UNIVERSITY OF GLASGOW

THE IMMUNE RESPONSE IN MICE AND SHEEP TO AN AROMATIC-DEPENDENT MUTANT OF SALMONELLA TYPHIMURIUM AND EXPRESSED HETEROLOGOUS ANTIGENS

by

Francis Richard Brennan

Department of Biochemistry,

Moredun Research Institute,

Edinburgh, EH17 7JH

and

Department of Bacteriology and Immunology

University of Glasgow

Glasgow G11 6NT

A thesis submitted for the degree of Doctor of Philosophy

December 1992

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ABSTRACT

An aromatic-dependent [aroA] mutant of an ovine isolate of Salmonella typhimurium, S25/1, was shown to be avirulent and have a much reduced capacity to invade the organs of both conventional mice and sheep when compared with the virulent parent strain. Oral vaccination with the aroA strain resulted in the production of Salmonella-specific immune responses in both mice and sheep, and the magnitude of these responses was related to the levels of tissue colonisation by the avirulent salmonellae.

In vaccinated mice, high levels of Salmonella-specific IgM, IgG and IgA were detected in the serum, specific intestinal IgA was produced and there was evidence of specific cellular immunity. In vaccinated sheep, specific serum IgM was produced although specific IgG responses were very low and there was no evidence of specific coproantibody or specific T cells. Despite the different immune responses engendered in mice and sheep, both species were highly protected from high lethal challenge doses of the wildtype strain.

The ability of the S25/1aroA strain to deliver recombinant heterologous antigens to the immune system of both mice and sheep was investigated. Strains expressing the major outer membrane protein [MOMP] of Chlamydia psittaci were shown to colonise murine tissues to similar levels as the non-recombinant strain and produced MOMP in vivo. MOMP-specific serum antibody and MOMP-specific intestinal IgA were detected in some mice, although

these responses were low when compared with those to Salmonella, and there was no evidence of MOMP-specific T cells. In conventional lambs, no specific responses to MOMP could be detected, although the responses to Salmonella were very low in these animals.

The aroA strain was also constructed to produce the leukotoxin [cytotoxin] of Pasteurella haemolytica A1, however in vivo expression of the protein was very low. Consequently, only a small number of mice were seen to produce leukotoxin-specific antibody, and specific cell-mediated immunity was absent. When the strain was administered to germ-free lambs, reversion to virulence was seen which revealed instabilities in the vaccine strain, and in vivo expression of the protein was virtually lost after 24 h. Although there was no evidence of leukotoxin-specific antibody, leukotoxin specific cellular responses were demonstrated. However the low responses to Salmonella and the reversion to virulence seen in these lambs question the specificity of these responses.

This study therefore demonstrated the ability of a live Salmonella aroA strain to engender specific immune responses and to confer protection against experimental salmonellosis. However, although the vaccine vector potential of the strain was not clearly demonstrated, some of the problems associated with expression of foreign antigens in Salmonella were revealed allowing optimal strategies for expression to be applied in future studies.

DEDICATION

For my father and mother who have given me their support, both loving and financial, throughout my time at university.

AKNOWLEDGEMENTS

I am deeply indepted to Dr. David Baird of this Institute for his supervision throughout the course of the work described in this thesis. I am also grateful to Dr. Allan Mowat, University of Glasgow, for his constructive criticism and guidance and particularly for his help in the preparation of the manuscript. Thanks also goes to Dr. Joanna Oliver for her practical help and supervision in the first year of the work. I should also like to thank Dr. Alan Herring and Dr. Alex Lainson for their many useful discussions.

I am grateful also to Mr. David Henderson, Department of Clinical Studies, and members of his staff, in particular Mr. Jim Williams, Mrs Lorna Evans and Miss Elspeth Scott for the provision and care of animals and for their invaluable advice and assistance. My thanks also goes to Mr. Gordon Bell for the provision of isolators [often at short notice!] and members of his staff, in particular Mrs. Ann Woolgar, for much needed technical assistance throughout the course of this Ph.D.

My special thanks goes to the late John Gilmour for his help and for performing the histopathology studies when other things were more important. He is sorely missed. Thanks also goes to Mr. B. Easter and Mr. A. Inglis for photographic services and Mrs. E. Imrie for preparation of media.

Finally, I would like to express my gratitude to my many colleagues at the Moredun Research Institute [MRI], Edinburgh, too numerous to mention, for the support and encouragement given to me in undertaking this work.

The work described in this thesis was supported by a grant awarded by the Scottish Office Agriculture and Fisheries Department.

DECLARATION

The S25/1*aroA* strain and its recombinant strain derivatives were constructed by Dr. J.J. Oliver, MRI, with the exception of the pAL12 strain which was constructed by Miss Pamela Dalgleish, MRI. Otherwise the work presented in this thesis was carried out by myself.

FRANCIS RICHARD BRENNAN

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AP	=	alkaline phosphatase
*aro*A	=	aromatic A gene
B cell	=	B lymphocyte
BDV	=	Border Disease Virus
BRL	=	Bethseda Research Laboratories
BSA	=	bovine serum albumin
CD	=	cluster of differentiation antigen
CFA	=	complete Freunds adjuvant
CFU	=	colony forming units
CHO	=	Chinese hamster ovary
CMI	=	cell-mediated immunity
ConA	=	Concanavalin A
CPE	=	cytopathic effect
CPM	=	counts per minute
CSP	=	circumsporozoite protein
CTL	=	cytotoxic T lymphocyte
DHB	=	dihydroxybenzoate
DMSO	=	dimethyl sulfoxide
DTH	=	delayed-type hypersensitivity
EBs	=	elementary bodies
EBSS	=	Earle's balanced salt solution
EDTA	=	ethylenediaminotetraacetic acid
ELISA	=	enzyme-linked immunosorbant assay
FACS	=	fluorescence-activated cell sorting
FCS	=	foetal calf serum
FBS	=	foetal bovine serum
FHA	=	filamentous haemagglutinin
ga	=	gauge
*gal*E	=	galactose E gene
GALT	=	gut-associated lymphoid tissue
h	=	hour
HBSS	=	Hank's balanced salt solution
HRP	=	horseradish peroxidase
ICFA	=	incomplete Freunds adjuvant
IFN-g	=	interferon-gamma
Ig	=	immunoglobulin
IL	=	interleukin
IMDM	=	Iscove's Modification of Dulbeccos Medium
Ity gene	=	immunity to typhimurium gene
K	=	kilodalton
LB	=	Luria-Bertani
LC	=	light chain
LD_{50}	=	lethal dose 50
LPS^{d}	=	lipopolysaccharide
Lps	=	defective gene for LPS response
LT-B	=	subunit B of heat-labile toxin
LTT	=	lymphocyte transformation test
min	=	minutes
MHC	=	major histocompatibility complex
mIgA	=	membrane IgA
MOMP	=	major outer membrane protein
MRI	=	Moredun Research Institute

MS	=	mannose-sensitive
NBL	=	Northumbria Biologicals Ltd.
NK cells	=	natural killer cells
NMS	=	normal mouse serum
no. CFU/g	=	number of colony forming units per g
no. CFU/ml	=	number of colony forming units per ml
OAE	=	ovine abortion of ewes
OD	=	optical density
OPD	=	o-phenylene diamine
PABA	=	p-aminobenzoic acid
PAGE	=	polyacrylamide gel electrophoresis
PBMC	=	peripheral blood mononuclear cells
PBS	=	phosphate-buffered saline
PBST	=	PBS containing 0.1% Tween
PCV	=	packed cell volume
%DW	=	percentage dry weight
PMSF	=	phenyl methyl-sulfonyl fluoride
PNPP	=	p-nitrophenyl phosphate
rec MOMP	=	recombinant MOMP
rec leukotoxin	=	recombinant leukotoxin
RES	=	reticuloendothelial system
RT	=	room temperature
SCID	=	severe combined immune-deficient
SDS	=	sodium dodecyl sulphate
SFV	=	Semliki Forest Virus
SI	=	stimulation index
sIgA	=	secretory IgA
STI	=	soyabean trypsin inhibitor
TBS	=	Tris-buffered saline
TBST	=	TBS containing 0.1% Tween
T cell	=	T lymphocyte
$TCID_{50}$	=	50% tissue culture infective dose
TcR g-d	=	T cell receptor gamma-delta
Th	=	T helper
TNF	=	tumour necrosis factor
UDP	=	uridine di-phosphate
Vi antigen	=	virulence antigen
VD	=	variable domain
VS	=	variable segment
xid	=	x-linked immune-defective gene
XLD	=	xylose lactose deoxycholate

Chapter 1

General Introduction

1.1 Introduction

The Potential of Live Vaccines as Carriers of Cloned Heterologous Antigens to the Immune System

Potentially protective antigens have been identified for many parasites, pathogenic viruses and bacteria and pure antigen can be made by recombinant DNA technology for assessment in vaccination strategies. These proteins are poorly immunogenic unless they are administered with adjuvant, and parenteral delivery of purified proteins often fails to elicit strong serum antibody responses and is ineffective in eliciting secretory immune responses at mucosal surfaces and effective cell-mediated immunity [CMI]. In contrast, certain live vaccines such as *Vaccinia* and *Salmonella* are potent stimulators of the immune system without the need for adjuvant. Such live vaccines are less expensive to produce commercially than killed or subunit vaccines and do not produce the violent side-effects often associated with killed vaccines. Thus, strategies for expression of recombinant proteins in these live vaccines are being developed.

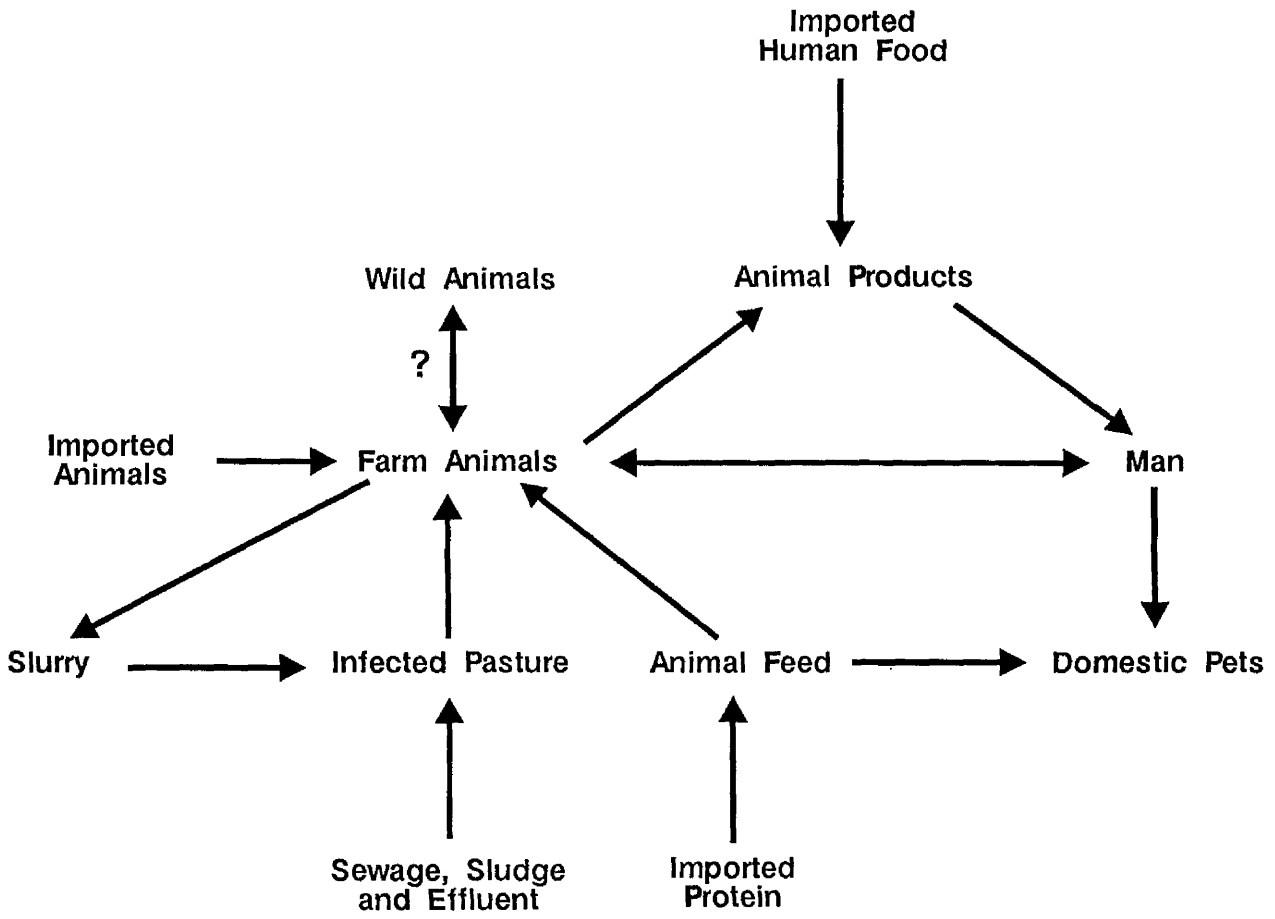
Several features of the *Vaccinia* vaccine limits its usefulness as a vaccine delivery system. It does not appear to be that effective as a carrier system, it has to be administered by injection, and the size of the virus limits the size of the recombinant protein

that can be introduced into the system. Oral Salmonella vaccines are of particular interest as one can use the natural route of infection, namely the oral route, which results in direct stimulation of the gut-associated lymphoid tissue [GALT] and induction of specific intestinal responses. Live avirulent salmonellas are able to traverse the gut and invade the reticuloendothelial system [RES] where they can persist for up to 70 days, facilitating the induction of specific systemic humoral and cell-mediated immune responses. The fact that vaccination with Salmonella, unlike Vaccinia, may give useful protection against a currently important disease is also significant. Furthermore, because of the close relationship between Salmonella and Escherichia coli [E.coli] the same techniques that have been developed for expression and overproduction of recombinant proteins in E.coli are at least partly applicable to Salmonella.

1.2 The Genus Salmonella

Salmonellosis is a worldwide infectious disease of humans and animals. It is an agricultural problem, causing great economic loss to the farming industry and the infected animals constitute a vast reservoir for the disease in humans. Humans may become infected with salmonellas by the consumption of contaminated water or animal products eg. poultry, eggs, beef, pork and milk, and animals may become infected with salmonellae by contact with human waste products. Thus the diseases in animals and man are linked, and may be summarised in the 'Salmonella cycle' [Figure 1.1].

Figure 1.1 The Salmonella Cycle



There are over 2000 different serotypes of salmonellae, described on the basis of somatic [O], flagellar [H] and capsular [Vi] antigens in the Kaufmann-White scheme [Le Minor, 1984], but only a small number are encountered frequently in disease.

As a member of the family Enterobacteriaceae, salmonellae may have the following characteristics: they are Gram-negative rods, which may be motile due to the possession of peritrichous flagella or non-motile. They do not produce spores, and are facultative anaerobes, producing acid from glucose. They do not ferment lactose or sucrose.

1.3 Host Specificity

Salmonella may be considered in 3 groups based on their association with human and animal hosts [Williams and Miles, 1975]. One group comprises host-adapted serotypes, including S.typhi and paratyphi, which cause disease only in man; S.gallinarum and S.pullorum which cause disease only in poultry; and S.abortusovis, which causes disease only in sheep. A second group, which can be termed host-restricted, includes S.dublin and S.choleraesuis which cause disease mainly in cattle and swine respectively, but are opportunistic pathogens of other animals. The third group consists of salmonellas that are not host-restricted and cause disease in all types of animals. Most salmonellae fall into this group, including S.typhimurium, which is the most frequent cause of salmonellosis in the United Kingdom. [Wray, 1985; Humphrey et al., 1988].

The vaccine strain used in experiments described in this thesis was constructed from an ovine isolate of S.typhimurium, so in the following sections particular references will be made to this serotype.

1.4 The Disease

Salmonellosis occurs in 3 major forms: enteritis, systemic infection [typhoid in humans] and abortion, which only occurs in some animals. Some animal species are more prone to a particular form of the disease than others. Septicaemic spread of salmonellae may produce meningitis or septic arthritis.

There appears to be an association of serotype with a particular disease syndrome. S.typhimurium is commonly associated with enteritis, S.choleraesuis with septicaemia, S.typhi and S.paratyphi with enteric fever [typhoid] and S.dublin with abortion.

Salmonella can be studied in laboratory rodents. S.typhi is only virulent for primates thereby precluding easy and extensive murine manipulations. However, mice infected with S.typhimurium develop a typhoid fever-like syndrome [MacKaness et al., 1966] and as a consequence, the S.typhimurium mouse model has been used to test both the relative efficacy of live attenuated and killed vaccines and immunity against systemic infection with Salmonella species.

1.5 Invasiveness and Pathogenicity - Interaction of Salmonella with the Cells of the Immune System

Much of the information on the pathogenesis of Salmonella has come from the experimental models of mouse typhoid caused by S.typhimurium and S.enteriditis [Collins, 1971, 1972, 1974; Carter and Collins, 1974]. Salmonellae usually enter by the oral route. After encountering the mechanical and chemical defences of the host they are deposited in the intestine where they invade the enterocytes [Wray and Sojka, 1977; Turnbull, 1979]. The acidity of the stomach and the presence of the normal commensal gut flora, reduce the establishment and growth of salmonellae in the intestine [Nurmi and Rantala, 1973; Impey et al., 1982].

Concepts of mucosal penetration by salmonellae have largely been formulated by Takeuchi [1967] who found that when salmonellae came within a critical distance of the brush border of enterocytes, the microvilli and tight junctions underwent degeneration. The salmonellae then enter the cells through the microvilli or through the gap junctions between the enterocytes [Takeuchi and Sprinz, 1967] and pass through to the lamina propria where they come into contact with a wide array of immune effector cells, including macrophages, B lymphocytes [B cells], T lymphocytes [T cells], eosinophils and mast cells. Macrophages engulf the bacteria [Takeuchi and Sprinz, 1967] and there is a subsequent inflammatory response, characterised by the presence in the intestine and lumen of large numbers of neutrophils [Nakoneczna and Hsu, 1980, 1983].

During the next 24 hours [h], bacteria in the lumen and the enterocytes are cleared, but those in the lamina propria persist. Bacteria in the mucosa and submucosa are transported in the lymphatics to the regional lymph nodes. From the lymph nodes the salmonellas travel in the efferent lymph vessels into the blood circulation, from which they are filtered out by the specialised phagocytes of the RES, particularly those in the liver and the spleen. Failure to contain the infection may result in septicaemia and dissemination, often resulting in pneumonia, meningitis and septic arthritis [Wray and Sojka, 1977].

As we have seen, antigens of Salmonella encounter the cells of the immune system following direct penetration of the gut mucosa. Salmonella antigens and whole Salmonella bacteria may also reach subepithelial lymphoid tissue through absorption from the lumen via specialised epithelial cells [M cells] in Peyer's patches which are distributed throughout the ileal mucosa [Owen et al., 1986]. The M cells are thought to engulf the antigens by phagocytosis and pass them to the B cells, T cells and macrophages in the layer below.

1.6 Bacterial Virulence Factors - An Association with Structure

The ability of an organism to invade, multiply and cause pathological damage in a host, as well as avoiding host defences, is dependent on the virulence factors that it possesses. These may be in the form of surface-associated structures or released soluble compounds, and will be discussed in this section.

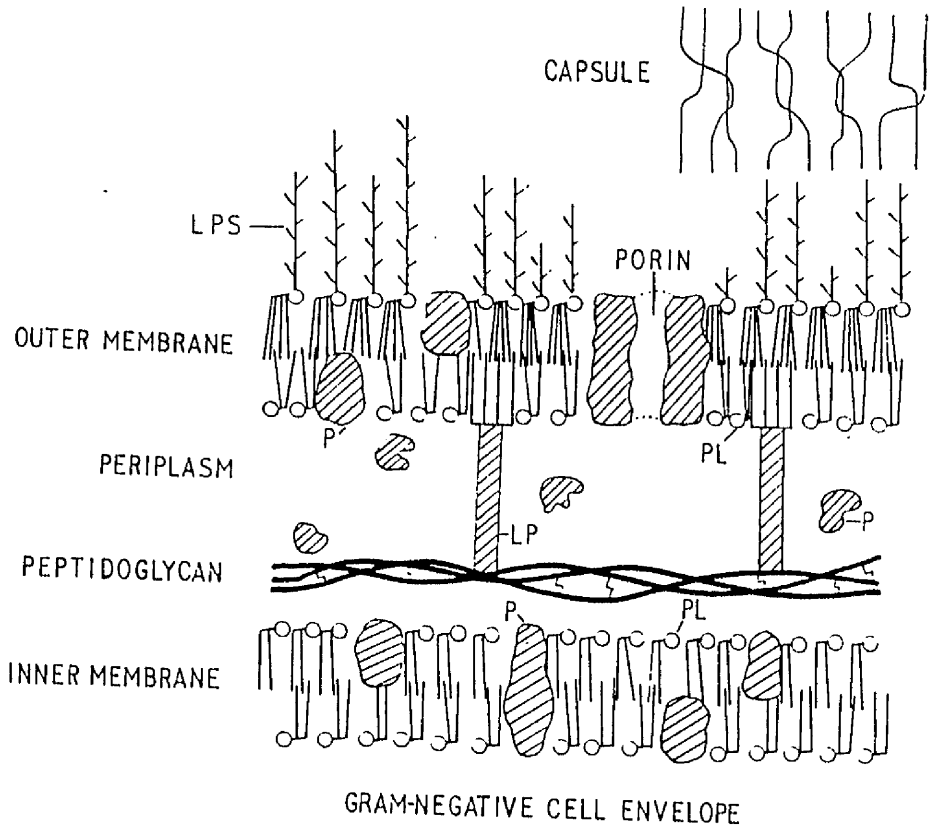


Figure 1.2[A] A schematic diagram of the Gram-negative cell envelope.
LPS= lipopolysaccharide, P= protein
LP = lipoprotein, PL= phospholipid

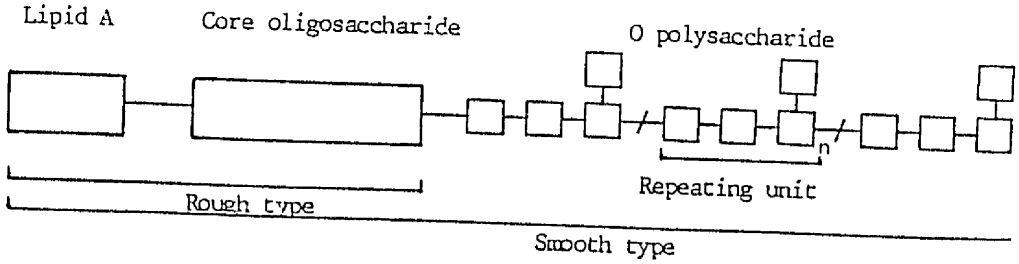


Figure 1.2[B] A schematic diagram of the lipopolysaccharide molecule.

Gram-negative bacteria have a cell envelope consisting of a distinct outer membrane which lies external to a thin layer of peptidoglycan and the underlying cytoplasmic membrane [Figure 1.2A]. The structures of the envelope that may play an important role in Salmonella infection are discussed below.

1.6.1 Lipopolysaccharide [LPS]

The O-antigen and its associated lipid A constitute LPS, which is unique to the outer membrane of Gram-negative organisms. The lipid A and lipopoprotein provide the lipid phase of the outer leaflet of the bilayer, whilst the O-polysacharide antigen protrudes from the surface [Figure 1.2B]. The O-antigen consists of O-specific chains made up of repeating oligosaccharide units attached to a core oligosaccharide [Wilkinson, 1977; Lüderitz et al., 1982a,b]. The structure of the core is practically identical in all salmonellas, but varies between genera [for review, see Roantree, 1967]. The serological specificity is determined by the O-antigens and there is a correlation between O-specific antigens and the virulence of salmonellas [Roantree, 1967; Nakano and Saito, 1969; Edebo and Norman, 1970]. In Salmonella strains differing in only their O-antigens, virulence in mice is dependent on their specific O-antigen composition [Valtonen, 1970; Valtonen et al., 1975; Lyman et al., 1977]. Mutants of Salmonella that have lost part or all of the O-antigen [rough mutants] are less virulent than their parents with complete LPS [Mintz and Deibel, 1983]. Rough mutants are taken up more readily by macrophages than smooth strains

[Liang-Takasaki et al., 1982], being more sensitive to opsonisation with antibody and complement [Thjøtta and Waller, 1932; Rowley, 1954; Liang-Takasaki et al., 1983; Saxén et al., 1987]. Following phagocytosis, rough mutants have less protection against lysosomal attack, and are more sensitive to the bactericidal action of granule products than smooth strains [Tagesson and Stendhal, 1973; Modrzakowsky and Spitznagel, 1979].

1.6.2 Toxins

Intestinal fluid secretion and cell destruction observed during Salmonella infection [Gianella et al., 1976; Gianella, 1979] may be the result of toxin production by the organism. There are 3 types of toxin, 2 being cell-associated and the other an exotoxin. The endotoxin is an integral part of the outer membrane of Gram-negative organisms and is equivalent to LPS and the cytotoxin is found in the outer membrane between the peptidoglycan and the outer envelope. The Salmonella exotoxin is stored in the periplasmic space between the peptidoglycan and the outer envelope.

1.6.2[a] Endotoxins

Endotoxins are heat-stable lipopolysaccharides. The lipid A part, consisting of fatty acids linked to 2 molecules of N-acetyl glucosamine, is where the toxicity resides [Rietchel et al., 1984]. Lipid A by itself is insoluble in water and hence lacks biological activity, so the polysaccharide moiety of LPS contributes to toxicity by increasing the aqueous solubility of lipid A.

Endotoxin is thought to cause most of the systemic effects of infection by Gram-negative bacteria, including thrombosis through activation of the coagulase system and destruction of vascular endothelium. It causes shock with characteristic symptoms including fever, changes in white blood cell counts, respiratory collapse and reduction in blood pressure, with death often a consequence.

The effects of endotoxin on immune regulation are also great. It activates macrophages, resulting in production of secretory products such as tumour necrosis factor [TNF], interleukin [IL]-1 and prostaglandins, all implicated in shock [for review see Lüderitz et al., 1982b]. LPS is a T cell-independent B cell mitogen, so it can evoke polyclonal B-cell activation [Anderson et al., 1973; Rosenstreich et al., 1973], possibly resulting in autoimmune disorders such as arthritis [Cooke et al., 1983]. Endotoxin may also cause the placental changes preceding abortion, as a result of Salmonella infection [Hall, 1973, 1974].

1.6.2[b] The Cytotoxin

Reitmeyer et al. [1986] have reported the existence of a cytotoxin in Salmonella characterised by its ability to cause rounding and detachment of Chinese hamster ovary [CHO] cells. Situated in the outer membrane, it would be in an ideal position for direct

contact with host cells. Indeed, one could speculate a role for it in enterocyte invasion and destruction as seen in gastroenteritis, or as an antiphagocytic device following physical contact with macrophages.

1.6.2[c] Exotoxins

Exotoxins are known to be responsible for the pathogenic effects seen in a number of diseases. In diarhoeal diseases caused by enteropathogens such as Vibrio cholerae [V.cholerae] and E.coli the exotoxin is known as an enterotoxin. The existence of a Salmonella enterotoxin has been controversial [Stephen et al., 1985]. Enterotoxic activity has been demonstrated in culture filtrates and extracts [Sandefur and Peterson, 1976; Sedlock and Deibel, 1978; Peterson et al., 1981; Wallis et al., 1986], and the results of test systems set up suggest that such an enterotoxin may resemble shiga-toxin [O'Brien et al., 1982] and E.coli heat-stable toxin, but most evidence suggests that it resembles cholera toxin [Sandeflur and Peterson, 1977; Jiwa, 1981; Wallis et al., 1986; Chopra et al., 1987a,b]. However, Shukla and Sharma [1985] have demonstrated an enterotoxin in several strains of Salmonella, including S.typhimurium, that is not related antigenically to cholera toxin. The discrepancy in the nature of Salmonella enterotoxin suggests that there could be a range of toxins as is observed in E.coli.

1.6.3 Capsules

External to the envelope components, many Gram-negative bacteria, including Salmonella, possess exopolysaccharide layers called the glycocalyx [Costerton et al., 1981]. Exopolysaccharides can be divided into homo- and hetero-polysaccharides. Homopolysaccharides, seen in Salmonella species, are composed of single-sugar, long-chain molecules such as cellulose, levan and glucan polymers. Heteropolysaccharides, such as those seen in Klebsiella species, are composed of 2 or more monosaccharides and frequently contain uronic acids. The capsule of S.typhi has been shown to be responsible for bacterial virulence and was therefore termed the Vi [for virulence] antigen. Vi+ strains are more serum-resistant and less readily phagocytosed by macrophages [Felix and Pitt, 1951] and cause more cases of infection in human volunteers [Hornick et al, 1970] than Vi- strains. Capsules have been postulated to protect bacteria from the environment by a number of mechanisms. They may reduce phagocytosis by reducing macrophage-Salmonella collisions required for optimal phagocytosis. Capsules may also provide steric hindrance to the opsonic effects of antibody and complement bound to the bacteria, thereby reducing macrophage attachment. The shielding from the immune system of subcapsular determinants which may be more antigenic may also be affected by capsules. However, in some cases where capsules are potent

immunogens, phagocytosis may be enhanced.

1.6.4 Flagella

Flagella are used in the serotyping of some bacterial species such as Salmonella species and E.coli and are responsible for the motility of an organism. A flagellum is a 10-20 nanometres filament of protein embedded in the cytoplasmic membrane.

Flagella play a role in the in vitro invasiveness of S.typhimurium by facilitating attachment to cells [Jones et al., 1981; Khoramian et al., 1990]. The motility of the organism also facilitates invasion and survival within macrophages in vitro [Tomita et al., 1981; Tomita and Kanegasaki, 1982; Fields et al., 1986]. However the contribution of flagella to in vivo virulence has been more controversial. Past reports suggested that flagella are required for full expression of virulence of S.typhimurium in mice [Carsiotis et al., 1984; Weinstein et al., 1984]. However more recent studies have shown that non-flagellated strains of S.typhimurium are as virulent as flagellated strains [Carsiotis et al., 1989; Lockman and Curtiss III, 1990; Jones et al., 1992].

One virulence factor conferred by flagella that is well understood is the ability flagellate bacteria to avoid immune recognition through phase variation of their flagellar H antigen, which is

characterised by the existence of 2 types of H antigen in a particular clone [reviewed by Iino, 1969]. The phenomeno was first described for S.typhimurium but has since been recognised in many other species.

1.6.5 Fimbriae

Fimbriae are filamentous appendages of many Gram-negative bacteria and are shorter, thinner and straighter than flagella. They consist of a protein called pilin and are not involved in motility [Otow, 1975]. Fimbrial antigens have been shown to facilitate mucosal attachment by enteric pathogens [Stocker and Mäkelä, 1986; Isaacson and Kinsel, 1992] and hence have been called adhesins. Adherence is assumed to be a prerequisite for invasion [Freter, 1981], and loss of adhesiveness results in a decrease in virulence [Gahring et al., 1990]. A variety of fimbriae have been identified on various Salmonella serotypes [Muller et al., 1991]. S.typhimurium possesses Type I fimbriae which are referred to as mannose-sensitive [MS] adhesins becauses they bind to D-mannose-containing receptors on cell surfaces and the binding is inhibited by D-mannose. In vitro studies showed that the MS adhesins are involved in attachment of S.typhimurium to HeLa and HEp-2cells [Tavendale et al., 1983] and to rat enterocytes [Lindquist et al., 1987]. However the role of MS fimbriae in the pathogenesis of S.typhimurium in vivo is controversial. Fimbriated strains were shown to be better than non-fimbriated forms in

causing infection in mice [Duguid et al., 1976; Tanaka and Katsube, 1978]. Conversely, it has been shown recently that fimbriated strains are less invasive in mice than non-fimbriated strains [Lockman and Curtiss III, 1992].

1.6.6 Virulence Plasmids

Autonomous plasmids of Salmonella have been shown to encode essential virulence determinants. Such virulence plasmids have been reported in S.typhimurium [Baird et al., 1985] and in other serotypes [Terakado et al., 1983; Nakamura et al., 1985; Barrow et al., 1987; Kawahara et al., 1988; Barrow and Lovell, 1988]. Studies on the role of these plasmids have suggested their involvement in mannose-resistant adhesion and invasion of HeLa cells [Jones et al., 1982], resistance to the bactericidal action of serum and complement [Helmuth et al., 1985; Vandenbosch et al., 1987] and in establishing a progressive systemic infection in the RES [Gulig and Curtiss III, 1987; Hackett and Wyk, 1986; Heffernan et al., 1987]. A gene, mka, has been isolated on the S.typhimurium virulence plasmid and has been shown to be necessary for intracellular growth [Taira and Rhen, 1989].

1.7. Host Resistance to Salmonella

Natural resistance to S.typhimurium infection is regulated by several genes. In the first few days following infection, control of virulent S.typhimurium is an inherent property of macrophages and is regulated by the Ity [immunity to typhimurium] gene [Hormaeche, 1979b; O'Brien, 1986] which is expressed in

macrophages [Lissner et al., 1983]. The suceptibility allele Ity^s is present in strains of mice such as BALB/c and C57BL/6. The mechanism of action of the Ity gene is unknown. There is debate as to whether the Ity gene regulates bacterial growth [Hormaeche et al., 1981] or bacterial killing [Lissner et al., 1983; Van Dissel et al., 1985] of salmonellae within macrophages. Its action is not dependent on the activation of macrophages by T cells [Briles et al., 1986; O'Brien and Metcalf, 1982] or by LPS [Briles et al., 1986]. Equivalent genes that regulate macrophage function in Mycobacterium bovis [bcg] and Leishmania donovani [lsh] infection have also been found [Taylor and O'Brien, 1982; Skamene et al., 1982; Brown et al., 1982]. These genes have been mapped to mouse chromosome 1 where the Ity gene is also located, suggesting that all 3 genes may be identical.

Susceptibility to S.typhimurium is also increased in C3H/HeJ mice that carry the Lps^d [defective for LPS response] gene [O'Brien et al., 1980], as they are resistant to the mitogenic effects of endotoxin [O'Brien et al., 1985]. The production of cytokines such as TNF and IL-1 in response to endotoxin, which have both been implicated in resistance to S.typhimurium infection [Tite et al., 1991; Morrissey and Charrier, 1991], is decreased in Lps^d mice [Beutler and Cerami, 1986]. The Xid [X-linked immune-defective] gene also renders mice more susceptible to S.typhimurium as they are B cell-defective and produce only low levels of Salmonella-specific Immunoglobulin [Ig] G whereby they succumb in the late phase of disease. A gene linked to the Major

Histocompatibility Complex [MHC], H-2^b [Hormaeche et al., 1985] and another gene present in DBA/2 mice and C57L mice also increase the susceptibility of mice to S.typhimurium in the late phase of infection [O'Brien et al., 1984].

1.8. Acquired Immunity to Salmonella

1.8.1 T-Independent Mechanisms

Strains of mice containing the Ity^s and Lps^d genes, as well as SCID [severe combined immune-deficient] and athymic mice, have been used to study the relative contributions of specific and non-specific mechanisms of immunity to S.typhimurium. Temperature-sensitive mutants of Salmonella have been used to study acquired immunity in genetically susceptible mice. Genetically susceptible [Ity^s, H-2^b] mice develop resistance to reinfection with the same kinetics as genetically resistant A/J mice [Ity^r, H-2^a] but exhibit a defect in bacterial clearance in the late phases of primary infection [Nauciel, 1990; Nauciel et al., 1988, 1990]. This suggests that resistance to reinfection and clearance of the primary infection may be mediated by different mechanisms and that non-specific immune mechanisms are important in the early stages of infection.

Immunity to facultative intracellular bacteria is assumed to be a result of macrophage activation by T cell-derived IFN-g [Nathan et al., 1983; Hahn and Kaufmann, 1981; Fukazawa et al., 1983]. in SCID mice which have no T or B cells, S.typhimurium infection still results in macrophage activation by IFN-g [Bancroft et al., 1987]. Also, in vivo treatment with anti-cluster of differentiation

antigen [CD] 4 or anti-CD8 antibody does not suppress resistance to reinfection in the early stage [Nauciel, 1990] in contrast to the effects of anti-IFN-g treatment which greatly reduced resistance [Nauciel and Espinasse-Maes, 1992]. Taken together these findings suggest that in the early stage of infection, resistance is independent of T cells and is regulated by IFN-g. The source of this IFN-g for macrophage activation is now thought to be natural killer [NK] cells [Bancroft et al., 1989; Nauciel, 1990; Nauciel and Espinasse-Maes, 1992].

Administration of anti-asialo $GM1^+$ [a marker on NK cells] to SCID mice blocked IFN-g production in vitro, macrophage activation in vivo and increased mortality rates [Bancroft et al., 1989; Nauciel, 1990]. The role of NK cells in resistance to S.typhimurium is further supported by the findings of Schafer and Eisenstein [1992] who demonstrated that protection against virulent S.typhimurium challenge induced by an aroA strain of S.typhimurium was mediated by NK cells.

NK cells have been shown to produce IFN-g in response to stimulation with bacterial antigen [Welsh, 1984; Klimpel et al., 1988] in the the presence of macrophages [Kawase et al., 1983; Blanchard et al., 1986; Bancroft et al., 1989]. It is thought that NK cells produce IFN-g following stimulation with Salmonella in the presence of macrophage-derived TNF [Bancroft et al., 1989; Nauciel, 1990; Nauciel and Espinasse-Maes, 1992]. Both these cytokines have been shown to effect macrophage activation [Kagaya

et al., 1989; Bermudez and Young, 1988] resulting in enhanced bacterial killing through the generation of reactive oxygen intermediates [Edwards III et al., 1992]. As cytotoxic cells, NK cells may also augment resistance to Salmonella infection through direct killing [Garcia-Peñarrubia et al., 1989] or by antibody-dependent cell cytotoxicity [Lowell et al., 1979].

The T-independent hypothesis of acquired resistance to Salmonella assumes that macrophages are able to kill the salmonellae following activation with IFN-g from NK cells. However there is much controversy regarding the ability of salmonellae to survive and multiply within host phagocytes. Descriptions of salmonellae as facultative intracellular parasites [Suter, 1956; Collins, 1974; Buchmeier and Heffron, 1989; Gharing et al., 1990; Vladoianu et al., 1990] imply that they are able to achieve this, perhaps by preventing macrophage phagosome-lysosme fusion [Buchmeier and Heffron, 1991]. However others have shown that macrophages from peritoneal exudates of infected mice can kill ingested salmonellae [Nakoneczna and Hsu, 1980, 1983; Van Zwet et al., 1975; Lin et al., 1989]. Killing within neutrophils as well as within macrophges has also been demonstrated [Briles et al., 1981; Beckerdike et al., 1974; Rest et al., 1978; Modrakowski and Spitznagel, 1979]. Vladoianu et al. [1990] and Buchmaier and Heffron [1989] studied intracellular survival within a wide range of macrophage types and concluded that intracellular survival by Salmonella may depend on

the macrophage source. However the isolation of genes that control the ability of macrophages to kill salmonellae suggests that the bacteria are susceptible to the anti-bacterial mechanisms that exist within macrophages.

1.8.2 T-Dependent Mechanisms

Previous studies have demonstrated a role for T cells in resistance to S.typhimurium [Blanden et al., 1966; Collins, 1974; Lindberg and Robertsson, 1983]. Although T cells appear not to be involved in the resistance to reinfection in the early stages, they do play a role in the clearance of the primary challenge from the spleen [Nauciel 1990]. In euthymic mice, levels of salmonellae in the spleen decreased between weeks 2 and 3 after challenge, whereas levels continued to increase in athymic mice [Nauciel, 1990]. Furthermore, the ability to clear salmonellae more rapidly from the spleen can be adoptively transferred into naive mice with CD4+ cells from Salmonella-vaccinated mice, and administration of anti-CD4 antibody greatly reduces the ability of vaccinated mice to clear the salmonellae from the spleen. CD4+ T cells are likely to exert their effects through the production of IFN-g for macrophage activation [Mossman and Coffman, 1987], the induction of delayed-type hypersensitivity [DTH] responses and in the production of cytokines that augment B cell proliferation [Hahn and Kaufmann, 1981].

In vivo and in vitro depletion experiments showed that the depletion of CD8+ cells slightly but significantly depressed the clearance of salmonellae from the spleen [Nauciel, 1990]. The involvement of CD8+ T cells as well as CD4+ T cells in immunity to Salmonella is consistent with the results of genetic restriction analysis performed by this laboratory. They localised the region of the H-2 complex involved in the control of Salmonella clearance to the D region, which encodes Class I MHC molecules and to a chromosomal segment between K and E which encodes Class II MHC molecules suggesting a role for both T cell subsets in immunity to Salmonella [Nauciel et al., 1990].

The role of CD8+ cytotoxic T lymphocytes [CTLs] may be related to their ability to lyse infected target cells and/or produce IFN-g [Kaufmann, 1988]. However, for CD8+ cells to be invoved in immunity to Salmonella, the generally accepted rules for antigen presentation would have to be broken. Antigen association with Class 1 is generally restricted to proteins that are newly synthesised within the cell [Monaco, 1992]. Antigens taken up by phagocytosis are exogenous and are presented by Class 2 MHC molecules. So for CD8+ CTLs to be involved in immunity to Salmonella, exogenous microbial antigens must be presented by Class 1 molecules. Evidence exists for the association of exogenous peptides with Class 1 molecules [Staerz et al., 1987]. Some bacteria produce compounds, e.g. Listeria produce listeriolysin-O, which destroy the phagosome [Gaillard et al., 1987] thereby

exogenous antigen enters the cytoplasm where it may gain access to Class 1 processing and recycling pathways. Furthermore, live Salmonella vaccine strains have been shown to elicit CD8+ T cells to expressed heterologous antigens providing further evidence for the ability of exogenous antigens to be presented with Class 1 molecules [Sadoff et al., 1988; Tite et al., 1990; Aggerwal et al., 1990].

1.8.3 The Role of Specific Antibody in Salmonella Infection

Adoptive transfer experiments have demonstrated a role for B cells in protection from Salmonella [Hochadel and Keller, 1977; Morris, 1976] and this is supported by the susceptibility of Xid mice to S.typhimurium infection [O'Brien et al., 1981]. Passive immunisation experiments have demonstrated the effectiveness of specific antibody against crude extracts of rough bacteria [Kussi et al., 1979] and antigens against the O-antigen of LPS [Svenson and Lindberg., 1981; Svenson et al., 1979] in affording protection from virulent challenge. However, the level of protection afforded by antibodies is strongly influenced by experimental conditions, such as the mouse strain and route of inoculation [Collins, 1969; Nauciel et al., 1981; Eisenstein et al., 1984]. The multifactorial effects of antibodies should allow them to play an important role in both the T-independent and T-dependent mechanisms of acquired resistance to S.typhimurium discussed previously.

Brown and Hormaeche [1989] examined the specificity of the antibodies produced during Salmonella infection. They found that antibodies were produced with specificity for more than 30 antigens, including LPS, outer membrane proteins, lipoprotein, flagella and capsule. Such antibodies would play a role in negating the virulence properties of these structures and in neutralising bacterial toxins and adhesins [Jones et al., 1988].

Saxèn and Mäkelä [1982] compared the efficiencies of different anti-Salmonella Ig isotypes in providing protection of mice from salmonellosis. They found that IgM antibodies were much more protective than IgG or IgA [Saxèn et al., 1984] even though the concentrations of each were similar. The efficiency of IgM is due to the enhanced activation of complement by the IgM bound to the bacterium, promoting opsonisation via the C3b receptors on macrophages [Rabinovitch, 1975] and direct complement lysis.

IgM is also a stong agglutinating antibody. These findings were supported by Duran and Metcalf [1987] who found that S.typhimurium stimulates clonal expansion of cells secreting IgM, IgG_2 and IgA, but very little IgG_1 is produced. However Collins et al. [1988] showed that intradermal inoculation of heat-killed S.dublin protected rats against a lethal intraperitoneal challenge in the absence of a good O or H agglutination response. As the protective factor in this case was IgG_{2a}, agglutinating antibodies may not be essential for protection. IgG is an opsonic antibody, binding via

its Fc region to Fc receptors on phagocytes, promoting phagocytosis of the salmonellae. Specific opsonic antibody may also bind in this way to the Fc receptors on T cells, K cells and NK cells thereby mediating cell cytotoxicity.

As well as the production of serum IgG and IgM, there is also a significant rise in the levels of specific sIgA in the gut following oral administration of Salmonella [Bartholomeuz et al., 1986, 1990; Forrest, 1988] which is important in protection [Hohmann et al., 1979, Michetti et al., 1992]. Following oral vaccination, bacterial antigen is taken up by M cells and presented to the immune cells in the Peyer's patches which are subsequently primed. Following priming, the B cells switch on membrane IgA [mIgA] production, and the mIgA+ B cells leave the Peyer's patches and return to the blood via the mesenteric lymph nodes and the thoracic duct. It is thought that at this time T helper [Th] 2 cells produce IL-5 which acts on B cells inducing them to switch from mIgA production to secretory IgA [sIgA] production. This sIgA is transported across the gut epithelium, during which it dimerises, and the sIgA dimer is deposited in the lumen [reviewed by McGhee et al., 1992]. As it is not opsonic and does not combine with complement, the major anti-bacterial activity of sIgA appears to be inhibition at the mucosal surface. It is resistant to proteolysis and hence forms an "antiseptic paint" preventing mucosal penetration, effecting neutralisation of enterotoxins and reducing bacterial motility.

1.9 Live Salmonella Vaccines

Despite some success with killed Salmonella vaccines when used as whole-cell vaccines [Hertzberg et al., 1972; Marecki et al., 1975; Eisenstein and Angermann, 1978; Angermann and Eisenstein, 1980] or as subunit vaccines [Kuusi et al., 1979; Killion and Morrison, 1986], live Salmonella vaccines provide the best protection against challenge with virulent salmonellae [Collins, 1974; Lindberg and Robertsson, 1983]. The most widely tested live Salmonella vaccines are galE and aroA mutants which are discussed below.

1.9.1 Galactose E Gene [gal E] Mutants

Gal E gene-defective mutants of Salmonella were first isolated by Fukasawa and Nikaido [1959] as galactose-sensitive mutants which undergo lysis in its presence. They are characterised by a block in the enzyme uridine di-phosphate [UDP]-galactose-4-epimerase and consequently cannot convert glucose to galactose, a component of Salmonella core oligosaccharide and O-repeat units. Without an external supply of galactose, only incomplete cell wall LPS is formed [Osborn et al., 1962, 1968] and the organisms are avirulent and poorly immunogenic. When galactose is supplied exogenously, as is the case for vaccine preparation, the organisms synthesise normal LPS and become immunogenic but lyse due to the intracellular accumulation of toxic amounts of UDP-galactose and galactose-1-phosphate. Strains grown on galactose can therefore establish a limited infectiom in mice, before eventually being cleared.

One gal E strain of S.typhi, designated Ty21a, was found to afford protection against virulent Salmonella challenge in the mouse typhoid model [Germanier and Furer, 1971]. In human field trials in Egypt and Chile, which are areas endemic for typhoid, Ty21a was shown to afford up to 96% protection, lasting for up to 3 years [Wahdan et al., 1982; Levine et al., 1987; Black et al., 1983; Ferreccio et al., 1989]. Oral administration of Ty21a to human volunteers produces strong specific mucosal sIgA responses [Sarasombath et al., 1987; Cancellieri and Fara, 1985; Bartholomeuz et al., 1986, 1990] serum antibody [Sarasombath et al., 1984; Bartholemeuz et al., 1990; Forrest et al., 1988], and strong CMI [Sarasombath et al., 1987].

1.9.2 Aromatic A Gene [aroA] Mutants

Another approach to the development of attenuated mutants was prompted by the work of Bacon et al. [1951] who surveyed biochemical mutants of S.typhi and found that strains dependent on aromatic compounds or purines were unable to kill mice, even though they still possessed a wide array of virulence determinants. 30 years later this observation was followed up by Hoiseth and Stocker [1981] who constructed S.typhimurium strains with genetically defined mutations in the aroA gene. The aroA gene codes for one of a series of enzymes involved in the synthesis of chorismic acid. This is the only intermediate from which bacteria can synthesise all their aromatic metabolites including the aromatic amino acids enterochelin, dihydroxybenzoate [DHB], and p-aminobenzoic acid

[PABA], which is a precursor for folate and nucleotide synthesis. A block in this pathway, making a Salmonella strain dependent on external aromatic metabolites should cause a loss of virulence in vivo because PABA and DHB are not found in mammalian tissues, and no efficient alternative route exists for their synthesis in bacteria.

Hoiseth and Stocker [1981] made use of the tendency of a tetracycline-resistance transposon, Tn10, to cause DNA alterations within the transposon itself, sometimes extending into the adjacent chromosomal genes such as aroA, causing inactivation of this gene and development of aromatic-dependence. The S.typhimurium aroA-defective mutants were found to be highly attenuated in mice and have proved to be effective vaccines against virulent Salmonella challenge [Hoiseth and Stocker, 1981; Killar and Eisenstein, 1985; O'Callaghan et al., 1988]. Vaccination results in the production of specific serum and mucosal humoral immune responses [Sigwart et al., 1989] and specific CMI [Killar and Eisenstein, 1985] to the vaccine strain.

Following the success of the studies in mice, aroA strains of Salmonella were tested in larger animals and have given promising results as live [oral or parenteral] vaccines in calves [Robertsson et al., 1983; Smith et al., 1984a,b], sheep [Mukkur et al., 1987; Mukkur and Walker, 1992] and poultry [Cooper et al., 1992], though none of these vaccines have been adequately field tested.

The results with aroA S.typhimurium and aroA S.dublin encouraged the development of aroA mutants of S.typhi with the purpose of vaccine trials in humans. A second attenuating mutation to an aroA strain is generally considered necessary if candidate strains are ever to be used commercially, as single transduction events could result in reversion to wildtype. Mutation to a purine requirement causes reduced virulence in S.typhi [Bacon et al., 1950] and in other serotypes [McFarland and Stocker, 1987]. However, strains with mutations in both the aroA and purA genes are less effective as vaccines in mice than single mutants as they are less able to persist in the host tissues [O'Callaghan et al., 1988; Sigwart et al., 1989]. Alternatively, strains with mutations in different aro genes are as invasive as single mutant strains [Dougan et al., 1988], and human trials with these strains are underway [Tacket et al., 1992].

1.10 Expression of Heterologous Antigens in Live Salmonella Vaccine Strains

In evaluating the potential of vaccine vectors such as galE and aroA mutants of Salmonella, certain important questions must be answered. Is the vaccine safe with no chance of reversion to wildtype? Is the vaccine effective in eliciting specific immune responses to both the vaccine strain and the heterologous antigen? What is the location of the heterologous antigen when expressed by the vaccine strain e.g cytoplasmic, cell surface or extracellular and is this important in the elicitation of an immune response?

Initially, Salmonella vaccine strains were used to express antigens from related enteric pathogens. Formal et al. [1981] expressed Shigella sonnei antigens into Ty21a, and mice vaccinated with the hybrid strain raised antibodies to both the Shigella and Salmonella antigens. Clements and El-Morshidy [1984] expressed the non-toxic B subunit of heat-labile enterotoxin [LT-B] of E.coli from Ty21a, and a good anti-toxin response developed in mice following intraperitoneal injection. LT-B expressed from both S.enteriditis [Clements et al., 1986] and S.typhimurium [Maskell et al., 1987] aroA strains elicited both sIgA and serum IgG as did the K88 fimbrial adhesin of enterotoxygenic E.coli expressed from galE and aroA strains of S.typhimurium. [Stevenson and Manning, 1985; Dougan et al., 1986; Maskell et al., 1986]. Combined Salmonella vaccines have also elicited humoral and secretory responses to outer membrane proteins of Brucella abortus [Stabel et al., 1990] and group B meningococci [Tarka et al., 1989].

All the aforementioned bacterial antigens were expressed on the surface or secreted extracellularly by the vaccine strain. In contrast, most of the potentially protective protein antigens from viruses and parasites which have been expressed in salmonellae accumulate in the cytoplasm of the vaccine strain. Although it is difficult to obtain surface expression of these hybrid proteins, Salmonella vaccines of this type can can elicit immune responses to cloned intracellular antigens. When administered orally to mice,

specific humoral and cellular immune responses were evoked to E.coli B-galactosidase, expressed from aroA S.typhimurium, illustrating that surface expression may not be necessary for the development of immunity [Brown et al., 1987].

Furthermore, Guzmán et al. [1991] expressed the filamentous haemagglutinin [FHA] of Bordetella pertussis from aroA S.typhimurium and detected FHA-specific IgA and IgG in the lungs of mice following oral immunisation, demonstrating the ability of live Salmonella vaccines to prime at one mucosal surface thereby eliciting antibody production at another. The FHA was expressed in the cytoplasm of the vaccine strain as insoluble inclusion bodies suggesting that the insoluble nature of the protein does not affect its immunogenicity.

Sadoff et al. [1988] expressed the circumsporozoite protein [CSP] of Plasmodium berghei from the cytoplasm of a live Salmonella strain and demonstrated CSP-specific CMI and protection from sporozoite challenge in mice in the absence of anti-CSP antibodies. Further studies showed that immunity was mediated through the induction of specific CD8+ cells, since in vivo elimination of CD8+ cells abrogated protection. [Sadoff et al., 1988; Aggarwal et al., 1990]. A 69 K [kilodalton] protein of Leishmania major expressed from the cytoplasm of aroA S.typhimurium elicited specific CD4+ T cells in mice which produced IL-2 and IFN-g following antigen stimulation in vitro. [Yang et al., 1990].

In this thesis, I examined the the immune response to both the major outer membrane protein [MOMP] of Chlamydia psittaci [C.psittaci] and the leukotoxin [cytotoxin] of Pasteurella haemolytica A1 [P.haemolytica A1] expressed from the cytoplasm of an aroA strain of S.typhimurium. The following sections discuss the economic importance of both Pasteurella and Chlamydia and the biological and immunological importance of the proteins selected for expression.

1.11 C.psittaci, Ovine Abortion of Ewes [OAE] and the MOMP

C.psittaci is a member of the genus Chlamydia. It has a broad spectrum of hosts and has been recovered from a wide range of domestic and wild birds, and mammals. C.psittaci is the causative agent of ovine enzootic abortion [OEA], which is the major cause of lamb loss in sheep farming in the UK [Aitken, 1983] and OEA strains of C.psittaci can cause abortion and severe illness in pregnant women [Buxton et al., 1986]. Man can also become infected with avian strains of C.psittaci which cause a serious and sometimes fatal respiratory illness called psittacosis [Schachter and Dawson, 1978].

C.psittaci is an obligate intracellular parasite with a complex developmental cycle that takes place within the cytoplasm of the host cell. In most cases C.psittaci causes only a subclinical infection, but under conditions of stress, illness or during pregnancy, the organism can invade mucosal surfaces and gain entry

to other organs and tissues, causing clinical disease. As a result, C.psittaci can cause epidemics in farm animals with serious economic consequences. Most notable among these are respiratory disease and enteritis in domestic poultry, abortion in ruminants, arthritis in lambs and calves, and pneumonia and conjunctivitis in various species.

OEA causes abortion of the foetus about 2-3 weeks before the end of the normal gestation period. There are no clinical signs of the disease until abortion occurs, when the foetal membranes appear necrotic with gross thickening of intercotyledonary spaces and are rich in chlamydial elementary bodies [EBs], the infectious form of the organism. Lambs are usually born dead or are weak and die within a few days.

The initial commercial vaccine against OEA consisted of inactivated EBs [McEwen et al., 1951] and although this vaccine did not eliminate the disease, it reduced the incidence of OEA to an economically acceptable level for many years up until the mid-1970s [Foggie, 1973]. However, outbreaks of OEA have increased and so more effective vaccine strategies are being sought.

Current chlamydial vaccine design is based on the major outer membrane protein [MOMP], which is the most abundant protein in the cell envelope of the EB. It is a 39-40 K protein which comprises 60% of the protein mass in the outer membrane [Caldwell et al., 1981; Salari and Ward, 1981] and is by far the best characterised

chlamydial antigen. Antigenically, MOMP is very complex and possesses a hierarchy of epitopes bearing genus-, species-, subspecies- and serotype-specificity [Caldwell et al., 1981; Caldwell and Schachter, 1982; Stephens et al., 1982, 1984, 1988; Newhall et al., 1986]. Structural studies of MOMPs by peptide mapping suggest that each MOMP represents a serological mosaic of antigenic determinants located on 5 conserved regions alternating with 4 variable segments [VS][also referred to as variable domains [VD][Caldwell and Judd, 1982; Ma et al., 1987]. Molecular analysis of the MOMP gene of C.trachomatis serotypes have confirmed these findings and have located these epitopes to specific VS of the MOMP [Baher et al., 1988; Stephens et al., 1988; Conlon et al., 1988]. VS1 and VS2 contain neutralising antibody recognition sites [Baehr et al., 1988] and VS3 has been shown to contain T cell epitopes that provide help for the production of these neutralising antibodies [Allen et al., 1991]. Also it has been shown that of 9 peptides delineating the MOMP protein, only the one encompassing VS3 was recognised by Chlamydia-specific T cell lines [Ishizaki et al., 1992].

Antibodies to specific epitopes on MOMP demonstrably interfere with the infection process at a stage after EB internalisation [Caldwell and Perry, 1982] and C.trachomatis infectivity can be neutralised with anti-MOMP Ig [Caldwell and Perry, 1982] and MOMP-specific monoclonal antibodies [Peeling et al., 1984]. However, CMI plays the most important role in the resolution of C.trachomatis pneumonitis in mice [Ramsey et al., 1989], and in a mouse model

of C.psittaci, CMI was better correlated with protection than humoral immunity [Buzoni-Gatel et al., 1987]. Both CD4+ and CD8+ cells were shown to be important [Williams et al., 1984], although CD8+ cells may have the dominant role [Buzoni-Gatel et al., 1992]. Other animal models of Chlamydia infection have shown that protection from reinfection is mediated by mucosal antibody and is T cell-dependent [Williams, 1988; Rank, 1988].

A MOMP-enriched vaccine has been shown to protect against abortion following experimental challenge of ewes with live organisms [G.E. Jones, personal communication], however the mechanisms invoved in protection have yet to be elucidated. Although there are elevated levels of MOMP-specific antibody in post-abortion animals, and ewes do not abort twice, passive transfer experiments demonstrated that post-abortion serum antibodies were not protective against OEA [G.E. Jones, personal communication]. MOMP-specific T cells are present in post-abortion animals [Dawson et al., 1986a,b; Huang, 1988; McCafferty, 1992] but their role in protection from OEA has yet to be elucidated.

1.12 P.haemolytica and the Leukotoxin

Amonst the infectious diseases affecting livestock, respiratory diseases are of particular importance, causing great economic loss in the sheep farming industry throughout the world. Pneumonic pasteurellosis, caused by the Pasteurella species P.haemolytica or P.multicida, is the most common form of fatal respiratory disease

in sheep, accounting for 70% of pneumonias [MAFF, 1980]. P.haemolytica has been shown to be the more significant of the 2 species [Gilmour, 1978, 1980] and was the one examined in this thesis.

P.haemolytica is a small Gram-negative pleomorphic rod being aerobic and facultatively anaerobic and is haemolytic on sheep-blood agar. Strains of P.haemolytica can be classified into 2 biotypes A and T, which denote arabinose fermentation and trehalose fermentation respectively. Within these biotypes there are 15 different serotypes [Fraser et al., 1982], identifiable by an indirect haemagglutination test [Biberstein et al., 1960].

Smith [1961] showed that the disease in sheep caused by P.haemolytica fell into 2 distinct syndromes, each of which is generally associated with one of the 2 biotypes. Biotype A causes enzootic pneumonia affecting sheep of all ages [Montgomerie et al., 1938]. Morbidity and mortality may be up to 10% of the flock and sheep which recover may remain chronically infected. Stress factors may play an important role in the development of disease, and factors such as climate, dipping, viral infection and anti-helminthic treatment have all been postulated as predisposing the animals to disease [Gilmour, 1978]. The T biotypes cause a systemic disease which is a causes of death in older sheep [Stamp et al., 1955].

Despite some success with both live and heat-killed Pasteurella vaccines [Dungal, 1931; Cameron, 1966] and the widespread use of the currently available formalin-killed whole-cell vaccines Heptovac-P [Hoechst, UK, Ltd] and Carovac [Pitman-Moore, UK], doubts about their efficacy have been expressed [Gilmour, 1980; Gilmour et al., 1983] and deaths of both ewes and lambs in vaccinated flocks have been reported [ADAS, 1984]. There appears to be a need for more effective vaccine strategies.

One of the most important virulence factor of all P.haemolytica serotypes is the leukotoxin which is an exotoxin that specifically kills leukocytes of sheep [Sutherland et al., 1983, 1985], cattle [Benson et al., 1978] and goats [Chang et al., 1982; Richards et al., 1982], but not those of non-ruminants [Sutherland et al., 1983]. As the leukotoxin is lethal for cells involved in immunity, it is likely to be an important pathogenic determinant. In experimental pasteurellosis of sheep, high numbers of the organism are recovered from the lungs in the early stages of disease [Sharp et al., 1978; Rushton et al., 1979] which may be due to in the impaired alveolar macrophage function as a result of leukotoxin activity. The leukotoxin has also been shown to inhibit the production of chemotactic factors by bovine alveolar macrophages [Markham et al., 1982].

Leukotoxin activity can be neutralised by antibodies produced in calves by vaccination with live P.haemolytica [Baluyut et al., 1981] and several studies have correlated resistance to bovine pasteurellosis with antibody titres to the cytotoxin [Shewen and Wilkie, 1982, 1983; Cho et al., 1984; Gentry et al., 1985]. Vaccines incorporating leukotoxin have therefore been tested for their ability to induce protective immunity from Pasteurella.

Leukotoxic activity is present in bacterium-free culture supernatant from logarithmic phase cultures, and vaccination of calves with P.haemolytica culture supernatant has been shown to induce resistance to experimental challenge [Gentry et al., 1985; Mosier et al., 1986, 1989; Shewen and Wilkie, 1987]. However this culture supernatant contained other soluble antigens, as well as leukotoxin. Conlon et al. [1991] were unable to protect lambs from pasteurellosis using only recombinant leukotoxin [rec leukotoxin] although the leukotoxin increased the efficacy of a whole cell vaccine. Therefore aroA S.typhimurium expressing leukotoxin would be unlikely to protect against pasteurellosis unless it was administered with other Pasteurella components.

1.13 Objectives of Thesis

1) To compare the virulence of the aroA and wildtype strains of an ovine isolate of S.typhimurium, S25\1, in mice and lambs.

2) To examine the immune responses elicited to the aroA vaccine strain following oral vaccination of mice and lambs.

3) To demonstrate the ability of the aroA strain to protect mice and lambs from virulent challenge with wildtype S25/1.

4) To assess the vaccine vector potential of the aroA strain by examining the immune responses in mice and lambs to expressed heterologous antigens [MOMP and leukotoxin] following oral inoculation.

Chapter 2

Materials and Methods

2.1 Chemicals

All chemicals used throughout the course of the work described in this thesis were of analytical grade from BDH Chemicals Ltd, Poole, Dorset, UK, unless otherwise stated.

2.2 Bacterial Methods

2.2.1 Culture Media

The formulae of media are listed in Appendix 1.

2.2.2 Storage of Bacterial Strains and Culture Conditions

Bacteria from a single colony were grown overnight at 37^{o}C in Luria-Bertani [LB] broth. 1.4 ml of culture was added to 0.6 ml of glycerol [50%v/v] in 1.5 ml Nunc plastic vials [Gibco], and stored at -70^{o}C. Strains were grown by scratching the surface of the frozen material with a sterile loop and subculturing on LB agar or LB broth as required. Bacteria were routinely cultured in LB broth with shaking or on LB agar at 37^{o}C for 16 h. Antibiotics were added to the media when required.

2.2.3 In Vitro Growth Rates

To determine the growth rates of all the strains used, 1 ml of an overnight LB broth culture was sub-cultured into fresh LB

broth and incubated at 37°C with shaking. Aliquots were taken at various times and the UV absorbance measured at 600nm using a single beam UV digital spectrophotometer [Cecil Instruments, Cambridge, UK]. The results are shown in Appendix 6.

2.2.4 Preparation and Determination of the Viable Count of an Inoculum Culture

A 16-h culture of the bacterial strain was spun on a Beckman J2-21 centrifuge at 7500g for 10 minutes [min] to pellet the cells. The cells were washed twice for 10 min in phosphate-buffered saline [PBS][see Appendix 5] and resuspended in the appropriate volume of PBS. Viable counts were estimated by measuring the UV absorbance of culture at 600nm using a single beam digital spectrophotometer. The number of colony forming units [no. CFU] per ml [no. CFU/ml] equivalent to specific Optical Density [OD] $_{600nm}$ readings were determined using the spreading plate method as described. Ten-fold dilutions of the samples [of known OD_{600nm}] were prepared in PBS and 100 ul aliquots were spread on each of 3 dry LB agar plates, with or without antibiotics, depending on the strain. After incubation of the plates at 37°C for 16 h, the colonies were counted and the no. CFU/ml was calculated from the average of the 3 plates.

2.2.5 Identification Of Bacteria

Confirmation that a particular organism was a Salmonella species was obtained by examining sugar fermentation using API 50 CHE

galleries [obtained from API Laboratory Products Ltd, Basingstoke, Hampshire, UK]. The galleries were inoculated according to the manufacturers instructions, incubated at 37°C, and examined after 3, 6, 24, 48 and 96 h.

The S.typhimurium serotype of an organism was confirmed by the detection of Salmonella H antigens or Salmonella O antigens using commercial slide agglutination tests [Difco].

The wildtype strain of S.typhimurium was distinguished from the aroA strain by plating onto XLD [Xylose Lactose Deoxycholate] medium where the wildtype strain produced black colonies due to the production of H_2S, and the aroA strain [H_2S-] produced pinky yellow colonies.

The wildtype and mutant strains were also distinguished by comparing their ability to grow on minimal agar and on minimal agar containing the appropriate aromatic compounds and amino acids [see Appendix 1F]. Virulent, aromatic-independent strains will grow on minimal agar irrespective of the presence of aromatic compounds whereas avirulent, aromatic-dependent strains will not grow on minimal agar unless the aromatic matabolites are added.

2.2.6 Construction of Bacterial Strains

Construction of an Aromatic-Dependent Mutant of an Ovine Isolate of S.typhimurium

The work made use of a transposon insertion, aroA554::Tn10, from the weakly pathogenic strain LT2, originally used by Hoiseth and Stocker [1981] to demonstrate that mutation of the aroA gene of S.typhimurium prevented the production of important aromatic compounds thus rendering the strain avirulent.

The aroA554::Tn10 region from LT2 was transduced into S25/1 [see Table 2.1] using the high-transducing Salmonella-specific phage P22 HT105/1 int. The transposon Tn10 encodes for tetracycline resistance and allowed transductants to be easily selected. S25/1aroA554::Tn10 was grown on Bochner media [Bochner et al., 1980] which selects for tetracycline-sensitivity. Single colonies resulting from this selection process were further screened to ensure tetracycline-sensitivity, a dependence on the major aromatic compounds and a smooth phenotype. An isolate with the correct genotype was grown in liquid selection media and repeatedly subcultured and plated to ensure that no reversion to wildtype occurred. Hereinafter, this isolate is referred to as S25/1aroA.

Construction of Plasmid-Bearing Strains

Plasmids pC, pD and pAL12 used in this thesis were all pUC8 derivatives [see Table 2.1] and were constructed by inserting the appropriate DNA fragment into the multiple cloning site of pUC8 [see Figure 2.1]. The pUC8 plasmid without an insert of foreign DNA was also used.

Attempts to transform S25/1aroA with the plasmids were inefficient owing to the smooth phenotype and tended to select for rough variants. For this reason the plasmids were transferred to S25/1aroA by first transforming the rough S.typhimurium strain LB5010. Transformants, obtained by selecting for ampicillin-resistance were grown on medium containing glucose and galactose, allowing the lesion in the galE gene of LB5010 to be overcome and a smooth phenotype to be obtained. P22 HT105/1 int was then used to transduce the plasmids into S25/1aroA.

The resulting strains S25/1aroA[pC], S25/1aroA[pD], S25/1aroA[pAL12] and S25/1aroA[pUC8], abbreviated to pC, pD, pAL12 and pUC8 respectively, were checked for possession of the appropriate plasmid [see 2.3] and Western blotting was performed on whole cell lysates to ensure the expression of the appropriate plasmid encoded protein [see 2.5]. Slide agglutination tests were performed on all the strains to check that a smooth phenotype had been maintained.

Table 2.1 BACTERIAL STRAINS, BACTERIOPHAGE AND PLASMIDS

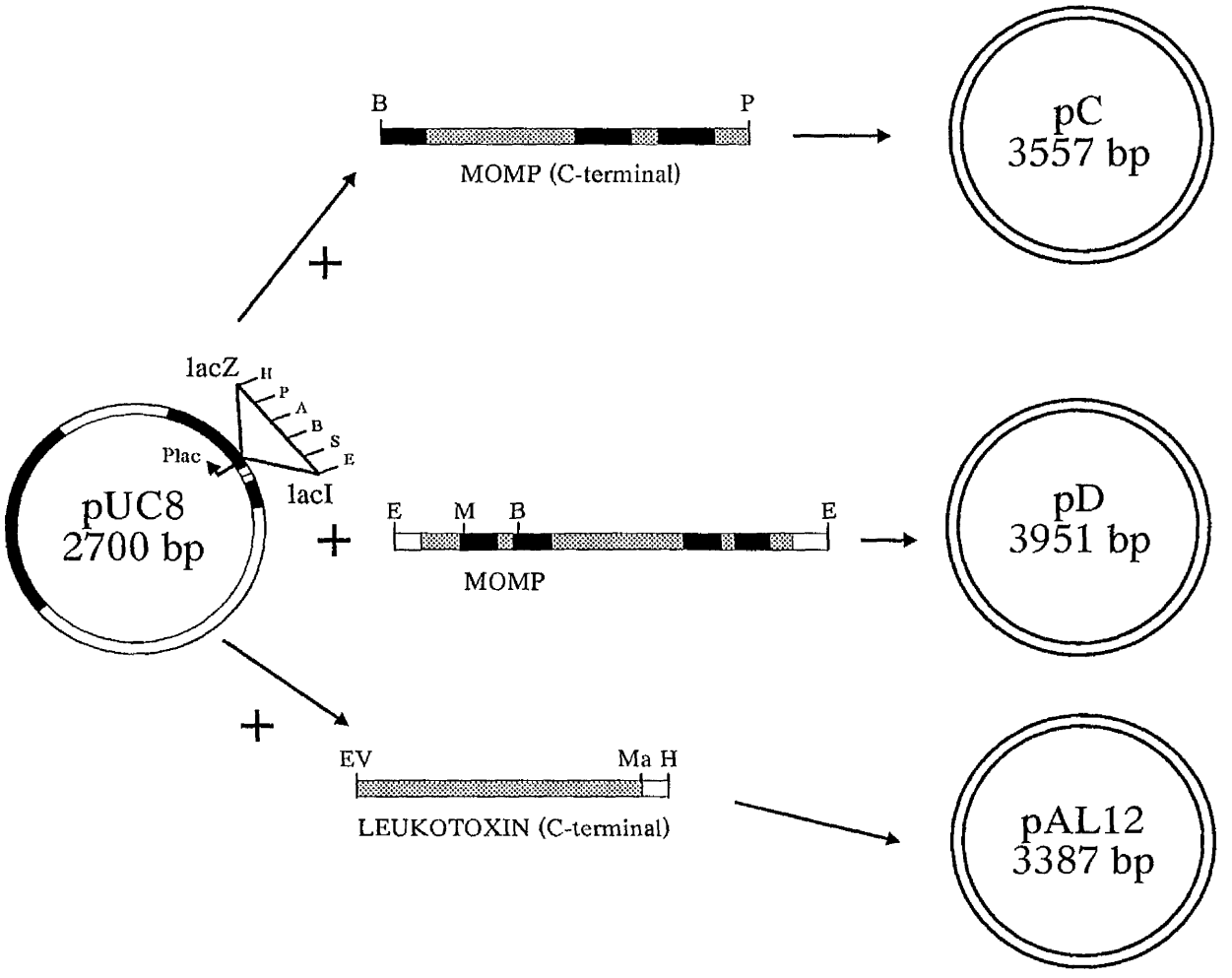
STRAIN	DESCRIPTION	SOURCE
LT2*aroA*554::Tn*10*	Weakly pathogenic strain of *S.typhimurium* with a Tn*10* [tetracycline resistance] insertion in the *aroA* gene.	Prof. G. Dougan, Imperial College, London
S25/1	Virulent ovine isolate of *S.typhimurium*.	Prof. K. Linklater, Scottish Agricultural Colleges
LB5010	Rough mutant [*galE*] of *S.typhimurium*. The mutation prevents the formation of complete LPS when grown on tryptic-soy broth without sugars [1]	Prof. G. Dougan, Imperial College, London
S25/1*aroA*	Derivative of S25/1 rendered avirulent by a mutation in the *aroA* gene preventing the synthesis of important aromatic compounds.	Dr. J. J. Oliver, MRI, Edinburgh
P22 HT105/1 *int*	High-transducing phage specific for smooth strains of *Salmonella* containing O-antigen factor 12.	Dr. G. D. Baird, MRI, Edinburgh
pUC8	General purpose cloning vector [2] carrying ampicillin-resistance and a variety of unique restriction sites located between the *lac* promoter-operator region and the a region of the *lacZ* gene.	Pharmacia, Milton Keynes, UK
pC	pUC8 with 857bp Bam HI-Pst I fragment from 3' end of the major outer membrane protein (MOMP) gene of *Chlamydia psittaci* encoding amino acid residues 140-367 of the protein.	Dr T. W. Tan, MRI, Edinburgh

pD	pUC8 with 1251bp Eco RI fragment of MOMP encoding amino acid residues 1-367 of the protein (does not include the leader peptide sequence).	Dr. T. W. Tan, MRI, Edinburgh
pAL12	pUC8 with 687bp Eco RV-Mae I fragment of the leukotoxin gene encoding amino acid residues 721-950 [the carboxy-terminal end] of the protein.	Dr. A. Lainson, MRI, Edinburgh

<u>References</u>

1. McLachan and Sanderson, 1985.
2. Vieira and Messing, 1982.

Fig 2.1 Construction of Recombinant Plasmids



1. pC - Derived by replacing the Bam HI-Pst I region of the multiple cloning site of pUC8 with a 857 bp fragment encoding the C-terminal part of the Major Outer Membrane Protein (MOMP) of Chlamydia psittaci.

2. pD - Derived by inserting a 1251 bp EcoR1 fragment carrying the complete MOMP gene into the Eco R1 site of pUC8.

3. The coding region of the genes are represented by hatched shading with the solid region depicting the variable domains for both pC and pD.

4. pAL 12 - Derived by inserting a 687 bp Eco RV/Mae I fragment (containing a Hind III site), carrying the C-terminal part of the leukotoxin gene, into the Hind III site of pUC 8.
The coding region of the gene is represented by hatched shading.

5. A-Acc I, B-Bam HI, E-Eco RI, EV-Eco RV, H-Hind III, M-Msp I, Ma-Mae I, P-Pst I, S- sma I.

2.3 Nucleic Acid Methodology

2.3.1 Preparation of Small Quantities of Plasmid DNA from Bacteria by Alkaline Lysis

10 ml of LB broth, containing ampicillin, was inoculated with a single colony of the recombinant strain [either pD, pC, pAL12 or pUC8] harbouring the plasmid. Following overnight incubation, 1.5 ml of the culture was placed in an Eppendorf tube and spun in a microfuge for 2 min at 5000g. The supernatant was removed, the pellet dried by draining the tube and the bacterial cells disrupted by resuspending the pellet in 100 ul cold lysis mix, containing 4 mg/ml lysozyme [Sigma], 50 mM glucose, 10 mM ethylenediaminetetraacetic acid [EDTA] and 25 mM Tris-HCl [pH 8.0] with vortexing. After 5 min at room temperature [RT], 200 ul alkaline sodium dodecyl sulphate [SDS] [0.2 M NaOH, 1% SDS] was added to the suspension, which was then mixed by inverting the tube several times and left on ice for 5 min. 150 ul of cold 3 M potassium acetate was then added with mixing and the tube left on ice for a further 5 min to remove the chromosomal DNA. The tube was then spun for 2 min at 5000g in a microfuge and the proteins precipitated by adding an equal volume of phenol:chloroform [mixed 1:1], vortexing and spinning for 2 min at 13000 rpm in a microfuge. The supernatant, containing plasmid DNA, was transferred to a fresh Eppendorf tube and 2 volumes of 100% ethanol added with vortexing. After 5 min at RT the tube was spun for 5 min at 5000g in a microfuge and the supernatant removed. To wash the plasmid DNA, 1 ml of 70% ethanol was

added with vortexing and the tube was then spun at 5000g in a microfuge for 5 min. The supernatant was removed and all traces of liquid removed from the pellet, which was then resuspended in 50ul of 1X TE [10 mM Tris base, 1 mM EDTA], pH 8.0.

2.3.2 Restriction Enzyme Digestion of Plasmid DNA

Restriction enzymes were obtained from Boehringer Mannheim, Lewes, Sussex, UK, unless otherwise stated. Reactions typically contained 0.2-1 ug of DNA [Maniatis et al., 1982]. To 7 ul of plasmid DNA solution was added 2ul of 0.15 mg/ml DNase-free RNase, followed by 1 ul of the appropriate 10X digestion buffer [Boehringer Mannheim]. 1 ul of the appropriate restriction enzyme was added and the contents of the tube were mixed by vortexing. Digestion was allowed to proceed at 37^{o}C for 2 h.

2.3.3 Agarose Gel Electrophoresis of Plasmid DNA

After digestion, plasmid DNA was analysed in 0.6% [w/v] agarose gels in a horizontal system using a Wide Mini Sub Cell apparatus [Biorad, Hemel Hempstead, Herts, UK]. Agarose gels were prepared by boiling Ultra Pure, low melting point agarose powder [Bethseda Research Laboratories [BRL], Gaithersburg, MD, USA] in Tris-borate buffer [see Appendix 5] until the solution was clear. The molten agarose was cooled and the gel was poured. The DNA samples were mixed with 4 ul of gel loading dye [4 mM urea, 50% [w/v] sucrose, 50mM EDTA, 0.1% [w/v] bromophenol blue] prior to loading onto the gel. Electrophoresis was carried out at RT in Tris-borate buffer containing 0.5 ug/ml ethidium

bromide [Sigma], after which time, the nucleic acid was visualised on a UV transilluminator of 302 nm. The fluorescing bands were photographed with a Polaroid MP-4 Land camera, using a Polaroid type 667 film and a yellow Kodak Wratten filter.

2.4 Preparation of Bacterial Lysates for Sodium Dodecyl Sulphate [SDS]-Polyacrylamide Gel Electrophoresis [PAGE]

A single Salmonella colony was inoculated into LB broth [containing ampicillin when culturing recombinant strains] and incubated at 37°C with shaking for 18 h. 1 ml of culture was placed in an eppendorf and spun at 5000g for 5 min. The supernatant was discarded and the pellet resuspended in Laemmli sample buffer containing 2-ME and bromophenol blue [see Appendix 2] and stored at -20°C for future electophoresis and immunoblotting [see below].

2.5 Protein Methodology

2.5.1 Separation of Proteins by Reducing SDS-PAGE

This procedure was carried out using a modification of the method of Laemlli [1970]. For mini gels [80mm x 60mm], the Biorad apparatus [Mini Protean II] was used, in conjunction with an EPS 500/400 powerpack [Pharmacia Ltd, Milton Keynes, Herts, UK], according to the manufacturers instructions. The compounds and solutions used in the preparation and running of gels are listed in Appendix 2. Samples for electrophoresis generally consisted of whole bacterial cell preparations [prepared as described above] or preparations consisting of

predominantly one protein such as MOMP or leukotoxin. Bacterial lysates were resuspended in 100 ul of sample buffer and MOMP or leukotoxin preparations were diluted 1:2 in sample buffer. All samples were further denatured by boiling for 2 min, prior to loading onto the gel. Samples and prestained molecular weight markers [Biorad] were loaded onto the stacking gel and run at 200 v until the dye front had reached the bottom of the resolving gel.

2.5.2 Electrotransfer and Immunoblotting

This procedure was based on the method of Burnette [1981] using a Tris [20 mM]-glycine [150 mM] blot buffer containing 20% methanol. SDS-PAGE was carried out as described before and the gel was equilibrated for 15 min in cold blot buffer, before electro-transfer onto nitrocellulose [Schleicher and Schuell, Dassel, Germany]. Briefly, the nitrocellulose was cut to size and equilibrated with transfer buffer for 20 min. Electrotransfer was carried out on Biorad blotting apparatus [Biorad] between pre-rinsed graphite electrodes in a transfer sandwich containing from anode to cathode: 3 sheets of 3 mm filter paper [Whatman], pre-soaked in transfer buffer; One sheet of pre-wetted nitrocellulose; equilibrated gel; 3 sheets of filter paper pre-soaked in blot buffer. Care was taken to eliminate air bubbles between the individual layers by rolling with a pasteur pipette. The transfer was performed with a constant voltage of 100 v for 1 h during which the system was cooled with an ice block. After the transfer, the blotted

nitrocellulose was rinsed with distilled water and stained in Ponceau S stain [Sigma] for 2-5 min, to check that transfer had taken place. The major bands were marked with a pen and the Ponceau stain was removed through several changes of distilled water. The nitrocellulose was then washed x3 in Tris-buffered saline [TBS][10 mM Tris-HCl, 0.95% [w/v] NaCl, pH 7.4], after which time it was ready for immunoscreening.

2.5.3 Screening of Blotted Proteins with Specific Antisera or Monoclonal Antibodies

The nitrocellulose blot was incubated overnight at RT in TBS containing 50% horse serum to block sites that had not been bound by protein. After 3 washes with TBS containing 0.1% Tween [TBST] the appropriate antiserum or monoclonal antibody, diluted in PBS containing 3% foetal calf serum [FCS][Sigma], was added in a volume sufficient to cover the nitrocellulose. When serum was being tested for the presence of antibodies specific for the blotted proteins, the blot was cut into strips, and each strip probed individually with an individual serum sample. The blot was then incubated for 2 h at RT, after which time the blot was washed x5 with TBST to remove unbound antibody. The appropriate conjugate was added to the blot for 90 min. Anti- mouse IgG conjugates were either alkaline phosphatase [AP]-labelled or horseradish peroxidase [HRP]-labelled [both Sigma]. Anti-sheep IgG conjugates [both AP- and HRP-labelled] were produced by Mr. A. Dawson, MRI. Following a further 5 washes in TBST, the enzyme substrate was added. For the AP substrate, the buffer

was a solution containing 100 mM Tris [pH 9.5], 50 mM $MgCl_2$, 100 mM NaCl and 66 ul of nitroblue tetrazolium [Sigma]. After the solution had dispersed throughout the buffer, 33 ul of 5-bromo-4-chloro-3-indolyl phosphate [Sigma] was added, and the reaction was stopped by submerging the blot in PBS containing 50mM EDTA after the colour had developed. For the HRP substrate, 1 mg/ml of diaminobenzoate [Sigma] was added to 20 ml of 10mM Tris-HCl and a few drops of H_2O_2 added. The reaction was stopped by submerging the blot in distilled water.

2.6 Animal Experiments

2.6.1 Mice

All mice used throughout the course of the work were BALB/c [H-2^d] of either sex and aged 6-12 weeks, unless otherwise stated. Mice were housed in cages [max 5/ small cage, 20/ large cage] at 22^{o}C, with 10-12 h lighting and fed and watered *ad libitum*. Mice were anaesthetised using halothane [ICI Pharmaceuticals, Macclesfield, Cheshire, UK].

2.6.2 Lambs

Conventional Blackface Suffolk crosses, aged 6-9 months and of either sex, were used in experiments invoving the non-recombinant strains of *Salmonella* [Chapter 4]. Lambs were housed in an open pen and fed on hay and ruminant A nuts [Dalghetty, Edinburgh, UK].

Conventional 10 day-old Finn lambs were used in the experiment involving the pD and pC strains of Salmonella [Chapter 5], housed in negative-pressure maintenance isolators [Moredun Animal Health, Edinburgh] and fed 4 times daily with UHT milk.

Germ-free Finn lambs were used in the experiment involving the pAL12 strain of Salmonella [Chapter 6]. Pregnant ewes underwent hysterectomy, and the sealed uterus was passed through a germicidal trap into an isolator bubble. Using sterile techniques, the lambs were transferred into positive-pressure maintenance isolators, which were converted to negative pressure immediately prior to infection, 5 days post-partum. The lambs were fed 4 times daily with UHT milk.

Before use, rectal swabs were taken from all lambs and inoculated into 10 ml of selenite broth enrichment media [see Appendix 1] which was subsequently incubated at 37^{o}C with shaking for 16 h. A loopful of broth was streaked onto an XLD plate and any lambs which were found to be excreting salmonellas were discarded.

2.6.3 Inoculations

Bacterial strains were administered orally to mice using 1ml syringes [Becton Dickinson [BD], Dun Laoghaire, Co.Dublin, Ireland.] with 21 gauge [ga] needles [BD], that were sheared of the tips using wire cutters, filed and covered with medical

grade catheter tubing [Dural Plastics and Engineering, Dural, NSW, Australia.]. Oral inoculations did not exceed doses of 200 ul. Oral inoculation of lambs was performed using a 10 ml syringe [BD] to administer doses of up to 10 ml. Intravenous administration involved warming the mouse in a light box, or massaging the tail, to allow an increasd flow of blood to the tail, and injecting up to 50 ul into the tail vein using a 26 ga needle [BD]. Intraperitoneal inoculations were carried out using 26 ga needles to administer doses of up to 1 ml. Intradermal injections involved injection of 100 ul of the appropriate preparation into the neck [mice] or flank [sheep] skinfold with a 26 ga needle. Following all injections, the point of needle insertion was swabbed with 70% alcohol to clean the wound.

2.6.4 Blood Collection

Blood was collected from the tail vein of mice. The mice were either warmed in a light box or the tail was massaged, after which time the tail vein was nicked with a scalpel blade and the blood was collected into capillary tubes [Hawksley, Lancing, Sussex, UK] which were then sealed with Crystaseal wax [Hawksley]. The blood was spun for 5 min on a micro-haematocrit centrifuge [Hawksley] and the part of the tube containing the serum was removed using a diamond-tipped pen and the serum collected. Blood was also removed from mice following cutting of the hepatic vein of anaethetised animals, when it was not

necessary to keep the animals alive. Lambs were bled from the jugular vein into Vacutainer tubes [BD] using Vacutainer needles [BD]. The tubes were spun at 300g for 5 min and the serum collected. All serum was stored at -20°C.

2.6.5 Lavage Technique for the Collection of Murine sIgA

Mice were fed every 15 min for 1 h with 0.5 ml 48.5 mM polyethylene glycol [molecular weight 3350] [Sigma]. 20 min after the last feed, the mice were culled and the small intestines and caeca washed out twice with 5 ml of ice-cold 50 mM EDTA containing 0.1 mg/ml soyabean trypsin inhibitor [STI][Sigma] [EDTA/STI]. After bringing the samples up to 6 ml with ice-cold PBS, they were vortexed vigorously, transferred to 50 ml centrifuge tubes and spun at 2500g for 10 min on a Beckman J2-21 centrifuge, to remove large debris. 3 ml of the supernatant was then transferred to round-bottomed polycarbonate centrifuge tubes and spun at 69,000g at 4°C for 20 min on a Beckman R-7 ultracentrifuge. 30 ul of phenylmethyl-sulfonylfluoride [PMSF][Sigma] in 95% ethanol, and 30 ul of 2% sodium azide were then added to 2 ml samples of clarified supernatant and after incubation for 15 min on ice, 100 ul of FCS [Sigma] was added. The samples were then aliquoted in 1 ml volumes and stored at -70°C.

2.6.6 Isolation of Ovine Coproantibody

Faecal samples from lambs were collected into plastic tubs, weighed and an exact volume of ice-cold EDTA/STI solution added.

Samples were transferred to Stomacher "80" bags [Seward Medical, London, UK] and homogenised in a Stomacher "80" lab blender [Seeward]. Homogenates were then transferred to 50 ml centrifuge tubes which were spun at 25,000g for 10 min on a Beckman J2-21 centrifuge. The brown supernatant, termed faecal extract, was aliquoted in 1ml volumes and stored at -20^{o}C following addition of 1% sodium azide to a concentration of 0.01%.

2.6.7 Measurement of Specific DTH Responses

In mice, specific DTH responses were determined following injection of antigen into the hind footpads. Prior to testing, the thickness of both hind footpads in mm was measured using dial-gauge calipers [Kroeplin, Germany]. Anaesthetised mice were injected into the left hind footpad with 50 ul of a heat-killed preparation of S25/1*aro*A containing approximately 2 x 10^{8} CFUs/ml which had been killed by boiling for 20 min. The opposite hind footpad was injected with 50 ul of saline. 24 and 48 h following injection, the footpad thickness was measured as before and the increase in thickness of the footpads determined by subtracting the pre-injection value from the post-injection value. The antigen-specific increase in thickness was obtained by subtracting the increase in the opposite footpad which had been injected with saline.

In lambs, the same procedures were performed following the intradermal injection of heat-killed S25/l*aroA* or saline into the flank skinfold.

2.6.8 Production of Antibody to Specific Antigen in Mice by Administration with Complete Freunds Adjuvant [CFA]

Preparations of MOMP or leukotoxin were diluted 1:2 with sterile distilled water before being mixed in an equal volume of CFA [Sigma]. The mixture was vortexed until the suspension was fully emulsified, when a clear layer [representing the CFA] was no longer formed. The emulsion was injected either intraperitoneally, or into the neck skinfold of mice. 14 days later the same antigen preparation was emulsified in Incomplete Freunds Adjuvant [ICFA][Sigma] as before and injected in the same way. After a further 7-14 days, the mice were bled and the sera stored at -20^{o}C.

2.7 Pathogenesis Studies

2.7.1 Clinical Evaluation of Mice and Lambs After Infection

Following inoculation of both mice and lambs, the appetite, respiratory state and demeanour of the animals was closely observed. In lambs, the rectal temperatures were recorded daily and the no. CFU per gram [no. CFU/g] of faeces of the inoculated strain was determined for at least 7 days post-infection. The percentage dry weight [%DW] of the faeces was also determined by weighing faecal samples and cooking in a

microwave until all the water had evaporated. The weights of the dried samples were then expressed as a percentage of the original weight. The presence of haemoconcentration was investigated in lambs by determining the packed cell volume [PCV] of the blood. Blood was placed in a capillary tube and spun for 5 min on a micro-haematocrit centrifuge. The PCV of the blood was obtained using an Hawksley micro-haematocrit reader.

2.7.2 Enumeration of Bacterial Growth in Mice and Lambs

At intervals after inoculation with Salmonella, mice and lambs were culled and tissue and faecal samples taken. From mice, the liver, spleen, Peyer's patches and various regions of the gut were examined and samples of heart, lung, hepatic lymph node, spleen, kidney, mesenteric lymph node, abomasum, ileum, caecum and colon were taken from lambs. All samples were taken into Stomacher "80" bags which had been pre-weighed. Tissue samples from lambs were also fixed in formalin for future histopathological studies. Following weighing of the samples, 9 ml of PBS was added and the samples homogenised in a stomacher "80" lab blender, then poured into plastic universal containers [Bibby Sterilin Ltd, Stone, Staffs, UK]. 1 ml of homogenate was plated onto XLD medium and left to air dry. All plates were incubated for 18 h at 37^{o}C, before estimations of viable counts were made. Counts were expressed as the no. CFU\g of organ [or faeces].

If the organism could not be detected after direct plating onto XLD agar, 1 ml of organ homogenate was inoculated into 10 ml of selenite broth. Following overnight culture at 37°C with shaking, 100 ul of culture was spread onto XLD and the plate incubated overnight at 37°C.

In lambs, the organism was detected in blood either by direct plating of 1 ml of whole blood onto XLD agar, or by inoculation of 1 ml of whole blood into nutrient broth [see Appendix 1] or into selenite broth, followed by overnight culture and subsequent spreading onto XLD agar plates.

2.7.3 Estimation of the Viable Counts of Ampicillin-Resistant Colonies of S25/laroA in the Tissues and Faeces of Mice

In mice, the proportion of bacteria retaining the ampicillin-resistance plasmid was determined in each organ. XLD plates containing the colonies were dried for 15 min at 37°C and an exact impression of the colonies were made onto paper filters [Whatman International Ltd, Maidstone, Kent, UK]. A round plastic block was covered with 2 sheets of tissue paper on top of which was placed a filter, held firmly in place with a metal band-clamp. The surface of the dried XLD plate was placed carefully onto the filter and pressure applied. The XLD plate was removed and the impressions of the colonies could be seen on the filter. A dry LB agar plate containing ampicillin was placed on top of the filter, and pressure applied as before. Both the

XLD and LB-ampicillin plates were then incubated at 37°C for 16 h. After this time, the number of colonies on each plate type were counted and the percentage of colonies that were ampicillin-resistant was recorded.

2.8 Immunological Assays

2.8.1 Enzyme-Linked Immunosorbent Assays [ELISAs]

2.8.1.1 Preparation of Coating Antigens

The recombinant MOMP [rec MOMP] protein [kindly donated by Dr.A Herring, MRI] was produced in S25/1 and purified by gel filtration in a Bio-gel P-60 column [Biorad]. The protein fraction was resolubilised in 10mM $NaPO_4$, pH 6.8, containing 0.1% SDS and 1ug/ml used for coating. Two antigen preparations were used for the detection of anti-*Salmonella* antibodies. Initially a live bacterial suspension containing 3 x 10^8 CFU/ml of S25/1*aro*A was used to coat plates. Subsequently, plates were coated with 1 ug/ml of LPS, extracted from a culture of S25/1*aro*A [see below].

2.8.1.1a Extraction of LPS from a Culture of S25/1aroA

The LPS was extracted from the S25/1*aro*A salmonellae by the hot phenol water extraction method [Westphal and Jann, 1965]. A 1 litre overnight culture of S25/1*aro*A was spun down at 3300g for 45 min, washed twice in PBS, and lyophilised. The dried cells were resuspended in 50 ml of distilled water and then mixed with an equal volume of 90% [v/v] phenol at 68°C. The mixture was shaken vigorously for 10 min at 68°C, then cooled down quickly

on ice before centrifugation at 3300g to deposit the cells. The resulting upper aqueous layer was removed without disturbing the interface and an identical volume of distilled water was added to the remaining mixture before a second phenol extraction was carried out. Again the aqueous layer was removed and the 2 aqueous layers combined and dialysed against running water for 48 h. After centrifugation at 3300g for 30 min to remove any debris, the volume of the sample was then reduced by half on a rotary evaporator for 1-2 h. The concentrated sample was spun at 368,000g for 90 min on a Beckman R-7 ultracentrifuge. After 2 washes in PBS, the pellet containing LPS was resuspended in 5 ml PBS, freeze dried and was stored at 4°C.

2.8.1.2 Measurement of Antibody by ELISA

The full composition of buffers used in the following protocol are listed in Appendix 3. 100 ul of antigen in bicarbonate coating buffer, pH 9.6, was added to each of 96 wells of a flat-bottomed microtitre plate [type 129A, Dynatech Laboratories, Billinghurst, Sussex, UK] and incubated for a minimum period of 2 h at RT. The plate was then washed x4 with PBS containing 0.1% Tween 20 [PBST], with the final wash being left on for 1 h. The plate was then dried on tissue paper, after which 100 ul of test sample was added in duplicate to the wells and doubling dilutions were made down the columns of the plate with ELISA buffer, pH 7.2 [Shafren and Tannock, 1989]. Following a 1 h incubation at 37°C, the samples were removed and the plate washed x4 with PBST. Following drying of the

plate, 100 ul of conjugate, appropriately diluted in PBST containing 0.25% bovine serum albumin [BSA] was added to the wells and the plate incubated at RT for 90 min. AP-labelled conjugates, specific for murine Igs, were obtained from Sigma and diluted 1:1000. HRP-labelled conjugates, specific for ovine Igs, were produced at MRI by Mr. A. Dawson and diluted 1:8,000, 1:10,000 and 1:5,000 for IgM, IgG and IgA-specific conjugates respectively. The conjugate was removed and the plate washed x4 with PBST. Following drying, 100 ul of the appropriate substrate was added to the wells. Where the conjugate was AP-labelled, the substrate was 1ug/ml p-nitrophenyl phosphate [PNPP] [Sigma] in 10% diethanolamine buffer, pH 9.8, and the reaction was stopped after 30 min at 37^{o}C by the addition of 50 ul/well of 3M NaOH. In assays incorporating HRP-labelled conjugates, the substrate was 0.1mg/ml O-phenylenediamine [OPD] [Sigma], in citrate phosphate buffer, pH 5, to which was added 0.1 ul/ml of 30% [w/v] H_2O_2 [Sigma], and the reaction was stopped after 20 min at 37^{o}C with 50 ul/well of 2M H_2SO_4. Plates were read on a "Titertek Multiskan" multichannel spectrophotometer [Flow Laboratories, Irvine, Ayrshire, UK] at a wavelength of 405nm for assays where the substrate was PNPP, and at a wavelength of 492nm where the substrate was OPD. In murine assays, the OD of infected mouse serum was corrected by subtracting the OD of normal mouse serum [NMS] while in ovine assays, the OD of infected lamb serum was corrected by subtracting the OD of the pre-infection serum from the same lamb.

2.8.2 Tissue Culture Experiments

2.8.2.1 Media

A full list of the contents of media are given in Appendix 4.

2.8.2.2 Preparation of Murine Spleen Cells

Spleens were asceptically removed from mice and placed in sterile plastic universal containers containing 2 ml of supplemented Hank's balanced salt solution [sHBSS]. The spleens were placed in plastic Petri dishes and the cells flushed out by injecting 10 ml sHBSS. The cell suspension was spun twice at 300g and the pellet resuspended in sHBSS for counting. The cells were counted after the addition of 20 ul of cell suspension to 20 ul of 0.1% nigrosin in an improved Neubauer chamber and after one final wash, the cell concentration was adjusted to 2 x 10^6/ml in supplemented RPMI 1640 medium [sRPMI 1640].

2.8.2.3 Preparation of Ovine Peripheral Blood Mononuclear Cells [PBMC]

Blood was collected in 20 ml Vacutainers containing 200 ul of preservative-free heparin [1000u/ml]. After thorough mixing, the blood was transferred to plastic universal containers and spun at 450g for 30 min. The "buffy coat" containing the lymphocytes was then removed with a Pasteur pipette, layered on top of 5 ml of Lymphoprep [Nycomed Pharma As, Oslo, Norway] and centrifuged at 650g for 30-40 min. The cells at the interface were washed

by centrifugation x3 at 300g for 6 min in sHBSS, resuspended in supplemented Iscove's Modification of Dulbecco's Medium [sIMDM] and counted in an improved Neubauer chamber [see 2.8.2.2]. The cell concentration was adjusted to 2 x 10^6/ml in sIMDM.

2.8.2.4 Separation of B and T Lymphocytes using Nylon Wool

Scrubbed nylon fibre [3 denier, 3.81 cm [Fenwal Labs, Deerfield, IL, USA] was boiled in 6 changes of distilled water for 1 h for each change, rung out and left to dry. 600 mg of washed nylon wool was packed into a 10 ml syringe, and after adding 6 ml of PBS to wet the nylon, the plunger was replaced. The column was then stored until use. For purification of cells, the column was washed through with approximately 50 ml of sRPMI 1640 and, after the wool was completely soaked , the outlet catheter was closed using a crocodile clip and sRPMI 1640 was added until a liquid head of approximately 1 ml had formed. The top of the column was sealed with sterile tape and the column was incubated for 2 h at 37°C in 5% CO_2. After incubation of the column, a maximum of 10^7 spleen cells in 2 ml of sRPMI 1640 at 37°C was layered gently onto the column, followed by 1 ml of warm medium. The column was resealed and returned to the incubator in an upright position for a minimum of 45 min. "Enriched" T cells were then eluted with 20 ml of medium, spun down, counted and resuspended to a concentration of 2 x 10^6 cells/ml in sRPMI 1640. The column was then washed through with 50 ml of warm medium to remove the null cells. B cells were eluted by adding a further 10 ml warm medium and

rapidly inserting the plunger and compressing the wool as hard as possible. This was repeated twice and the "enriched" B cells collected, spun down, counted and resuspended to a concentration of 2 x 10^6 cells/ml in RPMI 1640 medium.

2.8.2.5 Lymphocyte Transformation Tests [LTTs]

Lymphocytes were stimulated *in vitro* with Concanavalin A [ConA] [Sigma], LPS [extracted from *S.minnesota*] [Sigma], dilutions of a washed culture of heat-killed S25/1*aroA* [containing 5 x 10^8 CFU/ml] or with different preparations of soluble antigen [MOMP and leukotoxin] which were g-irradiated prior to use. One preparation of MOMP was separated by SDS-PAGE and blotted onto nitrocellulose. The MOMP was then removed from the nitrocellulose for use in LTTs as described. The nitrocellulose strip containing the protein was first placed onto a piece of filter paper, covered in cellophane and 2 mm^2 pieces excised using a scalpel blade and a ruler which had been washed in 70% ethanol to precipitate contaminating proteins. The squares were incubated in 0.5 ml of dimethyl sulphoxide [DMSO] [Sigma] for 1 h at RT to dissolve the nitrocellulose. The nitrocellulose was resolubilised [to allow removal of the DMSO] by the addition of 1 ml of sterile 0.05M bicarbonate buffer, pH 9.6, added dropwise to the bijoux during vigorous mixing in a vortex mixer. After 1 h at RT, the DMSO was removed by washing x3 in HBSS on a microfuge at 5000g for 5 min. The nitrocellulose was resuspended in 1 ml of sRPMI 1640 medium and stored at -20^{o}C.

To perform the LTT, 100 ul of a cell suspension containing 2 x 10^6 cells/ml in sIMDM [see Appendix 4] was added to the wells of 96-well, flat-bottomed microtitre plates [Nunc, Paisley, Strathclyde, UK]. 100 ul of antigen or mitogen preparation was added to the cells in triplicate, giving a final volume of 200 ul/well. The plate was incubated for 4-6 days at 37°C in 5% CO_2 in air, after which time the cells were pulsed with 0.5 uCi/well tritiated thymidine [Amersham International plc, Amersham, Bucks, UK]. Following a further 18-h incubation, the cells were harvested using an automatic cell harvester [Flow] and the radioactivity incorporated into DNA was determined by counting on a liquid scintillation counter [Canberra Packard, Pangbourne, Berks, UK]. Triplicates where the standard errors were greater than 20% were discarded. The results for stimulated cells were expressed either as net counts per minute [CPM] or as a stimulation index [SI], calculated by subtracting or dividing the counts obtained using unstimulated cells respectively.

2.8.2.6 Cytokine Assays

Cell supernatants were removed for analysis of IFN-g or IL-2 activity after 1-4 days of culture with specific antigen as described above, and stored at -20°C.

2.8.2.6[a] IFN Assays

Bioassays were used to measure levels of IFN-g. Murine IFN assays were carried out using the murine L929 fibroblast cell line as target cells, while ovine IFN was measured using ST-6 cells, an ovine adenocarcinoma of the small intestine. Both cell types were maintained in sIMDM in 20 ml tissue culture flasks [Nunc] and passaged twice weekly. After removal of culture medium, the cells were washed twice with PBS that had been warmed to 37°C in a waterbath and 5 ml of trypsin [0.25%]/versene [0.02%] in IMDM was added for 4 min at 37°C in 5% CO_2. The released cells were washed x3 in sHBSS by centrifugation at 300g, counted and adjusted to the appropriate concentration in sIMDM [see 2.8.2.2]. 5 x 10^3 ST-6 cells, or 3 x 10^3 L929 cells in 100 ul of sIMDM were plated out in flat-bottomed, 96-well microtitre plates [Costar[UK]Ltd, High Wycombe, Bucks, UK] and after incubation for 24 h [L929] or 72 h [ST-6] at 37°C in 5% CO_2 in a humidified incubator, the medium was removed and 100 ul of test supernatant was added in duplicate to the cells. In some cases, the test samples were serially diluted in serum-free sIMDM to titrate any IFN that was present. Following overnight incubation, the medium was removed from the plate and replaced with 200 ul of sIMDM/2% FCS containing 100 50% tissue culture infective doses [$TCID_{50}$] of Semliki Forest Virus [SFV] [supplied by Dr. A.G. Morris, Warwick, UK], which had been titrated previously on foetal lamb kidney cells and the concentration calculated by the Spearman-Karber method [Grist et al., 1966]. The plate was

returned to the incubator for 48 h when the amount of cytopathic effect [CPE], evident by the destruction of the monolayer as a result of the rounding of the cells, was evaluated visually using an inverted microscope. The titre of IFN-g was given as the inverse of the highest dilution which reduced CPE by 50%. In ovine assays, various dilutions of a standard preparation of recombinant ovine IFN-g [supplied by Dr. M. Scott, Wellcome, Beckenham, Kent, UK] and protective to ST-6 at 1 u/ml] were used as a positive control. Recombinant murine IFN-g, produced in the supernatant of transfected CHO cells [supplied by Dr. A.G. Morris], served the same purpose in murine assays.

To determine that the IFN in the supernatants was IFN-g, prior to the assay, the samples [including the positive controls] were incubated for 2-3 h at 37^{o}C in 5% CO_2 with neutralising monoclonal anti-IFN-g antibodies. For the murine assay, an IgG rat anti-mouse IFN-g monoclonal antibody [R46A2, [Spitalny and Havell, 1984], supplied by Dr A. McI. Mowat, Western Infirmary, Glasgow, UK] was used, while a mixture of 2 murine monoclonal antibodies [IFN9 and IFN2 [Wood et al, 1990] supplied by Dr. P.R. Wood, Parkville, Australia] originally raised against bovine IFN-g, but which neutralise ovine IFN-g [Entrican et al. 1992], was used in ovine assays. A control monoclonal antibody, specific for Border Disease Virus [BDV] and with no specificity for IFN-g, was added to the samples and

positive controls in parallel with the IFN-g specific monoclonal antibodies, and the differences between the titres of the respective samples with or without the anti-IFN-g monoclonal antibodies was interpreted as being due to IFN-g.

2.8.2.6[b] Measurement of IL-2 Activity

This assay was based on the ability of IL-2 [both murine and ovine] to cause the proliferation of the mouse cytotoxic T cell line, CTLL, which is dependent on IL-2 and IL-4 for growth and survival in culture. CTLL cells [obtained from Dr Tony Lammas, University of Birmingham, UK] were maintained in sIMDM in 24-well tissue culture plates [Nunc] in 5% CO_2 at 37^{o}C and fed every 3 days with 20 u/ml recombinant IL-2 [Sera-Lab Ltd, Crawley Down, Sussex, UK]. The cells were harvested 3 days following feeding with IL-2, washed twice in sHBSS by centrifugation at 300g and resuspended at 1 X 10^5/ml in sIMDM. 100 ul of cell suspension was added to individual wells of a 96-well microtitre plate [Nunc, Gibco] and 100 ul of test lymphocyte supernatant [from both and murine and ovine cells] added in triplicate. The plates were incubated for 1-2 days at 37^{o}C in 5% CO_2, after which 0.5 uCi of tritiated thymidine was added to each well and the plates returned to the incubator for 18 h and incorporation into DNA assayed as described previously [see 2.8.2.5].

As CTLL cells may also be dependent on IL-4, the presence of IL-2 in the supernatants was confirmed by incubation with an anti-mouse IL-2 monoclonal antibody [obtained from Dr. R.

Bujdoso, Royal Veterinary College, Edinburgh, UK] for 1-3 h before performing the assay. A control monoclonal antibody, specific for BDV, was added as a control and the differences between the titres of the respective samples with or without the anti-IL-2 monoclonal antibody was interpreted as being a result of the presence of IL-2.

2.8.2.7 Analysis of Cell Surface Phenotype by Flow Cytometry

Flourescence-Activated Cell Sorting [FACS] analysis was used to determine which subpopulation of ovine T cells was responding to specific antigen in an LTT. 1 ml of ovine PBMC, at a concentration of 2 x 10^6/ml in sIMDM, were cultured in 24-well tissue culture plates [Nunc] with 1 ml of a heat-killed S.typhimurium suspension containing 2 x 10^8 CFU /ml [240 ug/ml protein]. After 5-7 days in culture at 37^{o}C in 5% CO_2, the cells were washed and counted. 1 ml aliquots, containing 1 x 10^6/ml cells in supplemented Earle's balanced salt solution [sEBSS] were dispensed into polypropylene centrifuge tubes [Labsystems [UK] Ltd., Basingstoke, Hants, UK], pelleted by centrifugation and 100 ul of the appropriate monoclonal antibody added. The monoclonal antibodies used were mouse anti-sheep CD4, CD8, T cell receptor gamma-delta [TcR g-d] [all kindly gifted by Dr. C. McKay, Basel Institute for Immunology, Switzerland], Ig light chain [LC] and anti-BDV as a negative control [both kindly provided by D. Deane, MRI]. After 1 h on ice, the cells were washed twice in sEBSS at 300g, resuspended in 50 ul of a 1:25 dilution of fluorescein-labelled rabbit

anti-mouse IgG conjugate [Dako Ltd., High Wycombe, Bucks, UK], and incubated on ice in the dark for 1 h. Following a further 2 washes, 30 ul of propidium iodide was added to each tube for 2-5 min to allow identification and gating out of dead cells during analysis. After 2 washes in sEBSS, the cells were fixed in 400 ul of 1% paraformaldehyde in sEBSS and stored at $4^{o}C$ in the dark until ready to be read on a FACSAN Flowcytometer [BD]. Cytometric analysis was kindly performed by D. Deane, MRI, using Lysis 1 software [BD].

2.9 Statistical Analysis

All analysis was done using the "Minitab" statistical computer-programme package [Minitab, State College, Pa, USA]. Significance testing between grouped data was done using the two-sample Student's t-test, unless otherwise stated.

Chapter 3

The Immune Response in Mice following Oral Vaccination with S25/1aroA

3.1 Introduction

The aim of this project was to test the safety and efficacy of an aromatic-dependent mutant of S.typhimurium in sheep. However before doing this, it was necessary to test out the vaccine strain in inbred rodents. Murine infection with the natural mouse pathogen S.typhimurium is the most widely accepted model for studying immunity to typhoid fever and has been used to test both the relative efficacy of live attenuated and killed Salmonella vaccines.

In this chapter, I investigated the ability of a live aroA vaccine strain, constructed from an ovine isolate of S.typhimurium [S25\1], to confer immunity from salmonellosis in BALB\C mice. The relative virulence and invasiveness of the aroA strain was determined by comparison with the wildtype strain. The persistence of the vaccine strain in the tissues was determined following inoculation of high and low doses, and was correlated with both the vaccine-specific primary humoral and cell-mediated immune responses engendered, and to the levels of protection observed following wildtype challenge.

3.2 Results

3.2.1 Virulence of the Wildtype and aroA Strains of S25/1 for Mice

Forty mice were randomly allocated into 4 groups of 10 mice. Group A was inoculated orally with 2 x 10^7 CFU of the wildtype S25\1 strain, while Group B received an identical dose of the S25/1aroA strain. Groups C and D received oral doses of 2 x 10^2 CFU of the wildtype and S25/1 aroA strains respectively. The mortality rate of each of the 4 groups is recorded in Table 3.1. Mice infected with the higher dose of the wildtype strain became ill after 3-5 days, developing a scruffy appearance, diarhoea, and loss of appetite, and all died within 8 days. 5 mice receiving the lower dose of the wildtype strain died within 8 days of challenge. Thus as few as 200 wildtype S25/1 organisms are capable of establishing a lethal infection in mice. However, none of the mice receiving either the high or low doses of the S25/1aroA strain died or developed any clinical symptoms. These results confirm the results of preliminary studies with the wildtype and aroA strains of S25/1 which showed a difference of 7 log values between the Lethal Dose 50s [LD_{50}s] of the 2 strains [J.J. Oliver, unpublished observations].

3.2.2 Comparison of the Invasive Capacities of the Wildtype and aroA Strains of S25\1 in Mice

I next assessed the invasive capacities of the aroA and wildtype strains. 50 mice were allocated into 2 groups of which one was inoculated orally with 5 x 10^7 CFU of the wildtype strain and the other with an identical dose of the aroA strain. 5 mice from each

TABLE 3.1

Mortalities following Oral Inoculation with the Wildtype and aroA Strains of S25/1

	DAYS POST-CHALLENGE							
	1	2	3	4	5	6	7	8
GROUP A	0	0	0	2	1	1	3	3
GROUP B	0	0	0	0	0	0	0	0
GROUP C	0	0	0	0	0	1	3	1
GROUP D	0	0	0	0	0	0	0	0

GROUP A received 10^7 CFU wildtype S25/1

GROUP B received 10^7 CFU S25/1*aroA*

GROUP C received 10^2 CFU wildtype S25/1

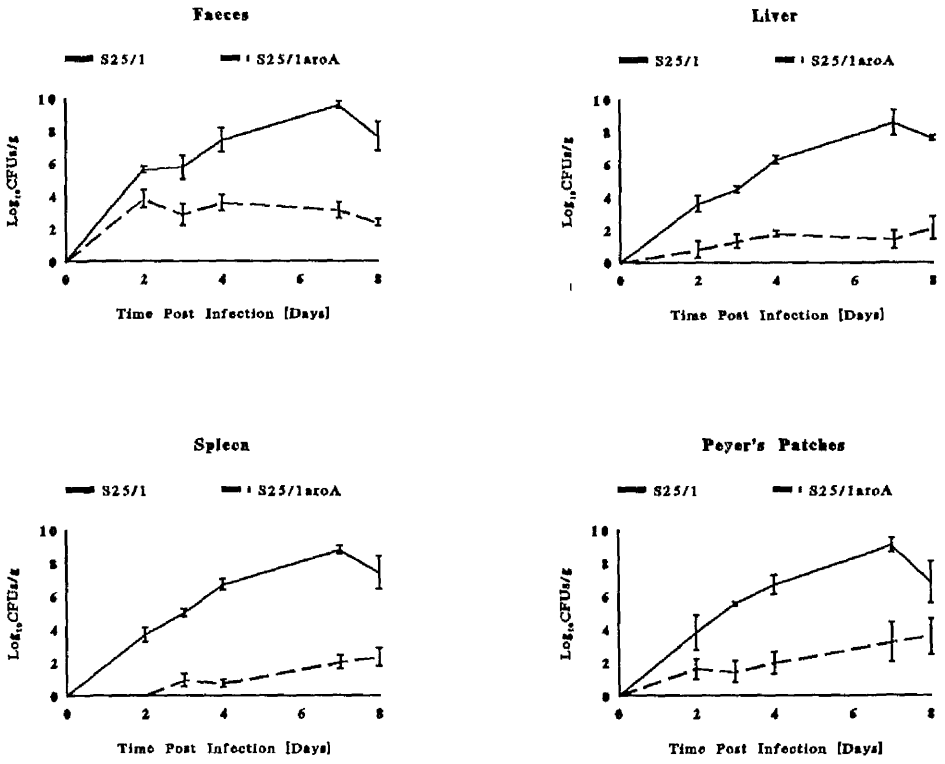
GROUP D received 10^2 CFU S25/1*aroA*

group were culled on each of days 2, 3, 4, 6 and 8 post-infection. The organs were removed and the no. of CFU\g was calculated. The results are displayed in Figure 3.1. As before, all mice given virulent organisms became ill after day 4. In parallel, there was progressive splenic enlargement and by day 8, the livers and spleens became yellow in appearance and developed large necrotic lesions. Congealed blood could be seen throughout the length of the gut which was blackened as a result of necrosis and/or ischaemia.

By day 2, the spleens, Peyer's patches and livers were already harbouring large numbers of the organism and high numbers were being excreted in the faeces. The levels in the tissues and faeces continued to rise until the experiment was terminated on day 8, consistent with the development of severe enteritis and systemic typhoid.

In contrast, the mice given the aroA strain showed no clinical symtoms at any time and the spleens and guts looked normal. In parallel, only small numbers of the organism could be recovered from any tissues, and although substantial levels were detectable in the faeces 24-48 h post-infection, the levels fell rapidly thereafter. Thus the S25/1aroA strain has a much reduced capacity to invade and colonise murine tissues.

Figure 3.1 Comparison of the Invasive Capacities of the Wildtype and aroA Strains of S25/1 in Mice After Oral Infection



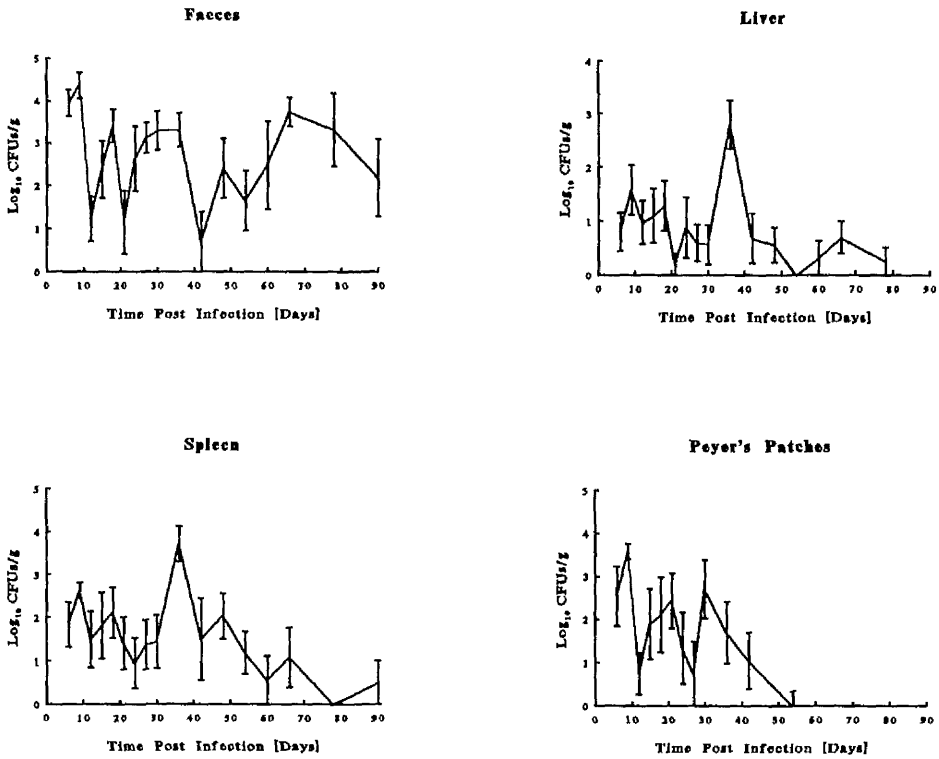
Mice were inoculated orally with 5 x 10^7 CFU of either the wildtype or the aroA strain. 5 mice from each group were culled on the stated days post-infection and the mean no. CFU/g for each group determined on each time-point for faeces, liver, spleen and Peyer's patches. Levels are expressed as $\log_{10}$ CFU/g.

3.2.3 Persistence of S25/1aroA in the Organs of Mice After Oral Inoculation with High and Low Doses

Having demonstrated the reduced invasive capacity of the aroA strain compared with wildtype, it was sought to determine the length of time that the aroA strain could persist in the murine tissues following oral inoculation of both high and low doses. Mice were infected orally with either 5×10^7 CFU or 5×10^2 CFU of S25/1aroA and 5 mice from each group culled at regular intervals until day 42 for the low dose and day 90 for the high dose. Organs were removed and the no. CFU\g of tissue determined. In the mice given the lower dose, the organism was absent from the liver, spleen and Peyer's patches of all the mice at all of the times tested. Low levels [10-100 CFU /g] were detected in the faeces of 5/65 mice tested. It would appear that a dose of 5×10^2 CFU of the S25/1aroA strain was insufficient to effect invasion of the Peyer's patches and subsequent colonisation of the internal organs.

The levels recovered from mice given 5×10^7 CFU S25/1aroA are shown in Figure 3.2. The number of organisms recovered from faeces was maximum 6-9 days post-infection. The levels were high in all of the mice at this time. From day 12 there was a considerable drop in the levels, which were often vastly different in individual mice. High numbers could still be isolated from some mice on termination of the experiment, 90 days post-infection.

Figure 3.2 Persistence of S25/laroA in the Faeces and Tissues of Mice After Oral Infection



Mice were inoculated orally with 5 x 10^7 CFU of the S25/laroA strain. 5 mice were culled on the stated days post-infection and the mean no. CFU/g determined on each time-point for faeces, liver, spleen and Peyer's patches. Levels are expressed as $\log_{10}$ CFU/g.

The levels of S25\1aroA in the livers of the mice was variable. Levels were detected on day 6 and rose steadily, peaking on day 18 after which time there was a gradual decrease in numbers. However, the organism could still be recovered from some mice on day 78 post-infection. The levels of bacteria recovered from the spleens were very similar to those found in the liver. Levels could be detected 6 days post-infection, peaking on day 9 after which there was a gradual decrease. Again, bacteria could still be recovered from some mice up to 66 days post-infection. On days 78, 84 and 90, all the spleens tested were free from bacteria. The organism was recovered from the Peyer's patches on day 6. The highest levels were seen on day 9, after which the levels dropped, although substantial numbers were seen until day 42. By day 60, the organism could no longer be isolated.

This unusual persistence of the organism in the faeces could be a result of the mice continually re-infecting themselves by coprophagy, rather than as a consequence of the initial infection. To test this, in a separate experiment, 6 mice were inoculated orally with 7 X 10^7 CFU S25/1aroA. The mice were housed individually in cages in which the bottoms had been removed and replaced with stainless steel grills. The grills allowed the faeces to fall through the bottom, where it was collected onto plastic trays and examined for the presence of S25/1aroA over a period of 70 days. The no. CFU\g of faeces was calculated on a

TABLE 3.2

Recovery of Salmonellae from the Faeces of Mice Infected Orally with 10^7 CFU of S25/laroA and Housed Separately in Grill-Bottomed Cages

No. CFU/g of Faeces

	DAYS POST-INFECTION							
MOUSE	3	7	15	24	34	41	48	70
1	NT	$1x10^4$	$2x10^3$	$4x10^4$	$4x10^3$	$3x10^3$	200	300
2	$1x10^4$	$1x10^4$	$6x10^4$	$1x10^4$	$8x10^3$	$5x10^3$	$4x10^3$	$1x10^3$
3	NT	$1x10^5$	$8x10^4$	$2x10^4$	$2x10^4$	NT	$3x10^3$	900
4	$2x10^3$	$5x10^4$	$4x10^4$	NT	NT	NT	$4x10^3$	$2x10^3$
5	$1x10^4$	$7x10^3$	$4x10^4$	$2x10^4$	NT	NT	$2x10^3$	0
6	NT	$1x10^5$	$7x10^4$	0	$6x10^4$	0	$4x10^3$	200

NT= Not Tested

0 = Not detected following direct plating onto XLD medium.

6 mice, housed in grill-bottomed cages, received 7×10^7 CFU of S25/1aroA and the no. CFU/g of faeces determined at times post-infection.

number of time-points for each mouse. It was found that the organism could still be recovered from the faeces of all mice 48 days post-infection, and from 5 of the mice on day 70 [Table 3.2].

3.2.4 Primary Immune Responses in Mice After Oral Inoculation with High and Low Doses of S25/laroA

I next examined the correlation between the levels of colonisation by the aroA vaccine strain described above, with the generation of specific primary immune responses. The levels of specific serum and mucosal antibody, as well as the specific T cell responsiveness, were examined in the mice receiving either 5×10^7 CFU or 5×10^2 CFU of the S25/laroA.

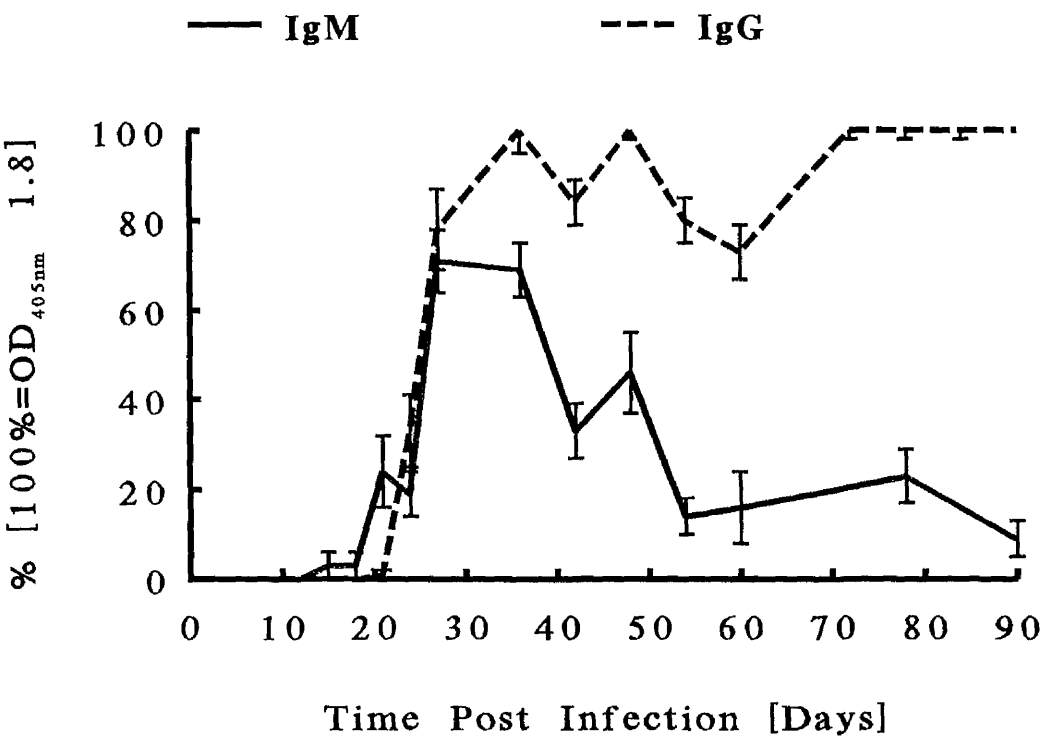
3.2.4.1 Specific Antibody Responses in Mice Infected with 5×10^7 CFU S25/laroA

ELISAs were developed to detect serum IgM, IgG and IgA antibodies and mucosal sIgA antibodies against S25/laroA LPS. The changes in the levels of IgM and IgG with time post-infection are shown in Figure 3.3. Serum IgA levels are shown in Figure 3.5, together with the sIgA levels.

Serum IgM

In most animals, serum IgM antibodies began to appear 18-21 days post-infection, although levels were detected as early as day 15 in

Figure 3.3 Salmonella-Specific Serum Antibody Responses in Mice Inoculated Orally with 10^7 CFU of S25/1 aroA



Mice were orally inoculated with 5 x 10^7 CFU of the S25/1aroA strain and the sera tested in an ELISA for both Salmonella-specific IgM and IgG at times post-infection. Each point represents the mean OD_{405nm} of 5 mice at a 1:200 serum dilution. Mean OD_{405nm} values are expressed as a percentage where 100% represents OD_{405nm} 1.8

some mice. Serum IgM rose to very high levels by days 21-28 in all mice, remaining high until day 37 when levels declined considerably. However, some IgM could still be detected in all the mice tested on day 90.

Serum IgG

Levels of IgG generally appeared slightly later [1-5 days] than those of IgM in the same mice. From day 21, the levels continued to rise steadily in all mice, reaching a plateau from day 28-35. There was very little variation in the levels of IgG produced by the mice with all mice producing high levels. At the end of the experiment, 6 mice were retained to determine how long the titres of serum IgG remained at this high level plateau. At 250 days post-infection, it was found that 3\6 of the mice retained these high IgG levels, 2\6 of the mice had reduced levels [OD_{405nm} 1.3-1.4] and 1 mouse had no detectable IgG at this time.

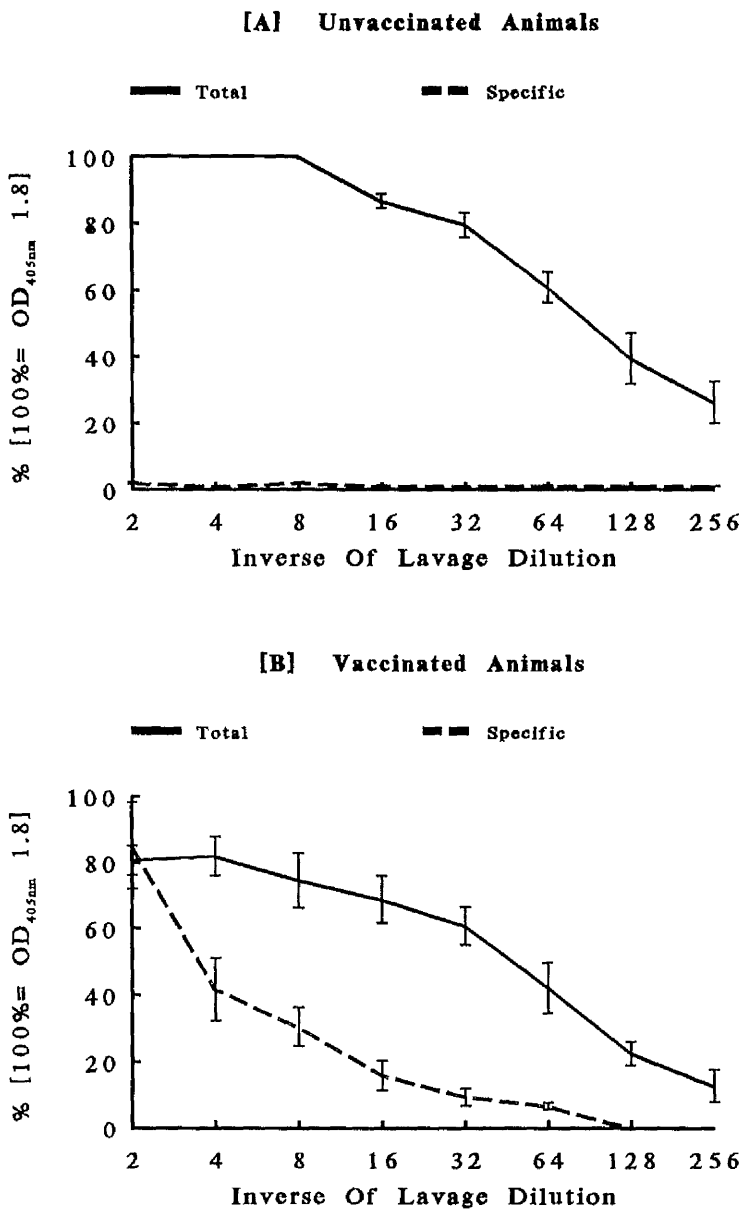
Serum IgA

Serum IgA levels appeared slightly later than IgG levels and continued to rise steadily in all mice until day 36, after which time there was a gradual decrease in the levels, with only low levels present on termination of the experiment on day 90.

Mucosal IgA

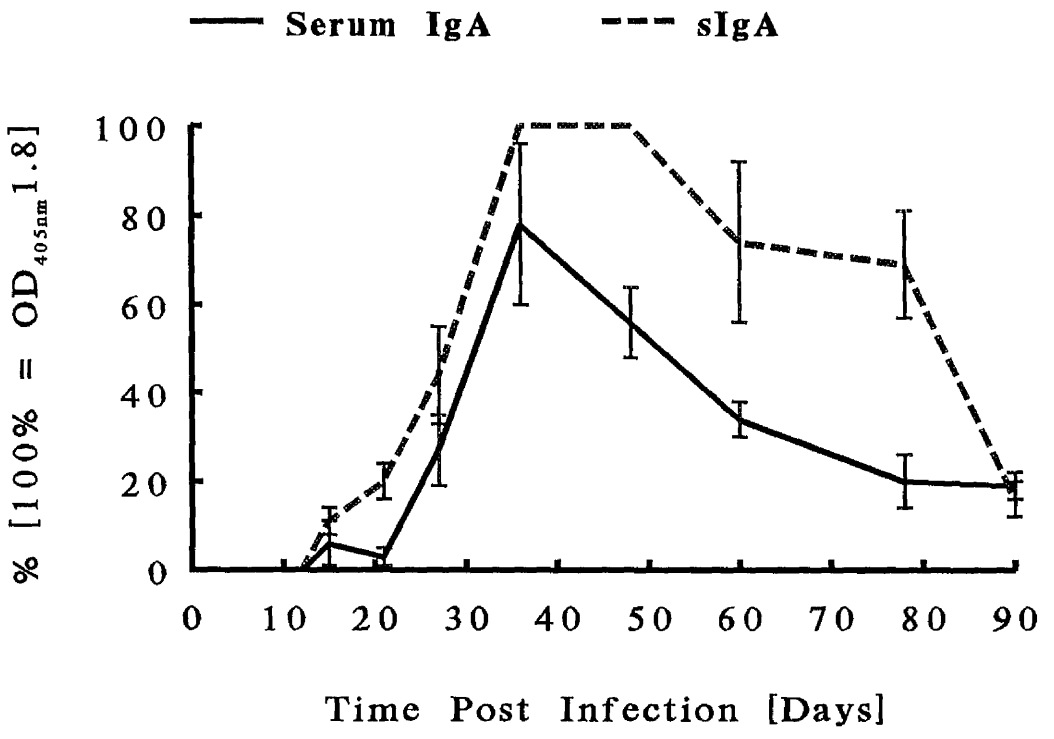
Gut lavages were taken from both unchallenged mice and from mice vaccinated 28 days previously. The levels of total and sIgA in the gut are shown in Figure 3.4. The lavage samples of both vaccinated

Figure 3.4 Comparison of the Levels of Salmonella-Specific and Total sIgA in the Intestines of aroA-Vaccinated and Unvaccinated Mice



The levels of total and Salmonella-specific intestinal IgA were examined in [A] unvaccinated mice, and [B] mice that had been vaccinated previously with 5×10^7 CFU S25/laroA. Each graph shows the mean OD of doubling dilutions of lavage samples from 5 mice. OD values are expressed as a percentage where 100% represents OD_{405nm} 1.8.

Figure 3.5 Salmonella-Specific Intestinal and Serum IgA in Mice Inoculated Orally with 10^7 CFU of S25/1aroA



Mice were inoculated orally with 5 x 10^7 CFU of the S25/1aroA strain and the sera and gut washes tested in an ELISA for Salmonella-specific IgA at times post-infection. Each point represents the mean OD_{405nm} of 5 mice at a 1:200 serum dilution or 1:4 lavage dilution.

TABLE 3.3

Confirmation of the Specificity of Anti-Salmonella IgM Antibodies Detected by ELISA

Post-Vaccination Serum

coating antigen	S25/1aroA LPS				E.coli LPS			
OD_{405nm}	1.21	1.25	1.30	1.20	0.18	0.18	0.17	0.18

Normal Mouse Serum [NMS]

coating antigen	S25/1aroA LPS				E.coli LPS			
OD_{405nm}	0.20	0.19	0.18	0.20	0.20	0.19	0.16	0.19

Sera from 4 uninfected mice [NMS] and 4 mice infected orally with 5×10^7 CFU S25/1aroA 30 days previously [post-vaccination serum] were diluted 1:100 and tested for specificity against both Salmonella and E.coli LPS in an ELISA.

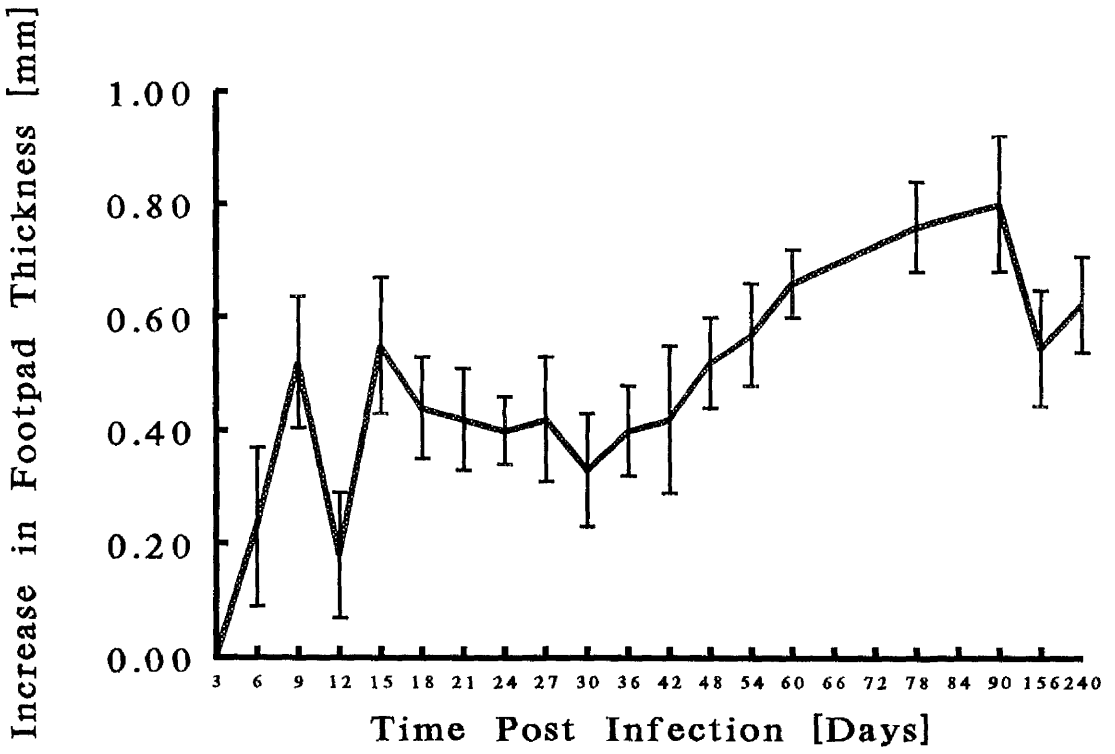
and unvaccinated mice contained high levels of non-specific sIgA as expected. However only the vaccinated mice contained Salmonella-specific sIgA. The time course of the sIgA response showed that the levels of specific sIgA were low until 3 weeks post infection when they started to rise rapidly [Figure 3.5]. At day 34, the levels reached a plateau, falling around 48 days post-infection. Interestingly the kinetics of the sIgA response mirrored that of the serum IgA with sIgA levels appearing slightly earlier. Intestinal sIgA and serum IgA levels also correlated well in individual animals. Both sIgA and serum IgA could still be detected on day 90, albeit at low levels, suggesting that the S25/1aroA vaccine strain was still stimulating the gut mucosa at this late stage.

The specificity of the antibody responses to the immunising organism was determined by testing sera from 4 unvaccinated an 4 vaccinated mice [removed on day 28] on plates coated with either Salmonella LPS or E.coli LPS [Sigma]. The responses are shown in Table 3.3. None of the sera from infected mice had IgM antibodies against the LPS derived from E.coli [ODs were similar to those obtained with NMS] confirming the specificities of the response to the immunising organism. Identical results were obtained for IgG and IgA [results not shown].

3.2.4.2 Salmonella-Specific DTH Responses in Mice following Oral Inoculation with 5×10^7 CFU S25/1aroA

The specific DTH responses of the mice to S.typhimurium were

Figure 3.6 Salmonella-Specific DTH Responses in Mice Inoculated Orally with 10^7 CFU of S25/1aroA



Mice were inoculated orally with 5 x 10^7 CFU of the S25/1aroA strain and the Salmonella-specific increases in footpad thickness determined at the stated times post-infection. Each point represents the mean increase in mm of 5 mice. 3 unvaccinated mice produced increases of 0, 0.04 and 0.01mm, highlighting the specificity of the test.

measured as an indication of the T cell responsiveness of the mice to the vaccine strain. The increases in thickness of the hind footpads of the vaccinated mice following injection of heat-killed S25/1aroA are shown in Figure 3.6. Large specific increases in skin thickness were detected as early as 9 days after infection and remained constant until 6 weeks post-vaccination, and then increased until the termination of the experiment on day 90. All 6 mice maintained after this time still had large responses on day 191 [mean 0.52mm+/-0.07mm].

3.2.4.3 Evaluation of the Specific Cellular Responses in Mice After Oral Inoculation with 5 x 10^7 CFU of S25/1aroA Using the LTT

Further studies were undertaken to characterise the T cell responses elicited by the vaccine strain. Mice inoculated orally with 5 x 10^7 CFU of the S25/1aroA strain were culled on day 30 post-vaccination, and the spleen cells removed and cultured for 5 days in vitro with heat-killed S25/1aroA in an LTT. The proliferative responses of vaccinated and unvaccinated mice are shown in Tables 3.4A and 3.4B respectively. All vaccinated mice produced high proliferative responses to specific antigen that were significantly higher [$p<0.05$] than those of the unvaccinated mice. However, 4 unvaccinated mice had proliferative responses similar to those found in a number of the vaccinated mice. One possible explanation for these results could be that the conventional uninfected mice used in these experiments had intestinal flora which cross-reacted with S.typhimurium.

TABLE 3.4A

Proliferative Responses of Spleen Cells from S25/laroA-Vaccinated Mice following Stimulation with Salmonella Antigen in an LTT

MOUSE NO.	1	2	3	4	5	6
MAX. CPM	56,773	51,178	64,438	25,471	53,920	23,472
MAX. SI	269	913	209	454	804	326
MOUSE NO.	7	8	9	10	11	12
MAX. CPM	27,684	29,400	43,936	33,900	31,188	33,031
MAX. SI	124	544	708	305	234	206

12 mice were inoculated orally with 5 x 10^7 CFU of S25/laroA and 30 days later, spleen cells were cultured with Salmonella antigen in an LTT and the proliferative responses recorded.

TABLE 3.4B

Proliferative Responses of Spleen Cells from Unvaccinated Mice following Stimulation with Salmonella Antigen in an LTT

MOUSE NO.	1	2	3	4	5	6
MAX. CPM	2,751	658	22,140	32,662	3,340	25,026
MAX. SI	41	6	220	355	20	116

MOUSE NO.	7	8
MAX. CPM	18,107	5,368
MAX. SI	177	41

Spleen cells from 8 unvaccinated mice were cultured with Salmonella antigen in an LTT and the proliferative responses recorded.

This hypothesis was tested by assaying the responses of spleen cells from unvaccinated germ-free mice. The results are shown in Table 3.5. The responses were not significantly different from those of the unvaccinated conventional mice. Again, 3 of the mice produced responses equal to those obtained with vaccinated mice [see Table 3.3] while the other 3 produced the expected lower levels. Thus, reduction of the intestinal flora of normal mice had no effect on their ability to respond to stimulation with the Salmonella antigen.

An alternative explanation for the high proliferative responses in unvaccinated mice was that the high levels of LPS in the antigen preparation caused polyclonal stimulation of the B cells in certain spleen cell cultures. To investigate this possibility, spleen cells from 5 uninfected mice were pooled and passed over nylon wool to separate the individual lymphocyte populations. As shown in Table 3.6, intact spleen cells responded to both ConA and LPS, while the enriched B cell population responded well to LPS and poorly to ConA and the reverse was true for the enriched T cell population. The intact spleen cells also responded strongly to stimulation with the S25/1aroA preparation as did the purified B cell population. However, the enriched T cell population also responded to the antigen, although this was significantly less [$p<0.01$] than the responses of intact spleen cells. Thus the antigen preparation appeared to stimulate both B and T cells in the spleen cell population from uninfected mice.

TABLE 3.5

Proliferative Responses of Spleen Cells from Unvaccinated Mice Placed in an Isolator following Weaning

	MOUSE NUMBER					
	1	2	3	4	5	6
MAX. CPM	3,984	5,936	39,358	10,290	31,162	12,097
MAX. SI	92	121	634	228	426	104

Spleen cells were cultured with *Salmonella* antigen in an LTT and the proliferative responses recorded.

TABLE 3.6

Proliferative Responses of Enriched Lymphocyte Populations from Unvaccinated Mice following Stimulation with Salmonella Antigen in an LTT

	MAX. CPM		
	Unseparated	B Cells	T Cells
ConA	42,243 +/- 2,231	7,261 +/- 897	76,735 +/- 1354
LPS	32,126 +/- 2,326	48,693 +/- 1745	4,221 +/- 532
S25/1 aroA	38,563 +/- 1,874	31,035 +/- 2,564	21,392 +/- 2,132

Whole spleen cells, as well as both nylon-wool-enriched B and T cell populations from uninfected mice were cultured with ConA, LPS and Salmonella antigen in an LTT, and the proliferative responses recorded. Each value represents the mean of 3 mean triplicate CPM values +/- 1 standard error.

The results of LTTs were inconclusive in illustrating Salmonella-specific T cell responsiveness in vitro in vaccinated animals and contrasted with the specific DTH responses in vivo. To study the presence of Salmonella-specific T cells further, I examined for the presence of IFN-g and IL-2 in supernatants of spleen cells stimulated with ConA or antigen.

3.2.4.4 IFN-g

Lymphocyte supernatants were tested for the presence of IFN by examining their ability to confer an antiviral state on a murine fibroblast cell line, L929. Tables 3.7A and 3.7B show the IFN-g titres of supernatants from 12 vaccinated and 12 unvaccinated mice respectively. The vaccinated mice were the same mice shown in Table 3.4A, culled 30 days post-vaccination. Of the unvaccinated mice, mice 1-6 are the same as mice 1-6 in Table 3.4B and mice 7-12 are those mice shown in Table 3.5. Supernatants of unstimulated cells from each mouse were used as negative controls. Unstimulated cells from either vaccinated or unvaccinated mice did not produce IFN of any kind, at any time, while ConA-stimulated cells from both groups all produced low amounts of IFN-g. Cells from all 12 vaccinated mice produced high levels of IFN-g following stimulation with all 4 antigen dilutions, while only one of the 6 conventional unvaccinated mice [mice 1-6] produced IFN-g, at very low levels and only at the higher antigen dilutions. Interestingly, 3 of these mice had also responded in the LTT. Of the 6 unvaccinated mice that were placed in an isolator [mice 6-12], again only 1 of 6

TABLE 3.7A

Production of IFN-g by Spleen Cells from S25/laroA-Vaccinated Mice following Stimulation with Salmonella Antigen

IFN-g TITRES

	MOUSE NUMBER											
	1	2	3	4	5	6	7	8	9	10	11	12
Unstimulated	0	0	0	0	0	0	0	0	0	0	0	0
ConA	4	4	2	<2	4	4	2	2	4	2	2	<2
S25/1aroA [60ug]	>8	>8	>8	>8	>8	2	0	2-4	4	>4	>4	4
S25/1aroA [24ug]	4-8	2	4	2	2	4	0	>8	>8	>4	>4	>4
S25/1aroA [12ug]	8	8	>8	2	2	8	4	8	2	>4	>4	>4
S25/1aroA [6ug]	4	4	4	<2	2	4	2-4	4	2	>4	>4	>4

Spleen cells from 12 mice, vaccinated 30 days previously with 5×10^7 CFU S25/1aroA, were cultured without antigen, with ConA and with decreasing dilutions of Salmonella antigen for 4 days when the titres of IFN-g in the supernatants were determined.

TABLE 3.7B

Production of IFN-g by Spleen Cells from Unvaccinated Mice following Stimulation with Salmonella Antigen

IFN-g TITRES

	MOUSE NUMBER											
	1	2	3	4	5	6	7	8	9	10	11	12
Unstimulated	0	0	0	0	0	0	0	0	0	0	0	0
ConA	2	4	8	2	4	4	2	4	<2	<2	2	2
S25/1aroA [60ug]	0	0	<2	0	0	0	0	0	<2	0	0	0
S25/1aroA [24ug]	0	0	<2	0	0	0	0	0	<2	0	0	0
S25/1aroA [12ug]	0	0	<2	0	0	0	0	0	<2	0	0	0
S25/1aroA [6ug]	0	0	0	0	0	0	0	0	<2	0	0	0

Spleen cells from 12 unvaccinated mice were cultured without antigen, with ConA and with decreasing dilutions of Salmonella antigen for 4 days when the titres of IFN-g in the cell supernatants were determined.

mice produced IFN-g, although 3 responded strongly in an LTT. Thus the production of IFN-g did not correlate well with the proliferative responses of lymphocytes from unvaccinated mice and appears to be a better indication of specific T cell responsiveness after infection. In later experiments [results not shown], it was shown that mice examined 80 days post-vaccination with 5×10^7 S25/1aroA produced similar levels of IFN-g to those described above on day 30, highlighting the specific T cell responsiveness in these animals at such a late stage. These findings are thus consistent with the strong DTH responses in vaccinated mice at both times.

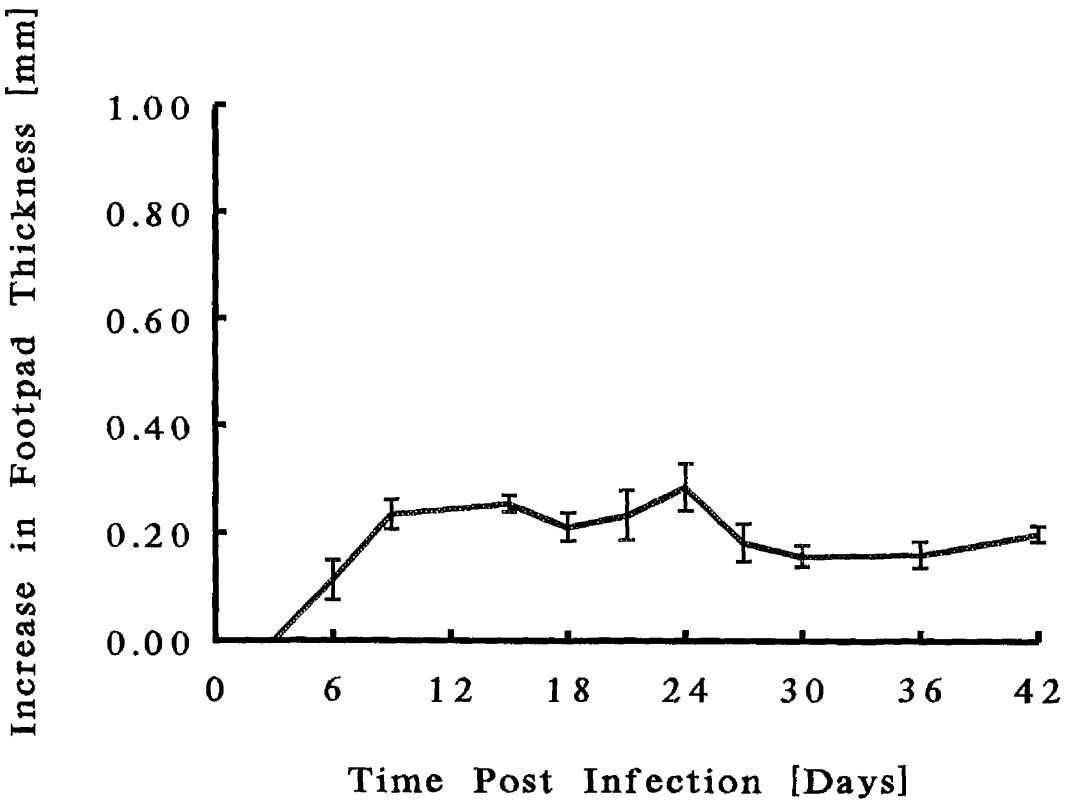
3.2.4.5 IL-2

I also attempted to detect IL-2 in the same supernatants from the cultures described above, using a CTLL bioassay. However this proved unsuccessful as the cell line did not grow well in culture and I was unable to obtain reproducible results.

3.2.4.6 Specific Immune Responses in Mice Infected with 5×10^2 CFU of S25/1aroA

In the mice which received 5×10^2 CFU S25/1aroA, no Salmonella-specific antibody of any isotype could be detected in either the serum or the intestine. However, small DTH responses were seen in most mice 6 days post-infection [Fig 3.7], suggesting a low level of T cell responsiveness in these animals.

Figure 3.7 Salmonella-Specific DTH Responses in Mice Inoculated Orally with 10^2 CFU of S25/1aroA



Mice were inoculated orally with 5 x 10^2 CFU of the S25/1aroA strain and the Salmonella-specific increases in footpad thickness determined at the stated times post-infection. Each point represents the mean increase in mm of 5 mice.

TABLE 3.8

Proliferative Responses and IFN-g Production to Salmonella Antigen by Spleen Cells from Mice Infected Orally with 5 x 10^2 CFU of S25/1 aroA

	MOUSE NUMBER					
Max. CPM	23,220	14,440	28,386	21,550	10,220	8,138
Max. SI	124	78	145	105	63	87
IFN-g TITRE	0	0	2	<2	0	0

Mice were inoculated with 5 x 10^2 CFU S25/1aroA and 35 days later, spleen cells were cultured with Salmonella antigen in an LTT, and the proliferative responses examined. The IFN-g titres of the cell supernatants were also determined.

Spleen cells were removed from 6 of these mice on day 35 and examined for proliferative responses and IFN-g produced after stimulation with Salmonella antigen in an LTT [Table 3.8]. The proliferative responses were significantly less [$p<0.01$] than those of mice given 5 x 10^7 CFU of S25/1aroA [Table 3.4A] and not significantly different from the responses of unvaccinated mice [Table 3.4B]. Furthermore, only 2 mice produced IFN-g and at very low levels.

3.2.5 Oral Immunisation with S25/1aroA Protects Mice from Subsequent Challenge with a Lethal Dose of Wildtype S25\1

The results described above show that 5 x 10^7 CFU of the vaccine strain was capable of establishing a limited infection in mice and induced specific primary immune responses, whereas 5 x 10^2 CFU did not colonise mice and induced little or no specific immunity. I next examined whether the levels of specific immune responsiveness generated by the 2 doses correlated with the ability to confer protection from a lethal challenge dose of the wildtype strain. 4 groups of mice were challenged with 5 x 10^7 CFU of the wildtype S25/1. Groups 1 and 2 [10 mice each] had been vaccinated 40 days previously with 2 x 10^7 CFU and 2 x 10^2 CFU respectively of S25/1 aroA. Group 3 [5 mice] had been challenged 40 days previously with a sublethal dose [2 x 10^2 CFU] of wildtype S25/1, and Group 4 comprised an unvaccinated control group. The mortality rates are shown in Table 3.9. All the mice which had been infected first with 2 x 10^7 CFU S25/1aroA [Group 1] were protected from virulent challenge and showed no clinical signs of infection. However like

TABLE 3.9

Ability of Primary Oral Infection with the Wildtype or aroA Strains of S25/1 to Protect Against Lethal S.typhimurium Challenge

	DAYS POST-INFECTION							
	1	2	3	4	5	6	7	8
GROUP B	0	0	0	0	0	0	0	0
GROUP C	0	0	0	0	0	2	3	0
GROUP D	0	0	0	2	1	3	2	2
GROUP E	0	0	0	0	4	2	2	2

Primary Immunisation:

GROUP B : 10^7 CFU S25/1aroA

GROUP C : 10^2 CFU wildtype S25/1

GROUP D : 10^2 CFU S25/1aroA

GROUP E : unvaccinated control

Mice were vaccinated with the stated doses, and 40 days later were challenged with 5×10^7 CFU wildtype S25/1 and the lethalities recorded.

the unvaccinated mice, all the mice which had received a primary infection of 2 x 10^2 CFU of either the aroA or wildtype strains developed severe clinical signs and succumbed to infection between days 4 and 8 post-infection. Clearly, the higher dose of the vaccine strain was required to evoke protection of the mice from virulent challenge. However it is important to note that sublethal doses of the virulent strain were also incapable of evoking protective immunity to a lethal challenge dose.

3.3 Discussion

The initial studies described in this chapter sought to characterise the infection in mice following administration of the virulent wildtype S25\1 strain. The minimum LD_{50} following oral challenge of BALB\c mice was estimated to be 10^2 CFU. This is in accordance with the findings of Hormaeche [1979a] who found that BALB\c mice were the most susceptible of all the mouse strains assessed by i.v. challenge. The similarity in the intravenous and oral LD_{50}s of virulent S.typhimurium highlights the ability of the strain to multiply in the gut lumen and to invade the enterocytes.

My comparative studies with the S25/1aroA vaccine strain showed at least a 7 log doses difference in the minimum oral LD_{50} for the aroA and wildtype strains in BALB\c mice. This is consistent with previous work showing that mice can tolerate doses of up to 10^{11} CFU of an aroA strain [Maskell et al., 1987]. Despite its lack of virulence, the S25/1aroA strain colonised the RES, with significant numbers of bacteria being recovered from the livers and

spleens. Although the *in vivo* growth rate was much slower and overall levels much lower than the virulent parent strain, the vaccine strain could still be isolated from the livers and spleens 80 days post-infection and from the Peyer's patches after 60 days. Similar findings have been reported by Dougan *et al*. [1986] and Maskell *et al*. [1986], although they could not recover the strain consistently beyond 6 weeks post-infection. Others have failed to show such longterm persistence of *aro*A *S.typhimurium* in the tissues following oral vaccination, with the organism being totally cleared as early as day 14 in some systems [Hayes *et al*., 1991]. However these workers used C3H/He/Ola mice which are less susceptible to *Salmonella* [Hormaeche, 1979a].

All mice vaccinated orally with 10^7 CFU of S25/1*aro*A produced *Salmonella* LPS-specific antibody in the serum and the intestine and levels of serum IgG were still detected 250 days after challenge. Levels of intestinal IgA peaked on day 42 but were still detectable on day 90. Hohmann *et al*. [1979] decribed similar kinetics of the sIgA response after oral inoculation with *Salmonella*. However, Hayes *et al*. [1991], failed to detect specific sIgA [and serum IgA] following vaccination with an *aro*A mutant of *S.typhimurium* which they attributed to low level intestinal persistence of the vaccine strain in C3H/He/Ola mice. The sustained sIgA response to S25/1 *aro*A was expected, as the Peyer's patches and faeces of the mice harboured the vaccine strain for 66 and 90 days respectively. The persistence of the organism in the faeces was a result of primary challenge and not coprophagy by the mice.

An interesting finding from the studies of sIgA production was the similarity in the antibody profiles for serum amd intestinal IgA, suggesting that mucosal sIgA produced in response to local stimulation with the oral vaccine may have leaked to the circulation or that there was simultaneous priming of the local and systemic immune systems by the vaccine strain. However, the levels of serum IgA declined rapidly [with the sIgA levels] unlike the serum IgG levels which remained high, suggesting that there was limited systemic priming of IgA-producing B cells.

Such an association of serum and gut IgA has not been reported in humans following oral vaccination with Ty21a [Labrooy et al., 1980; Bartholemeuz et al., 1990]. They found no correlation between the peak levels of anti-typhoid IgA in intestinal fluid and the anti-typhoid serum polymeric IgA antibody or total anti-typhoid serum antibody. The inability of Hayes et al. [1991] to detect either gut or serum IgA in the presence of specific IgG, coupled with the results with S25/1aroA, suggests that there can be systemic priming of IgG-producing B cells without a serum IgA response and that serum IgA may only be detectable in the presence of an intestinal IgA response.

As well as producing strong humoral responses, mice vaccinated with 10^7 CFU of S25/1aroA produced specific DTH responses in vivo which remained strong 6 months after vaccination. However it is controversial whether systemic DTH correlates with protective

immunity, as although early reports demonstrated a close relationship between the DTH response in mice and resistance to Listeria and Mycobacteria [MacKaness, 1967; Collins and MacKaness, 1968], later reports contradict these findings by showing that both resistant and susceptible strains of mice showed positive DTH response to S.typhimurium and that immune BALB/c mice did not produce a specific DTH response [Hormaeche, 1979a].

Due to the controversy surrounding DTH and protective immunity, I also performed LTTs using spleen cells from mice infected orally with S25/1aroA. These mice had strong proliferative responses to Salmonella antigen up to 70 days post-vaccination. However unvaccinated mice also responded to the antigen due to LPS contamination in the preparation. Non-specific responses of this kind were not found using peritoneal exudate T cells from aroA-vaccinated immune C3H/HeJ mice whose B cells cannot respond LPS [Killar and Eisenstein, 1985]. I therefore separated T and B cells to try and characterise the proliferative response in unvaccinated mice. Both B and T cell-enriched populations responded to the Salmonella antigen suggesting that the proliferative responses were a consequence of both LPS stimulation of B cells and non-specific T cell proliferation, perhaps as a result of cross-reactive antigens. However there is the possibility that low levels of B cells were present in the purified T cell population although this contamination would have to have been great to account for the high proliferative responses obtained.

In addition, I examined for the production of the cytokines IFN-g and IL-2. Only cells from vaccinated mice produced IFN-g, suggesting that there was indeed a specific T cell response in vaccinated mice. Production of IL-2 by the same cells was not determined as the assay proved unreliable. If this production of IFN-g by specific T cells also takes place *in vivo*, it may be an important factor in establishing protection against virulent *S.typhimurium* challenge as it has shown that recombinant IFN-g is able to activate macrophages for increased anti-*Salmonella* activity [Kagaya *et al*, 1989] and administration of anti-IFN-g to mice greatly enhances the virulence of *S.typhimurium* [Muotiala and Makela, 1990].

My studies also demonstrated the ability of S25/1*aro*A to protect against virulent oral challenge with a high lethal dose of the wildtype strain. Mice vaccinated orally with 10^7 CFU of S25/1*aro*A were protected from 10^7 CFU of the wildtype strain. In contrast, the mice receiving either 10^2 CFU of *aro*A or wildtype S25\1, which were not colonised, succumbed to a lethal virulent challenge dose. Thus, colonisation of the internal organs by the S25/1*aro*A strain appears to be a prerequisite for the development of protective immunity by the vaccine strain. Although it is impossible to conclude from my results the nature of the protective mechanisms, the strong vaccine-specific humoral and cell-mediated immune responses in vaccinated mice would suggest that both antibody and T cells are required for optimal levels of protection.

Chapter 4

The Immune Response in Lambs following Oral Vaccination with S25/laroA

4.1 Introduction

Following the characterisation of the mucosal and systemic immune responses elicited in mice after oral inoculation with the S25/1 aroA strain, I next assessed the behaviour of the same strain in sheep, as the vaccine potential of the S25/1aroA strain was to be exploited in this species.

The experiments described in this chapter were designed to compare the invasive capacities of the wildtype and aroA strains of S25/1 in lambs and to determine whether the aroA vaccine strain could protect against virulent challenge. In parallel, the persistence of the mutant strain in the tissues was determined following inoculation of different doses and was correlated with the vaccine-specific humoral and cell-mediated immune responses produced.

4.2 Results

4.2.1 Estimation of the Lethal Dose of the Wildtype S25\1 Strain for Lambs

I first determined the lowest dose of the wildtype strain that was lethal for lambs to allow studies comparing the invasiveness of the

wildtype and aroA strains. In addition, I wished to establish a dose for wildtype challenge of aroA-vaccinated animals.

3 lambs, numbered 1-3, were infected orally with 7×10^{11}, 6×10^{10} or 8×10^{9} CFU of the wildtype strain respectively. The clinical symptoms of all 3 lambs after infection were similar, although the onset of severe symptoms was dose-dependent. All 3 lambs developed elevated temperatures [increases of 1.9^{o}-2.3^{o}C] 24-48 h after challenge, which remained high until death. Diarrhoea, as represented by a severe drop in the %DW of faeces, was seen and there was an increase in the %PCV of the blood. Salmonellae could also be isolated from the blood from day 3 in lambs 1 and 2. Weight loss [7-9% of body weight] and lethargy were observed and there was excretion of high numbers of salmonellae in the faeces [10^{5}-10^{8} CFU/g].

Lambs 1, 2 and 3 were culled in extremis on days 3, 4 and 7 respectively and the no. CFU/g of tissue determined [Table 4.1]. Lambs 1 and 2 contained high levels of the organism in all the tissues tested. Lamb 3 harboured the organism in all tissues except the bronchial lymph node, but only the levels in the gut were comparable to those recovered from the previous 2 animals.

4.2.2 Oral Infection of 5 Lambs with 9×10^{9} CFU of Wildtype S25\1

Having established that 8×10^{9} CFU of the wildtype S25\1 strain was capable of causing a lethal infection, another 5 lambs were given a similar dose to characterise the infection further. 5 lambs

TABLE 4.1
Recovery of Wildtype S25/1 from the Organs of Lambs After Oral Infection

No. CFU/g of Tissue

LAMB NO.	1	2	3
DOSE OF S25/1	7×10^{11} CFU	6×10^{10} CFU	8×10^{9} CFU
CULLED*	DAY 3	DAY 4	DAY 7
HEART	1×10^{6}	4×10^{5}	500
LUNG	1×10^{8}	1×10^{6}	4×10^{4}
BRONCHIAL LN	1×10^{8}	2×10^{6}	0
LIVER	1×10^{7}	1×10^{7}	5×10^{3}
HEPATIC LN	9×10^{7}	5×10^{6}	4×10^{5}
SPLEEN	4×10^{6}	1×10^{6}	4×10^{3}
KIDNEY	4×10^{6}	4×10^{5}	600
MESENTERIC LN	3×10^{8}	1×10^{8}	5×10^{6}
ILEUM	9×10^{7}	1×10^{6}	2×10^{5}
CAECUM	2×10^{8}	2×10^{8}	6×10^{8}
COLON	4×10^{8}	1×10^{8}	6×10^{6}

LN = Lymph Node
0 = Organism not detected, even after enrichment

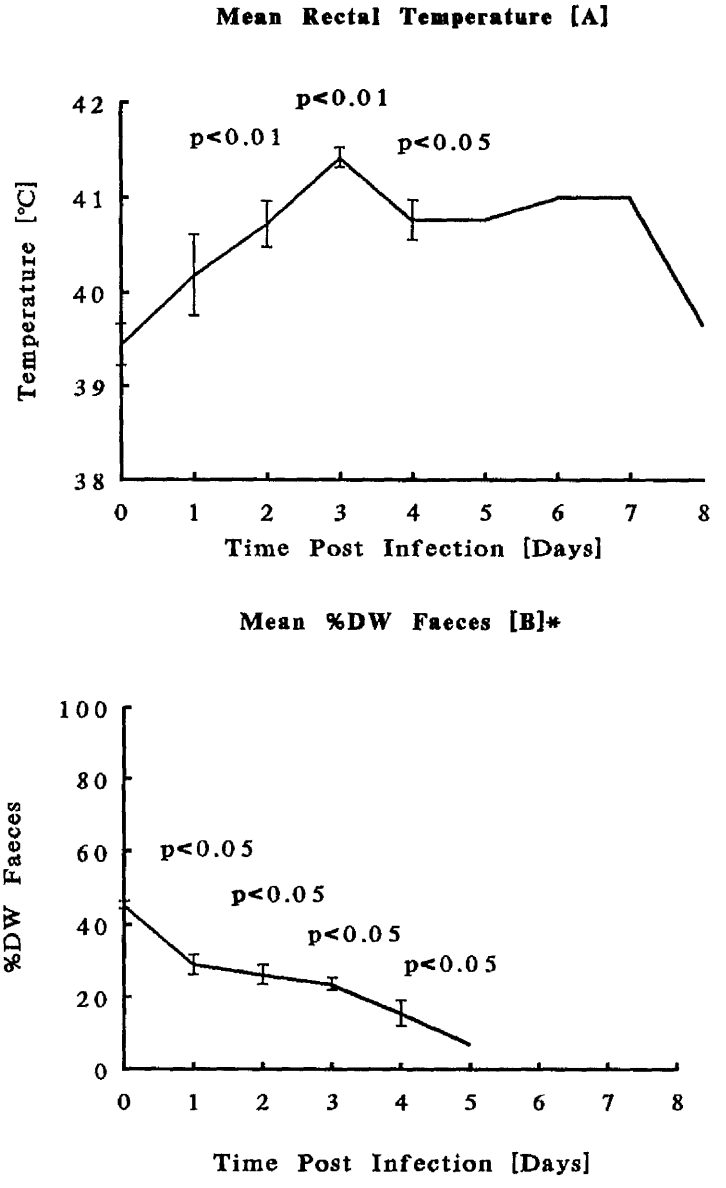
3 lambs [Nos. 1-3] were challenged orally with 7×10^{11}, 6×10^{10} or 8×10^{9} CFU of wildtype S25/1 respectively, culled *in extremis* on the stated days post-infection*, and the no. CFU/g of tissue determined for each lamb.

were infected orally with 9 x 10^9 CFU of the wildtype S25\1 strain and culled in the acute phase of infection on days 2, 3, 4, 5 and 7 for lambs numbered 4-8 respectively. Lambs 4-7 developed a lethal infection as described above, with elevated temperatures 24-48 h post-infection, and a gradual decrease in %DW of faeces which culminated in persistent diarrhoea in those animals that survived beyond 3 days [Figure 4.1]. There was a significant increase in the %PCV of the blood of these lambs [$p<0.05$] rising from a mean pre-infection level of 36.6% [range 33.4 to 40.1] to 44.1% [range 41.2 to 47.3]. Although the organism was not found in the blood, high levels [10^6-10^8 CFU\g] of virulent salmonellae could be isolated from the faeces of all these animals. Lamb 8 did not appear clinically ill at any time, although elevated temperatures [increases of 1.6-2.5^{o}C] were recorded.

Lamb 4, culled 48 h post-infection, contained high levels of the organism in all regions of the gut, but the internal organs were either free of the organism or harboured very low levels, suggesting that the passage of the organism out of the gut required at least 48 h. Lambs 5, 6 and 7 harboured high numbers of the organism in all tissues [Table 4.2]. In lamb 8, only the hepatic lymph node harboured the challenge strain, presumably accounting for the absence of severe disease in this animal.

Histopathological studies, kindly performed by Dr John Gilmour, MRI, showed that in lambs 4 and 6 , villi were absent, while lambs 6 and 7 showed evidence of follicle depletion of the Peyer's

Figure 4.1 Clinical Responses in Lambs Inoculated Orally with 10^9 CFU of Wildtype S25/1



5 lambs [No. 4-8] were inoculated orally with 9 x 10^9 CFU of wildtype S25/1 and the mean rectal temperature [A] and mean %DW of faeces [B] determined on each day post-challenge. *The %DW values of Lamb 8 were discarded as they were markedly different from the other 4 lambs.

TABLE 4.2
Recovery of Wildtype S25/1 from the Organs of Lambs After Oral Infection with 10^9 CFU

No. CFU/g of Tissue

LAMB NO.	4	5	6	7	8
CULLED*	DAY 2	DAY 3	DAY 7	DAY 5	DAY 7
HEART	0	1×10^4	2×10^4	5×10^3	0
LUNG	0	5×10^5	5×10^3	1×10^5	0
BRONCHIAL LN	0	3×10^5	5.5×10^3	2×10^4	0
LIVER	950	1×10^5	406	5×10^3	0
HEPATIC LN	7×10^5	4.5×10^3	88	0	1×10^4
SPLEEN	375	7×10^5	5×10^4	3×10^3	0
KIDNEY	0	2.5×10^3	0	93	0
MESENTERIC LN	6×10^5	2.5×10^7	3×10^6	4×10^6	0
ILEUM	1×10^7	1×10^8	3×10^8	3×10^8	+
CAECUM	7.5×10^7	2.5×10^8	4×10^7	2.5×10^7	NT
COLON	4×10^6	2×10^8	4×10^6	6×10^6	NT

LN = Lymph Node
\+ = Organism only detected after enrichment in selenite broth
0 = Organism not detected, even after enrichment
NT = Not tested

5 lambs [No. 4-8] were infected orally with 9×10^9 CFU of wildtype S25/1, culled *in extremis* on the stated days* post-infection and the no. CFU/g of tissue determined for each lamb.

patches and necrosis of the overlying epithelium. In lambs 4 and 5, which were culled earlier, the GALT was normal, suggesting that damage to the Peyer's patches is a sign of progressive disease. In all 5 lambs, there was extensive liver damage in the form of hepatocyte necrosis and there were large numbers of neutrophils in the lymph nodes of lambs 4, 5 and 6.

4.2.3 Oral Infection of Lambs with 10^9 CFU S25/1aroA

To compare the relative virulence of the wildtype and aroA strains, 7 lambs were inoculated orally with an identical dose [9 x 10^9 CFU] of S25/1aroA and culled on days 3, 6, 9, 15, 20, 25 and 30 for lambs numbered 9-15 respectively. No increase in rectal temperature was noted in any of the lambs and the %DW of faeces and the %PCV of the blood remained constant throught the course of the experiment. Within 24-48 h post-infection, large numbers of the organism could be found in the faeces [10^4-10^5 CFU\g], but after 3 days organisms were rarely isolated from the faeces and were never isolated from the blood of any of the animals at any time. On necropsy, the organism could be found in the mesenteric lymph node of 6 of the 7 lambs [Table 4.3]. However, isolation of the organism from the other organs was infrequent, with only 1 or 2 of the organs tested from each lamb harbouring only very low levels. There was no histological evidence of the gastrointestinal or liver damage that was noted in the lambs given the wildtype strain.

TABLE 4.3
Recovery of S25/laroA from the Organs of Lambs

After Oral Infection with 10^9 CFU

No. CFU\g of Tissue

Lamb No.	9	10	11	12	13	14	15
CULLED*	DAY 3	DAY 6	DAY 9	DAY 15	DAY 20	DAY 25	DAY 30
HEART	0	680	0	0	0	0	0
LUNG	0	25	0	0	0	0	0
BRONCHIAL LN	0	0	0	0	0	0	0
LIVER	50	0	0	0	0	13	10
HEPATIC LN	0	260	0	0	0	0	0
SPLEEN	0	3	0	0	10	+	0
KIDNEY	0	6	0	0	0	+	0
MESENTERIC LN	215	35	25	5	10	0	14
ABOMASUM	0	0	0	0	0	0	+
ILEUM	+	+	0	0	0	0	0
CAECUM	+	+	0	0	0	0	0
COLON	+	+	+	0	0	15	0

LN = Lymph Node
+ = Organism only detected after enrichment in selenite broth
0 = Organism not detected, even after enrichment

7 lambs [No. 9-15] were inoculated orally with 9×10^9 CFU of S25/1 aroA, culled on the stated days* post-infection and the no. CFU/g of tissue determined for each lamb.

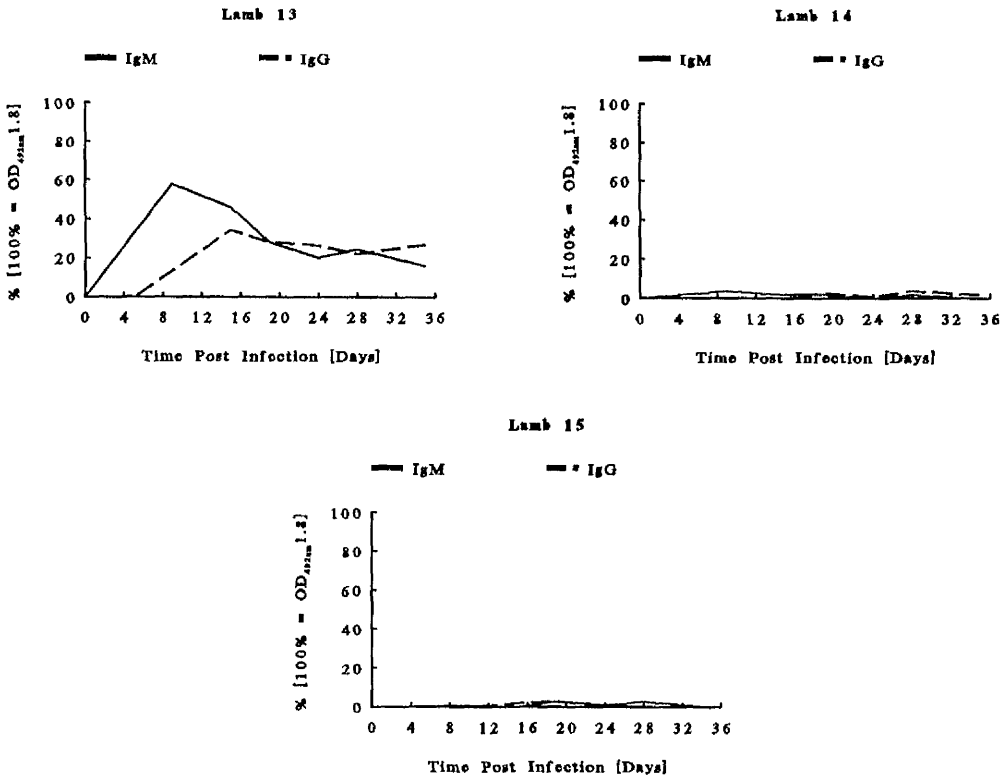
4.2.3.1 Humoral Immune Responses in Lambs After Oral Infection with 10^9 CFU of S25/1aroA

The sera from the 3 lambs that were maintained beyond 15 days [lambs 13, 14 and 15] were examined for the presence of antibodies to the vaccine strain. Lambs 14 and 15 produced no specific serum IgM or IgG antibody, however lamb 13 produced both specific IgM and IgG antibodies [Figure 4.2]. IgM appeared on day 5, peaked between days 8 and 11 and gradually decreased thereafter, although detectable levels were still seen on termination of the experiment on day 35. IgG was first produced between 5 and 9 days post-infection, rising until day 15 and was still present on day 35.

4.2.3.2 Cell-Mediated Immune Responses in Lambs After Oral Infection with 10^9 CFU of S25/1aroA

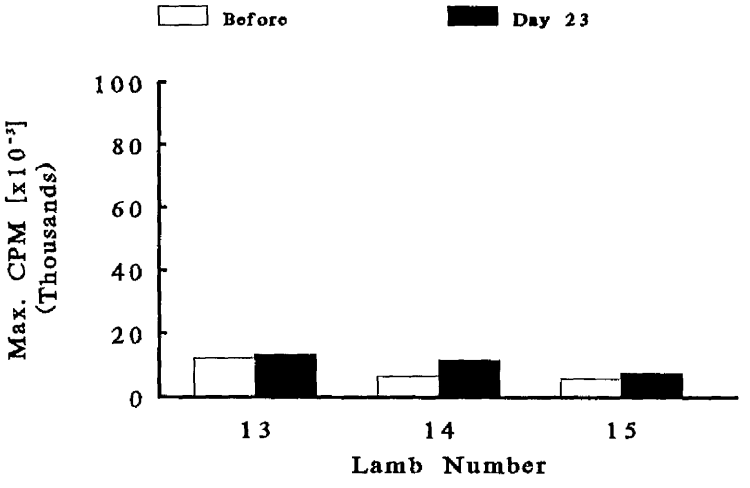
Initially, I attempted to determine the specific DTH response following oral vaccination with S25/1aroA. However, large non-specific increases in skinfold thickness were recorded in all lambs challenged with heat-killed S25/1aroA irrespective of whether they had been vaccinated, making the results impossible to interpret. I therefore attempted to examine the *in vitro* responsiveness of the PBMC to *Salmonella* antigen in an LTT, 23 days after infection [Fig 4.3]. However the lymphocytes from all 3 infected lambs responded poorly and these responses were not

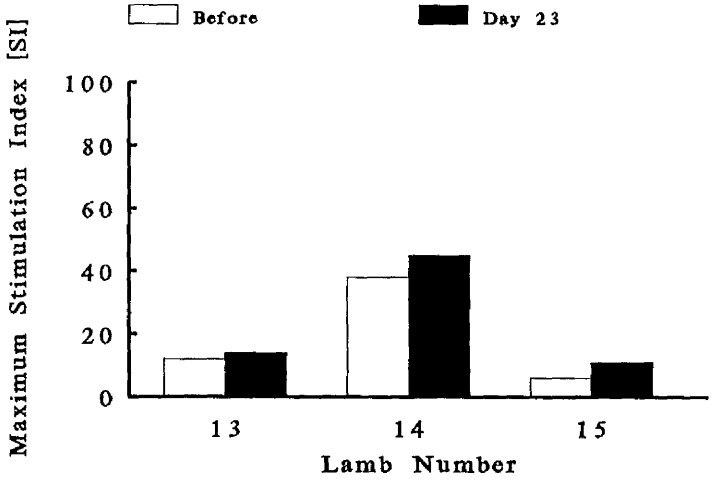
Figure 4.2 Salmonella-Specific Serum Antibody Responses in Lambs following Oral Inoculation with 10^9 CFU of S25/1aroA



The sera of 3 lambs [no. 13-15], inoculated orally with 9 x 10^9 CFU of S25/1aroA, were tested in an ELISA at times post-infection. The results show the OD_{492nm} for IgM and IgG at a 1:40 dilution of serum. ODs are expressed as a percentage where 100% represents OD_{492nm} 1.8.

Figure 4.3 Salmonella-Specific Proliferative Responses of PBMC from Lambs Inoculated Orally with 10^9 CFU of S25/1 aroA





PBMC from each of the 3 lambs were cultured with Salmonella antigen in an LTT before, and 23 days after oral infection with 9 x 10^9 CFU S25/1aroA and the proliferative responses compared.

significantly higher than those recorded using PBMC taken from the same lambs before infection. Thus, a single oral infection with the aroA strain was not effective in eliciting Salmonella-specific cell-mediated immune responses.

4.2.4 Oral Infection of Lambs with a Triple Dose of 10^{11} CFU of S25/1aroA

In an effort to obtain greater colonisation of the lambs by the S25/1 aroA strain and thus perhaps generate greater cellular and humoral immunity, both the size and number of the infecting dose were increased. 6 lambs [numbered 16-21] were infected orally on each of days 0, 2 and 4 with 5×10^{11} CFU of S25/1aroA. None of the lambs developed severe clinical symptoms. A temperature rise was seen [1.5-2.5^{o}C] in 2 of the lambs 24 h after receiving either the first [lamb 16] or second [lamb 17] dose, but this returned to normal after a further 24 h and was not observed in any of the additional 15 lambs used in section 4.2.4.1. None of the 6 animals described here developed diarrhoea or showed a visible softening of the faeces. In the subsequent experiments, 1 lamb out of 15 developed diarrhoea with a drop in %DW of faeces [from 43%-11%]. However, the remaining 14 showed no change in the %DW of faeces throughout the course of the experiment. No haemoconcentration was recorded in any of these groups of lambs.

Within 24-48 h, large numbers of salmonellae [10^{3}-10^{5} CFU\g] were found in the faeces of all 6 lambs, but after 5-6 days, the organism was seldom isolated. The organism was never isolated from

TABLE 4.4

Recovery of S25/1aroA from the Organs of Lambs After Oral Infection with 3 Doses of 10^{11} CFU

No. CFU\g of Tissue

Groups	DAY 6 [a]			DAY 12 [b]		
Lamb	16	17	18	19	20	21
HEART	2	180	10	400	457	40
LUNG	0	290	0	50	3×10^6	689
BLN	8	60	0	0	55	0
LIVER	26	1×10^3	50	111	721	139
HLN	28	12	15	0	801	23
SPLEEN	0	28	7	10	283	4
KIDNEY	0	440	6	13	636	700
MLN	180	2×10^3	2×10^3	3×10^3	86	122
ABOMASUM	+	40	4×10^3	238	615	1×10^3
ILEUM	+	+	+	0	0	0
CAECUM	+	+	+	0	0	0
COLON	+	+	+	0	0	0

BLN = Bronchial Lymph Node
HLN = Hepatic Lymph Node
MLN = Mesenteric Lymph Node
+ = Organism only detected after enrichment in selenite broth
0 = Organism not detected, even after enrichment

Lambs were inoculated orally with 3 doses of 10^{11} CFU of S25/1aroA on days 0, 2 and 4. 3 lambs were culled on days 6 [a] and day 12 [b] and the no. CFU/g of tissue determined for each lamb.

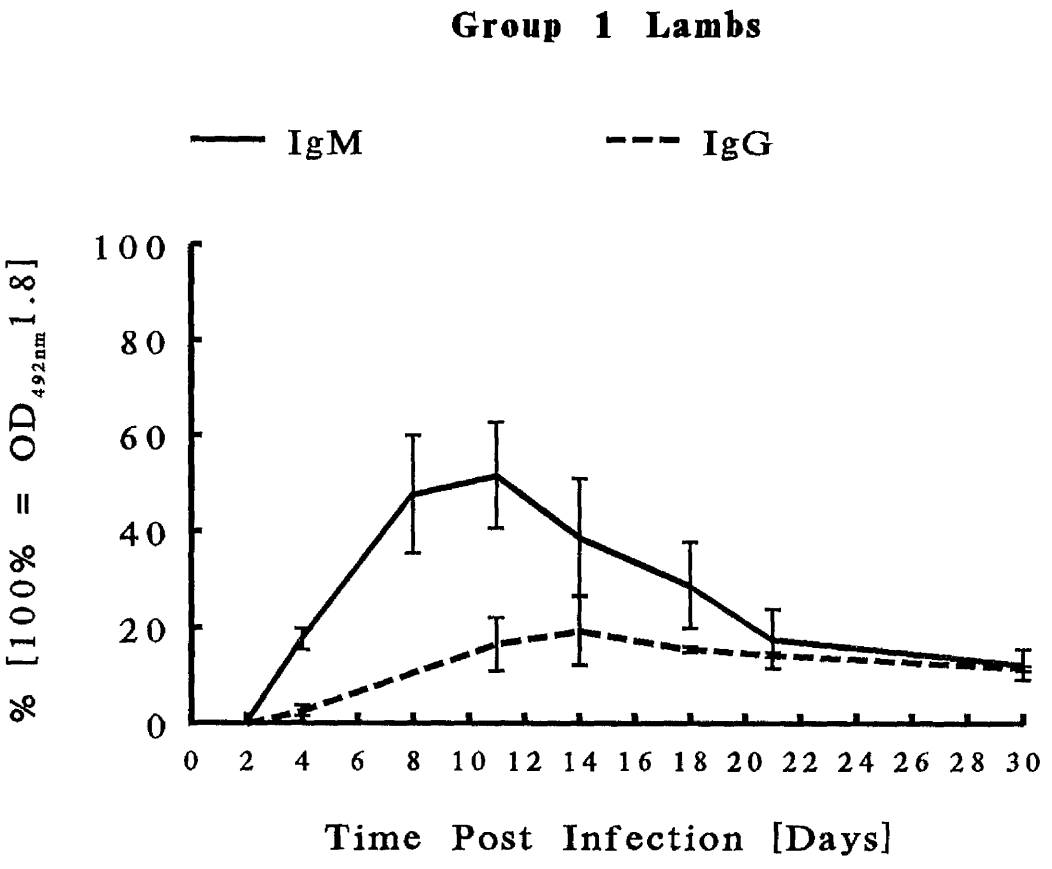
the blood even after culture in selenite broth enrichment media. 3 lambs were culled on days 6 [lambs 16-18] or 12 [lambs 19-21] post-infection and the no. CFU/g of tissue determined. The mesenteric lymph nodes of all the lambs contained substantial levels of the vaccine strain [Table 4.4] and varying levels of colonisation occurred in the the majority of the other tissues taken from each lamb at both times. These results indicate the relative efficacy of this multiple vaccination strategy. Moreover, histological studies revealed that there was no cell damage in the GALT or liver and there were no neutrophils present in the lymph nodes.

4.2.4.1 Humoral Immune Responses in Lambs After Oral Infection with Multiple Doses of 10^{11} CFU of S25/1aroA

3 separate groups of 5 lambs [numbered 22-26; 27-31 and 32-36 for Groups 1-3 respectively] were vaccinated with the aroA strain for use in the studies examining protection against the wildtype S25/1 strain described in section 4.2.7.

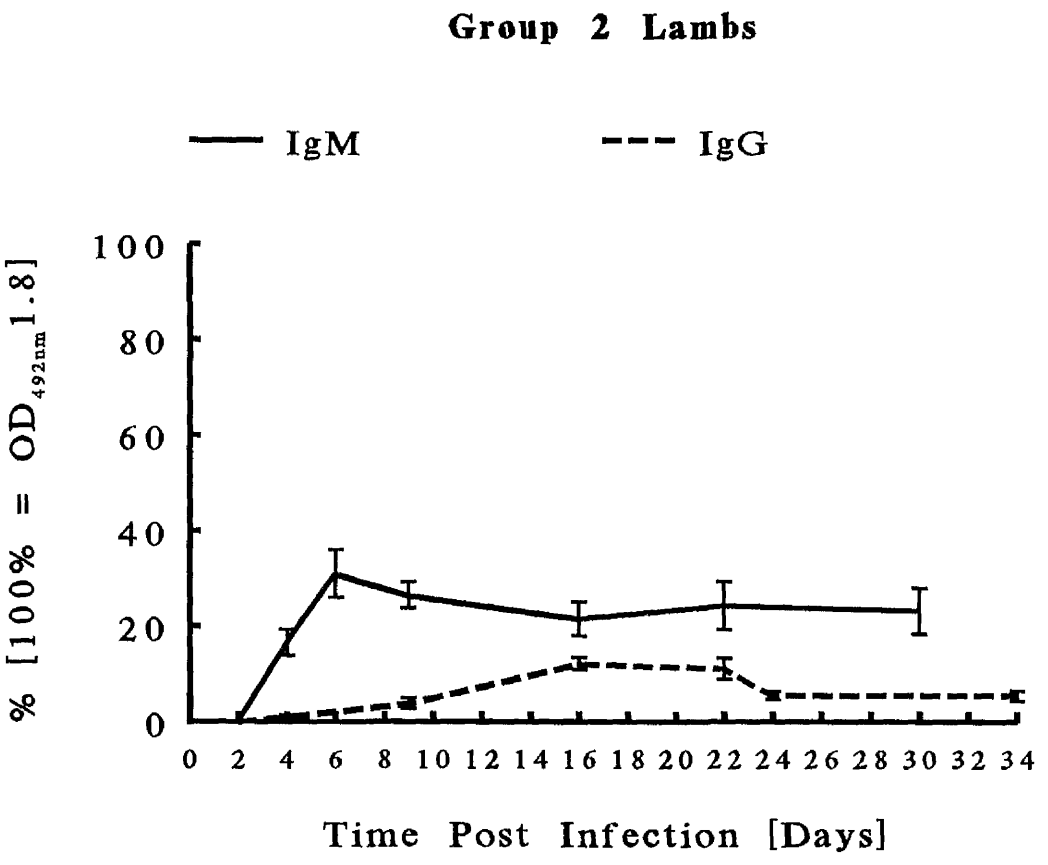
Lambs were inoculated orally with 5-9 x 10^{11} CFU S25/1aroA on days 0, 2, 4 and 24 and the serum examined for the presence of both vaccine-specific IgM and IgG at times post-infection [Figures 4.4.1-4.4.3]. All lambs produced detectable levels of IgM. The lambs in Group 1 produced considerably higher levels than those in either of the other 2 groups. IgM first appeared 5 days post-infection, reaching peak levels between days 8 and 12 and gradually decreasing thereafterwards. However detectable levels

Figure 4.4.1 Salmonella-Specific Serum Antibody Responses in Lambs Inoculated Orally with 10^{11} CFU of S25/laroA



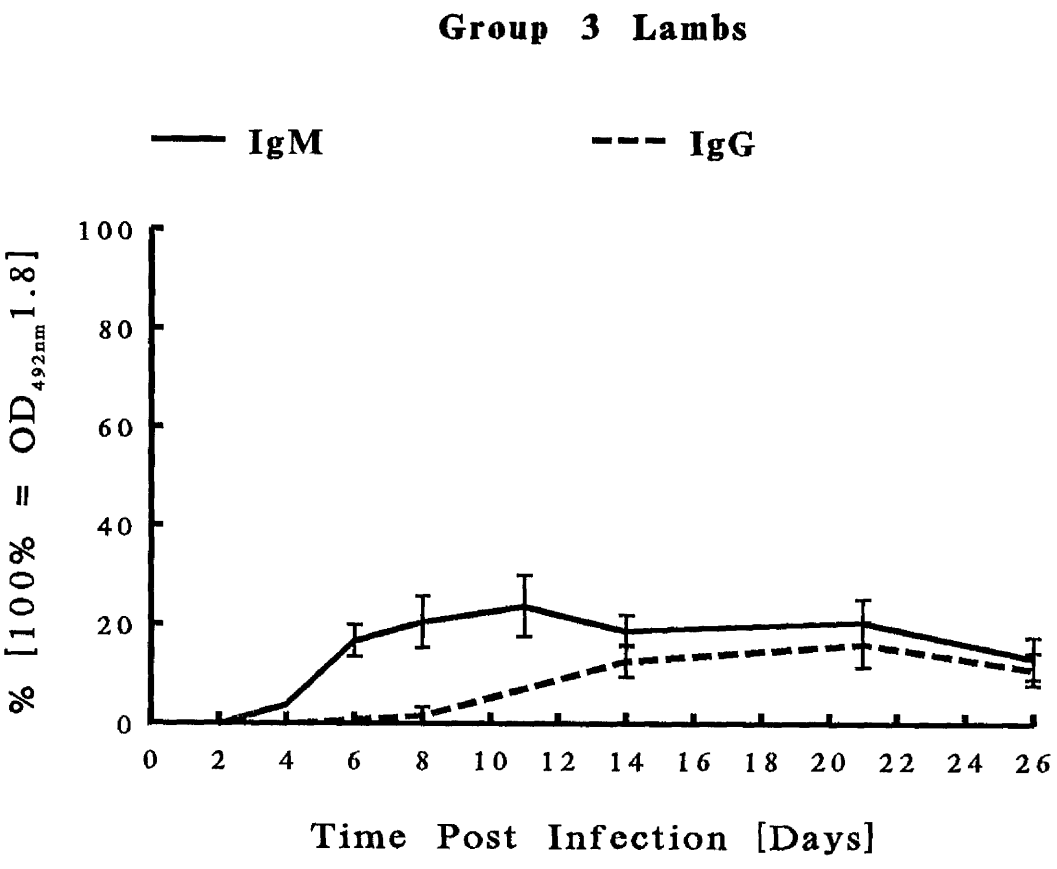
Lambs were inoculated orally with 4 doses of 5-9 x 10^{11} CFU of S25/1aroA on days 0, 2, 4 and 24 and the sera tested in an ELISA at times post-infection. Each point represents the mean OD_{492nm} of 5 sheep [Group 1] at serum dilutions of 1:200 and 1:80 for IgM and IgG respectively. Mean OD values are expressed as a percentage where 100% represents OD_{492nm} 1.8.

Figure 4.4.2 Salmonella-Specific Serum Antibody Responses in Lambs Inoculated Orally with 10^{11} CFU of S25/laroA



Lambs were inoculated orally with 4 doses of 5-9 x 10^{11} CFU of S25/laroA on days 0, 2, 4 and 24 and the sera tested in an ELISA at times post-infection. Each point represents the mean OD_{492nm} of 5 sheep [Group 2] at serum dilutions of 1:80 and 1:20 for IgM and IgG respectively. Mean OD values are expressed as a percentage where 100% represents OD_{492nm} 1.8.

Figure 4.4.3 Salmonella-Specific Serum Antibody Responses in Lambs Inoculated Orally with 10^{11} CFU of S25/laroA



Lambs were inoculated orally with 4 doses of 5-9 x 10^{11} CFU of S25/1aroA on days 0, 2, 4 and 24 and the sera tested in an ELISA at times post-infection. Each point represents the mean OD_{492nm} of 5 sheep [Group 3] at serum dilutions of 1:40 and 1:16 for IgM and IgG respectively. Mean OD values are expressed as a percentage where 100% represents OD_{492nm} 1.8.

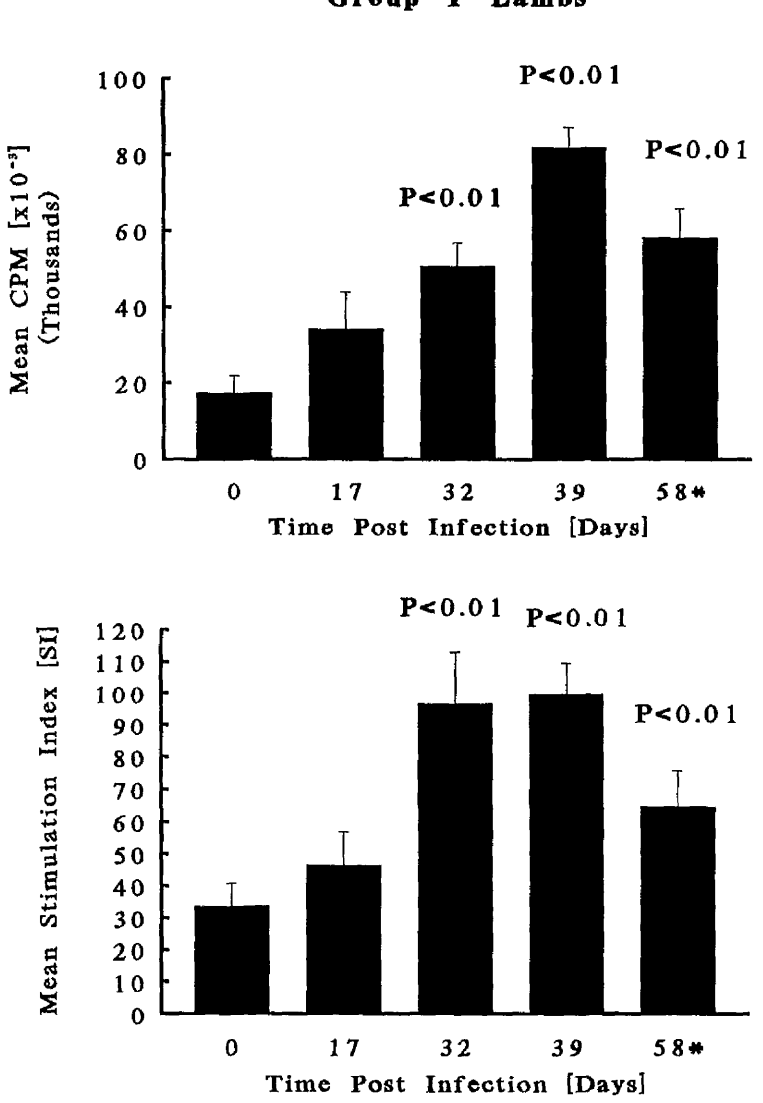
present on day 35. The levels of IgG in all of the 15 lambs was low in comparison to IgM, but were usually seen around 8 days post-infection, and remained relatively constant throughout the course of the experiments.

Only 2 of the 15 lambs [1 from Group 1 and 1 from Group 2] produced detectable levels of specific intestinal IgA on day 16, but the levels were very low in both animals and the remaining 13 lambs produced no specific coproantibody at any time post-infection.

4.2.4.2 Cell-Mediated Immune Responses After Oral Infection with Multiple Doses of 10^{11} S25/1aroA

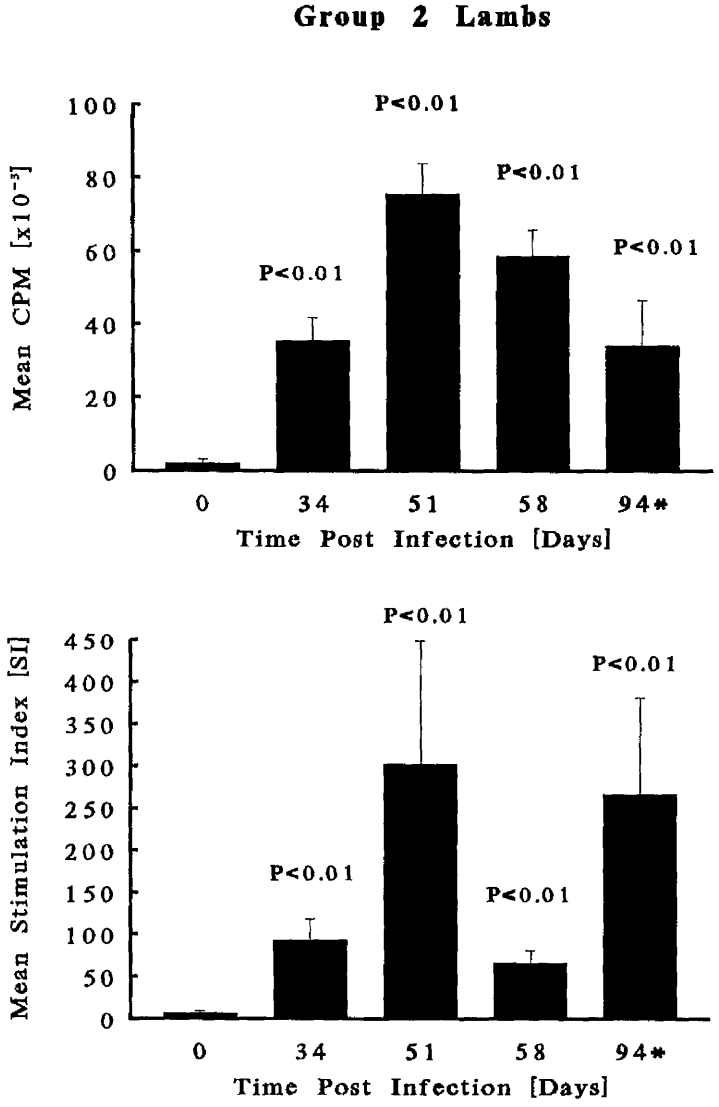
Lymphocytes were removed from the 15 lambs before and at various times following infection with the S25/1aroA strain, and assayed for specific proliferation against the vaccine strain. The results are shown in Figures 4.5.1-4.5.3 which represent Groups 1-3 respectively. All 15 lambs showed strong proliferative responses to the vaccine strain. In each of the 3 groups, all the responses recorded post-infection were significantly greater than the pre-infection responses, with the exception of the day 17 responses in Group 1. The high levels of lymphocyte responsiveness continued in all of the animals until challenge with virulent S25/1 on days 40, 87 and 40 for Groups 1, 2 and 3 respectively. Challenge did not result in a significant increase in the proliferative responses in Groups 1 and 2 [Group 3 lambs not tested].

Figure 4.5.1 Salmonella-Specific Proliferative Responses of PBMC from Lambs Inoculated Orally with 10^{11} CFU of S25/1aroA



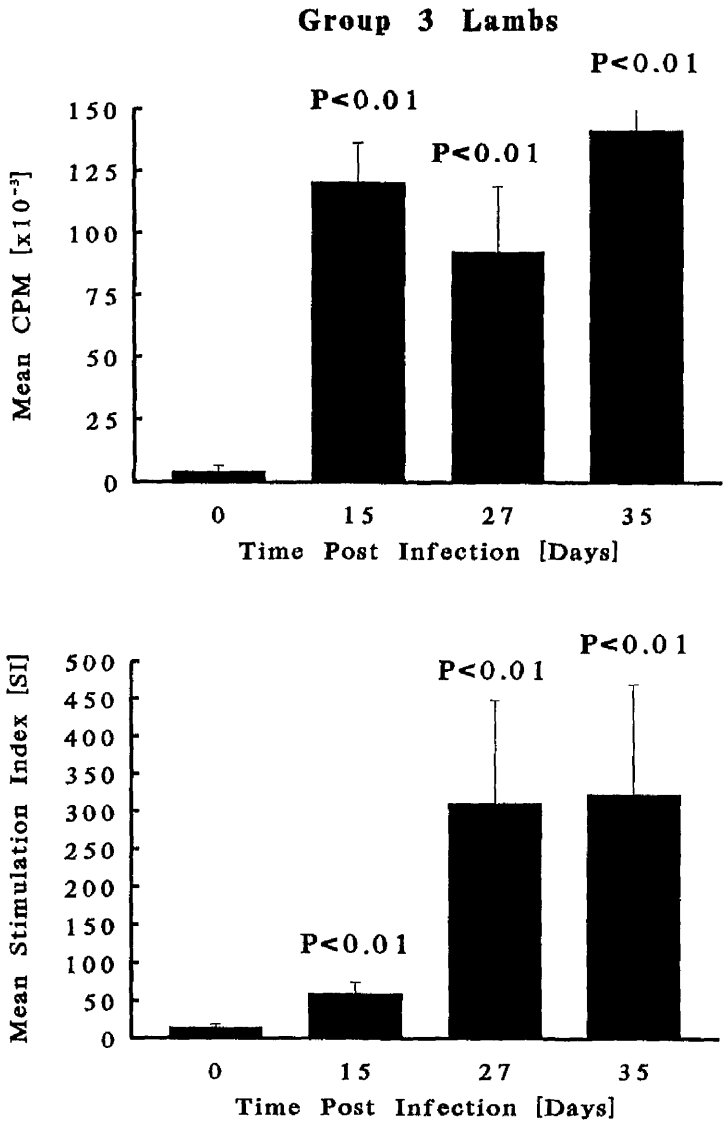
5 lambs [Group 1] were vaccinated orally with 4 doses of 5-9 x 10^{11} CFU of S25/1aroA on days 0, 2, 4 and 24 and the PBMC cultured with Salmonella antigen in an LTT before, and at various times after vaccination, and after challenge* with 8 x 10^{9} CFU wildtype S25/1. Both the mean CPM and SI are given for each time-point.

Figure 4.5.2 Salmonella-Specific Proliferative Responses of PBMC from Lambs Inoculated Orally with 10^{11} CFU of S25/laroA



5 lambs [Group 2] were vaccinated orally with 4 doses of 5-9 x 10^{11} CFU of S25/1aroA on days 0, 2, 4 and 24 and the PBMC cultured with Salmonella antigen in an LTT before, and at various times after vaccination, and after challenge* with 1 x 10^{10} CFU wildtype S25/1. Both the mean CPM and SI are given for each time-point.

Figure 4.5.3 Salmonella-Specific Proliferative Responses of PBMC from Lambs Inoculated Orally with 10^{11} CFU of S25/laroA



5 lambs [Group 3] were vaccinated orally with 4 doses of 5-9 x 10^{11} CFU of S25/1aroA on days 0, 2, 4 and 24 and the PBMC cultured with Salmonella antigen in an LTT before, and at various times after vaccination. Both the mean CPM and SI are given for each time-point.

4.2.5 Production of IFN-g by PBMC from Lambs Inoculated with S25/1 aroA following Stimulation with Specific Antigen in an LTT

To study the presence of Salmonella-specific T cell responses further, I examined for the presence of IFN-g in supernatants of PBMC cultures from the 3 groups of lambs following stimulation with antigen and ConA.

Supernatants were removed after 24, 48 and 96 h of culture with antigen. The responses of the lambs in Group 1 were variable at each time post-infection [Table 4.5]. However, all the lambs produced IFN-g at some stage, although the titres were very low. The lymphocytes from all of the lambs in Group 1 also produced IFN-g following mitogenic stimulation with ConA. None of the PBMC supernatants from any of the lambs in Groups 2 and 3 contained IFN of any kind, at any time post-infection, suggesting that there was no specific T cell responses in these animals. Also, none of the ConA supernatants from any of the lambs in these 2 groups produced IFN-g, suggesting that the the PBMC from these lambs inherently produced low levels of IFN-g whether stimulated with antigen or mitogen.

4.2.6 Phenotype Analysis of Cells Responding to Salmonella Antigen in an LTT

As all 15 lambs had produced strong proliferative responses to Salmonella but not IFN-g, this suggested that the proliferation was not due to T cells. I therefore attempted to determine the

TABLE 4.5
Titres of IFN-g Produced by PBMC from Lambs Inoculated with S25/1 aroA following Stimulation with Salmonella Antigen

GROUP 1 LAMBS

Titres of IFN-g

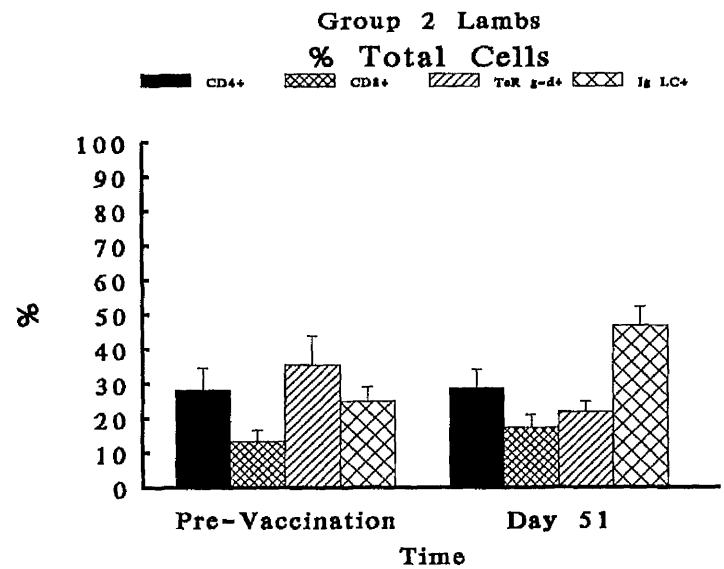
	TIME POST-VACCINATION WITH S25/1aroA																
Stimulant	PREVACCINATION						DAY 24						DAY 35				
Unstimulated	0	0	0	0	0		0	0	0	0	0		0	0	0	0	0
ConA [10ug\ml]	2	2	2	<2	<2		2	2	2	2	0		2	2	2	2	2
S25/1aroA [60ug\well]	0	0	0	0	0		0	0	8	8	0		0	0	0	2	<2
S25/1aroA [24ug\well]	0	0	0	0	0		0	8	16	16	0		4	0	2	0	4
S25/1aroA [12ug\well]	0	0	0	0	0		0	8	16	16	0		2	0	2	0	8
S25/1aroA [6ug\well]	0	0	0	0	0		0	0	0	8	0		2	0	2	0	4
LAMB	1	2	3	4	5		1	2	3	4	5		1	2	3	4	5

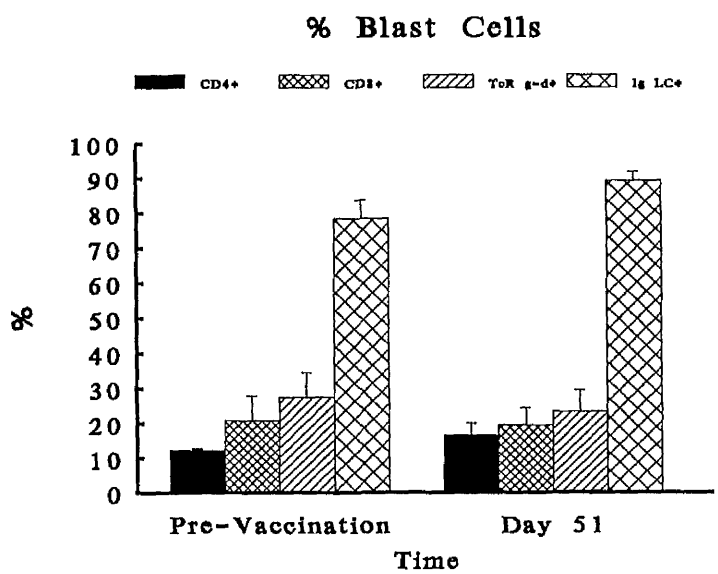
Group 1 lambs were inoculated orally with 10^{11} CFU of S25/1aroA on days 0, 2, 4 and 24. Before vaccination, and on days 24 and 35, PBMC from each lamb were cultured without antigen, with ConA and with decreasing dilutions of Salmonella antigen for 24, 48 and 96 h when titres of IFN-g in cell supernatants were determined. Only the 96-h results are shown as they were virtually identical to the 24- and 48-h readings.

phenotype of the cells that were proliferating in response to the Salmonella antigen. The percentages of the total cells and blast cells that expressed either CD4, CD8, the TcR g-d or the Ig LC were determined for lambs in Groups 2 [Fig. 4.6.1] and 3 [Fig.4.6.2] following stimulation of PBMC with Salmonella antigen, before and after vaccination with the aroA strain. The responses of both groups were very similar and were considered together. Before vaccination, the proliferative responses in the LTTs were very low [see Figs 4.5.2 and 4.5.3], and 26% of the total cells were B cells of which 78% were blast cells [Figs. 4.6.1 and 4.6.2]. After vaccination, proliferative responses were high and there was approximately a 2-fold increase in the percentage of B cells, of which approximately 90% were blast cells. There was no increase in either the total number of CD4+, CD8+ or TcR g-d+ cells, or in the number of blast cells expressing these molecules following vaccination.

It would seem that most of the proliferation to Salmonella antigen in the LTTs was due to B cells, which would explain the absence of IFN-g in response to antigen in these 2 groups and the low levels of serum IgG.

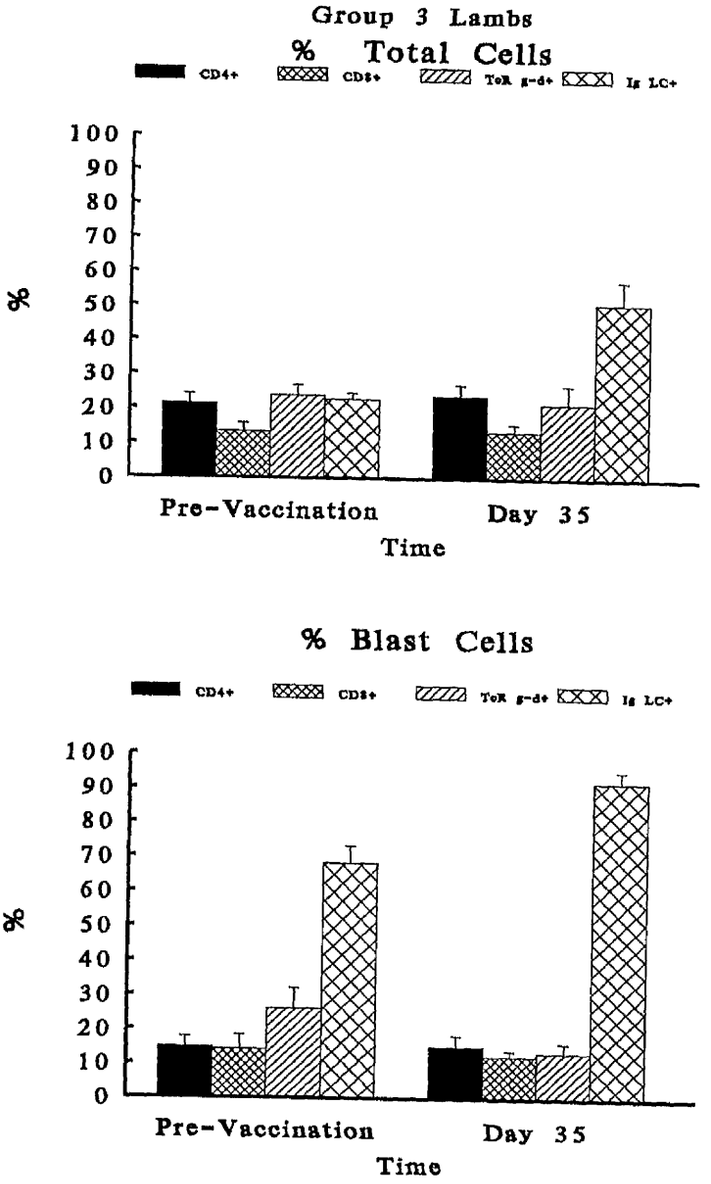
Figure 4.6.1 Phenotype of Cells Responding to Salmonella Antigen in the LTTs





PBMC from 5 lambs [Group 2] were stimulated with Salmonella antigen before and 51 days after vaccination with 4 doses of 5-9 x 10^{11} CFU S25/1aroA. The mean percentage of both the whole [total] cells and the blast cells that were CD4+, CD8+, TcR g-d+ and Ig LC+ were compared at these 2 time-points.

Figure 4.6.2 Phenotype of Cells Responding to Salmonella Antigen in the LTTs



PBMC from 5 lambs [Group 3] were stimulated with Salmonella antigen before and 35 days after vaccination with 4 doses of 5-9 x 10^{11} CFU S25/1aroA. The mean percentage of both the whole [total] cells and the blast cells that were CD4+, CD8+, TcR g-d+ and Ig LC+ were compared at these 2 time-points.

4.2.7 Challenge Of Vaccinated Lambs with Lethal Doses of Virulent S25\1

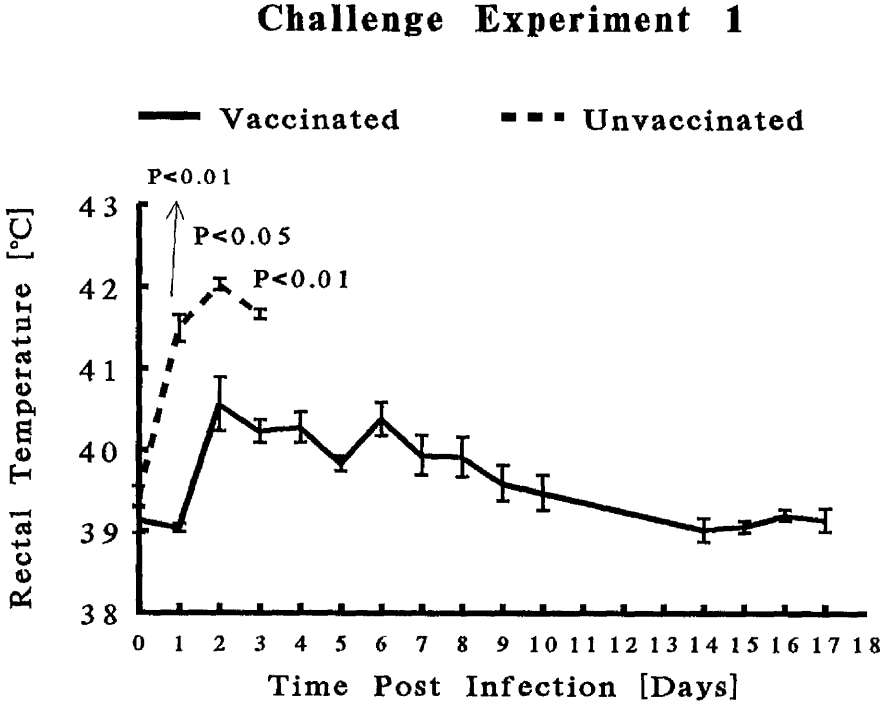
The 3 groups of lambs vaccinated orally with 10^{11} CFU of S25/1 aroA, were challenged with virulent S25\1 in 3 separate challenge experiments.

4.2.7.1 Challenge Experiment 1

The lambs in Group 1 were challenged orally with 8 x 10^9 CFU of virulent S25\1, 40 days after the first vaccination dose. A control group of 5 unvaccinated lambs [numbered 37-41] was challenged in the same way. The mean rectal temperature responses after challenge are shown in Figure 4.7. After 48 h, pyrexia was observed in all the vaccinated lambs [0.7-2.4^{o}C], but they all looked well and retained healthy appetites throughout the course of the challenge. By 72 h, the temperatures of the lambs had dropped, although pre-vaccination temperatures were not reached in all the lambs until 14 days post-challenge. The mean rectal temperatures were significantly lower in the vaccinated lambs than in the unvaccinated lambs on each day after infection.

Only 1 lamb [22] developed diarhoea [from days 7-12], but it remained clinically well throughout and, of the other 4 lambs, one produced normal faecal pellets throughout the course of the challenge and the other 3 showed only a slight softening of the faeces. When the lambs were culled on day 18 after challenge, most

Figure 4.7 Rectal Temperature Responses in Vaccinated and Unvaccinated Lambs After Oral Challenge with 10^9 CFU of Wildtype S25/1



5 lambs [Group 1] vaccinated orally 40 days previously with 4 doses of 5-9 x 10^{11} CFU of S25/1aroA, and 5 unvaccinated lambs were challenged orally with 8 x 10^9 CFU of wildtype S25/1 and the mean rectal temperatures for the 2 groups recorded on each day post-challenge.

TABLE 4.6
Recovery of the Wildtype Strain from aroA-Vaccinated and Unvaccinated Lambs Challenged with 10^9 CFU of Wildtype S25/1

No. CFU/g of Tissue

GROUP	VACCINATED						UNVACCINATED				
LAMB NO.	22	23	24	25	26		37	38	39	40	41
HEART	0	6	0	0	0		4	0	10^3	250	0
LUNG	0	0	0	0	0		41	12	437	0	10^3
BRONCHIAL LN	0	0	0	0	6		0	66	10^4	0	10^3
LIVER	0	0	0	0	0		87	19	10^3	21	51
HEPATIC LN	0	0	0	0	0		172	28	487	+	294
SPLEEN	0	0	0	0	0		8	4	10^5	50	95
KIDNEY	0	0	0	0	0		+	0	10^3	5	286
MESENTERIC LN	0	0	2	4	0		10^4	10^4	10^3	10^4	10^4
ILEUM	0	0	0	0	0		400	6	10^6	153	184
CAECUM	0	0	0	15	0		10^6	10^5	10^7	10^6	10^3
COLON	0	0	0	3	0		10^3	10^7	10^7	10^3	10^3

LN = Lymph Node
\+ = Organism only detected after enrichment in selenite broth
0 = Organism not detected, even after enrichment
NT = Not Tested

5 lambs vaccinated 40 days previously with 4 doses of 10^{11} CFU S25/1*aroA*, and 5 unvaccinated lambs were challenged orally with 8 x 10^9 CFU of wildtype S25/1. The unvaccinated lambs were culled *in extremis* on day 3 and the vaccinated lambs were culled on day 18 when the no. CFU/g of tissue were determined for each lamb.

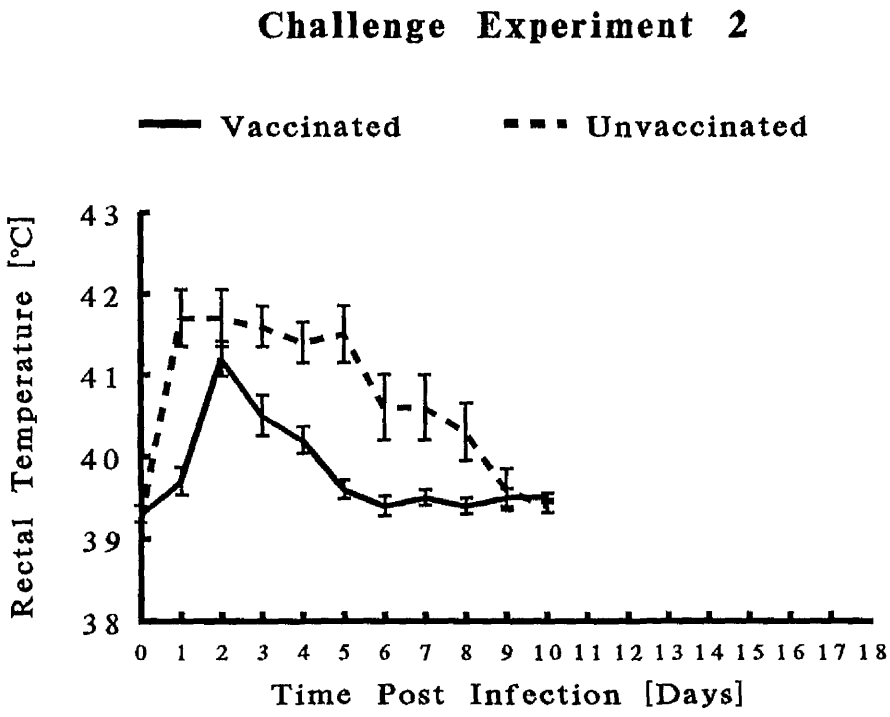
of the tissues tested from the 5 animals were free of the challenge strain and where the strain was recovered, it was only present at very low levels [Table 4.6]. The aroA vaccine strain could still be cultured from the organs of 3 of the lambs at this time.

In contrast to these mild clinical responses to virulent challenge in vaccinated animals, unvaccinated lambs developed a lethal infection as described earlier [4.2.1]. Pyrexia, loss of appetite and lethargy were observed and the lambs were culled in extremis on day 3. At this time, the colon, caecum and mesenteric lymph nodes of all the lambs harboured large numbers of virulent salmonellae, but lamb 39 was the only one to harbour high numbers in other tissues [Table 4.6].

4.2.7.2 Challenge Experiment 2

The lambs in Group 2 were inoculated orally with 1 x 10^{10} CFU of virulent S25\1, 87 days post-vaccination with S25/1aroA. 2 unvaccinated control animals [numbered 42 and 43] were challenged in the same way. The mean rectal temperatures of the vaccinated and unvaccinated groups are shown in Fig 4.8. After 48 h, pyrexia was observed in all of the vaccinated lambs [1.2- 2.0^{o}C], although they remained bright and had good appetites throughout the course of the challenge. Temperatures had returned to normal by day 5 in all vaccinated lambs. The mean rectal temperatures were lower in the vaccinated lambs than in the unvaccinated lambs on day 1 and days 3-8 after infection. All lambs showed a drop in the %DW of

Figure 4.8 Rectal Temperature Responses in Vaccinated and Unvaccinated Lambs After Oral Challenge with 10^{10} CFU of Wildtype S25/1



5 lambs [Group 2] vaccinated orally 87 days previously with 4 doses of 5-9 x 10^{11} CFU of S25/1*aroA*, and 2 unvaccinated lambs were challenged orally with 1 x 10^{10} CFU of wildtype S25/1 and the mean rectal temperatures for the 2 groups recorded on each day post-challenge.

TABLE 4.7
Recovery of the Wildtype Strain from aroA-Vaccinated and Unvaccinated Lambs Challenged with 10^{10} CFU of Wildtype S25/1

No. CFU\g of Tissue

GROUP	VACCINATED						UNVACCINATED	
LAMB NO.	27	28	29	30	31		42	43
HEART	0	0	0	0	0		0	0
LUNG	0	0	0	0	0		0	30
BRONCHIAL LN	0	0	0	0	0		0	0
LIVER	0	0	0	0	0		800	241
HEPATIC LN	2	0	0	0	0		326	10^3
SPLEEN	0	2	0	0	0		420	0
KIDNEY	0	0	0	0	0		0	0
MESENTERIC LN	0	0	6	0	0		10^3	10^5
ILEUM	0	0	0	0	0		+	800
CAECUM	0	0	0	0	0		0	0
COLON	0	3	0	0	0		+	400

LN = Lymph Node
\+ = Organism only detected after enrichment in selenite broth
0 = Organism not detected, even after enrichment
NT = Not Tested

5 lambs inoculated orally 87 days previously with 4 doses of 10^{11} CFU of S25/1aroA, and 2 unvaccinated lambs were challenged orally with 1 x 10^{10} CFU of wildtype S25/1. Vaccinated lambs were culled on day 18 and the unvaccinated lambs on days 13 and 18 for lambs 42 and 43 respectively, when the no. CFU/g of tissue were determined for each lamb.

faeces [mean decrease of 10%], but none scoured. On day 18, only 4 of the organs tested from all of the lambs harboured detectable levels of the virulent strain [Table 4.7] and only 3 contained levels of the S25/1aroA vaccine strain.

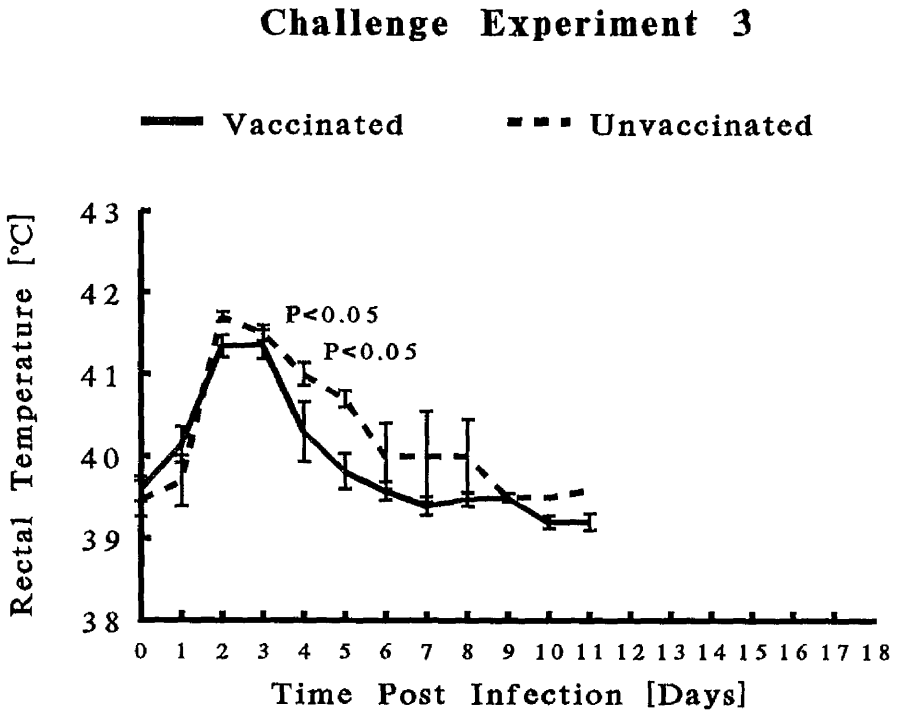
After 24 h, a dramatic rise in rectal temperature was seen in both unvaccinated lambs [increases of 2.1°C and 2.8°C for lambs 42 and 43 respectively]. By day 3, the temperature of both the lambs was off the thermometer scale [>42°C] and they showed a loss of appetite although they never looked as clinically ill as the unvaccinated lambs challenged previously. However lamb 42 began to scour on day 7 but within 2 days was producing faecal pellets again. Lamb 43 showed an appreciable drop in the %DW of faeces between days 4 and 7 [from 52% to 19%] but was producing normal pellets by day 9. The temperatures of of both lambs had returned to pre-vaccination readings by day 10. Lamb 42 was culled on day 13 and lamb 43 on day 18 and the no. CFU\g of tissue determined [Table 4.7]. Only the mesenteric lymph nodes harboured substantial levels of the virulent organism. The failure of these 2 lambs to develop a lethal infection similar to that found in the unvaccinated animals in the previous section given a lower challenge dose, was surprising. However it should be noted that one lamb given the wildtype strain in section 4.2.2 also failed to develop a lethal infection.

4.2.7.3 Challenge Experiment 3

The lambs in Group 3 were inoculated orally with 2 x 10^{11} CFU of wildtype S25/1, 40 days post-vaccination with S25/1aroA. A control group of 3 unvaccinated lambs [numbered 44-46] was challenged in the same way. The mean rectal temperatures of both vaccinated and unvaccinated lambs are shown in Figure 4.9. After 48 h, a dramatic increase in the rectal temperature of all 5 vaccinated lambs was seen [range 1.5-2.4^{o}C]. However the animals remained bright and retained their appetite throughout the experiment. By day 6, the rectal temperatures of all the vaccinated animals had returned to pre-vaccination readings. One lamb[34] started to scour on day 5, but within 24 h was pelleting again. The other 4 lambs produced faecal pellets throughout the course of the challenge. On day 14 only the mesenteric lymph node of lamb 33 was shown to harbour the challenge strain, albeit at very low levels [Table 4.8]. The aroA vaccine strain was detected in trace amounts in only 3 of all the organs tested.

All 3 unvaccinated lambs showed a dramatic increase in rectal temperature, and there was an appreciable drop in the %DW of faeces. However the mean rectal temperatures were only significantly higher [$p<0.05$] than the vaccinated lambs on days 4 and 5. Lamb 4 was culled in extremis on day 4 and the virulent organism was recovered in high numbers from all tissues tested [Table 4.8]. Of the remaining lambs, lamb 45 remained bright throughout the study and by day 14, its temperature and %DW of

Figure 4.9 Rectal Temperature Responses in Vaccinated and Unvaccinated Lambs After Oral Challenge with 10^{11} CFU of Wildtype S25/1



5 lambs [Group 3] vaccinated orally 40 days previously with 4 doses of 5-9 x 10^{11} CFU of S25/1*aroA*, and 3 unvaccinated lambs were challenged orally with 3 x 10^{11} CFU of wildtype S25/1 and the mean rectal temperatures for the 2 groups recorded on each day post-challenge.

TABLE 4.8
Recovery of the Wildtype Strain from aroA-Vaccinated and Unvaccinated Lambs Challenged with 10^{11} CFU of Wildtype S25/1

No. CFU\g of Tissue

GROUP	VACCINATED						UNVACCINATED		
LAMB NO.	32	33	34	35	36		44	45	46
HEART	0	0	0	0	0		10^3	0	0
LUNG	0	0	0	0	0		10^7	0	0
BRONCHIAL LN	0	0	0	0	0		10^7	10^3	0
LIVER	0	0	0	0	0		10^7	+	0
HEPATIC LN	0	0	0	0	0		10^8	350	0
SPLEEN	0	0	0	0	0		10^8	0	0
KIDNEY	0	0	0	0	0		10^6	35	0
MESENTERIC LN	0	0	0	29	0		10^8	10^5	10^3
ILEUM	0	0	0	0	0		10^8	NT	NT
CAECUM	NT	NT	NT	NT	NT		10^9	NT	NT
COLON	NT	NT	NT	NT	NT		NT	NT	NT

LN = Lymph Node
+ = Organism only detected after enrichment in selenite broth
0 = Organism not detected, even after enrichment
NT = Not Tested

5 lambs inoculated orally 40 days previously with 4 doses of 10^{11} CFU of S25/1*aro*A, and 3 unvaccinated lambs were challenged orally with 2 x 10^{11} CFU of wildtype S25/1. The vaccinated lambs were culled on day 14 and the unvaccinated lambs were culled on days 5, 9 and 14 for lambs 44, 45 and 46 respectively, when the no. CFU/g of tissue were determined for each lamb.

faeces had returned to normal and only the mesenteric lymph node harboured virulent salmonellae. Lamb 46 started to look clinically ill on day 6, despite normal temperature, and was culled *in extremis* on day 9. However, only the mesenteric lymph node contained substantial amounts of the challenge organism. This was the first animal that had succumbed to infection without excreting or harbouring in the tissues, large numbers of the challenge strain.

The results of these challenge studies have shown that the S25/1*aro*A strain can protect lambs from a lethal challenge dose of wildtype S25/1, however the failure of some of the unvaccinated lambs to develop a lethal infection has highlighted the variability in the susceptibility of lambs to experimental salmonellosis.

4.3 Discussion

The initial studies described in this chapter sought to characterise the infection established in lambs following oral challenge with wildtype S25\1. Lambs inoculated with 10^9 CFU or higher succumbed to infection and high numbers of the organism were recovered from the tissues. These findings are in contrast to those of Brown *et al*. [1976], who estimated an oral LD_{50} for *S.typhimurium* in lambs to be 2×10^{11} CFU, suggesting that the S25/1 isolate was particularly virulent for sheep.

Following these studies, parallel studies were undertaken using the S25/1 *aro*A vaccine strain. Lambs inoculated orally with 10^9 or 10^{11} CFU S25/1*aro*A did not develop any of the clinical symptoms

associated with the virulent strain, harboured only low levels of the organism in the tissues, and did not succumb to infection. The relative virulence of the wildtype and aroA strains reflected the fact that only the wildtype organism grew in large numbers in the gut wall, menteric lymph nodes and other tissues.

The immune responsiveness of animals given the aroA strain reflected the levels of organ colonisation. Lambs given 10^9 CFU of the mutant strain, harboured very few organisms in the tissues and consequently were immunologically unresponsive to the vaccine strain. Lambs given 4 doses of 10^{11} CFU of the vaccine strain harboured greater numbers of the aroA strain in the tissues and all lambs produced Salmonella-specific serum IgM. However levels of Salmonella-specific IgG were much lower than IgM levels and Salmonella-specific coproantibody [sIgA] was virtually absent.

Mukkur et al. [1987] detected Salmonella-specific serum antibody following oral vaccination with aroA S.typhimurium. However susequent studies by the same group showed that only intravenous administration of the aroA strain produced specific serum antibody [Begg et al., 1990]. The specific serum antibody responses described in this chapter are encouraging in view of these results. The absence of specific sIgA in the lambs may not be surprising in view of the findings of Lascelles et al. [1988] who were unable to detect Salmonella-specific IgA-producing cells in intestinal lymph

following repeated oral vaccination with an aroA S.typhimurium strain. The lack of a mucosal immune response may be a result of low level mucosal stimulation by the aroA strain [discussed in Chapter 7].

The PBMC from the lambs given 10^{11} of the S25/1aroA strain showed strong specific responses following vaccination. The SIs often varied considerably from one time-point to another post-infection as a result of variation in background counts between assays. The CPM values were relatively unaffected by background variability and may give a clearer indication of lymphocyte responsiveness. As sheep lymphocytes are unresponsive to the mitogenic effects of LPS [Burrells and Wells, 1977] compared to murine lymphocytes [Peavey et al., 1974], the polyclonal stimulation of B cells by the LPS in the heat-killed antigen preparation, which clouded specific responses in the mouse studies, was not a problem here, perhaps as a result of tolerance through repeated exposure to bacterial endotoxin.

Unlike earlier studies [Mukkur et al., 1987], I was unable to detect specific DTH responses to aroA S.typhimurium after oral inoculation. However subsequent studies have confirmed my findings [Begg et al., 1990]. The choice of LPS as a measure of T cell responsiveness by these groups was a curious one, as it is a T cell- independent B cell mitogen. The increases in skinfold thickness to the LPS observed by Mukkur et al. [1987] was probably an inflammatory response to the pyrogenic LPS.

Although strong Salmonella-specific responses were observed in LTTs, very few lambs produced IFN-g, suggesting that it was the B cells, which do not produce IFN-g, that were responding. The other possibility was that a population of T cells that does not produce IFN-g, such as Th2 cells, was responding specifically in the LTT. The lack of IFN-g production following ConA stimulation was surprising. However, such findings have been reported by others for both normal mouse and sheep lymphocytes [A. McI, Mowat, M. Bruce, MRI, personal communications] where IFN-g production may be dependent on the time of exposure of the cells to ConA. The fact that those sheep that produce IFN-g to antigen stimulation also produced IFN-g to ConA, suggests that some sheep inherently produce greater levels of IFN-g than others, perhaps as a result of increased mucosal stimulation by gut flora which may make the lymphocytes more responsive to antigen or mitogen. These differences may also reflect the outbred nature of the animals.

Phenotypic analysis showed that the cells responding to antigen were predominantly B cells. This could account for the lack of IFN-g in PBMC culture superntants from these lambs, predominance of IgM in the serum and lack of IgG\IgA, as these latter responses are T cell-dependent. However the levels of proliferation to Salmonella antigen in the LTTs were too high to be solely a result of B cell proliferation. IL-2 and IL-4 from Th cells would be required to effect such B cell proliferation, but phenotype analysis did not reveal the presence of such T cells. One

possibility is that T cell proliferation did occur in these cultures, but that this was before the time I observed the B cell proliferation on day 4. However, there was no increase in the absolute numbers of T cells suggesting that this was not the case.

Despite the absence of strong vaccine-specific responses, oral vaccination with 4 doses 10^{11} CFU S25/1aroA protected lambs from oral challenge with 8×10^{9}, 1×10^{10} and 2×10^{11} CFU of S25\1, with much reduced clinical symtoms following challenge. However the results were clouded somewhat by the failure of 3 of 10 unvaccinated lambs [2 given 1×10^{10} CFU and 1 given 2×10^{11} CFU] to succumb to infection. Similar problems were encountered by other workers. Mukkur et al. [1987] showed that doses of 1×10^{9} CFU of virulent S.typhimurium were sufficient to cause mortality in all unvaccinated animals. However when this experiment was repeated using the same challenge dose, none of the unvaccinated animals succumbed to infection and a ten-fold higher dose had to be given to invoke death in all the unvaccinated animals [Begg et al., 1990]. Thus, there appears to be considerable variation in the susceptibility of these outbred animals to a lethal infection with wildtype S.typhimurium. Nevertheless, when considering all the challenge studies carried out throughout this chapter, the results of the protection studies were encouraging. A total of 18 unvaccinated lambs were challenged with a potentially lethal dose of the wildtype strain, of which 14 succumbed to infection while

none of 15 vaccinated lambs died. However, since aroA-vaccinated lambs were protected in the absence of strong vaccine-specific responses, the nature of the protective response operating in these animals is unclear.

Chapter 5

The Immune Response to the Major Outer Membrane Protein [MOMP] of C.psittaci Expressed by the S25/1aroA Vaccine Strain

5.1 Introduction

The previous 2 chapters highlighted the ability of the S25/1aroA vaccine strain to colonise the organs of mice and sheep and induce protective immunity against wildtype challenge in these species. The next stage was to examine whether this vaccine strain could act as a vector for delivery of a heterologous antigen.

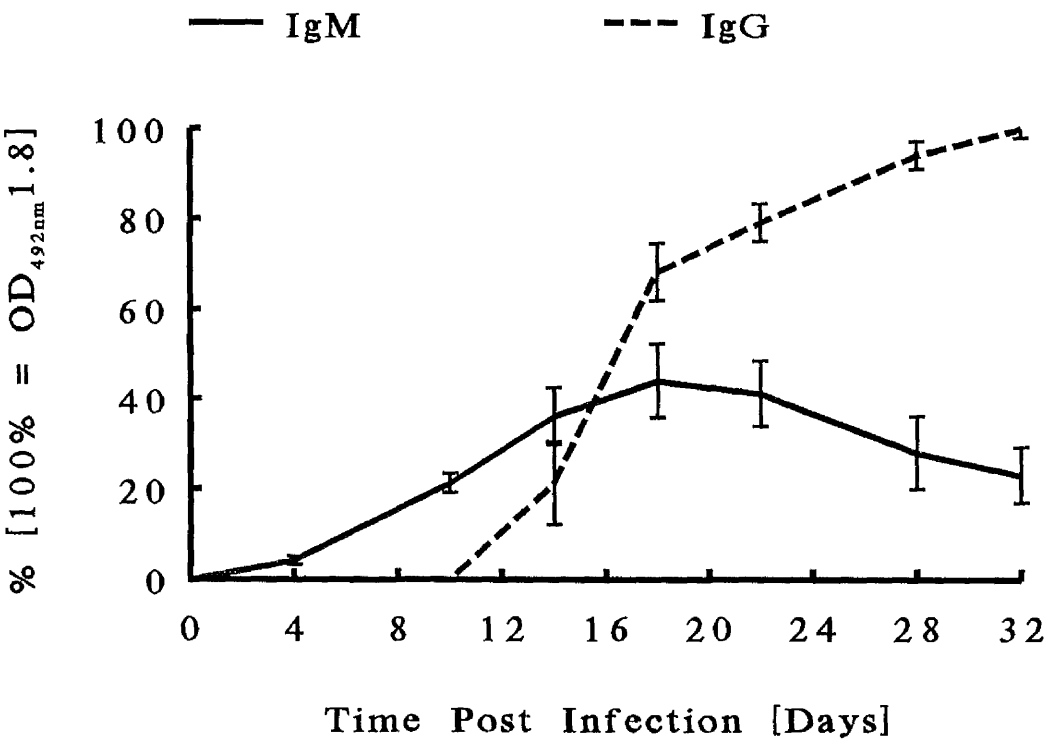
In this chapter, the ability of the S25/1aroA strain to deliver the MOMP of C.psittaci to the immune system of both mice and lambs was examined. Two recombinant Salmonella strains were available. The pD strain expressed the first 4 variable domains of the MOMP and the pC strain expressed domains 2-4 constituting a truncated form of the MOMP. The in vivo expression of the protein was monitored and an attempt was made to relate this to the production of MOMP-specific immune responses.

5.2 Results

5.2.1 Immunogenicity of Recombinant MOMP in Mice

Initial studies sought to determine if the recombinant MOMP [rec MOMP] protein itself was immunogenic in mice to give some idea of the responses likely to be induced when presented by the vaccine strain.

Figure 5.1 Levels of MOMP-Specific Serum IgM and IgG in Mice After Immunisation with MOMP in CFA



Mice were culled at times post-infection and the sera examined in an ELISA in which the coating antigen was SDS-purified rec MOMP. Each point represents the mean OD_{405nm} obtained with the sera of 5 mice. The mean OD_{405nm} is expressed as a percentage where 100% represents OD_{405nm} 1.8. Serum was diluted 1:100 and 1:400 for IgM and IgG respectively.

The primary immune response to 4 ug of rec MOMP in CFA was examined. MOMP-specific IgM started to appear as early as 3 days following injection of the antigen preparation and rose steadily thereafter, peaking on day 18 [Figure 5.1]. IgG levels started to rise on day 10 and continued to rise, remaining high until the termination of the experiment on day 32. The levels of IgM were considerably lower than IgG levels.

T Cell Responses

Spleen cells from 6 immunised and 4 control mice were examined on day 36 for their *in vitro* responsiveness to either Elementary Bodies [EBs] of *C.psittaci* [which represent native MOMP] or with synthetic peptides of MOMP, VD2 and VD4 [constructed and kindly gifted by Dr. A. Herring, MRI], which contain sequences of the second and fourth variable domains of MOMP respectively. Recombinant MOMP was not used, as the purified rec MOMP protein used to challenge the mice [and used in the ELISA] contained high levels of SDS from the purification procedure which was toxic to the lymphocytes. The responses to all 3 preparations in the MOMP-challenged mice were not significantly greater than those in the unvaccinated group [results not shown].

5.2.2 Specific Immune Responses in Mice following Infection with Live C.psittaci

As no T cell responses were detectable after immunisation with purified MOMP, it was important to determine if murine T cells would recognise native MOMP and/or the peptides after infection

TABLE 5.1
Specific Humoral and Cellular Responses of Mice Infected with Live EBs of C.psittaci

GROUP [a]	MOUSE	ANTIBODY TITRE TO MOMP [b]	CPM to EBs	SI to EBs	IFN to EB STIMULATION
1	1	32	36,314	36.5	NT
	2	128	5,510	8.5	NT
	3	128	8,320	6.0	NT
2	1	4096	25,965	21.5	+
	2	256	16,590	25.7	+
	3	64	3,504	10.5	+
	4	NT	55,626	17.2	NT
3	1	128	2,277	37.7	+
	2	128	12,335	63.2	+
4	1	32	12,028	69.3	+
	2	128	11,931	26.8	+
	3	128	42,961	74.6	+
5	1	16	62,606	36.5	+
	2	128	49,592	48.9	+
6 UNVACC	1	0	820	4.5	-
	2	0	1020	5.2	-

+/- = Presence/absence of IFN in EB-stimulated spleen cell supernatants.
NT = Not Tested.

Mice infected with live C.psittaci were culled on day 36 and the sera assayed in a rec MOMP ELISA and the proliferative responses and production of IFN to stimulation with Elementary Bodies [EBs] by spleen cells was determined. [a] Groups 1-5 received $1x10^6$ Inclusion Forming Units [IFU], $4x10^5$ IFU, $2x10^5$ IFU, $1x10^5$ IFU and $5x10^4$ IFU respectively. Group 6 was the uninfected control group. [b] Titres are expressed as the inverse of the highest serum dilution with OD_{405nm} greater than NMS.

with the live organism. Mice were infected intraperitoneally with different doses of live EBs of an ovine abortion strain, S26/3, of C.psittaci [kindly gifted by M.McCafferty, MRI]. 36 days post-challenge, all mice produced MOMP-specific antibodies although the levels did not correlate with the size of the infecting dose [Table 5.1].

T Cell Responses

On day 36, spleen cells were also cultured with EBs and synthetic peptides VD2 and VD4 in an LTT. None of the Chlamydia-challenged or the unvaccinated mice responded to either of the peptides [results not shown]. However, all of the Chlamydia-challenged mice responded strongly to the EB preparation [Table 5.1] and the responses of the unvaccinated mice were negligible by comparison. The levels of lymphocyte responsiveness did not correlate with the infecting dose, nor with the antibody levels in the same mouse. The majority of responsive cultures also produced IFN, although the neutralisation assays were not performed to prove the identity of IFN-g [Table 5.1]

5.2.3 Inoculation of Mice with S25/1aroA Expressing the MOMP of C.psittaci

The previous sections indicated that both recombinant and native MOMP could be recognised by the murine immune system, and I went on to examine if it would be possible to evoke an immune response to the MOMP expressed by a Salmonella vaccine strain. First, I

investigated if the presence of the plasmid and /or the production of a foreign protein altered the growth of the S25/1aroA strain *in vitro* and *in vivo*, or its capacity to evoke a *Salmonella*-specific immune response in the murine host.

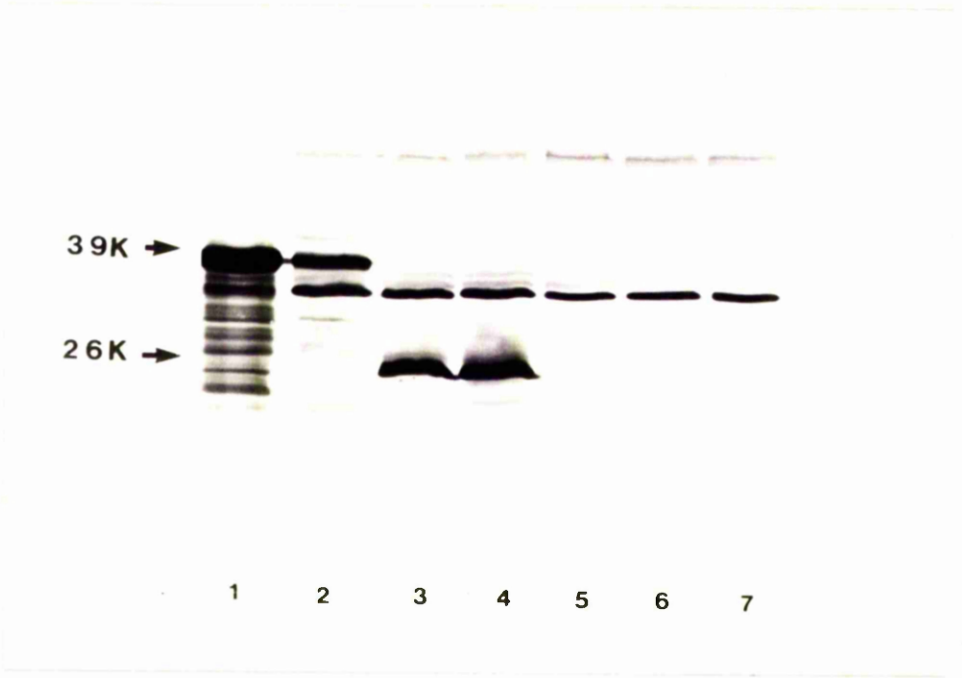
5.2.3.1 In Vitro Growth Rates of the Recombinant Strains

The relative *in vitro* growth rates of the strains are shown in Appendix 6. The growths of the pD, pC and pUC8 strains did not vary significantly from that of the S25/1 aroA strain without a plasmid.

5.2.3.2 Production of rec MOMP by the pD and pC Strains

Both the pD and pC strains were shown to contain plasmids, with inserts of the correct size [see Table 2.1] and samples of each strain were checked for the production of MOMP. Bacterial cell lysates were separated by SDS-PAGE, blotted onto nitrocellulose and probed with the MOMP-specific monoclonal antibody, 4/11. Lysates of the pUC8 strain [without an insert] were also run as was a preparation of rec MOMP [produced in *Salmonella*]. The results are shown in Figure 5.2. The pD strain [lane 2] was shown to express the full 39K rec MOMP protein. Both the pC strain isolates [Lanes 3 and 4] were shown to produce the 26K truncated form of the MOMP, but in slightly greater quantities than the MOMP produced by the pD strain.

Figure 5.2 **Production of MOMP by the pD and pC Stock Strains**



Lane 1 : rec MOMP
2 : pD [isolate 1]
3 pC [isolate 1]
4 pC [isolate 2]
5 pUC8 [isolate 1]
6 pUC8 [isolate 2]
7 S25/1*aroA*

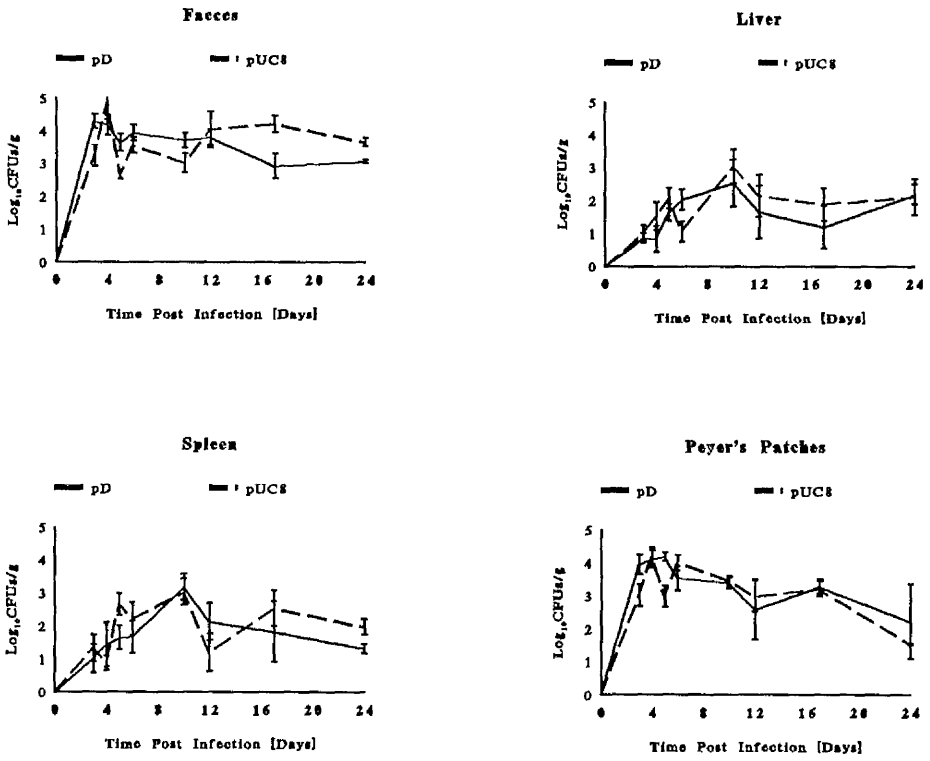
5.2.3.3 Invasiveness of the pD and pUC8 Strains in Vivo

Mice were allocated randomly into groups, one of which was inoculated orally with 5 x 10^8 CFU of the pD strain and the other with an identical dose of the control pUC8 strain. 4-5 mice from each group were culled on each of days 3, 4, 5, 6, 10, 12, 17 and 24 and the no. CFU/g of tissue determined for the faeces, liver, spleen and Peyer's patches. The results are shown in Figure 5.3. There was no significant difference in the levels of the pD and pUC8 strains recovered from the mice at any time, which were similar to those obtained previously with the S25/1aroA strain without a plasmid [see 3.2.3].

5.2.3.4 Maintenance of the Plasmid and MOMP Expression in Vivo

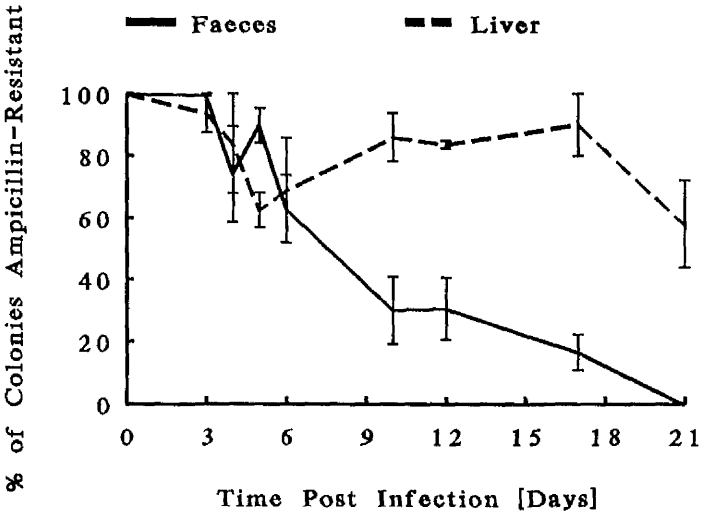
I next determined if the recombinant strains maintained expression of the MOMP gene in vivo, using ampicillin-resistance as a marker. Mice were orally inoculated with 3 x 10^8 CFU of the pD strain. 3-5 mice were culled on each of days 3, 4, 5, 6, 10, 12, 15 and 20 and the percentage of the colonies that were ampicillin-resistant was determined. The results are shown in Figure 5.4. On days 2 and 3, most of the recovered colonies were ampicillin-resistant. However, a significant level of plasmid segregation occurred after day 6. This continued until day 21, when virtually all the recovered colonies were sensitive to ampicillin. The liver sustained the level of plasmid expression for longer than the other organs.

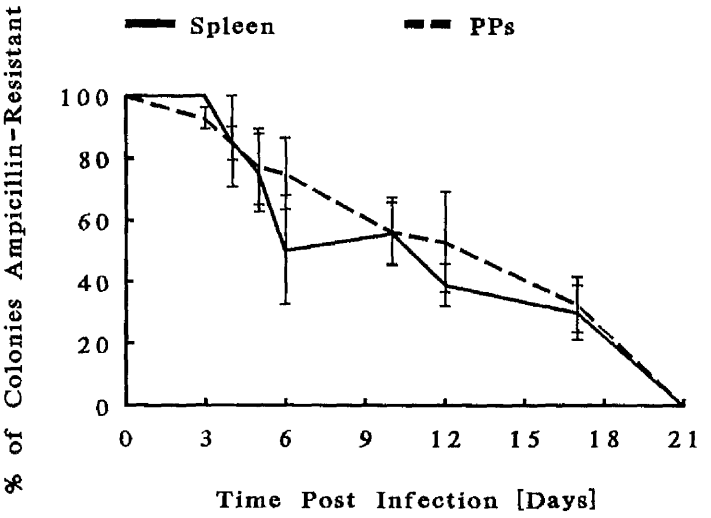
Figure 5.3 Recovery of the pD and pUC8 Strains fom Mice After Oral Infection



2 groups of mice were established of which one was inoculated orally with 5 x 10^8 CFU of the pD strain and the other with an identical dose of the pUC8 strain. 4-5 mice were culled from each group on the stated days post-infection and the mean no. CFU/g determined on each time-point for faeces, liver, spleen and Peyer's patches. Levels are expressed as $\log_{10}$ CFU/g.

Figure 5.4 Levels of Plasmid Expression by the pD Stain In Vivo After Oral Infection of Mice





Mice were inoculated orally with 3 x 10^8 CFU of the pD strain. 3-5 mice were culled on the stated days post-infection and the mean percentage of colonies recovered from the faeces, liver, spleen and Peyer's patches [PPs] that were ampicillin-resistant was determined for each time-point.

[A]

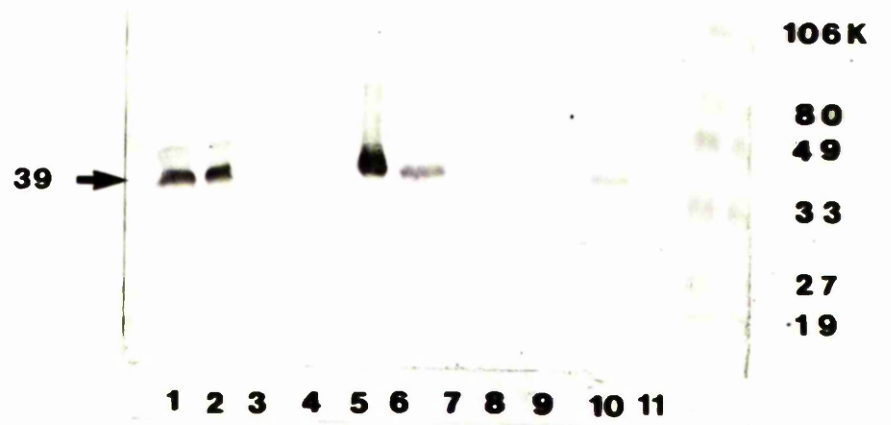
Lane 1 : pD stock isolate 1
2 : pD stock isolate 2
3 : pUC8 stock isolate 1
4 : pUC8 stock isolate 2
5 : rec MOMP
6 : pD, recovered from Peyer's patches, day 2
7 : pD, recovered from faeces, day 5
8 : pD, recovered from liver, day 7
9 : pUC8, recovered from liver, day 2
10: pD, recovered from Peyer's patches, day 9
11: pUC8, recovered from liver, day 5

[B]

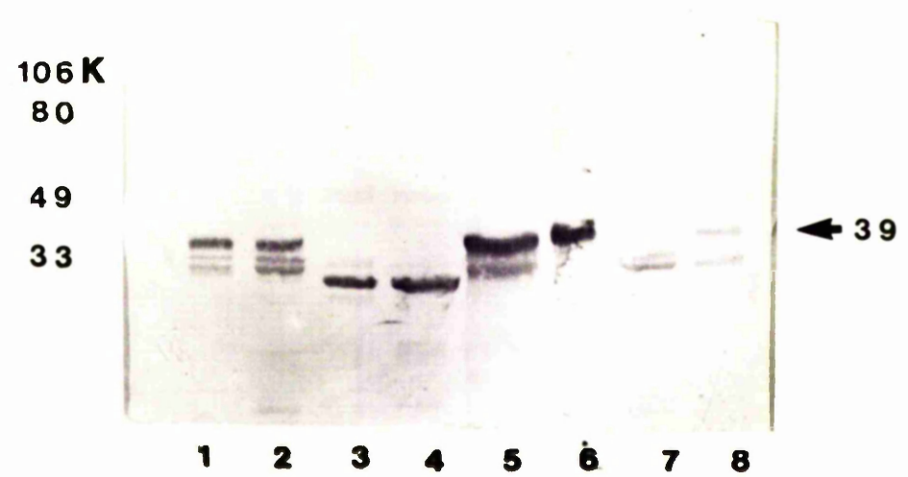
Lane 1: pD, recovered from liver, day 10
2 pD, recovered from faeces, day 15
3 pUC8, recovered from Peyer's patches, day 4
4 pUC8, recovered from Peyer's patches, day 5
5 pD, recovered from spleen, day 2
6 rec MOMP
7 pUC8 stock isolate 1
8 pD stock isolate 1

Figure 5.5 Production of MOMP by the pD Strain Recovered from Mice After Oral Infection

[A]



[B]



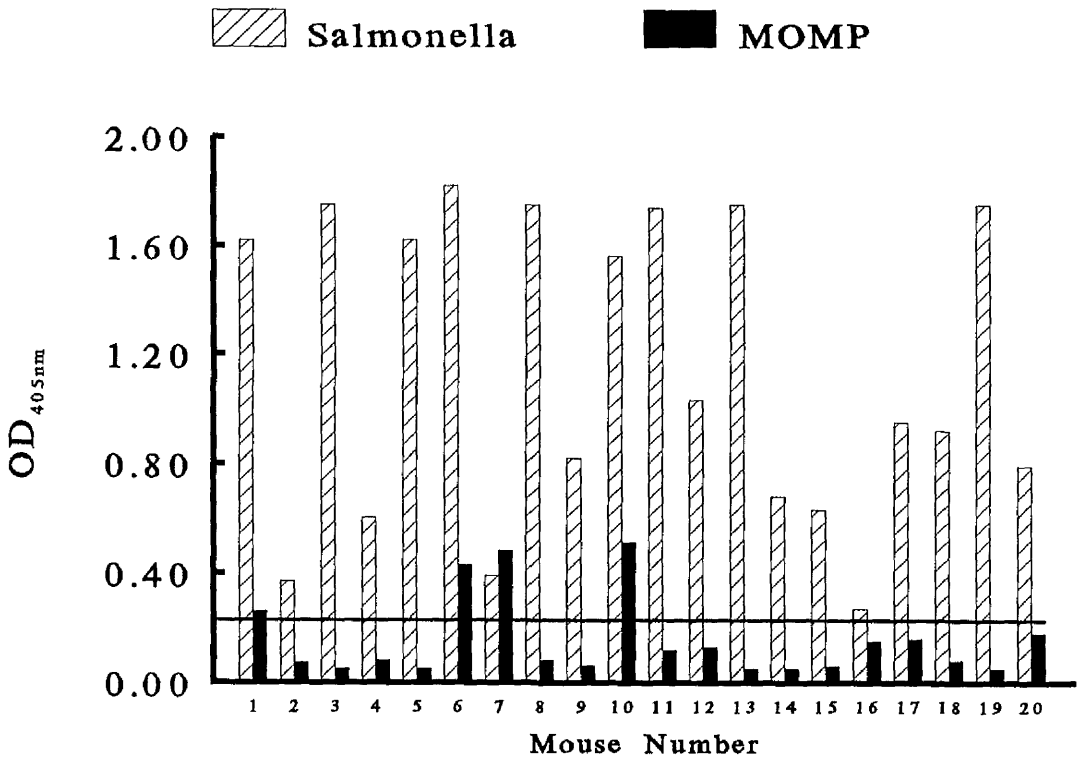
In an effort to reduce plasmid segregation *in vivo*, mice were fed 0.07 mg/ml ampicillin in their drinking water 48 h prior to inoculation and every 3 days thereafter, until termination of the experiment. It was found that this treatment had no effect on the levels of plasmid segregation [results not shown].

Bacterial lysates were made of ampicillin-resistant colonies recovered at the above times post-infection, and blotted proteins were probed with the 4/11 monoclonal antibody. The results are shown in Figure 5.5[A+B]. The pD colonies isolated on days 2,5,7,9, 10 and 15 post-inoculation all produced a unique band at 39 K representative of the rec MOMP protein and this band was absent from the pUC8 colonies. Thus the rec MOMP is expressed by the pD strain recovered from mice.

5.2.3.5 MOMP-Specific Immune Responses in Mice After Oral Infection with the pD Strain

Mice were inoculated orally with 5×10^8 CFU of either the pD or the pUC8 strain. 30 days post-challenge, the mice were culled and the serum examined for the presence of rec MOMP-specific and *Salmonella*-specific antibodies. The results are shown in Figure 5.6. All the mice produced detectable levels of IgG to *Salmonella*, although the levels varied considerably between individual mice. However, only 4 mice produced MOMP-specific IgG levels above those

Figure 5.6 Salmonella- and MOMP-Specific Serum IgG in Mice After Oral Infection with the pD and pUC8 Strains



[a] Each pair of columns represents an individual mouse. [b] Mice 1-10 were inoculated orally with 5×10^8 CFU of the pD strain and mice 11-20 received an identical dose of the pUC8 strain. [c] Serum was removed 30 days post-infection and diluted 1:200 for Salmonella and 1:16 for MOMP. [d] Hatched columns show IgG to Salmonella and filled columns show IgG to MOMP. [e] OD_{405nm} values of the tests were obtained by subtracting the OD_{405nm} of NMS. A line was drawn across the highest OD value of pUC8-infected mice and values above this were taken to be MOMP-specific.

found in mice vaccinated with the non-MOMP-expressing pUC8 strain. The levels of IgG to MOMP were much lower than for Salmonella and there was no correlation between the levels of antibodies to Salmonella and MOMP in individual mice.

T Cell Responses

On day 35, spleen cells from immunised mice were examined for in vitro responses against both Salmonella and MOMP. On this occasion, I used rec MOMP, prepared following blotting of 10ug of SDS-solubilised rec MOMP [to remove the SDS][see 2.8.2.5], as well as EBs and the synthetic peptides VD2 and VD4.

As shown in Chapter 3 for the S25/1aroA strain, all mice infected with either the pD or pUC8 strains produced strong proliferative responses to heat-killed Salmonella antigen [results not shown] which was accompanied by the production of IFN-g [titres of 4-16]. Table 5.2 shows the proliferative responses of 3 pD-infected, 3 pUC8-infected mice and 3 unvaccinated mice. Mouse 3 responded to EBs more strongly than any of the other mice. This mouse also responded to the VD2 peptide, as did mouse 2 which also reponded to VD4. None of the 3 pUC8-infected mice [mice 4-6] responded to either of the synthetic peptides or to the EBs. However 1 of the unvaccinated mice [mouse 8] also responded to both synthetic peptides, suggesting that the responses of mice 2 and 3 were non-specific. All 3 pD-infected mice responded to rec MOMP, however one of the pUC8-infected mice also responded suggesting that again these responses were non-specific. This

TABLE 5.2

Proliferative Responses of Spleen Cells from Mice Inoculated Orally with the pD or pUC8 Strains

CPM

	pD-infected [a]			pUC8-infected [b]			Control [c]		
MOUSE	1	2	3	4	5	6	7	8	9
EBs	859	528	2,003	63	33	332	11	593	422
VD2	0	1,311	1,591	323	0	276	49	3,376	25
VD4	0	1,524	548	173	0	116	0	2,239	0
recMOMP	3,855	767	720	328	0	3,028	0	320	0

SI

	pD-infected [a]			pUC8-infected [b]			Control [c]		
MOUSE	1	2	3	4	5	6	7	8	9
EBs	5	5.5	20	1.5	1.5	4	1.5	5	5
VD2	<1	12	16	5	<1	3.5	1	28	1
VD4	<1	14	6	3	<1	2	<1	19	1
recMOMP	11.5	6.5	6.5	4	<1	26.5	<1	2.5	1

[a] Mice received 5×10^8 CFU of the pD strain. [b] Mice received 5×10^8 CFU of the pUC8 strain. [c] Uninfected control. All mice were culled on day 35 and the proliferative responses of the spleen cells to EBs, VD2, VD4 and rec MOMP examined in an LTT.

assay was repeated with many more mice, with similar findings. Thus, it would seem that the MOMP expressed by the pD strain was incapable of eliciting specific T cell responses, however it may be that the rec MOMP preparation available may not have been ideal for the detection of MOMP-specific responses in mice.

5.2.3.6 MOMP-Specific Immune Responses of Mice Inoculated Repeatedly with the pD and pC Strains

In an effort to engender greater immune responses to the MOMP, mice were given multiple doses of the recombinant strains. Also, as strains harbouring the pC had been shown to produce slightly greater amounts of recombinant protein [see Figure 5.2], some mice were infected with this strain on this occasion. In addition, highly purified urea-solubilised rec MOMP had become available, which would maximise the chances of detecting MOMP-specific lymphocyte responses.

One group of mice was inoculated orally with 5×10^9 CFU of the pD strain on days 0 and 14 and with an identical dose of the pC strain on days 7 and 21. A control group received 5×10^9 CFU of the pUC8 strain on days 0, 7, 14 and 21. On days 21, 28 and 35, mice were culled and the serum and spleens removed and on this occasion, gut washes were also taken to examine for specific intestinal responses.

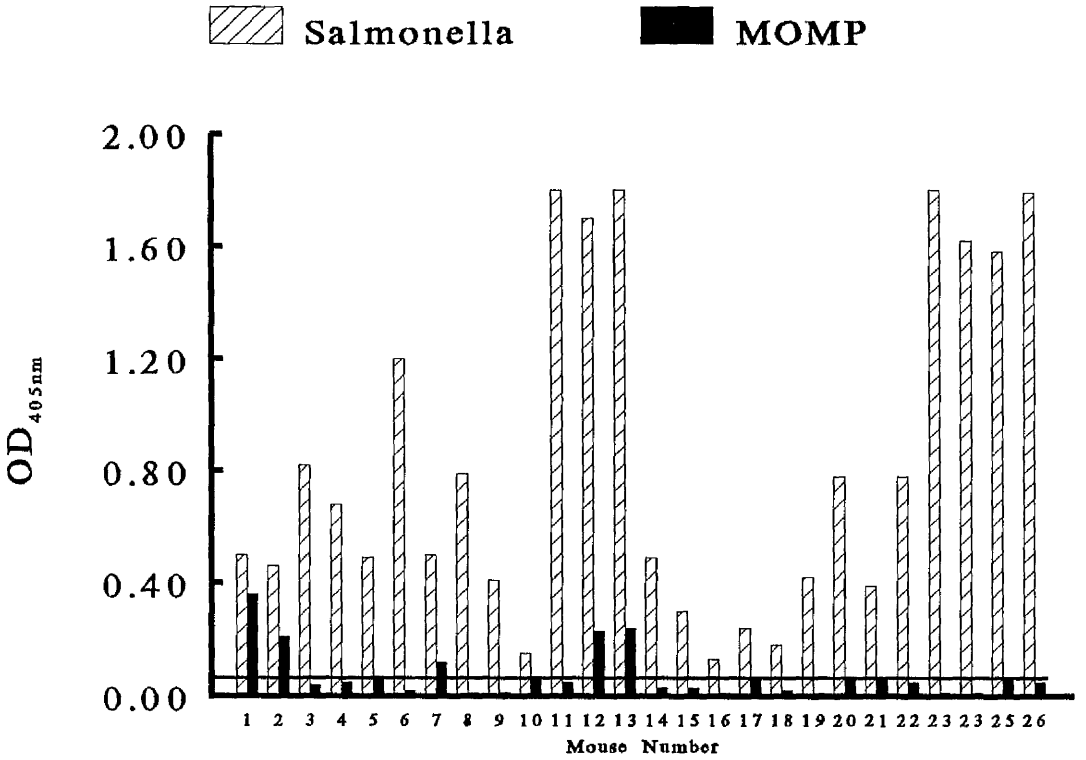
All mice in both groups produced Salmonella-specific IgG which were low on day 21, but had risen by day 28 and had reached high levels by day 35 [Figure 5.7]. However, only 5 mice given the pD/pC strains produced antibody to MOMP that was greater than those in the control mice infected with pUC8. As in the previous experiment, there was little correlation between the levels of Salmonella-specific antibody and MOMP-specific antibody.

The levels of Salmonella-specific and MOMP-specific intestinal IgA are shown in Figure 5.8. All infected mice had Salmonella-specific intestinal IgA with these being highest on day 35. Only 5 of the 15 mice given the pD/pC strains produced MOMP-specific IgA higher than that obtained in the gut washes from mice given the non-MOMP-expressing pUC8 strain, and again the levels were very low compared with the levels of Salmonella-specific IgA.

T Cell Responses

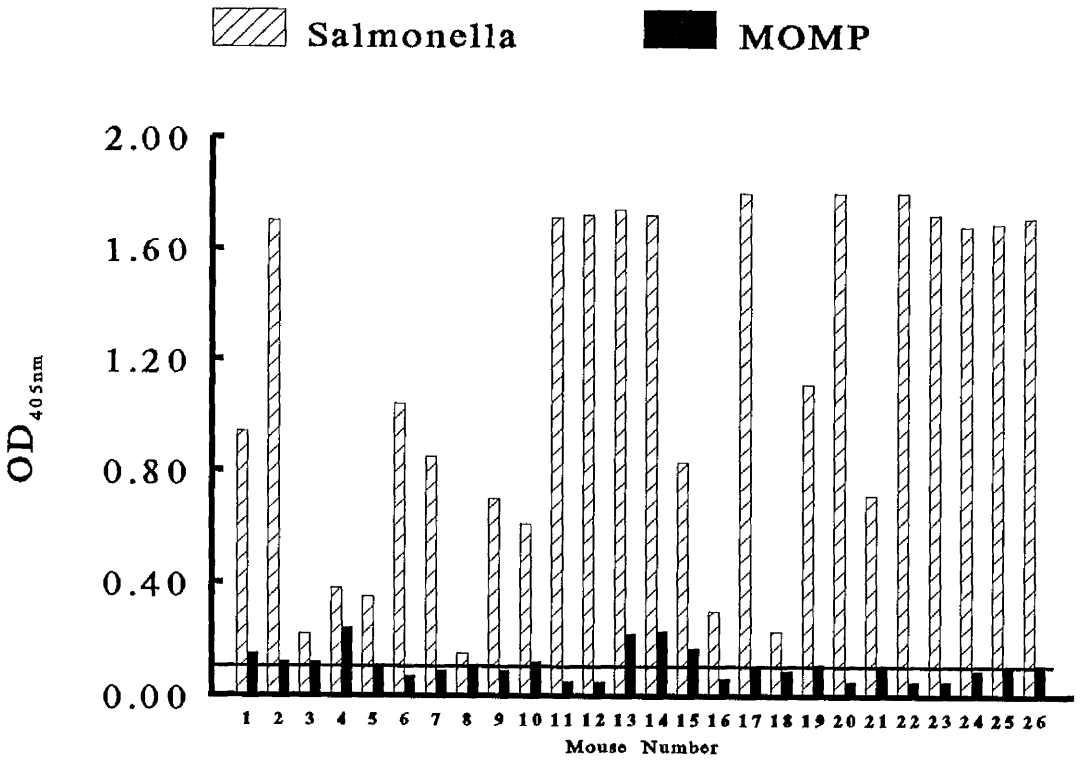
Spleen cells from mice culled on day 35 were removed and cultured with urea-solubilised purified rec MOMP [produced in Salmonella and gifted by M. McCafferty, MRI] and with synthetic peptides VD2 and VD4 in an LTT. The responses to Salmonella were also examined. The responses of 5 pD/pC-vaccinated and 3 pUC8-vaccinated mice are shown in Table 5.3. All mice produced strong proliferative responses to Salmonella which was associated with the production of IFN-g [titres of 8-16]. All the mice showed some responses to the rec MOMP, irrespective of whether they had been vaccinated with

Figure 5.7 Salmonella- and MOMP-Specific Serum IgG in Mice After Oral Infection with the pD/pC Strains or with the pUC8 Strain



[a] Each pair of columns represents an individual mouse. [b] Mice 1-15 received 2 doses each of the pD and pC strains and mice 16-26 were given identical doses of the pUC8 strain. [c] Mice 1-5 and 16-18 were culled on day 21, mice 6-10 and 19-22 on day 28 and mice 11-15 and 23-26 on day 35. [d] sera was diluted 1:100 for Salmonella and 1:16 for MOMP. [e] Hatched columns represent IgG to Salmonella and filled columns show IgG to MOMP. [f] OD_{405nm} values of the tests were obtained by subtracting the OD_{405nm} of NMS. A line was was drawn across the highest OD value of the pUC8-infected mice and values above this were taken to be MOMP-specifc.

Figure 5.8 Salmonella- and MOMP-Specific Intestinal IgA in Mice After Oral Infection with the pD/pC Strains and with the pUC8 Strain



Legend as for Figure 5.7, except: [a] Gut lavages were diluted 1:2 for both Salmonella and MOMP. [b] OD_{405nm} values of the test were obtained by subtracting the mean OD of gut lavage from 5 uninfected mice.

TABLE 5.3

Proliferative Responses of Spleen Cells from Mice Inoculated Orally with the pD and pC Strains or with the pUC8 Strain

CPM

	pD/pC-infected [a]					pUC8-infected [b]		
MOUSE NO	1	2	3	4	5	6	7	8
rec MOMP	2,438	6,557	9,981	4,427	4,607	4,339	9,131	8,421
VD2	411	1,067	1,794	737	1,402	532	1,050	868
VD4	238	877	1,349	713	1,524	477	832	752

SI

	pD/pC-infected [a]					pUC8-infected [b]		
MOUSE NO	1	2	3	4	5	6	7	8
rec MOMP	33	48	125	131	58	78	69	100
VD2	6	9	24	23	18	11	8	11
VD4	4	7	18	22	20	10	6	10

[a] received 5 x 10^9 CFU of the pD strain on days 0 and 2. [b] received the same vaccine regime as [a] with the pUC8 strain. Mice were culled on day 35 and the proliferative responses of the spleen cells to urea-purified MOMP, VD2 and VD4 determined in an LTT.

the pD/pC strains or the non-MOMP-expressing pUC8 strain and there were no clear differences between the groups [Table 5.3]. This assay was repeated twice with similar results. None of these responses were accompanied by IFN-g production, suggesting that the proliferation observed may have been due to stimulation of B cells by residual amounts of Salmonella proteins or LPS in the antigen preparation. Proliferative responses to the peptides were low with no clear differences between the 2 groups [Table 5.3].

5.2.3.7 Challenge of Mice with Live C.psittaci following Oral Vaccination with the pD Strain

Although there was only limited evidence of MOMP-specific responses in vaccinated mice, it was decided to challenge them with C.psittaci. 2 groups of 10 mice, vaccinated 40 days previously with the pD strain and the pUC8 strains respectively, were challenged intraperitoneally with 5×10^6 IFU of C.psittaci S26/3. No mortalities were recorded in either of the 2 groups, suggesting that BALB/c mice are resistant to chlamydial infection.

I therefore examined the susceptibility of other mouse strains to C.psittaci challenge and found that 6-week-old CBA mice were the most susceptible, dying within 8 days of challenge. However in subsequent experiments with these mice, mortalities were not consistently recorded, hence the challenge studies were abandoned.

5.2.4 Sheep Experiments

As the primary aim of this thesis was to develop a safe multivalent vaccine for sheep, despite the poor responses to the MOMP expressed from the pD and pC strains in mice, I decided to examine the responses to MOMP expressed from the same strains in sheep.

5.2.4.1 Immune Responses in Ewes After Challenge with Live C.psittaci

I first sought to determine if ovine T cells could recognise native MOMP following a natural infection with C.psittaci. PBMC from ewes that had been challenged with 1.3×10^6 IFU of a mixed inoculum of C.psittaci strains S26/1 and A22 one year previously were cultured with EBs, peptides VD2 or VD4, and urea-purified rec MOMP in an LTT. The results are shown in Table 5.4. All 4 challenged ewes responded strongly to EBs and rec MOMP although the responses to the protein were much lower. These responses were much greater than those of the unvaccinated animal. None of the ewes responded to peptide VD2, but ewes 1, 3 and 4 responded strongly to VD4.

5.2.4.2 Immunogenicity of Rec MOMP in Sheep

I next examined the immunogenicity of rec MOMP in sheep when given with adjuvant. Ewes were immunised 4 weeks apart with 25ug rec MOMP in alum and Marcol/Aralacel A and the sera tested in a rec MOMP ELISA. All 4 ewes produced very high levels of MOMP-specific IgG with titres of 2400, 4800, 2400 and 9600 for ewes 1, 2, 3 and 4 respectively. As before, the PBMC from each ewe were also

TABLE 5.4
Proliferative Responses of PBMC from Ewes Challenged with Live EBs of C.psittaci

CPM

	C.psittaci-challenged [a]				Control[b]
EWE NO.	1	2	3	4	5
EBs	44,444	26,742	28,360	38,420	7,230
recMOMP	6,018	6,076	8,920	10,040	893
VD2	1,090	50	1,110	1,360	920
VD4	14,804	0	9,360	10,140	921

SI

	C.psittaci-challenged [a]				Control[b]
EWE NO.	1	2	3	4	5
EBs	53	72	27	32.6	8
recMOMP	8	17	8.5	8.5	1
VD2	2.2	1	1	1	1
VD4	18	1	9	8.5	1

[a] Ewes were challenged 17 months previously with 1.3×10^6 IFU of a mixed inoculum of C.psittaci strains S26/3 and A22. [b] Unvaccinated control animal. PBMC were cultured with EBs, urea-purified rec MOMP and the synthetic peptides in an LTT and the proliferative responses determined.

TABLE 5.5
Proliferative Responses of PBMC from Ewes Immunised with rec MOMP in Adjuvant

CPM

	rec MOMP-immunised [a]				Control[b]
EWE NO.	1	2	3	4	5
EBs	39,375	38,183	93,871	80,854	4.320
rec MOMP	88,097	138,970	163,306	128,673	7,220
VD2	645	1,724	4,032	6,284	830
VD4	1,945	29,295	9,505	17,330	740

SI

	rec MOMP-immunised [a]				Control[b]
EWE NO.	1	2	3	4	5
EBs	45	12	27	32	4.5
rec MOMP	99	41	46	50	8
VD2	1.7	1.5	2.1	3.4	1.5
VD4	3.2	10	3.6	7.6	1

[a] Ewes were immunised intradermally, 2 months previously with 25ug of MOMP in alum. [b] Unvaccinated control animal. The proliferative responses of the PBMC from each lamb to EBs, urea-purified rec MOMP and the synthetic peptides were determined in an LTT.

cultured with EBs, synthetic peptides VD2 and VD4 and urea-purified rec MOMP in an LTT. The responses of an unvaccinated animal were also examined. The results are shown in Table 5.5. All 4 immunised animals responded strongly to the EBs and the responses to rec MOMP were even greater. The responses to the peptides were more varied. Only ewe 4 responded to VD2 and this response was very small. However, ewes 2, 3 and 4 clearly reponded to VD4. The responses of the unvaccinated animal to all the antigen preparations were negligible. Thus there were generally better responses to MOMP and the peptides [particularly VD4] in sheep than in mice, especially at the T cell level.

5.2.4.3 Oral Inoculation of Lambs with the PD and PC Strains

The results of both the rec MOMP-challenged and Chlamydia-challenged ewes demonstrate the highly immunogenic nature of MOMP in sheep. They also showed that the antigen preparations I used to detect MOMP-specific responses should have been appropriate in the mouse studies. I next went on to examine the immune responses of sheep to rec MOMP expressed by the aroA vaccine strains.

8 conventional lambs were placed into negative-pressure isolators [2/isolator] 7 days post-partum and randomly allocated into 2 groups of 4 lambs. One group of lambs was inoculated orally with 4 x 10^9 CFU and 1 x 10^{11} CFU of the pD strain on days 0 and 4 and with 5 x 10^{10} CFU and 8 x 10^{11} CFU of the pC strain on days 2 and 15. A second group of lambs was given identical doses of the pUC8

TABLE 5.6
Proliferative Responses of PBMC from Lambs Inoculated Orally with the pD and pC Strains or with the pUC8 Strain

CPM

	pD- and pC-infected [a]				pUC8-infected [b]			
	1	2	3	4	1	2	3	4
Salmonella	2,043	2,084	2,966	1,019	1,862	905	541	0
EBs	264	1,025	303	149	311	321	604	117
rec MOMP	3,224	49	242	72	0	0	175	0
VD2	1,701	0	0	102	0	0	0	43
VD4	1,877	0	281	39	0	272	1,546	0

SI

	pD- and pC-infected [a]				pUC8-infected [b]			
	1	2	3	4	1	2	3	4
Salmonella	5	20	2	7	11	6	2.4	1
EBs	1.5	10	2	2	1	4	2.5	1.6
rec MOMP	8	1	1	1	1	1	1.7	1
VD2	3.2	1	1	1	1	1	1	1
VD4	5	1	3	1	1	1	4.9	1

[a] Lambs received 4×10^9 CFU and 1×10^{11} CFU of the pD strain on days 0 and 4 respectively, and 5×10^{10} CFU and 8×10^{11} CFU of the pC strain on days 2 and 15 respectively. [b] Lambs received identical doses as [a] of the pUC8 strain. PBMC were removed on day 25 and cultured with Salmonella antigen, EBs, urea-purified MOMP and the synthetic peptides in an LTT, and the proliferative responses recorded.

strain. None of the lambs produced detectable levels of Salmonella-specific IgM or IgG at any time post-infection and none given MOMP-expressing strains produced detectable levels of MOMP-specific IgM or IgG. In parallel, the PBMC had very low Salmonella-specific proliferative responses, and only one lamb produced a proliferative response to rec MOMP that was greater than the those of the pUC8-vaccinated lambs [Table 5.6]. Thus the pD and pC strains were incapable of inducing either Salmonella-specific or MOMP-specific responses in these lambs.

5.3 Discussion

The experiments in this chapter demonstrated that the MOMP of C.psittaci was immunogenic for both mice and sheep. Both species produced MOMP-specific humoral responses when immunised with rec MOMP in adjuvant or with live chlamydial EBs. In sheep, specific cellular responses against rec MOMP, EBs and also synthetic peptides of MOMP, in particular VD4 were detected in both MOMP-immunised and Chlamydia-infected animals. The VD2 peptide did not prove to be as effective which is surprising, as epitope mapping and computer epitope prediction data suggest that both these peptides contain T cell epitopes [Tan, 1990]. The stronger T cell responses to rec MOMP in MOMP/CFA-immunised ewes than in Chlamydia-infected ewes demonstrates that, while MOMP is not presented very well during live infection with EBs, it is highly immunogenic on its own. The reduced responses to EBs may be a result of masking of MOMP epitopes by LPS and other surface components making them less immunoaccessible.

In murine assays, specific T cell responses could be detected only against EBs and these occurred only in Chlamydia-challenged mice. The lack of T cell responses to VD2 and VD4 in mice is consistent with the results of Ishizaki et al. [1992] who generated Chlamydia-specific mouse T cell lines which responded only to the VD3 region of MOMP, but not to VD2 or VD4. In view of the high IgG titres in MOMP/CFA-immunised mice, it is surprising that MOMP-specific T cells were not demonstrated. However, the urea-purified rec MOMP used to detect responses in ewes was not available at the time of these mouse studies and may have been successful in detecting MOMP-specific responses.

Care should be taken in comparing the responses in MOMP-immunised mice and sheep. Mice were given only a single immunising dose of rec MOMP whereas ewes were also given a secondary boosting dose, which may account for the different responses in the 2 species. However, the stronger responses in sheep suggests that in sheep there are less stringent criteria governing the presentation of the MOMP protein to the immune system. Ovine antigen-presenting cells may be able to process the MOMP more effectively into suitably-sized peptides for MHC Class II association whether it is in the cell wall of EBs as a whole protein preparation or in peptide form. Also, there may antigen-presenting cell types present in sheep but not mice, that can present MOMP more effectively. Consequently a greater repertoire of MOMP-specific T cells would be generated. Additionally, there may be epitopes of

MOMP that are recognised by the ovine immune system and not by the murine immune system.

Having established the immunogenicity of MOMP, the pD and pC strains were assessed for their ability to generate rec MOMP-specific immune responses after oral immunisation of mice. Only a small number of mice produced MOMP-specific antibodies in the gut and serum and the levels were much lower than the levels of S.typhimurium-specific antibodies, consistent with the much lower mass of chlamydial epitopes present in the vaccine strain. Protective studies to assess the protective capacity of the detectable MOMP-specific antibodies against C.psittaci infection proved entirely unsuccessful, as naive mice were relatively resistant to a lethal infection. Others have also found it difficult to kill mice with C.psittaci [G.E. Jones. personal communication]. Anderson et al. [1986] was able to effect mortality in mice with C.psittaci but only at very high doses of EBs which were not available to me.

In lambs orally infected with both the pD and pC strains, Salmonella-specific antibody was not detected in the serum and this was parallelled by very low specific lymphocyte proliferation. It was not surprising then that MOMP-specific responses were not produced. The responses to Salmonella were surprising in view of the results obtained in Chapter 4, but this may reflect the fact that the latter lambs were almost 1 year-old compared with the 7 day-old lambs used in this chapter. Thus the age of the lambs may

govern the invasiveness of the strain and its capacity to elicit immune responses. However the low immunogenicity of the vaccine strain in lambs is more likely to account for the absence of specific responses.

The low level responsiveness of the mice to MOMP was interesting in view of the high level of responsiveness to the vaccine strain itself. It may be that the large Salmonella-specific respones, in particular to the LPS, were masking the response to the MOMP. Alternatively, Salmonella may not have the correct adjuvant qualities to boost MOMP-specific responses. I considered the possibility that other factors such as route of inoculation, the in vivo loss of plasmid and the solubility of the expressed antigen could be responsible. However, organisms expressing the rec MOMP protein were recovered up to day 15 following challenge and total plasmid segregation was not seen until day 20. Molina and Parker [1990] demonstrated plasmid segregation as early as day 1 post-infection but specific immune responses to the heterologous antigen still occurred, implying that the immune system only requires a short time for antigen recognition. These findings make it unlikely that plasmid segregation accounted for the low responses in my system.

Another explanation for poor MOMP-specific responses could be low level expression of the protein by the vaccine strain. However, both strains, in particular the pC strain, produced high levels

of the protein *in vitro*, while the colonies of the pD strain recovered from mice also produced MOMP *in vivo*.

The most likely explanation for the low immunogenicity of the recombinant strains may reflect the insoluble nature of the expressed MOMP protein. Within the cytoplasm of the vaccine strain, the rec MOMP forms large insoluble inclusion bodies, visible by electronmicroscopy. It has been reported by others that insoluble proteins are not immunogenic in the cytoplasm of *Salmonella* [Taylor *et al.*, 1986; Maskell *et al.*, 1986] whereas soluble proteins are [Brown *et al.*, 1987]. These differences may reflect effects on the processing and presentation of the MOMP to T cells. The inclusion bodies consist of linear molecules of MOMP that are crosslinked together and these may be more resistant to proteolysis within antigen processing compartments. Thus peptides of the appropriate size for association with MHC Class II molecules and subsequent recognition by T cells may not be formed. However, Guzmán *et al.* [1991] expressed the FHA of *B.pertussis* as cytoplasmic inclusion bodies in *Salmonella* and detected FHA-specific responses in both the serum and lung demonstrating that the insolubility of the antigen does not always prevent the generation of specific immune responses.

One means of avoiding problems of protein insolubility would be to obtain surface expression of the inserted antigen. Several strategies have been developed to achieve this and are discussed in Chapter 7.

Chapter 6

The Immune Response to the Leukotoxin of P.haemolytica A1 Expressed by the S25/laroA Vaccine Strain

6.1 Introduction

The second heterologous antigen which was available for expression by the aroA vaccine strain was the leukotoxin of P.haemolytica A1. A 33 K protein, representing the C-terminal end of the leukotoxin, was expressed cytoplasmically in a soluble form, to circumvent the possible problems of protein insolubility associated with the MOMP protein. The behaviour of the pAL12 strain was monitored in vivo and vaccine-specific immune responses were examined in both mice and sheep. I also attempted to determine how maintenance of the plasmid and protein expression was related to the specific humoral and cell-mediated immune responses elicited by the leukotoxin.

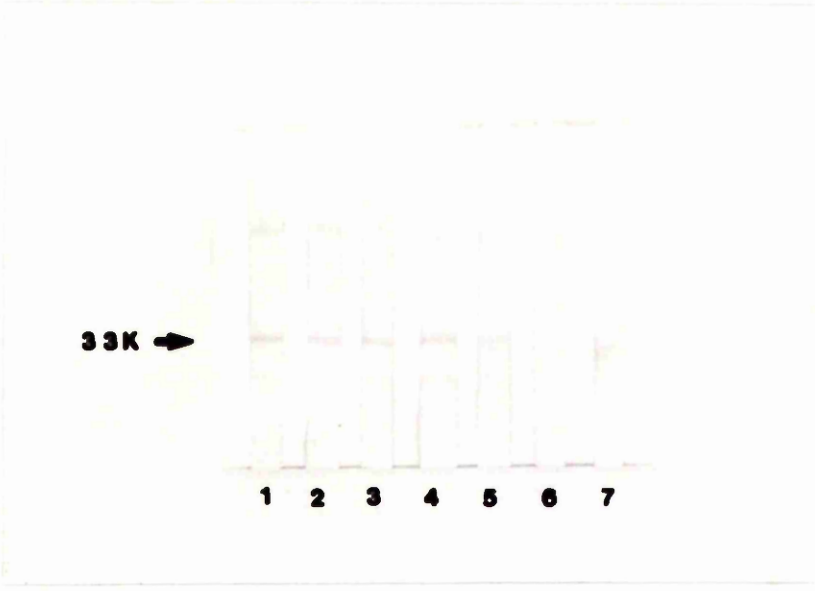
6.2 Results

Initially, I examined the immunogenicity of the leukotoxin itself in mice, to give some indication of the responses likely to occur when expressed by the Salmonella vaccine strain.

6.2.1 Immunogenicity of Leukotoxin in Mice

Mice were injected intraperitoneally with native leukotoxin in CFA, prepared from either the pellet fraction or the supernatant fraction of a centrifuged 8-h culture of P.haemolytica A1. The sera of individual mice were examined for leukotoxin-specific IgG

Figure 6.1 **Leukotoxin-Specific Antibody in Mice After Immunisation with Leukotoxin in CFA**



Lane : 1-5 serum from mice immunised with leukotoxin in CFA
6 NMS
7 leukotoxin-specific monoclonal antibody

by immunoblotting against recombinant leukotoxin [rec leukotoxin] [produced in E.coli]. Although an ELISA was available for the detection of leukotoxin-specific antibody, it was not used, as the sensitivity was poor and there was only a limited supply of rec leukotoxin available for coating. The results are shown in Figure 6.1. Mice immunised with either the supernatant fraction [lanes 1-2] or the pellet fraction [lanes 3-5] produced antibodies which reacted with the 33 K leukotoxin protein [although the response in lane 5 was poor]. A similar band was produced by the monoclonal antibody, MM601B [lane 7], but was absent in the strip probed with NMS [lane 6].

Spleen cells from both immunised and control mice were removed and stimulated with purified rec leukotoxin in an LTT [Table 6.1]. The responses of the immunised mice were significantly greater [$p<0.05$] than those of the unvaccinated mice with 4 out of 5 [1, 3, 4 and 5], given either the pellet or the supernantant fraction, responding specifically to the leukotoxin. There were no responders within the unvaccinated group. All of the immunised mice also produced IFN in vitro in response to stimulation with rec leukotoxin, while none of the 5 control animals produced IFN. Due to a short supply of antigen, there was insufficient supplies of supernatant to test in a IFN-g neutralisation assay. Collectively, these studies indicate that the leukotoxin is fully immunogenic in mice.

TABLE 6.1

Rec Leukotoxin-Specific Responses of Spleen Cells from Mice Immunised with Native Leukotoxin in CFA

	Leukotoxin-immunised [a]					Unvaccinated [b]			
MOUSE NO.	1	2	3	4	5	1	2	3	4
CPM	4,426	2,958	6,879	7,672	2,940	872	208	804	1,340
SI	6.2	4	8	10	6.5	1.8	1.7	2.1	3.1
IFN TITRE	2	2	4	4	<2	0	0	0	0

[a] Mice were immunised intraperitoneally on day 0 with native leukotoxin in CFA. [b] Unvaccinated control group. Mice were culled on day 28 and spleen cells cultured with rec leukotoxin in an LTT. Both the proliferative responses and the IFN titres are shown.

6.2.2 Oral Inoculation of Mice with the pAL12 Strain

I then went on to examine if it would be possible to evoke detectable immune responses to the leukotoxin when produced by the pAL12 *Salmonella* vaccine strain.

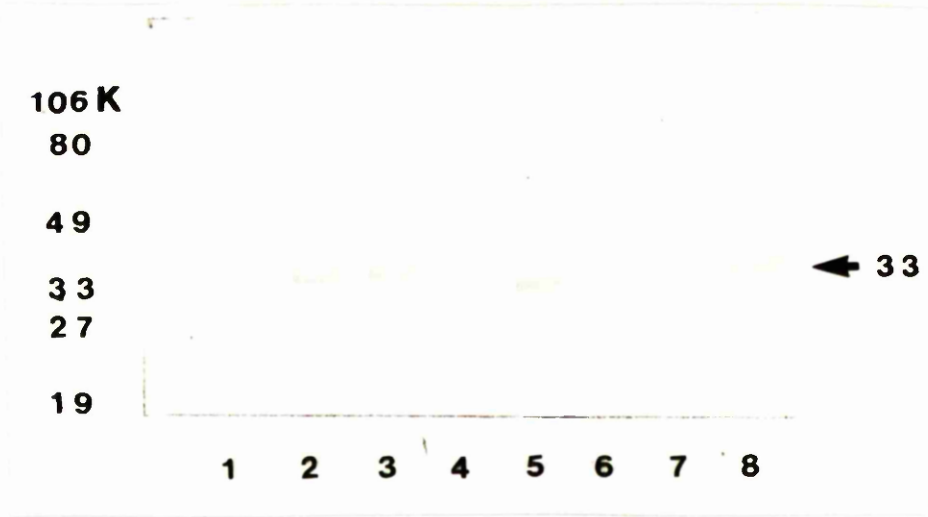
6.2.2.1 Production of Rec Leukotoxin by the pAL12 Strain

The plasmid DNA from the pAL12 strain was shown to contain the correct size of insert by restriction endonuclease analysis. 5 samples of pAL12 strain isolates were checked for the production of leukotoxin. Bacterial cell lysates were separated by SDS-PAGE and blotted onto nitrocellulose, which was probed with the leukotoxin-specific monoclonal antibody, MM601B. Lysates of the non-transformed pUC8 strain were also run [lanes 6 and 7], as was a preparation of rec leukotoxin pAL12 [produced in *E.coli*] [lane 8]. The results are shown in Figure 6.2. All 5 isolates of the pAL12 strain produced a unique band of 33 K which corresponded with the rec leukotoxin and which was absent in the pUC8 strain isolates.

6.2.2.2 Maintenance of the Plasmid in Vivo

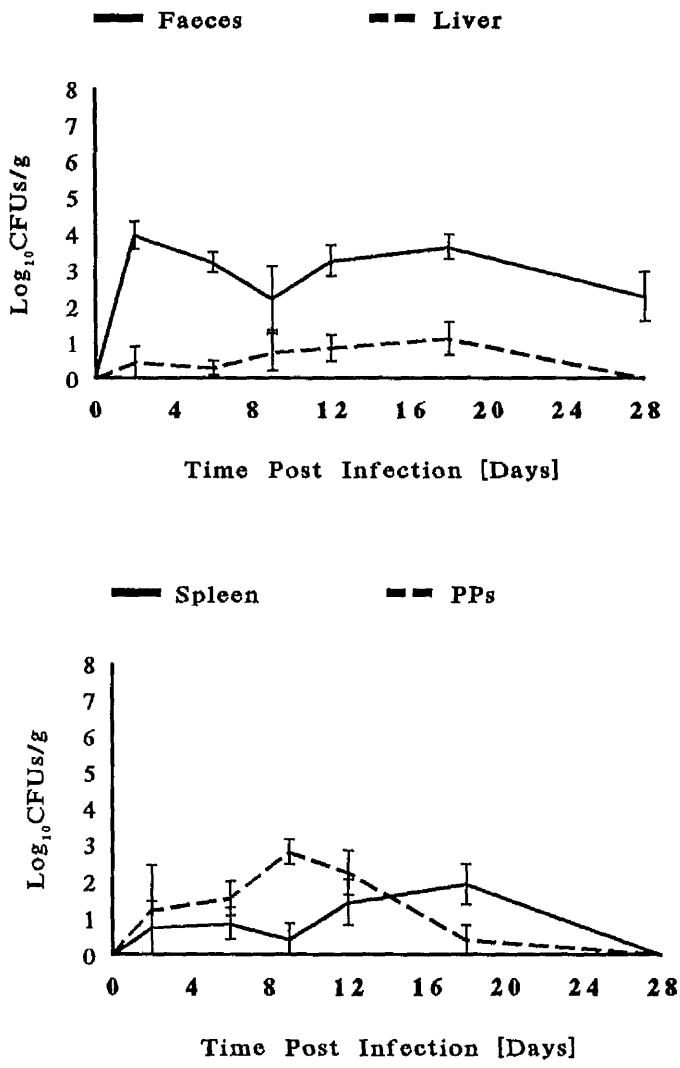
Mice were inoculated orally with 5×10^8 CFU of the pAL12 strain and culled on days 2, 6, 9, 12, 18 and 28 and the no. CFU\g of organ determined. The results are shown in Fig 6.3. The levels of colonisation were much reduced compared with those described previously for the S25/1*aroA* strain as well as the MOMP-expressing pD and pC strains, with only small numbers being recovered from the

Figure 6.2 Production of Leukotoxin by the pAL12 Stock Isolates



Lane : 1-5 pAL12 stock isolates
6+7 pUC8 stock isolates
8 rec leukotoxin

Figure 6.3 Recovery of the pAL12 Strain from Mice After Oral Infection



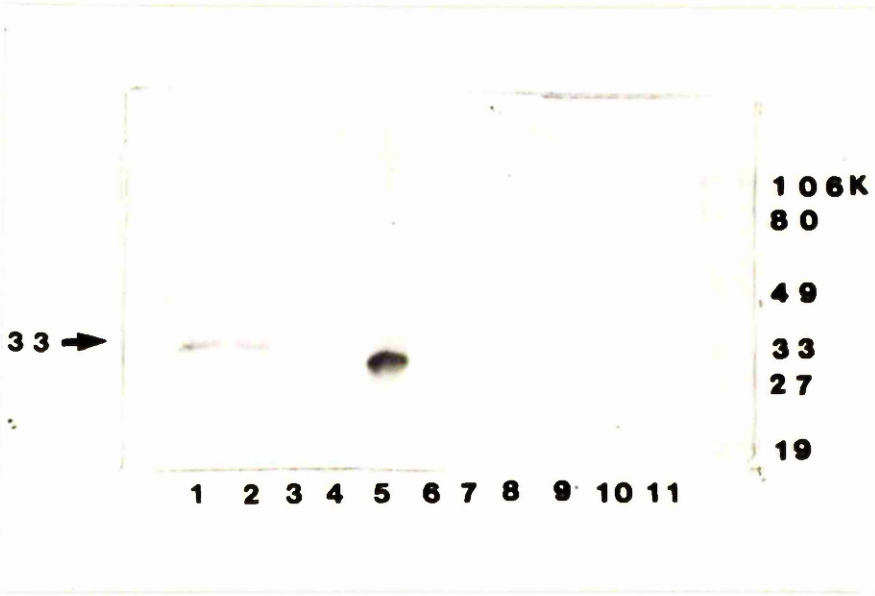
Mice were inoculated orally with 5×10^8 CFU of the pAL12 strain. 3-5 mice were culled on the stated days post-infection and the mean no. CFU/g determined on each time-point for faeces, liver, spleen and Peyer's patches [PPs]. Levels are expressed as $\log_{10}$ CFU/g.

livers, spleens and Peyer's patches. The pAL12 strain also had a considerably reduced *in vitro* growth rate than all of the other strains used in this thesis [Appendix 6], perhaps explaining its poor ability to grow *in vivo*.

To determine the retention of the plasmid *in vivo*, the colonies recovered from the mice were replica-plated from XLD agar onto LB agar containing ampicillin. As early as day 6, there was significant plasmid segregation, as only poor growth was observed in the presence of ampicillin. By day 10, no ampicillin-resistant colonies could be recovered from the spleens and by day 15, all the colonies recovered from all the organs were sensitive to ampicillin. In addition, very few bacterial lysates prepared from ampicillin-resistant colonies were found to contain leukotoxin when probed with leukotoxin-specific monoclonal antibody, MM601B [Figure 6.4]. Only colonies isolated on day 6 post-inoculation [lanes 9-11] displayed very feint bands corresponding to the protein. Thus It would seem that not only does the organism grow slowly *in vitro*, but the plasmid is lost very rapidly from the bacterial cell *in vivo*.

The above experiment was repeated with a further 10 mice which were culled on days 2, 3, 5 and 8 post-infection in an effort to try and detect colonies that were clearly producing the leukotoxin

Figure 6.4 Production of Leukotoxin by pAL12 Colonies Recovered from Mice After Oral Infection



Lane : 1+2 pAL12 stock isolates
3+4 pUC8 stock isolates
5 rec leukotoxin
6 pAL12, recovered from faeces, day 6
7 pAL12, recovered from liver, day 6
8 pAL12, recovered from liver, day 12
9 pAL12, recovered from Peyer's patches, day 6
10 pAL12, recovered from Peyer's patches, day 6
11 pAL12, recovered from spleen, day 6

protein. The results [not shown] were even less encouraging than in the previous experiment. Very few ampicillin-resistant colonies were obtained on any of the days tested and efforts to subsequently inoculate LB-ampicillin broth proved unsuccessful.

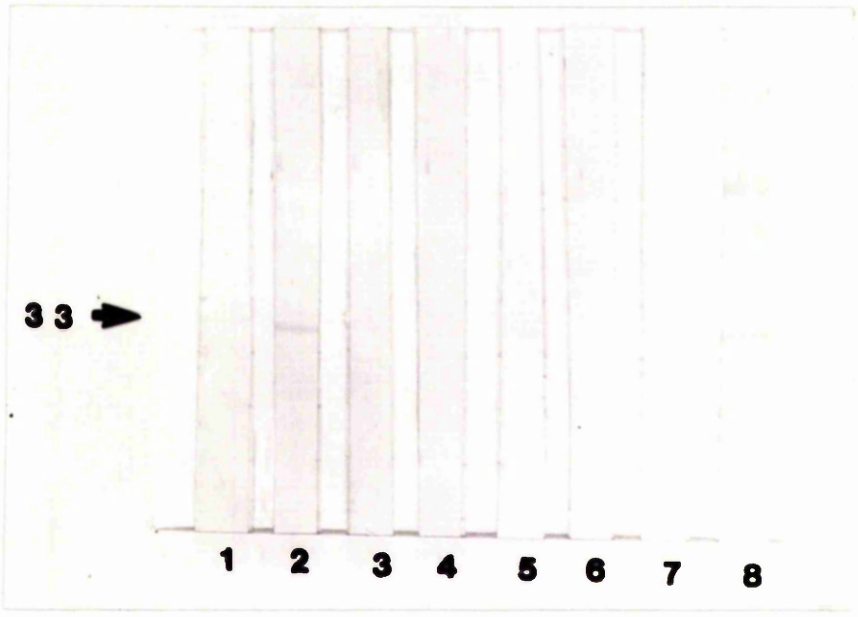
6.2.2.3 Leukotoxin-Specific Immune Responses in Mice After Oral Infection with the pAL12 Strain

On day 25, 4 of the above mice [1st group] were culled and the serum examined for the presence of leukotoxin-specific antibodies by probing strips of blotted pAL12 leukotoxin [Figure 6.5]. 1 out of 4 mice given the pAL12 strain [lane 2] produced a single detectable band at 33 K identical to that produced by the serum from a leukotoxin\CFA-immunised mouse [lane 8]. In a further experiment, 1 of 8 mice infected with the pAL12 strain produced leukotoxin-specific antibody.

T Cell Responses

Spleens cells from the same mice showed no significant proliferative responses or IFN production to the rec leukotoxin [Table 6.2]. Similar results were obtained in mice vaccinated with the pAL12 strain 50 days before. Thus, the leukotoxin expressed by the pAL12 strain was unable to elicit specific CMI and specific antibody was seen in only a small proportion of the mice.

Figure 6.5 Leukotoxin-Specific Antibody in Mice After Oral Inoculation with 10^8 CFU of the pAL12 Strain



Lane 1-4 : serum from 4 mice inoculated orally with 5 x 10^8 CFU of the pAL12 strain

5-7 : serum from 3 mice given 5 x 10^8 CFU of the pUC8 strain

8 : serum from mouse immunised with leukotoxin in CFA

TABLE 6.2

Rec Leukotoxin-Specific Responses of Spleen Cells from Mice Orally Inoculated with the pAL12 or pUC8 Strains

[A] Mice Culled 25 Days Post-Vaccination

	pAL12-infected [a]					pUC8-infected [b]		
CPM	1,120	1,229	1,353	1,377	1,258	1,220	1,234	1,510
SI	3.7	4.2	3.4	2.5	4.2	2.8	3.3	5.1
IFN TITRE	0	0	0	<2	0	0	0	0

[B] Mice Culled 50 Days Post-Vaccination

	pAL12-infected [a]							
CPM	1,232	800	1,240	960	750	800	836	741
SI	2.9	2	2.9	1.5	2.5	2.3	2.3	2.1
IFN TITRE	0	0	0	0	0	0	0	0

Mice were inoculated orally with 5×10^8 CFU of the pAL12 strain [a] or with an identical dose of the pUC8 strain [b] on day 0. Mice were culled on day 25 [A] and day 50 [B] and spleen cells cultured with rec leukotoxin in an LTT. Both the proliferative responses and the IFN titres are shown.

6.2.3 Sheep Experiments

Despite the failure of the pAL12 strain to elicit leukotoxin-specific immune responses in mice, I thought it important also to perform similar experiments in lambs.

6.2.3.1 Confirmation that Rec Leukotoxin Lacks Biological Activity in Vitro

Leukotoxin has been shown to be toxic for ovine but not murine macrophages [Sutherland et al., 1983]. Before administration of the pAL12 strain to lambs, it had to be confirmed that the rec leukotoxin produced by the Salmonella strain lacked this cytotoxicity. Both the pAL12 and pUC8 strains were added separately to ovine alveolar macrophages [prepared by J. Wood, MRI] at a bacteria:cell ratio of 100:1 for 30 min at 37^{o}C. After this time the percentage cell death was calculated by the trypan blue exclusion test. Neither of the bacterial strains lysed the ovine alveolar macrophages, with 84% of the cells treated with the pAL12 strain being viable compared with 86% viability using the pUC8 strain.

6.2.3.2 Oral Inoculation of Lambs with the pAL12 Strain

In all these experiments, germ-free lambs were used to circumvent the problems of pre-existing immunity to Pasteurella in conventional animals [W. Donachie, personal communication].

TABLE 6.3

Recovery of Bacteria from the Organs of Germ-Free Lambs After Oral Infection with the pAL12 or pUC8 Strains

No. CFU/g

GROUP	pAL12-infected [a]				pUC8-infected [b]	
CULLED	DAY 7	DAY 7	DAY 3	DAY 7	DAY 4	DAY 7
HEART	$1x10^4$	$6x10^4$	$4x10^6$	$1x10^5$	$1x10^6$	NT
LIVER	$6x10^3$	$3x10^4$	$7x10^6$	$4x10^5$	$8x10^6$	24
SPLEEN	$1x10^4$	$3x10^4$	$1x10^7$	$8x10^6$	$2x10^7$	$1x10^5$
MLN	$1x10^5$	$4x10^4$	$1x10^6$	$1x10^7$	$5x10^6$	$3x10^6$
KIDNEY	$2x10^4$	$6x10^4$	$1x10^6$	$6x10^5$	$1x10^6$	3
ILEUM	$3x10^6$	$7x10^4$	$1x10^8$	$1x10^8$	$2x10^8$	NT
COLON	NT	NT	$1x10^8$	NT	NT	NT
JOINTS	$2x10^3$	$1x10^5$	NT	$4x10^4$	NT	$1x10^4$

NT= Not Tested

MLN= Mesenteric Lymph Node

Lambs were inoculated orally with 4×10^7 CFU of the pAL12 strain [a], or with an identical dose of the pUC8 strain [b]. Lambs were culled on the stated days post-infection and the no. CFU/g of tissue determined.

8 lambs were inoculated orally with 4 x 10^7 CFU of the pAL12 strain. A control group of 4 lambs was given an identical dose of the pUC8 strain. 48 h after challenge, the lambs displayed a loss of appetite and there was a dramatic increase in the rectal temperatures of all the lambs [ranging from 40.4-41.7^{o}C]. All the animals developed severe diarrhoea, with watery faeces containing mucus and sometimes blood. The faeces from all the lambs contained high levels of salmonellae when examined using XLD medium. However, the colonies obtained on this agar were black, rather than the pinky-yellow colonies expected from the aroA strains, suggesting that the vaccine strain had reverted to virulence in the lambs. This was confirmed when 6 of the lambs were culled *in extremis* on days 3-7. The guts of all these lambs were inflamed, the lymph nodes were greatly enlarged and all their tissues harboured large numbers of virulent black colonies [Table 6.3]. Virulent salmonellae were recovered from blood and from the hind knee-joint, indicating the presence of septicaemia and septic arthritis.

6.2.3.3 Immune Responses in Lambs After Oral Infection with the pAL12 Strain

All 6 surviving lambs [4 given the pAL12 strain and 2 given the pUC8 strain] produced *Salmonella*-specific IgM by day 15, although the levels varied greatly in individual lambs [Table 6.4]. The levels of *Salmonella*-specific IgG were very low in all the lambs.

TABLE 6.4

Titres of Salmonella-Specific Antibody in Lambs After Oral Infection with the pAL12 or pUC8 Strains

TITRE

	pAL12-Infected [a]				pUC8-Infected [b]	
LAMB NO	1	2	3	4	5	6
IgM	512	512	320	640	80	40
IgG	8	4	4	8	2	2

Lambs were inoculated orally with 4×10^7 CFU of the pAL12 [a] or the pUC8 [b] strains on day 0 and titres of Salmonella-specific IgM and IgG determined on day 15. Titres of individual lambs are expressed as the inverse of the highest dilution of test serum that produced an OD_{492nm} reading greater than the pre-vaccination reading.

I next examined for leukotoxin-specific antibody. No bands were produced by any of the pre-vaccination sera [Figure 6.6] confirming the lack of previous exposure to Pasteurella in these germ-free animals. However sera from both the pAL12- and pUC8-infected lambs reacted with the 33K protein, making it impossible to determine if leukotoxin-specific responses had transpired. As several other bands were also seen in all these sera, these represent anti-Salmonella antibodies which were cross-reacting with E.coli proteins in the pAL12 preparation used in the blotting.

PBMC from the same 6 lambs were tested for reactivity against Salmonella and leukotoxin [both rec and native] in an LTT. All lambs responded to the Salmonella antigen [Table 6.5], although there was great variation in the responses of individual lambs as in the previous chapter. Lambs 1, 2 and 3 given the pAL12 strain clearly responded to rec leukotoxin and the latter 2 lambs also responded to the native leukotoxin. Lamb 4 was the only pAL12-infected lamb that did not respond to either preparation. Neither of the pUC8-infected lambs responded to either of the 2 antigen preparations. The Salmonella-specific responses did not correlate well with the responses to leukotoxin within individual lambs.

Figure 6.6 Leukotoxin-Specific Antibody in Lambs After Oral Inoculation with 4×10^7 CFU of the pAL12 Strain

LANE NO.	LAMB NO.	VACCINE STRAIN	TIME OF SAMPLING
1	1	pAL12	pre-vaccination
2	1		day 15
3	2	pAL12	pre-vaccination
4	2		day15
5	5	pUC8	pre-vaccination
6	5		day 15
7	6	pUC8	pre-vaccination
8	6		day 15
9	3	pAL12	day 24
10	4	pAL12	day 24
11	5	pUC8	day 24
12	6	pUC8	day 24

TABLE 6.5

Rec Leukotoxin-Specific Responses of PBMC from Lambs Inoculated Orally with the pAL12 or pUC8 Strains

CPM

	pAL12-infected [a]				pUC8-infected[b]	
	1	2	3	4	5	6
Salmonella	27,277	8,220	4,088	1,013	2,666	2,539
rec leukotoxin	1,076	1,075	1.094	50	70	104
native leukotoxin	338	3,162	2,911	0	223	163

SI

	pAL12-infected [a]				pUC8-infected[b]	
	1	2	3	4	5	6
Salmonella	272	41	21.5	3	31	37
rec leukotoxin	11.3	11.5	6.7	1	1.8	2
native leukotoxin	4.2	20	16	1	2.7	4.1

Lambs were inoculated orally with 4×10^7 CFU of the pAL12 strain [a] or the pUC8 strain [b] on day 0. PBMC were removed on day 15 and the proliferative responses to Salmonella, rec leukotoxin and native leukotoxin determined in an LTT.

6.2.4 Studies on the Reversion to Virulence

In view of the potential problems that could arise with the S25/1*aro*A strain, I decided to investigate the reversion to virulence of the pAL12 vaccine strain in lambs in more detail.

6.2.4.1 Demonstration of the Aromatic-Independency and Ampicillin-Sensitivity of Recovered Colonies

All the black colonies on XLD agar recovered from the faeces and organs of each of the vaccine-infected lambs grew on minimal agar, with or without addition of aromatic compounds, whereas the original stock strain and the pAL12 colonies recovered from conventional mice would only grow on the supplemented minimal agar. Thus the vaccine strain had lost its dependence on aromatic compounds following passage in lambs. This was associated with loss of the plasmid, as the black colonies obtained from the lambs had also lost their ability to grow on LB agar containing ampicillin. This loss of ampicillin-resistance was seen as early as 18 h following inoculation of the strain into lambs.

6.2.4.2 Oral Inoculation of Germ-Free Mice with the S25/1aroA Strain and its Recombinant Derivatives

One difference between the mice and lambs used in these experiments was that the mice which survived the infection were not germ-free, while the susceptible lambs were. I therefore investigated whether the germ-free environment allowed reversion to the virulent form by infecting germ-free mice with all the vaccine strains used in this thesis. Faecal samples were removed from each of the mice on days

2, 4 and 6 and 1 ml plated onto XLD agar and minimal agar with and without aromatic metabolites [see Appendix 1]. All the XLD plates from all the mice showed confluent growth of yellow colonies which were H_2S- when tested by API and would only grow on minimal agar that was supplemented with the aromatic compounds. 1 mouse [given the S25/1aroA strain] died on day 11 and was seen to harbour 2-5 x 10^7 CFU/g in the liver and spleens. These colonies were yellow on XLD and did not grow on unsupplemented minimal agar. None of the remaining mice became ill. Thus, the strains did not revert to virulence in germ-free mice.

6.3 Discussion

Specific humoral and cell-mediated immune responses were evoked when native leukotoxin was administered with adjuvant to mice, but none of the mice inoculated orally with the pAL12 strain produced leukotoxin-specific lymphocyte responses and only 2 of 12 mice given the pAL12 strain produced leukotoxin-specific antibody.

There are a number of possible reasons for the low level responsiveness to leukotoxin expressed by the pAL12 strain in mice. Firstly, the pAL12 strain had a reduced *in vitro* growth rate compared with the other aroA strains and was recovered from the organs at lower levels than the other aroA strains. However, all the mice produced *Salmonella*-specific humoral and cellular responses indicating that the invasiveness of the strain should have been sufficient to potentiate the generation of leukotoxin-specific responses. As the leukotoxin is produced

intracytoplasmically by the salmonellae as a soluble protein, thereby circumventing the problems encountered with insoluble antigens [Maskell et al., 1986], the most likely reason for the low leukotoxin responses appears to have been plasmid loss from the pAL12 strain, which was seen as early as 2 days post-infection and resulted in limited in vivo production of the protein.

In germ-free lambs given the pAL12 [or pUC8] strain, reversion to wildtype was seen, causing severe clinical symptoms and death. In these lambs, leukotoxin-specific antibody was not produced, although specific cellular responses were detected. However the specificity of these responses was questioned by the low responses to Salmonella in these lambs which may be due to oral tolerance following administration of high doses of bacteria to young lambs. More likely, the low responses are due to the low imunogenicity of the vaccine strain. The lack of leukotoxin-specific responses in the lambs would not be surprising in view of the reversion events which were associated with plasmid loss and limited production of leukotoxin in vivo.

The reasons for this reversion to virulence are unclear. In earlier chapters, conventional lambs and mice showed no signs of disease when given the aroA vaccine strains and only low level colonisation by aromatic-dependent bacteria was observed. In contrast, here almost all of the pAL12 colonies recovered from the germ-free lambs after 18 h were biochemically wildtype. This rapid switch to aromatic-independence argues against the possibility that low

numbers of revertants gradually multiplied in the lambs until numbers capable of causing a lethal infection were reached. Reversion was also not due to the presence of the the aromatic metabolites in the milk, as the pD and pC strains given to conventional lambs of the same age and fed in the same way did not revert [Chapter 5].

One way that the aroA strains could revert to virulence would be to regain the deleted piece of the aroA gene by transduction from other intestinal bacteria. However, the absence of gut flora in the germ-free animals would surely have precluded from such DNA transfer events from taking place.

The aroA gene in our mutant strain has not been sequenced and hence the size of the deletion, if any, is undefined. The only way that a strain could be tetracycline-sensitive and yet aromatic-dependent without a deletion of aroA gene sequences would be if the transposon caused an insertion mutation rather than a deletion. Transposons have been shown to be lost from host DNA but some of their sequences remain which interrupt the coding sequences of the host DNA [insertion mutations]. Insertion mutations are known to revert, as there is selection for the loss of the insertion sequences, which would allow normal gene function. Rapid multiplication in the gut of germ-free animals would have provided

optimal conditions for loss of insertion sequences if they exist in our strain. However repeated *in vitro* culture of the strain did not reveal any such revertants, suggesting that insertion sequences do not cause the attenuation of the S25/1*aroA* strain.

If the germ-free nature of the lambs facilitated the reversion events, why did the strain not revert in germ-free mice? Although species differences seem the most obvious reason, the faeces of the infected germ-free mice contained both *Salmonella* and *Pseudomonas* species, implying that the mice were not entirely germ-free to begin with which may account for the failure of the strain to revert in these mice. It will therefore be important to repeat these studies with the the *aroA* strains in genuine germ-free mice.

Chapter 7

General Discussion

The results outlined in this thesis have demonstrated the effectiveness of the S25/1aroA strain in eliciting strong specific immune responses in both mice and sheep and conferring immunity to salmonellosis in these species. However its potential as a vector for vaccination against other antigens was not clearly demonstrated, as immune responses to MOMP and leukotoxin incorporated into S25/1aroA were very low in mice and absent in sheep. Furthermore, the S25/1aroA strain reverted to virulence in germ-free lambs, further undermining its potential as a candidate vaccine vector.

Although the S25/1aroA strain protected both mice and sheep from wildtype challenge, Salmonella-specific intestinal IgA and CMI occurred in mice but not sheep, suggesting that the mechanisms governing protection may be different in these species. This could reflect differences in the gastrointestinal physiology of mice and sheep which may influence the interaction of the salmonellae with the immune system. Sheep digest food by rumination and orally administered aroA salmonellae are likely to sit in the rumen for up to 5 h, by which time they may be competed out by other bacteria or regurgitated back up the oesophagus for secondary mastication. As a result, many organisms are likely to be destroyed thereby reducing the numbers that enter the small intestine and can

subsequently stimulate the GALT. Rumination may therefore account for absence of intestinal IgA and the low level of colonisation of the internal organs which I found in sheep given S25/1*aroA*. In contrast, the stomach contents in mice pass rapidly to the small intestine therefore increased numbers of salmonellae are available for uptake by Peyer's patches and stimulation of mucosal immune responses.

The startling differences in the immune responsiveness of mice and sheep to the vaccine strain suggest that *Salmonella* infection in mice may not always be an ideal system for testing candidate vaccine strains destined for use in other species. Nevertheless the mouse system does seem to be relevant for humans as oral administration of the live *Salmonella* vaccine strain Ty21a results in the production of both mucosal and systemic antibody and strong CMI [Cancellieri and Fara, 1985; Sarasombath *et al*., 1984, 1987; Bartholomeuz *et al*., 1990].

The absence of specific mucosal antibody to the vaccine strain in lambs may limit the usefulness of the *aroA* strain as a vaccine vector in this species, particularly for the delivery of heterologous antigens derived from mucosal pathogens such as *V.cholerae*, *Shigella*, enterotoxigenic *E.coli* and numerous gastrointestinal parasites. Stimulation of the GALT can also result in the generation of specific protective IgA responses at other mucosal sights such as the lung [Guzmàn *et al*., 1991] and such lung responses are likely to prevent the development of lung

consolidation seen in lambs infected with e.g. P.haemolytica [W. Donachie, personal communication]. The lack of vaccine-specific intestinal IgA responses in sheep suggests that the respiratory IgA responses likely to be important in protection against this infection may also not be generated by the pAL12 strain. Similarly, the aroA vector did not stimulate systemic CMI in sheep thus reducing the possible usefulness of these vectors in vaccinating this species against obligate intracellular parasites such as Chlamydia where protective responses are likely to be T cell-mediated. Strategies should be adopted to try and increase the mucosal and cellular responses to the S25/1aroA strain following oral vaccination. The S25/1aroA strain could be administered orally with adjuvants to try and evoke specific mucosal immune responses. Husband et al. [1979] detected high numbers of Salmonella-specific IgA-producing B cells in the intestinal lamina propria of sheep following oral immunisation with killed antigen only if the animals had first been immunised intraperitoneally with antigen in CFA. It may be that such a vaccination regime is necessary to elicit specific mucosal antibody in sheep to the live S25/1aroA strain and the use of an adjuvant might also stimulate greater Salmonella-specific CMI. However any vaccination procedure involving intraperitoneal injection would be of very limited field use.

The strong Salmonella-specific responses in mice allowed me to examine the potential use of aroA strains as carriers of heterologous antigens. However, only very poor MOMP-specific and

leukotoxin-specific antibody responses were detectable in immunised mice and no antigen-specific CMI was evoked. These experiments thus highlight possible difficulties in expressing foreign genes in an immunogenic form by live Salmonella vaccine strains. The low or absent responses to MOMP and leukotoxin expressed from S25/1aroA were not a result of an innate lack of imunogenicity of the proteins, as both the mice and the sheep made specific immune responses to the proteins when administered with adjuvant. The low response to the vaccine-expressed MOMP may reflect a combination of its intracytoplasmic expression and its insolubility within the vaccine strain. In contrast, the low or absent response to the leukotoxin was most likely a result of rapid loss of the pAL12 plasmid in vivo thus limiting the expression of the protein. Some of the strategies currently under investigation to circumvent these problems of antigen expression by Salmonella vectors are discussed below.

Although the immune system can recognise foreign proteins expressed from the cytoplasm of Salmonella strains [Brown et al., 1987], one possibility would be to obtain surface expression of MOMP or the leukotoxin in S25/1aroA. This can be achieved by expressing antigens as fusion proteins with E.coli outer membrane proteins. Mice produce very strong responses to outer membrane proteins such as OmpA protein and lipoprotein [Brown and Hormaeche, 1989] and it is not clear whether this is due to their surface location or intrinsic immunogenicity. Epitopes of foot-and-mouth disease virus, hepatitis B virus, polio virus and Plasmodium falciparum are all

immunogenic in Salmonella when expressed as fusions with outer membrane proteins such as OmpA, PhoE and LamB [Leclerc et al., 1989; O'Callaghan et al., 1990; Agterberg et al., 1990; Schorr et al., 1992]. However MOMP epitopes expressed on the surface of S.typhimurium aroA by fusion with the LamB protein produced only low MOMP-specific responses, very similar to those described in this thesis [Hayes et al., 1991]. Nevertheless these studies used C3H/He/Ola mice which are less susceptible to Salmonella infection [Hormaeche, 1979a] and clear the organism very rapidly. In addition, these workers employed only short sequences of MOMP compared with the full length MOMP gene expressed in our system and obtained relatively low Salmonella-specific responses with no Salmonella-specific IgA in the gut. As my studies showed long-term persistence of the organism in the murine tissues with strong vaccine-specific responses, it would now be important to examine immunity to the full MOMP protein when expressed as a surface protein with LamB. Alternatively, surface expression of MOMP or leukotoxin could be achieved by incorporating them into the flagella of the S25/1aroA strain, as both LT-B and hepatitis B virus epitopes are immunogenic in Salmonella when expressed in this way [Newton et al., 1989, 1991; Wu et al., 1989]. Studies to obtain surface expression of MOMP in S25/1aroA are already underway. The pUC8 plasmid containing the MOMP gene with a leader peptide has been transformed into S25/1aroA. However, although the

MOMP protein was surface-expressed in high quantities, this proved lethal for the salmonellae and hence new strategies are currently being developed to obtain non-lethal surface expression [A.Herring, personal communication].

Plasmid expression of foreign antigens in Salmonella may also not be an ideal expression system for potential multivalent vaccines. As well as its instability in vivo, the pUC8 plasmid possesses an antibiotic-resistance gene which is not permissable according to Food and Drug Administration Standards for live vaccines. Hence if S25/1aroA is to be used as a multivalent vaccine in sheep or other larger animals, other ways of expressing foreign proteins must be exploited. Nakayama et al. [1988] described the use of recombinant plasmids expressing foreign antigens that did not contain drug-resistance markers hence the problems of in vivo deselection were also avoided. Normally lethal mutations were made in the asd gene [coding for diaminopimelic acid] in the Salmonella chromosome. A plasmid containing the intact asd gene and the spa A gene of Streptococcus sobrinus was transformed into the asd mutant strain which balanced the effect of the mutation. Thus only plasmid-containing organisms survived in vivo and it would be interesting to investigate the use of this strategy for expression of MOMP and leukotoxin in S25/1aroA.

A second approach to avoid the problems of plasmid instability would be to express the leukotoxin from the chromosome of S25/1aroA. Such chromosomal expression has been effected in a novel

expression system which utilises an invertible DNA sequence resulting in spontaneous segregation of cells with very high levels of expression of the recombinant antigen; the system was capable of inducing antibodies to cholera toxin CT-B [Yan et al., 1990]. Another way of expressing foreign genes from the chromosome of the salmonellae is by homologous recombination, using cloning vectors with flanking regions of homology to host genes such as his [Hone et al., 1988; Clements and Cardenas, 1990] or aroC [Strugnell et al., 1990]. The aroC gene system has the added advantage of creating an additional attenuating lesion in the vaccine strain. This expression system would be particularly useful in the S25/1aroA strain which harbours only a single mutation and, as I found, was capable of reverting to virulence in germ-free animals. Thus in one step, the S25/1aroA strain could be made to express the MOMP or leukotoxin from the chromosome and additionally be given a second attenuating mutation. As these double mutants are no less invasive than single mutants [Dougan et al., 1988], this system provides a suitable alternative to previously described double mutants which are less invasive than single mutant strains and are ineffective in stimulating Salmonella-specific immune responses [O'Callaghan et al., 1988; Sigwart et al., 1989].

The discussed strategies for circumventing plasmid instability and for obtaining surface expression of the proteins should be applied. However before MOMP, leukotoxin or other antigens are expressed in S25/1aroA, it may prove beneficial to express well-defined

antigens such as nucleoprotein, LT-B and B-galactosidase from the S25/1aroA strain, solely to demonstrate its vector potential.

The reversion seen in germ-free animals gives further credence to the view that if live Salmonella vaccines are to be given to humans or large animals, double mutant strains must be used to further reduce the chances of reversion to virulence. It would be important in future studies to sequence the aroA gene in the S25/1aroA strain before and after reversion, to provide a molecular explanation for its reversion to virulence. When the strain I used was constructed, several isolate colonies were produced that were all tetracycline-sensitive and aromatic-dependent. However it is possible that each individual mutant isolate harbours a different deletion as a result of the transposon mutagenesis. These other isolates should also be sequenced and tested for reversion in germ-free lambs and comparisons made with the isolate used in this thesis. Thus it may be possible to predict the possibility of reversion to virulence from the extent of the deletion in the aroA gene of individual isolates.

Once the S25/1aroA strain has been fully characterised at the molecular level, experiments should be set up to define the protective response against Salmonella in sheep. The effect on protection from wildtype challenge in vaccinated and unvaccinated lambs following administration of specific monoclonal antibodies against IgG, IgM and IgA, as well as against T cell subsets and IFN-g, might demonstrate the relative importance of these immune

mechanisms. Also, lymphocyte populations from vaccinated animals should be isolated by cell sorting and the ability of passively transferred B and T cells to confer protection from virulent challenge determined. Although these experiments have been performed extensively in mice [Morris et al., 1976; Eisenstein et al., 1984; Paul et al., 1985; Killar and Eisenstein, 1985; Hormaeche et al., 1990; Muotiala and Mäkelä, 1990] and have shown that immunity to Salmonella is both T cell-dependent and T cell-independent, no such studies have been performed in sheep and hence the mechanisms of protection are completely unknown.

It seems unlikely that the relatively low levels of Salmonella-specific IgM that I detected in S25/1aroA-vaccinated lambs could be the sole protective factor and that either CMI or local intestinal responses are not involved. That Salmonella itself is a facultative intracellular parasite would suggest that cellular immunity should be involved. My failure to detect systemic T cells and only low levels of serum antibody following oral vaccination with aroA S.typhimurium is supported by previous findings [Mukkur et al., 1987; Begg et al., 1990; Mukkur and Walker, 1992] and suggests that systemic immune responses are not essential for protection. Together with the absence of intestinal IgA responses, these findings might suggest an important role for mucosal T cells in protection. The failure to recover the wildtype organism from most of the systemic organs following oral challenge

of aroA-vaccinated lambs indicates that strong vaccine-specific local immune mechanisms must be operating in vaccinated animals. The mucosal T cell response following vaccination could be investigated by examining the Salmonella-specific responses of T cells in intestinal lymph following cannulation of the intestinal lymphatic duct of vaccinated animals. Also, T cells from the mesenteric lymph node, intestine and Peyer's patches as well as intraepithelial lymphocytes should be removed from aroA-vaccinated lambs and examined for Salmonella-specific responses.

In conclusion, it is hoped that the work described in this thesis will provide the groundwork for developing more effective systems of expression of foreign proteins in Salmonella vaccine strains. It has highlighted the quite different immune responses in mice and sheep following oral vaccination, and has outlined the need for careful consideration of both the origin and the nature of the antigen before expression in these vaccine strains. Furthermore it has emphasised the need for detailed molecular characterisation of candidate strains to avoid problems of reversion to virulence which clearly may undermine the safety and effectiveness of aroA strains of Salmonella.

REFERENCES

ADAS [1982]. Animal Health Report of the Chief Medical Officer. HMSO [Pub]. 116-117.

Adlam, C., Knights, J.M., Mugridge, A., Lindon, J.C., Baker, P.R.W., Beesley, J.E., Spacey, B., Craig, G.R. and Nagy, L.K. [1984]. Purification, characterisation and immunological properties of the serotype-specific capsular polysaccharide of Pasteurella haemolytica [serotype A1] organisms. Journal of General Microbiology 131, 2415-2426.

Aggarwal, A., Kumar, S., Jaffe, R., Hone, D., Gross, M. and Sadoff, J. [1990]. Oral Salmonella: Malaria circumsporozite recombinants induce specific CD8+ cytotoxic T cells. Journal of Experimental Medicine 172, 1083-1090.

Agteberg , M., Fransen, R. and Tommassen, J. [1990]. Expression of Escherichia coli PhoE protein in avirulent Salmonella typhimurium aroA and galE strains. FEMS Microbiology Letters 50, 295-299.

Aitken, I.D. [1983]. Enzootic [Chlamydial] abortion. In: Diseases of sheep. W.B. Martin [Ed.]. Blackwell Scientific Publishers, Oxford, UK. pp119-123.

Allen, J.E., Locksley, R.M. and Stephens, R.S. [1991]. A single peptide from the major outer membrane protein of C.trachomatis elicits T cell help for the production of antibodies to protective determinants. Journal of Immunology 147, 674-679.

Anderson, I.E. [1986]. Comparison of the virulence in mice of some ovine isolates of Chlamydia psittaci. Veterinary Microbiology, 12, 212-220.

Anderson, J., Melchers, F., Galanos, C. and Lüderitz, O. [1973]. The mitogenic effect of lipopolysaccharide on bone marrow-derived mouse lymphocytes. Journal of Experimental Medicine 137, 943-953.

Angerman, C.R. and Eisenstein, T.K. [1980]. Correlation of the duration and magnitude of protection against Salmonella infection afforded by various vaccines with antibody titers. Infection and Immunity 27, 435-443.

Bacon, G.A., Burrows, T.W. and Yates, M. [1950]. The effects of biochemical mutation on the virulence of Bacterium typhosum: the virulence of mutants. British Journal of Experimental Pathology 31, 714-724.

Bacon, G.A., Burrows, T.W. and Yates, M. [1951]. The effects of biochemical mutation on the virulence of Bacterium typhosum: the loss of virulence of certain mutants. British Journal of Experimental Pathology 32, 85-96.

Baehr, W., Zhang, Y.-X., Joseph, T., Su, H., Nano, F.E., Everett., K.D.E. and Caldwell, H.D. [1988]. Mapping antigenic domains expressed by Chlamydia trachomatis major outer membrane protein genes. Proceedings of the National Academy of Sciences [USA] 85, 4000-4004.

Baird, G.D., Manning, E.J. and Jones, P.W. [1985]. Evidence for related virulence sequences in plasmids of Salmonella dublin and Salmonella typhimurium. Journal of General Microbiology 131, 1815-1823.

Baluyut, C.S., Simomson, R.R., Bemrick, W.J. and Maheswaran, S.K. [1981]. Interaction of Pasteurella haemolytica with bovine neutrophils. Identification and partial characterisation of a a cytotoxin. American Journal of Veterinary Research 42, 1920-1926.

Bancroft, G.J., Schreiber, R.D., Bosma, G.C., Bosma, M.J. and Unanue, E.R. [1987]. A T cell-independent mechanism of macrophage activation by interferon-g. Journal of Immunology 139, 1104-1107.

Bancroft, G.J., Sheehan, K.C.F., Schreiber, R.D. and Unanue, E.R. [1989]. Tumour necrosis factor is involved in the T cell-independent pathway of macrophage activation in SCID mice. Journal of Immunology 143, 127-130.

Barrow, P.A. and Lovell, M.A. [1988]. The association between a large molecular mass plasmid and virulence in a strain of Salmonella pullorum. Journal of General Microbiology 134, 2307-2316.

Barrow, P.A., Simpson, J.M., Lovell, M.A. and Binns, M.M. [1987]. Contribution of Salmonella gallinarum large plasmid toward virulence in fowl typhoid. Infection and Immunity 55, 388-392.

Bartholomeusz, R.C.A., Labrooy, J.T., Ey, P.L., Pyle, D., Shearman, D.J.C. and Rowley, D. [1990]. The serum polymeric IgA antibody response to typhoid vaccination; its relationship to the intestinal IgA response. Immunology 69, 190-194.

Bartholomeusz, R.C.A., Labrooy, J.T., Johnson, M., Shearman, D.J.C. and Rowley, D. [1986]. Gut immunity to typhoid: The immune response to a live oral typhoid vaccine Ty21a. Journal of Gastroenterology and Hepatology 1, 61-67.

Beckerdite, S., Mooney, C., Weiss, J., Franson, R. and Elsbach, P. [1974]. Early and discrete changes in permeability of Escherichia coli and certain other Gram-negative bacteria during killing by granulocytes. Journal of Experimental Medicine 140, 396-409.

Begg, A.P., Walker, K.H., Love, N.V. and Mukkur, T.K.S. [1990]. Evaluation of protection against experimental salmonellosis in sheep immunised with 1 or 2 doses of live aromatic-dependent Salmonella typhimurium. Australian Veterinary Journal 67, 294-298.

Benson, M.L., Thompson, R.G. and Valli, V.E.O. [1978]. The bovine alveolar macrophage II. In vitro studies with Pasteurella haemolytica. Canadian Journal of Comparative Medicine 42, 368-369.

Bermudez, L.E.M. and Young, L.S. [1988]. Tumour necrosis factor alone or in combination with IL-2, but not IFN-g, is associated with macrophage killing of Mycobacterium avium complex. Journal of Immunology 140, 3006-3013.

Beutler, B. and Cerami, A. [1986]. Cachectin and tumour necrosis factor are two sides of the same biological coin. Nature 320., 584-588.

Biberstein, E. L., Gills, M.G. and Knight, H. [1960]. Serological types of Pasteurella haemolytica. Cornell Veterinary 50, 283-300.

Black, R., Levine, M.M., Young, C., Rooney, J., Levine, S., Clements, M.L., O'Donnell, S., Hughes, T., Germanier, R. and Chilean Typhoid Committee [1983]. International Symposium on Enteric Infections in Man and Animals: Standardisation of Immunological Procedures, Dublin, Ireland, 1982. In: Development of Biological Standards 53, 9-14. S. Karger, Basel.

Blanden, R.V., MacKaness, G.B. and Collins, F.M. [1966]. Mechanisms of acquired resistance in mouse typhoid. Journal of Experimental Medicine 124, 585-599.

Blanchard, D.K., Djeu, J.Y., Klein, T.W., Freidman, H. and Stewart II, W.E. [1986]. Interferon-g induction by lipopolysaccharide :dependence on interleukin 2 and macrophages. Journal of Immunology 136, 963-970.

Bochner, B.R., Huang, H., Schieven, G. and Ames, B.N. [1980]. Positive selection for loss of tetracycline-resistance. Journal of Bacteriology 143, 926-933.

Briles, D.E., Benjamin, Jnr, W., Posey, B., Michalek, S.M. and McGhee, J.R. [1986]. Independence of macrophage activation and expression of the alleles of the Ity [immunity to typhimurium] locus. Microbial Pathogenesis 1, 33-41.

Briles, D.E., Lehmyer, J. and Forman, C. [1981]. Phagocytosis and killing of Salmonella typhimurium by peritoneal exudate cells. Infection and Immunity 33, 380-388.

Brown, A. and Hormaeche, C.E. [1989]. The antibody response to salmonellae in mice and humans studied by immunoblots and ELISA. Microbial Pathogenesis 6, 445-454.

Brown, A., Hormaeche, C.E., Demarco de Hormaeche, R., Winther, R., Dougan, G., Maskell, D.J. and Stocker, B.A.D. [1987]. An attenuated aroA Salmonella typhimurium vaccine elicits humoral and cellular immunity to cloned B-galactosidase in mice. Journal of Infectious Diseases 155, 86-92.

Brown, D.D., Ross, J.G. and Smith, A.F.G. [1976]. Experimental infections of sheep with Salmonella typhimurium. Research in Veterinary Science 21, 335-340.

Brown, I.N., Glynn, A.A. and Plant, J. [1982]. Inbred mouse strain resistance to Mycobacterium lepraemurium follows the Ity/lsh pattern. Immunology 47, 149-156.

Buchmeier, N.A. and Heffron, F. [1989]. Intracellular survival of wild-type Salmonella typhimurium and macrophage-sensitive mutants in diverse populations of macrophages. Infection and Immunity 57, 1-7.

Buchmeier, N.A. and Heffron, F. [1991]. Inhibition of macrophage phagosome-lysosome fusion by Salmonella typhimurium. Infection and Immunity 59, 2232-2238.

Burnette, W.N. [1981]. "Western Blotting": electrophoretic transfer of proteins from sodium dodecyl sulphate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated Protein A. Analytical Biochemistry 112, 195-203.

Burrells, C. and Wells, P.W. [1977]. In vitro stimulation of ovine lymphocytes by various mitogens. Research in Veterinary Science 23, 84-86.

Buxton, D. [1986]. Potential danger to preganant women of Chlamydia psittaci from sheep. Veterinary Record 118, 510-511.

Buzoni-Gatel, D., Guilloteau, L., Bernard, F., Bernard, S., Chardes, T. and Rocca, A. [1992]. Protection against C.psittaci in mice conferred by Lyt-2+ T cells. Immunology 77, 284-288.

Buzoni-Gatel, D., Rodolakis, A. and Plommet, M. [1987]. T cell-mediated and humoral immunity in a mouse Chlamydia psittaci systemic infection. Research in Veterinary Science 43, 59-63.

Caldwell, H.D., Kromhout, J. and Schachter, J. [1981]. Purification and partial characterisation of the major outer membrane protein of Chlamydia trachomatis. Infection and Immunity 31, 1161-1176.

Caldwell, H.D. and Judd, R.C. [1982]. Structural analysis of chlamydial major outer membrane proteins. Infection and Immunity 38, 960-968.

Caldwell, H.D. and Perry, L.J. [1982]. Neutralisation of Chlamydia trachomatis infectivity with antibodies to the major outer membrane protein. Infection and Immunity 38, 745-754.

Caldwell, H.D. and Schachter, J. [1982]. Antigenic analysis of the major outer membrane protein of Chlamydia spp. Infection and Immunity 35, 1024-1031.

Cameron, C.M. [1966]. The haemagglutination test and immunity to Pasteurella haemolytica. Journal of the South African Veterinary Medicine Association 37, 165.

Cancellieri, V. and Fara, G.M. [1985]. Demonstration of specific IgA in human faeces after immunisation with live Ty21a Salmonella typhi vaccine. Journal of Infectious Diseases 151, 482-484.

Carsiotis M., Stocker, B.A.D., Weinstein, D.L. and O'Brien, A.D. [1989]. A Salmonella typhimurium virulence gene linked to flg. Infection and Immunity 57, 3276-3280.

Carsiotis, M., Weinstein, D.L., Karch, H., Holder, I.A. and O'Brien, A.D. [1984]. Flagella of Salmonella typhimurium are a virulence factor in infected C57B1/6J mice. Infection and Immunity 46, 814-818.

Carter, P.B. and Collins, F.M. [1974]. The route of enteric infection in normal mice. Journal of Experimental Medicine 139, 1189-1203.

Chang, Y-F., Richards, A.B. and Renshaw, H.W. [1982]. A toxic agent derived from Pasteurella haemolytica. In: Proceedings of the Third International Conference on Goat Production and Disease. Tuczon, AZ, USA. pp295.

Cho, H.J., Bohac, J.G., Yates, W.D.G. and Ohmann, H.B. [1984]. Anti-cytotoxin activity of bovine sera and body fluids against Pasteurella haemolytica cytotoxin. Canadian Journal of Comparative Medicine 48, 151-155.

Chopra, A.K., Houston, C.W., Peterson, J.W. and Mekalanos, J.J. [1987a]. Chromosomal DNA contains the gene coding for Salmonella enterotoxin. FEMS Microbiology Letters 43, 345-349.

Chopra, A.K., Houston, C.W., Peterson, J.W., Prasad, R. and Mekalanos, J.J. [1987b]. Cloning and expression of the Salmonella enterotoxin gene. Journal of Bacteriology 169, 5095-5100.

Clements, J.D. and Cardenas, L. [1990]. Vaccines against enterotoxigenic bacterial pathogens based on hybrid Salmonella that express heterologous antigens. Research in Microbiology 141, 981-983.

Clements, J.D. and El-Morshidy, S. [1984]. Construction of a potential live oral bivalent vaccine for typhoid fever and cholera-Escherichia coli-related diarrhoeas. Infection and Immunity 46, 564-569,

Clements, J.D., Lyon, F.L., Lowe, K.L., Farrand, A.L. and El-Morshidy, S. [1986]. Oral immunisation of mice with attenuated Salmonella enteriditis containing a recombinant plasmid which codes for the production of the B subunit of heat-labile Escherichia coli enterotoxin. Infection and Immunity 53, 685-692.

Collins, F.M. [1969]. Effect of specific immune mouse serum on the growth of Salmonella enteriditis in nonvaccinated mice challenged by various routes. Journal of Bacteriology 97, 667-675.

Collins, F.M. [1971]. Mechanisms in antimicrobial immunity. Journal of the Reticuloendothelial Society 10, 58-99.

Collins, F.M. [1972]. Salmonellosis in orally infected specific pathogen-free C57B1 mice. Infection and Immunity 5, 191-198.

Collins, F.M. [1974]. Vaccines and cell-mediated immunity. Bacteriological Reviews 38, 371-402.

Collins, F.M. and MacKaness, G.B. [1968]. Delayed Hypersensitivity and Arthus reactivity in relation to host resistance in Salmonella-infected mice. Journal of Immunology 101, 830-845.

Collins, P., Parsons, K.P. and Jones, P.W. [1988]. The role of immunoglobulin and peritoneal phagocytes in the protection of rats against Salmonella infection. Research in Veterinary Science 44, 329-334.

Conlon, J.W., Clarke, I.N. and Ward, M.E. [1988]. Epitope mapping with solid phase peptides: identification of type-, subspecies-, species- and genus-reactive antibody binding domains on the major outer membrane protein of Chlamydia trachomatis. Molecular Microbiology 2, 673-679.

Conlon, J.A., Shewen, P.E. and Lo, R.Y.C. [1991]. Efficacy of recombinant leukotoxin in protection against pneumonic challenge with live Pasteurella haemolytica A1. Infection and Immunity 59, 587-591.

Cooke, A., Lydyard, P.M. and Roitt, I.M. [1983]. Mechanisms of autoimmunity: a role for cross-reactive idiotypes. Immunology Today 4, 170-175.

Cooper, G.L., Venables, L.M., Nicholas, R.A.J., Cullen, G.A. and Hormaeche, C.E. [1992]. Vaccination of chickens with chicken-derived Salmonella enteriditis phage type 4 aroA live oral Salmonella vaccines. Vaccine 10, 247-254.

Costerton, J.W., Irvin, R.T. and Cheng, K-J. [1981]. The role of bacterial surface structures in pathogenesis. CRC Critical Reviews of Microbiology 303-338.

Dawson, M., Venables, C. and Wilsmore, A.J. [1986a]. Immune responses of sheep experimentally infected with ovine abortion isolates of Chlamydia psittaci. In: Agriculture: Chlamydial diseases of ruminants. Aitken, I.D. [Ed]. Commission of the European Communities Publication. Luxembourg. pp97-105.

Dawson, M., Zaghloul, A. and Wilsmore, A.J. [1986b]. Ovine enzootic abortion: experimental studies of immune responses. Research in Veterinary Science 40, 59-64.

De Libero, G. and Kaufmann, S.H.E. [1986]. Antigen-specific Lyt2+ cytolytic T lymphocytes from mice infected with the intracellular bacterium Listeria monocytogenes. Journal of Immunology 137, 2688-2694.

Dougan, G., Chatfield, S.N., Pickard, D., Bester, J., O'Callaghan, D. and Maskell, D. [1988]. Construction and characterisation of vaccine strains of Salmonella harboring mutations in two different aro genes. Journal of Infectious Diseases 158, 1329-1335.

Dougan, G., Sellwood, R., Maskell, D.J., Sweeney, K., Liew, F.Y., Beesley, J. and Hormaeche, C.E. [1986]. In vivo properties of a cloned K88 adherence antigen determinant. Infection and Immunity 52, 344-347.

Duguid, J.P., Darekar, M.R. and Wheater, D.W.F. [1976]. Fimbriae and infectivity in Salmonella typhimurium. Journal of Medical Microbiology 9, 459-473.

Dungal, N. [1931]. Contagious pneumonia in sheep. Journal of Comparative Pathology and Therapeutics 44, 126-143.

Duran, L.W. and Metcalf, E.S. [1987]. Clonal analysis of primary B cells responsive to the pathogenic bacterium Salmonella typhimurium. Journal of Experimental Medicine 165, 340-358.

Edebo, L. and Norman, B. [1970]. Virulence and immunogenicity of mutant strains of Salmonella typhimurium. Acta Patologica et Microbiologica Scandinavica Section B 78, 75-84.

Edwards III, C.K., Ghiasuddin, S.M., Yunger, L.M., Lorence, R.M., Arkins, S., Dantzer, R. and Kelley, K.W. [1992]. In vivo administration of recombinant growth hormone or gamma interferon activates macrophages: enhanced resistance to experimental Salmonella typhimurium infection is correlated with generation of reactive oxygen intermediates. Infection and Immunity 60, 2514-2521.

Eisenstein, T.K. and Angerman, C.R. [1978]. Immunity to experimental Salmonella infection: studies on the protective capacity and immunogenicity of lipopolysaccharide, acetone-killed cells, and ribosome-rich extracts of Salmonella typhimurium in C3H/HeJ and CD-1 mice. Journal of Immunology 121, 1010-1014.

Eisenstein, T.K., Killar, L.M. and Sultzer, B.M. [1984]. Immunity to infection with Salmonella typhimurium: mouse-strain differences in vaccine- and serum-mediated protection. Journal of Infectious Diseases 150, 425-435.

Entrican, G., McInnes, C.J., Rothel, J.S. and Haig, D.M. [1992]. Kinetics of ovine interferon-gamma production. Detection of mRNA and characterisation of biological activity. Veterinary Immunology and Immunopathology 33, 171-178.

Felix, A. and Pitt, M.R. [1951]. The pathogenic and immunogenic activities of Salmonella typhi in relation to its antigenic constituents. Journal of Hygiene 49, 92-110.

Ferreccio, C., Levine, M.M., Rodriguez, H., Contreras, R., Chilean Typhoid Committee [1989]. Comparative efficacy of two, three, and four doses of Ty21a live oral typhoid vaccine in enteric-coated capsules: A field trial in an endemic area. Journal of Infectious Diseases 159, 766-769.

Fields, P.I., Swanson, R.V., Haidaris, C.D. and Heffron, F. [1986]. Mutants of Salmonella typhimurium that cannot survive within the macrophage are avirulent. Proceedings of the National Academy of Sciences, USA 83, 5189-5193.

Foggie, A. [1973]. Preparation of vaccines against enzootic abortion of ewes. A review of the research work at the Moredun Research Institute. Veterinary Bulletin 43, 587-590.

Formal, S.B., Baron, L.S., Kopecko, D.J., Washington, O., Powell, C. and Life, C.A. [1981]. Construction of a potential bivalent vaccine strain: introduction of Shigella sonnei form 1 antigen genes into the galE Salmonella typhi Ty21a typhoid vaccine strain. Infection and Immunity 34, 746-750.

Forrest, B.D. [1988]. Identification of an intestinal immune response using peripheral blood lymphocytes. Lancet, January 16, 1988, 81-83.

Fraser, J., Laird, S. and Gilmour, N.J.L. [1982]. A new serotype [biotype T] of Pasteurella haemolytica. Reviews of Veterinary Science 32, 127-128.

Freter, R. [1981]. Mechanisms of association of bacteria with mucosal surfaces. CIBA Foundation Symposium. 80, 36-55.

Fukosawa, T. and Nikaido, H. [1959]. Galactose-sensitive mutants of Salmonella. Nature 184, 1168-1169.

Fukasawa, Y., Kagaya, K. and Ishibashi, Y. [1983]. Effect of delayed-type hypersensitivity reaction and transferred lymphokine on the resistance of mice to Salmonella typhimurium. Infection and Immunity 39, 986-989.

Gahring, L.C., Heffron, F., Findlay, B.B. and Falkow, S. [1990]. Invasion and replication of Salmonella typhimurium in animal cells. Infection and Immunity 58, 443-448.

Gaillard, J-L., Berche, P., Mounier, J., Richard, S. and Sansonetti, P. [1987]. In vitro model of penetration and intracellular growth of Listeria monocytogenes in the human enterocyte-like cell line Caco-2. Infection and Immunity 55, 2822-2829.

Garcia-Penarrubia, P., Koster, F.T., Kelley, R.O., McDowell, T.D. and Bankhurst, A.D. [1989]. Antibacterial activity of human natural killer cells. Journal of Experimental Medicine 169, 99-113.

Gentry, M.J., Confer, A.W. and Panciera, R.J. [1985]. Serum neutralisation of cytotoxin from Pasteurella haemolytica serotype 1 and resistance to experimental bovine pneumonia pasteurellosis. Veterinary Immunology and Immunopathology 9, 239-250.

Germanier, R. and Furer, E. [1971]. Immunity in experimental salmonellosis II. Basis for the avirulence and protective capacity of galE mutants of Salmonella typhimurium. Infection and Immunity 4, 663-673.

Gianella, R.A. [1979]. Importance of the intestinal inflammmatory reaction in Salmonella-mediated intestinal secretion. Infection and Immunity 23, 140-145.

Gianella, R.A., Rout, W.R., Formal, S.B. and Collins, H. [1976]. Role of plasma filtration in the intestinal fluid secretion mediated by infection with Salmonella typhimurium. Infection and Immunity 13, 470-474.

Gilmour, N.J.L. [1978]. Pasteurellosis in sheep. Veterinary Record 102, 100-102.

Gilmour, N.J.L. [1980]. Pasteurella haemolytica infections in sheep. The Veterinary Quarterly 2, 191-197.

Gilmour, N.J.L., Martin, W.B., Sharp, J.M., Thompson, D.A., Wells, P.W. and Donachie, W. [1983]. Experimental immunisation of lambs against pneumonic pasteurellosis. Reviews of Veterinary Science 35, 80-86.

Griffin, F.M. [1977]. In: Biological Amplification Systems in Immunology. Day, N.K. and Good, R.A. [Eds]. Plenum, N.Y., USA. pp85-113.

Grist, N.R., Ross, C.A.C., Bell, E.J. and Stott, E.J. [1966]. In: Diagnostic Methods in Clinical Virology. Blackwell Scientific Publications, Oxford, UK. pp44-45.

Groschel, D., Paas, C.M.S. and Rosenberg, B.S. [1970]. Inherited resistance and mouse typhoid I. Some factors which affect the survival of infected mice. Journal of the Reticuloendothelial Society 7, 484-499.

Gulig, P.A. and Curtiss III, R. [1987]. Plasmid-associated virulence of Salmonella typhimurium. Infection and Immunity 55, 2891-2901.

Guzmán, C.A., Brownlie, R.M., Kadurungamuwa, J., Walker, M.J. and Timmis, K.N. [1991]. Antibody responses in the lungs of mice following oral immunisation with Salmonella typhimurium aroA and invasive Escherichia coli strains expressing the filamentous heamagglutinin of Bordetella pertussis. Infection and Immunity 59, 4391-4397.

Hackett, J. and Wyk, P. [1986]. Molecular cloning of a gene encoding a 23-kDa outer membrane polypeptide from the cryptic plasmid of Salmonella typhimurium. FEMS Microbiology Letters 36, 251-256.

Hahn, H. and Kaufmann, S.H.E. [1981]. The role of cell-mediated immunity in bacterial infection. Reviews of Infectious Diseases 3, 1221-1250.

Hall, G.A. [1973]. Changes in the rat placenta following inoculation with Salmonella dublin. American Journal of Pathology 72, 103-114.

Hall, G.A [1974]. An investigation into the mechanism of placental damage in rats inoculated with Salmonella dublin. American Journal of Pathology 77, 299-310.

Hayes, L.J., Conlon, J.W., Everson, J.S., Ward, M.E. and Clarke, I.N. [1991]. Chlamydia trachomatis major outer membrane epitopes expressed as fusions with LamB in an attenuated aroA strain of Salmonella typhimurium; their application as potential immunogens. Journal of General Microbiology 137, 1557-1564.

Heffernan, E.J., Feirer, J., Chikami, G. and Guiney, D. [1987]. Natural history of oral Salmonella dublin infection in BALB/c mice: effect of an 80-kilobase-pair plasmid on virulence. Journal of Infectious Diseases 155, 1254-1259.

Helmuth, R., Stephan, R., Bunge, C., Hoog, B., Steinbeck, A. and Bulling, B. [1985]. Epidemiology of virulence-associated plasmids and outer membrane protein patterns with seven common Salmonella serotypes. Infection and Immunity 48, 175-182.

Hertzberg, M., Nash, P. and Hino, S. [1972]. Degree of immunity induced by killed vaccines to experimental salmonellosis in mice. Infection and Immunity 5, 83-90.

Hochadel, J.F. and Keller, K.F. [1977]. protective effects of passively transferred immune T or B lymphocytes in mice infected with Salmonella typhimurium. Journal of Infectious Diseases 135, 813-823.

Hohmann, A., Schmidt, G. and Rowley, D. [1979]. Intestinal and serum antibody responses in mice after oral immunisation with Salmonella, Escerichia coli and Salmonella-Escherichia coli hybrid strains. [1979]. Infection and Immunity 25, 27-33.

Hoiseth, S.K. and Stocker, B.A.D. [1981]. Aromatic-dependent Salmonella typhimurium are non-virulent and effective as live vaccines. Nature 291, 238-239.

Hone, D.M., Attridge, S., Van den Bosch, L. and Hackett, J. [1988]. A chromosomal integration system for stabilisation of heterologous genes in Salmonella-based vaccine strains. Microbial Pathogenesis 5, 407-418.

Hormaeche, C.E. [1979a]. Natural resistance to Salmonella typhimurium in different inbred mouse strains. Immunology 37, 311-318.

Hormaeche, C.E., Mastroeni, P., Arena, A., Uddin, J. and Joysey, H.S. [1990]. T cells do not mediate the initial suppression of a salmonella infection in the RES. Immunology 70, 247-250.

Hormaeche, C.E. [1979b]. Genetics of natural resistance to salmonellae in mice. Immunology 37, 319-327.

Hormaeche, C.E., Harrington, K.A. and Joysey, H.S. [1985]. Natural resistance to salmonellae in mice: control by genes within the major histocompatibility complex. Journal of Infectious Diseases 152, 1050-1055.

Hormaeche, C.E., Pettifor, R.A. and Brock, J. [1981]. The fate of temperature-sensitive Salmonella mutants in vivo in naturally resistant and susceptible mice. Immunology 42, 569-576.

Hornick, R.B., Greisman, S.E., Woodward, T.E., DuPont, H.L., Dawkins, A.T. and Snyder, M.J. [1970]. Typhoid Fever: Pathogenesis and Immunologic Control [1st of 2 parts]. New England Journal of Medicine 283, 686-691.

Huang, H.-S. [1988]. The ovine immune responses to Chlamydia psittaci. M.Phil thesis. University of Edinburgh, UK.

Humphrey, T.J., Mead, G.C. and Rowe, B. [1988]. Poultry meat as a source of human salmonellosis in England and Wales. Epidemiology and Infection 100, 175-184.

Husband, A.J., Beh, K.J. and Lascelles, A.K. [1979]. IgA-containing cells in the ruminant intestine following intraperitoneal and local immunisation. Immunology, 37, 597-601.

Iino, T. [1969]. Genetics and chemistry of bacterial flagella. Bacteriological Reviews 33, 454-475.

Impey, C.S., Mead, G.C. and George, S.M. [1982]. Competitive exclusion of salmonellas from the chicken caecum using a defined mixture of bacterial isolates from the caecal microflora of an adult bird. Journal of Hygiene 89, 479-490.

Isaacson, R.E. and Kinsel, M. [1992]. Adhesion of Salmonella typhimurium to porcine intestinal epithelial surfaces: Identification and characterisation of two phenotypes. Infection and Immunity 60, 3193-3200.

Ishizaki, M., Allen, J.E., Beatty, P.R. and Stephens, R.S. [1992]. Immune specificity of murine T cell lines to the major outer membrane protein of Chlamydia trachomatis. Infection and Immunity 60, 3714-3718.

Jiwa, S.F.H. [1981]. Probing for enterotoxigenicity among the salmonellae: an evaluation of biological assays. Journal of Clinical Microbiology 14, 463-472.

Jones, B.D., Lee, C.A. and Falkow, S. [1992]. Invasion by Salmonella typhimurium is affected by the direction of flagellar rotation. Infection and Immunity 60, 2475-2480.

Jones, G.W., Rabert, D.K., Svinarich, D.M. and Whitfield, H.J. [1982]. Association of adhesive, invasive and virulent phenotypes of Salmonella typhimurium with autonomous 60-megadalton plasmids. Infection and Immunity 38, 476-486.

Jones, G.W., Richardson, L.A. and Ulhman, D. [1981]. The invasion of HeLa cells by Salmonella typhimurium: reversible and irreversible bacterial attachment and the role of bacterial motility. Journal of General Microbiology 127, 351-360.

Jones, P.W., Collins, P. and Aitken, M.M. [1988]. Passive protection of calves against experimental infection with Salmonella typhimurium. Veterinary Record 123, 536-541.

Jones, P.W., Collins, P., Brown, G.T.H. and Aitken, M.M. [1982]. Transmission of Salmonella mbandaka to cattle from contaminated feed. Journal of Hygiene 88, 255-263.

Kagaya, K., Watanabe, K. and Fukazawa, Y. [1989]. Capacity of recombinant gamma interferon to activate macrophages for Salmonella-killing activity. Infection and Immunity 57, 609-615.

Kaufmann, S.H.E. [1988]. CD8+ T lymphocytes in intracellular microbial infections. Immunology Today 9, 168-174.

Kawahara, K., Haraguchi, Y., Tsuchimoto, M., Terakado, N. and Danbara, H. [1988]. Evidence of correlation between 50-kilobase plasmid of Salmonella choleraesuis and its virulence. Microbial Pathogenesis 4, 155-163.

Kawase, I., Brooks, C.G., Kuribayashi, K., Olabuenaga, S., Newman, W., Gillis, S. and Henney, C.S. [1983]. Interleukin-2 induces g-interferon production: participation of macrophages and NK-like cells. Journal of Immunology 131, 288-292.

Khoramian, F.T., Harayama, S., Kutsukake, K. and Pechère, J.C. [1990]. Effect of motility and chemotaxis on the invasion of Salmonella typhimurium into HeLa cells. Microbial Pathogenesis 9, 47-53.

Killar, L.M. and Eisenstein, T.K [1985]. Immunity to Salmonella typhimurium infection in C3H/HeJ and C3H/HeNCrlBR mice: studies with an aromatic-dependent live S.typhimurium strain as a vaccine. Infection and Immunity 47, 605-612.

Killion, J.W. and Morrison, D.C. [1986]. Protection of C3H/HeJ mice from lethal Salmonella typhimurium LT2 infection by immunisation with lipopolysaccharide-lipid A-associated protein complexes. Infection and Immunity 54, 1-8.

Klimpel, G.R., Neisel, D.W., Asuncion, M. and Klimpel, K.D. [1988]. Natural killer cell activation and interferon production by peripheral blood lymphocytes after exposure to bacteria. Infection and Immunity 56, 1436-1441.

Kuusi, N., Nurminen, M., Saxen, H., Valtonen, M. and Makela, P.H. [1979]. Immunisation with major outer membrane proteins in experimental salmonellosis of mice. Infection and Immunity 25, 857-862.

Labrooy, J.T., Davidson, G.P., Shearman, D.J.C. and Rowley, D. [1980]. The antibody response to bacterial gastroenteritis in serum and secretions. Clinical and Experimental Immunology 41, 290-296.

Laemlli, U.K. [1970]. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lascelles, A.K., Beh, K.J., Mukkur, T.K.S. and Willis, G. [1988]. Immune response of sheep to oral and subcutaneous administration of a live aromatic-dependent mutant of Salmonella typhimurium [SL1479] [1988]. Veterinary Immunology and Immunopathology 18, 259-267.

Leclerc, C., Charbit, A., Molla, A. and Hofnung, M. [1989]. Antibody Response to a foreign epitope expressed at the surface of recombinant bacteria: importance of the route of immunisation. Vaccine 7, 242-248.

Le Minor, L. [1984]. Genus Salmonella. In: Bergey's Manual of Systemic Bacteriology Vol 1. Holt, J.G. and Krieg, N.R. [Eds]. Williams and Wilkins, Baltimore, USA. pp427-458.

Levine, M.M., Black, R.E., Clements, M.L., Cisneros, L., Saah, A., Nalin, D.R., Gill, D.M., Craig, J.P., Young, C.R. and Ristaino, P. [1982]. The pathogenicity of nonenterotoxigenic Vibrio cholerae Serogroup O1 Biotype El Tor isolated from sewage water in Brazil. Journal of Infectious Diseases 145, 296-299.

Levine, M.M., Ferreccio, C., Black, R.E., Germanier, R. and the Chilean Typhoid Committee. [1987]. Large-scale field trial of Ty21a live oral typhoid vaccine in enteric-coated capsule formulation. Lancet 1, 1049-1052.

Liang-Takasaki, C-J, Grossman, N. and Leive, L. [1983]. Salmonellae activate complement differentially via the alternative pathway depending on the structure of their lipopolysaccharide O-antigen. Journal of Immunology 130, 1867-1870.

Liang-Takasaki, C-J, Mäkelä, P.H. and Leive, L. [1982]. Phagocytosis of bacteria by macrophages: changing the carbohydrate of lipopolysaccharide alters interaction with complement and macrophages. Journal of Immunology 128, 1229-1235.

Lin, F.R., Hsu. H.S., Mumaw, V.R. and Moncure, C.W. [1989]. Confirmation of the destruction of salmonellae within murine peritoneal exhudate cells by immunocytochemical technique. Immunology 67, 394-400.

Lindberg, A.A. and Robertsson, J.A. [1983]. Salmonella typhimurium infection in calves: cell-mediated and humoral immune reactions before and after challenge with live virulent bacteria in calves given live or inactivated vaccines. Infection and Immunity 41, 751-757.

Lindquist, B.L., Lebenthal, E., Lee, P.C., Stinson, M.W. and Merrick, J.M. [1987]. Adherence of Salmonella typhimurium to small-intestinal enterocytes of the rat. Infection and Immunity 55, 3044-3050.

Lissner, C.R., Swansson, R.N. and O'Brien, A.D. [1983]. Genetic control of the innate resistance of mice to Salmonella typhimurium: expression of the Ity gene in peritoneal and splenic macrophages isolated in vitro. Journal of Immunology 131, 3006-3013.

Lockman, H.A. and Curtiss III, R. [1990]. Salmonella typhimurium mutants lacking flagella or motility remain virulent in BALB/c mice. Infection and Immunity 58, 137-143.

Lockman, H.A. and Curtiss III, R. [1992]. Virulence of non-Type 1-fimbriated and non-fimbriated nonflagellated Salmonella typhimurium mutants in murine typhoid fever. Infection and Immunity 60, 491-496.

Lowell, G.H., Smith, L.F., Artenstein, M.S., Nash, G.S. and MacDermott Jnr, R.P. [1979]. Antibody-dependent cell-mediated antibacterial activity of human mononuclear cells.I. K lymphocytes and monocytes are are effective against Meningococci in cooperation with immune sera. Journal of Experimental Medicine 150, 127-137.

Lüderitz, O., Freudenberg, M.A., Galanos, C., Lehmann, V., Rietschel, E.T. and Shaw, D.H. [1982a]. Current Topics in Membranes and Transport 17, 79-151.

Lüderitz, O., Galanos, C. and Rietschel, E.T. [1982b]. Endotoxins of Gram-negative bacteria. Pharmacology and Therapeutics 15, 382-402.

Lyman, M.B., Stocker, B.A.D. and Roantree, R.J. [1977]. Comparison of the virulence of O:9,12 and O:4,5,12 Salmonella typhimurium his transductants for mice. Infection and Immunity 15, 491-499.

MacKaness, G.B [1967]. The relationship of delayed hypersensitivity to acquired cellular resistance. British Medical Bulletin 23, 52-54.

MacKaness, G.B., Blanden, R.V. and Collins, F.M. [1966]. Host-parasite relations in mouse typhoid. Journal of Experimental Medicine 124, 573-583.

McCafferty, M.C. [1992]. Ovine cell-mediated immunity to Chlamydia psittaci. PhD thesis, University of Edinburgh, UK.

McEwen, A.D., Stamp, J.T. and Littlejohn, A.I. [1951]. Enzootic abortion of ewes. II. Immunisation and infection experiments. Veterinary Record 63, 197-201.

McFarland, W.C. and Stocker, B.A.D. [1987]. Effect of different purine auxotrophic mutations on mouse virulence of a Vi-positive strain of Salmonella dublin and of two strains of Salmonella typhimurium. Microbial Pathogenesis 38, 129-141.

McGhee, J.R., Mestecky, J., Dertzbaugh, M.T., Eldridge, J.H., Hirasawa, M. and Kiyono, H. [1992]. The mucosal immune system: from fundamental concepts to vaccine development. Vaccine 10, 75-88.

McLachlan, P.R. and Sanderson, K.E. [1985]. Transformation of Salmonella typhimurium with plasmid DNA: differences between rough and smooth strains. Journal of Bacteriology 161, 442-445.

Ma, J.J., Chen, K.C.S. and Kuo, C-C. [1987]. Identification of conserved regions for species and subspecies specific epitopes on the major outer membrane protein of Chlamydia trachomatis. Microbial Pathogenesis 3, 299-307.

MAFF [1980]. Animal Health. 1978. Report of the Chief Veterinary Officer. 118-120.

Maniatis, T., Fritsch, E.F. and Sambrook, J. [1982]. In: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

Marecki. N.M., Hsu, H.S and Mayo, D.R. [1975]. Cellular and humoral aspects of host resistance in murine salmonellosis. British Journal of Experimental Pathology 56, 231-243.

Markham, R.J.F., Ramnaraine, M.L.R. and Muscoplat, C.C [1982]. Cytotoxic effect of Pasteurella haemolytica on bovine polymorphonuclear leucocytes and impaired production of chemotactic factors by Pasteurella haemolytica-infected alveolar macrophages. American Journal of Veterinary Research 43, 285-288.

Maskell, D.J., Sweeney, K.J., O'Callaghan, D., Hormaeche, C.E., Liew, F.Y. and Dougan, G. [1987]. Salmonella typhimurium aroA mutants as carriers of the Escherichia coli heat-labile-enterotoxin B subunit to the murine secretory and systemic immune systems. Microbial Pathogenesis 2, 211-221.

Maskell, D., Sweeney, K., O'Callaghan, D., Liew, F.Y., Fairweather, N. and Dougan, G. [1986]. Attenuated salmonellae as vaccines and carriers of foreign antigens. In: Bacterial Vaccines and Local Immunity- Ann. Sclavo 1-2, pp133-141.

Michetti, P., Mahan, M.J., Slauch, J.M., Mekalanos, J.J. and Neutra, M.R. [1992]. Monoclonal secretory immunoglobulin A protects mice against oral challenge with the invasive pathogen Salmonella typhimurium. Infection and Immunity 60, 1786-1792.

Mintz, C.S. and Deibel, R.H. [1983]. Effect of lipopolysaccharide mutations on the pathogenesis of experimental Salmonella gastroenteritis. Infection and Immunity 40, 236-244.

Modrzakowski, M.C. and Spitznagel, J.K. [1979]. Bactericidal activity of fractionated granule contents from human polymorphonuclear leukocytes: antagonism of granule cationic proteins by lipopolysaccharide. Infection and Immunity 25, 597-602.

Molina, N.C. and Parker, C.D. [1990]. Murine antibody responses to oral infection with live aroA recombinant Salmonella dublin vaccine strains expressing filamentous hemagglutinin antigen from B.pertussis. Infection and Immunity 58, 2523-2528.

Monaco, J.J. [1992]. A molecular model of MHC class 1-restricted antigen processing. Immunology Today 13, 173-179.

Montgomerie, R.F., Basworth, T.J. and Glover, R.E. [1938]. Enzootic pneumonia in sheep. Journal of Comparative Pathology 51, 87-107.

Morris, J.A., Wray, C. and Sojka, W.J. [1976]. The effect of T and B lymphocyte depletion on the protection of mice vaccinated with a galE mutant of Salmonella typhimurium. British Journal of Experimental Pathology 57, 354-360.

Morrissey, P.J. and Charrier, K. [1991]. Interleukin-1 administration to C3H/HeJ mice after but not prior to infection increases resistance to Salmonella typhimurium. Infection and Immunity 59, 4729-4731.

Mosier, D.A., Confer, A.W., Hall, S.M., Gentry, M.J. and Panciera, R.J. [1986]. Enzyme-linked immunosorbent assay for detection of serum antibodies to Pasteurella haemolytica cytotoxin [leukotoxin] in cattle. Journal of Clinical Microbiology 24, 218-222.

Mosier, D.A., Simons, K.R., Confer, A.W., Panciera, R.J. and Klinkenbeard, K.D. [1989]. Pasteurella haemolytica antigens associated with resistance to pneumonic pasteurellosis. Infection and Immunity 57, 711-716.

Mosmann, T.R. and Coffman, R.L. [1987]. Two types of mouse helper T-cell clones. Immunology Today 8, 223-227.

Mukkur, T.K.S., McDowell, G.H., Stocker, B.A.D. and Lascelles, A.K. [1987]. Protection against experimental salmonellosis in mice and sheep by immunisation with aromatic-dependent Salmonella typhimurium. Journal of Medical Microbiology 24, 11-19.

Mukkur, T.K. and Walker, K.H. [1992]. Development and duration of protection against salmonellosis in mice and sheep immunised with live aromatic-dependent Salmonella typhimurium. Research in Veterinary Science 52, 147-153.

Muller, K.H., Collinson, S.K., Trust, T.J. and Kay, W.W. [1991]. Type 1 fimbriae of Salmonella enteriditis. Journal of Bacteriology 173, 4765-4772.

Muotiala, A. and Makela, P.H. [1990]. The role of IFN-g in murine Salmonella typhimurium infection. Microbial Pathogenesis 8, 135-141.

Nakano, M. and Saito, K. [1969]. Chemical components in the cell wall of Salmonella typhimurium affecting its virulence and immunogenicity in mice. Nature 122, 1085-1086.

Nakamura, M., Sato, S., Ohya, T., Suzuki, S. and Ikeda, S. [1985]. Possible relationship of a 36-megadalton Salmonella enteriditis plasmid and virulence in mice. Infection and Immunity 47, 831-833.

Nakayama, K., Kelly, S.M. and Curtiss III, R. [1988]. Construction of an Asd+ expression-cloning vector: stable maintenance and high level expression of cloned genes in a Salmonella vaccine strain. Bio/Technology 6, 693-698.

Nakoneczna, I. and Hsu., H.S. [1980]. The comparative histopathology of primary and secondary lesions in murine salmonellosis. British Journal of Experimental Pathology 61, 76-84

Nakoneczna, I. and Hsu, H.S. [1983]. Histopathological study of the protective immunity against murine salmonellosis induced by killed vaccine. Infection and Immunity 39, 423-430.

Nathan, C.F., Murray, H.W., Wiebe, M.E. and Rubin, B.Y. [1983]. Identification of interferon-g as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. Journal of Experimental Medicine 158, 670-689.

Nauciel, C. [1990]. Role of CD4+ T cells and T-independent mechanisms in acquired resistance to Salmonella typhimurium infection. Journal of Immunology 145, 1265-1269.

Nauciel, C. and Espinasse-Maes, F. [1992]. Role of gamma interferon and tumour necrosis factor alpha in resistance to Salmonella typhimurium. Infection and Immunity 60, 450-454.

Nauciel, C., Ronco, E., Cuenet, J-L. and Pla, M. [1988]. Role of H-2 and non H-2 genes in control of bacterial clearance from the spleen in Salmonella typhimurium-infected mice. Infection and Immunity 56, 2401-2411.

Nauciel. C., Ronco, E. and Le Faou, A. [1981]. Role of humoral immunity in the protection of mice against an intravenous challenge of Salmonella typhimurium. Annales de l'Institut Pasteur/Immunologie 132D, 225-236.

Nauciel, C., Ronco, E. and Pla, M. [1990]. Influence of different regions of the H-2 complex on the rate of clearance of Salmonella typhimurium. Infection and Immunity 58, 573-574.

Newhall V, W.J. and Basinski, M.B. [1986]. Purification and structural characterisation of chlamydial outer membrane proteins. In: Proceedings of the Sixth International Symposium on Human Chlamydial Infection. Oriel, D. et al. [Eds]. Cambridge University Press. pp93-96.

Newton, S.M.C., Jacob, C.O. and Stocker, B.A.D. [1989]. Immune response to cholera toxin epitope inserted in Salmonella flagellin. Science 244, 70-72.

Newton, S.M.C., Kotb, M., Poirier, T.P., Stocker, B.A.D. and Beachey, E.H. [1991]. Expression and immunogenicity of a streptococcal M protein epitope inserted in Salmonella flagellin. Infection and Immunity 59, 2158-2165.

Nurmi, E. and Rantala, M. [1973]. New aspects of Salmonella infection in broiler production. Nature 241, 210-211.

O'Brien, A.D. [1986]. Influence of host genes on the resistance of inbred mice to lethal infection with Salmonella typhimurium. Current Topics in Microbiology and Immunology 124, 37-48.

O'Brien, A.D., LaVeck, G.D., Thompson, M.R. and Formal, S.B. [1982]. Production of Shigella dysenteriae type 1 cytotoxin by Escherichia coli. Journal of Infectious Diseases 146, 763-769.

O'Brien, A.D. and Metcalf, E.S. [1982]. Control of early Salmonella typhimurium growth in innately Salmonella-resistant mice does not require functional T lymphocytes. Journal of Immunology 129, 1349-1351.

O'Brien, A.D., Rosenstreich, D.L., Scher, I., Campbell, G.H., MacDermott, R.P. and Formal, S.B. [1980]. Genetic control of the susceptibility to Salmonella typhimurium in mice: role of the Lps gene. Journal of Immunology 124, 20-24.

O'Brien, A.D., Scher, I. and Metcalf, E.S. [1981]. Genetically-conferred defect in anti-Salmonella antibody formation renders CBA/N mice innately susceptible to Salmonella typhimurium infection. Journal of Immunology 126, 1368-1372.

O'Brien, A.D., Taylor, B.A. and Rosenstreich, D.L. [1984]. Genetic control of natural resistance to Salmonella typhimurium in mice during the late phase of infection. Journal of Immunology 133, 3313-3318.

O'Brien, A.D., Weinstein, D.A., Soliman, M.Y. and Rosenstreich, D.L. [1985]. Additional evidence that the Lps gene locus regulates natural resistance to S.typhimurium in mice. Journal of Immunology 134, 2820-2823.

O'Callaghan, D., Charbit, A., Martineau, P., Leclerc, C., Van der Werf, S., Nauciel, C. and Hofnung, M. [1990]. Immunogenicity of foreign peptide epitopes expressed in bacterial envelope proteins. Research in Microbiology 141, 963-969.

O'Callaghan, D., Maskell, D., Liew, F.Y., Easmon, C.S.F. and Dougan, G. [1988]. Characterisation of aromatic- and purine-dependent Salmonella typhimurium: attenuation, persistence and ability to induce protective immunity in BALB\c mice. Infection and Immunity 56, 419-423.

Ornellas, E.P., Roantree, R.J. and Steward, J.P. [1970]. The specificity and importance of humoral antibody in the protection of mice against intraperitoneal challenge with complement-sensitive and complement-resistant Salmonella. Journal of Infectious Diseases 121, 113-123.

Osborn, M.J. [1968]. Biochemical characterisation of mutants of Salmonella typhimurium lacking glucosyl or galactosyl lipopolysaccharide transferases. Nature 217, 957-960.

Osborn, M.J., Rosen, S.M., Rothfield, L., Zelesnick, L.D. and Horecker, B.L. [1962]. Lipopolysaccharide of the Gram-negative cell wall. Science 145, 783-789.

Ottow, J.C.G. [1975]. Ecology, physiology, and genetics of fimbriae and pili. Annual Reviews in Microbiology 29, 79-108.

Owen, R.L., Pierce, N.F., Apple, R.T. and Cray Jnr., W.C. [1986]. M cell transport of Vibrio cholerae from the intestinal lumen into Peyer's patches: A mechanism for antigen sampling and for microbial transepithelial migration. Journal of Infectious Diseases 153, 1108-1118.

Paul, C., Shalala, K., Warren, R. and Smith, R. [1985]. Adoptive transfer of murine host protection to salmonellosis with T cell growth factor-dependent, Salmonella-specific T cell lines. Infection and Immunity, 48, 40-43.

Pardon, P., Popoff, M.Y., Coynault, C., Marly, J. and Miras, I. [1986]. Virulence-associated plasmids of Salmonella serotype typhimurium in experimental murine infection. Annales de l'Institut Pasteur/Microbiologie 137B, 47-60.

Peavey, D.L., Adler, W.H., Shands, J.W. and Smith, R.T. [1974]. Selective effects of mitogens on subpopulations of mouse lymphoid cells. Cellular Immunology 11, 86-98.

Peeling, R., MacLean, W.I. and Brunham, R.C. [1984]. In vitro neutralisation of Chlamydia trachomatis with monoclonal antibody to an epitope on the major outer membrane protein. Infection and Immunity 46, 484-488.

Peterson, J.W., Houston, C.W. and Koo, F.C.W. [1981]. Influence of culture conditions on mitomycin C-mediated bacteriophage induction and release of Salmonella toxin. Infection and Immunity 32, 232-242.

Rabinovitch, M. [1975]. Macrophage spreading in vitro. In: Mononuclear Phagocytes in Immunity, Infection and Pathology. Van Furth, R. [Ed]. Blackwell Scientific, Oxford, UK. pp369.

Ramsey, K.H., Newhall V, W.J. and Rank, R.G. [1989]. Humoral immune response to chlamydial genital infection of mice with the agent of mouse pneumonitis. Infection and Immunity 57, 2441-2446.

Rank, R.G. [1988]. Role of the immune response. In: Microbiology of Chlamydia. A.L. Baron [Ed]. CRC Press Inc., Boca Raton, FL, USA. pp217-234.

Rest, R.F., Cooney, M.H. and Spitznagel, J.K. [1978]. Bactericidal activity of specific and azurophil granules from human neutrophils: studies with outer-membrane mutants of Salmonella typhimurium. Infection and Immunity 19, 131-137.

Reitmeyer, J.C., Peterson, W. and Wilson, K.J. [1986]. Salmonella cytotoxin: a component of the bacterial outer membrane. Microbial Pathogenesis 1, 503-510.

Rietschel, E.T., Wollenweber, H.W., Russa, R., Brade, H. and Zahringer, V. [1984]. Concepts of the chemical structure of lipid A. Reviews of Infectious Diseases 6, 432-438.

Richards, A.B., Chang, Y-F., Hanson, T.D. and Renshaw, H.W. [1982]. Interaction of goat peripheral blood leukocytes and Pasteurella haemolytica. In: Proceedings of the Third International Conference on Goat Production and Disease. Tuczon, AZ, USA. pp295.

Roantree, R.J. [1967]. Salmonella 'O' antigens and virulence. Annual Review in Microbiology 21, 443-466.

Robertsson, J.A., Lindberg, A.A., Hoiseth, S. and Stocker, B.A.D. [1983] Salmonella typhimurium infection in calves: protection and survival of virulent challenge bacteria after immunisation with live or inactivated vaccines. Infection and Immunity 41, 742-750.

Robertsson, J.A., Svenson, S.B. and Lindberg, A.A. [1982]. Salmonella typhimurium infection in calves: delayed specific skin reactions directed against the O-antigenic polysaccharide chains. Infection and Immunity 37, 737-748.

Rosenstreich, D.L., Navotny, A., Cheud, T. and Mergenhagen, S.E. [1973]. In vitro transformation of mouse bone-marrow-derived [B] lymphocytes induced by the lipid component of endotoxin. Infection and Immunity 8, 406-411.

Rowley, D. [1954]. The virulence of strains of Bacterium coli for mice. British Journal of Experimental Pathology 35, 528-538.

Rushton, B., Sharp, J.M., Gilmour, N.J.L. and Thompson, D.A. [1979]. Pathology of an experimental infection of specific-pathogen-free lambs with parainfluenza virus type 3 and Pasteurella haemolytica. Journal of Comparative Pathology 89, 321-329.

Sadoff, J.C., Ripley Ballai, W., Baron, L.S., Majanon, W.R., Brey, R.N., Hochmyer, W.T., Young, J.F., Cryz, S.J., Ou, J., Lowell, G.H. and Chulay, J.D. [1988]. Oral Salmonella typhimurium vaccine expressing circumsporozoite protein protects against malaria. Science 240, 336-338.

Salari, S.H. and Ward, M.E. [1981]. Polypeptide composition of Chlamydia trachomatis. Journal of General Microbiology 123, 197-207.

Sandefur , P.D. and Peterson, J.W. [1976]. Isolation of skin permeability factors from culture filtrates of Salmonella typhimurium. Infection and Immunity 14, 671-679.

Sandefur , P.D. and Peterson, J.W. [1977]. Neutralisation of Salmonella toxin-induced elongation of Chinese hamster ovary cells by cholera antitoxin. Infection and Immunity 15, 988-992.

Sarasombath, S., Banchuin, N., Sukasol, T., Vanadurongwan, S., Rungpitarangsi, B. and Dumavibliat, B. [1987]. Systemic and intestinal immunities after different typhoid vaccinations. Asian Pacific Journal of Allergy and Immunology 5, 53-61.

Sarasombath, S., Suvaltev, S., Sukasol, T., Nungpitarangsi, R. and Manasatit, S. [1984]. Relapsing typhoid fever: report of a case with immunological studies. Asian Pacific Journal of Allergy and Immunology 2, 103-106.

Saxèn, H. and Mäkela, O. [1982]. The protective capacity of immune sera in experimental mouse salmonellosis is mainly due to IgM antibodies. Immunology Letters 5, 267-272.

Saxèn, H., Mäkelä, O. and Svenson, S.B. [1984]. Isotype of protective anti-Salmonella antibodies in experimental mouse salmonellosis. Infection and Immunity 44, 633-636.

Saxèn, H., Reima, I. and Mäkelä, P.H. [1987]. Alternative complement pathway activation by Salmonella 'O' polysaccharide as a virulence determinant in mice. Microbial Pathogenesis 2, 15-28.

Schachter, J.J. and Dawson, C.R. [1978]. In: Human Chlamydial Infections, PSG Publishing Company Inc., Littleton, MA, USA.

Schafer, R. and Eisenstein, T.K. [1992]. Natural killer cells mediate protection induced by a Salmonella aroA mutant. Infection and Immunity 60, 791-797.

Schorr, J., Knapp, B., Hundt, E., Kupper, H.A. and Amann, E. [1991]. Surface expression of malarial antigens in Salmonella typhimurium: induction of serum antibody response on oral vaccination of mice. Vaccine 9, 675-681.

Sedlock, D.M. and Deibel, R.H. [1978]. Detection of Salmonella enterotoxin using rabbit ileal loops. Canadian Journal of Microbiology 24, 268-273.

Shafren, D.R. and Tannock, G.A. [1989]. Development and application of an ELISA technique for the detection of antibody to avian encephalomyelitis viruses. Research in Veterinary Science 46, 95-99.

Sharp, J.M., Gilmour, N.J.L., Thompson, D.A. and Rushton, B. [1978]. Experimental infection of specific-pathogen-free lambs with parainfluenza virus type 3 and Pasteurella haemolytica. Journal of Comparative Pathology 88, 237-243.

Shewen, P.E. and Wilkie, B.N. [1982]. Cytotoxin of Pasteurella haemolytica acting on bovine leukocytes. Infection and Immunity 35, 91-94.

Shewen, P.E. and Wilkie, B.N. [1983]. Pasteurella haemolytica cytotoxin neutralising activity in sera from Ontario beef cattle. Canadian Journal of Comparative Medicine 47, 497-498.

Shewen, P.E. and Wilkie, B.N. [1987]. Vaccination of calves with leukotoxic culture supernatant from Pasteurella haemolytica. Canadian Journal of Veterinary Research 52, 30-36.

Shukla, S.K. and Sharma, V.D. [1985]. Detection of Salmonella enterotoxin using rabbit ileal loops. Indian Journal of Experimental Biology 23, 653-654.

Sigwart, D.F., Stocker, B.A.D. and Clements, J.D. [1989]. Effect of a purA mutation on efficacy of Salmonella live- vaccine vectors. Infection and Immunity 57, 1858-1861.

Skamene, E.P., Gros, A., Forget, P.A.L., Kongshavn, C., St. Charles, C. and Taylor, B.A. [1982]. Genetic regulation of resistance to intracellular pathogens. Nature 297, 506-509.

8

Smith, B.P., Reina-Guerra, M., Stocker, B.A.D., Hoiseth, S.K. and Johnson, E.H. [1984a]. Vaccination of calves against Salmonella dublin with aromatic-dependent Salmonella typhimurium. American Journal of Veterinary Research 45, 1858-1861.

Smith, B.P., Reina-Guerra, M., Stocker, B.A.D., Hoiseth, S.K. and Johnson, E.H. [1984b]. Aromatic-dependent Salmonella dublin as a parenteral modified live vaccine for calves. American Journal of Veterinary Research 45, 2231-2235.

Smith, G.R. [1961]. The characteristics of two types of Pasteurella haemolytica associated with different pathological conditions of sheep. Journal of Pathology and Bacteriology 81, 431-440.

Smith, H.W. [1965]. The immunisation of mice, calves and pigs against Salmonella dublin and Salmonella choleraesuis infections. Journal of Hygiene 63. 117-135.

Smith, R.A., Esa, A. and Stiff, M. [1982]. Transfer of Salmonella resistance and delayed hypersensitivity with murine-derived transfer factor. Infection and Immunity 36, 271-276.

Spitalny, G.L. and Havell, E.A. [1984]. Monoclonal antibody to murine gamma interferon inhibits lymphokine-induced antiviral and macrophage tumouricidal activities. Journal of Experimental Medicine 159, 1560-1565.

Stabel, T.J., Mayfield, J.E., Tabatabai, L.B. and Wannemuehler, M.J. [1990]. Oral immunisation of mice with attenuated Salmonella typhimurium containing a recombinant plasmid which codes for production of a 31-kilodalton protein of Brucella abortus. Infection and Immunity 58, 2048-2055.

Staerz, V.D., Kara Suayama, H. and Garner, A.M. [1987]. Cytotoxic T lymphocytes against a soluble protein. Nature 329, 449-451.

Stamp, J.T., Watt, J.A.A. and Thomlinson, A.G. [1955]. Pasteurella haemolytica septicaemia of lambs. Journal of Comparative Pathology and Therapeutics 65, 183-196.

Stephen, I., Wallis, T.S., Starkey, W.G., Candy, D.C.A, Osborne, M.P. and Hadden, S. [1985]. Salmonellosis: in retrospect and prospect. In: Microbial Toxins and Diarrhoeal Disease. Ciba Foundation Symposium. No. 112, 175-192. Pitmam Medical, Turnbridge Wells, UK.

Stephens, R.S. and Kuo, C.-C. [1984]. Chlamydia trachomatis species-specific epitope detected on mouse biovar outer membrane protein. Infection and Immunity 45, 790-791.

Stephens, R.S., Tam, M.R., Kuo, C.-C. and Nowinski, R.C. [1982]. Monoclonal antibodies to Chlamydia trachomatis: antibody specificities and antigen characterisation. Journal of Immunology 128, 1083-1089.

Stephens, R.S., Wagar, E.A. and Schoolnik, G.K. [1988]. High resolution mapping of serovar-specific and common antigenic determinants of the major outer membrane protein of Chlamydia trachomatis. Journal of Experimental Medicine 167, 817-831.

Stocker, B.A.D. and Makela, P.H. [1986]. Genetic determination of bacterial virulence with special reference to Salmonella. Current Topics in Microbiology and Immunology 124, 149-172.

Strugnell, R.A., Maskell, D., Fairweather, N.F., Pickard, D., Cockayne, A., Penn, C. and Dougan, G. [1990]. Stable expression of foreign antigens from the chromosome of Salmonella typhimurium vaccine strains. Gene 88, 57-63.

Su, G-F., Brahmbhatt, H.N., Wehland, J., Rhode, M. and Timmis, K.N. [1992]. Construction of stable lamB-shiga toxin B subunit hybrids: analysis of expression in Salmonella typhimurium aroA strains and stimulation of B subunit-specific mucosal and serum antibody responses. Infection and Immunity 60, 3345-3359.

Suter, E. [1956]. Interaction between phagocytes and pathogenic microorganisms. Bacteriology Reviews 20, 94-132.

Sutherland, A.D., Gray, E. and Wells, P.W. [1983]. Cytotoxic effect of Pasteurella haemolytica on ovine bronchoalveolar macrophages in vitro. Veterinary Microbiology 8, 3-15.

Sutherland, A.D. and Donachie, W. [1985]. Cytotoxic effect of serotypes of Pasteurella haemolytica on sheep bronchoalveolar macrophages. Veterinary Microbiology 11, 331-336.

Svenson, S.B., Nurminen, M. and Lindberg, A.A. [1979]. Artificial Salmonella vaccines: O-antigenic oligosaccharide protein conjugates induce protection against infection with Salmonella typhimurium. Infection and Immunity 25, 863-872.

Svenson, S.B. and Lindberg, A.A. [1981]. Artificial Salmonella vaccines: Salmonella typhimurium O-antigen-specific oligosaccharide-protein conjugates elicit protective antibodies in rabbits and mice. Infection and Immunity 32, 490-496.

Tacket, C.O., Hone, D.M., Curtiss III, R., Kelly, S.M., Losonsky, G., Guers, L., Harris, A.M., Edelman, R. and Levine, M.M. [1992]. Comparison of the safety and immunogenicity of aroC aroD and cya crp Salmonella typhi strains in adult volunteers. Infection and Immunity 60, 536-541.

Tagesson, C. and Stendhal, O. [1973]. Influence of the cell surface lipopolysaccharide structure of Salmonella typhimurium on resistance to intracellular bacterial systems. Acta Pathology and Microbiology Scandinavia 81, 480-483.

Taira, S. and Rhen, M. [1989]. Identification and genetic analysis of mkaA - a gene of the Salmonella typhimurium virulence plasmid necessary for intracellular growth. Microbial Pathogenesis 7, 165-173.

Takeuchi, A. [1967]. Electron microscope studies of experimental Salmonella infection. I. Penetration into the intestinal epithelium by Salmonella typhimurium. American Journal of Pathology 50, 109-119.

Takeuchi, A. and Sprinz, H. [1967]. Electron microscope studies of experimental Salmonella infection in the preconditioned guinea pig. II. Response of the intestinal mucosa to the invasion by Salmonella typhimurium. American Journal of Pathology 51, 137-146.

Tan, T.W. [1989]. Biochemical, immunological and genetic characterisation of the major outer membrane protein from an ovine abortion strain of Chlamydia psittaci. PhD thesis, University of Edinburgh, UK.

Tanaka, Y. and Katsube, Y. [1978]. Infectivity of Salmonella typhimurium for mice in relation to fimbriae. Japanese Journal of Veterinary Science 40, 671-681.

Tarka, E., Muotiala, A., Karvonen, M., Saukkonen-Laitinen, K. and Sarvas, M. [1989]. Antibody production to a meningococcal outer membrane protein cloned into a live Salmonella typhimurium aroA vaccine strain. Microbial Pathogenesis 6, 327-335.

Tavendale, A., Jardine, C.K.H., Old, D.C. and Duguid, J.P. [1983]. Haemagglutinins and adhesion of Salmonella typhimurium to HEp-2 and HeLa cells. Journal of Medical Microbiology 16, 371-380.

Taylor, B.A. and O'Brien, A.D. [1982]. Position on mouse chromosome 1 of a gene that controls resistance to Salmonella typhimurium. Immunology 36, 1257-1260.

Taylor, D.W., Cordingley, J.S., Dunne, D.N., Johnson, K.S., Haddow, W.J., Hormaeche, C.E. and Butterworth, A.E . [1986]. Molecular cloning of schistosome genes. Parasitology, 91, S73-81.

Taylor, H.R., Whittum-Hudson, J., Schachter, J., Caldwell, H.D. and Prendergast, R.A. [1988]. Oral immunisation with chlamydial major outer membrane protein [MOMP]. Investigative Ophalmology and Visual Science 29, 1847-1853.

Terakado, N., Sekizaki, T., Hashimoto, K. and Naitoh, S. [1983]. Correlation between the presence of a fifty-megadalton plasmid in Salmonella dublin and virulence for mice. Infection and Immunity 41, 443-444.

Thjotta, Th. and Waller, E. [1932]. Dissociation and sensitiveness to normal serum in dysentery bacilli of type III. Journal of Bacteriology 25, 301-316.

Thompson, D.A., Fraser, J. and Gilmour, N.J.L. [1977]. Serotypes of Pasteurella haemolytica in ovine pasteurellosis. Research in Veterinary Science 22, 130-131.

Tite, J.P., Gao, X.-M., Hughes-Jenkins, C.M., Lipscombe, M., O'Callaghan, D., Dougan, G. and Liew, F.-Y. [1990]. Antiviral immunity induced by recombinant nucleoprotein of influenza virus, III. Delivery of recombinant nucleoprotein to the immune system using attenuated Salmonella typhimurium as a live carrier. Immunology 70, 540-546.

Tite, J.P., Dougan, G. and Chatfield, S.N. [1991]. The involvement of tumour necrosis factor in immunity to Salmonella infection. Journal of Immunology 147, 3161-3134.

Tomita, T. and Kanegasaki, S. [1982]. Enhanced phagocytic response of macrophages to bacteria by physical impact caused by bacterial motility or centrifugation. Infection and Immunity 38, 865-870.

Turnbull, P.C.B. [1979]. Food poisoning with special reference to Salmonella: its epidemiology, pathogenesis and control. In: Clinics in Gastroenterology Infections of the GI Tract. Lambert, H.P [Ed]. W.B. Saunders, Toronto, Canada. pp663-714.

Valtonen, V.V. [1970]. Mouse virulence of Salmonella strains: the effect of different smooth-type O side-chains. Journal of of General Microbiology 64, 255-268.

Valtonen, M.V., Plosila, M., Valtonen, V.V. and Makela, P.H. [1975]. Effect of the quality of the lipopolysaccharide on mouse virulence of salmonella enteriditis. Infection and Immunity 12, 828-832.

Vandenbosch, J.L., Rabert, D.K. and Jones, G.W. [1987]. Plasmid-associated resistance of Salmonella typhimurium to complement activated by the classical pathway. Infection and Immunity 55, 2645-2652.

Van Dissel, J.T., Leijh, P.C.J. and Van Furth, R. [1985]. Differences in initial rate of intracellular killing of Salmonella typhimurium by resident peritoneal macrophages from various mouse strains. Journal of Immunology 134, 5404-3410.

Van Zwet, T.L., Thompson, J. and Van Furth, R. [1975]. Effect of glucocorticosteroids on the phagocytosis and intracellular killing by peritoneal macrophages. Infection and Immunity 12, 699-705.

Vieira, J. and Messing, J. [1982]. The pUC plasmids: an M13 mp7 derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19, 259-268.

Vladoianu, I-R., Chang, H.R. and Pechère, J-C. [1990]. Expression of host resistance to Salmonella typhi and Salmonella typhimurium: bacterial survival within macrophages of murine and human origin. Microbial Pathogenesis 8, 83-90.

Wahdan, M.H., Serie, C., Censier, Y., Sallam, S. and Germanier, R. [1982]. A controlled field trial of live Salmonella typhi strain Ty21a oral vaccine against typhoid: three year results. Journal of Infectious Diseases 145, 292-295.

Wallis, T.S., Starkey, W.G., Stephen, J., Hadden, S.J., Osborne, M.P. and Candy, D.C.A [1986]. Enterotoxin production by Salmonella typhimurium strains of different virulence. Journal of Medical Microbiology 21, 19-23.

Watson, D.L. [1976]. The effect of cytophilic IgG_2 on phagocytosis by ovine polymorphonuclear leukocytes. Immunology 31, 159-165.

Weinstein, D.L., Carsiotis, M., Lissner, C.R. and O'Brien, A.D. [1984]. Flagella help Salmonella typhimurium survive within murine macrophages. Infection and Immunity 46, 819-825.

Welsh, R.M. [1984]. Natural killer cells and interferon. Critical Reviews of Immunology 5, 55-93.

Westphal, O. and Jann, K. [1965]. Bacterial lipopolysaccharides. Extraction with phenol water and further applications of the procedure. In: Methods in Carbohydrate Chemisitry vol.5. Whistler et al. [Eds]. Academic Press, New York, USA. pp83-91.

Wilkinson, S.J. [1977]. In: Surface Carbohydrates of the Prokaryotic Cell. Sutherland, I.W. [Ed]. Academic Press, New York, USA. pp97-171.

Wilson, G.S. and Miles, A. [1975]. Topley and Wilson's Principles of Bacteriology, Virology and Immunity, 6th edition: Edward Arnold, London.

Williams, D.M., Schachter, J., Coalson, J.J. and Grubbs, B. [1984] Cellular immunity to the mouse pneumonitis agent. Journal of Infectious Diseases 149, 630-639.

Williams, D.M. [1988]. Stimulation of immune response. In Microbiology of Chlamydia. A.L. Baron [Ed]. CRC Press Inc., Boca Raton, FL, USA. pp209-216.

Wood, P.R., Rothel, J.S., McWaters, P.G.D. and Jones, S.L. [1990]. Production and characterisation of monoclonal antibodies specific for bovine gamma interferon. Veterinary Immunology and Immunopathology 25, 37-46.

Wray, C. [1985]. Is salmonellosis still a serious problem in veterinary practice? Veterinary Record 116, 485-489.

Wray, C. and Sojka, W.J. [1977]. Reviews of the progress of dairy science: bovine salmonellosis. Journal of Dairy Research 44, 383-425.

Wu, J.Y., Newton, S., Judd, A., Stocker, B. and Robinson, W.S. [1989]. Expression of immunogenic epitopes of hepatitis B surface antigen with hybrid flagellin proteins by a vaccine strain of Salmonella. Proceedings of the National Academy of Sciences, USA. 86, 4726-4730.

Yan, Z.X., Reuss, F. and Meyer, T.F. [1990]. Construction of an invertible DNA segment for improved antigen expression by a hybrid Salmonella vaccine strain. Research in Microbiology 141, 1003-1004.

Yang, D.M., Fairweather, N., Button, L.L., McMaster, W.R., Kahl, L.P. and Liew, F.W. [1990]. Oral Salmonella typhimurium [aroA-] vaccine expressing a major leishmanial surface protein [gp63] preferentially induces T helper 1 cells and protective immunity against leishmaniasis. Journal of Immunology 145, 2281-2285.

APPENDICES

Appendix 1

1 Bacterial Culture Media

All media were sterilised by autoclaving unless otherwise stated.

1A Luria-Bertani [LB] Medium

Per litre :	Bacto-tryptone [Difco Laboratories Detroit, MI, USA.]	10g *1
	Bacto-yeast extract [Difco]	5g *2
	NaCl	5g *3
	Bacto-agar [Difco]	5g *4

For agar [LB agar], all of the above.

For broth [LB broth], 1, 2 and 3 only.

Antibiotics may be added to broth or agar to a final concentration of:

Ampicillin [Sigma Chemical Company, Poole, Dorset, UK.]	60ug/ml
Tetracycline [Sigma]:	50ug/ml

1B Nutrient Broth

Per litre :	Nutrient broth No.2 [CM67] [Oxoid, Basingstoke, Hampshire, UK.]	25.0g

1C Xylose Lactose Deoxycholate [XLD] Medium

Per litre :	XLD [Oxoid CM467]	53.0g

Sterilised by melting in a microwave.

1D Selenite Broth

Per litre :	Selenite broth base [Oxoid CM395]	19.0g
	Sodium biselenite [Oxoid L121]	4.0g

1E Blood Agar

Per litre :	Blood agar base [Gibco, Paisley, Strathclyde, UK].	40.0g
	44ml whole sheep blood containing Trisodium citrate.	

1F Minimal Agar

Per litre :	Bacto agar [Difco]	15.0g
	Thiamine B1 [1mg/ml]	2ml
	20% Glucose	10ml
	Spizizen salts 5X	200ml

The bacto-agar was dissolved in 700 ml distilled water and autoclaved. After cooling, the other 3 compounds were added and the final volume made up to 1 litre with distilled water.

When testing bacterial strains for aromatic metabolite dependence, the following compounds were added to 1 litre of minimal agar prior to pouring.

100ul PABA [100mg/ml][Sigma] in 100% ethanol.

100ul DHB [100mg/ml][Sigma] in 100% ethanol.

20ml Tryptophan [2mg/ml]. Filter sterilise.

20ml Tyrosine [2mg/ml]. Filter sterilise.

20ml Phenylalanine [2mg/ml]. Autoclave.

Appendix 2

2 Materials for SDS-PAGE

2A Sample Buffer

For 8ml:		
	Distilled water	4.0ml
	0.5M Tris-HCl, pH 6.8	1.0ml
	Glycerol	0.8ml
	10% [w/v] SDS	1.6ml
	2-b-mercaptoethanol	0.4ml
	0.05% [w/v] bromophenol blue [Sigma]	0.2ml

2B Stacking Gel Preparation For 4% gels.

For 10ml:		
	Distilled water	6.1ml
	0.5M Tris-HCl, pH 6.8	2.5ml
	10% [w\v] SDS	100ul
	Acrylamide/bis [30% stock]	1.3ml
	10% ammonium persulphate	50ul
	TEMED [Sigma]	50ul

2C Separating Gel Preparation For 7.5% gels.

For 10ml:		
	Distilled water	4.85ml
	1.5M Tris-HCl, pH 8.8	2.5ml
	10% [w\v] SDS	100ul
	Acrylamide/bis [30% stock]	2.5ml
	10% ammonium persulphate	50ul
	TEMED	5ul

2D Tris-Glycine Electrode Buffer, pH8.3

For 1 Litre:		
	Tris-[hydroxymethyl]-methylamine	30.3g
	Glycine	144.0g
	SDS	5.0g

Made up to 1 litre with distilled water. Sterilised by autoclaving and stored at $4^{o}C$.

Methanol may be added to 20%.

Appendix 3

3 Buffers for Use in ELISAs

3A Antigen Coating Buffer: 0.03M Carbonate Buffer, pH 9.6

For 1 litre:		
	Di-sodium carbonate.	1.59g
	Sodium hydrogen carbonate.	2.93g
	Sodium azide.	0.20g

Made up to 1 litre with distilled water. Diluted 1 in 2 prior to use.

3B ELISA Buffer pH 7.2.

For 1 litre:		
	Tris hydrochloride	15.79g
	Sodium chloride	29.22g
	Ethylenediamine tetra-acetic acid [EDTA]	0.372g
	Bovine serum albumin [BSA] [Sigma]	20.0g
	Triton-X 100 [Sigma]	30ml
	Tween 20	30ml

Made up to 1 litre with distilled water.

3C Substrate Buffer: 10% Diethanolamine Buffer, pH 9.8.

For 1 litre:		
	Diethanolamine.	100ml
	Magnesium chloride x 6 H_2O	0.102g
	Sodium azide	0.200g
	Distilled water	800ml

1M hydrochloric acid added [approximately 60ml] to adjust to the the correct pH. Made up to 1 litre with distilled water and stored in the dark.

For use with p-nitrophenyl phosphate [PNPP] substrate.

3D Substrate Buffer: Citrate Phosphate Buffer, pH 5.

[A]	Citric acid	21.01g\1
[B]	Di-sodium hydrogen phosphate	28.40g\1

Both reagents made up separately to 1 litre with distilled water and 48.5mls of [A] was added to 51.5mls of [B] producing a pH of approximately 5. For use with O-phenylenediamine [OPD] substrate.

Appendix 4

4 Tissue Culture Media

4A Supplemented RPMI 1640 Medium [sRPMI 1640]

For 500ml:420ml RPMI 1640 without L-glutamine [Gibco]
5ml 1M HEPES
10ml [2%] Penicillin\Streptomycin [10,000u/ml][Sigma]
10ml [2%] Glutamine [0.1M][Gibco]
5ml [1%] Preservative-free heparin [1000u/ml][Sigma]
50ml [10%] Foetal bovine serum [FBS] [Sigma], heat-inactivated at $56^{o}C$.
50ul 5 x 10^{-3}M 2-mercaptoethanol [Sigma]

4B Supplemented Iscove's Modification of Dulbecco's Medium [sIMDM]

For 500ml:460ml IMDM [1X][Gibco]
50ul 5 X 10^{-3}M 2-mercaptoethanol [Sigma]
10ml [2%] Penicillin/streptomycin [10,000u/ml]
5ml [1%] Preservative-free heparin [1000u/ml]
25ml [5%] FBS [Northumbria Biologicals Ltd [NBL], Northumberland, UK]

4C Supplemented Hank's Balanced Salt Solution [sHBSS]

For 500ml:483ml HBSS [1X] [see below]
5ml [1%] Preservative-free heparin [1000u/ml]
10ml [2%] FBS [NBL]
2ml Sodium bicarbonate [8%] containing phenol red [0.4%][Gibco]

[HBSS] [10X]

This solution was prepared as a 10X.

Solution A

Magnesium sulphate	5.0g
Distilled water	1000ml

Solution B

Magnesium chloride	5.0g
Distilled water	1000ml

Solution C

Calcium chloride	9.3g
Distilled water	1000ml

Solution D

Sodium chloride	400.0g
Potassium chloride	20.0g
Di-sodium hydrogen orthophosphate	7.6g
Potassium di-hydrogen orthophosphate	3.0g
Distilled water	2000ml

Solutions A, B, C and D were mixed together before being dispensed into litre amounts and stored at $4^{o}C$.

HBSS [1X]

This solution was prepared by diluting the 10X stock ten-fold with distilled water.

HBSS [10X]	500ml
0.4% Phenol red	25ml
Distilled water	4475ml

The solution was dispensed into 500ml amounts before being autoclaved at 10lbs for 20 min.

4D Supplemented Earle's Balanced Salt Solution [sEBSS]

For 500ml: 50ml EBSS [10X] [see below]
10ml [2%] FBS [NBL]
0.5g [0.1%] sodium azide
Volume made up to 500ml with sterile distilled water.

EBSS [10X]

This solution was prepared as a 10X

For 1 litre:

Solution A

Calcium chloride	2.6g
Distilled water	200ml

Solution B

Magnesium sulphate	2.0g
Distilled water	200ml

Solution C

Sodium chloride	68g
Potassium chloride	4.0g
Sodium di-hydrogen orthophosphate	1.45g
Distilled water	600ml

Each solution was filter sterilised and the 3 were added together.

Appendix 5

5 Additional Solutions

5A Phosphate Buffered Saline [PBS] pH 7.2

For 1 litre:	Disodium hydrogen phosphate	1.15g
	Potassium di-hydrogen phosphate	0.20g
	Sodium chloride	8.00g
	Potassium chloride	0.20g

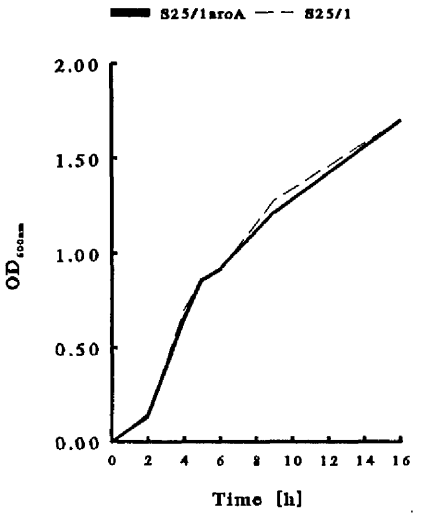
Made up to 1 litre with distilled water. Sterilised by autoclaving.

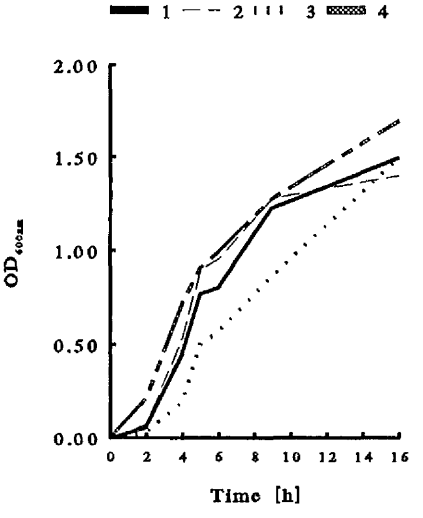
5B Tris-Borate Buffer, pH8.3

For 1 litre:	Tris [89mM]	10.76g
	EDTA [2.5mM]	0.93g
	Boric acid [89mM]	5.50g

Made up to 1 litre with distilled water. Sterilised by autoclaving.

Appendix 6 In Vitro Growth of the Salmonella Strains





1 = pD; 2 = pC; 3 = pAL12; 4 = pUC8.

1 ml of an overnight LB broth culture of each of the strains were subcultured into fresh LB broth and incubated at 37°C with shaking. Aliquots were taken at the stated times and the UV absorbance measured at 600_{nm}.