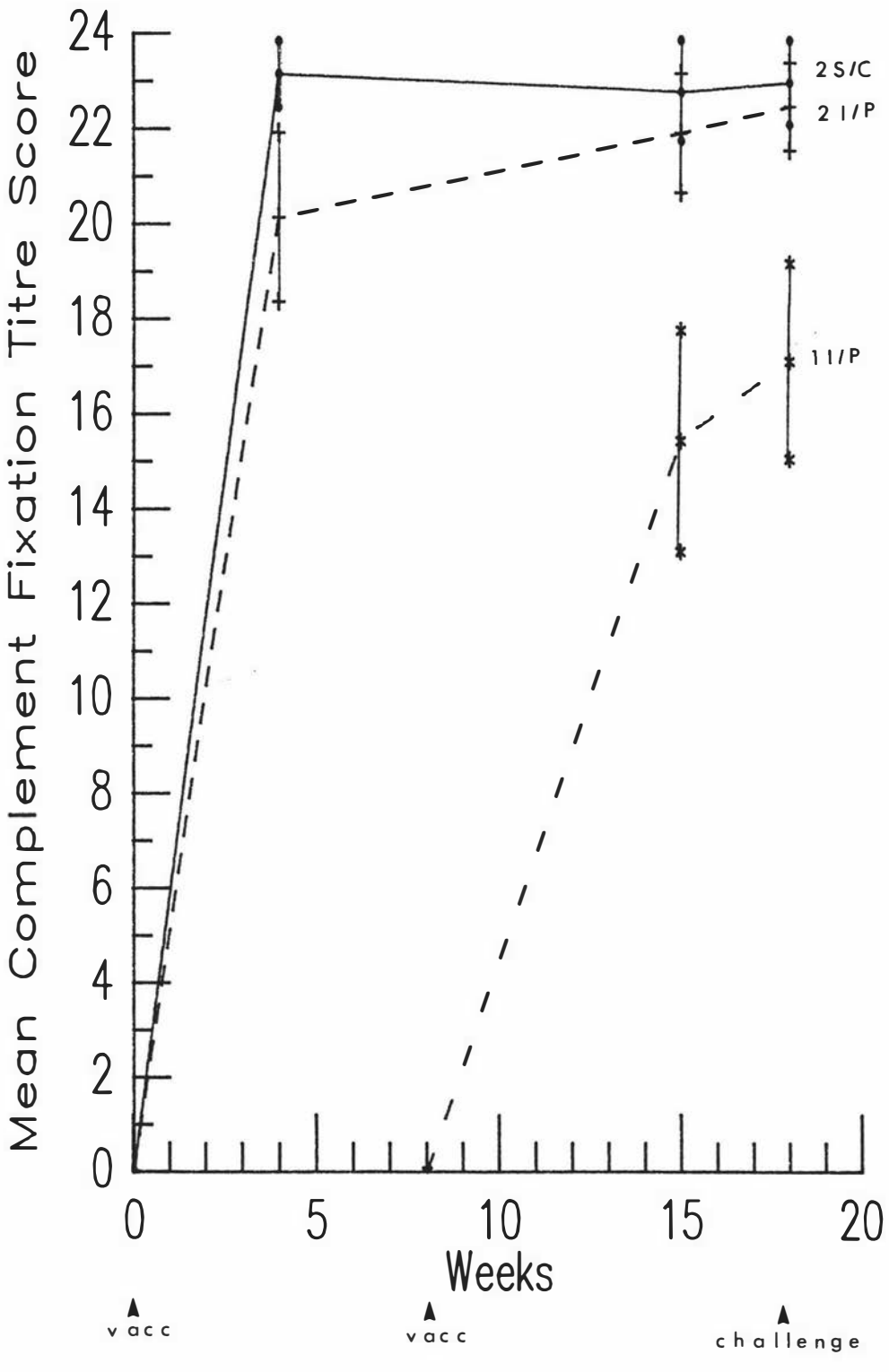
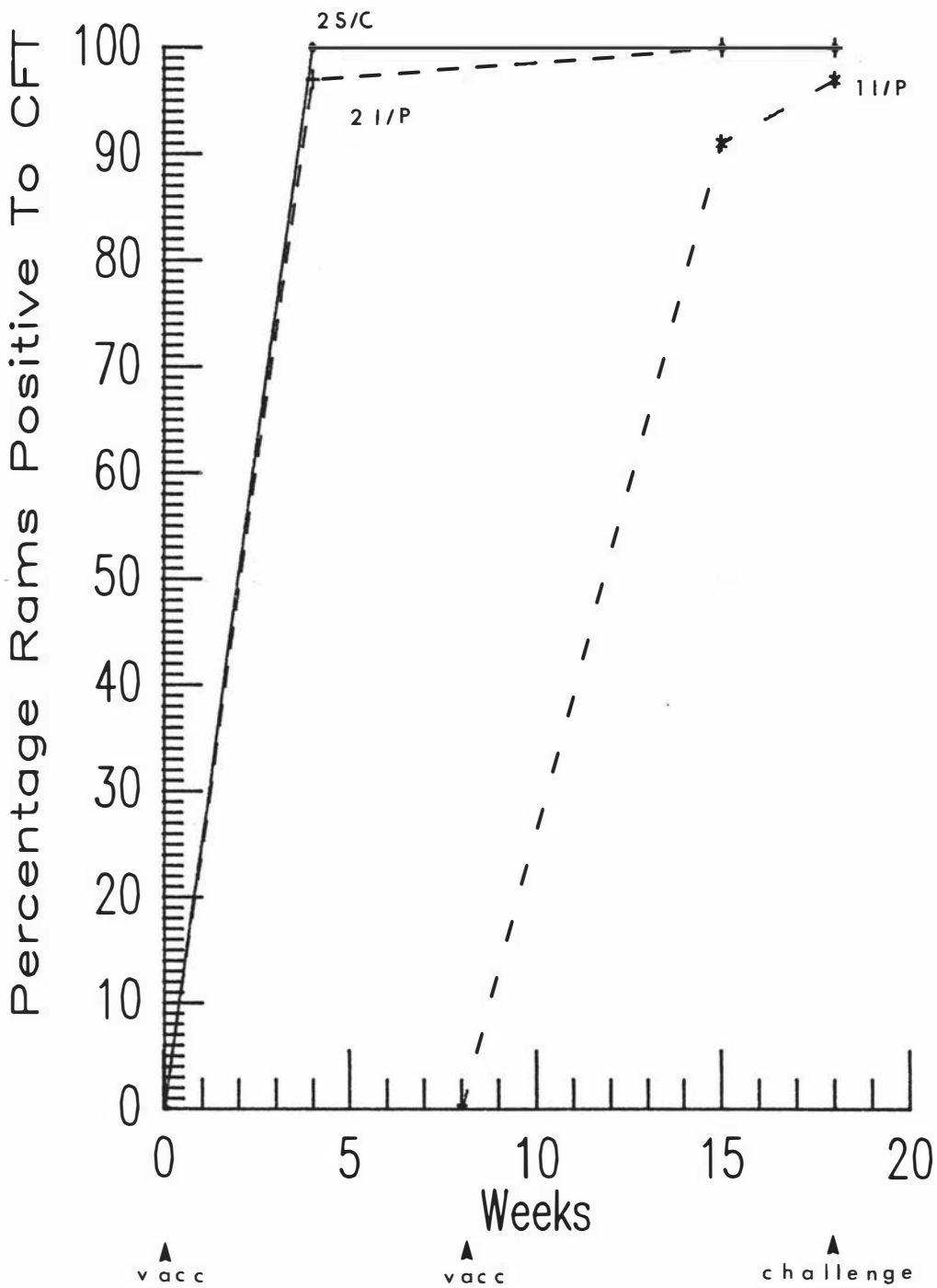

Mean Complement Fixation Titre Score
24
22
20
18
16
14
12
10
8
6
4
2
0
0
5
10
15
20
Weeks
2 S/C
2 I/P
1 I/P
vacc
vacc
challenge

Figure 7.2

Percentage of rams having a positive reaction to the complement fixation test following the administration of *Brucella ovis* vaccine either twice by the subcutaneous route, or once or twice by the intraperitoneal technique.


Percentage Rams Positive To CFT
100
90
80
70
60
50
40
30
20
10
0
2 S/C
2 I/P
1 I/P
0
5
10
15
20
Weeks
vacc
vacc
challenge

Serological Response to Challenge

The proportion of rams in the control group which had a positive reaction to the complement fixation test (Figure 7.3) was calculated on a cumulative basis to account for the removal of infected rams. Twenty out of 35 (57%) of the unvaccinated rams developed a positive complement fixation titre. All but two of these rams were excreting Brucella ovis in their semen by the end of the experiment.

The number of unvaccinated rams developing titres increased as the challenge dose increased (Table 7.2).

The length of time that elapsed between challenge and the development of a positive complement fixation titre in unvaccinated rams tended to reduce as the number of bacteria in the challenge dose increased (Table 7.2).

Therefore, as the dose of infective bacteria increased, the percentage of animals that developed positive complement fixation titres also increased, and the period between challenge and subsequent seroconversion decreased.

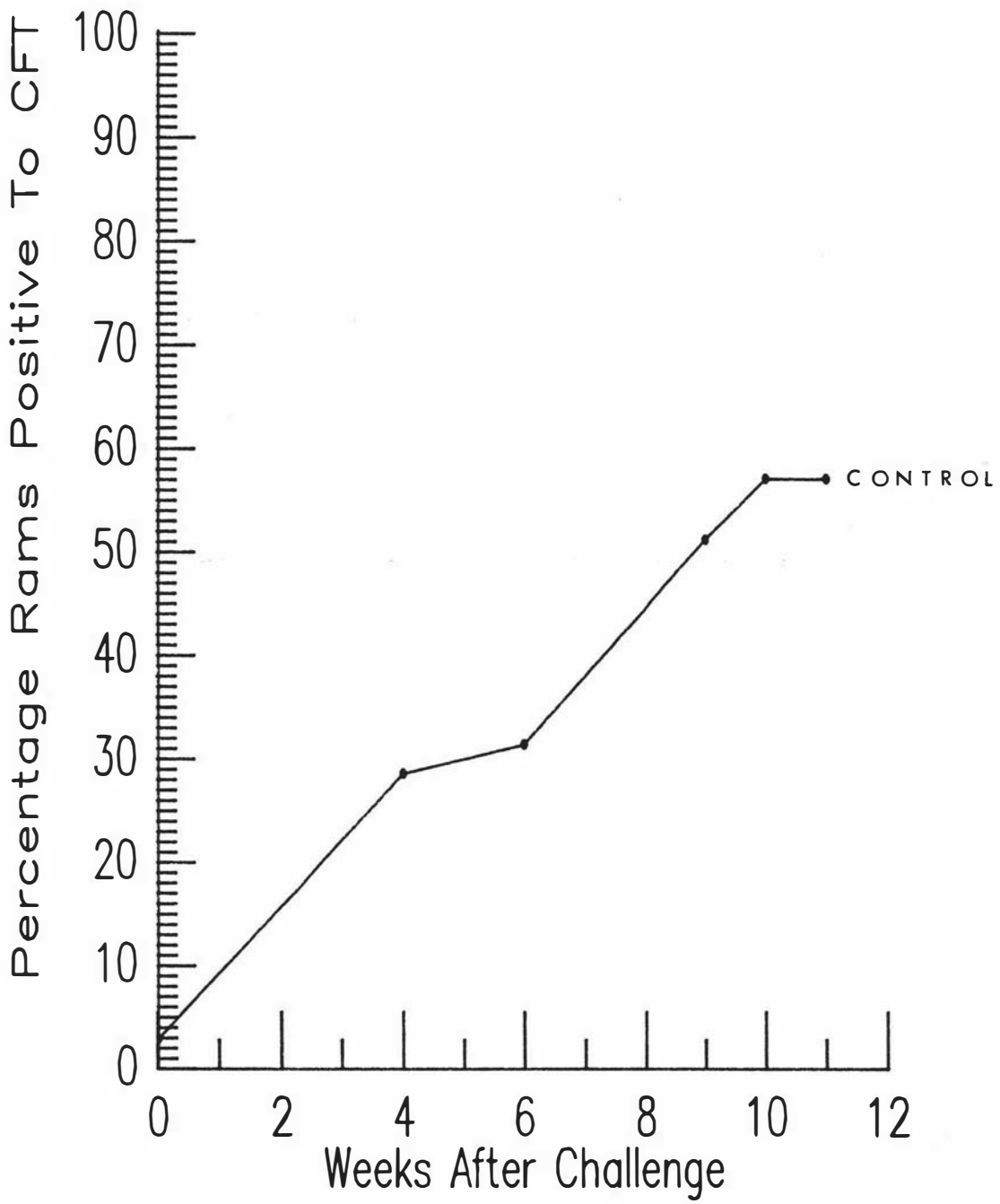
Table 7.2

Number Of Unvaccinated Rams Which Developed A Positive Reaction To The Brucella ovis Complement Fixation Test After The Administration Of Intravenous Challenge Doses Of Varying Magnitude

Challenge Dose (Approximate Number of Brucella ovis Bacteria)	Weeks After Challenge 4	6	9	10	Total
1.23×10^3	0	0	1	1	1 (out of 5)
1.23×10^4	0	0	2	2	2 (out of 5)
1.23×10^5	1	1	2	3	3 (out of 5)
1.23×10^6	1	2	3	4	4 (out of 5)
1.23×10^7	3	3	5	5	5 (out of 5)
1.23×10^8	5	5	5	5	5 (out of 5)
Total	10	11	18	20	20 (out of 35)

Figure 7.3

Percentage of unvaccinated rams having a positive reaction to the complement fixation test following the administration of intravenous Brucella ovis challenge doses of varying magnitude.


Percentage Rams Positive To CFT
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
Weeks After Challenge
CONTROL

Bacteriology

Of a total of 138 rams which were challenged, 38 (approximately 28%) excreted Brucella ovis in their semen. More of the rams which had received a high challenge dose became infected than those which had received a low dose (Table 7.3). The unvaccinated control group had a greater proportion of rams succumbing to infection than any of the vaccinated groups. This difference was significant at the 5% level ($P<0.05$) for the groups vaccinated by the intraperitoneal technique (1 i/p and 2 i/p) and significant at the 0.1% level ($P<0.001$) for the group vaccinated twice by the subcutaneous method (2 s/c).

There were no significant differences between any of the three vaccinated groups in the total number of rams that became infected after challenge.

As would be expected, there was a significant trend of increasing numbers of animals becoming infected as the challenge dose was increased (Table 7.3). This was true both for the total number of animals challenged at each dose, and for each individual treatment group.

At a dose of 1.23×10^7 Brucella ovis bacteria, all five unvaccinated rams in the control group became infected, but none of the five rams in the 2 s/c group challenged at this dose succumbed to infection. This difference was significant at the 5% level ($P<0.05$), and was the only significant difference between treatment groups for animals challenged at the same dose. It was difficult to demonstrate significance in this type of comparison because of the extremely small numbers of animals involved (only four or five in each group).

Generally, the greater the bacterial concentration of the challenge suspension, the sooner the rams began to excrete Brucella ovis in their semen (Figure 7.4). It was also noted that unvaccinated rams were the first to begin excreting Brucella ovis in their semen. Next, those rams vaccinated by the intraperitoneal technique began to excrete the organism, and then finally those

vaccinated twice by the subcutaneous route (Figure 7.5).

The rams vaccinated twice by the subcutaneous method required the highest challenge dose before they began to succumb to infection, and the unvaccinated rams were susceptible to the lowest "minimum infective dose" (Figure 7.6).

The first isolation of Brucella ovis was from a semen sample collected just three weeks after challenge. The greatest number of new cases (16) was recorded seven weeks after challenge, and at the last test, 11 weeks after challenge, no new cases of infection were detected (Figure 7.7).

Table 7.3

Number Of Rams Which Became Infected In Each Treatment Group After The Administration Of Intravenous *Brucella ovis* Challenge Doses Of Varying Magnitude

Challenge Dose (Approximate Number of *Brucella ovis* Bacteria)	Treatment Group				Total Infected (%)	Total Number Challenged
	2 s/c	2 i/p	1 i/p	control		
1.23×10^2	0	0	0	0	0 (0%)	20
1.23×10^3	0	0 *	0	1	1 (5%)	19
1.23×10^4	0	0	0	2	2 (10%)	20
1.23×10^5	0	0	0	2	2 (10%)	20
1.23×10^6	0	1	1	3	5 (25%)	20
1.23×10^7	0	2	4	5	11 (55%)	20
1.23×10^8	4	4 *	4	5	17 (90%)	19
Total Infected	4	7	9	18	38	138
Percentage Infected	11%	21%	26%	51%	28%	
Number Challenged	35	33	35	35	138	

NB "*" - indicates only 4 animals in this group (otherwise 5).

Figure 7.4

Time (in weeks) following the administration of intravenous Brucella ovis challenge doses of varying magnitude before the first isolation of Brucella ovis from the semen of vaccinated and unvaccinated rams.

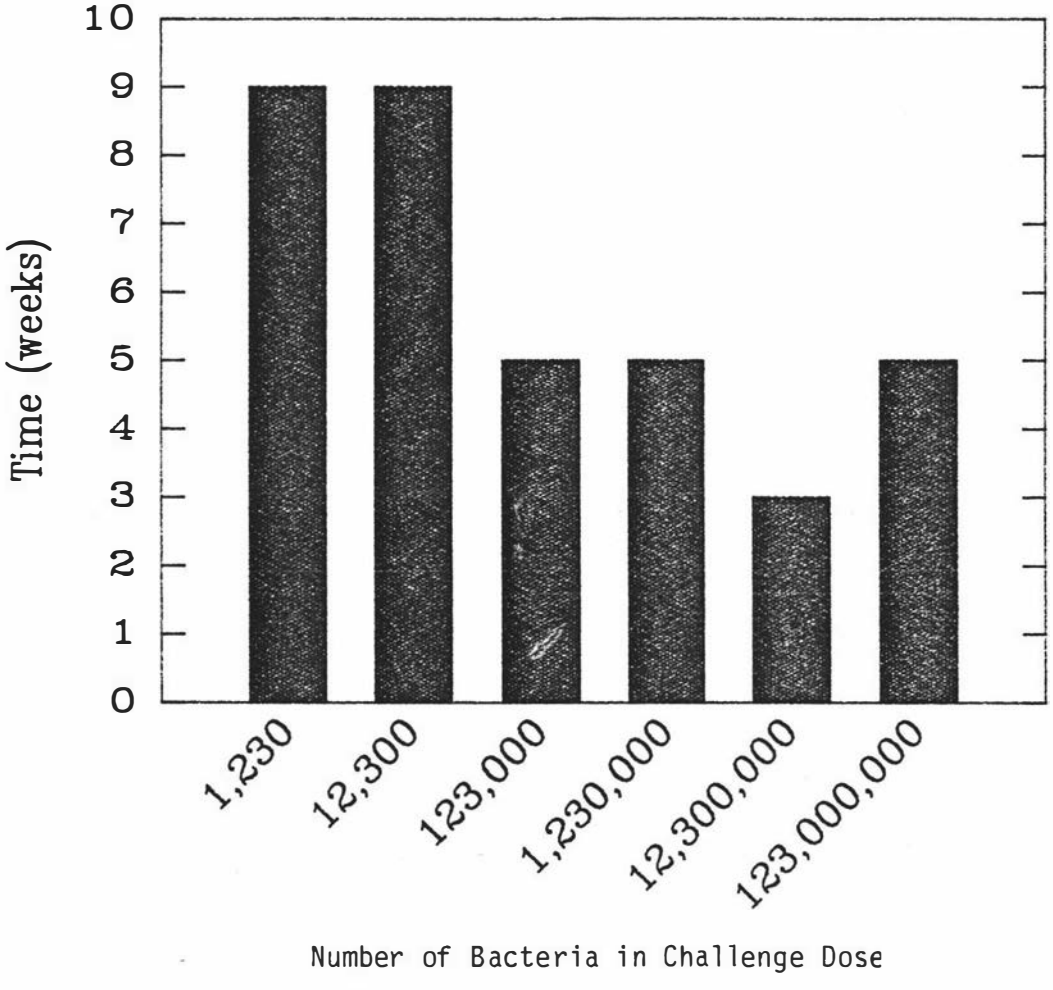

Time (weeks)
0
1
2
3
4
5
6
7
8
9
10
1,230
12,300
123,000
1,230,000
12,300,000
123,000,000
Number of Bacteria in Challenge Dose

Figure 7.5

Time (in weeks) following the administration of intravenous Brucella ovis challenge doses of varying magnitude before the first isolation of Brucella ovis from the semen of unvaccinated rams or rams vaccinated either once or twice by the intraperitoneal technique or twice by the subcutaneous method.

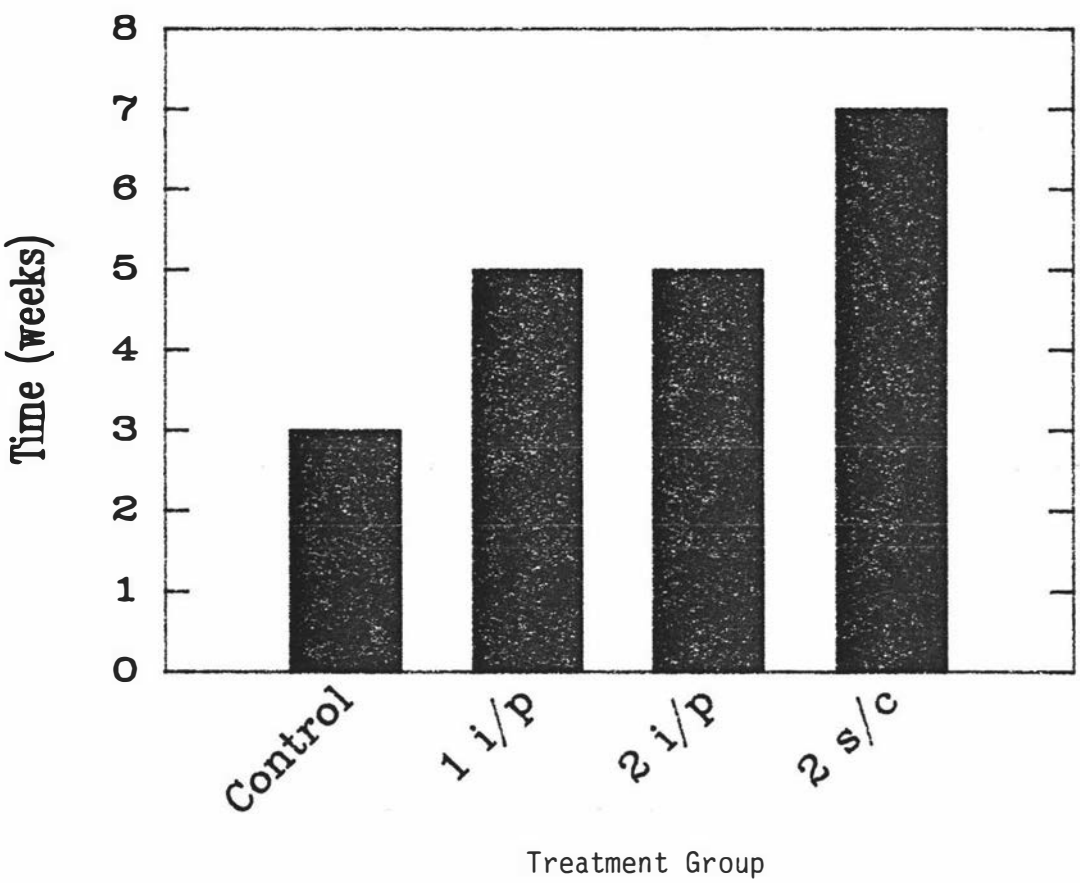

Time (weeks)
0
1
2
3
4
5
6
7
8
Control
1 i/p
2 i/p
2 s/c
Treatment Group

Figure 7.6

Lowest dose of intravenous Brucella ovis organisms which caused infection in unvaccinated rams, rams vaccinated once or twice by the intraperitoneal technique, and rams vaccinated twice by the subcutaneous method.

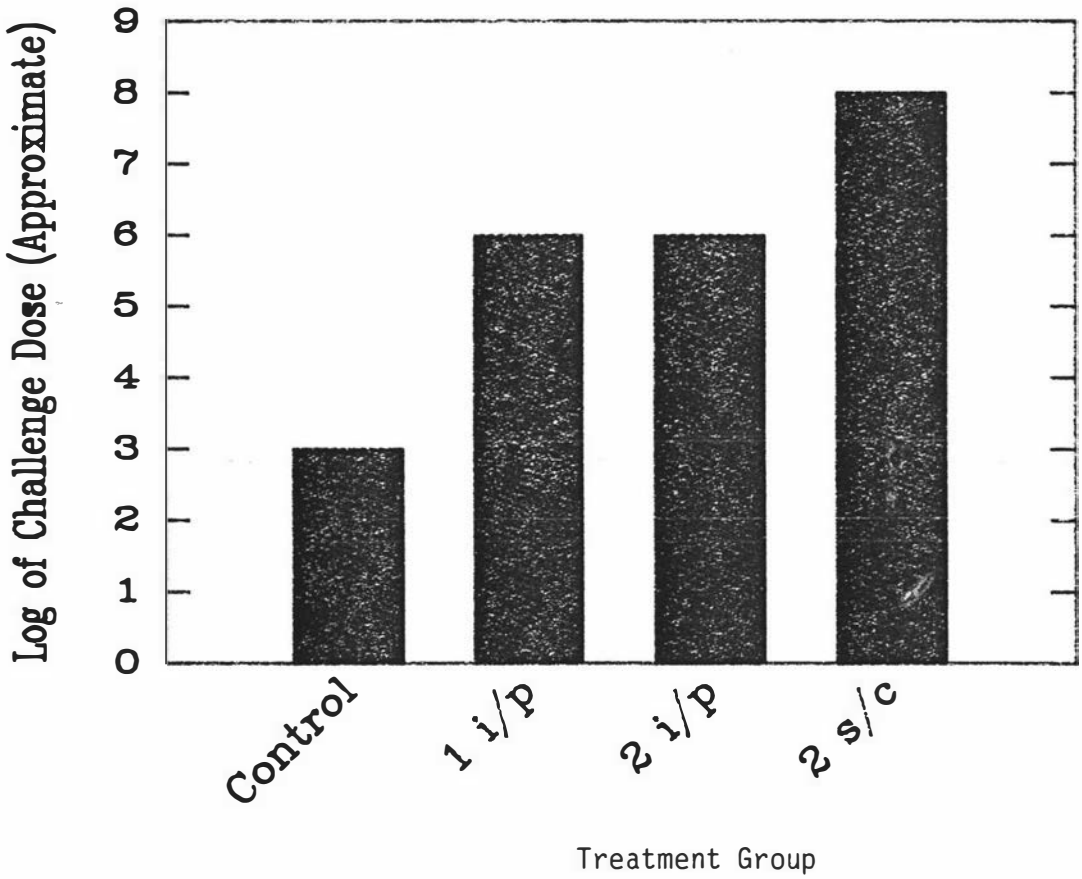
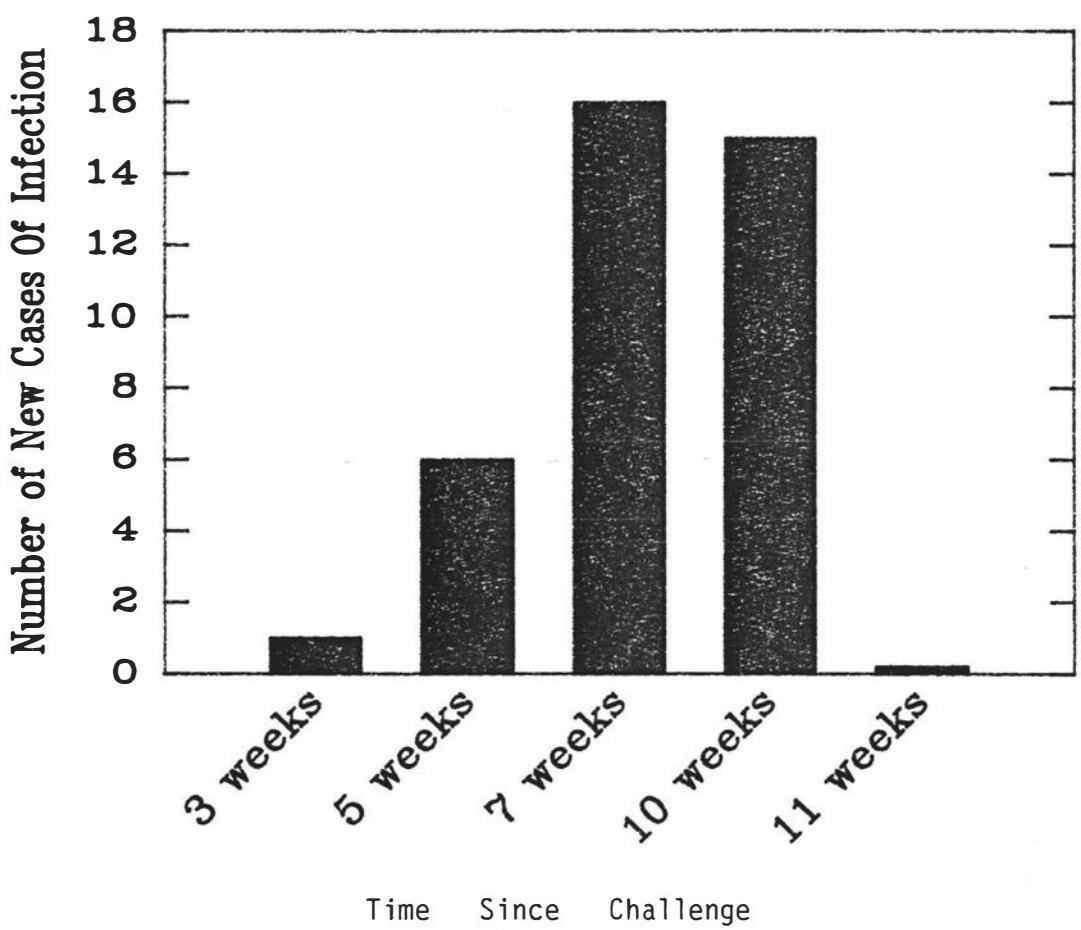

Log of Challenge Dose (Approximate)
0
1
2
3
4
5
6
7
8
9
Control
1 i/p
2 i/p
2 s/c
Treatment Group

Figure 7.7

Number of new cases of Brucella ovis infection detected at various times after the administration of a range of intravenous challenge doses of Brucella ovis organisms.


Number of New Cases Of Infection
18
16
14
12
10
8
6
4
2
0
3 weeks
5 weeks
7 weeks
10 weeks
11 weeks
Time Since Challenge

ID50 Estimates

Using the method of Reed and Muench (1938), the estimates of the ID50 for each of the treatment groups were as follows:

Unvaccinated	(control)	–	9.1×10^4 bacteria
One intraperitoneal vaccination	(1 i/p)	–	5.3×10^6 bacteria
Two intraperitoneal vaccinations	(2 i/p)	–	8.9×10^6 bacteria
Two subcutaneous vaccinations	(2 s/c)	–	5.2×10^7 bacteria

The estimate of the dose that would be required to infect 50% of rams vaccinated once by the intraperitoneal technique was 58 times greater than that for unvaccinated animals. The ID50 of rams vaccinated twice by the intraperitoneal technique was 98 times greater, and that of rams vaccinated twice by the subcutaneous method 571 times greater than the "standard ID50" of these unvaccinated animals.

As there was no simple method of assessing the accuracy of the estimates obtained by this method, it was decided to use an additional statistical method which would allow 95% confidence limits to be placed on the resultant estimates of the ID50.

A "Logit" transformation (Nelder and Wedderburn, 1972) was applied to the experimental data. This was accomplished using the "Genstat" statistical package on the "Prime" computer at Massey University.

Briefly, the package fits a line to the transformed data, and the ID50 estimate is read from the point on the line corresponding to a 50% infection rate. When separate lines were fitted for each of the four treatment groups, (i.e. the lines for each treatment group were not assumed to be parallel), the line was found to have a better "fit" to the relevant data than when all the data was used to form a line of one gradient (parallelism assumed), which then, using different

intercepts, gave estimates for all of the groups.

Unfortunately, as there were only two data points for the group vaccinated twice subcutaneously, no separate line could be fitted for this group, and in order to obtain an estimate of the ID50 of this group, a combined regression line had to be used. When this was done, the ID50 estimates with their 95% confidence limits were as shown in Table 7.4.

There were no statistically significant differences among the three vaccinated groups, but all of the three vaccinated groups had significantly higher ID50 estimates than the unvaccinated control group.

The ID50 estimates obtained by the Reed and Muench method all fell between the corresponding estimate and lower 95% confidence limit calculated by the Logit method, indicating that these two methods of estimating the ID50 give comparable results.

It was possible to increase the amount of data input for the ID50 estimations by using the information generated from the previous challenge trial (Chapter 6). Although the challenge dose in the previous trial (1.63×10^8) had been slightly different to the corresponding dose in this trial (1.23×10^8), this additional dose rate could be easily incorporated into the programme, so that information from eight different challenge doses could be used in making the ID50 estimates. This increased the number of animals in the analysis to 178, 45 from each of the control, 2 s/c and 1 i/p groups, and 43 from the 2 i/p group. The ID50 estimates and 95% confidence limits that resulted from the analysis of this enlarged set of data are shown in Table 7.5

When these figures are compared to those in Table 7.4, it can be seen that the estimates resulting from the larger set of data have smaller confidence intervals, indicating that the estimates themselves are more accurate. This is because of the larger number of animals involved in the estimates.

Therefore, the best estimates that can be made of the ID50s from the information available in this project are those shown in Table 7.5.

In terms of the number of "standard ID50" units (i.e. the ID50 estimate of the control group), the vaccinated groups had ID50s that were estimated to be as follows:

1 i/p group - 71 x "standard ID50" (i.e. 71 x control)

2 i/p group - 158 x "standard ID50"

2 s/c group - 716 x "standard ID50"

Therefore, although the differences between the vaccinated groups could not be demonstrated to be statistically significant, it can be suggested that the double intraperitoneal technique may be twice as effective as the single intraperitoneal technique, and that using two doses of vaccine administered by the subcutaneous method may be ten times more effective than using a single dose of vaccine administered by the intraperitoneal technique.

Whichever method of vaccination was used, it significantly increased the dose that would be needed to infect 50% of the animals above that which would have been required had the animals not been vaccinated at all. The magnitude of this increase, however, depended on the method of vaccination that was used.

Table 7.4

Brucella ovis ID50 Estimates And Their 95% Confidence Limits For Unvaccinated Rams And For Rams Vaccinated Either Once By The Intraperitoneal Method, Twice By The Intraperitoneal Method Or Twice By The Subcutaneous Technique Estimated By The LOGIT Transformation.

Treatment Group	Lower 95% Confidence Limit	ID50	Upper 95% Confidence Limit
control	1.7×10^4	9.8×10^4	5.4×10^5
1 i/p	1.2×10^6	6.6×10^6	4.1×10^7
2 i/p	2.0×10^6	1.2×10^7	8.1×10^7
2 s/c	1.5×10^7	9.4×10^7	9.0×10^8

Table 7.5

Brucella ovis ID50 Estimates And Their 95% Confidence Limits For Unvaccinated Rams And For Rams Vaccinated Either Once By The Intraperitoneal Method, Twice By The Intraperitoneal Method Or Twice By The Subcutaneous Technique Estimated By The LOGIT Transformation.

Treatment Group	Lower 95% Confidence Limit	ID50	Upper 95% Confidence Limit
control	1.8×10^4	9.5×10^4	4.9×10^5
1 i/p	1.4×10^6	6.7×10^6	2.9×10^7
2 i/p	3.2×10^6	1.5×10^7	6.2×10^7
2 s/c	1.8×10^7	6.8×10^7	2.7×10^8

Palpation

Of the 138 rams challenged, 25 (18%) developed epididymitis. Two of the rams which developed lesions did not excrete Brucella ovis in their semen. Of the 38 rams that excreted the organism therefore, 23 (61%) developed epididymitis. The problems of interpreting the findings of a physical examination of the genitalia have been discussed previously (Chapter 6). In each of the four treatment groups, the first lesions were detected seven weeks after challenge. The magnitude of the challenge dose did not appear to have any significant effect on the proportion of infected rams which developed lesions of epididymitis.

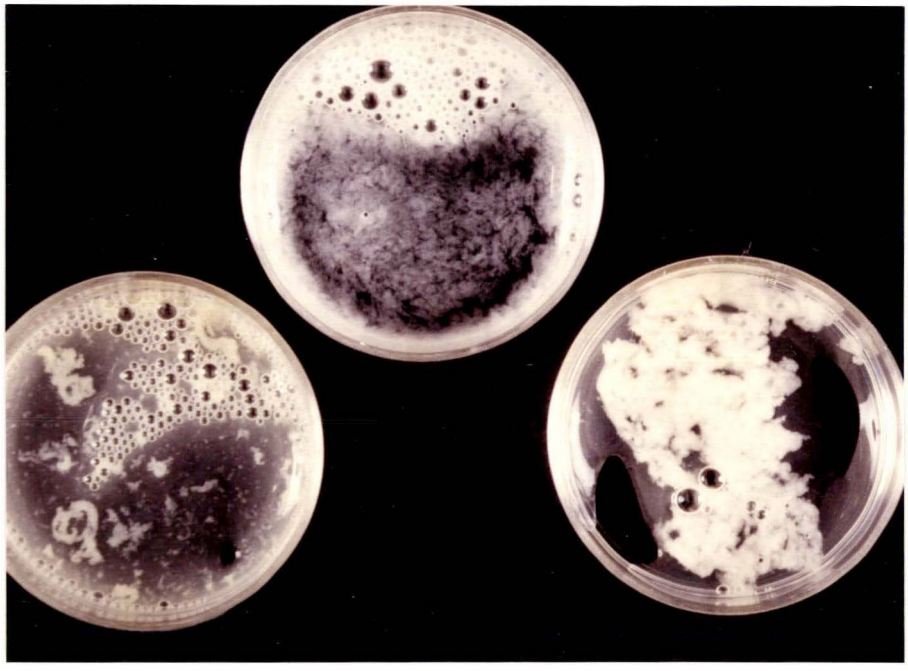
Semen Smears

Of the 138 rams that were challenged, 32 (23%) had acid-fast bacteria detected in their semen on at least one occasion after challenge. Brucella ovis was isolated from 40 semen samples originating from 38 rams. Of these 40 samples, 33 (82.5%) were found to contain acid-fast bacteria during microscopic examination of a Modified Ziehl-Neelsen stained semen smear.

The semen of infected rams, in contrast to the creamy appearance of normal semen, was often a thin, slightly cloudy, colourless fluid containing clots of purulent exudate (Plate 7.1).

Plate 7.1

Semen samples from rams challenged by the intravenous administration of Brucella ovis bacteria. The sample at the top of the photograph shows the normal appearance of semen from an uninfected ram. The moving spermatozoa make patterns form in the liquid. The two samples below are from infected rams, and consist of purulent exudate suspended in a cloudy fluid composed mainly of accessory sex gland secretions.



DISCUSSION

In the previous challenge trial (Chapter 6), no significant increase in resistance due to vaccination could be demonstrated in rams challenged using 1.63×10^8 Brucella ovis organisms administered by the intravenous route. The only difference observed in the vaccinated animals was an increase in the "incubation period" before excretion of bacteria in the semen began. In this present experiment, however, approximately 51% of the non-vaccinated animals became infected after challenge, but only 19% of the vaccinates. This difference was highly significant ($P<0.001$). This reaffirmed that the vaccine has a significant beneficial effect, removing some of the doubts raised by the findings of the previous experiment (Chapter 6).

Serological Response to Vaccination

Following vaccination, the mean complement fixation titres of rams vaccinated in different ways developed in exactly the same pattern as had been seen in all the other trials involving vaccination (Chapter 3, Chapter 6). There did not appear to be any obvious relationship between the serological titre of an individual ram at the time of challenge and the likelihood of subsequent infection. Some rams having the maximum titre score of 24 became infected, and some rams having very low titres when challenged resisted infection. Possible reasons for this inconsistency have been discussed previously (Chapter 6).

Serological Response to Challenge

All the unvaccinated rams which became infected developed a positive reaction to the complement fixation test after challenge. These titres persisted for the duration of the experiment. There were two rams that developed a positive reaction to the test but never excreted Brucella ovis in their semen. Both of these rams remained negative to the complement fixation test until ten weeks after challenge, when one had a titre of 4^2 and the other a titre of 4^3 to

the complement fixation test; reactions which had developed later than those of all the other rams which became positive. There is the possibility that these reactions could have been the result of natural exposure to infected rams, rather than being the direct result of experimental challenge. At the next test, 11 weeks after challenge, one of these two rams still had a positive titre of 4^3, but the other had returned to negative status. This type of transient serological response was also seen in one of the rams in the pilot trial (Chapter 5) which also never excreted Brucella ovis in the semen. This phenomenon, which was also noted by Burgess et.al. (1982) in flocks of naturally infected rams in Australia, was discussed previously (Chapter 5).

The failure to isolate Brucella ovis from the semen in both the rams in this project that displayed this type of transient serological reaction supports the hypothesis that these rams do not become infected, excrete the organism, and subsequently recover, but merely have a transient antibody response when briefly exposed to bacteria which fail to establish an infection.

The other possibility that must be considered is that these transient responses might be false positive reactions to the test. Although there were three false positive results in this trial from unvaccinated rams prior to challenge, this explanation for the phenomenon described is unlikely.

There was considerable variation in the time it took for individual unvaccinated rams to become positive to the complement fixation test after challenge. Many, especially those in the high challenge dose groups, became positive to the test within four weeks of challenge. All the unvaccinated rams which had Brucella ovis isolated from their semen had a positive reaction to the test by nine weeks after challenge. This has implications for accreditation programmes based on the Brucella ovis complement fixation test. The requirement of the programme currently in use in New Zealand that tests to qualify for accreditation should be performed no less than eight weeks apart, is based on the commonly held premise that the incubation period of the disease is approximately eight weeks. From

the results of this experiment, it appears that eight weeks would encompass the incubation period in the majority of rams, but a small proportion may take slightly longer to seroconvert. Rams such as these may be the cause of a small number of "breakdowns" in accreditation status.

Diagnosis of Infection

By the time Brucella ovis was isolated from their semen, all the unvaccinated rams had developed a positive reaction to the complement fixation test, confirming the high sensitivity of this serological test.

Examination of smears detected infection in 82.5% of semen samples from which Brucella ovis was subsequently isolated. All of the samples that had Brucella ovis detected in the stained smear had the organism isolated on culture, confirming the superiority of culture over smears as a diagnostic technique.

Frequently, infected animals delivered poor quality semen samples consisting mainly of clear fluid from accessory sex glands. These also sometimes contained clots of purulent material. This type of sample made it difficult to produce a good quality smear of the correct density, and the resultant slides were of limited diagnostic value. Any bacteria present were reduced to such a low concentration that they were almost impossible to find on microscopic examination. This was a major cause of negative results in the smear test of semen samples from which Brucella ovis was isolated.

Of the semen samples from which Brucella ovis was isolated, only 45% came from rams which had epididymitis at the same time. Of the rams that excreted the organism, only 61% developed epididymitis during the experiment. This reemphasises the danger of relying solely on palpation as a basis for the diagnosis of infection in individual animals.

The best methods of diagnosing infection, therefore, were the culture of semen and the complement fixation test. Direct microscopic examination of semen smears alone can be expected to underestimate the number of animals excreting the organism by about 20%, and a single physical examination of the genitalia may underestimate it by up to 55%.

The culture of semen samples, although labour-intensive, is a good diagnostic technique provided the quality of the samples is good. In the early stages of infection, large numbers of bacteria are present in the semen, and a positive diagnosis can be obtained within four days of the sample having been collected. However, a negative result is more difficult to interpret, and a dirty sample resulting in contaminated plates gives no information at all. Intermittent shedding of the bacteria is known to occur in chronic cases (Worthington et.al., 1985), and some infected animals might not be detected by the culture of a single semen sample.

The fact that some of the rams began to shed Brucella ovis bacteria in their semen within three weeks of being challenged has some interesting implications for the application of accreditation and eradication programmes. In this experiment the challenge was given by the intravenous route, and infection by natural means would probably have a longer incubation period. It is nevertheless clear that to eradicate the disease from infected flocks, frequent testing at intervals of two to three weeks is very desirable.

There were some animals which did not begin to excrete the organism until ten weeks after inoculation. This indicates that the commonly accepted eight-week incubation period of the disease may be exceeded in some cases. This may be significant for accredited flocks introducing new rams. An eight week "quarantine" period during which the new stock are isolated from other animals may in some cases be insufficient. There is the possibility that although the rams are free of serological and bacteriological evidence of the disease at the end of this period, they may subsequently become excretors, reinfecting the flock to which they are then introduced.

Effect of Challenge Dose

The total number of Brucella ovis bacteria to which an animal was exposed influenced the outcome of the challenge in several ways. In regard to the serological response, the greater the number of bacteria in the challenge dose, the greater the proportion of animals which developed a positive reaction to the complement fixation test. A higher challenge dose also reduced the time it took for the animals to develop a positive titre after challenge.

When considering infection rates, it was noted, not surprisingly, that more animals became infected at the higher challenge doses. Also, the time between challenge and the isolation of Brucella ovis from the semen was reduced as the challenge dose increased.

In the natural situation therefore, the variability seen in the incubation period of the disease, and in the morbidity in different flocks could be explained, at least in part, by differences in the "severity" of challenge to which the animals have been exposed.

Effect of Vaccination

It was demonstrated in the previous experiment (Chapter 6) that prior vaccination delayed the onset of bacterial excretion in the semen after challenge. This effect was confirmed by the results of this experiment. The first isolation of Brucella ovis from an unvaccinated ram was made three weeks after challenge, whereas it was five weeks before the first isolation was made from the rams vaccinated by the intraperitoneal technique, and seven weeks before infection was confirmed in the rams vaccinated by the subcutaneous route. Possible reasons for this effect were discussed in Chapter 6.

Vaccination also reduced the numbers of rams succumbing to infection. Although the small numbers of animals involved made it difficult to show statistical significance, it was strongly suggested that the efficacy of the subcutaneous route was greater than that of

the intraperitoneal method, and that two doses of vaccine, at least when administered by the intraperitoneal method, were more effective than one dose given by that route.

Minimum Infective Dose

For unvaccinated animals, the minimum intravenous infective dose of Brucella ovis was remarkably low. A mere 1,230 bacteria were sufficient to cause infection in one animal.

Vaccination appeared to raise this minimum infective dose considerably. When one or two doses of vaccine were given by the intraperitoneal technique, rams did not become infected until a challenge dose of 1.23 million bacteria were used.

Two doses of vaccine administered by the subcutaneous route raised this threshold dose to 123 million bacteria.

ID50

It is common for the efficacy of a commercially produced vaccine to be expressed in terms of the number of standard ID50s or LD50s which need to be used before vaccinated animals will succumb to challenge. The results of this experiment allow the efficacy of the Brucella ovis vaccine to be expressed in this way. In the groups of rams vaccinated either once or twice by the intraperitoneal technique, the most susceptible animal to become infected did so at a challenge dose equivalent to nearly 13 times the estimated ID50 of the unvaccinated animals (i.e. the standard ID50). No animals were infected at the nearest lower dose, so it could be said that one or two doses of Brucella ovis vaccine administered by the intraperitoneal technique protected all animals up to a dose of approximately 1.3 ID50s. This does not appear to be a very high level of protection.

For rams vaccinated twice by the subcutaneous route, a dose of 1,300 ID50s was required before any infection occured. Thus, it could be said that two doses of vaccine given by the subcutaneous route protected animals up to a dose of approximately 130 ID50s.

A similar picture of the comparative effectiveness of the three different vaccination techniques was revealed when the ID50 estimates of the groups were expressed in terms of standard ID50s.

Using the upper confidence limit of the "standard ID50" of unvaccinated rams, and the lower confidence limits of the ID50 estimates for each of the three vaccinated groups, the most conservative estimates of the effects of each vaccination regime can be calculated. These indicate that one dose of vaccine administered by the intraperitoneal technique raises the ID50 by at least three times, two intraperitoneal doses raise it by at least seven times, and two subcutaneous doses raise it by at least 37 times that of unvaccinated animals.

These differences between the various methods of vaccination could not be shown to be statistically significant. However, when the total percentages of rams to become infected in each treatment group were compared, the difference between the 2 s/c group and the controls was found to be much more statistically significant ($P<0.001$) than the difference between either of the i/p groups and the controls ($P<0.05$). The relative efficacy of the different methods of vaccination was reflected in the consistent pattern of serological differences demonstrated in both the serological investigation (Chapter 3) and the previous challenge trial (Chapter 6) as well as this experiment. This uniform consistency strongly supports the hypothesis that real differences exist between the various techniques.

The reasons for the lack of statistical significance can probably be explained by the low numbers of animals contributing to the ID50 estimates, and the wide range of challenge doses that had to be used because of a lack of any prior estimates on where the 50% end point was likely to lie. This meant that large numbers of vaccinated animals in the lower challenge dose groups were "wasted", as not

becoming infected, they contributed little to the estimation of the ID50. Now that the approximate ID50 of the various groups is known, it would be easier to get a more accurate estimate by testing larger numbers of animals over a small range of challenge doses lying close on either side of the estimated ID50. If this was done, a statistically significant difference in the ID50s of rams vaccinated in different ways would in all probability be demonstrated.

Challenge Dose in Natural Transmission

An obvious question to be addressed when considering the infective dose of organisms to be used in an experimental situation is: what numbers of bacteria are involved in natural challenge? As the bacteria are excreted in semen, and as natural transmission occurs by direct contact (Brown et.al., 1973), venereal transmission through homosexual activity is commonly accepted to be the most likely method of ram-to-ram infection. This type of activity between rams was frequently seen to occur spontaneously in the paddocks among the animals in this experiment. This being so, several factors would therefore be expected to have an effect on the number of bacteria involved in natural exposure. These include: the concentration of bacteria in the semen, which is affected by the duration of infection and the phenomenon of intermittent excretion; the volume of the ejaculate; the frequency of homosexual contact; the permeability of the rectal mucosa; and the nature of the rectal contents.

It would seem reasonable to assume that a much larger number of bacteria would be required to achieve infection by this route than were needed when the bacteria were placed directly into the blood stream, as was the case in this experiment.

To establish some information concerning semen as a source of infective bacteria, a viable count of Brucella ovis organisms was carried out on a semen sample obtained by electroejaculation from one of the unvaccinated rams in this trial nine weeks after challenge. This ram, in the early stages of an acute infection, was found to be shedding approximately 1×10^9 Brucella ovis bacteria in each

millilitre of semen. As the average volume of a ram's ejaculate collected by electroejaculation is 2-3ml (Gunn et.al., 1942), one episode of homosexual contact could deposit up to at least 3×10^9 Brucella ovis bacteria or, expressed another way, 31,579 "standard ID50s" in the rectum of a susceptible ram.

Buddle (1958) found that none of the methods of vaccination against Brucella ovis infection that he tested under natural challenge conditions was 100% effective in preventing infection. The status of the currently used vaccine in this respect is unknown but some projection can be made by considering the most resistant rams in this experiment. The group of rams vaccinated twice by the subcutaneous route had an estimated ID50 equivalent to 716 "standard ID50s". Even allowing for the large proportion of bacteria that would be prevented from reaching the blood stream by the physical barrier of the rectal mucosa, the possibility still exists that, in certain circumstances, sufficient challenge could be experienced from natural transmission as to cause infection even in vaccinated animals.

CONCLUSION

1) The number of Brucella ovis bacteria required to cause infection in 50% of susceptible unvaccinated rams when administered intravenously (i.e. the intravenous ID50) is estimated to be approximately 9.5×10^4, with 95% confidence limits of 1.8×10^4 and 4.9×10^5.

2) One dose of Brucella ovis vaccine administered by the intraperitoneal method increases the ID50 by approximately 71 times. Two doses of vaccine administered in this way increase it 158 times. Two spaced doses of Brucella ovis vaccine administered by the subcutaneous route increase it 716 times.

3) In unvaccinated rams, an intravenous challenge dose as low as 1,230 bacteria is sufficient to cause infection.

4) Increasing the challenge dose increases the proportion of rams infected and decreases the incubation period before Brucella ovis is excreted in the semen.

5) Vaccination reduces the proportion of rams infected and increases the incubation period before the organism is excreted in the semen.

6) At high challenge doses, even vaccinated animals become infected. This threshold occurs at lower doses for rams vaccinated by the intraperitoneal technique than it does for those vaccinated twice by the subcutaneous method.

7) Under certain circumstances, natural challenge could cause infection even in vaccinated rams. This is more likely to occur in animals vaccinated once than it is in those vaccinated twice. It is also more likely to occur in those rams vaccinated by the intraperitoneal technique than it is in those vaccinated by the subcutaneous method.

8) Using two spaced doses of Brucella ovis vaccine given by the subcutaneous route is a more effective way of protecting animals against infection than using a single dose of vaccine given by the intraperitoneal technique.

GENERAL DISCUSSION

Vaccination of rams has long been used as a method of controlling Brucella ovis infection. In recent years, there has been considerable debate over the rational use of the inactivated Brucella ovis vaccine available in New Zealand, whether a single dose is sufficient, and what route of administration should be used. There was concern about the efficacy and adverse effects of the various procedures being used. Of these procedures, the two most common were the single-dose intraperitoneal technique and the two-dose subcutaneous method. This project was undertaken to evaluate the various techniques used in order to try to resolve some of the confusion surrounding the use of the vaccine.

The question of whether vaccination should be undertaken at all is a contentious one. The availability of reliable serological diagnostic tests, such as the Brucella ovis complement fixation test and the enzyme linked immunosorbent assay (ELISA) make eradication of the disease on a farm, district or even a national basis a practical option. For example, this approach has been tried with great success in Tasmania (Ryan, 1964). In the long term, national eradication may be the best solution, as it would eliminate the need for costly control programmes involving repeated serological testing, the culling of affected rams and, in some cases, vaccination. A nationwide eradication scheme, however, requires much organisation, and in the short term is quite costly. Because vaccinated animals react to the serological tests in the same way as infected animals, vaccination would have no place in such a scheme.

In New Zealand, a voluntary accreditation scheme has been used for several years as a method of ovine brucellosis control. The objective of this programme is to identify uninfected flocks, and it is aimed primarily at ram breeding flocks, although others are not excluded. In the case of flocks found to be infected at an initial test, the farmers concerned may undertake to follow a policy of eradication. This entails the identification of infected rams by

serological and other tests, with appropriate action being taken until freedom from infection is achieved. Such schemes have been well received, particularly by farmers producing rams for sale. Recently, the New Zealand Ministry of Agriculture and Fisheries, together with the New Zealand Veterinary Association and sheep industry representatives, agreed on a set of guidelines for the operation of the flock accreditation scheme. These guidelines have been produced as an "Aglink" publication, and should help to standardise the procedures throughout New Zealand.

In spite of the success of voluntary schemes in eliminating infection in many flocks, total eradication is difficult to achieve on some properties, especially those in areas where large commercial flocks exist. The reasons for difficulty include poor fencing, infected neighbouring flocks, poor stock control, extremely large numbers of animals run on extensive areas, geographical isolation, lack of funds or labour, and farmers who are not interested in the scheme. In such situations, and while there is no official policy of national eradication, vaccination remains a useful method of controlling the disease. The efficacy of this vaccine, however has sometimes been brought into question (e.g. Bruere, 1982)

It is known that the organisms causing some diseases exist as a number of different antigenic types. Because immunization against one type does not necessarily protect against the others, vaccines against these diseases must include several different types of the organism in order to be effective. The Brucella ovis vaccine in use in New Zealand is made from two isolates of the organism. This is presumably to reduce the likelihood that the vaccine will fail to protect against any particular Brucella ovis variant that a vaccinated animal may encounter.

A limited study of Brucella ovis isolates has been carried out in Australia using an isoenzyme electrophoresis technique. These workers could not detect any genetic variation in 12 Brucella ovis isolates from various sources in New South Wales, South Australia and Victoria (A.J.M. Belfield and M.L. Adams, 1984, personal communication). The results of the bacterial restriction endonuclease

DNA analysis (BRENDA) carried out on seven Brucella ovis isolates encountered in this current project, also suggest that the existence of such genetic variants is unlikely. Accordingly, the inclusion of more than one "strain" of Brucella ovis in any vaccine preparation is probably unnecessary. However, more extensive studies using a larger number of isolates and possibly several endonucleases are required for a more definitive answer to the question of whether genetic variation between Brucella ovis isolates exists.

For the purposes of this study, however, it was concluded that the various isolates involved were all genetically similar, and that the vaccine should therefore be capable of stimulating specific immunity to the challenge organism.

The Brucella ovis vaccine has been used extensively in New Zealand for at least three decades, but currently only about 72,000 doses are used annually (W.G. Orbell, 1984, personal communication). It is quite likely that one reason why voluntary accreditation schemes were accepted so readily by ram breeders was that the alternative method of control, vaccination, was known to be associated with unsightly lesions at the site of subcutaneous administration in the neck, making rams unattractive to prospective purchasers.

It has been shown in this study that rams vaccinated by the subcutaneous route invariably develop an inflammatory response at the site of injection, irrespective of the cleanliness of the technique used. The lesions are usually large enough to be immediately obvious to the eye in a shorn animal, and in an unshorn sheep are easily detected by running a hand over the neck. They are persistent and most are still present a year after vaccination.

These subcutaneous lesions are chronic granulomatous reactions arranged around droplets of the oily Brucella ovis vaccine. A similar reaction also occurs following the use of other vaccines containing an oil-based adjuvant.

Clearly, this type of reaction is unacceptable to some animal owners, and this has implications not only for the manufacturers of the Brucella ovis vaccine, but also for the manufacturers of other vaccines incorporating this type of adjuvant. If a vaccine was developed which produced the same protection but did not result in local vaccine-induced lesions, it would have a distinct market advantage over the existing formulation. This is an area of future investigation which may prove to be of considerable economic as well as scientific benefit. The search for better vaccine adjuvants is a continual one, as illustrated by a recent paper by Afzal, Tengerdy, Ellis, Kimberling and Morris (1984) which discusses the use of Vitamin E as an adjuvant in a Brucella ovis vaccine.

In an attempt to avoid the development of visible reactions to the vaccine, an intraperitoneal technique of vaccination has been advocated (Quinlivan and Wallace 1975, 1979). Although the method seems simple enough to follow, it has been demonstrated in this project that in over 50% of cases, the use of the intraperitoneal technique as described by both Thomson et.al. (1969) and Quinlivan and Wallace (1979) fails to deposit the vaccine totally within the peritoneal cavity. In these cases, the vaccine may be deposited between the muscle and the parietal peritoneum, or within the musculature of the abdominal wall itself. The reasons why this occurs could be investigated in the future using endoscopic techniques to observe intraperitoneal injections being made using needles of various length and diameter.

Regardless of the site of deposition however, the vaccine always provokes the same type of granulomatous reaction as is seen at the subcutaneous injection site in the neck. A sterile peritonitis frequently results, which may be severe, resulting in fibrous adhesions between various abdominal organs. The lesion is persistent and almost one-third of the rams vaccinated by the intraperitoneal technique are found to have peritonitis when necropsied six months later.

There is the possibility that the peritonitis and intermuscular lesions resulting from the use of the intraperitoneal technique might necessitate trimming of the carcasses when rams are slaughtered at an abattoir. However, vaccination is usually carried out only on rams which are to be used for breeding purposes. This type of animal is rarely sent for slaughter at an abattoir and therefore this potential problem would be a minor one. Nevertheless, such problems have been reported to occur following the slaughter of groups of young rams which presumably had not been sold for breeding purposes. (Surveillance, 1978, 1980).

Although no obvious signs of distress were observed in animals vaccinated by the intraperitoneal technique, the extensive peritonitis observed in some of the animals suggested that this method of vaccination might cause discomfort. No effort was made to measure the pain caused by injection, but it was noted nevertheless that when animals were vaccinated by the intraperitoneal route they struggled more vigorously than those injected subcutaneously. The effects of any pain that occurs as a result of vaccination could be studied more extensively in the future.

In spite of the fact that there were no differences in the type of inflammation provoked by the vaccine when it was administered by either the subcutaneous or the intraperitoneal technique, there was a difference in the serological response to these two techniques. This difference was demonstrated consistently in all three trials involving vaccination (Chapters 3, 6 and 7). The rise in titre was more rapid and the final titre level of greater magnitude when the subcutaneous route of administration was used. This difference between the two techniques was apparent in three different serological tests used to detect antibody to Brucella ovis. The consistency of this difference is evidence of its validity.

The physiological basis for this disparity in the serological response to the vaccine when it is administered by the two different routes is unknown. It has been shown that the type of inflammatory reaction cannot account for this difference, as it is similar at both sites. However, there may be a difference in the speed of development

of this local tissue reaction. The timing may differ because of differences in the blood supply to the two sites, but a more likely possibility is a difference in the efficiency of the lymphatic drainage. These interesting aspects of the effect of site of deposition on the immunological efficiency of a vaccine could be investigated either by using fluorescent antibody techniques, or by injecting vaccine which has been labelled in some way. This would allow the site of the material in the tissues to be determined at various times after vaccination.

To obtain an adequate immunological response from a vaccine containing killed organisms, it is generally necessary to give a second dose a few weeks after the first (Herbert 1970). When the suggestion was made that the intraperitoneal technique of administration should be employed in order to avoid the visible lesions associated with the use of the subcutaneous route (Quinlivan and Wallace, 1975, 1979), the implication was that a single intraperitoneal injection of vaccine would confer adequate immunity against infection. The results reported here do not support that conclusion, but rather confirm the findings of Buddle (1962) and Buddle et.al. (1963) that two doses of the vaccine are more effective than one. This finding is difficult to explain having regard for the nature of the adjuvant, the success of which is thought to be due at least in part to the formation of a long-lived depot which stimulates the immune system over a protracted period. It may be, however, that the small repeated doses of antigen released are not great enough to trigger a secondary antibody response of the type which can be readily demonstrated in rams given a second dose of the vaccine.

A single-dose vaccination regime is obviously desirable as it is more convenient and less costly. In addition, a two-dose programme is sometimes difficult to organize, as the instructions indicate that it should begin four months before the period of risk. The manufacturers of the Brucella ovis vaccine, Coopers Animal Health N.Z. Ltd, recommend that vaccination should not be undertaken closer than two months before mating, and that two doses should be given at least eight weeks apart. This latter recommendation probably arose from the experiments of Buddle in the 1950s, in which an eight week interval

between the two doses of vaccine was the shortest period investigated. Since that time, there have been no trials using the vaccine at shorter intervals. In practice, farmers sometimes do not obtain all their rams until close to the time of mating, and veterinarians then find it difficult to fit in two vaccinations at the suggested interval.

It was discovered in this project that rams which received a single dose of vaccine administered by the subcutaneous route developed maximal complement fixation titres by about six weeks after vaccination. In the absence of more detailed work, there seems therefore to be no reason why the second dose of vaccine in a two dose regime should not be given approximately one month after the first, rather than having to wait eight weeks.

Furthermore, the complement fixation titres of animals vaccinated twice were at a peak approximately one month after the second dose of vaccine had been administered. This suggests that a one month interval between vaccination and the period of risk may be all that is required. It may be, however, that cell-mediated immunity develops more slowly than the humoral response, and therefore that the animals should be allowed a longer period in which to develop this immunity before being exposed to challenge. However, this seems unlikely to be the case. Kristensen, Kristensen and Lazáry (1982) stated that in general, the lymphocyte stimulation test is positive some days before the development of detectable antibody, and that a maximum response usually occurs between 10 and 30 days after the first application of antigen. This test measures the in-vitro transformation of lymphocytes in response to a specific antigen and is one way of evaluating cell-mediated immunity. These results indicate that the humoral and cell-mediated responses probably develop simultaneously. In order to investigate this question in relation to specific immunity to Brucella ovis, measurements of cell-mediated immunity, and challenge trials conducted at various intervals after vaccination could be used.

There is the possibility that the administration of the vaccine may cause a temporary deterioration in the health of the animal or in the quality of the semen. If this is the case, an interval of two months between vaccination and mating may indeed be warranted.

A key matter that must be considered when planning vaccination programmes is the period when animals are at the greatest risk from infection. The period of risk is presumed by the manufacturers of the vaccine to be at mating. Frequently, however, one of the greatest risk periods is when mobs of rams are mixed before mating commences.

All the rams in this project which were vaccinated twice, using the conventional subcutaneous route, retained a positive reaction to the complement fixation test and the ELISA test for over twelve months. It is recognised that rams can maintain a positive reaction to the complement fixation test for at least five years following vaccination (personal observation), a period which in New Zealand is often longer than the entire productive lifetime of the ram. In the trial reported in Chapter 3, however, the antibody titres of vaccinated animals were falling steadily one year after vaccination. Several animals that had been vaccinated only once had lost their positive titre status, and even one of the rams vaccinated twice (by the intraperitoneal method) had reverted to a "suspicious" complement fixation titre by the end of the twelve months.

It has been demonstrated in Chapters 6 and 7 that rams vaccinated against Brucella ovis infection may still be susceptible to challenge. For this reason, it may be prudent to consider annual revaccination for rams which are at high risk of infection. Further investigations of the long term effects of vaccination are needed to determine if such revaccination would be of any real value in terms of protection from infection. Many rams in New Zealand are only used for two or three breeding seasons, and a single two-dose vaccination programme may be sufficient to protect the majority of these rams throughout their productive life.

An unfortunate but unavoidable consequence of revaccination would be the addition of yet another local inflammatory lesion at the site of vaccination. However, measurements of the subcutaneous lesions at the site of primary and secondary vaccination in the neck suggest that although the primary inoculation results in a large and persistent lesion, subsequent injections may result in smaller lesions which resolve more quickly. Observations need to be made on larger numbers of animals in order to validate this suggestion.

The differences between the serological responses of rams vaccinated in various ways were reflected by similar differences in resistance to intravenous challenge. Although any form of vaccination using the inactivated Brucella ovis saline-in-oil product significantly increased resistance, two doses appeared to be more effective than one, and the subcutaneous technique more effective than the intraperitoneal method. The correlation between antibody titre and resistance in groups of animals was not always reflected in individuals. Some animals with relatively high titres succumbed to infection while others with low titres were resistant. However, it should be reemphasised that antibody titres are not always correlated with protection, since such factors as cell-mediated immunity and secretory IgA are also important. In the case of Brucella ovis infections, cell-mediated immunity is known to be extremely important in the protective response (Collins & Campbell 1982).

The measurement of the cell-mediated response to vaccination against Brucella ovis could be an area for future investigations. The association between this aspect of the response to vaccination and the resistance to subsequent challenge may prove to be much more direct than that between antibody response and resistance. Preliminary investigations made during this study suggest that the lymphocyte transformation test, which measures the in-vitro transformation of lymphocytes in response to a specific antigen, may be a useful way of investigating this aspect of the immune response.

The cell-mediated component of immunity may also be a factor in the phenomenon of transient serological responses to infection, which were seen in rams in Chapters 5 and 7. Although these two rams reverted to a negative antibody titre, they may have retained a level of cell-mediated immunity. It would be of value in future investigations to try to measure the cell-mediated response in such animals and also to determine their response to subsequent challenge with Brucella ovis bacteria. It may be that rams such as this have an innate genetic resistance to infection which is substantially greater than that of other rams.

Burgess et.al. (1982) considered that in recently infected flocks, a relatively high proportion of rams could be detected as positive to a complement fixation test although they would never become excretors of the organism. These workers classed these results as "false positive" reactions and considered them to be the result of "abortive infections". Of the 50 unvaccinated rams experimentally infected in the present project, 35 (70%) developed positive complement fixation titres. Of these 35, 32 (91%) excreted Brucella ovis in their semen. Two of the rams which did not excrete the organism reverted to a negative complement fixation titre after a short time. The third was killed two weeks after it developed a positive reaction to the test. Had it been allowed to live, there is every possibility that it would either have reverted to a negative titre or, alternatively, begun to excrete the organism. The conclusion that has to be drawn from these results is that the correlation between a positive complement fixation test and the excretion of Brucella ovis is very high. So-called "false positive" reactions resulting from "abortive infections" are probably a minor problem in the interpretation of serological results.

From the results reported in Chapter 7, the best estimate that could be made of the number of Brucella ovis bacteria required to infect 50% of unvaccinated rams following intravenous inoculation was 9.5×10^4 organisms. That figure was increased to 6.8×10^7 bacteria following the administration of two doses of vaccine, given by the subcutaneous route, eight weeks apart. Having regard for the results of the various serology and challenge trials, it was concluded that

the best method of vaccinating rams against Brucella ovis infection was to administer two spaced doses of vaccine by the subcutaneous route in the neck.

A more extensive study of the concentrations of bacteria that may be present in the semen of infected rams is needed, but from the one sample examined in this project, there was evidence that at least 3×10^9 bacteria could be deposited in the rectum of a ram by a single ejaculation during one episode of homosexual activity. Allowing for the fact that only a small proportion of bacteria is likely to reach the blood stream, there is still a very real possibility that even vaccinated rams may become infected by natural transmission. This conclusion is supported by the results of Buddle (1958), who found that even when the Brucella ovis vaccine was used in conjunction with live Brucella abortus Strain 19, some rams still became infected by natural challenge. There have also been occasional reports of "vaccine breakdown" associated with the use of the contemporary vaccine (Bruere 1982). It would be unreasonable to expect any vaccine to protect 100% of the animals on which it is used. However, if vaccination against Brucella ovis infection is to be undertaken, it would be sensible to use the vaccine in such a way as to allow its maximum efficiency to be expressed. The results of these studies indicate that the best technique is the administration of two doses of vaccine, a few weeks apart, by the subcutaneous route.

In this project, it was not possible to prove the existence of any statistically significant differences between the relative resistances to infection produced by the various methods of vaccination, despite such differences being strongly suggested. Now that approximate ID50 values for Brucella ovis administered by the intravenous route are known, the way is open for researchers, using these parameters as a basis, to determine more accurate estimates of the ID50, and to confirm the differences between methods of vaccination which in all probability exist.

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APPENDIX 1

MICROBIOLOGICAL METHODS

A) Microbiological Media

1. Blood Agar

a) Salt base: Dissolve 15g Agar and 5g Sodium Chloride in one litre of distilled water. Dispense into 400ml bottles. Autoclave 15 minutes at 15 lbs pressure. Pour 10ml in each sterile petri dish.

b) Blood base: Make up Columbia Blood Agar Base (Difco) as instructed on pack. Autoclave 15 minutes at 15 lbs pressure. When cooled to 45°C, add 5% defibrinated sheeps blood. Mix. Pour 10-15 ml over the salt base in each petri dish.

For blood agar slopes, use blood base. Pour 10ml into a sterile Universal bottle and allow to set at angle.

2. **Brucella ovis** Selective Medium (Modified Thayer-Martin Agar)

Dissolve 36g G.C. Medium Base (BBL) in 600ml distilled water. Autoclave and cool to 57°C. Dissolve 10g Haemoglobin (BBL) in 400ml distilled water. Autoclave and cool to 57°C. Dissolve contents of a 10ml vial of VCN Inhibitor (BBL) in 10ml of sterile distilled water. Dissolve 10mg Nitrofurantoin in 1ml distilled water OR 1ml acetone.

Keeping ingredients at 57°C, combine all together, then dispense into sterile disposable petri dishes.

3. Dextrose Oxidation/Fermentation Test Medium

Dissolve 9.4g of O.F. Basal Medium (Difco) and 1g Yeast Extract (Difco) in one litre distilled water. Adjust pH to 7.0., dispense into 200ml aliquots and autoclave 15 minutes at 15 lbs pressure. When cooled to 56°C add 10ml of 10% Sietz Filtered Dextrose (Difco) and dispense in 6-10ml aliquots into loose metal-capped test tubes.

4. Heart Infusion Broth

Add 25g Heart Infusion Broth (Difco) to one litre distilled water. Dispense 3ml aliquots into bijoux jars. Autoclave 15 minutes at 15 lbs pressure.

5. McConkey Agar

Make up McConkey Agar (BBL) according to instructions on label. Dispense into 400ml bottles, autoclave for 15 minutes at 15 lbs pressure and dispense onto plates.

6. Motility Test Medium

Combine 16g Motility Test Medium (Difco), 4g Nutrient Broth (BBL) and 1g NaCl in one litre distilled water. Immediately before dispensing into 15ml Universals, add 0.05g Tetrazolium Salt (BDH). Autoclave in Universals 15 minutes at 15 lbs pressure.

7. Nitrate Reagent A

Dissolve 0.2g of 5-amino 2-naphthalenesulfonic acid in 30ml glacial acetic acid and add to 120ml distilled water. Store away from sunlight.

8. Nitrate Reagent B

Dissolve 0.5g sulfanilic acid in 30ml glacial acetic acid and add to 120ml distilled water. Store away from sunlight.

9. Nitrate Test Medium

Dissolve 25g Indole Nitrate (Gibco) in one litre deionised water. Dispense in 10ml aliquots into Universal bottles containing Durham tubes. Autoclave 15 minutes at 15 lbs pressure.

10. Oxidase Reagent (Kovac's Reagent)

Dissolve 0.5g of Tetramethyl-p-phenylene-diamine-dihydrochloride in 100ml distilled water. Store in a brown bottle containing a little calcium sulphate, away from sunlight.

11. Peptone Saline

Dissolve 10g Peptone (Difco) and 5g NaCl in one litre distilled water. Dispense into 100ml bottles and autoclave.

12. Simmons Citrate Agar

Dissolve 24.2g Simmons Citrate Agar (Difco) in one litre distilled water. Dispense in 3ml aliquots into bijoux jars. Autoclave 15 minutes at 15 lbs pressure and allow to slope on trays while cooling.

13. Trypticase Soy Agar (TSA)

Dissolve 40g Tryptic Soy Agar (Difco) in one litre distilled water. Dispense in Universals, Autoclave 15 minutes at 15 lbs pressure, allow to slope on trays while cooling.

14. Urea Test Slopes (Christiansens Medium)

a) Agar - Dissolve 15g Bacto Agar in one litre distilled water. Dispense into 200ml bottles and autoclave 15 minutes at 15 lbs pressure. When blood heat add 30ml urea base to it, then dispense in 3ml aliquots into bijoux jars and allow to slope while cooling.

b) Urea base - Dissolve 29g Urea Agar Base (BBL) in 100ml distilled water. Sterilise by filtration and keep in fridge until required.

B) Biochemical Tests

These were the techniques employed in this study, some being modifications of the approved methods. The incubation was always carried out in an atmosphere of 10% CO_2, as this was a requirement for the growth of the Brucella ovis organism.

1. Citrate Utilisation Test

Inoculate surface of citrate agar slope using pure culture of organism to be tested. Incubate at 37°C in 10% CO_2 for up to six days. A positive result is indicated by growth of the organism and the development of an intense blue colour on the slope. A negative result is indicated by lack of bacterial growth and the colour of the medium remaining green.

Brucella ovis result - negative.

2. Dextrose Oxidation and Fermentation

Inoculate two tubes of O/F dextrose medium using the organism to be tested, by stabbing with an inoculating needle to within 0.5cm of the bottom of the medium. Seal one of the tubes by overlaying with 1-2ml of melted petrolatum to exclude oxygen. Incubate at 37°C for up to six days. A positive reaction is indicated by a change in the colour of the medium from blue/green to yellow. A colour change in the sealed tube indicates fermentation of the substrate by the organism, and in the unsealed tube it indicates oxidation.

Brucella ovis result - no fermentation or oxidation, although the unsealed tube became slightly discoloured at the top, probably as a result of the high CO_2 level.

3. Hydrogen Sulphide Test

Inoculate the surface of a blood agar slope with the organism to be tested. Suspend a strip of lead acetate paper above the slope, bending the edge of the paper strip over the lip of the bottle and securing it by replacing the cap loosely. Incubate at 37°C in 10% CO_2 for up to seven days, replacing the lead acetate strip daily. The production of H_2S is indicated by a brownish-black discolouration of the white lead acetate paper.

Brucella ovis result - negative.

4. Nitrate Reduction Test

Inoculate medium with pure culture. Incubate at 37°C in 10% CO_2 for five days. Look for the presence of gas in the Durham tube. If gas is present and the organism is a non-fermenter of glucose, the test is positive for denitrification, and is completed. Otherwise, proceed as follows: Add five drops of Nitrate Reagent A and five drops of Nitrate Reagent B. If a red colour develops within 1-2 minutes, it indicates that nitrate (NO_3^-) has been reduced to nitrite (NO_2^-) by the organism. If no colour develops, it indicates that nitrite (NO_2^-) is not present. If this is the case, add a pinch (approximately 20mg) of zinc powder to the contents. If a red colour develops within five to ten minutes, it indicates that nitrate (NO_3^-) is present, and has not been reduced by the organism. If no colour develops, it indicates that the organism has reduced nitrate to nitrite and then further reduced nitrite to non-gaseous products.

Brucella ovis result - no gas produced, no reduction of nitrate.

5. Motility Test

Inoculate with pure culture by stabbing the centre of the medium using an inoculating needle to a depth of 2cm. Incubate at 37°C for five days. Motility is indicated by turbidity of the medium or growth streaks outwards from the stab line, with a pink colouration where the bacteria are growing.

Brucella ovis result - non-motile.

6. Oxidase Test

Place a piece of Whatman No.1 filter paper in a petri dish. Add 2-3 drops of oxidase reagent to centre of paper. Using a platinum wire inoculating needle, smear a loopful of the colony onto the paper in a 3-6cm long line. A positive reaction is indicated by the development of a purple-black colour along the line within 10 seconds.

Brucella ovis result - negative.

7. Urease Test

Inoculate surface of urea agar slope with pure culture. Incubate two hours at room temperature and then for six days at 37°C. A positive reaction is indicated by the development of an intense pink-red colour on the slope. If the medium remains yellow, the result is negative.

Brucella ovis result - negative.

C) Staining Techniques

1. Gram Stain

a) Fix slide using gentle heat.
b) Flood with 0.5% solution of Methyl Violet and leave for 30 seconds.
c) Wash off with tap water, drain.
d) Flood with Lugol's Iodine and leave for 30 seconds.
e) Wash off with tap water, drain.
f) Flood slide with Acetone and immediately wash off with water. Drain.
g) Counterstain by flooding with a 0.5% solution of Safranin for 30 seconds.
h) Wash off with tap water, drain and air dry.

Result: Gram-positive organisms stain purple-blue. Gram-negative organisms such as Brucella ovis stain red-orange.

2. Modified Ziehl-Neelsen Stain

a) Allow slide to dry.
b) Flood slide with dilute Carbol Fuchsin (a 10% solution of concentrated Carbol Fuchsin) and leave for ten minutes.
c) Wash off with tap water and drain.
d) Decolourise with 0.5% Acetic acid for 15-20 seconds.
e) Wash off with tap water and drain.
f) Counterstain with a 1% solution of Methylene Blue for 30 seconds.
g) Wash off with tap water, drain and air dry.

Result: Positive (acid-fast) organisms such as Brucella ovis stain pink-red. All others stain blue.

APPENDIX 2

SEROLOGICAL METHODS

A) The Complement Fixation Test

Reagents

1) Buffer - Veronal buffered saline

2) Complement - Freeze-dried guinea-pig serum preserved in Richardsons solution, obtained from a commercial source. The dilution to be used in the test is determined by titration.

3) *Brucella ovis* antigen - Prepared at Wallaceville by a process of heat-extraction of *Brucella ovis* cells, described as the method for "Antigen 3" in a report in the New Zealand Veterinary Journal (Animal Health Division, 1983). The dilution to be used in the test is determined by titration.

4) Red Blood Cells - Sheeps blood is collected into sterile bottles half filled with Alsevers solution and stored at 4°C. The blood is washed three times in veronal buffered saline. The concentration, determined by microhaematocrit, is adjusted using veronal buffer so as to contain a 2% packed cell volume of red blood cells. The optical density of a 1/30 dilution of this suspension in veronal buffer should be approximately 0.57 at 541nm, and the optical density of a 1/16 dilution in distilled water (haemolysed) should be approximately 0.34 at 541nm. A fresh 2% solution of cells is prepared daily. For use in the complement fixation test, the 2% solution is diluted 50:50 with haemolysin.

5) Haemolysin - A commercial source of haemolysin is used. The optimal dilution for use in the test is determined by a haemolysis titration.

Method

1) On arrival at Wallaceville, the sera in their autoanalyser cups are inactivated by standing in a water bath for 50 minutes at 62°C - 63°C, to remove most of the anticomplementary activity.

2) A 50µl aliquot of serum is placed in each of the first 11 wells in the first row of a microtitre plate, one well per sample. A 50µl aliquot of a standard serum is placed in the last well of the row.

3) Twenty-five microlitres of veronal buffered saline is placed in all the remaining empty wells (i.e from the second row on).

4) Doubling dilutions of serum, from 1/2 to 1/128 are then made across the plate using a stirrer-diluter machine, so that there are 25µl of diluted serum in each of the wells on the plate.

5) To each well, 25µl of diluted complement is added.

6) To each well from the third row back, 25µl of diluted Brucella ovis antigen is added.

7) The plate is incubated at 4°C overnight, then left on the bench for 30 minutes.

8) To each well, 25µl of the haemolysin/red blood cell suspension is added, and the plate is first incubated standing for 15 minutes at 37°C, and then for a further 15 minutes using gentle agitation on a shaking machine, at 37°C.

9) The results are then read. The first two rows, containing no antigen, are checks for anticomplementary activity. If both are positive (i.e. not haemolysed), then the sample is considered anticomplementary and the result cannot be interpreted.

10) The results are read from the third row backwards. A reaction of 100% haemolysis is considered negative, 75% is a "+" (1) reaction, 50% is a "++" (2) reaction, 25% is a "+++" (3) reaction, and no haemolysis is a "++++" (4) reaction.

11) A titre of less than 4 is considered "negative", a titre of between 4.1 and 4.3 is "suspicious", and a titre of 4^2 or more is "positive". (See Chapter 3 for a description of the reporting convention).

12) The standard sera in the last of the twelve wells should have a titre of between 4.2 and $4^2 2$.

13) A standard serum plate is included in each batch of sample plates, it has three samples of the standard serum used in the last well of all the plates, and nine other sera of known titre.

B) The Enzyme Linked Immunosorbent Assay

Reagents

1) *Brucella ovis* Antigen - Heat extracted antigen as used for the complement fixation test.

2) Phosphate Buffer - A buffer of pH 7.2, made by combining 360ml of 0.1M Na_2HPO_4, 140ml of 0.1M KH_2PO_4, and 500ml deionized water, to make one litre.

3) Tween Phosphate Buffer - A buffer of pH 7.2 made by combining 360ml of 0.1M Na_2HPO_4, 140ml of 0.1M KH_2PO_4, 9g of NaCl, 0.5ml of "Tween 20", 1ml of 10% merthiolate, and 473.5ml deionized water, to make one litre.

4) Conjugate - Rabbit anti-bovine IgG, conjugated to peroxidase. It was diluted using Tween phosphate buffer. The dilution of conjugate to be used is determined by titration.

5) Substrate - A 1% solution of Ortho-phenylenediamine (OPD) in methanol was stored at -20°C. For use in the test, fresh substrate was prepared daily by combining 4ml of the OPD solution with 100ml of phosphate citrate buffer to which 30µl of 30% H_2O_2 had been added.

6) Phosphate Citrate Buffer - A buffer of pH 5 made by combining 25.7ml of 0.2M Na_2HPO_4, 24.3ml of 0.1M citric acid, and 50ml of deionized water.

Method

1) Microtitre plates are coated by adding 0.1ml of diluted Brucella ovis antigen in phosphate buffer to each well. The optimum antigen dilution is determined by titration. The plates are incubated overnight (about 18 hours) at 4°C.

2) The plates are then washed three times in flowing deionized water, dried in a 37°C incubator, and stored in sealed plastic bags over indicator silica gel.

3) Each serum to be tested is diluted 1/150 using Tween phosphate buffer, and 0.1ml is pipetted into a single well on the prepared plate. The plate is incubated for two hours at 37°C, and then washed three times in flowing deionized water.

4) To each well, 0.1ml of diluted conjugate is added, and the plate is incubated two hours at 37°C and then washed three times in deionized water.

5) To each well, 0.1ml of diluted substrate (OPD) is added, and after 16 minutes the reaction is halted by the addition of 0.05ml of 1M HCl to each well.

6) The plate is then read in a plate reader using a 490nm filter.

7) The value allocated to each test serum is read from a regression line derived from the results of various standard sera that are included on the plate.

8) During this study, a reading of 0-69 units was considered "negative", 70-89 units "suspicious" and 90 units or more "positive".

C) Gel Diffusion Test

Reagents

1) Agarose Gel - This gel is made of 3.49g sodium barbital, 3g NaCl, 3.75g glycine, 0.625g NaN_3, 0.25g CdCl and 0.45g "Litex" HSA Agarose, dissolved in one litre of water. The pH is adjusted to 7.5 using HCl.

2) Brucella ovis Antigen - The supernatant of a sonicated suspension of Brucella ovis cells is used as an antigen, as described by Worthington, Weddell and Penrose (1984).

Method

1) Four millilitres of molten agarose gel is pipetted onto a microscope slide and allowed to set.

2) A central well 4mm in diameter and six outer wells 5mm in diameter are cut out using a template. Each outer well is 4mm from the central well.

3) The central well is filled with 20µl of diluted antigen.

4) Two diametrically opposed wells are filled with 50µl of known positive serum, and the other four with 50µl of a serum to be tested.

5) The slide is incubated at 4°C in a humid atmosphere for 72 hours.

6) The slide is then read. Positive sera develop precipitin lines which match up with the lines of the known positive sera. Sera which give a "hook" on a positive control line are classed as "suspicious", and lines which cross each other indicate a non-specific reaction. If there are no lines, the serum sample is considered to be "negative".

APPENDIX 3

HISTOLOGICAL METHODS

A) Reagents

1. Fixative - 10% Formol Saline

To make up 50 litres of 10% Formol Saline, combine 400g NaCl and 5 litres of Formalin (a 40% solution of Formaldehyde) in 45 litres of distilled water.

2. Ehrlichs Haematoxylin Stain

Dissolve 6g Haematoxylin in 300ml Absolute Alcohol. Add 300ml distilled water, then 300ml Glycerol, then 30ml Glacial Acetic Acid. Finally, add Potassium Alum until a deposit of crystals forms at the bottom of the vessel.

3. Acid Alcohol

Add 10ml of concentrated HCl to two litres of 70% alcohol and mix.

4. Scotts Tapwater

Dissolve 50g Magnesium Sulphate and 2g Potassium Bicarbonate in two litres of tapwater. Keep in closed vessel.

5. Eosin Stain

Dissolve 4g Eosin in 400ml distilled water to make a 1% solution. Add 1ml of 1% Acetic Acid to every 50ml of the 1% Eosin solution before use.

6. Celestine Blue

Dissolve 5g Ferric Ammonium Sulphate (iron alum) in 100ml distilled water overnight. Add 0.5g Celestine Blue B, and boil gently for three minutes. Cool and filter. Finally, add 14ml glycerine.

7. Mayers Haemalum Stain

Dissolve 1g Haematoxylin in one litre of distilled water, heating gently if necessary. Add 50g Potassium Alum and dissolve. Add 0.2g Sodium Iodate, then 1g Citric Acid, and finally 50g Chloral Hydrate.

8. Van Gieson Stain

Combine 100ml of a saturated (approximately 1%) aqueous solution of Picric Acid with 10ml of a 1% solution of Acid Fuchsin. Use when fresh.

9. Sudan Black Stain

Combine 7g Sudan Black B with 500ml of 70% Alcohol and warm in a flask in a water bath for 30-60 minutes at 56°C. Keep well sealed and filter before use.

B) Methods

1. Haematoxylin and Eosin (H & E)

a) Dewax section in xylol for ten minutes, five minutes each in two changes of xylol.

b) Bring to water by rinsing for one minute each in absolute alcohol, then 70% alcohol, then water.

c) Stain in Ehrlichs Haematoxylin for ten minutes.

d) Rinse in tapwater.

e) Differentiate in Acid Alcohol for ten seconds.

f) Rinse in tapwater.

g) Blue in Scotts Tapwater for two minutes.

h) Rinse in tapwater.

i) Stain in Eosin for two minutes.

j) Rinse rapidly in water by dipping four times.

k) Dehydrate by immersing in 70% alcohol and then three changes of absolute alcohol.

l) Clear in three changes of xylol for one minute each.

m) Mount in DPX mountant (BDH).

2. Van Gieson Stain

a) Dewax in two changes of xylol, five minutes each.

b) Bring to water by rinsing one minute each in absolute alcohol, 70% alcohol and then water.

c) Stain with Celestine Blue for ten minutes.

d) Rinse in water.

e) Stain in Mayers Haemalum for ten minutes.

f) Rinse in water.

g) Rinse in Scotts Tapwater for two minutes.

h) Wash in water.

i) Stain in Van Gieson stain for seven minutes.

j) Rinse in water.

k) Dehydrate and Clear as for H & E stain.

l) Mount.

3. Sudan Black Stain

a) Bring to 70% alcohol by rinsing one minute in absolute alcohol and then one minute in 70% alcohol.

b) Stain in filtered Sudan Black Stain for 30 minutes in a closed vessel.

c) Rinse in 70% alcohol.

d) Wash in tapwater for three to four minutes.

e) Mount in glycerine jelly.

APPENDIX 4

ENUMERATION OF BRUCELLA OVIS ORGANISMS

This appendix describes three methods of counting Brucella ovis bacteria in suspension that were evaluated for use in this project, and the results of comparisons that were made of the techniques.

Production of the Bacterial Suspension

A Brucella ovis isolate was used to inoculate a blood agar plate and cultured at 37°C in an atmosphere of 10% CO_2 for four days. The bacterial growth was harvested using a sterile cotton wool swab and suspended in a small volume of Heart Infusion Broth which was then used to inoculate several Trypticase Soy Agar slopes containing 5% bovine serum.

After three days incubation at 37°C in 10% CO_2 the slopes were harvested by flushing each with 2ml sterile peptone saline. The resultant suspension was pooled, and counting began immediately.

1. Dark Field Microscopy

A Petroff-Hausser bacteria counting slide chamber was filled with the bacterial suspension to be examined. The chamber was placed under the high power objective (40 x magnification) of a dark-field microscope. As the eyepiece had a magnification of 10 x, the total power of magnification was 400 x.

Bacteria in ten of the small squares marked on the grid of the chamber in the selected field were counted and the numbers recorded. Another field of view was then selected and the number of bacteria in ten more small squares were counted. This was repeated until five samples, each consisting of ten small squares, had been counted.

The average number of bacteria in one small square was then calculated, and since it was known that each small square contained 0.5×10^{-7} ml of suspension, then each ml of the original suspension must have contained 2×10^{7} x average number of bacteria per small square.

If the number of bacteria in each square was very low or very high, the accuracy of the estimate was greatly reduced. It was found that the count was best made using a dilution which gave a density of between six and twelve bacteria in each small square. If dilution of the suspension was carried out, the calculation of the bacterial concentration in the original suspension was as follows: Estimated number bacteria/ml original suspension = Average number per small square x 2×10^{7} x dilution factor. For example, if the suspension had been diluted 1/100, the estimate would be: Average number bacteria per small square x 2×10^{9}.

Since the Brucellae are small bacteria (0.6-1.5µm x 0.5-0.7µm, Alton et.al. 1975), and the depth of the counting chamber relatively great (20µm), one depth of focus was not always sufficient to detect all the bacteria for making an accurate count of the numbers in the area of each square, and slight adjustment of focus was necessary. This inevitably lead to inaccuracies in counting, usually causing slight underestimation of the numbers of bacteria present.

2. Viable Count

Serial tenfold dilutions of the suspension to be counted were made using sterile peptone saline as a diluent. One ml of the original suspension was added to 9ml diluent to make a 1/10 dilution, 1ml of this added to 9ml diluent to make a 1/100 dilution, and so on as necessary (usually judged from the results of a previous dark-field count).

A graduated pipette was used to transfer 0.1ml of bacterial suspension into the centre of each of two blood agar plates. A glass spreader dipped in 95% alcohol and flamed, was cooled by touching it to the agar surface, and then used to spread the droplet evenly over the plate. This was repeated for the second plate.

Duplicate viable count plates like these were made for each of the dilutions and the original suspension. They were then inverted and incubated at 37°C in an atmosphere of approximately 10% CO_2. After four days, the bacterial colonies were usually large and distinct enough to be counted.

The lowest dilution (highest number of bacteria) which could still be counted was the best single dilution to use in the estimate, as it was usually the most accurate. Another way of making an estimate was to use the average of the counts from more than one dilution.

3. Spectrophotometry

The degree to which light is scattered by a suspension of cells is approximately proportional to its concentration, within a limited concentration range (Alton et.al. 1975). This "turbidity" can be measured by a spectrophotometer, which measures the proportion of a beam of light which is transmitted through a liquid (% transmittance), or inversely, the proportion "absorbed" or scattered (absorbance). The relationship between cell concentration and absorbance or transmittance is linear only within a limited range, and only a

limited range of densities can be measured by this method (Alton et.al. 1975).

It was important that the suspensions to be compared should be of a standard organism, grown in standard culture conditions for a standard time and suspended in a standard diluent. In the experiments that were made to evaluate this method of counting bacteria, a Bausch and Lomb Spectronic 20 spectrophotometer was used, at a wavelength of 420nm. The bacteria were from 72 hour cultures grown on TSA slopes at 37°C in 10% CO_2, harvested and diluted using sterile peptone saline. The same glass spectrophotometry tube was used throughout, and a blank of pure peptone saline was used to set the zero reading.

The aim of this method was to graph a standard curve of absorbance (or transmittance) against the actual number of organisms (in this case, viable count was used as the benchmark), from which a prediction of bacterial numbers could be made from the absorbance reading of any unknown suspension measured at a later stage.

Results

a) Spectrophotometry

The results of five separate experiments conducted to evaluate the three methods showed that in some cases the viable count predicted by the spectrophotometric method was very inaccurate, especially at the higher absorbance values, when the prediction was in some cases 100 x the value given by the actual viable count. In practice, several dilutions of the suspension were sometimes required before the concentration of suspension (and hence the absorbance) was in a range useful for prediction. This was wasteful of time and equipment, and therefore a practical disadvantage.

There was a wide range of bacterial concentrations which produced the same absorbance reading when the suspension was measured, reducing the value of the technique as a method of predicting viable count.

It was noted that there appeared to be a closer correlation between absorbance and dilution factor than between absorbance and viable count. This was a cause for concern, as it implied that the dilution of the suspension may have been contributing more to the absorbance reading than simply an altered bacterial concentration. There may have been an inherent turbidity in the suspension, not associated with bacterial numbers, which was affecting the absorbance.

It was clear then, that the spectrophotometric technique was associated with a good deal of unexplained variation, reducing the usefulness of this method.

b) Dark Field Microscopy

The accuracy of the dark field count as a method of predicting the viable count increased from the first experiment to the last as the operator became more experienced with the technique, and the closeness of the prediction was far less variable than that of the spectrophotometric method.

Comparison of Methods

The advantages and disadvantages of the three techniques can be summarised as follows:

1) Dark Field Microscopy

Advantages:

a) Quick procedure.
b) Immediate result.
c) Low cost.
d) Accuracy of prediction of viable count reasonably consistent (+ or - 10^{10}).
e) Small volume of suspension required.

Disadvantages:

a) Special equipment required - a dark field microscope and a bacteria counting chamber.
b) Experienced operator preferable.
c) Measures both viable and non-viable cells.

2) Viable Count

Advantages:

a) Only viable bacteria are counted.
b) High accuracy.
c) Equipment required is standard in most microbiology laboratories.
d) Small volume of suspension required.

Disadvantages:

a) Time consuming procedure to make the plates.
b) Expensive - cost of plates and running the incubator.
c) Four days to get a result.

3) Spectrophotometry

Advantages:

a) Immediate result.

b) Accuracy can be quite high.

c) Low running costs.

d) Equipment required (spectrophotometer) common in most laboratories.

Disadvantages:

a) Time consuming procedure.

b) Variable accuracy.

c) Both viable and non-viable bacteria measured.

d) Large volume of suspension required, which is not recoverable.

The viable count was the most accurate method, but the result is not available for four days, by which time the bacterial numbers may have altered considerably. If the suspension is to be used in a challenge experiment to infect experimental animals, an immediate estimate is needed to allow the suspension to be diluted to the required concentration for administration.

For an immediate estimate, either the dark-field count or spectrophotometry could be used. For this project, the dark-field count was more practical, being quicker to perform, more uniform in accuracy than the spectrophotometry technique, and needing only a small volume of suspension, leaving the remainder for use in subsequent procedures such as the challenge of experimental animals.

It was therefore decided that the viable plate count would be used as the standard count, and the dark field count as an immediate estimate for all experiments requiring evaluation of bacterial numbers.

APPENDIX 5

SEMEN COLLECTION

The ram was restrained in lateral recumbency on a low table. The penis was gently extruded from the prepuce and held gently but firmly using a strip of clean gauze. The glans and urethral process were placed just inside a sterile Universal bottle. An electroejaculator probe[1] similar to that described by Nichols and Edgar (1964) was inserted into the rectum of the ram. The probe was operated as suggested by Nichols and Edgar (1964). The switch was pressed and released alternately, four seconds on and then four seconds off, until the ram ejaculated. Most rams ejaculated after two or three stimulations when this rhythmic cycle was used. If after six or seven stimulations the ram had not ejaculated, it was rested for 30 minutes or more before a further attempt at collection was made. If there was again no ejaculation after six or seven stimulations, the attempt to collect semen from that ram was abandoned for the day.

In order to reduce contamination of the samples to a minimum, grossly contaminated wool around the prepuce was removed with scissors where necessary, the operators collecting semen washed and dried their hands between rams, the lid was placed on the sample bottle immediately after collection, and the bottles were kept in an upright position at all times after collection, as most of the contamination was around the top of the vessel.

NOTE

1) Ram Probe MK V - A.J. Thompson Manawatu Ltd. Box 1607 Palmerston North. (Modelled on the previously designed "Ruakura" ram probe).

APPENDIX 6

STATISTICAL METHODS

A) Group Comparisons

1) Confidence Limits

To evaluate the significance of differences in group means in this study, 95% confidence limits were used. The 95% confidence limits of the means were calculated as follows:

Upper and Lower 95% Confidence Limits = $\bar{x} \pm (t \times S.E)$

Where $\bar{x}$ = Sample mean

t = Student's "t" value for P=0.05 and (n-1) degrees of freedom, obtained from "t" distribution table.

$$S.E. = \text{Standard Error of the Mean} = \frac{S.D}{\sqrt{n}}$$

n = Sample Size (e.g. number of rams)

$$S.D. = \text{Standard Deviation} = \sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}$$

x = individual observation

2) Chi-squared Test

For qualitative data, the chi-squared method was used to test the significance of any differences between groups.

The chi-squared value, χ^2, was calculated from the following formula:

$$\chi^2 = \sum \frac{(O-E)^2}{E}$$

Where O = Observed value

and E = Expected value

Yate's correction was always employed in these calculations, as there was only one degree of freedom.

Once the χ^2 value had been calculated, statistical tables of the χ^2 distribution were consulted to determine whether the calculated value exceeded the appropriate critical value at the selected level of significance. In some of the results in this study, differences up to the 10% level of significance were reported. However, for testing the validity of any observed differences the 5% level of significance (P=0.05) was chosen; i.e. when the calculated value exceeded the critical value at this level for the appropriate number of degrees of freedom the difference was considered to be statistically significant. In these experiments, there was always one degree of freedom, and therefore the critical value for χ^2 was always 3.84.

B) ID50 calculations

1) The Method Of Reed And Muench (1938)

This method is a way of using the total number of animals in the experiment, rather than just those near the 50% end point. The effect is that of using, at the two critical dilutions between which the endpoint lies, larger groups of animals than were actually included at these dilutions. By tending to equalize chance variations, this method defines the point more nearly than would have been possible if it had simply been interpolated between the two nearest results. The method is illustrated in the table below, using the results of the unvaccinated rams in the ID50 experiment (Chapter 7).

Example of ID50 Calculation By The Method Of Reed And Muench Using Data From The Unvaccinated Rams In The Trial Described In Chapter 7

a Challenge Dose	b Number Infected	c Number Uninfected	d Total Infected	e Total Uninfected	f Percentage Infected
1.23×10^8	5	0	18	0	100%
1.23×10^7	5	0	13	0	100%
1.23×10^6	3	2	8	2	80%
1.23×10^5	2	3	5	5	50%
1.23×10^4	2	3	3	8	27%
1.23×10^3	1	4	1	12	8%
1.23×10^2	0	5	0	17	0%

Five rams were tested at each challenge dose, and numbers succumbing to or resisting infection are entered in columns "b" and "c". It is assumed that a ram succumbing to infection at a given dose would have succumbed to any higher dose, column "b" is therefore added

from the bottom up, and the subtotal for each dose is entered in column "d" as the accumulated number of rams succumbing to this and lower doses. Conversely, a ram resisting a given dose would have resisted it at any lower one, so column "c" is added from the top down, and the subtotals placed in column "e". Percentage infected is calculated from columns "d" and "e", and entered in column "f".

The 50% end point lies between 1.23×10^4 and 1.23×10^6, but nearer the former. The formula for the proportionate distance of the end point above the dose next below the 50% end point is:

$$\frac{50\% - (\text{infected at dose below})}{(\text{infected dose above}) - (\text{infected dose below})} = \text{proportional distance}$$

In this case, this is 0.434.

Since the doses are on a logarithmic scale, the final reading is calculated as follows:

Log of lower dose 4.0899
0.434 (proportional distance)
x log 100 (dilution factor) = 0.434 x 2 .... 0.868

Sum (log of 50% end point) 4.9579

ID 50% (antilog) ..... 9.1×10^4

2) The Logit Transformation

In pharmacological studies, if the percentage of subjects responding is plotted against dose (or, more usually, $\log_{10}$dose), a sigmoid curve generally results (Ashton, 1972). To obtain a straight line, a transformation of the scale on which response is measured becomes necessary.

For the logistic curve (a sigmoid curve) given by the equation:

$$P = \frac{1}{1 + e^{-(\alpha + \beta x)}}$$

the transformation is:

$$l = \text{logit } P = \ln \frac{P}{1 - P} = \alpha + \beta x$$

P is the probability of a response at dose x, and α and β are the parameters to be estimated, that is the intercept and the slope of the line respectively. The terms "ln" and "e" refer to the natural logarithm function and the base of the natural logarithm function respectively.

The transformation of the dependent variate, P, is made in such a way that if the transformed variate, l, is plotted against the independent variate, x (in this project, challenge dose), the points fall on a straight line, the intercept and slope of which are given by the parameters α and β respectively. This enables any point on the line in which we are interested (in this project, where P = 50%) to be read from the line. In an experiment, the standard error of the 50% endpoint can be calculated from the standard errors of the parameters α and β, thus confidence limits may be placed on estimates so that they may be compared to each other.