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ABSTRACT OF THESIS (Regulation 7.9)

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Title of Thesis A Study of Protein Antigens of Listeria monocytogenes

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Cell wall components of L. monocytogenes and the sub-species of L. ivanovii were analysed by SDS-PAGE and Western blotting. Preliminary studies demonstrated the protein nature of blotted material. Differences in the protein antigens of L. monocytogenes (serotypes 4a and 4b) and L. ivanovii (serotype 5) were established. Differences were not detected between proteins of serotypes 4a and 4b and serotype 7.

Cell wall proteins of L. monocytogenes grown at 20°C and 37°C were defined. Differences of expression of proteins were noted, particularly with regard to a complex of 3 proteins of approximate molecular weight 29,000D expressed at 20°C but not at 37°C. This complex was found to be referable to bacterial flagellin and the results support the microscopic evidence for temperature dependent expression of flagella.

The production of an anti-flagellin monoclonal antibody allowed analysis of flagellins from different serotypes. The monoclonal antibody detected the 29,000D flagellin of serotypes 1/2b, 3b, 4a, 4b and 4d but not the flagellins of serotypes 3c and 5 which had a slightly lower molecular weight.

A study of cross-reaction of L. monocytogenes proteins with proteins of other bacterial species was undertaken. Extensive cross-reaction was revealed between somatic antigens of L. monocytogenes and proteins of S. epidermidis, S. aureus, Str. fecalis and B. subtil

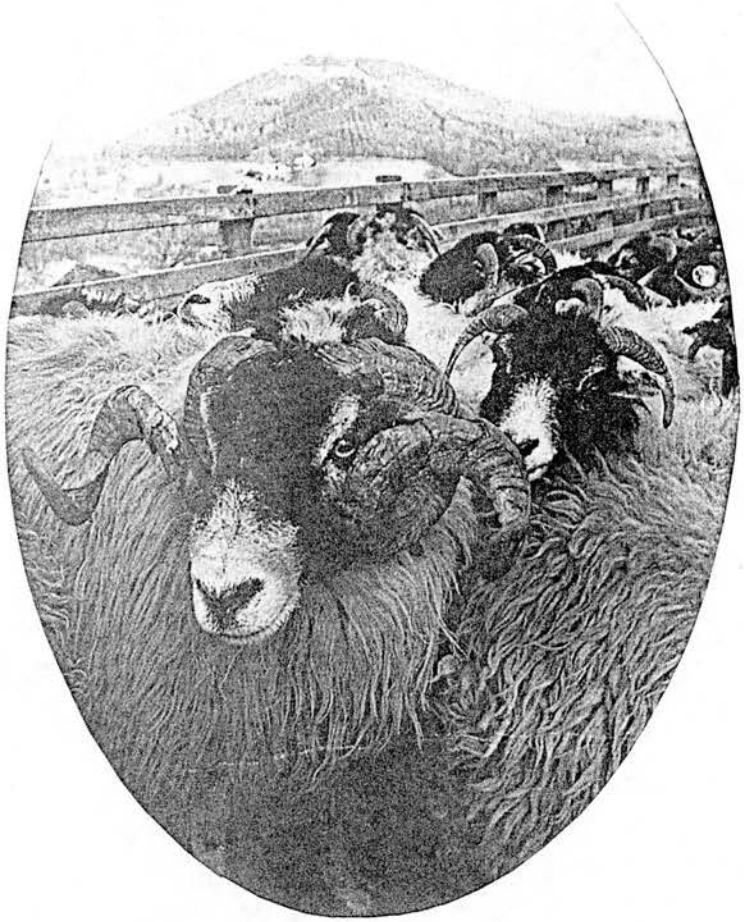
Serological studies revealed that convalescent serum from CBA mice recovering from a primary challenge of flagellate organisms (1 x LD50 organisms intraperitoneally or 100 x ID50 organisms by the oral route) did not elaborate antibody against L. monocytoger flagellin as determined by ELISA. In the same mice, antibody levels against L. monocytogenes cell wall proteins were low and inconsistent.

Serum antibody levels in 14 field cases of ovine listerial encephalitis were measured by ELISA. Titres against both L. monocytogenes cell wall protein and flagellin were of the same order as healthy control animals. Three out of 23 field cases had anti-listerial antibody in the CSF. For two of these cases serum samples from the same sheep were also available. By Western blotting it was shown that the same spectrum of antibody was present in both serum and CSF.

Sheep recovering from experimentally induced L. monocytogenes septicaemia did not have antibody levels, as measured by ELISA, significantly highter than normal uninfected controls.

LD50 and ID50 experiments were conduced in CBA mice using flagellate and aflagellate organisms of the same serotype. No difference in pathogenicity could be determined by these methods. From these experiments it was possible to define the two syndromes of murine Listeriosis, of early septicaemic death and late neurological dysfunction. Nervous symptoms were recorded in 30% of mice which died, and the syndrome occurred following either intraperitoneal or intragastric challenge with both flagellate and aflagellate organisms.

Repeated vaccination of CBA mice with adjuvanted flagella failed to protect them against a subsequent intraperitoneal challenge with flagellated organisms.



For Sid and Kitty Michaelson
..... and everything at
10 Salisbury Road
- xx

DECLARATION

I declare that this thesis has been composed entirely by myself, and that the work contained within it, except on occasions which are clearly stated, was performed by myself.

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CONTENTS

VOLUME 1

ABBREVIATIONS

BA	Blood agar
B cell	Bone marrow derived lymphocyte
CFA	Complete Freund's adjuvant
DTH	Delayed type hypersensitivity
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme linked immunoabsorbent assay
F'ab2	Antigen binding fragment
HRP	Horse radish peroxidase
IFA	Incomplete Freund's adjuvant
IgG	Immunoglobulin G
IgM	Immunoglobulin M
i.p.	Intraperitoneal
i.v.	Intravenous
KD	Kilo-dalton
LPS	Lipopolysaccharide
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
s.c.	Subcutaneous
SDS	Sodium dodecyl sulphate
spf	Specific pathogen free
T cell	Thymus derived lymphocyte
tsb	Trypticase soy broth
V.I.C.	Veterinary Investigation Centre,

CHAPTER 1

Listeria monocytogenes, with Particular Regard to Listeriosis in Ruminants: A Literature Review.

The history of listeriosis

Listeria monocytogenes infection was first described in 1926 by Murray, Webb and Swann who recovered the bacterium from the livers of sick rabbits and guinea pigs during an epizootic septicaemic disease. The disease was characterised by a leukocytosis of large mononuclear cells and so the newly discovered organism was designated *Bacterium monocytogenes*.

One year later an organism was isolated from lesions of hepatic necrosis in African gerbils (Pirie, 1927) and was named *Listerella hepatolytica*. Shortly after this it became apparent that the same organism was implicated in both diseases and a compromise of nomenclature led to the bacterium being known as *Listerella monocytogenes*.

Listerella monocytogenes was first recovered from a disease of man in 1929 by Nyfeldt where it was held responsible for an outbreak of infectious mononucleosis.

In 1931, Gill described an encephalitis of sheep in New Zealand known locally as "circling disease". Study of the histological lesions of the brain in these cases revealed, in many instances, a small Gram positive rod.

In a subsequent article, Gill (1933), described the isolation of a bacterium from the brains of affected animals, and by intranasal and intracarotid injection of live broth cultures he induced a condition which he believed clinically and histologically resembled field cases of the disease. Efforts to demonstrate a filtrable virus proved negative, suggesting a probable link between the isolated bacterium and the disease.

Four years later, the bacterium was placed in the recently defined genus Listerella, (Gill, 1937).

In 1940, Olafson, in the USA, reported the isolation of Listerella monocytogenes from the brains of cattle, sheep and goats in a series of twenty-five local outbreaks over a span of ten years. Hitherto a specific encephalitis of sheep had not been characterised in that country.

Listeriosis is now known to be of world-wide geographical distribution and importance. In Scotland, the prevalence of Listeria monocytogenes encephalitis, as reflected by submissions to the department of diagnostic histopathology of Moredun Research Institute, Edinburgh has more than doubled over the last five years, and in 1986 listerial encephalitis represented 21.2% of total submissions where a diagnosis could be made, and was the most frequently recorded cause of encephalitis.

Characteristics of Listeria monocytogenes

Since 1940, the organism has been known as *Listeria monocytogenes*, and is listed in the eighth edition of Bergey's manual with the family Lactobacillaceae under the heading, "Genera of Uncertain Affiliation". The genus comprises *Listeria monocytogenes*, together with *L. denitrificans*, *L. murrayi* and *L. grayi*. These latter species however are very rarely isolated from animal sources and are considered by many to occupy a separate genus. Following analysis of cell wall proteins, (Lamont *et al*: 1985), mycolic acids, cytochromes and DNA base composition, (Jones *et al*: 1985), and teichoic acids, (Fiedler *et al*: 1985), there is general agreement that *L. denitrificans* must be reclassified. The position with regard to *L. murrayi* and *L. grayi* is more equivocal, but major differences are apparent. However, the controversy which rages over the taxonomy of these two species forms no part of this thesis, which is confined to a study of *L. monocytogenes* and the sub-species, *L. ivanovii* (table 1.1).

The cultural characteristics of *L. monocytogenes* are described in standard bacteriology texts, (Wilson and Miles: 1964). It is a Gram positive, non sporing, diphtheroid organism of approximate length 2um. In old cultures it has a tendency to form short chains and to lose its Gram positive nature and assume varying degrees of Gram negative staining. It will grow over a wide range of temperatures (from 4°C to 45°C) with optimum growth between 35°C and 37°C. When grown at temperatures between 20°C and 25°C it is motile by means of peritrichous flagella and exhibits a

characteristic "pin wheel" motility when observed in broth cultures by phase contrast microscopy. Motility is not apparent in organisms grown at 37°C. L. monocytogenes is an aerobic or microaerophilic organism and although it does not form spores the organism can survive for months in soil, bedding, fodder and faeces under moist conditions. Dijkstra (1984) reports that naturally infected samples of brain tissue, silage, faeces, and milk suspended in tryptose phosphate broth yielded L. monocytogenes after several years of storage at 4°C.

L. monocytogenes forms small, translucent, smooth, greenish colonies with a narrow zone of β haemolysis on 5% sheep blood agar after overnight incubation. It is, however, notoriously difficult to isolate from clinical material, either because of incarceration in focal sites of infection and the intracellular distribution of the organism, or due to overgrowth by bacterial contaminants. However, since L. monocytogenes grows well at low temperatures, material kept refrigerated will often yield the organism after many months of negative sub-culturing. This is the "cold enrichment" technique first described by Gray et al (1948) and elaborated by Seeliger and Höhne (1979).

Biochemical reactions of L. monocytogenes are reviewed by Bottonne and Sierra, (1977) and seem to be uniform for the species despite variations of morphology and Gram staining.

Distribution

Listeriosis in animals and man has a world-wide geographical distribution, and outbreaks of infection have been reported in North America, Australasia, all parts of Europe, and Japan. There appears to be an increased prevalence of reported listeriosis from more temperate regions which may simply be a function of improved medical and veterinary diagnosis in these areas. As well as being a successful soil saprophyte, L. monocytogenes has also been isolated from a wide range of both domestic, laboratory and wild animal species. At the time of the review by Murray in 1955 isolation had been made from some twenty seven species, which included migratory birds. A later review by Hyslop (1974) reports the recovery of L. monocytogenes from reptiles, fishes, crustaceans, leeches, snails and a variety of terrestrial arthropods, as well as from clinical disease states of 50 species of mammals and birds. It is unknown or unclear whether all of these species are true carriers of disease or simply vectors but it is known that both man and animals can be symptomless carriers of infection, excreting the bacterium in faeces or in milk sometimes for long periods of time. Gronstol (1979a) found that in a healthy flock where there had been no cases of clinical listeriosis for the previous three years, 64% of ewes were excreting L. monocytogenes in the faeces at parturition and 41% were excreting the organism in the milk.

The longevity of the carrier state is exemplified by a case described by Donker-Voet (1965) who cultured L. monocytogenes from the mammary secretions of a dairy cow over a period of three years which included an eight week dry period. Although there was a subclinical mastitis, the milk from this animal appeared quite normal.

In a single sample survey, Kampelmacher and van Noorle Jansen, (1969) found that between 10% and 14% of clinically normal adult human populations were excreting L. monocytogenes in the faeces. In a survey where weekly samples were taken over a period of eight weeks, between 60% and 80% of individuals were found to be excreting L. monocytogenes (Kampelmacher and van Noorle Jansen, 1972). They found no increased incidence of excretion of the organism in groups of workers in animal related industries.

The incidence of listeriosis in the human population does not appear to have a direct zoonotic connection. When outbreaks of listeriosis occur both in humans and ruminants it is often associated with the consumption of contaminated foodstuffs. In ruminants, onset of listeriosis following the feeding of contaminated silage has often been reported (Wardrope and MacLeod, 1983: Dijkstra, 1984: Low and Renton, 1985). In man, causes of outbreaks have included eating coleslaw, pasteurized milk, and cheese (Schlech et al, 1983: Fleming et al, 1985: James et al, 1985).

Serotyping

The earliest serological studies were performed by Paterson (1940) who defined four serogroups based on possession of somatic [O] and flagellar [H] antigens. Types 1, 3 and 4 were defined according to [O] antigens and type 2 by a distinctive [H] antigen. This basic antigenic scheme of Paterson is still valid for serotyping today and, after serological examination of many thousands of isolates from different sources by the same techniques of absorption and agglutination, it has been extended to include serogroups 5, 6, and 7, and subdivided to form a total of 17 serotypes, (Table 1.1). Serotype 5, the sole member of serogroup 5, is described by Ivanov (1962) as a common isolate from sheep material and is distinguished from more typical strains of L. monocytogenes by producing wider zones of β haemolysis on sheep blood agar. Serotypes 4f, 4g and 6 are non-haemolytic, non-pathogenic strains of L. monocytogenes isolated from faecal material and environmental sources, and are sometimes referred to as Listeria innocua, (Donker-Voet, 1966).

In addition to classical serotyping L. monocytogenes may also be classified using bacteriophages, as reviewed by Rocourt (1986). Phages isolated from Listeria species and checked against other genera of bacteria were found to be listeria specific (Sword and Pickett, 1961: Rocourt et al, 1985). Moreover, although early work by Watson and Eveland, (1965) described a phage which appeared to have a lytic spectrum independent of serotype, later work has shown that there is a direct correlation between phage type and

serogroup, particularly with regard to serogroups 1/2 and 4 (Audurier et al, 1977). Audurier et al (1984) suggests that certain [O] antigens may act as surface receptors for phage attachment.

Phage typing is used as an adjunct to serotyping in epidemiological studies. Using 70 phages it has been possible to type 77% of isolates of L. monocytogenes and elucidate a total of 116 different lysotypes. In the face of such complexity, for practical purposes, a battery of 30 bacteriophages is recommended (Rocourt et al, 1985).

Paterson (1940) concluded that there was no relationship between serotype and either zoological species of the host or geographical distribution. It is now apparent that the vast majority of isolates of L. monocytogenes from pathogenic material belong to serogroups 1/2, 3 and 5 and to serotypes 4a and 4b, and that the distribution of these serotypes throughout the world is variable. In their review, (1979), Seeliger and Hohne state that serotype 4b comprises 65-80% of all clinical isolates in North America, whereas in Scandinavia serotype 1/2a predominates. According to Seeliger and Finger (1983), in the United Kingdom, representatives of serogroups 1/2a and 4b are isolated with equal frequency.

However, in agreement with the observation of Paterson, E.G.D. Murray (1955) wrote, "the serotypes, of which there are four, are not in any way restricted to a particular host and do not show any correlation with clinical, pathological, or other features of the disease. These variations are peculiar to the host and are not influences imposed by the strain of listeria involved". After a further thirty years of research this statement has not been refuted.

The Disease

Listeriosis of man, and the various domestic and laboratory species was reviewed by Hyslop and Osbourne, (1959), and by Gray and Killinger, (1966). In domestic rodents and rabbits the disease MOST commonly presents as a septicaemia with focal hepatic necrosis. In rabbits and chinchillas it is associated with a pathognomonic haemorrhagic necrotic metritis which either occurs post partum or, more commonly, leads to abortion. In rabbits, disease is typically associated with a peripheral blood leukocytosis.

L. monocytogenes can cause disease of domestic fowl, geese and turkeys. In chickens the disease is predominantly a septicaemic one, often with conspicuous lesions of myocardial degeneration, (Cole, 1941).

In man, L. monocytogenes can cause two separate syndromes. Listeriosis is a disease of pregnancy and/or the neonatal period, and infection in utero can cause abortion, stillbirth, or early death of the foetus with miliary granulomatous lesions. A meningoencephalitic syndrome which occurs in previously healthy neonates within the first two weeks of life is considered to be contracted either during passage through the birth canal or as a nososcomial infection , (Larsson, Cronberg and Winblad, 1979).

Listeriosis is otherwise a rare disease of man and generally occurs as a meningitis following immunosuppressive therapy in transplant recipients (Ganz et al, 1975: Niklasson et al, 1978), or as a complication of malignant disease (Louria et al, 1967).

L. monocytogenes also causes distinct syndromes in ruminants. Listerial encephalitis is a disease of adult cattle and sheep and is only rarely recorded in pre-ruminant animals. Lambs which succumb to listerial infection generally manifest a septicaemic form of the disease. Wardrope and MacLeod, (1983) report an outbreak of cerebral listeriosis in five week old lambs which they related to a period of decreased milk production by the ewes and consequent ingestion of large amounts of silage which were found in the rumen post mortem. A case of listeriosis in a two week old lamb described by Kidd and Terlecki, (1966) is the only unequivocal report of simultaneous septicaemia and encephalitis.

The incidence of listerial encephalitis in a flock is rarely greater than 8%, (Vandegraaff et al, 1981; Wardrope and Macleod, 1983), and the two syndromes of listerial encephalitis and listerial abortion are usually epidemiologically distinct. Nevertheless, Gitter et al, (1965) record an outbreak of encephalitic and septicaemic listeriosis in sheep of the same flock, and Low and Renton, (1985) describe a particularly severe outbreak of listeriosis in a flock of 196 breeding ewes which resulted in encephalitis, septicaemia and abortion of adult animals, causing 11% mortality and leaving 48% of ewes barren. These cases are reported as a contradiction of the general pattern of disease.

As determined by the pattern of monthly submissions to the Moredun Research Institute, the highest prevalence of clinical disease occurs in the spring, coinciding with all the physiological changes associated with late pregnancy and parturition, and at a time when sheep are housed and being fed silage.

Both Gronstol, (1979b) and Fenlon, (1985) show that the frequency of isolations of L. monocytogenes from silage increases with increasing pH and decreasing quality. They isolated the organism from between 40% and 60% of silage samples where the pH was greater than 5.0. Gronstol, (1980a), in a comparative study of serum factors between silage fed and hay fed sheep, reports a decrease in serum protein and circulating lymphocytes and an increase in serum iron in the silage fed group. He speculates that these factors may make animals fed silage more susceptible to disease as well as silage being the probable vehicle of infection. However, since the protein fraction which was reduced was undefined, and the circulating lymphocytes though lower were within the normal range, and since no animal in either group developed listeriosis, this hypothesis is not substantiated.

Feeding of silage is not a prerequisite for infection and clinical disease, as these may occur at pasture. Vandegraaff, Borland and Browning, (1981) record an outbreak in Victoria, Australia where the peak incidence of disease followed a period of heavy rain with severe flooding of the pasture. Vandegraaff et al (1981) remark upon the evidence of concurrent debilitating disease including copper deficiency, and chronic parasitism.

Gronstol and Overas (1980a; 1980b) infected groups of sheep respectively with Eperythrozoon ovis and Ehrlichia phagocytophilia and then failed to induce listerial encephalitis following nasal and oral administration of the bacterium. Similarly, Gronstol, (1980b) could not induce encephalitis in sheep which he had previously immunosuppressed with drugs.

Ovine listerial encephalitis: pathology, pathogenesis, and experimental reproduction

The pathology of ovine listerial encephalitis is described by Sullivan (1985). Lesions are confined to the brain where there is little gross change except for congestion of the meninges and pin-point grey foci of malacia in the medulla.

The histological appearance and location of lesions within the CNS are pathognomonic. According to Innes and Saunders, (1962), "there is probably no other bacterial encephalitis where a diagnosis may be rendered with confidence on the basis of histopathology". The typical lesions consist of perivascular cuffs, predominantly of mononuclear leukocytes, in conjunction with focal areas of neuron degeneration and neuronphagia which are associated with accumulations of mononuclear cells and polymorphonuclear leukocytes. Bacteria may be seen in association with these microabscesses both extracellularly or within phagosomes of polymorphonuclear leukocytes. Other lesions include focal gliosis and varying degrees of infiltration of the meninges with mononuclear leukocytes. These lesions are distributed from the thalamus and diencephalon back to the cranial spinal cord, but with a particular predilection for the medulla and the pons.

The pathogenesis of listerial encephalitis has been a subject of dispute. The body of opinion is that the bacterium gains access to the brain via the axons of cranial nerves. In studies of field cases of spontaneous listerial encephalitis Asahi

et al, (1957) describe neuritis of cranial nerve roots, and Urbaneck, (1962a) and Groch, (1976) found lesions of the trigeminal nerve consistent with an ascending neuritis. Charlton and Garcia, (1977), and Charlton (1977) found that sixteen out of seventeen field cases studied had neuritis of one or more cranial nerves and found Gram positive bacilli within intact axons by both light and electron microscopy. Asahi et al, (1957) succeeded in reproducing an encephalitis in goats, which clinically and histologically resembled field disease, either by inoculation of the lips or by feeding contaminated foodstuffs mixed with abrasive material calculated to traumatise the buccal mucosa. Urbaneck, (1962b), and Schleicher et al, (1968) reproduced the encephalitic syndrome by inoculation of cranial nerves. Schleicher et al describe a topographic relationship between the site of infection, the spread of neuritis and the pathology observed in the brain stem.

Barlow and McGorum (1985), made a study of neuropathological material submitted to the department of Histopathology of the Moredun Institute over a three year period. They remark on an age distribution of listerial encephalitis, with the highest incidence of disease amongst young (less than 1 year old) and older (2 to 6 years old) animals , but with very few cases in animals between 1 and 2 years of age. From this they hypothesise that the peaks of prevalence of disease are related to ages at which sheep are cutting, changing, or losing teeth, and at these times such sheep are vulnerable to infection through a breach in the buccal mucosa.

The same authors managed to reproduce clinical encephalitis in 6 out of 21 (28.5%) of sheep experimentally inoculated into the pulp cavity of either an incisor or a premolar tooth. Furthermore in 67% of these sheep they observed a histological encephalitis which they considered characteristic of L. monocytogenes infection both in nature and distribution of lesions. They conclude that this work represents a successful test of the feasibility of their hypothesis.

The original hypothesis derived from the field data is not a strong one, since it takes insufficient account of other factors influencing their calculations. The work does however represent one of the few successful experimental reproductions of the disease in sheep, and is notable for the novelty of the route employed.

After studying 15 field cases of listerial encephalitis, Cordy and Osebold, (1959) proposed a different hypothesis. They observed that lesions developed in association with small blood vessels, and that parenchymal lesions were most extensive in the depths of the brain stem, in the reticular formation. Furthermore, these lesions bore no relationship to meningeal and ependymal surfaces, or to cranial nerve roots, and were consistent with a haematogenous spread of the organism.

A third pathogenesis is proposed by Borman et al, (1960) who reproduced an encephalitis in sheep by inoculation of peripheral nerves and described lesions consistent with spread of infection via perineural connective tissue sheaths to the C.S.F. In

the face of this latter hypothesis however, it has been noted that L. monocytogenes is very rarely isolated from C.S.F., and that meningitis is not a primary or consistent feature of the disease in sheep.

Small animal models of infection

Apart from some encouraging results in mice produced by Asahi et al (1957), and Cordy and Osebold, (1959), which will be fully discussed below, there has been little further success in establishing a useful small animal model of infection. Moreover, despite legions of mice being used over the last 30 years in studies of the immune response to infection with L. monocytogenes the encephalitic syndrome has rarely been reported.

Ovine listerial abortion, pathology, pathogenesis, experimental reproduction.

Infection of pregnant sheep with L. monocytogenes may cause abortion (usually in late pregnancy), still-births, or weak lambs that die of listeric septicaemia in the postnatal period (Dennis, 1966). The pathology of listeric abortion is reviewed by Ladds et al, (1974). In field cases of abortion there is gross focal necrosis of cotyledons with ulceration and oedema of intercotyledonary areas. Microscopically there is extensive oedema of the placenta with diffuse leukocyte infiltration and necrotic vasculitis of the chorioallantois. The lesions of experimental listerial abortion induced by intravenous inoculation are

described by Molello and Jensen (1964) and claimed to be similar to those of natural cases. According to Gray, (1958) no symptoms were observed in sheep following experimentally induced abortion and delivery of infected lambs. The outbreak of listeric abortion reported by Low and Renton, (1985) which was responsible for a 10% mortality of ewes was atypical in its severity which was probably associated with the concurrent L. monocytogenes septicaemia observed.

Aborted foetuses may be either mummified or oedematous. The most consistent lesions observed are miliary foci of hepatic necrosis, 'sawdust liver', with perhaps similar lesions in lymph nodes and spleen (Gitter et al, 1965). After a study of both experimental and naturally occurring listeric abortion Jeleff and Djurov, (1969) concluded that the lesions in both instances were similar and were consistent with a disorder of circulation.

Despite the ubiquitous nature of L. monocytogenes and the apparently common reservoir of infection, listeriosis in sheep is a sporadic disease and outbreaks of listerial encephalitis in flocks typically cause a morbidity/mortality of around 8% or less. It seems likely, therefore, that the vast majority of infections are modified by the immune response of the host.

The immune response to infection with L. monocytogenes.

L. monocytogenes is a facultative intracellular parasite and has been used extensively in mouse model systems for the elucidation of the pathways of the cellular immune response (Mackaness, 1962; Miki and Mackaness, 1964; Mackaness, 1969; Mackaness and Hill, 1969).

Mackaness, (1962) demonstrated that the ability of strains of L. monocytogenes to survive in macrophage monolayers was directly related to their virulence as determined by the estimation of bacterial populations in the liver and spleen four hours following intravenous inoculation. By enumerating bacteria in liver and spleen at various times following challenge he established the growth kinetics for an intravenous infection which has been confirmed by subsequent workers (Mitsuyama et al, 1978). Briefly, 80 - 90% of an intravenous inoculum is sequestered in the liver and spleen within 10 minutes of infection where there ensues a log 10 decrease in bacteria over the following two hours. This is followed by a log linear increase (generation time four hours) in recovery of bacteria from both organs. After three days, bacterial growth ceases and rapid inactivation occurs. Mackaness (1962) remarked that the onset of this antibacterial response corresponded with massive accumulations of mitotic macrophages in liver and spleen. He noted that macrophages from the peritoneal cavity of immune mice had an increased listericidal capacity in vitro when compared to normal, resident peritoneal macrophages, (Mackaness, 1962), and that transfer of these to peritoneal cavities of normal mice afforded significant protection against a simultaneous peritoneal challenge, (Mackaness, 1964). Conversely he could demonstrate no protection at all by passive transfer of immune serum. In an effort to demonstrate cytophilic antibody he transferred urea extract of immune spleen cells and freeze-thawed immune peritoneal cells which were equally ineffective at conferring protection.

Mackaness concluded that the resistance developed by mice during infection with L. monocytogenes depended upon the altered state of host macrophages. Working with mice vaccinated with L. monocytogenes, Brucella abortus, or Bacillus Calmette-Guerin, (BCG), Mackaness, (1964) demonstrated that the antibacterial immunity which developed was not directed exclusively against the organism which provoked it. This 'non-specific' nature of the effector arm of the immune response is well documented (Armstrong and Sword, 1964; Coppel and Youmans, 1969). The process involved in the induction of the immune response however is highly specific. An anamnestic response can only be evoked by challenge with the homologous organism (Mackaness, 1964).

Mackaness, (1969) was also the first to demonstrate specific passive protection against a lethal challenge of L. monocytogenes using immune splenic lymphoid cells. He established that the protective capacity of donor cells was dependent upon the number of cells, and the time of cell transfer following immunisation of donors. Protection was first apparent using 4 day immune cells, reached an optimum at 7 days and declined thereafter. This corresponded closely to the onset, peak, and gradual decay of specific bacterial immunity seen in vaccinated animals (Mackaness, 1962).

Termination of infection with L. monocytogenes is followed by heightened specific resistance which depends on the host's capacity to generate lymphocytes faster and in larger numbers than a naive host, (North, 1975). Short lived replicating T cells are responsible for macrophage activation and bacterial elimination

during both primary and secondary infection,and the activity of these cells decays rapidly after the organism has been removed. The anamnestic response is initiated by a residual population of stable non-replicating small T lymphocytes, (North and Diessler, 1975).

Immunity in mice is apparently of short duration. Kearns and Hinrichs (1977), using Swiss Webster mice showed that immunity to challenge with 10 x the mean lethal dose (LD_{50}) of organisms given intravenously was beginning to decline two weeks after primary vaccination, and had completely disappeared after 5 weeks. They also found that immunity was independent of vaccinal dose, and that its duration could not be extended by repeated vaccination.

Adoptive transfer of listerial immunity in mice occurs optimally when immune donors and the host are homologous at the I region of the H-2 complex, (Zinkernagel et al, 1977). In vitro studies have also shown that macrophage activation by listeria immune T cells works best when mouse cells are of a homologous haplotype with regard to immune response genes, (Farr, Dorf and Unanue, 1977). For the initiation of a lymphocyte response, an initial binding step is necessary between receptive T cells and listeria pulsed macrophages bearing 1-A region gene products, (Ziegler and Unanue, 1979).

The ability to grow T lymphocytes in culture and the development of identification systems using specific surface antigens as markers, (Lyt:L3T4) have enabled workers to define the immune response more precisely, (Dialynas et al, 1983: Sperling Kaufmann and Hahn, 1984: Kaufmann et al 1985).

The precise phenotypic expression of specific listerial resistance by macrophages is disputed. Campbell, (1986) disputes the role of an 'activated' macrophage and suggests that it is the influx of large numbers of recruited phagocytes which mediate protection. This hypothesis is partly based on the work of Czuprynski *et al*, (1984) who showed that peritoneal exudate cells from normal mice elicited by an inflammatory agent, and peritoneal exudate cells from hyperimmunized mice elicited with listerial antigen were equally capable of killing bacterial cells *in vitro*. Czuprynski *et al*, (1985a) also demonstrated that mice injected with immune T cells recruited more macrophages to the peritoneal cavity after challenge with listerial antigen than did mice injected with non immune T cells, but that macrophages again did not differ in their ability to kill *L. monocytogenes* *in vitro*.

This contradicts the findings of Harrington-Fowler *et al*, (1981) who found that non specifically elicited peritoneal macrophages from normal mice were unable to control the growth of *L. monocytogenes* in vitro but that elicitation of peritoneal macrophages from immune mice with heat killed organisms rendered them listericidal.

The difficulties of interpreting the results of macrophage listericidal assays is compounded by the variety of different protocols used. Harrington-Fowler *et al*, (1981) for example, used adherent cells harvested eighteen hours after elicitation and incubated during the post phagocytic phase with penicillin, whereas Czuprynski *et al*, (1985a) used suspension cultures in an antibiotic free assay harvested at 48 hours following elicitation and used at a different macrophage/parasite ratio. True postphagocytic events in the various macrophage populations remain unclear.

Delayed Type Hypersensitivity (DTH)

Delayed Type hypersensitivity is frequently associated with infection with facultative intracellular bacteria. Mackaness, (1962) noted the close temporal relationship between the development of a DTH response and the onset of bacterial immunity. By passive transfer experiments using splenic lymphocytes, he later demonstrated that the level of DTH evoked was proportional to the degree of protection conferred (Mackaness: 1969). Passive transfer of DTH was shown to be a T cell dependent process by Youdim et al, (1973) and Pearson and Osebold, (1974), who abrogated protection by mixing transferred cells with anti θ antiserum,raised against mouse thymocytes.

Osebold, Pearson and Medin, (1974) demonstrated that antimicrobial resistance could exist in the absence of a measurable DTH response in the footpads of mice. They concluded that all committed lymphoid cells do not release the same array of lymphokines, and suggest that DTH and cellular immunity are mediated by different populations of lymphocytes.

Latterly, work using T cell clones has helped to clarify the situation. Kaufmann and Hahn (1982) demonstrated that a single T lymphocyte clone was capable of listeria-specific proliferation, interleukin secretion, and bystander help for B cells in vitro, as well as protection against a live listeria challenge and DTH response against listeria antigen in vivo. Sperling, Kaufmann and Hahn, (1984) showed that a single T cell clone was capable of producing both macrophage activating factor and migration inhibition factor.

Genetically determined listerial resistance

Superimposed upon this well defined picture of the specific murine immune response to listeriosis is a genetic susceptibility/resistance of certain inbred strains (Review, Kongshavn 1985). Genetic differences between the various strains have been described by Cheers and McKenzie, (1978); and Skamene and Kongshavn, (1979) who observed from 10-100 fold differences of LD_{50} between Listeria susceptible (S) and Listeria resistant (R) strains. S-strain mice include CBA, DBA/1, DBA/2, Balb/c and A/J mice. NZB and C57/BL mice are comparatively resistant. The phenotypic expression of genetically determined resistance resides in the early non specific phase of the immune response which follows primary infection of the naive animal. Kongshavn (1985) concludes that the differences observed between levels of infection in liver and spleen of the various mouse strains during the first few days post inoculation is therefore a reflection of the efficiency of macrophage production, emigration and accumulation at the site of microbial deposition.

Further evidence that this relative, 'natural' resistance to L. monocytogenes is a macrophage mediated phenomenon is provided by Stevenson et al 1980: and Sadarangani et al 1980.

It is clear from experiments measuring anti-listerial resistance in H-2 congenic and recombinant strains of mice that 'natural' resistance to L. monocytogenes is not linked to the major histocompatibility complex (Cheers and McKenzie, 1978; Skamene et al, 1979).

Studies by Cheers and McKenzie, (1978) and Cheers et al, (1980) using progeny of C57Bl (R-strain) and Balb/c (S-strain) mice have shown that a single dominant autosomal gene (the Lr gene) is the major determinant of resistance/susceptibility to listeriosis. This was affirmed by Skamene and Kongshavn, (1979) and Skamene et al, (1979) who used the A/J mouse as the S-strain prototype. Gervais et al, (1984) used recombinant inbred strains derived from C57Bl and A/J progenitors to study the distribution pattern of the resistance trait. The results from 13 inbred strains suggested an allelic difference at a major locus (Lr-1) is responsible for resistance/susceptibility to L. monocytogenes. In addition, a further, unlinked gene (Lr-2) was postulated which modified the level of susceptibility and rendered susceptible mice either fully or semi-susceptible.

The precise phenotypic expression of early 'natural' immunity is also in question. Harrington-Fowler et al, (1981) showed that the pattern of bacterial replication in in vitro cultures of peritoneal macrophages from Balb/c, C57Bl/6 and an outbred strain of mice corresponded to the bacterial growth in livers and spleens of these animals. They suggested therefore that differences between mouse strains resided in the ability of non immune macrophages to handle the organism.

Czuprynski et al, (1985b) remarked that inflammatory macrophages from C57Bl/6 mice exhibited a slight but significantly enhanced ability to kill L. monocytogenes in vitro as compared to inflammatory macrophages from A/J mice but attributed the superior resistance of the C57BI/6 strain to an enhanced recruitment of cells.

Wood et al (1986) in a comparative study of C57Bl/10, CBA and Balb/c strains observed no difference of in vitro chemotaxis of peritoneal cells and no difference in the numbers of macrophages recruited following intraperitoneal inoculation of L. monocytogenes. Using an assay system which measured the release of radiolabelled DNA from L. monocytogenes as an indicator of bactericidal reactivity of cells they noticed a greatly superior activity of macrophages from the C57BI/10 strain.

It is possible that the differences observed by Czuprynski et al (1985a) and Wood et al (1986) relate to the fact that Czuprynski used A/J mice as the S-strain prototype which are known to be deficient in the fifth component of complement, whereas the latter workers used Balb/c mice which are complement sufficient.

The Polymorphonuclear Leucocyte (PMNL) Response

The early infiltration of PMNL's into infective foci has been observed (Mackaness, 1962; Mandel and Cheers, 1980) but the importance of PMNL's in effective defence has not been fully researched. Czuprynski, (1984) demonstrated that PMNL's elicited to the peritoneum by sterile irritants were as capable of killing L. monocytogenes as were elicited macrophages, and that they were present after four hours elicitation with a peak of listericidal activity within the first 24 hours. Conversely, Wood et al, (1986) found that the bactericidal activity of peritoneal exudate cells, harvested six hours following elicitation and which contained 50% PMNL's, was not markedly different from control populations of resident cells. They point out that this apparent contradiction of the data of Czuprynski may have been due to the difference of their bactericidal assay, and the fact that they did not use opsonins.

To summarise: the immune response in mice is mediated primarily by macrophages recruited to the site of microbial deposition by committed lymphocytes. The induction of immune T cells is a highly specific antigen driven process which requires homology of immune response genes between antigen presenting cells and T lymphocytes. Recovery from infection is followed by a transient phase of enhanced immunity.

An inherent difference in susceptibility of various inbred strains exists. This relates to differences of macrophage recruitment and/or activation during the early non-specific stage of the immune response. Resistance/susceptibility has an apparently simple, Mendelian pattern of inheritance unrelated to the MHC.

The Serological Response

The serological response to L. monocytogenes has been used not only as a means of serotyping as described above but also as a diagnostic measure and an epidemiological tool. Early serological studies were reviewed by Gray and Killinger, (1966) who concluded that although the sera of several species had been studied by various workers, "the only real contribution made by many was an expansion of the bibliography". They point out that many workers disregard the fact that not only does L. monocytogenes commonly autoagglutinate but also shares common antigens with other ubiquitous bacterial species.

The most commonly used test throughout the last 40 years has been the tube agglutination test using either [O] antigen preparations (heat killed organisms) or [H] antigen preparations (formol killed organisms).

It is possible to overcome the problem of autoagglutination of antigen preparations by ultrasonication (Gray: unpublished data, Gray and Killinger,1966) and render other non-agglutinable strains agglutinable by treatment with trypsin (Osebold et al, 1965), such that clear end points are achieved, but the overwhelming disadvantage of this test is its non specificity. Seeliger (1958a) found that [O] antigens of serotypes 1 and 3 gave marked cross reaction with both Staphylococcus aureus and with Lancefield's group D enterococci. Welshimer, (1960) showed cross-reaction between formol killed L. monocytogenes and S. epidermidis. Gray (unpublished data, Gray and Killinger, 1966) claimed that agglutination titres of 1:640 in cattle serum against L. monocytogenes serotype 1 [O] antigen were rendered negligible by absorption with S. aureus. He similarly reports serotype 1 [O] antigen titres of 1:640 in a closed flock of sheep, and negligible titres in cattle sera taken several weeks after abortion of fetuses infected with serotype 1. Seeliger, (1958b) observed that in both hyperimmunized and naturally infected animals the increase in titre of [O] agglutinating antibody preceded any increase in [H] antibody which made it imperative to check both in suspect cases. Because a large percentage of the normal animal population with no history of listeriosis has variable titres of agglutinating antibodies, the cut-off between positive and negative is decided arbitrarily.

Therefore, it is generally agreed that the agglutination test is of little value except where a rise in titre can be demonstrated during the course or convalescence of a disease which is suggestive of listeriosis (Gray and Killinger, 1966; Potel, 1963).

Work by Osebold and Aalund, (1968) suggests that agglutination tests may be made more specific by the prior reduction of the sera under test with 2-mercaptoethanol which effectively removes IgM antibodies. In extensive tests on human, bovine and ovine sera from clinically normal individuals with no known previous exposure to L. monocytogenes all 512 sera contained 2-mercaptoethanol sensitive (IgM) antibodies. Less than 2% of samples contained agglutinating IgG antibodies following reduction. They found that animals vaccinated with live culture synthesised IgG antibodies, that IgG titres were significantly higher in cattle and sheep from exposed herds, and that although titres were low they were consistently produced. However, since the test does not measure early antibody, it probably has more value for epidemiological investigation than for the diagnosis of early acute infection.

Precipitation by agar gel diffusion has also been used. Muraschi and Tompkins, (1963) describe Ouchterlony plate tests using aqueous ether extracts of various strains of L. monocytogenes and offer this as a useful alternative to conventional serotyping. They do not investigate the possible use of this test for diagnosis, and Seeliger, (1958a) demonstrates cross - reacting precipitating antigens between Streptococcus fecalis and L. monocytogenes.

Other tests which have been tried include looking for complement fixing antibodies against trichloroacetic acid extracts, (Seeliger, 1958a) or lysates prepared by freeze-thawing of merthiolate killed cultures, (Patocka et al, 1956). Seeliger, (1958a) claims that the complement fixation test is comparatively specific provided that antiserum is first absorbed with S. aureus cells.

Sachse and Potel, (1957) worked on a haemagglutination test but found marked cross-reaction between L. monocytogenes serotype 1 and Group A and D streptococci and enterococci. Neter et al, (1960), also using a haemagglutination test could not demonstrate antibody which was species specific for L. monocytogenes since all antibody could be completely absorbed by S. aureus and B. subtilis.

More recently, Potel, (1979) used bacterial agglutination reactions in conjunction with growth-inhibition and agglutination-immobilization tests and determined that where the agglutination test detected both [O] and [H] antibodies, the growth inhibition test was a test for [O] antibodies and the agglutination-immobilization test detected flagellar antibody. He implemented these tests in a field study of four flocks including 237 sheep. Three flocks had no cases of listeriosis within the previous three years whereas flock four had been exposed to L. monocytogenes within the previous six months. Results showed very low titres in any flock tested by any of the methods. Potel concludes that determining levels of infection in flocks by these serological methods is not possible.

Kuhlmann-Berger and Potel, (1985) used these three tests to study the kinetics of antibody response in sheep vaccinated with mouse avirulent R forms of serotypes 1/2a and 4b and concluded that only by using the agglutination-immobilization test which measures [H] antibody was a significant increase in titre recorded one month after vaccination of unprimed animals, which persisted for some three months.

North and Diessler, (1975) failed to detect an increased titre during primary infection of mice, using a haemagglutination test with ammonium sulphate precipitate of culture supernatant as the adsorbed antigen. At this time, mice showed a strong DTH response to a foot pad inoculation of the same antigen. They also found that the antibody response to a second infection resembled the kinetics of a primary response and generated mercaptoethanol sensitive antibody.

Vaccination

Very early vaccination trials demonstrated the inefficacy of 'bacterins' and killed organisms to confer immunity, and demonstrated the requirement for a sublethal inoculation of live, virulent organisms, (Graham et al, 1940; Graham and Levine, 1943; Osebold and Sawyer, 1957; Osebold et al, 1959).

Later work has confirmed that the efficacy of vaccination relates directly to the replication of the organism in the host for the first 3-4 days following infection. Coppel and Youmans, (1969)

failed to protect mice using L. monocytogenes cells killed either by heat or by ultraviolet radiation. They were equally unsuccessful using a streptomycin dependent strain or a listerial ribosomal fraction, but did find that large quantities of an attenuated strain afforded protection. Conversely, le Garrec et al, (1981) increased the immunity of mice to listeriosis, by vaccination with irradiated organisms. This was only possible however by using trehalose dimycolate as an adjuvant.

There is a live attenuated vaccine commercially available in Bulgaria. This vaccine (for use in sheep) has been developed by Ivanov (1985) by attenuating field strains of serotypes 1/2b and 4b by multiple passage through rabbits.

This vaccine has had a limited trial outside Eastern Europe. In Norway , Gudding et al (1985) vaccinated 1665 sheep from a total of 70 flocks with the imported vaccine. Half of each flock was left unvaccinated to serve as controls. The percentage mortality for the two groups during the ensuing winter season were given as 0.265 for the 'vaccinates' and 0.775 for the non vaccinated animals, with a statistical difference given of $p<0.05$. The derivation of the statistical data is not revealed.

There are major discrepancies between the tables and the text of this communication which considerably discredit the data given. Moreover, the use of such small sample numbers to investigate a disease of low prevalence does not allow serious evaluation of this vaccine.

Van der Meer et al, (1977) and Van Dijk et al, (1980) claimed to have protected mice by use of heat killed organisms. Their claims however were discredited five years later by von Koenig et al, (1982) who showed that the temperature they had used to heat kill bacteria was insufficient and allowed enough organisms to survive and cause infection and subsequent immunity in the mice used.

Audurier et al, (1980) and Audurier et al, (1981) showed that the pathogenicity of L. monocytogenes was related to its ability to colonise the spleen and lymph nodes during the first three days following infection. Using serotype 1/2a, Hof, (1984) extended these observations and showed that wide variations in both pathogenicity, and the ability to parasitise mice could exist within a single serotype. He demonstrated that a smooth haemolytic strain multiplied in the spleen of normal mice for three days, caused chronic infection in nude mice, and conferred long lasting immunity. A rough haemolytic strain of the same serotype was unable to multiply in the spleens of naive mice even when these were treated with dextran sulphate, was slowly eliminated by nude mice, and evoked a weaker immune response. Finally, a smooth, non haemolytic serotype was rapidly eliminated both in dextran sulphate compromised mice and nude mice, and evoked no measurable protective immunity. However Hof did show that protective immunity once established could be recalled equally well by any of the strains used.

This demonstration by Hof of a common immunogenic principle was confirmed by Kaufmann, (1984). He designated strains of L. monocytogenes either 'persistent' or 'non persistent' according to their ability to parasitise the spleens of infected mice. Although 'persistent' strains induced stronger immune responses, heat killed cells of all L. monocytogenes strains were equally capable of causing antigen induced proliferation of protective T cell clones. They concluded that the various serotypes share a common protective epitope which is not effective in some strains because of rapid elimination from the host.

The protective effects of listeria cell products is unclear. Petit and Unanue, (1974) showed that sterile broth culture supernatants were mitogenic for B lymphocytes both in vitro and in vivo and caused a transitory state of non-specific resistance to listeriosis as determined by spleen counts 48 hours after infection. They related increased resistance to morphological change of peritoneal cells harvested 2-5 days following intraperitoneal (ip) inoculation of broth supernatants. The increased resistance they observed was of the same order as that produced by E. coli endotoxin. The active principle of the preparation has not been defined.

Paquet et al, (1982) demonstrated that a crude L. monocytogenes cell wall preparation enhanced resistance of mice to a subsequent challenge with Candida albicans or a mammary carcinoma. They also found that cell walls were mitogenic for spleen cells in vitro at a level comparable to LPS. Again the active principles of the crude cell wall preparation have not been defined.

In 1974, Rodriguez, McClatchy and Campbell protected mice against 25 LD_{50} L. monocytogenes by giving crude cell wall fraction together with LPS 7 days before a challenge inoculum. However, Baker and Campbell, (1978) went on to show that a cell wall fraction treated with proteases and nucleases decreased the resistance of mice to a challenge infection when given intraperitoneally up to three days before an ip challenge.

Much of Listeria cellular immunology over the last decade has been done by Kaufmann using undefined Listeria whole cell antigens. Kearns and De Freitas, (1983) used a water soluble intracellular extract from mechanically disrupted L. monocytogenes to cause in vitro proliferation of protective T cells but, again, did not resolve the different antigenic components involved. This thesis includes a preliminary study of protein antigens of different serotypes of L. monocytogenes and their role in the humoral immune response.

The Gram Positive Cell Envelope

Gram positive cell wall structure is reviewed by Leive and Davis, (1980). Briefly, in sections fixed by osmium tetroxide, the Gram positive cell envelope exhibits a homogenous electron dense wall structure 20nm - 80nm thick surrounding a typical trilaminar cytoplasmic membrane of 7.5nm. Freeze-etching of some Gram positive species shows that this electron dense layer is composed of two layers. Furthermore, negative staining of some Gram positive cells reveals a surface pattern of regular hexagonal tightly packed spherical subunits which have been identified as proteins.

The electron dense layer is composed of 'peptidoglycan' which can be readily hydrolysed by lysozyme to yield its constituent substituted disaccharides. The basic features of peptidoglycan are of a backbone of alternating residues of N-acetyl glucosamine and N-acetyl muramate in B 1 -4 linkage and cross linked by peptide bridges between the residues of N-acetyl muramate to give a three dimensional structure. Variation in peptidoglycan of different species is primarily afforded by the structure and extent of the peptide cross linkages, (figs 1.1 and 1.2).

A further component of Gram positive cell walls is the teichoic acids; the biochemistry and structure of which are reviewed by Rose, (1976). Basically, these polymers are chains of up to thirty residues of ribitol (ribitol teichoic acids) or glycerol (glycerol teichoic acids) linked by phosphodiester linkages and held to the wall by covalent attachment to the N-acetyl muramate residue of the peptidoglycan (figs 1.1 and 1.3). Teichoic acids attached to membrane glycolipids are known as the lipoteichoic acids (fig 1.2). The walls of almost all Gram positive species contain either ribitol or glycerol teichoic acids. Ribitol residues may have linked D-alanine residues and various sugar substituents. Teichoic acids are strongly negatively charged and are thought to play a crucial role in the attraction of magnesium ions needed for enzyme activity and maintenance of membrane integrity. Wall teichoic acids are also surface-active components which greatly increase antigenic diversity and form specific receptor sites for binding of bacteriophages and antibodies.

Studies by Electron Microscopy

By electron microscopy, Smith and Metzger, (1962) claimed to demonstrate a capsule-like structure surrounding L. monocytogenes cells which had been treated with "capsular immune serum" to which ferritin had been conjugated. It is difficult to understand their conclusions, since the use of a capsular specific antiserum already presupposed that a capsule existed. The existence of true capsules could not be demonstrated by Seeliger and Bockemuhl, (1968). Edwards and Stevens, (1963) suggested that capsules seen by electron microscopy were an artefact due to adsorption of substances to the outermost layer of the cell wall brought about by treating cells prior to fixation with salt solutions.

North, (1963) made an ultrastructural study of late log phase broth cultures of L. monocytogenes. He reported a 20nm thick trilaminar cell wall consisting of two 3nm electron dense bands separated by a lighter staining middle layer of about 12nm. In older cultures cell walls appeared thinner and more diffuse with the three layered structure being less apparent.

North also defined a relatively thick (11nm) three layered cytoplasmic membrane within the cell wall, and separated from the wall by a space which was "periodically interrupted by outfoldings of the cytoplasmic membrane". He suggested that this space was likely to be an artefact due to shrinkage during the dehydration process.

Edwards and Stevens, (1963) agree with North with regard to the appearance of the cell wall but define a complex five layered structure for the cytoplasmic membrane which accounts for the 'space' observed by North. By treating cells with hypertonic solution they demonstrate a typical three layered unit membrane immediately adjacent to the cytoplasm. Outside this, however they define a further electron dense layer closely opposed to the cell wall and separated from the unit membrane by a light zone across which are many electron dense bridges. (These 'bridges' probably correspond to the plasmalemma 'outfoldings' described by North).

Ghosh and Murray, (1967) studied the fine structure of different serotypes of L. monocytogenes in relation to protoplast formation. They too defined the cell wall as a three layered structure, and also remarked upon the existence of a cross-bridged layer between the cell wall and the plasma membrane. These bridging structures have also been observed in B. subtilis, (Glauert et al: 1964). Ghosh and Murray however showed that the plasma membrane of stable protoplasts consisted only of the inner three layers (ie: the normal components of a limiting membrane) and concluded that the bridging layer was a cell wall component.

Ghosh and Murray also showed that different strains of L. monocytogenes exhibited a variable susceptibility to lysozyme which was not related to serotype. Moreover, pretreatment of bacteria with lipase considerably enhanced the sensitivity of cell walls to lysozyme, and one strain was lysed by lipase alone. They speculated that the cross links of the bridging layer have a lipid component.

All workers demonstrate a complex system of branching tubular cytoplasmic structures which appear to be contiguous with the cytoplasmic membrane, closely associated with nuclear material and numerous at the site of septum synthesis.

Biochemistry and Immunochemistry of Listeria monocytogenes cell walls

A general description of Gram positive cell wall structure is given by Leive and Davis (1980), and according to Fiedler et al (1985); "the cell wall of Listeriae is typical of a Gram positive eubacterium."

An accepted model of peptidoglycan and teichoic acid arrangement in the gram positive cell wall is given schematically in fig 1.1. Peptidoglycan gives the bulk and rigidity to the cell wall, whereas the function of the teichoic acids is unclear. Since they are strongly negatively charged ions it is hypothesised that they have a role in regulating the supply of cations to the membrane; and in some species, viz Lactobacilli, they are the dominant cell wall antigens, (Rogers: 1983).

Biochemical analysis of the peptidoglycan component of thirty two strains of L. monocytogenes by Fiedler et al, (1984) showed that all strains contained the cross-linked mesodiaminopimelic acid murein variant of the basic structure shown in fig 1.3. They also showed that each strain had polyribitol teichoic acid as the prevalent accessory cell wall polymer, (fig 1.4). They found a sufficient correlation within the teichoic acids of the different serogroups of L. monocytogenes to suggest that the teichoic acids are the immunochemical determinants of the different serotypes.

Ullmann and Cameron, (1969) studied the antigen specificity of different serotypes of L. monocytogenes. They extracted immunologically active carbohydrates of various kinds from cell walls. The predominant carbohydrates extracted from serotypes 1 and 2 were glucosamine and rhamnose. Galactose, glucosamine, and rhamnose were all extracted from serotype 3, and glucose and galactose from serotypes 4a and 4b. The major antigenic determinants as revealed by precipitin-inhibition tests using purified monosaccharide showed rhamnose to be the major determinant of serotypes 1 and 2. Precipitin reactions could not be detected with carbohydrates extracted from serotype 3 organisms using either homologous or heterologous antiserum, and the major determinants of serotypes 4a and 4b were galactose and glucose respectively.

Kamisango et al, (1983) isolated an immunologically active teichoic acid from the cell walls of the 'E.G.D.' laboratory strain of L. monocytogenes which accounted for 20% of weight of the cell wall. They deduced the chain to be approximately 120,000 D MW, containing equimolar amounts of N-acetyl glucosamine, rhamnose, ribitol and phosphorus. Precipitation-inhibition tests using purified monosaccharide again revealed rhamnose to be the major antigenic determinant. The absence of a D-alanine substituent common to most bacterial ribitol teichoic acid polymers renders the teichoic acids of L. monocytogenes unique.

From the combined studies of Rhuland and Fiedler (1985), and Fiedler et al (1985) it was concluded that the membrane attached lipoteichoic acids of L. monocytogenes were irrelevant in the differentiation of serotypes, but that the occurrence of proteins as somatic antigens needed careful investigation.

There has been very little work done to elucidate any of the protein antigens of Listeria cell walls. Delvallez et al (1979) studied a crude cell wall preparation of serotype 4b by crossed immunoelectrophoresis with the aim of preparing a listeria-specific antigenic fraction which could be used in serological studies. They defined an antigen present on all serotypes of L. monocytogenes and subsequently purified this antigen by affinity chromatography using monospecific antiserum. The monospecific antiserum precipitated a single band when used in a crossed immunoelectrophoresis assay against crude soluble extract of L. monocytogenes cell walls. These workers speculate that this is a listeria-specific antigen since no cross-reactions were observed using rabbit sera prepared against S. fecalis, S aureus, S. epidermidis, B. subtilis and C. pseudodiphtheriticum, and since monospecific anti-antigen 2 serum did not react with these bacterial species. Although the authors are optimistic about using this defined antigen either in a passive agglutination reaction or an enzyme-linked immunosorbent assay in order to detect human antibodies they have published no further data.

Using ion exchange chromatography and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) Antonissen et al, (1986) defined and purified a protein of approximately 20 kilodaltons (KD) molecular weight which induced a DTH response in mice. They remarked that, despite the 'drastic' conditions of antigen extraction with deoxycholate and subsequent ion exchange chromatography, the various fractions had retained their antigenic activity. They point out that since recognition of protein

antigens by T cells appears to depend mainly on amino acid sequence rather than secondary configuration, these purification and analytical procedures provide a useful means of examining individual antigens of the cellular immune response. Despite these observations, little other work has been published in this area.

Preparation of bacterial flagella and flagellin

Flagella are readily sheared from the bacterial cell body by mechanical forces and separated from somatic antigen by cycles of differential centrifugation. Whiteside and Rhodes-Roberts, (1985) separated flagella of Pseudomonas species by agitation for 60 minutes using a 'Griffin' shaker, and recovered flagella by repeated ultracentrifugation of supernatants and cell washings, and resuspension in borate buffer. Schmitt et al, (1974) sheared flagella of Pseudomonas rhodos by shaking vigorously with glass beads on a reciprocal shaker for 1 minute at 1,400 strokes per minute, and again recovered sheared flagella by ultracentrifugation. Richardson and Parker, (1985) removed flagella using a commercial blender and, by differential centrifugation through a caesium chloride gradient , separated flagellar core material from outer membrane components.

Structure of Prokaryotic Flagella

Studies of flagella from many bacterial species have revealed the same basic structure. Flagella consist almost entirely of linear arrays of the protein flagellin together with small quantities (1-5% w/w) of carbohydrate and lipid (Nossal and Ada,

1971). Rose, (1976) states that flagellar filaments are anchored within the plasma membrane and the cell wall to an undefined basal structure by flagellar hooks, which consist of proteins antigenically different from the filament. Flagellar hooks of Pseudomonas rhodos have been clearly demonstrated by Schmitt et al, (1974) by transmission electron microscopy. Rose, (1976) also describes individual flagellar molecules (flagellin) as either globular or ovoid subunits which are packed in an arrangement which varies in flagella from different species.

Preparation of bacterial flagellin

Bacterial flagella are readily depolymerized into monomeric flagellin molecules by dissociation with acid (Whiteside and Rhodes-Roberts, (1985), with detergents (Montie et al, 1982), and with heat (Koffler et al, 1957; Martinez and Rosenberg, 1964). Flagella may be repolymerised by the addition of saturated ammonium sulphate to form a 15% (v/v) solution. The ability of flagellin to spontaneously repolymerize is noted by both Nossal and Ada, (1971) and Rose, (1976). Stored flagellin will slowly polymerize at a rate dependent upon the amount of protein and salt present. This rate is enhanced by repeated freezing and thawing of the sample. The subunits reaggregate to form helical fibrils which, although larger, look like normal flagellar filaments by electron microscopy. Using these techniques Ibrahim et al, (1985a), Allison et al, (1985) and Richardson and Parker, (1985) prepared flagellins from Salmonella spp., Pseudomonas spp. and Vibrio cholerae respectively, which appear as one or two bands only on SDS polyacrylamide gels stained by Coomassie blue: a criterion judged by the authors to denote purity of preparation.

Molecular weights of bacterial flagellins are also variable with regard to different species, and commonly range between 30 and 50KD. Salmonella flagellin is of approximate molecular weight 40KD (Nossal and Ada, 1971). Schmitt et al, (1974) identified two 37KD isoprotein flagellin molecules in purified 'plain' flagella of Pseudomonas rhodos by SDS-PAGE, following reduction of sample with 1% SDS and 1% 2-mercaptoethanol. Approximate molecular weight determinations of flagellins of Pseudomonas aeruginosa, P. putida, and P. fluorescens were made by Whiteside and Rhodes-Roberts, (1985) from sedimentation coefficients and were found to be between 38 and 43KD. These values were in accordance with values obtained from estimates based on methionine content. Richardson and Parker, (1985) using SDS-PAGE and immunoblotting elucidated two flagellar core proteins of Vibrio cholerae of apparent molecular weight 47KD and 49KD.

Some general physiological and chemical characteristics of bacterial flagella are discussed by Rose, (1976). Nossal and Ada, (1971) use Salmonella flagella and flagellins as antigens in their studies of the immune response, and therefore define their biochemical characteristics fairly precisely.

Rose, (1976) states that although individual flagellins differ in their amino acid composition they typically contain either no detectable amounts, or very little cysteine, histidine, proline, tryptophan or tyrosine. In their study of Salmonella flagellin, Nossal and Ada (1971) could detect neither cysteine or tryptophan, and only small quantities of either methionine, proline,

phenylalanine or tyrosine. Many arginine and lysine residues were found to be present. In their study of P. aeruginosa, P. putida and P. fluorescens, Whiteside and Rhodes-Roberts, (1985) could find no tryptophan in the flagellins of any of the three type strains and trace amounts of cysteine in P. putida only. The predominant amino acids in the flagellins of the three type species tested were alanine and aspartic acid.

The linear arrangement of flagellin molecules in the flagellar filament is variable. The simple helical configuration observed by Schmitt et al, (1974) in the 'plain' flagella of Pseudomonas rhodos using optical diffraction agrees with the sub-unit arrangement proposed by Lowy and Spencer (1968). In contrast, the same strain of P. rhodos is capable of elaborating a 'phase variant', 'complex' flagellum consisting of an internal core surrounded by a closely adherent sheath of three helical bands. The authors observed that cells with plain flagella showed normal translational motion and that cells with complex flagella showed rapid spinning and speculate on the determination of function by structure.

The best known example of flagellar 'phase variation' within a genetically homogenous bacterial strain is given by Salmonella species which produce two antigenically distinct flagella, (Miles and Wilson 1964).

Other than observations of the temperature dependent motility of L. monocytogenes, (a phenomenon shared by P. paratuberculosis - Miles and Wilson, 1964), there has been little else published on the ultrastructural, physical and biochemical properties of the flagella of these organisms.

Serological Properties of Prokaryotic Flagella

The Kauffmann-White Scheme (described by Miles and Wilson, 1964) classifies Salmonella serotypes on the basis of the serological properties of their O and H antigens. Salmonella detection by immunoassay of O antigens is not possible because of close antigenic relationships with other members within the family Enterobacteriaceae. However, Ibrahim et al, (1985a) demonstrated the feasibility of a specific radioimmunometric assay for Salmonella detection using bacteria immobilized on titanous hydroxide and a battery of 10 antisera raised in rabbits against purified flagella of different Salmonella serotypes. None of the rabbit antisera under test exhibited cross-reaction with surface antigens and native flagella of sixteen other enterobacterial species which had been similarly immobilized.

However, in an accompanying paper, Ibrahim, (1985b), using a modified radioimmunometric techique, demonstrated cross-reactions between Salmonella flagella and the flagella of ten other species of Enteriobacteriaceae which had first been depolymerized by heat. The authors speculate that this demonstrated the unmasking of common antigenic determinants due to changes in the tertiary structure of the flagellin sub-units during depolymerization. This phenomenon is also mentioned by Nossal and Ada, (1971).

The work described by Whiteside and Rhodes-Roberts, (1985) is also an attempt to devise a means of rapid identification of Pseudomonas species using a single anti-flagellar antiserum. However, using the Ouchterlony double diffusion technique, although antisera produced against three different Pseudomonas flagellins were specific over a wide range of other species, certain strains of Pseudomonas remained undetectable.

The species specificity of flagellar antigens of L. monocytogenes has never been determined, and the use of affinity purified monospecific antiserum raised against listeria flagellins has never been investigated either as a means of serotyping or of speciation. Moreover purified flagellins have not, to date, been characterised for L. monocytogenes and their use in sensitive solid phase immunoassay (eg. ELISA) for serological study has not been examined. This thesis therefore incorporates some preliminary studies of L. monocytogenes flagella and flagellins in this respect.

Bacterial flagella and infection

Motility is an important attribute of virulence in Vibrio cholerae. Guentzel and Berry, (1975) showed a reduced virulence of non flagellate mutant strain of Vibrio cholerae compared with the parent motile strain which was associated with reduced capacity to absorb to the surface of the intestinal mucosa. Work by Attridge and Rowley (1983) also suggests that flagella are important with

regard to adherence. Moreover, Yancy et al 1979, and Resnick et al, 1980 have shown that vaccination of rabbits with flagellar components enhances protection against a challenge with virulent organisms.

Work done by Holder et al (1982) suggests that motility of Pseudomonas aeruginosa is also associated with virulence. Craven and Montie, (1981) demonstrated a correlation between pathogenicity and motility of various strains. Holder et al, (1982) showed that immunization of mice with P. aeruginosa flagellar antigen enhanced the survival of challenged mice, probably due to immobilization of organisms within the tissues.

Carsiotis et al, (1984) have shown that flagella of Salmonella typhimurium are a virulence factor in infected C57Bl/6J mice, and in an accompanying paper, Weinstein et al (1984) suggest that this is due to enhanced survival of organisms within murine macrophages.

Campylobacter jejuni is an organism which undergoes a bidirectional transition between a flagellated (Fla+) and a non flagellated (Fla-) phenotype. In a study of one particular strain, Newell et al, (1982) found that the (Fla+) variant colonized the gastrointestinal tract of infected mice more readily than the (Fla-) derivative. Caldwell et al, (1985) observed that although in culture medium the equilibrium was in favour of nonflagellate organisms, passage through the rabbit intestine resulted in a shift from a (Fla-) to a (Fla+) phenotype.

The possible role of flagella in the invasive stage of infection with L. monocytogenes has not been investigated.

INTRODUCTION AND RESEARCH OBJECTIVES

This thesis has been funded by AFRC (The Agricultural and Food Research Council) and its principal motiviation was the study of ovine listerial encephalitis.

The aim of this comprehensive literature review is to collate data from bacteriological, serological and immunological studies in all species. It is considered that a wide knowledge of both the organism, and the immune response to it, is fundamental to a study of the problem in sheep.

From a study of this review the major research objectives in the field of veterinary medicine become self-evident.

Firstly, after 30 years of research there is still no serological test which will accurately and specifically reflect previous experience of the organism. Diagnosis continues to be by histopathology, preferably accompanied by bacteriological isolation. Morbidity/mortality ratios can not be determined since recovery of the animal always puts the original diagnosis in doubt. Epidemiological studies which relate prevalence of disease to previous exposure to the organism can not be done.

The second major research requirement is the establishment of an experimental model for listerial encephalitis. Vaccinal trials conducted by protection testing of experimentally infected animals

are a necessary adjunct to field trials. An animal model of listerial encephalitis is also essential for study of the pathogenesis and the accompanying immune response.

In this thesis protein antigens of L. monocytogenes were separated and characterised using the relatively new techniques of SDS-PAGE and Western blotting. Antigens were sought which were not only specific to L. monocytogenes but which were also highly antigenic during field exposure to the organism and which could be used in a solid phase immunoassay, ie. ELISA.

This thesis also describes novel ways of inducing a neurological disease in mice distinct from the septicaemic disease generally described. It is suggested that this syndrome should be fully investigated as a potential experimental model.

CHAPTER 2

MATERIALS AND METHODS

BASIC BACTERIOLOGICAL TECHNIQUE

Bacteria:

Serotypes of L. monocytogenes, 1/2b, 3b, 3c, 4a, 4b, 4d, 5 and 7 were obtained from the Central Veterinary Laboratory, Weybridge, Surrey, where they had been maintained on Dorset Agar Slopes. Two further strains, designated throughout this thesis as 4a(T) and 4b(T), were isolated from the brains of sheep with listerial encephalitis.

All cultures were checked for purity by inoculation on 7% sheep blood agar plates and examination of individual colonies by Gram's stain. Pure colonies were emulsified on the surface of blood agar plates with sterile skimmed milk, and 0.25ml aliquots were lyophilised and sealed under vacuum for storage.

C. pseudodiphtheriticum, B. subtilis, S. epidermidis and S. fecalis (NCTC 11136, NCTC 3610, NCTC 11047, NCTC 775) were all obtained from the National Collection of Type Cultures. S. aureus was isolated in this laboratory and identified using a standard laboratory identification kit. (API System S.A., Montalieu-Vereieu, France). E. rhusiopathiae was isolated at the Moredun Institute from the joint of a lamb with polyarthritis. All cultures were checked for purity on blood agar then plated and lyophilised in aliquots in the same way as for L. monocytogenes.

Culture conditions:

Using sterile procedures, freeze dried vials were opened and the contents resuspended in 0.25ml of trypticase soy broth (tsb) taken from a volume of 10ml. The resuspended bacteria were then transferred to the 10ml volume and plated out on to 7% sheep blood agar plates which were then incubated at 37°C for 18 hours. Pure colonies were picked from these plates and were inoculated into 50ml quantities of tsb which were incubated at 37°C for 18 hours. If required, this was used as a seed broth for larger volumes. Large quantities of bacteria for antigen extraction were produced by incubating 1 l. of tsb with 5mls of the seed culture and incubating for 18 hours on an orbital shaker (L.H. Engineering, Stoke Poges, Bucks, U.K.). For extraction of flagella, bacteria were grown in 1 l. of tsb at 20°C for 24 hours without shaking.

For challenge experiments with live L.monocytogenes in CBA mice, bacteria were obtained from the late log phase of growth in broth culture at both 20 and 37°C. Organisms were washed, counted, resuspended in PBS with 30% glycerol, aliquoted, and stored in liquid nitrogen. A second count was made from each aliquot when it was withdrawn for use.

All bacterial counts made in this thesis depend on the number of colony forming units (cfu) grown on 7% sheep blood agar, calculated according to the method of Miles et al, (1938).

Relationship of bacterial numbers to optical density (OD) in broth culture.

This was done for 1 l.tsb broth cultures of L. monocytogenes serotype 4b(T) grown at 37°C on an orbital shaker. Two 1 l. flasks of tsb were inoculated with 5ml of a seed culture, and at hourly intervals two 1.0ml samples were removed from each flask. The number of cfu was determined from one sample, and from the second sample a determination of optical density O.D. was made using a photometer with a 420mm filter, (Vitatron Universal Photometer, Fisons, UK) calibrated to zero using sterile tsb. Optical Density was plotted against log10 cfu. This work was repeated for bacteria grown in broth at 20°C using a sampling interval of 2 hours.

PREPARATION OF ANTIGENS

Preparation of cell wall antigen (a) By pressure cell disruption

L. monocytogenes serotype 4a(T) was grown in tsb to the stationary phase. Cells were then pelleted by centrifugation at 4,500g for 25 minutes at 4°C, washed in phosphate buffered saline (PBS) (one fifth of the broth volume) and repelleted. The final pellet was resuspended in PBS at a concentration of 1 l. of bacterial cell growth per 20ml of PBS. This bacterial suspension was passed twice through a French Pressure Cell (Aminco American Instruments Co Inc, Silver Springs, Ma., USA) at a pressure of 8,000 psi. An estimate of percentage bacterial breakage was made by phase contrast microscopy. The suspension was then centrifuged at 4,500g for 25 minutes at 4°C and the pellet of unbroken bacteria was discarded.

The supernate was centrifuged at 40,000g for 45 minutes at 4°C and the resulting pellet was washed in distilled water, repelleted, resuspended in a small quantity of distilled water and freeze dried for storage.

(b) By sonication

Crude cell wall fractions of L. monocytogenes serotypes 1/2b, 3b, 3c, 4a, 4a(T), 4b, 4b(T), 5 and 7 were prepared from washed cells, grown in broth culture at 37°C to the stationary phase, by ultrasonic disintegration (MSE 150 watt Ultrasonic Disintegrator MK2, Sussex, England). Bacteria were suspended in PBS at a concentration of approximately 10^{10}/ml in sealed heat-resistant glass tubes, packed with crushed ice and given six two minute periods of sonication at an amplitude of 16 microns using a 19mm titanium probe. Following this, unbroken bacteria were removed by centrifugation at 4,500g for 45 minutes at 4°C. The supernatant was then centrifuged at 40,000g for 45 minutes and the resulting pellet was washed twice in PBS and frozen at -20°C or lyophilised for storage at room temperature.

SDS extraction of cell wall preparations

Lyophilised crude cell wall fractions made either by pressure cell disruption or by sonication were resuspended at a concentration of 1mg/ml in PBS containing 2.5% sodium dodecyl sulphate (SDS) and incubated at 45°C for 30 minutes. The suspension was then centrifuged for 30 minutes at 26,000g and the supernatant dialysed against distilled water for 18 hours at 4°C, aliquoted, and stored at -20°C.

Detergent extraction of whole bacterial cells

Cells which had been stored at -20°C were suspended in PBS containing either, (a) 0.5% SDS and 0.5% Triton-100, or (b) radio immune precipitation assay buffer, (RIPA (10mM phosphate buffer, (pH. 7.2) containing 0.15M NaCl, 1mM phenyl methyl sulphonyl fluoride, 1% octyl phenol ethylene oxide (Nonidet P-40 (Sigma Chemical Co. Ltd.)), 0.1% SDS and 1% sodium deoxycholate, (BDH Ltd))) at a dilution of 1ml per 100mg of wet cell pellet. Cells were then shaken vigorously on a microid wrist action shaker (Griffin George Ltd, England) for 30 minutes at 37°C. Insoluble cell material was removed by centrifugation at 4,500g$_{\wedge}$ for 20 minutes at room temperature. The supernatant was chilled to -4°C, and precipitated SDS was removed by further centrifugation at 4,500g$_{\wedge}$ for 20 minutes. The supernatants were then frozen at -20°C.

Preparation of crude flagella

L. monocytogenes grown in broth culture at 20°C were harvested by centrifugation at 4,500g$_{\wedge}$ for 20 minutes, washed twice in PBS and resuspended at a ratio of 5ml PBS/l of original broth culture. 10ml amounts of this suspension were added to stoppered glass universals together with 10, 2mm diameter glass beads. The glass universals were clamped vertically on a microid wrist action flask shaker and shaken vigorously for 30 minutes at 20°C. The bacterial suspension was then centrifuged at 4,500g$_{\wedge}$ for 20 minutes and the supernatant retained. The bacterial pellet was then washed twice with PBS by vigorous pipetting to remove sheared flagella trapped within the cell mass. The supernatant and cell washings were then pooled and centrifuged

at 14,000g for 40 minutes to clear remaining bacteria, and then centrifuged at 200,000g for 90 minutes to obtain a pellet of crude flagella.

Purification of flagellar proteins (a) By column chromatography

The crude flagellar pellet was solubilized by boiling for 3 minutes in PBS containing 1.0% SDS and 2.0% 2-mercaptoethanol. SDS and mercaptoethanol were then removed by dialysis against PBS with 0.02% azide for 18 hours at 20°C. The resulting dialysate was concentrated by centrifugation through the membrane of a microconcentrator (Centricon-10, Amicon). The solution was then applied to a Superose 12 gel filtration column (FPLC, Pharmacia) eluted with 0.02M tris and 1M sodium chloride at pH 7.5. Maximum loading was 200µl of solution containing 2mg of protein, and eluted at a flow rate of 0.2ml/minute. The major peaks were collected and analysed by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The peak containing flagellin was dialysed for 18 hours at 4°C against 0.1M tris and then applied in 0.5mg aliquots, at a flow rate of 2.0ml/ minute to a Mono Q ion exchange column (FPLC, Pharmacia) and eluted with a 0.1-0.4M sodium chloride gradient. The resulting peaks were again checked by SDS-PAGE.

(b) By urea-extraction

A crude flagellar pellet, as obtained above, was resuspended in 6M urea pH 2.6 at an approximate concentration of 1mg/ml and heated in a waterbath for 45 minutes at 50°C. The suspension was then centrifuged at 40,000g for 30 minutes and the supernatant dialysed for 48 hours

against 100 volumes of PBS with four buffer changes. Finally the material was centrifuged at 200,000g for 90 minutes, and 10µg of both the resuspended pellet and the supernatant was analysed by SDS-PAGE and silver staining. Uncontaminated flagellar bands of 29KD molecular weight were obtained from the supernatant material. This material was used as flagellar antigen in subsequent ELISA tests where indicated in this thesis.

SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS,SDS-PAGE

A discontinuous system was used, employing slab gels either 0.75mm or 1.5mm thick with a 12% separating gel and a 4% stacking gel with the buffer system of Laemmli (1970). Briefly, for the separating gel, a 12% acrylamide solution containing 0.33% N N'methylenebisacrylamide, and 0.5% SDS in 0.25M Tris-HCl buffer (pH 8.8) was polymerised by the addition of 1% ammonium sulphate and 0.1% N N N'N'-Tetramethylethylene, (Temed). This was overlaid by a similarly polymerised 4% acrylamide solution containing 0.1% N N' methylenebisacrylamide and 0.25% SDS in 0.125M Tris-HCl buffer (pH 6.8).

Samples were diluted 1:1 with sample buffer containing 2% SDS, 4.0% beta-mercaptoethanol, 10% glycerol and 0.001% bromophenol blue in 63 mM Tris-HCl (pH 6.8) and immersed for three minutes in a boiling water bath before application to the gel.

Gels were loaded in two ways. For Western blotting experiments in which several antigen preparations were to be reacted with a single antiserum the samples were loaded into wells formed with a

comb. In experiments where a number of different sera were to be tested against a single antigenic mixture, the gel was cast with a single large well and the subsequent blot developed as a number of strips.

Gel Electrophoresis was carried out using a running buffer containing 0.2M glycine, 0.25M Tris and 0.1% SDS. Gels were run under constant current conditions at a current density of 19mA/cm^2 for approximately 3 hours (until the marker dye front had migrated 10cm into the resolving gel).

Well loadings (µg bacterial protein/mm^2 of sample well area) are shown in the legends to the figures where appropriate.

Two sources of molecular weight marker were used:

BDH Chemicals Ltd., Poole, Dorset, UK.

Equine cytochrome C	(12.3KD)
Equine myoglobin	(17.2KD)
Bovine chymotrypsinogen	(25.7KD)
Hen egg ovalbumin	(45.0KD)
Bovine serum albumin	(66.25KD)
Hen egg ovotransferrin	(76-78KD)

Sigma Chemical Company, St. Louis, Missouri, USA.

Alpha-Lactalbumin	(14.2KD)
lyosyme	(14.3KD)
trypsinogen	(24.0KD)
carbonic anhydrase	(29.0KD)
Hen egg albumin	(45.0KD)
Bovine serum albumin	(66.0KD)
Phosphorylase b	(97.4KD)
Beta-galactosidase	(116.0KD)

The BDH molecular weight markers were used unless otherwise stated.

The molecular weights marked on the figures in this thesis are given for the position of migration of the molecular weight markers themselves. Specific molecular weights given in the text were calculated by reference to a standard curve derived from the plot of the relative mobilities of the reference proteins against the log 10 of their molecular weights (Shapiro et al, 1967). A later study by Weber and Osborn (1969) confirmed the reliability of this technique.

Fixing and Staining of Gels.

Coomassie Blue.

For this stain, gels were fixed for 18 hours in a solution of 30% methanol containing 25% trichloroacetic acid and 8.5% sulphosalicylic acid. They were then immersed for 30 minutes in 50% methanol with 10% acetic acid and 0.25% Coomassie Blue on a rocking platform - (1.5mm gels were stained for 1 hour).

Destaining was by immersion in a solution of acetic acid, methanol, and distilled water in a volume/volume ratio of 1:3:6 until the desired result was obtained.

Silver Staining

The method of Morrissey (1981) was used. Gels were fixed in 50% methanol with 10% acetic acid for 30 minutes followed by a further 30 minutes in 5% methanol with 7% acetic acid. A final fixing step of 30 minutes by immersion in 10% glutaraldehyde was employed. Following this, gels were washed in copious amounts of distilled water for a minimum of 4 hours. Gels were then immersed in a solution of dithiothreitol at 5µg/ml for 30 minutes and then for a further 30 minutes in a 0.1% solution of silver nitrate. Finally, after quickly rinsing in distilled water, bands were developed using a 3% solution of sodium bicarbonate with the addition of 0.5 µl/ml of 40% formaldehyde. The reaction was stopped by the addition of 2M citric acid (approximately 5ml citric acid/100 ml developer). To ensure good penetration of reagents all incubations were carried out on a rocking platform.

ELECTROBLOTTING

a. 'Wet'.

One technique of transfer to nitrocellulose was that of Burnette (1981) as modified by Herring and Sharp (1984). Gels were placed on nitrocellulose membrane of pore size 0.2μm (Shleicher and Schull, Dassel, West Germany), between two sheets of 3mm filter paper (Whatman Ltd., Maidstone, Kent, UK). This 'sandwich' was constructed by liberally wetting both filter paper and membrane with electroblotting buffer (20mM Tris, 154mM glycine, 20%v/v methanol, pH 8.3) before assembly, in order to avoid the formation of air bubbles. The position of the tracks and the dye front were marked on the membrane with ball point pen. The 'sandwich' was then compressed into an electroblotting cassette (E-C Electroblot, E-C Apparatus Corporation, St. Petersburg, Florida, USA), with the nitrocellulose towards the anode, and the cassette was placed in a tank containing electroblotting buffer. Buffer was continuously circulated across the cassette by means of a small pump. Transfer was allowed to proceed for 18 hours at a current density of 1.3mA/cm^2 and a voltage gradient of approximately 7.0 volts/cm.

b. 'Semi-dry'.

The technique used was as described by Burnette in 'Procedures for Western blotting: Electrophoretic transfer of proteins from acrylamide gels to nitrocellulose', a manual produced and distributed by 'Sartorius GmbH', D-3400 Gottingen, FDR. The apparatus used was built at the Moredun Research Institute based on

a design by Kyhse-Anderson (1984). This involves the compression and electrophoresis of a filter paper/nitrocellulose/polyacrylamide gel/filter paper sandwich between solid graphite electrodes using a three buffer system. Briefly, two sheets of 3mm filter paper were soaked in a buffer composed of 0.3M Tris in 20% methanol at pH 10.4 and were apposed to the graphite surface of the anode. A further sheet of filter paper was soaked in a buffer containing 25mM Tris in 20% methanol at pH 10.4 and placed on top of this. The nitrocellulose sheet, pre-wetted with distilled water, was placed on top and was overlaid by the polyacrylamide gel, taking care to exclude air bubbles. The sandwich was completed with two further sheets of filter paper soaked in a buffer of 25mM Tris and 40mM 6 amino-n-hexanoic acid at pH 9.4 which were laid on top and covered by the graphite plate of the cathode. Electrophoresis was completed by connecting the electroblotter to a power supply which delivered a current of $0.8mA/cm^2$ of the gel. Gels were run for 1 hour at room temperature.

Both 'wet' and 'semi-dry' techniques have been used throughout this thesis, but wherever comparisons between blots have been made, the same system has been used.

Development of Electroblots

Following transfer, blots were trimmed, and, where necessary, cut into strips. Blotted molecular weight standards were trimmed from the edge and developed with a dilute solution of Coomassie Blue (2µg/ml) in 30% ethanol and 10% acetic acid.

The reaction of the blotted proteins with antiserum was performed in six stages and, during all incubations and washes, blots were agitated gently on a rotary shaker at 37°C. Washing steps were performed between all stages.

(1). Non specific binding sites on the membranes were 'blanked' out using 50% v/v horse serum, except where a staphylococcal protein A detection technique was employed when a concentrated bovine serum albumin solution (5% w/v) was used. Blanking proteins were diluted with a 'washing buffer' of 8.7mM sodium phosphate buffer, pH 7.2, containing 0.5M NaCl, 0.5% 'Tween 80', and 1.0mM EDTA. This blanking step was carried out for 1 hour at 37°C.

(2). Blots were then incubated for a further hour in test antiserum. Test antiserum was diluted 1:40 in washing buffer with 1% 'blanking protein'.

(3). Blots were then incubated in detection antiserum. Two detection techniques were employed in the course of this study.

(a) Detection by Autoradiography

For detection of sheep antibody, affinity-column-purified rabbit IgG, specific for ovine F'ab2 was used. This had been prepared by Mr. A. Dawson of the Moredun Institute. Rabbit antibody was probed using Staphylococcal Protein A (Cowan strain: Sigma Chemical Co.). Both reagents were labelled with I^{125} using the chloroamine T reaction (Hunter and Greenwood, 1962) by Mr. N. Inglis of the

Moredun Institute, and were used at a previously determined optimal dilution in washing buffer containing 1% blanking protein. Incubation was for 1 hour at room temperature.

(b) Detection by peroxidase labelling

Donkey anti-sheep IgG and donkey anti-rabbit IgG horse radish peroxidase (HRP) conjugates were obtained from the Scottish Antibody Production Unit (SAPU), (Glasgow and West of Scotland Blood Transfusion Service, Law Hospital, Carluke, Lanarkshire, Scotland). Affinity purified sheep IgG anti-mouse IgG conjugate was prepared by Mr A. Dawson of the Moredun Institute. Conjugates were used at a dilution of 1/400 with 1% blanking protein. Incubation was for 1 hour at 37°C.

(4) A thorough wash was performed in 'washing buffer'.

(5) For Autoradiography, strips were dried under vacuum at room temperature and mounted on filter paper before being exposed to X-ray film (Kodak X-Omat S, Kodak, Liverpool, UK) in a radiographic cassette containing an intensifying screen.

For development of the peroxidase reaction strips were rinsed in distilled water and immersed in a 0.1M solution of Tris with 0.02% w/v diamino-benzidine and 0.2% hydrogen peroxide (pH 7.4) until bands appeared. Strips were then washed in copious amounts of water, dried under vacuum, mounted on filter paper and photographed.

Biotin- Avidin Peroxidase Technique

For enhanced staining of blots, a biotin/avidin amplification step was used. For this, a mouse antibody step was followed by incubation with an affinity purified biotinylated anti-mouse reagent prepared in a horse, which was in turn followed by incubation with peroxidase conjugated avidin D (Sera - Lab, Crawley Down, W. Sussex). Colour was developed in the usual way.

ENZYME-LINKED IMMUNOABSORBENT ASSAY (ELISA) TECHNIQUE

(a) Soluble Antigen

ELISA procedure using both cell wall antigen, and flagellar antigen was performed as described by Donachie and Jones (1982) with some modifications. A reagent volume of 200µl/well was used throughout the test, and all sera were diluted in PBS with 0.05% Tween 20 (PBS/Tween). Tests consisted of four separate incubation stages, with unbound reagents being removed from the wells, after each incubation, by thorough washing with PBS/Tween. The procedure was as follows:

Step 1. Flat-bottomed polystyrene 96 well microtitre plates were coated by an 18 hour incubation with coating antigen diluted in 0.1M carbonate/bicarbonate buffer (pH 9.6). Unless otherwise stated antigen was used at a dilution of 1µg/ml.

Step 2. Plates were washed three times with PBS/Tween and incubated with test and standard antiserum dilutions for 1 hour at 37°C.

Step 3. Plates were washed three times with PBS/Tween and incubated with the appropriate conjugated anti-immunoglobulin (see development of Western blots), in PBS/Tween at a pre-determined dilution for 1 hour at 37°C.

Step 4. Plates were washed three times with PBS/Tween, before enzyme substrate was added to the wells and incubated at room temperature for 15 minutes. The substrate used was a 0.08% solution of o-phenylenediamine (OPD) in citrate/phosphate buffer (0.05M citric acid, 0.1M Na_2HPO_4) with 0.08% hydrogen peroxide. The reaction was stopped by adding 50µl of 2.5 M H_2SO_4 to each well.

The results were recorded as light absorbance values at a wavelength of 492nm using an automatic multichannel spectrophotometer (Titertek Multiskan, Flow Laboratories, Irvine, Ayrshire, UK). Background readings were automatically subtracted from sample values by blanking the plate reader with wells where all reagents except test or standard sera had been added. Some results were translated in digital form to a punched tape in order to allow computer analysis.

(b) ELISA using whole bacterial cells

For this assay reagents were incubated in 96 well filtration plates containing a hydrophilic polyvinylidene fluoride membrane with a 0.2µm pore size (Millititer-GV, Millipore Products Division, Millipore Corporation, Bedford, Mass., USA). Plates were used in conjuction with the Millititer Vacuum Filtration Holder

(Millipore Corporation) which was attached to a vacuum pump driven by the mains water supply.

In simple terms, this system allows particulate material >0.2µm (bacterial cells in this case) to be retained within the wells and reacted in a step-wise manner. The porous membrane at the base of each well is designed to retain liquids of limited surfactant concentration (0.05% Tween 20) but to allow the unbound reagents to be drained away at the end of each incubation step and the particulate matter to be exhaustively washed by the simple application of a vacuum. The ELISA was conducted in the following stages:

Stage 1. Wells were filled (400µl) with 5% BSA in PBS/Tween (0.05% Tween) and incubated for two hours at 37°C. This step was included to minimise non-specific reaction.

Stage 2. A 100µl volume of washed formol killed L.monocytogenes suspended in 5% BSA/PBS/Tween was added to each well. Antigen was prepared from an 18 hour broth culture of serotype 4a(T) which was adjusted to an optical density of 0.1 as determined using a photometer with a 548 nm filter (Vitatron Universal Photometer).

Antigen was incubated together with 100µl volumes of test and standard antiserum, also diluted in 5% BSA/PBS/Tween for 1 hour at 37°C.

Stage 3. Wells were drained and washed 9 times with 250µl volumes of PBS/Tween.

Stage 4. 100µl volumes of donkey anti-rabbit conjugate (SAPU) were added to the wells at a previously determined optimal dilution and incubated for 1 hour at 37°C.

Stage 5. After drainage, a further wash step with 9 x 250µl volumes of PBS/Tween was performed.

Stage 6. Colour was developed at room temperature for 15 minutes using O-phenylenediamine and the reaction stopped with 2.5N H_2SO_4 as previously described.

Stage 7. Finally, individual filtrates were collected for colorimetric determination by placing a standard 96-well plate into the vacuum chamber and draining the contents of the upper plate through under vacuum. Transfer was assisted by using a 'guide' which when accurately placed made contact with the bottom of the well of the filtration plate and the centre of the receiving well below. The results were then recorded using an automatic multichannel spectrophotometer as described above.

PREPARATION OF ANTISERA

Antisera was raised against formol killed (fk) organisms in sheep (conventional and specific pathogen free (spf)), rabbits, and two inbred strains of mice (VM and C57 Bl). In addition, antiserum was raised in rabbits against fk organisms of other species, purified flagella of L. monocytogenes, and cell wall fraction of L. monocytogenes. For clarity therefore the vaccination protocols for each animal or group of animals is shown as a series of tables

(Tables 2.1-2.6). The number of fk organisms given is based on the number of cfu of live broth cultures in stationary phase which were subsequently killed by addition of 0.2% formaldehyde, and can therefore only be approximate. Adjuvanted vaccines were made by emulsification of equal volumes of bacteria or bacterial proteins in PBS with either complete or incomplete Freund's adjuvant (Difco Laboratories, Detroit, Michigan, USA) using a homogenizer (MSE, England).

Rabbit antiserum raised against heat killed (hk) L. monocytogenes 4a(T) was prepared by Bruce McGorum working at the Moredun Research Institute.

Spf lamb antiserum raised against formol killed Pasteurella haemolytica was obtained from Dr. W. Donachie at the Moredun Research Institute.

Absorption of antiserum

0.5ml aliquots of serum from Rab. 1 (Table 2.2) vaccinated with fk L. monocytogenes 4a(T) were diluted 1:1 with PBS containing 0.02% sodium azide. Each aliquot was used to resuspend 0.5l of washed fk bacterial cells in the stationary phase of growth. Serum and bacteria were mixed on an 'end-over-end' serum mixer for 1 hour at 37°C and then held overnight at 4°C. Bacteria were removed by centrifugation and the procedure repeated using a further 0.5l of bacterial growth.

Preparation of Affinity-Purified Anti-Flagellar Antiserum

One mg of 29KD Flagellar protein (as obtained by gel filtration and ion exchange chromatography) was coupled to 3ml of swollen activated CH-sepharose 4B (Pharmacia) by the method recommended by Pharmacia in "Affinity Chromatography, Principles and Methods". The bed volume was increased to 6ml by the addition of inactive sepharose 4B. The immunoabsorbent was packed in a column, 10x1cm, washed with 3 column volumes of eluting buffer (0.1 glycine/HCl pH 2.3) and then equilibrated with a neutral running buffer (0.1M phosphate with 0.5M NaCl).

Following equilibration, 2ml volumes of rabbit anti-flagellar antiserum (Table 2.7) were applied to this column at a flow rate of 0.33ml/minute. The column was then washed with running buffer until a steady base line was being recorded. The bound material was then eluted with the eluting buffer, collected, neutralised to pH 7 and stored at -20°C.

PRODUCTION OF MONOCLONAL ANTIBODY

(a) Vaccination Protocol

Adult female Balb/c mice were inoculated into each hind foot pad with a 10µl volume of vaccine containg 5µg of crude flagellar protein of serotype 4b(T), at intervals of two weeks for ten weeks. The vaccine used for primary vaccination was prepared by homogenising equal volumes of antigen in PBS with complete Freund's adjuvant and then homogenising this emulsion with an equal volume of 2% Tween 20. The

three subsequent inoculations were made using a vaccine made from equal parts of antigen with incomplete Freund's adjuvant. A final inoculation of 5µg of protein in PBS was given. Cell fusion was carried out three days after the fifth, and final, inoculation.

(b) Fusion Technique

Popliteal lymph nodes were aseptically removed from 3 mice into RPMI containing 1% penicillin/streptomycin, 1% Hepes, 2% glutamine and 10% serum (10% RPMI medium) and pressed through fine gauze with a rubber policeman to make a single cell suspension. The cells were washed twice in this medium and a third time in serum free RPMI medium. Cell numbers were determined and adjusted, and cells were added to an equal number of washed myeloma cells (NS-0). A mixed pellet was obtained by centrifugation which was slowly resuspended in a small volume of the serum free RPMI medium containing 50% v/v polyethylene glycol (MW 1500). Stirring gently, serum free RPMI was added to the cells to a final volume of 10ml. Finally, cells were pelleted by centrifugation, and resuspended in 60ml of the RPMI medium containing 20% serum, 0.068% hypoxanthine, 0.002% aminopterin and 0.002% thymidine (20% RPMI HAT medium) together with $2x10^5$/ml of normal spleen cells. All manipulations were conducted at 37°C. Cells were distributed in 100µl aliquots in flat-bottomed 96-well microtitre plates (Nunc. Gibco Ltd., Paisley, Scotland) and incubated at 37°C in 5% CO_2.

(c) Cloning Technique

After 7 days each well received a further 100µl of 20% RPMI HAT medium containing 20% of a mixed thymocyte conditioned medium. On day 12 individual wells were examined, clones identified, and supernatants screened by ELISA. Positive clones were transferred into 24 well costar plates (Nunc) and maintained in 20% RPMI conditioned medium until cell numbers were sufficient for cloning (1,000 cells/ml). Cells were cloned by limiting dilution in 96 well microtitre plates. Plates were checked every three days, and single clones were identified and fed with 20% RPMI conditioned medium. Positive clones were expanded, recloned twice, and frozen in liquid nitrogen.

Mixed thymocyte conditioned medium was obtained from mixed cultures of rat thymus and was prepared by Angela Shaw of the Moredun Institute who was also responsible for all the sterile manipulations detailed above.

(d) Production of Ascites

Adult female Balb/c mice were primed by intraperitoneal inoculation of 0.5ml of 2, 6, 10, 14 - Tetramethyl pentadecane (Pristane, Sigma, St. Louis, Mo, US.) One week later mice were given a 1ml volume of PBS containing $1x10^5$ cloned hybridoma cells into the peritoneal cavity. After a further two weeks mice were sacrificed by cervical dislocation and ascitic fluid was aseptically collected, centrifuged to remove cellular material, aliquoted and stored at -70°C.

(e) Isotype determination

The isotype of the monoclonal antibody used in this thesis was determined by ELISA to be IgG_2. This ELISA employed purified flagellin as the coating antigen, and HRP conjugated isotype specific anti-mouse reagents raised in rabbits as the probes, (Miles Scientific, ICN Biomedicals Ltd. High Wycombe, Bucks).

LABORATORY ANIMAL TECHNIQUES

(a) Mice

Inoculations:

Subcutaneous (s.c.) inoculations into mice were made without anaesthesia in the loose skin of the groin using a 25 gauge needle.

Intraperitoneal (i.p.) inoculations were also given without anaesthesia using a 25 gauge needle. The injection site was into the abdomen, slightly to the left of the umbilicus.

Intragastric inoculations were performed without anaesthesia using an 18 gauge, 1.5 inch long hypodermic needle from which the point had been filed. In order to ensure atraumatic introduction of the needle, the filed surface was covered by a short length of polyvinyl tubing which was moulded by heat on to the tip with a short length protruding. The modified needle was gently introduced

into the stomach by holding the head and neck of the mouse in full extension.

Exsanguination: Blood was taken from the posterior vena cava caudal to the kidneys through a laparotomy incision with the mouse freshly dead following CO_2 overdose.

Blood Sampling: Small quantities of whole blood were collected into buffer from the lateral tail veins. This involved warming mice over an electric light bulb to encourage peripheral vasodilation, puncturing the vein using a 25 gauge needle and aspirating blood from the wound using a micropipette.

Tissue Sampling for Bacteriology: Mice were sacrificed by carbon dioxide overdose and each organ aseptically removed into 9ml of peptone water (Oxoid). These were individually macerated in a Colworth stomacher (Seward, London) and serial ten fold dilutions were plated out for counting of viable cells by the method of Miles et al (1938).

(b) Rabbits:

Inoculations: Rabbits were inoculated intramuscularly in the mid femoral region of the hind limbs. Intravenous inoculations were given into the ear vein using a 23 gauge needle.

Exsanguination: Rabbits were anaesthetised with intravenous alphaxalone/alphadolone acetate (Saffan: Glaxovet, Middlesex, UK) and exsanguinated by cardiac puncture.

Blood Sampling: Quantities of up to 20ml were collected from the veins of the margin of the ear through a small incision. This was done without anaesthesia.

MISCELLANEOUS TECHNIQUES

Electron Microscopy

(a) Gold Shadowing

A drop of bacterial suspension, fixed with 0.5% glutaraldehyde, was placed on the carbon/pioloform coated grids. After 20-30 seconds the excess was removed and grids were allowed to air dry. Grids were then washed with several drops of distilled water. Shadowing was carried out in an Edwards 306 coating unit using gold/palladium metal. The shadow angle was 20°. Grids were examined in a Siemens Elmiskop 1A electron microscope.

(b) Negative Staining

Material was air dried on grids as described above, fixed in 0.5% glutaraldehyde and stained with 0.5% ammonium molybdate solution.

Electron microscopy,negative staining,and gold shadowing were done by Jim Menzies of the Moredun Institute.

Protein Assay

Protein estimations were determined using commercial assay reagents (BCA-Protein Assay, Pierce Chemical Company, Rockford, Illinois, US). This is a colorimetric method which uses a working reagent of $CuSO_4$ and bicinchoninic acid. Protein in the test sample reduces Cu2+ ions to Cu+ which subsequently chelate the bichinchoninic acid to form an intense purple colour. Colour change was measured in glass cuvettes in a negative control-blanked spectrophotometer (SP6-550/VIS Pye-Unicam, Cambridge, UK) at 562nm with a 1cm path length. Protein concentration was determined by reference to a standard curve calculated for bovine serum albumin. (Sigma).

Enzymic Digestion using Fungal Protease

For enzymic digestion of proteins, cell wall components of L. monocytogenes in PBS were incubated with type XI fungal protease, ('Proteinase K', Sigma Chemical Co. Ltd.) in a final solution containing 304µg of enzyme and 660µg of protein/ml of diluent. Incubation was at 37°C for 1 hour.

Whereas this chapter gives a broad account of materials and methods used, some detail of individual experiments is necessary and will be given throughout the rest of the text where appropriate.

CHAPTER 3.

A Study of the Cell Wall Proteins of L. monocytogenes 4b(T) by SDS-PAGE and Western Blotting.

INTRODUCTION

Little work has been published on the application of SDS-PAGE to the study of L. monocytogenes. In a taxonomic study, Lamont et al (1985) examined ultrasonically disrupted organisms of L. monocytogenes, L. welshimeri, L. seeligeri, L. innocua and L. ivanovii. However, only limited results are reproduced in this publication.

Even less data are available relating to Western blotting of electrophoresed proteins of L. monocytogenes. The paper presented by Paquet et al (1985) is not supported by data.

It is considered that this thesis represents one of the first comprehensive studies of Western blots of proteins of L. monocytogenes of different serotypes using antiserum from different species, and one of the first attempts to enumerate and characterise cell wall proteins.

SUMMARY

In this chapter:-

(a) The protein components of a cell wall fraction of L. monocytogenes serotype 4a(T), derived by disruption in a pressure

cell, were determined by gel electrophoresis and staining with Coomassie blue. To ascertain which of these components were antigenic following vaccination with whole formol killed organisms, components were transferred to nitrocellulose by Western blotting and were analysed using antiserum from both rabbits and sheep.

(b) Other extracts of L. monocytogenes serotype 4a(T), prepared by the different techniques described in chapter 2 were also examined.

(c) Preliminary studies were undertaken using the rabbit hyperimmune antiserum, absorbed with washed formol killed cells of homologous and heterologous organisms in an attempt to determine the expression of proteins at the L. monocytogenes cell surface.

(d) The protein nature of blotted components was determined by a proteinase digestion technique.

(e) Finally in this chapter, protein extracts of 9 different serotypes of L. monocytogenes are examined by SDS-PAGE and Western blotting.

Four serotypes, viz:- 4a(T), 4b(T), 5 (L. ivanovii), and 7, were subjected to a more detailed analysis using immune serum raised both in lambs, and in two strains of inbred mice, against each of the four serotypes. Blots of sonicated cell walls of each serotype were developed with homologous and heterologous antisera in order to elucidate differences of component proteins, and differences of response to vaccination.

Representatives of each of the major serotypes were chosen for analysis, and for more detailed study it was decided to choose

those four serotypes where major protein differences might most be anticipated: (serotypes 4a(T) and 4b(T), isolated from brains of encephalitic sheep, serotype 5, with a distinctive pattern of haemolysis and fermentation, and serotype 7, of uncertain pathogenicity).

RESULTS

SDS-PAGE and Western blot analysis of crude cell wall fraction (CWF) of serotype 4a(T) derived by pressure cell disruption.

At least fifteen polypeptides can be detected by Coomassie blue staining of SDS-PAGE preparations of crude cell wall fraction (CWF) derived by pressure cell disruption. These are indicated in Fig. 3.1 by arrows spanning the molecular weight range from 76 KD to just below 25 KD, with a major band being evident at the 66 KD molecular weight marker.

Western blot analysis using a hyperimmune rabbit serum raised against formol killed organisms (Table 2.2), shows that the same crude cell wall fraction consists of 9 discernible antigens (Fig. 3.2, track 2fk, "a"-"i"). Analysis using rabbit antiserum raised by inoculation of crude cell wall fraction (Table 2.5) reveals a very similar antigenic profile, with an additional band becoming visible between the 66 and 45 KD markers (arrow) and the apparent lack of antibodies directed against component "a" (Fig. 3.2, track 2 cwf). The track marked hk represents the same cell wall fraction examined using antiserum raised in rabbits against heat killed organisms. In this case, only three antigens are clearly visible (including 2 major ones) which appear to correspond in each instance with antigens detected by the other two sera, (track hk, arrows).

A Western blot of the same crude cell wall fraction was developed using aliquots of hyperimmune rabbit antiserum which had been absorbed both by L. monocytogenes, and by 6 other bacterial species, as described in chapter 2. The result is shown in Fig. 3.3. (In this instance, the two antigens previously designated "a" and "h" are not apparent in any of the developed strips). It is clear that absorbance with L. monocytogenes has resulted in removal of antibodies directed against the 7 components visible in the control strips. Antibodies are apparently not differentially removed following absorption with any of the other organisms.

The same antigen preparation was electrophoresed and examined using antiserum raised by inoculation of two mature sheep with formol killed organisms (Table 2.1). Results were obtained separately for each sheep, and are shown in Fig. 3.4. Strips 1 were incubated with prevaccinal antiserum and strips 2 and 3 developed with serum obtained at progressive stages of the vaccinal process. The ultimate response observed is almost identical for each sheep, with the exception of an extra band observed below the 45 KD marker in sheep 2 (arrow). From this result it would appear that almost the full repertoire of antibody response is present early in the vaccinal process but that repeated vaccination enhances the response either by increasing the titre and/or the affinity of the existing antibody.

Study of the autoradiographs shown in Figs. 3.2 and 3.4 demonstrates an apparent difference between the spectrum of antibodies produced by the rabbit and the sheep. The most obvious single difference is the protein of lowest molecular weight (30

KD) which is more readily detected using the ovine antiserum than antiserum from the rabbits. To study these, and other differences in more detail two contiguous strips of nitrocellulose containing electrophoresed CWF were examined using both the sheep and the rabbit hyperimmune antiserum. These strips (Fig. 3.5), probed in this instance with peroxidase labelled conjugate, suggest that the sheep have responded to more antigens in the 30 KD region than the rabbit. Other differences are also apparent, with the rabbit responding less strongly to antigens in the 66-29 KD molecular weight range. Moreover, by using a peroxidase probe instead of autoradiography a larger number of antigens became apparent both with the rabbit and the sheep hyperimmune serum.

Retrospectively, it was remarked that this 30 KD complex of antigens (antigen "i" in Fig. 3.2) had a similar electrophoretic mobility to listerial flagellin (see chapter 4). When CWF was "dot blotted" on to nitrocellulose and developed using affinity purified rabbit anti-flagellar antiserum followed by HRP conjugate a colour change was detected in excess of that obtained using HRP conjugate alone (Fig. 3.6). This further suggested that the CWF derived by pressure cell disruption had a flagellar component.

SDS-PAGE and Western blot analysis of different antigenic preparations of L. monocytogenes.

A comparative study of proteins made, 1.) by detergent extraction, and 2.) by bacterial cell disruption and differential centrifugation revealed some significant differences (Figs. 3.7 a and b).

Stained gels of either SDS/Triton or Ripa buffer extracted antigens showed very little difference in the distribution of component proteins. The single visible exception is a complex of three distinct bands of approximate molecular weight 100 KD (Fig. 3.7-b, track 2, arrow) where only two can be detected in the SDS/Triton extract of track 1.

The pressure cell disruption technique of antigen preparation has led to a selective concentration of major proteins in the four areas shown by arrows (track 3). This is more obvious in the Coomassie blue stained gel than in the silver stained gel.

The cell wall proteins obtained by sonication of cells, (track 4), show yet another distribution pattern. There has been an accumulation of protein corresponding to the upper 3 arrows of track 3 but in addition there are bands above the 76 KD marker and two major bands (marked by arrows) between 45 and 25 KD.

A Western blot of the same antigens examined with hyperimmune serum from a vaccinated sheep again shows a great similarity between antigens extracted using either SDS/Triton or Ripa buffer (Fig 3.8). These detergent extraction techniques have yielded an antigenic mixture containing a component of approximate molecular weight 40KD(arrow, track 1), corresponding in electrophoretic mobility to the arrowed bands in track 1 of Figs. 3.7a and b, which is not significantly present in preparations obtained by mechanical disruption. Methods of mechanical disruption have led

to a concentration of a complex of approximate molecular weight 30 KD which is especially prominent in the preparation obtained by pressure cell disruption.

SDS-PAGE of proteins of serotype 4a(T) obtained by extraction with Ripa buffer, and with SDS/Triton are shown at a higher well-loading in tracks 2 and 3 of Fig 3.9. The distribution of protein in this track is almost identical to that of tracks 1, 4 and 5 which represent proteins obtained from serotype 4a(T) by boiling whole organisms with SDS-mercaptoethanol solubilising buffer prior to loading on the gel. There are similarities both in the absolute number and distribution of bands as well as the relative quantity of each band present. Moreover, a Western blot of proteins obtained by the two different methods, and incubated with ovine hyperimmune serum reveals almost identical antigenic profiles (Fig. 3.10) with the exception of the band marked with an arrow . Gels and Western blots of similar preparation of serotypes 1/2b and 3b confirmed this result (data not shown).

Western blot analysis of L. monocytogenes antigens following enzyme digestion with 'proteinase K'.

When Listeria antigens, obtained by sonication of seven different serotypes, were incubated with the proteolytic enzyme 'proteinase K', all bands reacting with sheep hyperimmune serum were removed (Fig. 3.11). The track on the extreme right marked C 4aT represents sonicated cell wall which has not been exposed to enzyme. A similarly blotted protein profile was stained with Coomassie blue and, again bands were completely removed except

where undigested protein had been blotted (result not shown). When this procedure was repeated using detergent-extracted bacteria a similar result was obtained (Fig. 3.12). With the exception of a band of approximately 70 KD (arrow), and some faint banding below, all antigenically reactive listerial antigens, as detected by this method, have been removed from the enzyme treated sample run in track 2. A Coomassie blue stained gel of the same preparations yielded a very similar result with, in this case, no banding at all visible in the track where digested material had been run.

Analysis of different serotypes of L. monocytogenes by SDS-PAGE and Western blotting

Electrophoretic analysis of proteins derived by detergent extraction of bacteria of different serotypes revealed some interesting differences, as shown in the Coomassie blue stained gel of Fig 3.13. Differences include a strongly staining 76 KD polypeptide of serotype 1/2b which is much less prominent in the other serotypes examined. Similarly, serotype 3b has a prominent band of approximate molecular weight 35 KD (arrow) which is less obvious in the other serotypes. In addition, serotypes 4a, 4b, 4d and 5 each have a 55 KD polypeptide (4b, arrow) which, if present, is considerably less well defined in serotypes 4a(T), 1/2b, 3b, 3c and 7. Finally, a 43.7 KD molecular weight protein, or protein complex, (1/2b, arrow) is significantly present in all serotypes other than serotype 4a and 5.

When Western blots of detergent extracted material (Figs. 3.14 and 3.15), and sonicated cell wall preparations (Fig. 3.16) were examined with ovine hyperimmune serum other differences were apparent.

For ease of reference in this analysis, the most prominent antigens of the type strain 4a(T) have been reproduced schematically to the left of Figs 3.14 and 3.15, grouped, and labelled a - h.

group a: 2 bands - extracted from serotype 4a(T) only.

group b: 2 closely apposed bands - extracted from all serotypes except serotype 5.

group c: a three band complex - extracted from all serotypes

group d: a dark/light/dark/light complex of four antigens - clearly visible in all serotypes except 4a which may be a function of the lower well loading in this instance.

group e: 3 bands - extracted from all serotypes, although the lower band is clearly composed of two bands in serotypes 1/2b and 3b, and the band distribution in serotype 4a is equivocal, perhaps again due to lower well loading.

group f: 2 dark bands - extracted from all serotypes

group g: One or two dark bands of similar molecular weight in all serotypes except serotype 5, where a band is present of similar intensity but higher molecular weight (arrow).

group h: The three band complex of group h is variable, with only the middle band being consistently present.

Lower molecular weight bands (25 - 17 KD):

These are much more variable, with the single antigen just below the 25 KD mark being the only one which is consistently present (track 4a T - arrow). A complex of 3 antigens at the 17 KD molecular weight mark is consistent except for serotype 5 and, perhaps, 4a.

Though the protein loading of each track in Fig. 3.16 was not made equivalent, it is obvious, in a comparative study of crude cell wall fractions obtained by sonication, that serotypes 1/2b and 4b contain larger amounts of an antigen of approximate molecular weight 40 KD (arrows).

A more detailed comparison of serotypes 4a(T), 4b(T), 5 and 7 was made using blots of sonicated cell wall antigen probed with antiserum raised both in spf lambs, and in two inbred strains of mouse (Figs 3.17, 3.18 and 3.19).

From these results, antigens from each bacterial source, blotted on 4 separate nitrocellulose strips and incubated with the same antiserum, can be compared (eg. each strip labelled 4a from the 4 photographs shown in Fig 3.17).

In this way, it is shown that by using antiserum from the spf lamb vaccinated with serotype 4a(T), the quantity and distribution of antigens of serotypes 4a(T), 4b(T), and 7 are very similar, with some differences being apparent for serotype 5. Differences relate to the antigen complex immediately below the 66 KD marker which in serotype 5 appears to be composed of three equally spaced bands of equal intensity compared with a two band complex observed for the other three serotypes. In addition, there is a dissimilar distribution of antigens between 45 and 25 KD for serotype 5 compared with the three other serotypes. The two most prominent antigens in this region of serotypes 4a(T), 4b(T) and 7 are probably equivalent to each other, and are more widely spaced than the two most prominent antigens in the 45-25 KD region of serotype 5. In support of this result, antisera raised in the three other spf lambs against serotypes 4b(T), 5 and 7 also demonstrate the antigenic similarity between serotypes 4a(T), 4b(T) and 7 and, again, reveal a difference in distribution of antigens in the 45-25 KD region of serotype 5.

In conclusion, we can detect antigenic differences between serotype 5 and the other three serotypes of L. monocytogenes in the 45-25 KD molecular weight range using antiserum raised in each of the spf lambs. Such differences could not be clearly determined by using mouse antiserum from either of the two inbred strains since

mice, compared with sheep, recognised fewer antigens of L. monocytogenes, especially in the apparently variable 45-25 KD region, (Figs 3.18, and 3.19).

A second question arising from this work is whether the four different serotypes of L. monocytogenes evoke the same or different antibody responses following vaccination. This can partly be determined by comparing adjacent strips of the same antigen, each of which has been probed with a different antiserum, as shown for example in Fig 3.17 a-d.

The strongest antibody response in terms of the number of different antibodies produced has been evoked by vaccination with serotype 4b(T) (Fig 3.17). Vaccination with serotype 5 resulted in a poorer response directed against a protein of approximate molecular weight 50 KD, which is strongly reactive with antisera raised against serotypes 4a(T) and 4b(T). Vaccination with serotype 7 evoked no detectable antibody response against this 50 KD protein (Fig 3.17), yet it is evident that each of the four serotypes examined possesses this 50KD cell wall component.

It is tempting to assume that the different response evoked is due to the differences of antigen processing and presentation of the four different serotypes following vaccination. Such an assumption however can not be made in view of the small sample size from an outbred population. Pooled sera, each obtained from 10 mice from two inbred mouse populations, were therefore used to derive the results shown in Fig 3.18 and Fig 3.19.

However, since none of the groups of mice seroconverted to proteins in the 50KD range, differences in antigen presentation of this particular component could not be established.

Nevertheless, it was noticed that though each serotype possessed a single distinct antigen of MW>76, it was only those C57 Bl mice which were vaccinated with serotype 4b(T) which seroconverted to this protein. In addition, it is the C57 Bl mice vaccinated with serotypes 4b(T) and 7 which have produced the full range of antibody response to antigens between 45 and 25KD molecular weight.

The results obtained from the two mouse populations are very similar. Vaccination of VM mice with serotype 4b(T) again produced the strongest response to the >76 KD protein component, and vaccination with serotype 7 similarly induced a more readily detectable antibody response to 45-25 KD proteins than occured with other vaccinates including, this time, vaccination with 4b(T).

DISCUSSION

When the protein components of L. monocytogenes CWF made by pressure cell disruption, were inoculated into a rabbit, an antibody response was elicited to some of the major components as can be determined by the similarity of antigen distribution seen in Figs. 3.1 and 3.2 (track cwf 2). The simple conclusion is that the majority of the 15 proteins which have been indicated by arrows are potentially antigenic when inoculated with adjuvant into rabbits.

Further consideration of Fig. 3.2 shows that all three outbred rabbits have responded to bacterial antigen in a similar way, despite variation of vaccinal protocol. Rabbits receiving formol killed organisms and CWF recognise a total of eight out of nine antigens in common. The antigen of MW just greater than 45 KD has been recognized by the rabbit vaccinated with CWF but has not elicited a response in the animals receiving whole organisms. The rabbit vaccinated with heat killed organisms has developed antibodies directed against fewer components of the CWF, but recognizes a total of five antigens in common with the other two rabbits: three antigens denoted by arrows and a further two antigen only faintly visible on the photograph and corresponding to antigens "d" and "f". It is therefore apparent that the same cell wall components are antigenic when whole killed organisms are presented without adjuvant.

From Fig. 3.3, it can be seen that all antibody activity has been removed by absorption of serum with fk cells of L. monocytogenes. It can be postulated therefore that antigens "a" - "i" (with the exception of antigen "h" which is not visible in this blot) reside on the L. monocytogenes cell surface. The effects of non-specific absorption of antibody would seem to be adequately accounted for by the inclusion in this experiment of strips probed with serum absorbed with equivalent quantities of heterologous organisms. Moreover, the fact that there has been no apparent differential diminution of bands by absorption with each of the six bacterial species would suggest that these organisms do not have epitopes in common with these listerial antigens as a significant part of their cell surfaces.

The result shown in Fig. 3.4 shows that two outbred sheep have responded to L. monocytogenes antigen in a nearly identical manner. Moreover, when the responses of rabbits and sheep are compared (Fig. 3.5), there seems to be no variation of response to antigens of higher molecular weight (66 KD and above); but some diversity of response to lower molecular weight antigens (between "f" and "i"). It is of course, recognised that inter-species comparisons of this nature require further analysis of breed and/or strain differences within a single species.

It is apparent from a comparison of blots developed using either an iodinated probe or an HRP conjugate (Fig. 3.2 cf Fig. 3.5) that using an HRP conjugate gives a better resolution of bands. From Fig. 3.5 it is obvious that antigen "e" is a complex of bands, that antigen in the region "f" has become resolved into 3 distinct bands, and that two further bands have become apparent in the "f"-"g" region. From this result it was determined to develop the technique of HRP-conjugate detection for use on all subsequent blots.

The magnitude of the response of the two sheep to their first exposure to formol killed organisms could be misleading (Fig. 3.4). It must be remembered that the strips marked 2 in Fig. 3.4 do not represent a primary response to vaccination since these animals had each received approximately 5×10^6 organisms perineurally eight weeks previously as part of another experiment (Table 2.1). The strips marked 1 were incubated with antiserum taken before this date. The range of antibodies produced by sheep in response to a

primary vaccination with formol killed organisms would, at any rate, be difficult to assess in aged conventional animals due to the ubiquitous nature of L. monocytogenes in the environment.

Both methods of detergent extraction yielded an almost identical protein/antigen profile (Figs. 3.7a and b: Fig. 3.8). In addition proteins and antigens obtained by detergent extraction of cells were almost indistinguishable from those achieved by boiling whole cells in solubilising buffer; a method calculated to render all bacterial components soluble. It can therefore be assumed that detergent extracted protein obtained in this way represents total bacterial protein and is a convenient way of achieving a homogeneous solution of known protein concentration.

A study of Figs. 3.7a and b and Fig. 3.8 therefore allows a comparison of total bacterial protein with more specific cell wall preparations prepared either by pressure or by sonic disruption. Cell wall preparations were made by mechanical disruption and differential centrifugation with no particular regard for either the conservation or removal of cytoplasmic membrane components. It is reasonable to suppose that some cytoplasmic membrane components would be lost during preparation of crude cell wall fraction. This, and loss of cytoplasmic components may account for differences seen. Further study in this area should include SDS-PAGE and blot analysis of membrane preparations obtained by extraction with 'sarkosyl' which should selectively solubilise the cytoplasmic membrane (Filip et al, 1973). Antigenic differences

are also seen between the two cell wall preparations derived by mechanical disruption, and demonstrate the absolute necessity of using standard techniques for the comparative study of serotypes.

It might be inferred from Figs. 3.7a and b and Fig 3.8 that many proteins are highly antigenic (many epitopes eliciting antibody of high avidity). Some bands which are scarcely visible by either Coomassie blue or silver staining are strongly detected following transfer to nitrocellulose. This point is exemplified by the protein of approximate MW 30 KD seen in track 3. An alternative explanation would be that such antigens, though readily blotted, have only a small protein moiety, and are not strongly stained using specific protein stains.

From the results obtained in Figs. 3.11 and 3.12 it can be deduced that all the antigens under consideration are either proteins, or that they have a protein moeity. Whether or not these antigens are proteins, lipoproteins, or glycoproteins might be ascertained in further studies of gels stained with periodic acid Schiff and of blots stained with labelled lectins. In any event, the elucidation of such an abundance of proteinaceous antigens has rarely been reported for L. monocytogenes.

By Coomassie blue staining alone there are obvious differences in bacterial protein content between preparations of different serotypes. This result is an apparent contradiction of the results obtained by Lamont et al (1985) who reported similarity between L. monocytogenes (including representatives of all serotypes) and

other Listeria species (L. welshimeri, L. seeligeri, L. innocua and L. ivanovii) based on Coomassie blue stained SDS-PAGE gels of ultrasonically disrupted organisms.

Differences in distribution of protein components were also apparent by Western blotting. Using conventional sheep antiserum on detergent extracted antigen (Fig. 3.14) distinct differences could be detected between serotype 5 and other species in respect of antigens in groups b and g, and antigens of lower molecular weight (25 - 17 KD). Moreover, Western blots of sonicated cell wall fraction of the four serotypes chosen for closer analysis confirmed antigenic differences between serotype 5 and the other three serotypes. This result lends support to the inclusion of serotype 5 in the taxonomic schema as a distinct subspecies: Listeria ivanovii.

Conversely, however, Western blot analysis did not distinguish between the two serogroup 4 organisms (4a(T) and 4b(T)) and serotype 7.

There is scope for further analysis of this nature using antisera from many more individuals and other species. The above results suggest that mouse antiserum from inbred populations is not very suitable for this work because of the limited range of antibodies evoked by vaccination. For analysis of strain differences pooled sera from many individuals of an outbred population would have been more useful.

Though protein differences are apparent between serotype 5 and the other serotypes examined in terms of their molecular weight, we can not assume that this represents differences of antigenicity. It is quite possible that proteins of different molecular weight have identical epitopes. True serotype-specific antigenic determinants might only be definitively demonstrated by developing blots with cross-absorbed antisera.

Preliminary results using inbred strains of mice indicate that though different serotypes may contain common components (viz >76 KD protein); these can induce a variable serological response on vaccination. It is speculated that this reflects a variation of antigen handling of the different serotypes. This work might well be continued by using further inbred strains of mice or other species which would give a wider range of antibody response.

The results reported here are at variance with other electrophoretic protein analyses of L. monocytogenes. Ivanov and Massalski (1977) conducted disc electrophoresis of water soluble proteins of formol fixed organisms of various serotypes, including 4a, 4b and 4d, which were studied above. By this method he distinguished, for each serotype, between 13 and 19 separate proteins of undetermined molecular weight. Ivanov observed that each serotype had its "characteristic protein graph", yet remarked upon the "full similarity" between the various subtypes of serogroup 4, and the two serotypes studied of serogroup 5. Unfortunately, it is impossible to ascertain whether this interpretation is substantiated, or even what it might mean, because the reproduction of the results in this article is so poor.

Lamont *et al* (1985) studied SDS-PAGE gels of whole sonicated organisms and defined percentage similarity as = number of matching bands x 2 / total number of bands in both strains x 100. They found that this was between 79 and 98% for *L. ivanovii*, *L. innocua*, and all other serotypes of *L. monocytogenes*. Indeed from a purely visual appraisal of a well reproduced gel showing serotypes 1/2a, 6a, and 5, no differences can be detected. Albeit using different serotypes, this is in direct contradiction to Fig 3.13, where some major differences in the intensity of bands are apparent. Bands were so numerous in SDS-PAGE preparations of whole organisms (as can be seen from Fig 3.13) that it was considered that an estimation of percentage similarity based on presence or absolute absence of bands was not possible. It is felt that homology between different bands is more accurately assessed from Western blots. Western blotting showed expected differences of serotype 5 with other serotypes. However, the proteins of serotype 7 on gels and blots were similar in all respects to **serotypes 4a(T) and 4a(T).**

From the serotypes studied, it was considered that sufficient homology existed to warrant the use of cell wall proteins of one serotype in a diagnostic ELISA test.

CHAPTER 4.

Structure and Expression of Flagella of L.monocytogenes serotype 4b

INTRODUCTION

The temperature dependent motility of L. monocytogenes is widely reported in standard texts, Sonnewirth and McGee (1980), Wilson and Miles (1964). There are, however fewer references in the literature relating to the extent, number and arrangement of flagella at different temperatures. Griffin and Robbins (1944) studied 43 strains of L. monocytogenes and showed that all strains were motile at 20°C and that all but 7 were motile at 37°C. Of the same strains, they remarked that all strains were flagellated regardless of the temperature of incubation, though for two strains grown at 37°C less than 1% of cells had flagella. In the 7 strains where motility could not be determined they found that cells were nevertheless flagellated, with, in one instance, 18% of organisms possessing flagella.

Leifson and Palen (1955), showed that, at 20°C in semi-solid medium, 11 out of 81 different strains of L. monocytogenes yielded stable flagellar variants. These included non motile organisms which expressed normal flagella as seen by light microscopy.

In this thesis broth cultures of L. monocytogenes 4b(T), at both 20°C and 37°C were used for challenge experiments in mice. Washed pellets of organisms grown at 4°C, 20°C and at 37°C were examined

by electron microscopy. It was considered that this would allow a more reliable assessment of flagellation than an observation of motility.

This chapter also contains what is considered to be one of the first reported electron-microscopic studies of L. monocytogenes flagella.

Also in this chapter, a crude flagellar preparation has been examined by SDS-PAGE and Western blotting, and the major structural flagellin' sub-unit determined to be a protein complex of 29KD molecular weight.

Finally, the expression of 29KD 'flagellin' by organisms at 20°C and at 37°C is determined by SDS PAGE and Western blotting of whole organisms.

RESULTS

Temperature dependant expression of flagella of L.monocytogenes serotype 4b(T) as seen by transmission electron microscopy.

L. monocytogenes serotype 4b(T) was grown in tsb, without shaking at 4°, 20° and at 37°C, to the early stationary phase of growth, and a washed pellet of each culture was examined and compared by transmission electron microscopy. Bacteria grown at 20°C had many flagella distributed in a peritrichous manner with many points of attachment. Fig 4.1 shows three such bacteria and

is a typical example of the number of flagella present and their apparently random arrangement around the bacterial cell wall. By contrast, examination of organisms grown at 37°C revealed no fully formed flagella although a few flagella fragments could occasionally be seen both attached to cells, and detatched in association with the cell pellet (Fig 4.2, arrows). In pellets of bacteria grown at 4°C, and examined by this technique flagellar fragments were seen similar to those observed for bacteria grown at 37°C, (Fig 4.3).

In conclusion, it is only at room temperature (20°C) that flagella are evident in large numbers on all bacteria.

The appearance of flagella by negative staining and transmission electron microscopy

Flagella of L.monocytogenes are about 4-5µm in length, and by staining with 0.5% ammonium molybdate following fixation with 0.5% glutaraldehyde they are apparently a simple unsheathed structure of a uniform thickness of approximately 14 nanometres comprising a relatively electron lucent area bordered by dense bands (Fig 4.4). The sinusoidal wave form of flagella of L. monocytogenes described by Leifson and Palen (1955) using light microscopy could not be seen. This may have been due, in this instance, to the use of glutaraldehyde for fixation.

The Relationship of flagella to 29KD protein

By agitating a washed suspension of 20°C broth culture with glass beads, flagella could be sheared from the bacterial cell surface leaving the organisms apparently intact (Fig 4.5). The clear gelatinous precipitate obtained by ultracentrifugation of supernatant and cell washings was seen to be an almost homogenous mass of flagella (Fig 4.6). Approximately 2.0mg of crude flagellar protein per litre of broth culture could be recovered in this way. The crude flagellar material gave rise to only one discernible band of approximate molecular weight 29KD in polyacrylamide gels stained with Coomassie Blue (Fig 4.7). This 29KD protein is seen to comprise the bulk of the crude flagellar preparation by both silver staining of SDS polyacrylamide gels (Fig 4.8, track 1) and development of Western blots using hyperimmune antiserum (Fig 4.9, track 1). As the greater sensitivity of the latter two techniques revealed the presence of other bacterial components together with 29KD flagellar protein, further purification procedures were carried out.

Purification of 29KD proteins (a) By column chromatography

When crude flagellar protein was depolymerised and applied to a Superose 12 molecular sizing column, an elution profile of three distinct peaks was obtained (Fig 4.10).

The first, large peak (A) was consistently present and always appeared immediately following the void volume of the column. No protein could be detected in this peak either by colorimetric assay,

or by silver staining of gels, and it was considered to be an artefact due to residual SDS in the sample.

Silver-staining of SDS-PAGE preparations of peaks (B) and (C) showed that peak (B) contained only minor protein bands, whereas peak (C) contained 29KD flagellar protein. Peak (B) material was discarded, and Peak (C) was applied to a positively charged ion exchange column (Mono Q - Pharmacia) in a further attempt to remove contaminating material. Silver staining and Western blotting of peak (C) are shown in Figs 4.8 (track 2) and 4.9 (track 2).

When peak (C) from the Superose column was applied to the ion exchange column, three further peaks were obtained (Fig 4.11). Peaks 1, 2 and 3 were eluted with 0.16, 0.31 and 1.0 molar sodium chloride respectively. When examined by SDS-PAGE and silver-staining, each of the three peaks was found to contain flagellar protein. In addition, peak 1 was found to contain two bands of molecular weight less than that of the major flagellar proteins, and peak 3 to contain small amounts of a 43KD contaminating protein. These minor contaminants are demonstrated by small arrows in Fig 4.8.

When a similar protein profile was examined by Western blotting, the 29KD flagellar proteins are again seen to be the most prominent feature of each peak, (Fig 4.9). The contaminating components of peaks 1 and 3 are again demonstrated by small arrows.

It is evident from silver-staining and Western blotting that peak 2 contained 29KD listeria flagellin in a highly purified

state. Material from peak 2 was therefore used for the preparation of affinity purified mono-specific anti-flagellar antiserum.

Moreover, it is apparent from Figs 4.8 and 4.9 that listeria flagellin is comprised of at least two proteins of nearly identical molecular weight in this system; See Fig 4.8 lane 4 where large arrows clearly demonstrate the two bands.

Purification of 29KD proteins (b) By urea extracton

A preparative method of obtaining pure L. monocytogenes 29KD flagellin was sought which would yield sufficient material for all subsequent ELISA tests.

The method of urea extraction described in chapter 2 was successful.

The rationale behind this method was based on the work of Koffler et al, (1957) who showed that flagella of mesophilic bacteria (E.coli and P.vulgaris) were dissociated in the presence of 6M urea. For the present study a pellet of L. monocytogenes flagella (serotype 4b(T) was dissociated by treatment with 6M urea at 50°C for 45 minutes.

It was anticipated that prolonged dialysis against PBS would cause reaggregation of dissociated flagellin which could then be recovered by ultracentrifugation, according to the method described by Asakura (1970). However, when 1µg quantities of protein of both pellet and supernatant of the centrifuged dialysate were examined

by SDS PAGE and silver staining, the pellet revealed contaminating proteins only, whereas the supernatant showed 29KD flagellin with no visible contaminants (data not shown). This supernatant material was used in all subsequent ELISA assays.

Expression of 29KD protein ("flagellin") by L.monocytogenes 4b(T) at 20°C and 37°C

In a comparative study of L.monocytogenes grown both at 20°C and at 37°C using SDS-PAGE and staining with Coomassie Blue some major differences are apparent (Fig 4.12).

For this result, equal numbers of live organisms of serotype 4b(T), from late log phase cultures, were dissociated by boiling for 6 minutes in SDS/mercaptoethanol solubilising buffer, and loaded onto the gel in equal amounts.

Flagellin is the predominant protein elaborated by organisms grown at 20°C, whereas it is not an apparent feature of organisms grown at 37°C. Moreover, with the exception of a distinct protein of 43.7KD and two proteins of higher molecular weight, (72.4 and 75.9KD) L. monocytogenes grown at 20°C appears to express little other structural protein when compared to bacteria grown at 37°C.

By Western blotting using antiserum raised in an spf lamb against L. monocytogenes serotype 4b(T) these differences are emphasised (Fig 4.13-b).

Again, flagellin appears to be the largest single antigenic component of bacteria grown at 20°C. It is not clear how much flagellin is present in a similar number of bacteria grown at 37°C by using a polyclonal antiserum since there are at least 4 separate protein bands discernible occupying the 28-30KD molecular weight range. The protein at 43.7KD is again more prominent in bacteria grown at 20°C compared with organisms grown at 37°C. However, with the above two exceptions, all other protein antigens are present in smaller amounts in bacteria grown at the lower temperature. A distinct triad of antigens from L. monocytogenes cultured at 37°C can be seen in the molecular weight range of 70.8 - 73.3 KD (top arrow). In the same molecular weight range, only 2 antigens are discernible in the 20°C culture. Similarly, two antigens can be seen between 66 and 68.4KD at 37°C, where only one is present at 20°C,(middle arrow). At 20°C, few antigens of low molecular weight (<29KD) were detected. In the same region of blotted organisms grown at 37°C, bands were more clearly visible with two antigens of similar molecular weight being particularly prominent,(bottom arrow).

Finally, for a clearer assessment of flagellin content at the two temperatures, affinity-purified anti-flagellin rabbit antiserum and the monoclonal antibody Mab Flag 1 were used to develop Western blots of the two preparations, (Figs 4.13a and 4.13c respectively).

Using the affinity purified antiserum a vast difference is evident in the amount of flagellin present between bacteria grown at the two different temperatures, with a faint trace of flagellin at 37°C only just discernible.

Using the monoclonal antibody Mab Flag 1, a much narrower antigen band was visualised for bacteria grown at 20°C, and no antigen at all could be detected for bacteria grown at 37°C.

DISCUSSION

Hitherto, electron microscopic studies of L. monocytogenes have been primarily concerned with cell wall structure and cytoplasmic components, and have almost exclusively been conducted using bacteria grown at 37°C, Ghosh and Murray (1967), North (1963). As a result of this the ultrastructure of listeria flagella has been somewhat overlooked.

The electron micrographs shown in Figs 4.1-3 bear out the early results of Griffin and Roberts (1944) who, by flagellar staining and light microscopy of strains grown on semi-solid medium, concluded that "L. monocytogenes is peritrichous when grown at ordinary room temperatures and has a strong tendency to non-flagellation at 37°C". The same authors observed a maximum of four flagella on each organism. In their study of 81 different Listeria strains Leifson and Palen, (1955) stated that at 20°C on semi-solid agar the best flagellated strains showed many individuals with 5-6 flagella, but that commonly there were fewer. The strain 4b(T) as seen in Fig 4.1 shows a peritrichous distribution of flagella at 20°C (apparently more than the four described by Griffin and Robins, and more than can be seen in the pictures of Leifson and Palen). However, the electron microscope technique employed above did not reveal the typical sinusoidal wave pattern observed by Leifson and Palen (1955) such that the point of

attachment of every flagellum is very difficult to determine. This technique would be unsuitable for a determination of the exact number of flagella per organism.

Flagella of L. monocytogenes are of a length and diameter consistent with normal limits for prokaryotic flagella quoted in standard texts. Since all flagella examined were of the same apparently simple construction, and since the technique was calculated to cause minimum trauma it can be supposed that no flagellar sheath exists.

A denatured sample of the crude flagellar material seen in Fig 4.6 is composed predominantly of a 29KD complex. It can therefore be unequivocally stated that the flagellin subunits of L. monocytogenes are 29KD proteins. The crude flagellar preparation contains many impurities demonstrable by the more sensitive techniques of silver staining and Western blotting, the most prominent of which is a protein of approximate molecular weight 43KD. This could be either contaminating somatic antigen removed during the shearing process or some other flagellar component. Many of the flagella in the sheared mass had slightly thickened or bulbous ends consistent with the known morphology of flagellar hooks (Fig 4.6, arrows). It is possible that the 43KD contaminating antigen is referable to hook protein.

The major proteins of all three peaks eluted from the ion exchange column are in each instance of molecular weight 29KD. A likely explanation of this phenomenon is that each peak represents flagellin at different degrees of polymerisation. Abram and Koffler

(1964) showed that Bacillus flagellin polymerises into flagellar filaments at low ionic strengths. It is reasonable to suppose that 18 hours of dialysis of the sample against 0.1M Tris before application to the ion exchange column allowed some polymerisation of the flagellin monomer. This could account for differences in net positive charge and subsequent differences in their elution from the column.

It seems reasonably clear from the silver stain of the proteins of the peaks obtained from the Mono Q column that the flagellin is composed of two proteins of almost identical molecular weight. Isoelectric focussing of purified flagellin on an ampholyte gradient might serve to separate the flagellar proteins more distinctly. It is not clear whether the two proteins are both present in a single flagellar structure or whether they represent two types of flagella each composed of a single protein species. Nevertheless this finding is compatible with the observations of Schmitt et al (1974), who detected two flagellin components in Pseudomonas rhodos. Schmitt et al speculated that the presence of two slightly different monomers could function in the propagation of helical waves providing an alternative to the theory of assembly of identical sub-units proposed by Klug (1967).

Flagellin is a minor component of bacteria grown at 37°C as determined by SDS-PAGE and Western blotting, (Figs 4.12 and 4.13). It would appear therefore that L. monocytogenes grown at 20°C produce flagella de novo rather than simply assembling pre-existing flagellin from either the surface or the interior of the cell. The affinty-purified monospecific rabbit antiserum detected a trace of

flagellin only in the bacteria grown at 37°C. This result is compatible with the trace amounts of flagella seen by electron microscopy.

It can not be overlooked that organisms grown at 37°C might secrete free flagellin into broth supernatants. To settle this question would require an analysis of protein precipitates of such supernatants. This kind of study was undertaken by Paquet et al (1985) using peptone broth supernatants of serotype 4b grown at 37°C. They claimed that SDS-PAGE of ammonium sulphate precipitated proteins revealed bands corresponding to surface proteins of the same organisms. They remark particularly on the presence of a 60KD protein, but do not mention a 29KD complex. Unfortunately the gels from which their data was derived are not shown.

The result obtained using Mab Flag 1 (Fig 4.13-c) reveals only a single 29KD band in an SDS separated immunoblot of 20°C grown cells. The band is much narrower than that detected by the polyclonal sera. The result suggests that the two flagellar proteins observed in Figs 4.7 and 4.8 are dissimilar in at least 1 epitope.

Finally, it is established that flagellin of L. monocytogenes 4b(T) can be a potent antigen as determined from Fig 4.13-b. The antiserum used in this blot was obtained by vaccination of an spf lamb with formol killed organisms of serotype 4b(T) grown at 37°C, and yet it detects 29KD protein very strongly. This serum also reacted strongly with crude flagellar extract of serotype 4b(T) as seen in the following chapter, (Fig 5.10). It has been amply

demonstrated that this serotype expresses very little flagellin at this temperature. In addition the vaccine was prepared with very little concern for flagellar preservation. Nevertheless, this animal still mounted a considerable antibody response to flagellin.

There is overwhelming evidence that infection of sheep with L. monocytogenes is via contaminated foodstuffs. It is quite reasonable to suppose that the original inoculating dose is composed of organisms which express flagella.

Since bacterial flagellins are known to be potent stimulators of the immune response (Nossal and Ada, 1971) it was considered possible that L. monocytogenes flagella would elicit a measurable serological response in sheep following field infection. This is investigated in Chapter 7.

To be useful in serological assays it is required that L. monocytogenes flagella should be present on all pathogenic serotypes. Previous work based on agglutination of whole organisms, has indicated that all flagella of L. monocytogenes possess a common antigenic determinant (Paterson, 1939). In the following chapter, seven further serotypes are examined. Isolated flagellins are compared with flagellin from serotype 4b(T) by the relatively new techniques of SDS PAGE, Western blotting and dot blotting.

CHAPTER 5:

Comparison of Flagella of Different Serotypes of L. monocytogenes by Electron Microscopy, SDS-PAGE and Western Blotting.

INTRODUCTION

By cross-agglutination tests using washed, formol-killed cells of five serotypes of L. monocytogenes grown at 25°C, and unabsorbed antiserum, Paterson (1939) demonstrated a common flagellar antigen which he designated antigen B.

Subsequent tests using the same antisera absorbed with homologous and heterologous organisms of the same five strains revealed a further three flagellar antigens, (A, C and D), which were present on some isolates but not on others. In the same study, Paterson used monospecific antiserum against factors A, C, and D to analyse a total of 27 isolates of L. monocytogenes. He found that all the strains fell into one or other of three antigen combinations, namely AB, ABC and BD.

In the ensuing years no other method of antigenic analysis of L. monocytogenes flagella has been undertaken, and no further antigen combinations have been determined. Thirteen of the seventeen serotypes defined by agglutination tests bear the ABC combination (Table 1.1). This chapter analyses crude cell surface extracts of eight serotypes of L. monocytogenes grown at 20° C, by SDS-PAGE and by immune staining using both polyclonal monospecific antiserum and monoclonal antibody raised against purified flagellin of serotype 4b(T).

RESULTS

Comparison of different serotypes by transmission electron microscopy

When late log phase broth cultures grown at 20°C were examined by transmission electron microscopy no difference could be observed between serotypes 4a(T), 4b(T) and 5 with regard to structure quantity and distribution of flagella. Serotype 7 however was distinguishable by having no visible flagella.

Comparison of flagella of different serotypes by SDS-PAGE and silver staining

When cells of serotypes 4a(T), 4b(T) and 5 grown at 20°C are vigorously agitated with glass beads, flagellin is the predominant protein stripped from the cell surface as shown in Fig 5.1. It is not clear from this gel how much flagellin is expressed by serotype 7. A minor protein band only is present in the 29 KD region for this serotype. No 29 KD antigen could be detected for serotype 7 on a Western blot of an identical protein profile developed with affinity purified anti-flagellar antiserum raised against flagella of serotype 4b(T), (Fig 5.2).

Serotypes 4a(T), 4b(T) and 5 appear to have approximately the same amount of flagellin per µg of loaded protein and the flagella of each serotype again appear to be composed of two proteins of almost identical molecular weight. An additional observation from the above two results is that the flagellins of serotype 4a(T),

4b(T) and 5 apparently have slightly different electrophoretic mobilities. Taking measurements to the minimum distance of migration of the flagellin bands in Fig 5.1 the molecular weight was calculated for each serotype. Serotype 4a(T) was found to be 28.3 KD, Serotype 4b(T) was 29.0 KD and Serotype 5 was 27.7KD. Calculations from Fig 5.2 also showed a difference, with 4a(T) and 5 having an apparent molecular weight of 28.8 KD and with 4b(T) again slightly higher at 29.4 KD.

To elucidate these minor molecular weight differences, a further gel was run using a reduced loading such that resolution of flagellar proteins was improved (Fig 5.3). Furthermore, using the same gel, pairs of serotypes were mixed together such that observed differences were real and not due to distortion of the gel or the effects of parallax. Fig 5.3 shows no difference of molecular weight between flagellins of serotypes 4a(T) and 4b(T) (tracks 4a and 4b). However, Serotype 5, (track 5) again appears to be of a lower molecular weight and in this instance there is a difference of 1.1 KD when either the upper or the lower borders of the migrated flagellin complexes are compared.

Furthermore, a 10 fold reduction in loading of the samples demonstrates more clearly that there are two components of listeria flagellin. Silver staining of the gel showed a dark staining higher molecular weight polypeptide clearly separated, in serotypes 4a(T) and 4b(T), from a wider more pale staining polypeptide of slightly lower molecular weight. The different degree of silver impregnation into the two components of the flagellin complex was clearly apparent but is not readily demonstrated by monochrome photography.

The resolution of two polypeptides is not quite so clear with serotype 5 though two bands are still visible.

When serotypes 4a(T) and 4b(T) are mixed and run together, the flagellin bands are superimposed (Fig 5.3, track 4a-4b). However when 4a(T) or 4b(T) are run in combination with serotype 5 three distinct flagellin polypeptides are visible, a fine upper band with two wider bands directly beneath (tracks 4a-5 and 4b-5, arrows).

The monoclonal antibody Mab Flag 1 raised against 4b(T) flagellin showed specificity for both serotype 4a(T) and 4b(T) but not for serotypes 5 and 7 as determined by development of nitrocellulose dot blots (Fig 5.4).

The crude flagellar extracts of a further four serotypes (1/2b, 3b, 3c and 4d) were compared with the crude flagellar extract of serotype 4b(T). An equivalent protein loading of each serotype demonstrated that the double flagellar band was equally evident for each serotype as shown in Fig 5.5. Calculations of molecular weight for each of the serotypes using the standards shown and taking measurements from both the leading and the trailing edges of the two band complex show that serotype 3c has a molecular weight which is slightly lower than the four other serotypes by just over 1 Kilo-dalton. In order to make this small difference of molecular weight more demonstrable it was decided to run a further gel for an extended period of time using prestained molecular weight markers and continuing electrophoresis until the 25.7 KD chymotrypsinogen A component had approached the bottom of the slab. This took approximately double the time normally taken for electrophoresis.

Silver staining of this gel not only revealed a difference of molecular weight between serotype 3c and the other serotypes but in addition resolved the flagellar complex of each serotype into 3 components, (Fig 5.6).

When equivalent quantities of flagellin from serotypes 1/2b, 3b, 3c and 4d were run respectively with equal amounts of crude extract of serotype 4b(T), and silver stained, the result shown in Fig 5.7 was obtained. The triple band complex is retained for a combination of serotype 4b(T) with serotypes 1/2b, 3b or 4d, suggesting an identical electrophoretic mobility of all components. For the combination of 3c with 4b(T) however a complex of four bands was obtained. Moreover the monoclonal antibody Mab Flag 1 recognizes serotypes 4b, 1/2b, 3b and 4d but does not recognize serotype 3c (Fig 5.8).

It was found that the most successful separation of the three flagellar constituents was effected by prolonged electrophoresis through a 12% resolving gel, using prestained molecular weight markers to monitor migration of the sample through the gel. The well-loading compatible with maximum resolution of the three bands, (x, y and z) was low (0.5μg/mm^2 gel area) but the three components were readily detected in Western blots by affinity purified rabbit anti-flagellar antiserum (Fig 5.9 strip 3). The monoclonal antibody Mab Flag 1 clearly detected antigen 'y' when probed with HRP conjugated anti-mouse antibody (Fig 5.9 strip 1). Very close scrutiny of strip 1 revealed that antigen 'z' had also been detected. Antigen 'z' of strip 1 was much too faint to show up on a photograph, but to verify that it was detected by the monoclonal

antibody, a further strip was developed from the same Western blot using Mab Flag 1, followed by intermediate incubation with a biotinylated anti-mouse antibody and subsequent incubation with an avidin-peroxidase probe. This procedure was calculated to enhance the sensitivity of the test. From the result obtained (Fig 5.9 strip 2) it is clear that the monoclonal antibody recognizes an epitope present on both antigen 'y' and 'z'. Careful examination of the strips 1 and 2 revealed no staining corresponding to antigen 'x'.

Finally, of the antisera raised in spf lambs against the four serotypes, both anti 4a(T) and 4b(T) sera recognised type 4b(T) flagellin as shown in Fig 5.10. No anti-flagellin antibody at all was detected in serum from lambs vaccinated with serotypes 5 and 7. In addition, no anti-flagellin activity could be demonstrated in the lamb antiserum raised against serotype 5 in Western blots against the homologous serotype (data not shown).

DISCUSSION

From the above data we can infer that the strain of serotype 7 examined does not elaborate surface flagellins. Furthermore, by reducing well loadings it was possible to distinguish the flagellins of serotypes 1/2b, 3b, 4a, 4b and 4d from the 1.1 KD lower MW flagellins of serotypes 3c and 5. In addition, the results obtained using Mab Flag 1 provide evidence for an antigenic difference for the flagellins of serotypes 3c and 5

as well as the molecular weight difference already observed. It is obvious that flagellins of serotypes 1/2b, 3b, 4a, 4b and 4d express a common epitope not shared by the other two serotypes.

Table 5.1

Flagellar designation of L. monocytogenes serotypes after Paterson (1939)

serotype	H Antigens
1/2b	ABC
3b	ABC
3c	B D
4a	ABC
4b	ABC
4d	ABC
5	ABC
7	ABC

Table 5.1 summarizes from Table 1.1 what is known of flagellar antigens of the eight serotypes examined, as determined by serum agglutination of whole formol killed cells. From this it might be predicted that a difference of flagellar structure would be observed for serotype 3c, since this serotype does not possess flagellar antigen A.

However, the above result does reveal a difference with regard to serotype 5, which has not previously been recorded. Serotype 5

was described by Ivanov (1962) as a common isolate from sheep in Bulgaria. In his 1962 publication Ivanov states that serotype 5 possesses the ABC flagellar antigen combination. No differences between flagella of serotype 5 and other flagella possessing the ABC combination have ever been reported. The above results demonstrate the superiority of gel electrophoresis and Western blotting for serotyping, since by these techniques individual proteins can be visualised by highly specific antibody.

An obvious adjunct to the above study would be to analyse crude flagellar extracts on Western blots using polyclonal monospecific serum raised against antigens A,B,C and D.

The absence of flagella from serotype 7 is probably not remarkable since other workers have demonstrated the existence of stable, non-flagellated variations of L. monocytogenes, (Leifson and Palen, 1955). Further work is required to determine whether aflagellation is a common feature of serotype 7.

From the beginning it was noticed that the flagellar protein was composed of two polypeptides seen by both SDS-PAGE and Western blots, and subsequently it was found that three polypeptides (x, y and z) were resolved on extended electrophoresis.

A reasonable interpretation of this observation is that the higher molecular weight antigen 'x' represents flagellin and that antigens 'y' and 'z' represent modifications of flagellin brought about by the action of bacterial proteases released from the organism during the shearing stage of flagellar harvest. Though

specific protease inhibitors were not used during flagellar preparation, this was otherwise done in such a way as to minimise proteolytic action. For the preparation of purified flagella described in chapter 4, the crude flagella pellet obtained was immediately denatured by boiling in SDS/mercaptoethanol buffer; a treatment calculated to inactivate any released proteases. For the above results using crude flagella, the extracted material was immediately treated in a water bath at 60°C for 60 minutes. All material was aliquoted and stored at -20 °C or -70°C. However, despite these precautions it is recognised that proteolytic digestion might have taken place.

The result obtained using Mab Flag 1, with avidin-biotin amplification, suggests that antigens 'x' and 'y' are unrelated polypeptides, and thus it is unlikely that the polypeptide 'y' has been derived through proteolytic digestion of polypeptide 'x'. Conversely the presence of the epitope bound by Mab Flag 1 in the polypeptide 'z' suggests it is a closely related and possibly modified protein.

The necessity of using low protein concentration to achieve optimum resolution of flagellar proteins imposes a limitation on the above technique. It is realised that to determine the difference between antigens 'x' and 'y', and the relationship between antigens 'y' and 'z' with confidence would require an analysis of larger amounts of material. This would be possible using the technique of two dimensional electrophoresis or perhaps by separation of flagellar components on an immunoabsorbent column using the monoclonal antibody. An analysis of cyanogen bromide and

trypsin digests of each of the three band polypeptides would also determine, unequivocally the relationship between them.

In conclusion, preliminary studies show that unabsorbed polyclonal antiflagellin antibodies are not useful tools with which to distinguish L. monocytogenes serotypes by immunoblotting, but that differences could be determined by using a monoclonal antibody directed against the major components of the flagellar complex. These differences did not correspond to those anticipated from results of agglutination tests. Affinity purified monospecific polyclonal antiserum raised by inoculation of 29KD flagellin of serotype 4b(T) was equally able to detect flagellins of 6 other flagellated serotypes, including representatives of the serogroups commonly isolated from pathogenic material, viz; 1/2 and 4. 29KD flagellin from serotype 4b(T) was therefore used as an antigen fixed to the solid phase in an ELISA analysis of field material from animals which had died of L. monocytogenes encephalitis (chapter 7).

CHAPTER 6

A study of the specificity of protein antigens of Listeria monocytogenes using Western blotting, dot blotting, and ELISA technique.

INTRODUCTION

Previous serological tests for L. monocytogenes have been confounded by cross reaction of Listeria antigens with antigens of other bacterial species (as reviewed in chapter 1). Hitherto the cross reaction of proteins of L. monocytogenes has not been defined in terms of individual antigens. Since Western blotting allows us to visualise individual antigens, it was decided to determine if any single protein or group of proteins could be incorporated into a specific serological test.

In addition, ELISA tests were used in an attempt to quantify the cross reaction of crude cell wall fraction and cell surface antigens of L. monocytogenes with representatives of four different genera of bacteria.

Finally, preliminary studies were undertaken to define the specificity of flagellar antigens of L. monocytogenes.

RESULTS

Western blotting of heterologous antigens using Listeria immune antiserum from sheep.

All of the four heterologous bacterial species examined were shown to possess a number of cross-reacting antigens when Western blots prepared with antiserum from the spf lamb vaccinated with serotype 4a(T) were studied (Fig 6.1). Moreover, the four species also showed diversity in the distribution of molecular weights of these antigens. Streptococcus fecalis (SF) has 11 prominent cross reacting antigens between MW 114.8 and 28.8 KD. This particular antiserum reveals a different range of cross reacting antibodies directed against antigens of Staphylococcus epidermidis (SE) which span the 49.9-24 KD molecular weight range. The pattern of cross reacting antigens of S. epidermidis is dissimilar even to that observed for Staphylococcus aureus (SA); a bacterium of the same genus. Finally, a preparation of Bacillus subtilis (BS) is shown to have more than a dozen antigens which have epitopes in common with L. monocytogenes. Antiserum from the spf lamb vaccinated with serotype (4bT) gave a very similar result, and antiserum taken from either lamb before vaccination showed no reaction with any of the bacterial proteins when examined by this method (data not shown). Moreover, vaccination of an spf lamb kept under the same conditions, with Pasteurella haemolytica, elicited very few antibodies de novo against any components of the five species tested, (Fig 6.2).

Fig 6.3 shows the same proteins of the different bacterial species blotted and probed with antiserum from a conventional sheep both before and after vaccination with L. monocytogenes serotype 4a(T). This result demonstrates the presence of antibodies detecting 3 out of the 5 bacterial species, even in unvaccinated animals. A very similar result in terms of both intensity and distribution of bands was obtained using antiserum from a second conventional sheep, (data not shown).

Post vaccinal 4a(T) antiserum from a conventional sheep and a conventional rabbit show considerable similarity in distribution of cross-reacting antibodies, (Fig 6.4).

Consideration of Fig 6.5 shows that vaccination of a rabbit with formol killed S. fecalis elicited cross reacting antibodies against four antigens of L. monocytogenes: with molecular weights slightly above and below 45KD and below 12KD.

A rabbit vaccinated with B. subtilis produced antibodies which detected a total of 9 different listeria antigens by this method (Fig 6.5).

In addition, rabbit antiserum from the S.epidermidis vaccinate also clearly recognises 9 Listeria antigens. A common antigen of approximate molecular weight 12KD was detected using antiserum against S. epidermidis and B. subtilis; and a pair of antigens between 45KD and 25KD were detected with S. epidermidis antiserum in common with the S. fecalis vaccinate, (Fig 6.5).

Conversely, the rabbits given formol killed C. pseudodiphtheriticum with adjuvant, and adjuvant alone produced no significant quantities of antibody de novo following vaccination, although the rabbit receiving adjuvant alone did produce more antibody against a protein of approximately 45KD than was demonstrated in prevaccinal serum from the same rabbit (Fig 6.5).

Demonstration of Cross Reactivity of Cell Wall Components by ELISA

It was decided to quantify the cross reaction of antiserum from the rabbits vaccinated with heterologous organisms, using the cell wall fraction of serotype 4a(T) made by pressure cell disruption, in a simple ELISA test. The ELISA technique used was a direct one, involving incubation of antigen-coated plates with test antiserum, and followed by incubation with the anti-rabbit HRP-conjugated probe, as described in Chapter 2.

An initial chequerboard titration of dilutions of sensitising antigen against positive control antiserum was conducted according to the method of Burrells et al (1978). Dilutions of reagents giving approximate absorbance values of 0.9-1.0 were chosen as optimal. This corresponded to an antigen dilution of 1:400 (approximately 0.3µg/ml) and an initial dilution of positive antiserum of 1:800. Conjugate was used at an arbitrary dilution of 1:4000 in this test.

Pre-vaccinal and post-vaccinal serum samples from the four rabbits given heterologous organisms were included in this ELISA test at a dilution of 1:800. The average optical densities of the

paired samples are given in Table 6.1. For the purposes of this test it was decided to define O.D. 0.1 as an arbritrary negative baseline. (Sera are designated positive or negative accordingly).

Demonstration of Cross Reactivity of Cell Surface Components of L. monocytogenes by ELISA

It was then decided to quantify cross reactivity of L. monocytogenes cell surface antigens using an ELISA technique which employed formol killed whole organisms as the test antigen as described in Chapter 2. The number of organisms added to each well of the Millipore filter microtitre plates was standardised to a known optical density by photometry as described in the materials and methods. This dilution corresponded to the maximum number of organisms which did not prohibit emptying of wells. By chequerboard titration of positive control antiserum and conjugate it was determined that dilutions of antiserum beginning at 1:100 and of conjugate at 1:4,000 gave optimal absorbance values. At a 1:100 dilution of antiserum, the difference between positive control antiserum and prevaccinal serum from the four animals from which heterologous antiserum was derived was maximum. All pre-vaccinal antisera had an OD of < 0.1. It was arbitrarily decided to define titre as the last dilution of antiserum to give an optical density of greater than 0.1. According to these prescribed values, the titres of heterologous antisera against cell surface components of L. monocytogenes are as shown in Table 6.2.

A study of the species specificity of flagellar antigens of L. monocytogenes - by Western blot analysis using heterologous serum.

A Western blot of crude flagellar antigen was examined using both pre-vaccinal and postvaccinal antiserum from each of the rabbits. The result obtained is shown in Fig 6.6. was obtained.

Rabbits vaccinated with formol killed L. monocytogenes (Lm1) and monocytogenes cell wall fraction (Lm2) both recognised 29 KD flagellin. Of the other paired serum samples only that rabbit receiving complete freunds adjuvant alone showed an unequivocal increase in titre following vaccination. Post-vaccinal serum from rabbit Sf brought about a slightly more intense staining of 29KD flagellin than could be seen using pre-vaccinal serum from the same animal. Strips developed using serum from rabbit Cps showed bands of similar intensity both before and after vaccination, whereas antibody binding to 29KD protein could not be found either before or after vaccination with B. subtilis (strips Bs). Finally, the binding of antibody against flagellin, seen using pre-vaccinal serum from rabbit Se, had virtually disappeared after vaccination.

A study of species specificity of flagellar antigens of L. monocytogenes by dot blots.

2µg quantities of various bacterial antigens in extraction buffer (PBS with 0.1% Triton, 0.1% SDS) were spotted directly on to nitrocellulose paper, air dried, and tested with rabbit

anti-flagellar polyclonal antiserum both before and after affinity purification (Fig 6.7, strips 1 and 2).

Strip 4 represents bacterial proteins which have been examined using supernatant fluid from the hybridoma which was subsequently cloned to form Mab Flag 1. Strips 3 and 5 were developed using anti-rabbit conjugate and anti-mouse conjugate respectively, without using intermediate antibody. (Previous tests had determined that the presence of 0.1% Triton and 0.1% SDS did not prevent binding to nitrocellulose).

From Fig 6.7, it appears that there has been litle or no binding of anti-flagellin antibody to the dotted protein mixtures of any of the five bacterial species tested in excess of controls. The position with regard to S. aureus is perhaps more equivocal, requiring a sensitive quantitative test. Any increased binding seen for S. aureus might be due to non-specific FC binding of immunoglobulin to staphylococcal protein A which has been bound to the nitrocellulose membrane.

Strip 1 simply demonstrates the necessity to affinity purify antiserum from this rabbit before it could be used as an analytical reagent. Subsequent tests on western blots showed that before affinity purification this antiserum contained antibodies directed at components of L. monocytogenes other than the 29 KD complex.

DISCUSSION

The results reported above extend and confirm our knowledge of the cross-reactivity of L. monocytogenes and other bacterial antigens. In addition, the result shown in Fig 6.3 demonstrates that conventional sheep will undoubtedly have seen many of these antigens, making it necessary to use serum from spf animals in this kind of study.

When it is considered that : (a) prevaccinal antiserum from spf lamb 4a(T) showed no activity against any of the test antigens, and (b) a lamb vaccinated with P. haemolytica, kept in the same spf unit under the same regime developed only moderate amounts of antibody to only a few of the test antigens ; it can be concluded that vaccination with L. monocytogenes is responsible for all the activity seen in Fig 6.1 .

This being so, it can be seen that, with the possible exception of an antigen of approximate MW 114.8 KD, it would be difficult to choose any other protein antigen which is specific to L. monocytogenes.

We cannot be certain which (if any) of these cross-reacting proteins of other organisms causes a significant anti-listeria antibody response when presented to the immune system of sheep by vaccination. Rabbits vaccinated with heterologous organisms do show increased reactivity to listeria antigens as shown both by Western blotting (Fig 6.5) and by ELISA. Moreover, a rabbit

vaccinated with L. monocytogenes recognised a similar pattern of heterologous antigens as conventional sheep (Fig 6.4). It is therefore not unreasonable to suppose that sheep vaccinated with heterologous organisms would demonstrate a similar increase in titre against antigens of L. monocytogenes.

It must be remembered, however, that the above data give no indications of those antigens of heterologous species which are responsible for eliciting anti-listerial antibodies following a live infection. Studies using serum from sheep following, for example, outbreaks of staphylococcal skin disease or cases of caseous lymphadenitis infection (corynebacterium ovis) would be useful in this context.

Additional information provided by ELISA includes a crude quantification of cross-reaction. The result obtained using cell wall antigen (summarized in Table 6.1) is in agreement with the Western blot shown in Fig 6.5 where a cross reaction with total bacterial protein was demonstrated. The ELISA data clearly shows the degree of cross reaction elicited by the various organisms to be the following: S. epidermidis > B. subtilis > S. fecalis or C. pseudodiphtheriticum. However, when cell surface antigens alone are considered it can be seen that the titre of the B. subtilis immune serum is more than four times greater than that observed for the serum raised against S. epidermidis. It might be concluded that it is principally cell surface components which are responsible for serological cross reaction with B. subtilis.

Of the four rabbits given heterologous organisms, only that animal given S. fecalis had a very slightly increased titre to 29 KD protein (Fig 6.6). It can not be assumed that the faint banding observed following vaccination with S. fecalis has been elicited by vaccination. The fact that the prevaccinal serum from rabbit "Se" demonstrated activity against 29 KD protein which subsequently disappeared, and that a substantial titre existed against flagellin in Rabbit "Cps" even before vaccination amply demonstrates that conventional experimental rabbits of the MRI are being naturally exposed to flagellin or flagellin like antigens in the environment. Moreover, since rabbit CFA exhibited an unequivocal increase in titre following vaccination with adjuvant alone, it is possible that the mycobacterial component of Freund's complete adjuvant possesses flagellin-like epitopes which could stimulate anti-flagellar antibody. However, this result does suggest that none of the four heterologous species tested shares major epitopes with L. monocytogenes flagella. This interpretation is supported by the result of the dot blot shown in Fig 6.7. Fig 6.6 also demonstrates that subsequent studies would be easier to interpret if either spf or gnotobiotic animals were used.

In conclusion, it has been shown that cross-reaction of L. monoctyogenes cell wall protein antigens with other bacterial species is extensive. The bacterial species chosen include those which are commonplace in the environment of sheep. However, it is likely that the bacterial species examined represent only a small fraction of the total number of cross-reacting species. This suggests that the identification of a listeria-specific protein

antigen following a comprehensive study of other species would be extremely difficult. Moreover, if a specific antigen could be identified, then its subsequent purification from such a complex mixture would be a daunting task. Conversely, it is possible to purify flagellin with comparative ease and preliminary checks for species specificity are rather more encouraging.

The most important recent publication relating to the production of a listeria-specific antigenic fraction comes from Delvallez et al (1979). These workers demonstrated a total of 17 immunoprecipitates of the cell walls of L. monocytogenes using the technique of crossed immunoelectrophoresis. In a subsequent paper, Delvallez et al (1985) described the purification of one of these antigens ("antigen 2") to homogeneity - (a protein of 2 peptides, 40 and 60 KD).

By using double diffusion tests these workers went on to demonstrate that monospecific antiserum prepared against "antigen 2" did not precipitate crude soluble extracts of S. fecalis, S. aureus, S. epidermidis or C. pseudodiptheriticum and conversely, that antiserum produced against these organisms did not precipitate "antigen 2".

The objective of their study and the one conducted in this thesis is the same: viz, the selection of an antigen for diagnostic serology. For their part Delvallez et al (1985) reported that "antigen 2" formed a precipitin line together with antiserum taken from a human case of listeriosis 15 days after positive blood culture.

The conclusion from the above study using Western blotting is that the cross reaction between L. monocytogenes and other Gram positive bacteria is potentially enormous, and that it is unlikely that specific antigens will be identified by using this technique alone. Further ideas of how this problem might be tackled are fully discussed in the final chapter.

However, in the final analysis, the usefulness of any Listeria test antigen for diagnostic serology, or epidemiological studies, can only be made by examining the serological response of naturally infected animals. It is possible that the antibody response to certain selected antigens of L. monocytogenes following field infection is so high when measured by ELISA that any cross-reaction is negligible by comparison and can be accommodated below an arbitrary negative line.

The final two experimental chapters of this thesis therefore include a study of the serological response to some listeria antigens of experimentally and naturally infected animals.

CHAPTER 7

A Study of Antibody Response to Flagellar and Cell Wall Antigen in Sheep Infected with L.monocytogenes.

INTRODUCTION

Diagnosis of listerial encephalitis is a perennial problem in the UK, relying entirely upon histopathology. This is slow and labour intensive. It is also expensive (requiring the services of a skilled veterinary pathologist) and, of course, retrospective. Hitherto, tests for ovine listeriosis have not employed the antigen capture techniques of blotting and ELISA. Therefore, to determine whether these techniques had any potential, they were used here for the analysis of field and experimental material.

Blood and CSF were collected from sheep which had died of listerial encephalitis, and were subjected to ELISA as described in the materials and methods.

In addition, serum from 6 sheep which had been inoculated subcutaneously with log 10 increasing doses of live organisms were analysed by both Western blotting and ELISA technique.

RESULTS

Paired CSF and serum samples from 14 sheep were analysed, together with an additional 9 CSF samples. All samples originated from animals which had died of listerial encephalitis, the

diagnosis being made by the submitting veterinary investigation centre. All diagnoses were made by histopathology, and in 60% of cases L. monocytogenes was isolated (Table 7.1). The samples were included in an ELISA test at dilutions of 1:100 and 1:500 and were compared with a titration of a known positive antiserum, and a known negative antiserum. The standard positive and negative sera in this instance were obtained from a gnotobiotic lamb both pre and post vacination with serotype 4b(T) as described in the protocol shown in Table 2.3. Standard curves were produced from titrations of the positive antiserum against both the flagellar antigen and the CWF prepared from serotype 4a(T) by pressure cell disruption. These are reproduced in Figs 7.1a and 7.1b. The ELISAs were conducted according to the method described in chapter 2. Since the titre of most samples fell below 1:500 it is the results of samples included at the 1:100 dilution which are shown in table 7.1. Ten sheep (nos. 24-33) were randomly selected from a flock of adult ewes at the Moredun Research Institute and were included in the test. Table 7.1 shows reciprocal titres obtained when either purified flagella antigen or cell wall antigen was fixed to the solid phase.

Antibody titres against listeria flagella are low (maximum 360 - sheep no.1, serum) regardless of geographical origin of sample or age of animal from which it was taken. Only 3 out of 23 CSF samples have a detectable titre at all. The average titre of the serum of encephalitic sheep is in the same order as that obtained from apparently normal control animals (166 and 158 respectively). Titres against CWF antigen are similarly low with again only 3 out of 23 animals having detectable levels of antibody in the CSF. In

this instance the control animals have a slightly higher average titre than animals which died from listerial encephalitis (370 cf 302). It is interesting to note that the young 8 week old animal (sheep no.11) had no antibody to L. monocytogenes detectable either in serum or CSF.

From Table 7.1, it can be seen that sheep nos. 1 and 2 had antibody in the CSF apparently directed against components of L. monocytogenes. Though the quality of samples submitted by the V.I.C's was generally good it was noted that the CSF sample from sheep 1 was visibly contaminated with blood, and it was recorded in this instance that the sample had been taken from a cadaver. It was decided to use CSF and serum from these two animals on Western blots of cell wall fraction antigen. Any difference in detection of individual antigens of a CWF betwen CSF and serum of individual animals would indicate that antibody in the CSF had been produced locally and was not the result of post mortem contamination or contamination with blood during collection.

Fig. 7.2 shows that for sheep 1, two antigens are detected at the level of the top two molecular weight markers, and two are detected midway between the 45 and 25.7 KD MW markers, with the lower band being only faintly visible. Importantly, the four antigens are detected using CSF and serum alike. In a similar way, three antigens are apparently equally detected by both CSF and serum of sheep 2. (In this case, the upper 76 KD antigen has not

been detected). This result was obtained in the usual way using 4 ml of a 1:40 dilution of test serum/CSF in blot washing buffer as described in Chapter 2. Antigens were only visible after prolonged incubation (20 mins) with diaminobenzidine substrate.

Enough material was available from sheep 2 to repeat this test with a 10 fold higher concentration of test material (1:4 dilution) and using, in this case, a serotype 4a cell wall fraction as the test antigen (Fig 7.3). In this instance, two bands of the cell wall fraction are detected by both CSF and serum.

There is therefore, no evidence from Figs. 7.2 and 7.3 to suggest that antibody is produced locally in the CSF.

Finally, 6 sheep were challenged with a live inoculum of L. monocytogenes 4b(T) grown in broth culture at 37°C. Animals used were females of mixed breed between 8 and 12 months of age and were selected from a batch of 11 as having the lowest pre-infection antibody response to L. monocytogenes CWF as tested by ELISA. Each animal of three pairs was given respectively 10^9, 10^{10} and 10^{11} organisms suspended in a 10ml volume of PBS and inoculated subcutaneously at a single site in the neck just anterior to the shoulder. Rectal temperatures were recorded for 12 days following infection, and blood was taken for serology on days 0, 2, 4, 6, 10 and 17 for the two animals given 10^{11} organisms, and on days 0, 2, 4, 6, 8 and 17 for the other 4 animals. In addition, heparinised blood samples were taken from all animals on days 4 and 10 for bacteriology.

Only sheep A was obviously ill following infection. This was manifest on day 2 by depression, respiratory distress and a mucopurulent nasal discharge. A profuse growth of L. monocytogenes was recovered from heparinised blood samples of sheep A and B on day 4. No organisms were recovered from the other four sheep on day 4 and no organisms were isolated from any of the animals on day 10. All animals survived the experiment, with no neurological impairment becoming evident in the subsequent year.

Sub-cutaneous inoculation of live organisms produced a temperature rise in all six animals at each level of infection. This was detectable in 5 of the animals on the first day of infection, and reached a maximum in all animals by the third day, thereafter returning to normal (Fig 7.4).

As the rectal temperature decreased, the antibody response to the CWF antigen increased,(Fig 7.5). In each instance an increase in titre was observed after 96 hours of infection. Fig 7.5 shows a logarithmic increase in titre between day 4 and day 10 to reach reciprocal titres of 1534 and 2007 respectively. There is no significant increase in titre between day 10 and day 17. A similar logarithmic effect is seen for sheep D, E and F between days 4 and 8 with sheep F reaching a titre of the same magnitude as sheep A and B.

In addition, the Western blots shown in Fig 7.6 (a, b and c) demonstrate the development of antibodies to previously unrecognised epitopes, and the intensification of existing antibody-antigen reactions appearing after the first 4 days of

infection. This response is least evident in sheep C, which is in keeping with the rather low antibody titres detected for this sheep by ELISA (Fig 7.5).

After day 4, antibodies to 70-77KD epitopes either appear for the first time, or increase in concentration in each sheep, with the exception of sheep C. In sheep F these antibodies are already strongly present before vaccination. Antibodies directed against a complex of three antigens (MW 44-48KD) also develop, becoming especially prominent in sheep A and B. Other antibody-antigen reactions which are consistently intensified by infection are seen at 15 and 20KD.

The Western blot in Fig 7.7 shows the reaction of sheep A to a crude flagellar preparation of L. monocytogenes (serotype 4b(T)). Before inoculation, antibodies are already present to 29KD antigen and on day 10 the antibody level has apparently slightly diminished. There is certainly no evidence for there being an increased titre against flagellin in this animal.

DISCUSSION

Study of Table 7.1 shows that serological diagnosis of listerial encephalitis using protein antigens of L. monocytogenes in an ELISA test is not possible since animals which had died of the disease did not have titres in excess of background titres observed in normal healthy animals. There may of course have been an increase in the titre during the course of the disease from an even lower

pre-infection base line. Any increase, however, must have been very small, and certainly not sufficient for diagnostic purposes.

Unfortunately, it is not known how many of the CSF samples were taken from animals with a florid involvement of the meninges, but it can certainly be concluded that analysis of CSF from encephalitic animals is equally unhelpful for diagnosis. In a survey of 50 cases of listerial encephalitis submitted to Moredun Institute (47 ovine: 3 bovine) 54% of animals had significant accompanying meningitis of the cerebrum and 62% of the animals had a cerebellar meningitis. In 22% of animals, meningitis of the cerebellum was severe, with large numbers of infiltrated cells forming a continuous layer, (J.S. Gilmour, personal communication). In the same survey it was determined that plasma cells were not a common feature of the disease since 80-90% of animals had no more than occasional isolated plasma cells detectable in cerebral or cerebellar meninges, and where plasma cells were found they never constituted more than about 5% of the inflammatory meningeal infiltrate.

By analogy, it seems likely that a similar proportion (approximately 50%) of the 23 field cases which were examined in the present study would also have meningitis. The combined data from histopathological observation and antibody analysis (albeit from different animals) indicates that local antibody secretion to proteins of L. monocytogenes is not a significant feature of listerial meningitis in sheep.

There are limited data available which relates to the immunoglobulin content of the CSF of normal sheep. Reid et al (1971) using a radial diffusion technique measured the total immunoglobulin content of CSF of five control animals and recorded an average value of 3.2 mg/100 ml (range 2.2 - 3.8). They determined that this immunoglobulin was exclusively referable to the IgG subclass and that it compared with an average IgG serum concentration of 1200 mg/100ml in the same sheep; i.e. the ratio between CSF and serum IgG concentration was found to be in the order of 300-400.

The ratios of specific antibody in CSF and serum of sheep 1 and 2 against CWF, as determined by ELISA titres shown in Table 7.1, are in the order of 1:3. The apparent 100-fold increase in titre of immunoglobulin in the CSF of these animals is significant only if the samples were collected without contamination. In sheep 1 this was certainly not the case. In addition, Western blots provided no evidence that antibody was produced locally. A reliable way to check for local production of specific antibody in the CSF, is to compare CSF/serum antibody ratios with ratios of an antibody directed against a serum marker which is not involved in the disease process. This technique is described by Reid et al (1971) for the study of experimental infection with louping-ill but unfortunately can not be used for the study of field material.

The data in Table 7.1 do however conclusively show that serum antibody in diseased animals is in the same order as for control animals.

It cannot be determined from these results whether this apparent lack of antibody response is due to acute onset of disease following introduction of the organism, with animals succumbing before the occurrence of seroconversion, or whether it is due to lack of appropriate antigen presentation to B cells. In the 6 cases of experimentally induced ovine listerial encephalitis described by Barlow and McGorum, (1985) an incubation period of between 20 and 41 days was recorded (average 30 days). In addition, mice succumb to neurological disease following an intraperitoneal inoculation typically between 10 and 17 days (average 11.8 days) : see following chapter (Fig 8.1 and Table 8.4). These two lines of evidence suggest that listerial encephalitis has an extended prodromal phase, and make it a reasonable hypothesis that listerial proteins, though present, do not stimulate a B cell response.

The only serum sample which did not have detectable antibody against L. monocytogenes was derived from a two month old lamb, (sheep no. 11). We can speculate that the low level titres observed in all other animals are derived as much by contact with other organisms as an ageing process, as by infection with L. monocytogenes.

These results support the observations of Osebold and Aalund (1968) who compared agglutinating antibody titres from two different categories of flock, viz: flocks where listerial encephalitis was endemic, and flocks where it was not considered to be a problem. Using whole antiserum they found titres of the same

order in all animals regardless of origin. After reduction of serum with mercaptoethanol however, they determined that only 1.9% of normal flocks had titres against L. monocytogenes compared with 50% of animals from endemic flocks but with titres being very low in all cases.

Comparison of IgG titres between clinical material and control animals was not done in the present study. A proper comparison would require manipulation of the ELISA test since even a 50% reduction in titre would render many of the samples negative.

The results obtained in this chapter do not encourage the use of flagellar or cell wall antigens of L. monocytogenes for diagnostic serology in sheep where complete antiserum is used.

Subcutaneous inoculation of sheep with live organisms produced a pyrexia in all six animals. Four of the vaccinated sheep had a maximum temperature rise on day 3 following infection, with pyrexia being sustained over a two day period. The notable exception to this observation was sheep C which had a comparatively reduced and unsustained fever on day 1.

The non-specific immune-potentiating and pyrogenic effects of Gram positive bacterial cell wall products are well known. However, the pyrogenicity of various extracted muramyl-dipeptide derivatives in rabbits is observed early, within 6 hours of an intravenous inoculation (Kotani et al., 1979). The time of onset and duration of pyrexia observed in these sheep was later, co-inciding with the expected period of logarithmic growth in liver

and spleen of inoculated animals. The temperature response therefore implies a state of bacterial multiplication in all six animals in the period following inoculation. This is proven in sheep A and B by recovery of organisms from the blood on day 4.

An increase in antibody titre against L. monocytogenes CWF was observed in all six animals in the period following the pyrexia. All titres are low however, with a maximum recorded reciprocal titre of 2155 (sheep F, day 8). This is less than two times higher than the titre of 1105 which was recorded for one of the six randomly selected control sheep.

The range of titres recorded for the control sheep compared with experimentally infected sheep amply demonstrates the inadequacy of serology based on the analysis of a single sample. These results bear out the early observations of Gray and Killinger (1966) that a definitive diagnosis of Listeriosis depends upon a rise in titre preferably accompanied by appropriate clinical signs.

A study of Western blots shows that no antigen can be selected which induced a high antibody response de novo in all six animals following vaccination. In addition, the blot shown in Fig. 7.7 shows that an animal which has recovered from experimental septicaemia and which has seroconverted to L. monocytogenes cell wall fraction has not seroconverted to flagellin, and has antibody to flagellin already present in the serum.

It has to be concluded that there seems to be no particular advantage conferred by using these selected antigens in solid phase immunoassay for either diagnosis or epidemiological studies.

CHAPTER 8

A Study of Experimental Listeriosis in CBA mice.

INTRODUCTION

Experiments were performed using inbred mice to measure the LD_{50} for L. monocytogenes (serotype 4b(T)) following an intraperitoneal inoculation. Virulence correlates with motility for Vibrio cholerae, Pseudomonas aeruginosa and Salmonella typhimurium as discussed in Chapter 1. The primary motive of the following experiments was to investigate any effects that flagella might have on pathogenicity by comparing bacteria grown at both 37°C and 20°C. Sera were taken from surviving mice in order to measure antibody titres against both flagellar and CWF antigens.

Using the same strain of inbred mice, ID_{50} values were calculated from cfu counts of liver and spleen 72 hours following intragastric inoculation. Separate ID_{50} values were obtained for intragastric challenge with organisms grown at both temperatures. It was considered that this experiment would elucidate any differences of pathogenicity between flagellate and non-flagellate organisms, which might be related to their ability to colonise and cross the gut mucosa during the early stages of infection.

Finally, possible immunising effects of flagellar antigen were investigated in a simple mouse protection test.

RESULTS

LD_{50} experiments in CBA mice following ip. inoculation of L. monocytogenes (serotype 4b(T)) grown at 37°C and 20°C

A preliminary intraperitoneal challenge with graded doses of organisms grown at 37°C, using paired mice, established an approximate LD_{50} of between 10^4 and 10^5 organisms.

Consequently, for the following large scale LD_{50} experiments, groups of eight littermates were challenged with doses of organisms grown at 37°C which covered this range. In experiment 1, four groups of eight male 8-12 week old CBA mice, purchased from Harlan Olac Limited, (Bicester, Oxon.) were inoculated intraperitoneally with log 10 decreasing doses of L. monocytogenes grown at 37°C (0.8375×10^6 - 0.8375×10^3 cfu : Table 8.1(a)). For a comparative LD_{50} using organisms grown at 20°C, a further two challenge groups of eight mice, one above and one below this range, were included, in the eventuality that the LD_{50} following a challenge with flagellate organisms would be substantially different (Table 8.1(b)). Deaths were recorded for 21 days following challenge, after which the experiment was terminated. The LD_{50} has been calculated according to the method of Reed and Muench (1938). Experiment 1 shows a 6 fold difference of LD_{50} between organisms grown at the two temperatures, with organisms grown at 37°C being apparently more pathogenic.

This experiment was repeated (experiment 2 : Table 8.2) using 6-12 week old CBA mice which had been bred at the Moredun Institute. Mice were again randomised into groups of 8 and were challenged with graded doses of flagellate and non flagellate organisms. This experiment revealed an even greater difference in pathogenicity between the two groups, with the LD_{50} for flagellate organisms being more than 17 times greater than the LD_{50} for bacteria grown at 37°C.

However, for the primary object of accurate LD_{50} determination, the results from experiment 2 were especially disappointing. Even at the maximum dose of non-flagellate organisms given, ($0.6x10^6$ cfu), only 3 out of 8 mice died. Since this represents less than 50% of the mice in this group, an end point has obviously not been reached. Moreover, there is no linear dose response in experiment 2 for either the 37°C or the 20°C challenge groups. This also discredits the accuracy of the LD_{50} calculations for experiment 2, and will be discussed further at the end of this chapter.

Other interesting clinical, bacteriological and serological data accrued from this experiment which are given below.

Clinical observations of mice of experiments 1 & 2.

Animals were observed for 21 days following infection, and the time of death was recorded in each instance. In addition, mice were carefully observed twice daily for developing signs of neurological disorder. The results of these observations for both experiments

are given in Fig 8.1 (a-d). Where mice developed neurological disorder, this was unequivocal, and usually manifested by ataxia and inco-ordination, progressing to flaccid paralysis of either one or both of the front or back legs. Hind-limb paraplegia was associated with urinary incontinence. Occasionally both hind and fore-limb paralysis was noted in the same animal. One animal showed a tendency to roll when pushed into lateral recumbency: a clinical sign of infected mice described by Asahi et al (1957). One animal with hind-limb paraplegia had pronounced unilateral exophthalmos, and a further animal with both hind-limb paraplegia and unilateral fore-limb paralysis demonstrated excessive salivation. It appeared that the course of neurological disease was progressive with death resulting typically about 48 hours after the onset of clinical signs. However it was occasionally difficult to determine from clinical signs alone whether death was due to a progressive ascending neuritis or due to dehydration and debility caused by lack of food and water. In both experiments moribund mice were killed by cervical dislocation. Mice were deemed moribund when they had become recumbent or failed to respond to any external stimuli.

Bacteriological studies of mice in experiments 1 and 2.

Liver, spleen and brain from all survivors of experiment 1 (day 21) were aseptically removed, homogenised in peptone water, and inoculated on to sheep blood agar plates. No organisms were recovered from any of the animals.

Dead and moribund animals from experiment 2 were examined in the same way (where animals were moribund, blood samples were taken and 20µl aliquots streaked on to blood agar plates). The results of these investigations are shown in Table 8.3.

Despite the limited number of mice studied in experiment 2 it is apparent that a pattern has emerged which corresponds to the clinical evidence for early septicaemic and later neurological syndromes.

Where organisms have been isolated from CNS during early infection, in mice with no clinical neurological involvement, they have also, invariably, been isolated from liver and spleen. With the exception of a mouse dying on day 8 following infection, all early isolations from liver and spleen were profuse. Moreover, L. monocytogenes was recovered from blood in all cases of early death where blood culture was attempted. By comparison no organisms were recovered from spleens of the eight mice which died with neurological dysfunction. Moreover organisms were not recovered from the blood of six mice with neurological disase which were tested. Recovery of organisms from CNS and liver of mice dying with neurological dysfunction was not invariable, as no organisms were isolated at all from two mice which died showing this disorder.

Serological response of survivors from Experiments 1 and 2 to L. monocytogenes CWF and 29KD flagellar antigen by ELISA.

Survivors of experiments 1 and 2 were exsanguinated at the termination of the experiment (21 days after infection) and sera from each challenge group were pooled. Anti-flagellar antibody titres, and antibodies against L. monocytogenes somatic antigens (CWF) were measured by ELISA. Microtitre plates were therefore coated respectively with 1µg/ml of purified 29KD proteins from serotype 4b(T) or with CWF antigen of serotype 4a(T) (as made by pressure cell disruption - see chapter 2). Antisera from groups of mice were included in duplicate wells at doubling dilutions between 1:100 and 1:800. A pool of serum from ten uninfected litter mates served as negative controls for experiment 1 and a similar pool of eight uninfected mice was used in experiment 2 . For the flagellar assay, hybridoma supernatant from the cell fusion resulting in Mab Flag 1 was used as a positive control at a dilution calculated to give a maximum increase in O.D. following incubation with sheep anti-mouse IgG HRP conjugate at a recommended optimal dilution. For assay of antibody against cell wall fraction the control positive antiserum used was a titration of antiserum from C57/Bl mice vaccinated with fk L. monocytogenes 4b(T). The layout of the ELISA is shown in Fig 8.2. In ELISAs using flagellar antigen, and where serum from experiment 1 was titrated against CWF antigen the OD of a 1:100 dilution of negative antisera was well below 0.1 and this OD value was chosen as the negative baseline. Negative control serum from unchallenged animals in experiment 2 had an average OD value of 0.11 when included at a 1 in 100 dilution

against CWF antigen. In this assay an OD of 0.11 was chosen as a negative baseline.

By this technique, no anti-flagellar antibody at all could be found in any of the sera from any of the challenge groups in either experiment.

The serological responses of mice from experiment 1 and experiment 2 to CWF antigens are summarised in Tables 8.4(a), and 8.4(b) respectively.

Approximate reciprocal titres only are given. For example, groups recorded as >200 (groups 1, 3, and 9 of experiment 1) have titres which are greater than 1/200, but less than 1/400, at which serum dilution the OD's fall below the arbitrary baseline of 0.1. Reciprocal titres are low (0 - <800) for 17 out of 18 of the challenge groups, with only group 18 having a reciprocal titre in excess of 800.

ID_{50} of CBA mice following intragastric challenge of organisms grown either at 37°C or 20°C.

An experiment was set up to determine numbers of organisms recovered from liver and spleen following intragastric administration of log 10 increasing doses of either flagellate (20°C) or non-flagellate (37°C) organisms. Results from a preliminary probe experiment had determined that an intragastric dose of between 2.0 and 3.0 x 10^9 live organisms was required to kill 50% of the mice challenged, with some mice dying within the

early three day period following infection (Table 8.5). From this it was determined to give groups of six mice 10 fold dilutions of organisms starting at 3.0×10^9, with accurate counts obtained retrospectively. After 72 hours mice were killed by CO_2 overdose, livers and spleens were aseptically removed into 9ml of peptone water and whole organ counts were determined on duplicate blood agar plates by the method of Miles and Misra (1938). Results are shown in Table 8.6.

There is no linearity of dose response in terms of increased numbers of organisms isolated with increasing dose rate. At a sample time of 72 hours there seems rather to be a threshold effect where animals, if affected at all, have comparable numbers of organisms in liver and spleen. A dose-response effect is rather more apparent when the number of infected mice per challenge group is plotted against dose (Fig 8.3,a-d). The ID_{50} values shown in Fig 8.3 were calculated by the method of Reed and Muench (1938).

Serological response in CBA mice given intragastric inoculation of L. monocytogenes grown at 20°C and 37°C.

Survivors from the preliminary probe experiment, where mice were given approximately 3.0×10^9 cfu (20°C and 37°C) intragastrically were exsanguinated on day 21. Sera from three survivors of the 20°C challenge were pooled and sera from three survivors of the non-flagellar 37°C challenge were pooled and were compared with pooled sera from four uninfected littermates by ELISA. Purified 29KD protein and CWF were used as the coating antigens, and the layout of the ELISA was as shown in Fig 8.2. The results of this

assay are given in Table 8.5. Again, no flagellar antibody could be detected in either of the challenge groups, and only low titres were detectable against the cell wall fraction antigen.

A preliminary vaccination study to determine protective capacity of L. monocytogenes flagella

Male CBA mice, 8-12 weeks of age, bred at the Moredun Institute, were randomised into two groups of 18. Mice in 1 group were given 50µg of crude flagella of serotype 4b(T) in IFA subcutaneously on day 0. This procedure was repeated after 3 weeks and again after a further 2 weeks. Mice in the second group each received a total of 150µg of ovalbumin by the same protocol. Six days following the final inoculation, blood samples were taken from a representative four mice in each vaccinal group and the relative titre of antibody against 29KD protein determined by ELISA, as described above. Mice given ovalbumin had an OD of <0.1 when included at a 1 in 100 dilution. Mice given flagella had a reciprocal titre in excess of 12,800 using an OD of 0.1 as a negative baseline. Seven days following the final vaccination all mice were given 3 x 10^7 cfu i.p. of a broth culture of L. monocytogenes serotype 4b(T) grown at 20°C. Time of death was recorded, (Table 8.7).

Vaccination with flagella did not appear to modify the outcome of infection in any way. All mice in both groups died, with the majority of mice in both groups dying on the third day of infection.

DISCUSSION

The primary aim of the LD_{50} experiments (experiments 1 and 2) was to determine if a major difference in pathogenicity could be ascribed to the growth of L. monocytogenes at different temperatures. Previous investigations of this nature include work by Wood and Woodbine (1977) who used chick embryos as a model system to investigate the pathogenicity of broth cultures at various temperatures. Despite some contradictory results they concluded that there was an increased pathogenicity over organisms grown at 37°C when L. monocytogenes was grown at the lower temperatures of 22°C and 4°C. Experiments 1 and 2, using washed cells in the late log phase of growth, represent an attempt to study differences of pathogenicity unrelated to extracellular metabolic products. In contradiction of Wood and Woodbine, results from experiment 1 show that the LD_{50} for organisms grown at 20°C is 6.8 times higher than for organisms grown at 37°C. This result is apparently supported by experiment 2 where the difference in LD_{50} between organisms grown at the different temperatures is a factor of 17.2. (It is possible that the different values observed between experiments 1 and 2 represent inherent differences in the two populations of inbred mice).

Considering experiment 1, it would appear that there has been a dose-related response to infection, with progressively more animals dying with increasing dose. This pattern is not however, repeated in experiment 2 for organisms grown at either temperature. The same number of mice (3 out of 8) succumb to

infection in the top three groups of mice challenged with organisms grown at 37°C. Similarly, the mortality in three groups given flagellate organisms is exactly the same despite a hundred-fold difference in the number of organisms given (0.63×10^4 - 0.63×10^6 cfu). These data bear out the observations of Kautter et al (1963) who concluded from their studies of Swiss-Webster mice that LD_{50} titrations via the intraperitoneal, intravenous or intracerebral route were neither readily reproducible nor necessarily linear. They found this even though, in some instances, 50 mice per dose point were used. They go on to suggest that death is the wrong criterion for comparing the virulence of strains of L. monocytogenes and that infectivity would be a more logical index.

Despite the advantage of using inbred mice in this study, the dose-response curve in experiment 2 is still very poor. This sets the confidence limits for statistical analysis very wide, invalidating the LD_{50} calculations. The results therefore can do no more than suggest that L. monocytogenes grown at 20°C, expressing flagella, are less pathogenic than organisms of the same serotype grown at 37°C when measured by these means.

Kautter et al (1963), using Swiss Webster mice, found no difference in LD_{50} when L. monocytogenes (serotype 4b) was used for intraperitoneal challenge at 37 and 20°C. However, they do not state either the repeatability of this particular experiment, how many mice were used per dose point, or the state of flagellation of the challenge strain at the two different temperatures.

A somewhat unexpected finding arising from these two experiments was that two disease syndromes were being induced which could be distinguished by both clinical and bacteriological examination. An early septicaemic phase occurring in the first week following infection was followed by a neurological syndrome, by which time the organism has been cleared from the blood and the spleen. Residual organisms in the livers of some of these later animals could well be associated with liver abcesses which were often macroscopically visible in mice which had received large numbers of organisms.

The neurological syndrome is apparently produced equally by flagellate or non flagellate organisms. The syndrome is responsible for 37% of deaths in mice given organisms grown at 37°C , and 30% of deaths in mice given organisms grown at 20°C. This can be readily calculated from the combined data of both experiments as it is presented in Fig 8.1.

Moreover, it is apparent from Fig 8.4, which shows the incidence of the two different syndromes at each dose level, that both conditions may be induced by the same infecting dose.

Mice dying with neurological dysfunction represent one third of the total number of mice dying in the two experiments. Despite mice being used in widespread studies of L. monocytogenes over the last 30 years, the existence of two distinct syndromes has

rarely been reported. The work of Asahi et al (1957), and the somewhat contradictory findings of Cordy and Osebold (1959) are the two major investigations in this area.

Asahi et al induced a neurological syndrome in mice by submucosal and subcutaneous inoculation around the head, with a 90% incidence of histological encephalitis occurring following inoculation of lips and a 60% incidence following inoculation of gums. The neurological signs described (including time of onset and duration of disease) are compatible with observations made in experiments 1 and 2. It is, however, unclear what percentage of their mice developed overt neuropathological disease.

Cordy and Osebold induced histological encephalitis in 30% of mice inoculated by oral scarification and remarked that the mean survival time for mice with CNS lesions was longer than for mice with no CNS lesions, and that they showed fewer visceral lesions than animals dying early in infection without encephalitis. Moreover Cordy and Osebold induced encephalitis in 33% of animals by subcutaneous inoculation in the perineal region. However, they record that only 25% of animals with histological encephalitis showed well marked nervous symptoms other than depression.

Experiments 1 and 2 are remarkable for the high number of animals succumbing to nervous disorder following a simple intraperitoneal inoculation where no special care had been taken to place organisms in the vicinity of afferent nerve endings.

It is apparent from an appraisal of the work of Kautter et al (1963) that many hundreds of Swiss Webster mice were used in their experiments. Their work included LD_{50} determinations of over 60 strains of L. monocytogenes by oral, intravenous, intracerebral, and respiratory routes, with animals being observed for 14 days following infection. Despite this they state that, "the meningoencephalitic form of the disease typically found in nature was not observed".

The infecting organism used in the Cordy/Osebold study was serotype 4b, originally isolated from a case of ovine encephalitis. The organism used by Asahi and co-workers, though untyped, had been originally isolated from the CNS of a goat. The organism used for experiments 1 and 2 was serotype 4b(T), originally isolated from the brain of an encephalitic sheep and stored without further passage. It is interesting to speculate that the "neurotropic effect" observed in these experiments is a function of the infecting organism and that, in the majority of laboratory stains used, this effect is lost by extended mouse passage and/or maintenance on laboratory media. This could account for the low incidence of neurological disease which has been reported in mice since 1959.

Histological studies of the CNS of affected mice are required for a comparative study of listerial encephalitis of sheep and mice on the lines of that conducted by Asahi et al (1957). In the event of there being similarities (confirming the work of Asahi), the peritoneal route of infection represents a simple, cheap and

workable model for the study of listerial encephalitis in ruminants.

The results of experiments 1 and 2 shed little light on the disputed pathogenesis of listerial encephalitis, and in no way discredit the hypothesis of intraneural, centripetal spread proposed by Asahi et al. The peritoneal cavity is richly supplied with visceral afferent nerves with both central and spinal projections. By clinical appraisal alone it was apparent that both brain and cord lesions were involved: (unilateral exophthalmos and a tendency to roll is suggestive of a brain lesion whereas bilateral hind-limb flaccid paralysis and urinary incontinence is pathognomonic of a lesion of the cord). It is suggested that histological examination of splanchnic nerves and their connections in affected animals would help to clarify the pathogenesis.

Results from serological analysis of surviving mice from experiments 1 and 2 enable us to make some important conclusions. Convalescent naive mice recovering from an acute infection with both flagellate and non-flagellate organisms have no detectable circulating immunoglobulin against flagellar protein.

Titres produced against cell wall components were both low and inconsistent, bearing little apparent relation to either dose of organisms given, or the number of survivors in each group. Lack of response, even in the face of an infection which caused death in 50% of animals in the group, (Table 8.4a: group 2), bears out the observation of North and Diessler (1975) who, using a passive

agglutination test with a soluble Listeria antigen, also failed to detect an antibody response following primary infection. The only mice with a reciprocal titre in excess of 800 were the two survivors of group 18, and at a 1 in 800 serum dilution, the recorded OD for these two mice was 0.164, only 0.054 units above the negative baseline of 0.11.

It is possible that observed titres would be higher in an ELISA test where a homologous CWF antigen was used. (For the above tests it must be remembered that a CWF antigen of serotype 4a was used as a coating antigen, and serotype 4b was used as the challenging organism). However the extensive homology of antigens of serotypes 4a and 4b demonstrated in chapter 3 makes major differences unlikely. In addition, the standard positive antiserum which had been raised against serotype 4b(T) was highly reactive.

It is also possible that higher titres might be observed by using an antigen preparation which had not been prepared using SDS. The CWF in question was prepared by SDS extraction of cell walls, and subsequent removal of SDS by dialysis. If complete renaturation of all antigen binding sites did not occur following dialysis reduced antigen/antibody binding would be the result.

Further serological investigations should include a comparative study of the response of other strains of inbred mice, particularly Listeria-resistant strains in which the macrophages have a heightened responsiveness to infection: viz C57Bl/6. Secondly, the kinetics of antibody response to a secondary challenge might also be studied in inbred strains of mice. A third requirement is

further study of the serological response of sheep and cattle to a live infection. Similar results from these species would confirm that there are serious limitations on the use of Listeria protein antigens in either a diagnostic test or an epidemiological survey.

Comparatively little work has been done in laboratory species using the oral route of infection. Racz et al (1972) orally infected guinea pigs (previously starved and treated orally with calcium carbonate and intraperitoneally with opiate) and showed evidence for an apparent 'epithelial phase' of listerial infection. After 22 hours of infection they demonstrated dividing bacteria within intact epithelial cells of the ileum; both within vacuoles, and free within the cytoplasm. They also provide evidence for the spread of organisms between contiguous intact epithelial cells. They speculate that the gastrointestinal tract may be a portal of entry for infection.

The results shown in Table 8.4 confirm that very large numbers of organisms are required to induce lethal infection by the oral route. Audurier et al (1980) caused death in 80% of Swiss mice only when doses of 9.7×10^9 cfu of a virulent strain were administered to each animal. Similarly, in the probe experiment recorded in Table 8.5, dose rates in excess of 2.8×10^9 cfu resulted in only 50% of deaths over a 3 week period in a susceptible strain of mouse.

The values for ID_{50} for intragastric challenge with either flagellate or non-flagellate organisms calculated from the data shown in Fig 8.4 (a-d) vary by a factor of 2.9 for infection of the

liver and by only 1.6 for infection of the spleen. This preliminary result suggests that an oral infection of L.monocytogenes is equally invasive, regardless of temperature of incubation and the number of flagella present.

Moreover, The results shown in Table 8.5 albeit with very few mice do indicate that an intragastric challenge is capable of eliciting both the early and late disease syndrome.

Audurier et al (1981) recorded the isolation of L.monocytogenes from both spleen and mesenteric ganglia of mice 24 hours following an oral challenge. MacDonald and Carter (1980) isolated organisms from Peyers patches of orally challenged mice 24 hours after infection. The kinetics of infection in the spleen following oral administration are also described by Audurier et al (1981). From this and the extensive work already discussed in Chapter 1 it can be assumed that the number of organisms present 72 hours after infection, as recorded in Table 8.6 (a-d) represents the late logarithmic phase of division before the onset of specific immunity. It is suggested that further mouse experiments which compare the invasiveness of flagellate and aflagellate L. monocytogenes across the intestinal mucosa should include recovery of organisms from the mesenteric lymph nodes, and earlier killing times (viz 24 and 48 hours post inoculation).

Finally, repeated adjuvanted vaccination of mice with flagella did not protect them against a subsequent lethal challenge. The challenge dose given was approximately 17 times the LD_{50} as calculated for Moredun mice in experiment 2 (table 8.2b). It can be

concluded that any protection conferred by flagella presented in this way is marginal and would be better detected by experiments measuring ID_{50} and using smaller challenge doses.

To summarise this chapter; it was found that no major role in virulence or protection could be ascribed to the flagella of L. monocytogenes . Moreover, it was found that naive mice did not produce anti - flagellar antibody following infection with flagellated organisms, and that only low titres were produced against a somatic cell wall fraction. However, the fact that a neurological disease can be readily produced has exciting implications for research into this disease, particularly in the field of immunohistopathology. This will be fully discussed in the next chapter.

GENERAL DISCUSSION

This general discussion collates information obtained during the course of this study, and makes suggestions for future research which may provide additional useful data.

At its conception, the primary aim of this study was to identify, characterise and, if possible isolate genus-specific protein antigens of L. monocytogenes for incorporation in a diagnostic serological test. It was considered that a) the relatively new and sophisticated techniques of SDS-PAGE and Western blotting for antigen separation and selection, and b) the sensitivity of an ELISA for assay, would yield success where so many workers, over the last 30 years have so conspicuously failed (Kuhlmann-Berger and Potel, 1985).

The definition of flagella as specific bands on SDS-PAGE led directly to the development of a monoclonal antibody and an affinity purified polyclonal antiserum whereby the flagella of other serotypes could be characterised. An adjuvanted flagella vaccine and pathogenicity studies using flagellate and aflagellate organisms also developed from studies of flagellar proteins.

A consequence of the live challenge experiments was the induction of a neurological syndrome, the investigation of which should be made easier by the recent advances made in immunohistochemistry.

Proposals for future research on flagella, on neurological disease in mice, and on other matters arising from this thesis will be given later. The approach towards a diagnostic test will be discussed first.

The preparations used in ELISA were:-

(a) A cell wall fraction (CWF) made by pressure cell disruption of serotype 4a which contained:-

(i) At least 15 proteins (MW 76 - 20KD) visible by Coomassie blue staining of gels.

(ii) 9 proteins which stimulated an antibody response in rabbits given fk 4a.

(iii) 7 proteins present on the cell surface of L. monocytogenes and not abundantly present on the cell surface of representatives of five other bacterial genera.

(iv) A proportion of 29 KD flagellin.

In addition, other components such as peptidoglycan, teichoic acid, lipotechoic acid , and small amounts of undefined proteins (undetectable by SDS-PAGE) may also have been present in this preparation.

(b) 29 KD flagellin from serotype 4b.

Even though the components of the CWF antigenic mixture are not totally defined it is easy to see how the limited data available could be used to develop an ELISA of likely specificity. For

example, these proteins might be fixed to microtiter plates in ELISA or to nitrocellulose in a dot blot system and probed with a titration of sheep antiserum together with a standard dilution of the rabbit antiserum used in Fig. 3.3 in a competitive binding assay. The work of this thesis, however, amply demonstrates that such a test, though highly specific for Listeria antibodies, would be quite useless for the diagnosis of encephalitis since these animals did not have titres in excess of normal controls. Definition of genus specific antigenic determinants is of academic interest until it can be established which determinants induce antibodies following live infection. If we assume binding of all the components in the antigenic mixture we can say that cell wall proteins of L. monocytogenes 4a(T) are not useful for the diagnosis of encephalitis by serology. The antigenic homology observed between the different serotypes make it unlikely that other serotypes or mixtures of serotypes would be any more useful.

Serological diagnosis of ovine listerial encephalitis might be achieved by using preparations of lipoteichoic acids. Lipoteichoic acids are membrane associated amphiphilic molecules found in many gram-positive bacteria which can act as surface antigens (Wicken and Knox, 1975). They have been characterized for L. monocytogenes in key studies by Kamisango et al (1983) and Uchikawa (1986). In a recent report, Kamisango et al (1985) describe the development of a sensitive ELISA system for the detection of lipoteichoic acids of L. monocytogenes. This system involved using a coating antibody followed by biotinylated lipoteichoic acid

which was detected using an avidin peroxidase labelled probe. It might be possible to adopt such an antibody capture system for serological analysis. This is an open area for further research.

However, it is quite possible that animals with clinical listerial encephalitis do not seroconvert to any component of L. monocytogenes, and that a diagnosis can only be achieved using tests which measure the cell mediated immune response. The in vivo test of choice for cell mediated immunity (CMI) is the DTH skin test. A DTH response to various components of L. monocytogenes following foot pad inoculation of immunised mice has been described (Mackaness, 1962; Youdim et al, 1973; Osebold et al, 1974). It is not however known how DTH response correlates with overt clinical disease. It would be especially useful to investigate DTH response in mice with experimentally induced neurological disease. Positive DTH responses in affected mice might encourage the development of CMI tests for use in sheep. Conversely, negative responses in these mice might argue that the organism has reached the brain whilst largely evading the immune defences of the host and would argue for an intraneural route of infection.

Although investigation of DTH responses to specified listerial antigens would be a very valuable exercise, a diagnostic skin test for an acute disease such as listeriosis is unrealistic. It is likely that affected animals would die before the test could be read since the time course of the disease is only 3-4 days (Blood, Henderson and Radostits, 1979). An in vitro test of CMI could be devised based on antigen driven lymphocyte transformation or lymphokine production of peripheral blood samples. However, due to

the obvious impracticalities of using any such test in the field it seems likely that listerial encephalitis will continue to be diagnosed by histopathology.

Quite apart from diagnosis, it would be useful to have a test whereby the previous exposure of flocks to L. monocytogenes could be estimated. Recording the incidence of disease in such flocks would enable us to make a correlation betwen previous exposure and immunity and give indications of the feasibility and strategy of vaccination. The results obtained in this thesis using serum from experimentally infected mice and sheep at an estimated peak of antibody production suggest that a serological assay using a mixture of cell wall protein antigens is unhelpful. It might be that the important antigens of the L. monocytogenes 4a(T) cell wall fraction were present in the mixture in only small amounts, and that selection of these antigens would have given high specific titres. Before the use of protein antigens in ELISA can be totally discounted this would need to be investigated. One approach might be to raise monoclonal antibodies against cell wall proteins and use them to coat ELISA plates in an antigen capture technique. This project would have the added advantage of providing a battery of monoclonal antibodies for further bacteriological characterisation.

The situation with regard to flagella is much less equivocal, and it can be stated with some confidence from this thesis that flagellin can not be incorporated into a useful serological test

which will monitor a primary infection. This thesis also describes two methods of preparing purified flagellin, and it is suggested that the DTH response to flagellin should be investigated next.

Purification of flagella to homogeneity will allow, for the first time, a full investigation of their physical, chemical and immunological properties. In this context, isoelectric focussing of flagella on an ampholyte gradient might serve to separate the individual flagellin bands to allow analysis.

Using a battery of monoclonal antibodies, Newell (1986a) postulated six different epitopes on the flagella of Campylobacter jejuni. In a subsequent paper (Newell, 1986b) she reports on the serotype specificity of such monoclonal antibodies and on the development of an ELISA capture technique for the detection of flagellar antigen in faecal material. In this thesis, using only a single monoclonal antibody, an epitopic difference between serotypes of L. monocytogenes flagella was detected. This gives some optimism that a battery of monoclonal antibodies would reveal other differences which could be exploited in an alternative method of serotyping.

Flagella of the various serotypes might also be investigated using serotyping antibodies which had been prepared in the conventional way against formol-killed organisms (Seeliger and Hohne 1979). This would allow each of the four factors A, B, C and D to be related to the three flagellin bands. Additional

information on flagella structure could be obtained by gel analysis following enzyme digestion, the amino acid content might be determined, and ultimately the protein might be sequenced.

A role in pathogenesis or protection could not be determined for L. monocytogenes flagella in this thesis. However, it is possible that flagella are non specific stimulators of the immune response. Some of the most powerful immunomodulators in current use are products of bacterial cells. Muramyl dipeptide (MDP), a potent non specific stimulator of thymus dependent cell reactions, is a peptidoglycan derived from Mycobacterium tuberculosis. Lipopolysaccharide (LPS) is derived from gram negative cell walls and is widely used as a B cell mitogen. The various activities of MDP and LPS are reviewed respectively by Behling and Nowotny (1979) and by Kotani et al (1979). The precise mode of action is unclear and is beyond the scope of the work presented in this thesis. It is noted however that a feature of both of these mitogenic molecules is their polymeric construction as a sequence of identical repeating residues. Moreover, Butler et al (1979) demonstrated that the repeating polysaccharide moiety of LPS was able to stimulate T cells to a degree similar to that observed for the whole molecule. It has been shown that polyclonal activation of B cells can be brought about by multivalent antigens with identical "repeating" epitopes, (review: Vitetta et al, (1980). Though other bacterial flagellins are not routinely used as mitogens it is felt that the flagellin of L. monocytogenes should be checked in this respect.

More importantly, the *in vitro* response of Listeria- primed T cells to flagella of *L. monocytogenes* warrants full investigation.

Current models of infection for the study of the cell-mediated immune response include the use of purified protein derivative (PPD) in animals primed with either *M. tuberculosis* or B.C.G. However, PPD is a far from homogenous preparation, being a complex mixture of both proteins and polysaccharides, and to date it has not been discovered which particular components are responsible for the observed T cell proliferation (Ichiro et al, 1985). A more defined T-cell stimulating antigen is required whereby antigen presentation can be analysed in terms of specific epitopes.

Other work extending from this thesis include a full histological investigation of the neuropathological syndrome produced in mice. This phenomenon is by no means a new observation but has been considerably overlooked for more than 20 years. Preliminary investigations have shown that *L. monocytogenes* can be specifically detected in murine tissues following simple and routine fixation and processing techniques. This work is now poised for a full investigation of antigen localisation in CNS of mice which succumb to this syndrome. Histological localisation of antigen in murine CNS at successive stages of infection by means of a serial kill experiment would throw considerable light on the disputed pathogenesis of listerial encephalitis.

The syndrome described in this thesis was induced by infection with serotype 4b which had been isolated from the brain of an encephalitic sheep and used without further passage. The ability of other strains and serotypes to cause disease would be worth investigating.

Finally, with the recent advent of and monoclonal antibodies against surface antigens of the different murine T-cell subsets it will be possible, for the first time, to characterise those cells recruited to the CNS early during infection with L. monocytogenes, in the way described by Ceredig et al (1987) for lymphocytic choriomeningitis virus infection.

To summarise; three major avenues of further research into L. monotcytogenes are suggested by the work conducted in this thesis:-

(a) Further studies using SDS-PAGE and Western blotting should concentrate on defining antigens of cell mediated immunity.

(b) Flagellin should be investigated with regard to:-

  (i) measuring CMI

  (ii) mitogenicity

  (iii) finding a model system to investigate antigen presentation to T cells

(c) Histological investigation of the neuropathology produced in mice

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