Purification, immunogenicity and protective potency of the F1 antigen from *Yersinia pestis*

Thesis

PURIFICATION, IMMUNOGENICITY AND PROTECTIVE POTENCY OF THE F1 ANTIGEN FROM *YERSINIA PESTIS*

KAREN MARGARET M. REDDIN

A thesis in partial fulfilment of the requirements of the Open University for the degree of Doctor of Philosophy

February 1998

Collaborating Establishment:
Chemical and Biological Defence Establishment
Porton Down

Centre for Applied Microbiology and Research

PAGES NOT SCANNED AT THE REQUEST OF THE UNIVERSITY

SEE ORIGINAL COPY OF THE THESIS FOR THIS MATERIAL

DEDICATION

To David, Amelia and William for their constant encouragement and support.

Abstract

Yersinia pestis, an organism endemic in much of the world, is the causative agent of pneumonic and bubonic plague. The disease is severe with a high mortality rate. Most plague vaccines are formalised whole cells, which give limited and short-lived immunity. An acellular vaccine could prove to be more effective than using killed whole cells, without the associated side effects.

The 17.5kDa F1 antigen of *Y. pestis* has been shown to be immunogenic and protective in mice (Simpson *et al.*, 1990) and therefore it might be a suitable component of an acellular vaccine. In this study, the conditions for the production and purification of F1 from culture of *Y. pestis* were examined with a view to increasing the yield of F1 antigen. The optimal conditions were to grow *Y. pestis* MRE 1447 in a chemically defined medium at pH7.4 for 48h at 37^0C and these conditions were transferred to a 25-litre fermenter for the larger-scale production of F1 antigen. Typically, 40mg of F1 antigen of ≥90% purity was obtained from 1L of culture supernatant. The purified F1 was characterised using biochemical, structural and immunological methods to confirm the integrity of the purified antigen. Immunological methods using anti-F1 monoclonal and polyclonal antibody identified the purified antigen as F1. Structural studies on the glycosylation of the protein showed that no post-translational glycosylation of the protein could be detected by the methods used in this study. The *caf*1 sequence was successfully cloned into *Escherichia coli* to produce a recombinant F1 antigen. This recombinant antigen proved to be both immunogenic and protective but yields of recombinant protein were consistently low.

Purified culture-derived native F1 and a recombinant V antigen (produced at CBD, DERA) were used to assess controlled-release vaccine delivery systems in poly(lactide-co-glycolide) PLG microspheres and liposomes. Recombinant V was used in addition to F1 to assess a combination subunit vaccine with F1 given alone as an acellular vaccine.

F1 antigen encapsulated in PLG microspheres induced high serum antibody titres when injected i.p. in mice; mucosal IgA was also detected. Mice immunised with F1 in Alhydrogel or PLG microspheres were protected against sub-cutaneous challenge with *Y. pestis*. F1 antigen surface-labeled onto liposome vesicles stimulated high serum titres in

Balb/c mice; a mucosal response was also induced and mice were protected against sub-cutaneous challenge with up to 1 x 10^5 organisms. This strongly indicated that immunising with F1 formulated in PLG microspheres and liposomes induced a potent immune response and that protection was achieved after only one dose. However, increased protection was observed when a combination vaccine of F1 and rV was administered in PLG microspheres, liposomes or Alhydrogel given in multiple doses.

The efficacy of different delivery routes against sub-cutaneous *Y. pestis* challenge was also investigated. Administration by the i.p. route gave consistent protection against sub-cutaneous plague challenge. The i.n. route also proved effective for both PLG microspheres and liposomes when multiple doses were used. Oral delivery failed to give adequate protection against sub-cutaneous infection, even after multiple doses.

DECLARATION

I declare that the research presented in this thesis is all my own work, except where otherwise indicated, and has not been submitted elsewhere for a research degree.

KAREN M. REDDIN

ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr. A. Robinson and Dr. E.D. Williamson for their guidance and support during this study. I would also like to thank T. Easterbrook for his help and advice, Dr. R. Wait and R. Hartwell for their help in the characterisation of the antigen and B. Dowsett for the electron microscopy. Finally, I would like to thank all of my colleagues at CAMR and CBD with special thanks to Mary Matheson.

Publications and Communications

Publications from PhD thesis

Full Refereed Publications

A new improved sub-unit vaccine for plague: the basis of protection. 1995. Williamson, E.D., Eley, S.M., Griffin, K.F., Green, M., Russell, P., Leary, S.E.C., Oysten, P.C.F., Easterbrook, T., Reddin, K.M., Robinson, A. and Titball, R.W. FEMS Immunol. Med. Microbiol. **12** : 233-230.

Comparison of the immunological and protective responses elicited by microencapsulated formulations of the F1 antigen from *Yersinia pestis* 1998. Reddin, K.M., Easterbrook, T., Eley, S.M, Russell, P , Mobsby, V.A. , Jones, D.H., Farrar, G.H., Williamson, E.D. and Robinson, A. Vaccine. **16**: 761-767.

Poster presentations

Large-scale purification of the F1 antigen of *Yersinia pestis*. 1995. Reddin, K.M., Easterbrook, T.J., Robinson, A., Williamson, D. and Rhind-tutt, R.. Contrib. Microbiol. Immunol. **13** : 329-330.

Studies on the immunogenicity of the Pla protein from *Yersinia pestis*. 1995. Easterbrook, T.J., Reddin, K.M., Robinson, A. and Modi, N. Contrib. Microbiol. Immunol. **13** : 214-215.

Development of a liposomal acellular plague vaccine. 1996 Easterbrook, T.J., Reddin, K.M., Eley, S.M., Mobsby, V., Williamson, E.D. and Robinson, A. Presented at Liposome Advances : Progress in Drugs and Vaccine Delivery. University of London.

Other publications

Periplasmic superoxide dismutase in meningococcal pathogenicity. 1998. Wilks, K.E., Dunn, K.L.R., Farrant, J.L., Reddin, K.M., Gorringe, A.R., Langford, P.R. and Kroll, J.S. Infect. Immun. **66** : 213-217.

Abbreviations

α-MSH	Alpha-melanocyte stimulating hormone
Ail	Attachment invasion locus
ATP	Adenosine triphosphate
APCs	Antigen-presenting cells
ASCs	Antibody-secreting cells
AlOH	Alhydrogel
BALT	Bronchus associated lymphoid tissue
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
Ca	circa
CAMR	Centre for Applied Microbiology and Research
CBD	Chemical and Biological Defense Establishment
CCE	counterflow centrifugal elutriation
cfu	colony forming units
CH	Cholesterol
CTLs	Cytotoxic T lymphocytes
DCP	Dicetylphosphate
DERA	Defense and Environmental Research Agency
dH_2O	Distilled water
DIG	Dioxygenin
DMSO	Dimethylsulphoxide
DPPC	Dipalmitoylphosphatidylcholine
DPPS	Dipalmitoylphophatidylserine
DSPC	Distearoylphosphatidylcholine
ECACC	European Collection of Animal Cell Cultures
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunoabsorbent assay
ESI-MS	Electrospray ionisation mass spectroscopy
FAB-MS	Fast atom bombardment mass spectroscopy
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
GALT	Gut associated lymphoid tissue
GIT	Gastrointestinal tract

GLC	Gas liquid chromatography
GST	Glutathione-s-transferase
HMWP	High molecular weight protein
HPLC	High performance liquid chromatography
IEC	Ion exchange chromatography
IEF	Isoelectrofocusing
IFN-γ	Interferon gamma
Ig	Immunoglobulin
i.n.	intranasal
inv	invasin
i.p.	intraperitoneal
IPTG	Isopropylthiogalactopyranoside
Irp	Iron repressible proteins
ISCOMS	Immunostimulating complexes
kb	kilobase
kDa	kilodalton
LAL	Limulus Amebocyte Lysate
Lcr	Low calcium response
LPS	Lipopolysaccharide
Mab	Monoclonal antibody
MAC	Membrane attack complex
MBP	Maltose bindng protein
M cells	Microfold cells
MCS	Main cloning site
MDP	Muramyl dipeptide
MHC	Major histocompatibility complex
MPL	Monophosphoryl lipid A
MRE	Microbiological Research Establishment
m/z	mass-to-charge ratio
NCTC	National Culture Type Collection
NHSP	N-hydroxysuccinimide ester of palmitic acid
OD	Optical density
PAS	Periodic Acid Schiffs
PBS	Phosphate buffered saline
PC	Polycarbonate

PCR	Polmerase chain reaction
PCS	Photocorrelation spectroscopy
PEG	Polyethylene glycol
Pgm	Pigmentation
pI	Isoelectric point
Pla	Plasminogen activator
PLG	Poly(lactide-co-glycolide)
PMNs	Polymorphonuclear neutrophils
PPs	Peyer's patches
PsaA	Pilus adhesin or pH6 antigen
PSer	Phosphatidylserine
Pst	Pesticin
PVA	Polyvinyl alcohol
Rt	Retention time
s.c.	subcutaneous
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
s-IgA	Secretory IgA
SEM	Scanning electron microscopy
SM	Starting material
Tc	Transition temperature
TEM	Transmission electron microscopy
Th	T helper
UV	Ultra-violet
YadA	*Yersinia* adherence
Yops	*Yersinia* outer proteins
YSA	*Yersinia* selective agar

List of Figures

List of tables

Contents

Chapter 1

General Introduction

1.1 The Genus *Yersinia*

The genus *Yersinia* is part of the family Enterobacteriaceae. *Y. pestis, Y. pseudotuberculosis* and *Y. enterocolitica* are the three most important species of the genus with respect to pathogenicity for animals and humans. *Y. intermedia, Y. kristensenii, Y. fredericksenii, Y. aldovae* and *Y. rohdei* are widespread environmental bacteria, not usually associated with disease. *Y. mollaretti* and *Y. bercovieri* have been separated from *Y. enterocolitica* (Wauters *et al.*, 1987), although they formerly belonged to biogroups 3A and 3B respectively and their importance in pathogenesis is undetermined, but they seem to be devoid of virulence-linked properties. Another species, *Y. ruckeri*, is an important fish pathogen responsible for the red mouth disease in rainbow trout and some other fishes (Carniel and Mollaret, 1990).

The members of the genus *Yersinia* are facultatively anaerobic Gram-negative short rods which grow better at 25^0C than at 37^0C on media commonly used for Enterobacteriaceae (fig1.1). Colonies are lactose negative and only appear after 48h of growth. The bacteria have the ability to grow at 4^0C (cold enrichment); however, this property seems to be more beneficial for environmental species than for pathogenic ones (Carniel and Mollaret, 1990).

1.1.1 Diseases caused by *Yersinia*

Y. pestis, Y. pseudotuberculosis and *Y. enterocolitica* are the three species of the genus *Yersinia* that cause disease in humans. These three species differ considerably in invasiveness and vary in infection route; they also cause diseases of contrasting severity. However, they exhibit a common tropism for lymphoid tissue and are able to avoid lysis by complement, avoid phagocytosis by polymorphonuclear leucocytes or macrophages (Rodrigues *et al.*, 1992) and are capable of forming extracellular microcolonies in the infected tissues (Cornelius *et al.*, 1987). *Y. enterocolitica* and *Y. pseudotuberculosis* can cross the gastrointestinal mucosa to infect underlying tissue, but infections usually remain localised in the sub-mucosal area (Portnoy and Martinez, 1985; Bolin and Wolf-Watz, 1984). *Y. pseudotuberculosis* is essentially a rodent pathogen which can be transmitted orally and causes diarrhoea, emaciation and death as a result of septicaemia. *Y. enterocolitica* is a common human pathogen which causes gastrointestinal disorders of varying severity, from mild self-limiting diarrhoea to mesenteric adenitis resembling appendicitis; systemic involvement is unusual. Transmission is usually by consumption of contaminated food or water. Several cases of septic shock due to transfusion of blood

Fig. 1.1 Electron micrograph of *Y. pestis* strain MRE 1447

Strain MRE 1447 was grown in YPMH chemically defined media (Table 2.1). A 48h culture was diluted with YPMH containing 0.01% formaldehyde, after confirmation of non-viability cells were washed and resuspended in PBS. Bacterial suspensions were coated onto an electron micrograph grid and stained with 1% (w/v) PTA (pH7.2) and viewed by transmission electron microscopy using a magnification of x70 000.

contaminated with *Y. enterocolitica* have been reported (Tipple *et al.*, 1990). In Europe, pork was reported to be a source of infection by *Y. enterocolitica* (Tauxe *et al.*, 1987). Only a few well-defined serotypes of *Y. enterocolitica* are pathogenic, serotype 0:8 is often associated with disease.

Y. pestis, the causative agent of plague, is normally injected into the body by an insect bite and therefore does not have to penetrate a body surface unaided. However, after infection by this route, bacteria disseminate throughout the body and can eventually reach the lungs. Bacteria can then be spread from person-to-person via the respiratory route which is referred to as pneumonic plague (Bartelloni *et al.*, 1973) therefore, *Y. pestis* has the potential to cause epidemics.

1.1.2 Pathogenic mechanisms

Environmental effects on growth differ between species of *Yersinia* as they occupy their own environmental niche. Some pathogenic organisms become rapidly killed in nature following separation from their host. However, gastro-intestinal parasites are typically more sturdy and can often survive without net increase in cell number for prolonged periods of time. The enteropathogenic *Yersinia* are excellent examples of this, whereas *Y. pestis* is more fastidious and has evolved a distinct strategy of survival. The enteropathogenic *Yersinia* generally cause gastrointestinal disease in their natural hosts. As a consequence organisms are excreted for prolonged periods of time, thereby favouring ingestion by new hosts. This is not the case with *Y. pestis* which usually kills its host. Bubonic plague is manifested as an acute, intransigent and lethal disease which is dependent on mounting an immediate and overwhelming attack on the host before the immune system becomes capable of providing significant defence. Man sometimes becomes part of this cycle. *Yersinia* are known to interact with phagocytic cells of the host and they are obligate intracellular parasites which can multiply within the phagosome; in fact some virulence factors are only expressed after the bacteria have been phagocytosed.

In the first stages of *Y. enterocolitica* and *Y. pseudotuberculosis* infections, the bacteria attach to the intestinal mucosa followed by infection of underlying tissue. A toxin similar to the ST toxin of enterotoxigenic *Escherichia coli*, called Yst, is produced by *Y. enterocolitica* and may be responsible for the diarrhoea that is a common symptom of *Y. enterocolitica* infections. However, when *Y. enterocolitica* is grown *in vitro*, the gene for Yst is expressed only at temperatures lower than 30^0C (Amirmozafari and Robertson, 1993; Mikulskis *et al.*,

1994), therefore there has been a tendency to dismiss Yst as the cause of diarrhoea. Nevertheless, sufficient Yst could be present in bacteria entering the host to cause symptoms. There is also recent evidence to suggest that the pattern of temperature regulation of virulence factors may be different at different pH values (Straley and Perry, 1995). It is assumed that the bacteria cross the intestinal mucosa by first binding to the apical surface of gut epithelial cells; the cells in this region then engulf the bacteria, enabling them to cross the mucosa inside the phagocytic vesicle to reach the basal surface where bacteria are released by exocytosis. Such a model seems reasonable when bearing in mind the ability of these organisms to adhere to and be engulfed by cultured mammalian cells *in vitro* (Brubaker, 1991a). It remains unclear, however, as to the exact role of proteins that mediate invasion of cultured cells (invasion factors).

All three pathogenic species of *Yersinia* are serum resistant. In *Y. enterocolitica* and *Y. pseudotuberculosis*, serum resistance is an inducible trait, and bacteria become serum resistant only at 37°C (Salyers and Whitt, 1991). In *Y. pestis*, a protease, plasminogen activator (Pla) confers serum resistance (Sodeinde *et al.*, 1992). Among other functions, this extracellular protease can degrade the C3b and C5a components of complement. Therefore, Pla could contribute to serum resistance by preventing formation of the membrane attack complex (MAC) which damages the membrane of the pathogen, leading to lysis of the bacteria. In addition, Pla may have an antiphagocytic role in preventing opsonisation of bacteria by C3b and attraction of phagocytes by C5a.

Since iron is essential for bacterial growth and survival, *Yersinia* spp., like other bacterial species, have evolved strategies to obtain iron from the host. Pathogenic *Yersinia* spp. have multiple iron-acquisition systems which are induced at 37°C and allow the bacteria to obtain iron from haemin and transferrin, two abundant sources of iron in the human body. Among the first authors to provide evidence of the virulence-enhancing effect of iron in strains of *Y. pestis* were Jackson and Burrows (1956). They studied non-pigmented mutants of virulent strains of *Y. pestis* which were unable to absorb haemin from defined medium and consequently were non-virulent in mice and unable to proliferate freely *in vivo* because they were unable to derive iron from the host. However, the growth of these mutants could be augmented *in vivo* and virulence increased by injection of iron compounds with the organism. Many animal hosts can be infected by *Yersinia* spp., including mice, and developing animal models of infection has not been difficult, although these models do not necessarily mimic the human disease.

Yersinia spp. readily invade many types of cultured mammalian cells, including HeLa, Hep-2 and Henle cell lines. This trait has been intensively studied as a model for invasion of mammalian cells by bacteria (Isberg, 1989). However, results of tissue culture studies are not always consistent with the behaviour of bacteria *in vivo*.

1.1.3 Genetics of *Yersinia*

Yersinia spp. exhibit a complex virulence regulatory network which is partially controlled by temperature. The clinically significant *Yersinia* can multiply on appropriate media at temperatures ranging from about 5 - 24°C. However, marked physiological differences occur upon an increase in growth temperature from 26°C to 37°C, including the need for additional nutritional requirements and expression of virulence functions. The organisms possess novel regulatory functions that respond to temperature and to the presence of calcium and these are involved in virulence. Research into the molecular biology of this genus has shown that *Y. pestis* and *Y. pseudotuberculosis* exhibit nearly identical chromosomal DNA relatedness (Bercouvier *et al.*, 1980; Brubaker, 1972) and distinctions are mainly represented by differences in plasmid content (Ben-Gurion and Shafferman, 1981; Brubaker, 1991a). The chromosomal DNA of *Y.enterocolitica* has significantly less relatedness, indicating evolutionary divergence prior to that separating *Y. pestis* and *Y. pseudotuberculosis*. The fact that *Y. pestis* is very closely related to the much less invasive *Y. pseudotuberculosis* (90% DNA/DNA homology) has made it possible to look for genes expressed in *Y. pestis* but not in *Y. pseudotuberculosis* and *vice versa*, thus providing an insight into which factors might account for the greater invasiveness of *Y. pestis* in humans.

Yersinia as a genus are known to possess certain virulence factors, some of which are chromosomal and others are plasmid-mediated (table 1.1). A shared feature of all pathogenic *Yersinia* strains is the presence of 70- to 75-kbp Lcr (low calcium response) plasmids. Many of the genes necessary for virulence are encoded on these plasmids and have been cloned and identified. The products of these plasmid genes fall into four general categories: (i) adhesin protein (YadA), (ii) secreted antiphagocytic proteins and *Yersinia* outer proteins (Yops), (iii) proteins involved in processing and secretion of Yops (Ysc), and (iv) regulatory or Lcr proteins. Strains that have been cured of these plasmids are much less virulent. *Y. pestis* has three additional plasmids not found in *Y. enterocolitica* or *Y. pseudotuberculosis*. One is the 110-kbp plasmid (Fra) which encodes the fraction 1(F1) protein and murine toxin. The F1

Table 1.1 Virulence factors of *Y.pestis, Y.pseudotuberculosis* and *Y.enterocolitica*

VIRULENCE FACTOR	*Y.PESTIS*	*Y. PSEUDOTUBERCULOSIS*	*Y. ENTEROCOLITICA*
ca. 10-kb pst plasmid	Present	Absent	Absent
Plasminogen activator	+	0	0
Posttranslational degradation of Yops	+	0	0
ca. 70-kb Lcr plasmid	Present	Present	Present
Yops	+	+	+
YadA	0	+	+
V, W antigens	+	+	+
ca. 100-kb tox plasmid	Present	Absent	Absent
Fraction 1	+	0	0
Murine exotoxin	+	0	0
Chromosomal pigmentation (haemin storage at 26°C)	+	0	0
Host cell invasions inv product ail product	 0 0	 + 0	 + +
pH6 antigen	+	+	0
Catalase	+	+	0

protein is a component of an antiphagocytic protein capsule, whilst the murine toxin kills mice but has not been shown to have a role in human disease (Brubaker, 1991a). The second *Y. pestis* specific plasmid, is a 10kbp plasmid that encodes a plasminogen activator protease (Pla). Pla aids the dissemination of *Y. pestis* within the body and may also have a role in the insect-mediated transmission of the bacteria. The third *Y. pestis* specific plasmid is the Pst plasmid encoding the pesticin virulence determinant.

In *Y. enterocolitica* and *Y. pseudotuberculosis,* two invasion factors have been identified, invasin (encoded by the *inv* gene) and Ail (*a*ttachment-*i*nvasion *l*ocus) (Isberg and Leong, 1990; Miller, 1989, 1992). Both *inv* and *ail* are chromosomal genes. More recently, a plasmid-encoded protein originally identified as an adhesin, YadA (*Yersinia ad*herence), has been shown to mediate invasion of cultured cells and can thus be added to the list of potential invasion factors (Bliska *et al.*, 1993). Virulence factors which are shared by *Y. pestis* and either or both *Y. pseudotuberculosis* and *Y. enterocolitica*, include PsaA (a pilus adhesin), Yops (only a few of these have assigned functions) and LcrV or V antigen. These virulence mechanisms will be discussed later as *Y. pestis* virulence factors.

The medically significant *Yersinia* are closely related, therefore it follows that the genetic differences known to occur between them are likely to mediate the two distinct forms of infection. Table 1.1 summarises the similarities and differences in virulence factors in the three pathogenic species of *Yersinia.*

1.2 *Yersinia pestis*

1.2.1 The history of plague

Y. pestis is the causative agent of bubonic and pneumonic plague and the disease has a long, documented history. One of the first documented accounts of plague was when the disease struck the Roman empire and Western Europe during the time of the emperor Justinian in 542 A.D. and it raged on throughout the next century. Then for unknown reasons the disease disappeared until 1348, when it was reintroduced from the East. The disease was believed to have been introduced from Asia via trade routes to Europe. The first outbreaks of human plague are thought to have occurred in Russia, from where it spread down to the Black Sea. Bubonic plague then seems to have been transported aboard ships to the Mediterranean ports of Europe. The plague then spread further and most of Europe was affected before the

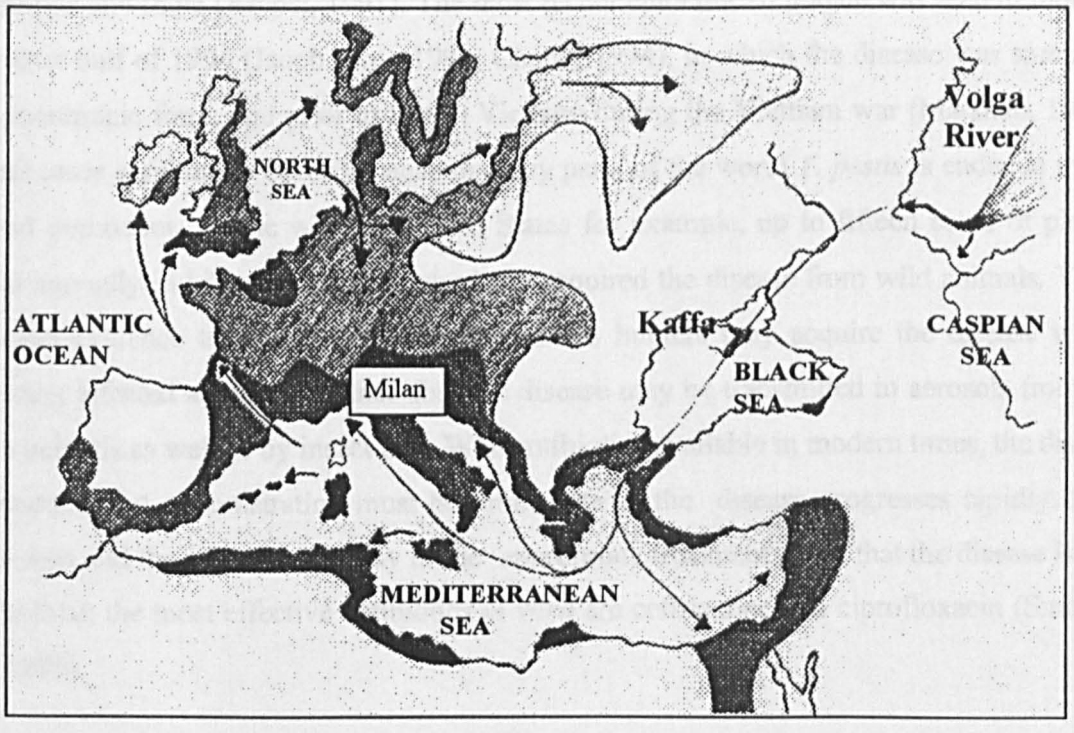
epidemic finally subsided in 1352 (Figure 1.2). Statistics are unreliable but it is generally supposed that about a third of the population of Western Europe perished during this epidemic. After this, episodes occurred into the late 17th and early 18th centuries and one of the last great outbreaks of bubonic plague occurred in 1894 in Hong Kong. Plague also broke out in Bombay in 1898 and in San Francisco in 1901. The latter incidences were presumably imported by merchant ships carrying plague-infected rats and fleas. Following this, the population of wild rodents in California and other western states may have become infected with the plague bacillus as a result of contact with imported rats and plague is still endemic in North America today.

Alexandre Yersin isolated the plague bacillus during the 1894 plague epidemic in Hong Kong. This epidemic was regarded as the return of an ancient, unpredictable, little understood and incurable disease, much as it is still regarded today. In June of 1894, Hong Kong was under quarantine after the first plague victims had been diagnosed. A number of scientists arrived to investigate the epidemic, including Alexandre Yersin, a little known bacteriologist at the time. They were tasked with isolating and identifying the organism that was causing the bubonic plague.

Yersin was the first scientist to look for the causative agent of plague in excised buboes and found them teeming with Gram-negative bacilli. There was some controversy, however, as another scientist, Kitasato, also working in Hong Kong investigating bubonic plague, claimed that the organism he had isolated was the plague bacillus. He, however, examined the heart, lungs, liver and spleen of plague victims and not the buboes characteristic of the disease. Kitasato insisted that his Gram-positive bacillus, and not Yersin's Gram-negative bacillus was the causative agent of plague. However, by 1898 Yersin's isolate was widely accepted as the plague bacillus and in France research commenced into the development of a vaccine. During his work in Hong Kong, Yersin established plague as a zoonosis transmitted from rat-to-rat and rat-to-human. However, he was not aware of the central role of the flea. This was discovered by another scientist from the Institut Pasteur, Paul-Louis Simond in 1897. Yersin also tested soil in plague-stricken areas and discovered that the bacteria are capable of lying in the ground for long periods without significant loss of viability.

In 1937, the plague bacillus was classified in the genus *Pasteurella*, following a proposal made by Rahn. However, Van Loghem (1945) reasoned that this classification was inappropriate because it placed the plague and pseudotuberculosis bacilli among the

Fig. 1.2 Map showing the spread of plague during the mid-14th century



The spread of plague was rapid, the disease originated in central Asia and was transmitted to Europe via the "Silk road", reaching Kaffa in about 1347. It was then carried by ship to the major ports of Europe and northern Africa. Most of Europe was affected before the epidemic finally subsided in 1352. Milan was the only major city at the time to escape plague.

Parvobacteriaceae, characterised by their exceptionally small size, whereas *Y. pestis* and *Y. pseudotuberculosis* are closely related and stand apart from the genus *Pasteurella* in general. Yersin studied at the Institut Pasteur for some of his career and because of this, Yersin's plague bacillus was originally named *Pasteurella pestis*, but, Van Loghem proposed to classify these two species in a new genus called *Yersinia*, in honour of the discoverer of the plague bacillus (Bendiner, 1989).

Cases of plague continue to be reported throughout the world in places such as South Africa, Argentina and India (Ampel, 1991). The most recent epidemic of plague was seen in India in the latter half of 1994 (Jacob John, 1994; Chand, 1994), in which the disease was spread in the pneumonic form, and prior to this in Vietnam during the Vietnam war (Marshall, 1967). These cases continue to occur because in many parts of the world *Y. pestis* is endemic in the rodent population. In the western United States for example, up to fifteen cases of plague occur annually in hikers or hunters who have acquired the disease from wild animals. There is some evidence to suggest that people such as hunters may acquire the disease whilst skinning infected animals and therefore the disease may be transmitted in aerosols from the dead animals as well as by insect bite. With antibiotics available in modern times, the disease is treatable but administration must be immediate as the disease progresses rapidly. Mis-diagnosis and the consequent delay in the appropriate treatment mean that the disease is still often fatal; the most effective antibiotics *in vitro* are ceftriaxone and ciprofloxacin (Smith *et al.*, 1995).

1.2.2 Pathogenesis of plague

The classical model of plague is that the disease exists in a primary animal reservoir, generally one of wild rodents. This epizootic spreads to the black rat (*Rattus rattus*), which is very sensitive to plague, and on its death its flea, *Xenopsylla cheopsis*, attaches itself to another available host, sometimes human. Inter-human transmission is possible, via the respiratory route in the case of pneumonic plague or via the human flea, although humans generally acquire the disease from rat fleas. Plague cannot persist endemically in human populations, though it does so in wild rodents. Rats which survive infection often become "carriers" and the rat fleas survive for long periods in the nests passing infection to new generations. As a result, occasional epidemics occur among the rodents greatly reducing their numbers. It is at such times that the two species of rats associated with man, the brown rat (*Rattus norvegicus*) and the black rat (*R . rattus*), become involved in epidemics; the black rat is more often associated with the great plague pandemics, possibly because it is more

inclined to invade ships and therefore extend the spread of disease, or because its fleas are more aggressive to man. The fleas which infest brown rats are *Ceratophyllus fasciatus*; those of the black rat are *X. cheopsis, X. astia* and *X. brasiliensis*. *X. cheopsis* is the flea usually associated with rat-to-man transmission of plague.

Y. pestis infections are therefore acquired in two ways, by transmission from rats or other wild animals to humans by flea bite, or by direct human-to-human transmission via aerosols. The flea bite injects the bacteria into the bloodstream and bacteria then travel in blood to the nearest lymph nodes. In the lymph nodes, *Y. pestis* is ingested by fixed macrophages, but the bacteria can survive and grow in normal inactivated macrophages and thus proliferate in the lymph nodes (Williams *et al.*, 1972). The inflammatory response to this proliferation produces the characteristic swelling, or bubo, that gives bubonic plague its name. Bacteria growing in the lymph nodes leak into the bloodstream; this stage of the disease is called septicaemic plague and lysis of the bacteria releases lipopolysaccharide (LPS), which causes septic shock. Eventually the bacteria reach the lungs, where they parasitise the lung macrophages and once the disease has reached this stage (called pneumonic plague) it can be transmitted by aerosols. Direct inhalation of the organisms in such aerosols produces a form of the disease that progresses much more rapidly than the flea-transmitted bubonic form, possibly because the aerosolised bacteria, in contrast to the flea-bite bacteria, already express all the virulence factors needed for colonisation of the human body and so provide a more overwhelming challenge to the host defence system. The fatality rate for pneumonic plague is high and death can occur within days. Humans with plague often develop necrotic lesions in peripheral blood vessels (probably as a result of LPS-induced intravascular coagulation) giving the skin a blackish appearance and leading to the term *black plague* or *black death.*

Wild-type strains of *Y. pestis* exhibit a consistent pattern of virulence in both natural and experimental hosts. The ability of *Y. pestis* to cause a fatal disease from peripheral sites of infection is an important characteristic of the species. In mice, *Y. pestis* is rapidly removed from the vascular system and emerges to colonise the liver, spleen and lungs. The bacteria then return to the bloodstream which results in pronounced bacteraemia. The number of *Y. pestis* organisms required to cause fatal infection in mice is significantly less than that for the enteropathogenic *Yersina*. This enhanced virulence may be due to the expression of virulence factors, not present in the enteropathogenic *Yersinia*.

1.3 *Y. pestis* Virulence Factors

Pathogenic bacterial species are adapted to multiply at the expense of a living host which simplifies the problem of nutrient acquisition for the bacteria but poses the problem of escaping from the response of the immune system of the host. To succeed in this, bacterial pathogens use a number of specific functions that they have evolved or acquired from other organisms. A bacterial infection is a sequential process in which the infecting organism must be able to withstand the different environmental conditions and host defence mechanisms it will encounter. To survive, the invading bacteria must therefore express their virulence functions at the appropriate time. Successful pathogens have therefore not only acquired virulence factors but must also subject these to a regulatory network. Therefore the virulence factors depend on the appropriate environmental stimuli to initiate expression.

Proof that a given determinant serves as a virulence factor requires demonstration that its mutational loss from the bacterium results in a loss or reduction in virulence. This definition becomes more difficult when virulence is mediated by two or more proteins encoded on a plasmid or within a chromosomal region known to undergo loss or deletion at high frequency; both of these situations occur in *Y. pestis*. However, research in recent years has made progress towards identifying the individual proteins involved in the disease process and their importance in virulence. *Y. pestis* harbours three plasmids that are required for the expression of virulence; a 9.5kb Pst plasmid which encodes the outer membrane protein Pla, a 75kb Lcr plasmid encoding the V antigen and Yops and the 110kb pFra plasmid which encodes the F1 antigen and murine toxin (table 1.1). Most of the mutant strains used to study virulence in *Y. pestis* show only a reduction in virulence and not a total loss of virulence, suggesting that the determinants may act in concert to contribute to full virulence. The most important virulence factors that contribute to disease will be discussed here.

1.3.1 F1 antigen

The F1 antigen is encoded on a 110kb plasmid and is a 17.5kDa surface protein, sometimes referred to as the capsular antigen. It is reported to be glycosylated, bearing galactose and fucose residues (Bennet and Tornabene, 1974; Glosnicka and Gruszkiewicz, 1980) and because a recombinant F1 antigen has been shown to induce a protective immune response in Balb/c mice (Simpson *et al.*, 1990) it might be a suitable component in an acellular vaccine to *Y. pestis*. F1 antigen is thought to consist of two sub-units, a protein linked to a polysaccharide (fraction 1A) and a free protein (fraction 1B). Fraction 1A, a 17.5kDa protein,

and fraction 1B, a 15kDa protein, are complexed to form a series of serologically similar aggregates (Glosnicka and Gruszkiewicz, 1980; Bennet and Tornabene, 1974; Baker *et al.*, 1952).

F1 may have a role in the ability of *Y. pestis* to evade phagocytosis. Janssen *et al.* (1958) showed that both virulent and avirulent organisms grown at 26^0C were capable of surviving inside neutrophil polymorphonuclear leukocytes and macrophages after inoculation into the peritoneum of guinea pigs. They reasoned that the survival and multiplication of bacteria inside phagocytic cells was more important than resistance to phagocytosis. Their experiments may resemble the conditions occurring in nature when *Y. pestis* lacking F1 antigen are injected into the mammalian host from a flea bite. It is only after the initial multiplication inside the host that the bacteria express the F1 antigen, enabling them to resist phagocytosis and disseminate throughout the body. However, Cavanaugh and Randall (1959) showed that *Y. pestis* lacking F1, when injected into an animal model, were ingested by neutrophil polymorphonuclear leukocytes which kill the bacteria but mononuclear phagocytes (these differentiate into macrophage) permitted intracellular multiplication.

Burrows (1963) found that virulence of naturally occurring $F1^-$ mutants was significantly reduced in guinea pigs. However, with advances in molecular biology it is possible to construct specific $F1^-$ strains to study virulence and this may have advantages over the use of naturally occurring mutants used in the past. Worsham *et al.* (1994) reported that mutants lacking the F1 antigen still had partial virulence and loss of it did not decrease the mouse lethality of *Y. pestis* but may have delayed time-to-death in mice that were infected with $F1^-$ recombinant strains. It is not known whether F1 is important in determining the high virulence of *Y. pestis* in humans but the fact that the time-to-death was delayed in mice when infected with $F1^-$ strains, indicates that F1 is important for invasiveness in the early stages of infection and possibly acts by preventing phagocytosis by neutrophils and monocytes. There is evidence that the F1 antigen as a surface component effectively protects bacteria *in vivo* against phagocytosis (Burrows and Bacon, 1956; Williams *et al.*, 1972). The mechanisms of the antiphagocytic action of F1 antigen have been investigated by Williams *et al.* (1972) who found that F1 interfered with the action of the complement components, C4 and C2. Complement proteins act together in a complex regulatory pathway that results in inactivation or efficient phagocytosis of bacteria and so any disruption of this could lead to prevention of phagocytosis, which in turn would aid dissemination of bacteria throughout the host.

1.3.2 V and W antigen

The V and W antigens of *Y. pestis* were first discovered by Burrows and Bacon (1956). Burrows (1958) showed that V^- strains were avirulent for mice and guinea pigs whereas V^+ strains were fully virulent. Une and Brubaker (1984), showed that virulence in *Y. pestis* was correlated with the expression *in vitro* of a temperature-dependent nutritional requirement for calcium (Ca^{2+}), associated with the ability to produce maximal levels of V and W antigens (Vwa^+). This phenotype is regulated by the Lcr plasmid which also promotes the synthesis of temperature-dependent Yops (Wong and Elberg, 1977).

V antigen induced in *Y. pestis in vitro* is found within the cytoplasm as a 37kDa protein; however, the origin of the W antigen, reported to be a 140kDa lipoprotein, has not yet been determined. The V antigen has been more intensively studied to determine its role in virulence and, more recently, its potential as a candidate vaccine antigen, but the physiological role of V in promoting disease is still not well understood. Straley and Brubaker (1981) isolated peptides associated with these virulence determinants by using methods of cell disruption and separation into cytoplasmic and outer membrane fractions using sucrose gradient centrifugation. Because they are not expressed on the cell surface, V and W antigens probably do not interact directly with host cell membrane surfaces during infection. However, Une and Brubaker (1984) concluded that V may facilitate intracellular growth because Vwa^+ strains plus anti-V serum or Vwa^- strains did not grow significantly within organs or appear in the vascular system; the bacteria had apparently been cleared. This hypothesis is supported by the fact that concentrations of Ca^{2+} and Mg^{2+} optimal for the synthesis of V antigen are similar to those in mammalian intracellular fluid (Straley and Brubaker, 1981). It is unclear how cytoplasmic V is released into the extracellular environment and whether the antigen is protective *in vivo* in man. Leary *et al.* (1995) and Williamson *et al.* (1995) have shown that V acts as a very effective protective antigen in mice when used as a vaccine antigen.

A commonly used method for detecting V antigen in *Y. pestis* is to culture the bacteria on agar that contains magnesium oxalate and is calcium-free. Virulent bacteria will express V antigen and not grow on this medium at 37^0C. Zahorchak and Brubaker (1982) studied *Y. pestis* grown under calcium-free conditions at 37^0C and showed that nucleic acid and protein synthesis had ceased. The calcium-deprived cells started growing again when placed at a

temperature of 26^0C, but at 37^0C, after the arrest of growth, even after the addition of calcium, growth could not be restored. Synthesis of these antigens is suppressed by cell division that occurs in the presence of calcium ions. After phagocytosis, however, the Vand W antigens are synthesised because the cytoplasm of mammalian cells contains insufficient calcium ions to support bacterial division. It is the V antigen that has been the focus of recent efforts to purify and characterise the protein, whereas, relatively little is known about the W antigen.

1.3.3 Plasminogen activator (Pla)

The primary sequence of Pla has been defined (Sodeinde and Goguen, 1989). Pla is a protease located in the outer membrane of the bacterial cell and probably has multiple functions. It has a fibrinolytic activity which prevents blood clotting and breaks down clots, thereby increasing invasiveness at the site of the flea-bite. Pla activates plasminogen by proteolytic activation to form plasmin, the blood protein that dissolves fibrin clots. A fibrin clot would limit the spread of bacteria by trapping them at the site of infection. Plasminogen would normally be activated only after the bacteria have been eliminated and the clot can safely be cleared. By prematurely activating plasminogen, the bacterial enzyme Pla prevents the fibrin clot from forming and removes one of the barriers to bacterial spread. *Y. pestis* lacking Pla are avirulent if injected in mice sub-cutaneously, but retain virulence if injected into the bloodstream (Sodeinde *et al.*, 1988).

Pla may also play a role in the ability of the flea to inject the bacteria into the new host. When a flea feeds, it ingests blood and the mouth parts of the flea trap clots causing a blockage that prevents the flea feeding again. At the surface of the human body, the temperature is high enough (35^0-37^0 C) for Pla and flea proteases to co-operate in dissolving the clot thereby disseminating plague bacteria trapped in the clot.

In addition to this, Pla degrades some of the Yops (section 1.3.4) and has the effect of limiting the amount of Yops on the surface and in the vicinity of the bacterial cell (Sodeinde *et al.*, 1992). This apparently self-defeating activity could be important if Yops are essential to protect *Y. pestis* from phagocytosis by polymorphonuclear neutrophils (PMNs) but need to be at a level which will allow uptake by circulating monocytes or macrophage that do not kill *Y. pestis*. PMNs are important short-lived phagocytic cells which engulf bacteria, destroy them and then die. Monocytes, in contrast, are long-lived phagocytic cells, that also

internalise and destroy infectious agents. They migrate out into the tissues where they become tissue macrophages. It is advantageous for *Y. pestis* to be engulfed by these monocytes as *Y. pestis* is known to be able to survive within these cells and to express additional virulence factors within the phagosome (Straley *et al.*, 1993; Straley and Brubaker, 1981). Therefore, modulating the amount of active Yops at the site of infection could be a means of balancing protecting bacteria from phagocytosis by PMNs yet still allowing them to be ingested by monocytes, which would further protect them from host defences and promote dissemination.

1.3.4 *Yersinia* outer proteins (Yops)

Yops are potentially important surface antigens that may be species specific or common to all three *Yersinia* spp. There are eleven Yops expressed by the Lcr virulence plasmid of *Y. pestis* (Straley and Cibull, 1989, Straley *et al.*, 1993). Yops were originally thought to be located in the outer membranes of *Yersinia* spp.(Straley and Brubaker, 1981; Portnoy *et al.*, 1981). However, research suggests that that they are not outer membrane proteins but are excreted proteins that can be membrane associated under some conditions (Straley *et al.*, 1993). Purification of Yops from *Y. pestis* has proved difficult when the organism was grown *in vitro* and may have been due to degradation of Yops in *Y. pestis* by Pla (Straley and Brubaker, 1981; Sodeinde *et al.*, 1992); however, when the Lcr plasmid of *Y. pestis* was transferred to *E. coli* or *Y. pseudotuberculosis*, several Yops were expressed that cross-reacted serologically with those of *Y. enterocolitica* (Wolf-Watz *et al.*, 1985; Portnoy *et al.*, 1984). Furthermore, a plague convalescent-phase serum recognised two of these cross-reactive Yops, indicating that *Y. pestis* expresses Yops during human infection (Bolin *et al.*, 1985).

Yops can be placed into two functional categories; those that interfere with signal transduction in host cells (i.e. the ability of host cells, particularly phagocytes to respond to environmental signals) and those that attack the host cell cytoskeleton (Salyers and Whitt, 1994), Yops M, K and L are unique to *Y. pestis* (Straley and Bowmer, 1986). Measurement of the LD_{50} of *Y. pestis* insertion mutants showed that at least two Yops (E and K) were necessary for virulence, whereas Yops J and L were not found to be essential for full virulence. The operons encoding Yops and V antigen are widely separated on the Lcr plasmid (Perry *et al.*, 1987). These co-ordinately regulated virulence genes probably represent a virulence regulon.

The genes that mediate the co-ordinate regulation of V and Yops lie within a 17kb region often called the Ca^{2+}-dependence region, because transposon insertions result in loss of the Ca^{2+} requirement for growth and have a greatly decreased expression of V and Yops (Goguen *et al.*, 1984). The maximal expression of Yops in the absence of Ca^{2+} and weak expression of Yops in its presence suggested that at least some of these proteins might be made when *Yersinia* are intracellularly located in a similar manner to the V antigen (Pollack *et al.*, 1986). However, there is evidence that two Yops, YopE and YopH are directly injected into the host cell cytoplasm by adherent bacteria. Purified Yops were not toxic when added exogenously to cultured mammalian cells, whereas, bacteria producing these proteins were cytotoxic when attached to these cells (Salyers and Whitt, 1994). The exact mechanism by which the proteins are "injected" is unknown.

Yops are highly heterogeneous with respect to both molecular weight and iso-electric point. This heterogeneity suggests possible multiple functions contributing towards the pathogenic properties of *Y. pestis*. The functions of some Yops are known and are summarised in table 1.2.

1.3.5 Pigmentation

Iron is essential for bacterial growth and survival, and an important host defence mechanism is to make iron unavailable to invading bacteria (Bullen *et al.*, 1978). To counteract this defence mechanism, pathogenic bacteria grow and survive in the low-iron environment of the tissues by possessing specific, high affinity mechanisms for obtaining iron. Organisms can obtain iron in several ways; (i) Production of high-affinity iron-chelating agents (siderophores, normally phenolate or hydroxamate compounds) which compete directly with host iron-binding proteins e.g. transferrin and lactoferrin; (ii) removal of iron from iron-binding proteins by direct interaction with bacterial surface receptors and (iii) degradation of iron-binding proteins. Furthermore, iron may become freely available to an organism following trauma in which there is haemolysis and/or a drop in tissue oxidation-reduction potential. Some organisms that produce haemolysis *in vivo* may thus liberate haem which could then be utilised as a source of iron.

In *Y. pestis*, the iron acquisition mechanisms are unclear and seem to depend upon growth conditions. *Y. pestis* has the ability to absorb exogenous haemin or the dye, Congo Red, at 26^0C from solid medium and thereby grow as coloured or pigmented colonies. This is

Table 1.2

Known functions of some *Y. pestis* outer proteins (Yops)

Yop	Molecular Weight	Protein	Function	Cellular location
YpkA	80kDa	Serine threonine kinase	Secreted protein, thought to interfere with phagocyte signal transduction	Extracellular
YopE	26kDa		Cytotoxic when injected directly into host cells; destroys actin monofilaments and host cell cytoskeleton	Injected directly into host cell cytoplasm by adherent bacteria
YopH	45kDa	Tyrosine phosphatase	Interferes with phagocyte signal transduction and therefore with the process of phagocytosis	Injected directly into host cell cytoplasm by adherent bacteria
YopN	34kDa		Membrane-associated protein, Ca^{2+} - sensing protein in vitro; function in vivo is unclear. This protein is involved in the regulation of expression and secretion of Yops and V antigen	Extracellular
Yop M	44kDa		Inhibits platelet aggregation, anti-inflammatory	Extracellular

referred to as Pgm^+ phenotype. In contrast Pgm^- mutants form white colonies on the same media and are avirulent in mice by sub-cutaneous infection unless animals receive sufficient iron to saturate serum transferrin. These Pgm^- isolates are typified by the *Y. pestis* EV76 strain which has been found to be avirulent in other animals and is used as a live plague vaccine in humans (Pollitzer, 1954). Pgm^- mutants are fully virulent in mice via the intravenous route.

Several researchers have examined siderophore production in *Y.* pestis. Wake *et al.* (1975) found evidence of siderophore production in *Y. pestis* by plating Pgm^+ and Pgm^- strains of *Y. pestis* onto deferrated brain heart infusion (BHI) agar. An activity diffused from some of the colonies which promoted growth of neighbouring organisms, which was interpreted as evidence of release of siderophores. These findings were disputed by Perry and Brubaker (1979). They found that on iron-limited media, *Y. pestis* failed to stimulate growth of *S. typhimurium* LT2 enb-7, a mutant capable of utilising numerous exogenous siderophores (Lucky *et al.*, 1972; Pollack *et al.*, 1970). In addition to this, when growth was limited in iron-deficient liquid medium (0.1 to 0.3μM Fe^{3+}), the organisms failed to produce detectable soluble phenolate or hydroxamate compounds. Decreasing the incubation temperature and further reducing the iron concentration failed to induce production of these compounds on solid medium. The organism exhibited significant growth under all but the most stringent conditions of iron deficiency, which suggested that iron was accumulated by some alternative process. Furthermore, they found that iron-starved *Y. pestis* were unable to utilise exogenous siderophores. Added Fe^{3+} was unable to promote growth of *Y. pestis* unless the haemin precursor protoporphyrin IX was present (Perry and Brubaker, 1979). The reasons for protoporphyrin requirement were not clear but the evidence suggested that haemin was the most effective nutritional source of iron and could serve as the sole source of iron for Pgm^+ *Y. pestis.*

Sikkema and Brubaker (1989) demonstrated the presence of outer-membrane peptides, peptide F and iron-repressible peptides IrpA - IrpE, that were involved in assimilation of iron; spontaneous mutation to Pgm^- resulted in loss of peptide F and IrpB - E. Recent research has provided evidence that assimilation of iron differs according to temperature, this is in common with other *Y. pestis* virulence factors. Straley and Perry, (1995) reported that at 26^0C, iron-deprived cells over-express HMWP1, HMWP2 proteins and the receptor for iron, yersiniabactin. Yersiniabactin in *Y. pestis* is iron-regulated protein C (IrpC). However, cells can also absorb exogenous haem at 26^0C and therefore be haem-loaded before infection and

not have an immediate requirement for iron at 37^0C. The full functioning of this iron-scavenging mechanism at 26^0C has not been verified and the iron acquisition mechanisms appear to be different at 26^0C compared to 37^0C. The exact mechanisms of iron acquisition at 37^0C are unclear and may involve receptors for iron-binding proteins.

1.3.6 Pesticin

The pesticin virulence factor is encoded on a small 9.5kb Pst plasmid. Pesticin is a bacteriocin which is produced by wild-type cells of *Y. pestis* and exerts an inhibitory effect on certain strains of *E. coli*, *Y. pseudotuberculosis*, *Y. enterocolitica* and non-pestinogenic strains of *Y. pestis* (Hu and Brubaker, 1974). It is a monomeric protein with a molecular weight of about 63kDa and is located in the cytoplasm. Hall and Brubaker (1978) showed pesticin acts by converting sensitive bacteria to non-viable osmotically stable spheroplasts. Pesticin is thought to enzymatically hydrolyse mureinlipoproteins in the walls of sensitive bacteria.

Loss of pesticin typically prevents dissemination from peripheral sites of infection (Brubaker, 1970) but pesticin-specific mutations on the Pst plasmid do not reduce virulence. Pesticin may have a role in iron assimilation. Pigmented (Pgm^+) *Y. pestis* cells which have spontaneously become non-pigmented (Pgm^-) because of a large deletion are resistant to pesticin (Perry *et al.*, 1990). These Pgm^- cells, as well as a specific Pgm^+, Pst^r mutant, no longer express the outer membrane iron-repressible proteins (IrpB-E) (Sikkema and Brubaker, 1989). Since the receptors for a number of bacteriocins and bacteriophages are outer membrane proteins which serve as receptors for high-affinity iron transport systems (Neilands, 1982), the above observations make the pesticin receptor a likely candidate for regulation of iron metabolism.

1.3.7 pH6 antigen (Psa A)

A pilus adhesin found in *Y. pseudotuberculosis* and *Y. pestis* but not in *Y. enterocolitica* is Psa A, sometimes referred to as a pH6 adhesin because it is produced maximally at pH6. Psa A was first characterised by Ben-Efraim *et al.* (1961) who found that the protein was expressed in all strains of *Y. pestis*, but only at temperatures above 34^0C and at a pH below 6.7. Lindler *et al.* (1990) described two loci, *psa*A and *psa*E, necessary for the expresssion of Psa A. The structural gene was found to be *psa*A, while *psa*E was required for the maximal expression of the antigen. Insertion mutations of these genes resulted in no expression or

greatly diminished expression of the antigen and a concomitant decrease in the virulence of *Y. pestis*. Lindler and Tall (1993) concluded that Psa A was the monomer of yersinial fimbriae and that other *psa* genes were involved in expression of this fimbrial structure. Computer analysis of the primary and secondary structure of the protein predicted a β-sheet structure (Zav'yalov *et al.* 1996).

Price *et al.* (1995) tried to determine whether expression of *psa*A was controlled at the transcriptional or post-transcriptional level. He examined PsaA$^+$ and PsaA$^-$ strains of *Y. pestis* grown under PsaA-inducing (pH6, 37^{0}C) or non-inducing conditions (pH7.4, 26^{0}C) (Ben-Efraim *et al.*, 1961) by probing Northern blots for the α-^{32}P-labelled *psa*A gene. Expression of the *psa*A transcript was found only in the induced cultures, suggesting that expression of *psa*A is regulated at the level of transcription by pH, temperature and *psa*E.

Proteins extracted from cultures were analysed by Western blot with absorbed anti-Psa A serum (Lindler *et al.*, 1990) and Psa A was detected only in the psaA$^+$ strain grown under inducing conditions. Their findings implied that pH was the initial stimulus for expression of *psa*A but that increased temperature was required for full expression of the Psa A gene. The antigen is associated with the two most invasive species of *Yersinia* suggesting that it may have some role in the disease process, perhaps as an adhesin (Straley, 1993). Psa A forms virulence-associated fimbriae on the surface of *Y. pestis* at pH6.7 in host phagolysosomes or extracellularly in abscesses such as buboes, characteristic of bubonic plague (Zav'yalov *et al.*, 1996). It is also possible that it may have a role as an adhesin in *Y. pseudotuberculosis* infection as this is an enteropathogenic organism which can cross the mucosal epithelium.

Zav'yalov *et al.* (1996) produced a recombinant *E. coli* strain expressing Psa A and studied the binding of this recombinant antigen to human, rabbit, sheep and mouse IgG. Different subclasses and whole IgG were used, also binding to Fab and Fc fragments was examined. The protein was found to react specifically with human IgG; it bound to IgG1,2 and 3 but not IgG4 and only bound to Fc fragments. Therefore, PsaA is a novel bacterial Fc receptor with limited species specificity. Earlier workers proposed that the primary function of *Y. pestis* fimbriae composed of Psa A was the mediation of specific attachment to macrophages (Straley, 1993). However, Zav'yalov's findings suggest that in humans, the specific attachment of *Y. pestis* to Fc-receptors of macrophages may be mediated by the formation of pseudo-immune complexes on the surface of the bacteria between Psa A fimbriae and normal

IgG. This covering of bacterial surface structures by normal IgG could prevent bacteria from recognition by specific antibodies, therefore enabling them to evade the immune system of the host.

1.3.8 Lipopolysaccharide (LPS)

Bacterial endotoxins are components of the Gram-negative outer membrane and include LPS, an amphipathic molecule, which represents an important virulence factor in Gram-negative infections. The lipid component of the LPS molecule, Lipid A, is the primary endotoxic structure and has immunomodulatory properties. In the disease process LPS causes intravascular coagulation, leading to septic shock.

The LPS of *Y. pestis* was first isolated in 1956 by a phenol-water extraction procedure and purified by precipitation with ethanol which resulted in relatively protein-free LPS (Davies, 1956; Luderitz, 1977). Since then the structure of *Y. pestis* LPS has been further investigated and debated. Hartley *et al.* (1974) examined hydrolysates of LPS and showed that the total carbohydrate content of the LPS consisted of glucose, heptose sugars, glucosamine and 2-keto-3-deoxyoctonate (KDO). These sugars are generally found in the core region of LPS from the *Enterobacteriaceae*. When applied to a Sepharose 4B column, the LPS was eluted with the void volume, indicating that the native LPS formed large aggregates. Some researchers reported that *Y. pestis* produced smooth LPS with long polysaccharide chains and demonstrated long, thread-like structures, 8-9nm in diameter when the LPS of *Y. pestis* was examined by electron microscopy. Smooth LPS is generally associated with intestinal pathogens, such as *E. coli* and *Salmonella* and is effective in protecting the bacterial surface from complement, as complement is activated by the lipid A of LPS which is embedded in the outer membrane of the bacterial cell. Some *E.coli* strains have both smooth LPS and a capsule and this may act together to shield the bacteria from the immune system (Michalek *et al.*, 1982), in *Y. pestis* the LPS and F1 antigen may have a similar role. However, other researchers examining the LPS of *Y. pestis* found that it possessed core components but lacked extended O-group structures and would therefore be classed as rough LPS (Porat *et al.*, 1992). More recent research confirmed this and also found that *Y. pestis* did not express long-chain LPS, this was in contrast to *Y. pseudotuberculosis* LPS (Chart *et al.*, 1995). The latter migrated as discrete bands, producing a "ladder pattern" on SDS-PAGE gels and it was possible to differentiate the two by SDS-PAGE and silver staining.

Y. pestis is an invasive organism and generally invasive pathogens have rough LPS, whereas,

enteric bacteria possess smooth LPS which may protect the organism from the harsh environment in the gut.

1.3.9 Murine toxin

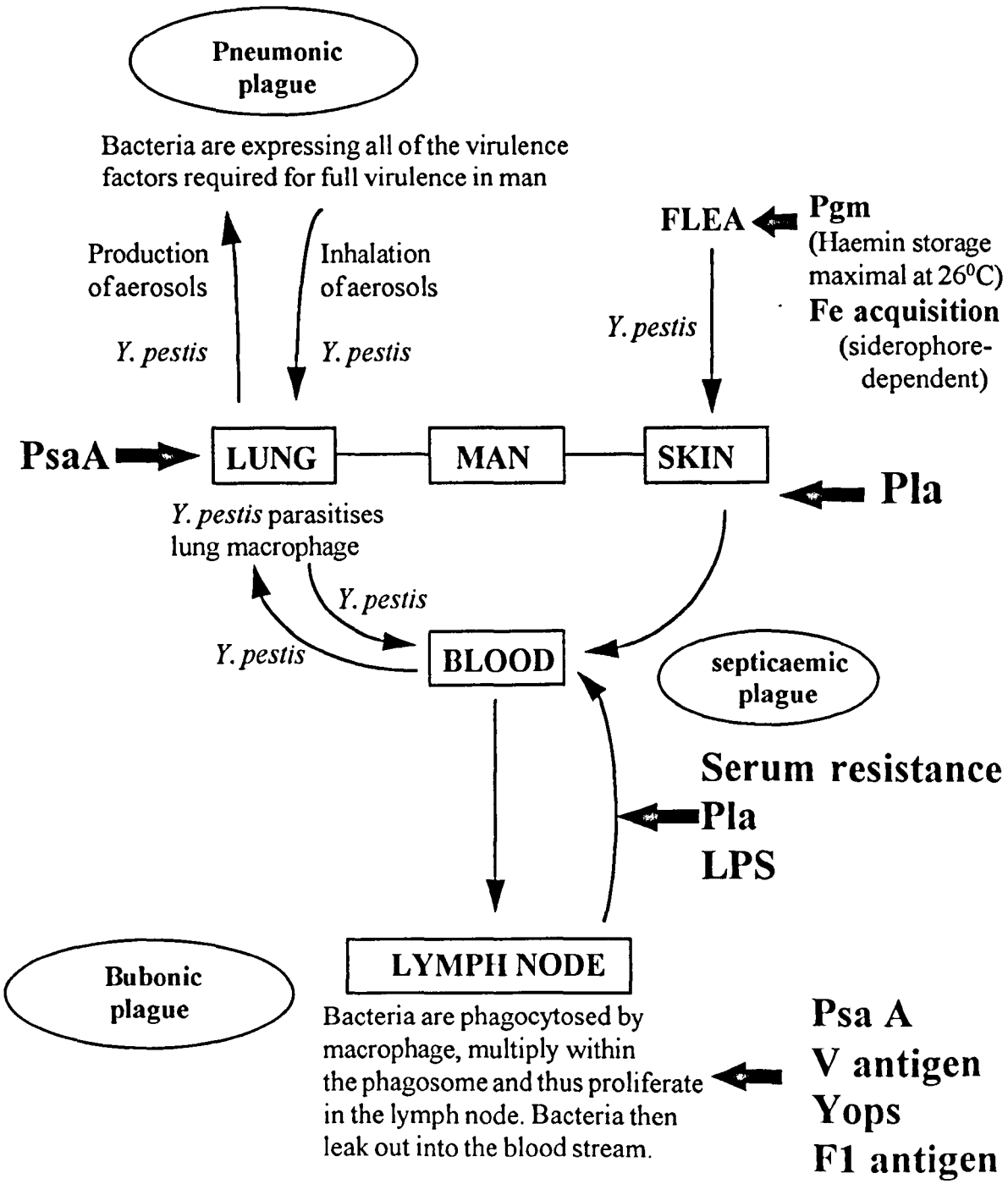
Murine toxin, a polymer of 12kDa protein sub-units, is also encoded on the 110-kb plasmid but less is known about its structure and mode of action. It is lethal for mice and rats but essentially non-toxic in other hosts. The significance of murine toxin in promoting disease is not well understood but it probably accounts for the more rapid death of mice infected with *Y.pestis* than when infected with the enteropathogenic *Yersinia* (Protsenko *et al.*, 1991).

1.3.10 Components required for full virulence of *Yersinia*

Some of the virulence factors described above are shared with *Y. pseudotuberculosis* and *Y. enterocolitica* which poses the question, why is *Y. pestis* so much more invasive than the other pathogenic *Yersinia*? *Y. pestis* has additional plasmids, one of which (Fra1) encodes the antiphagocytic capsular F1 protein, described earlier. Two other *Y. pestis*-specific virulence factors that may explain its greater invasiveness are haemin storage and plasminogen activator. In addition to being able to use haemin as an iron source, *Y. pestis* actively stores haemin and accumulates concentrations which are high enough to change the colour of colonies on agar plates containing haemin. Haemin storage occurs maximally at 26^0C, so that *Y. pestis* injected by flea-bite would be haemin loaded. The stored haemin may act as an iron reserve, but it is also possible that a haemin coat could give the bacteria some initial protection from the host's defences by making the bacteria appear like a host component. Another unique protein of *Y. pestis* is Pla which clearly has an important role in survival and dissemination of the bacteria in the body.

The virulence factors described are an example of the principle that the most successful pathogens have evolved virulence strategies that are successful in preventing the host clearance of bacteria but do not kill the host (balanced pathogenicity). It can be argued that a pathogen such as *Y. pestis,* with a high mortality rate, produces virulence factors that do not kill the host immediately but aid the spread of bacteria throughout the body to contribute to the spread of the disease and eventually kill the host. This would account for *Y. pestis* having occasional devastating effects on human populations. Whereas other pathogenic *Yersinia*, such as *Y. enterocolitica*, do not kill their hosts and thus maintain themselves at a constant level in the environment. The disease process in *Y.pestis* infection is summarised in fig. 1.3 and shows where the different virulence factors are considered to play a major role.

Fig. 1.3 Schematic summary of *Y. pestis* infection and the role of virulence factors



Virulence factors: Vantigen; Pla, plasminogen activator; serum resistance; iron acquisition; haemin storage; LPS; **Adhesion**: Psa A, pH6 antigen; **Antiphagocytic proteins**: Yops, yersinia outer proteins (secreted proteins); F1 antigen.

Certain of the virulence determinants described above have been found to be major antigens of *Y. pestis*. For high virulence, strains must be capable of producing not only F1 antigen but at least V and/or Pgm antigens in addition to F1. These antigens would seem to be the most important antigens determining virulence and certainly F1 and V antigen in native or recombinant form are capable of eliciting high titre antisera in mice and rabbits.

1.4 Regulation of Virulence

The behaviour of microbial pathogens is determined by genetic structure and by environment. When producing infection, the pathogens grow in the changing environment of the host tissues, and they produce the full complement of virulence determinants that are required to accomplish successive steps of infection; survival on and penetration of mucosal surfaces, multiplication in the tissues, interference with host defences and damaging the host. Therefore, study of the genetic control and expression of these antigens may provide much useful information about bacterial invasiveness.

Many *Y. pestis* virulence genes are regulated by temperature. For example, F1 antigen, is produced on incubation at 37^0C but not at 26^0C, and expression of some genes such as *psa* is affected by a combination of temperature and pH. However, little is known about the regulation of chromosomal genes. Most information about the regulation of virulence genes comes from studies of regulation of *yop* genes on the virulence plasmids. These genes are regulated not only by temperature but also by calcium concentration. Yops are expressed maximally at 37^0C in the absence of calcium. Calcium in millimolar concentrations (i.e. similar to those found in body fluids) considerably down-regulates transcription of the Yops genes at 37^0C, but expression is still greater than at lower temperatures. When bacteria are shifted from 25^0C to 37^0C in calcium-free medium (optimal conditions for Yops expression), growth of bacteria decreases considerably. During this period of growth restriction, Yops and V antigen (LcrV) are produced. Addition of calcium to the medium allows growth to resume at a normal rate. Therefore, growth at 37^0C is calcium dependent.

However, there is no proof that this phenomenon occurs *in vivo* and there is the possibility that calcium-dependent growth restriction is an *in vitro* artefact. The regulation of virulence genes in *Y. pestis in vivo* begins with initial colonisation, where the tissues surrounding a flea bite form an iron chelated environment for an invading bacterium. The transition in

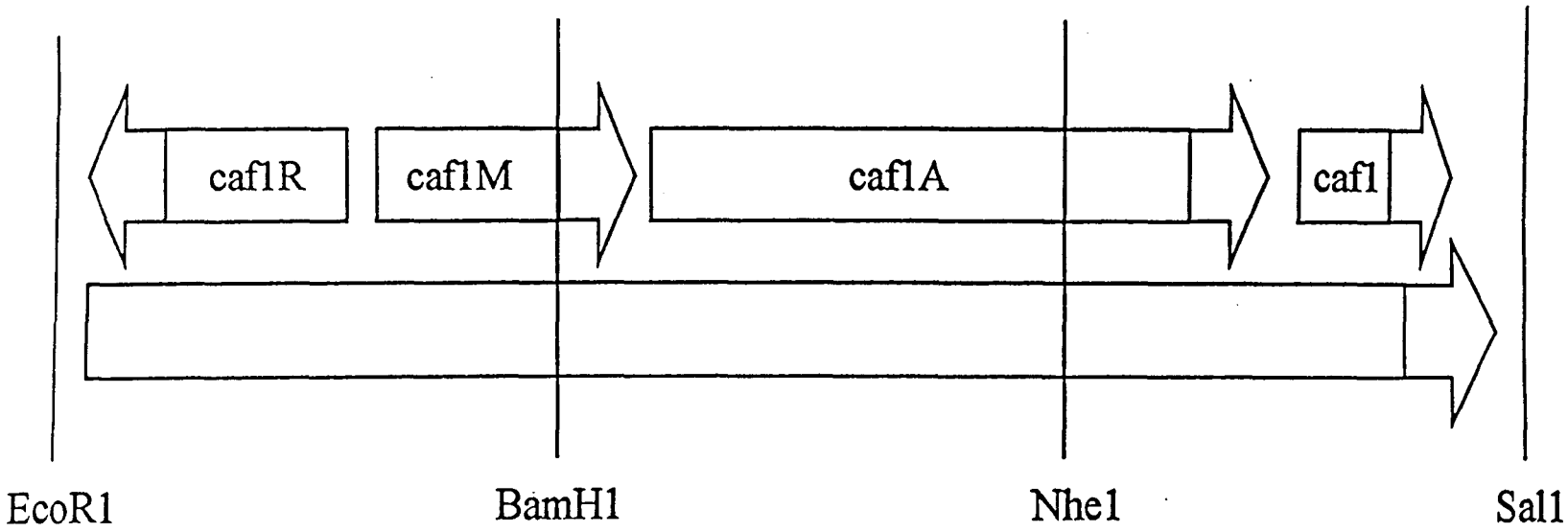
temperature on entering the mammalian host should turn off the 26^0C iron transport system and the second iron-transport system that is active at 37^0C would be induced to levels higher than those in the flea (Straley and Perry, 1995). After initial colonisation but early in infection, macrophages would not be fully activated and might support intracellular growth of bacteria. In the acidic phagosomal environment, *Y. pestis* might express the chromosomally encoded Psa A which could promote subsequent survival in the presence of PMNs attracted to the focus of infection. In enteropathogenic yersinia production of Yad A aids survival in PMNs; however, *Y. pestis* does not express Yad A because of a frame-shift mutation and is protected against phagocytosis by its capsular protein F1, which is regulated by increase in temperature from 26^0 to 37^0C.

Subsequently, growth of *Y. pestis* in peripheral tissues is primarily extracellular. Proteins expressed by the Lcr plasmid, especially V antigen, are important in inhibiting phagocytosis and also temper the inflammatory response and delay the development of an effective cell-mediated immune response (Straley and Perry, 1995). At 37^0C, millimolar concentrations of Ca^{2+} prevent full transcriptional upregulation at high temperatures and block the secretion of V antigen and Yops. This negative control may be overridden by *Y. pestis* becoming attached to a macrophage by an adhesin, possibly the Psa A adhesin, but this process is not well understood. Therefore wastage is prevented by blocking secretion and inhibiting full transcriptional upregulation until the proteins are needed. Dissemination to deeper tissues might be provided by the expression of proteins such as F1 and Pla.

1.4.1 Regulation of expression of F1 antigen

Most strains of *Y. pestis* possess the ca.110kb virulence plasmid pFra which mediates the expression of the F1 antigen as capsular material at 37^0C. The genes encoding the F1 structural protein are located in the *f1* operon which contains four loci, *caf*1R, *caf*1M, *caf*1A and *caf*1 (Figure 1.4) that have been assigned putative functions. The *caf*1 gene which encodes the F1 subunit, has been cloned and sequenced by Galyov *et al.* (1990). The *caf*1R gene encodes a regulatory protein (Karlyshev *et al.*, 1992) which seems to induce *caf*1M gene transcription leading to the production of the Caf1M and Caf1A proteins involved in capsule formation. The Caf1R protein may be involved in transduction of signals which might be temperature and/or other environmental factors, such as Ca^{2+} and Mg^{2+} ions concentration. The *caf*1A genes encode an assembly protein Caf1A (Karlyshev *et al.*, 1991) which was found to have a molecular weight of 93.2kDa. From the DNA sequence and deduced amino acid sequence, Caf1A has been found to have regions of homology with protein FaeD, PapC

Fig. 1.4 The Genetic Structure of the F1 Operon



The F1 operon and restriction sites

and FimD involved in the assembly of *E. coli* fimbriae. Deletion of the *caf*1A gene results in bacteria incapable of agglutinating with anti-plague anti-serum, implying that F1 has not been produced and that the Caf1A protein may be involved in capsule assembly. The Caf1A protein is located in the outer membrane and binds F1 antigen sub-units during the extracellular secretion process and is therefore important for anchoring the capsular protein to the cell surface.

The *caf*1M gene was sequenced by Galyov *et al.* (1992) who showed that the effective synthesis of the capsule layer of *Y. pestis* is mediated by the expression of the *caf*1M gene. Caf1M protein and the PapD protein of *E. coli* have homology in their primary structures and conserved amino-acid sequences. PapD acts as a chaperone protein in Pap pilus biogenesis, acting as a periplasmic transport protein, implying that Caf1M acts as a chaperone protein in capsule layer biogenesis (Zav'ylov *et al.*, 1995). More recent research has suggested that caf1M is important for the correct folding of F1, for aggregation of the antigen in the periplasm and transport of the F1 protein to the cell surface (Zav'ylov *et al.*, 1995). Examination of the structure of caf1M has defined two immunoglobulin-like domains, which is a characteristic feature of chaperone proteins.

1.4.2 Regulation of expression of V antigen

The low calcium response which leads to the production of the V antigen is mediated by a 75kb Lcr plasmid. This plasmid contains "Ca^{2+} dependence" genes that are thought to be responsible for detection of temperature and Ca^{2+} important in the regulation of expression of both V antigen and Yops, maximal expression of both V antigen and Yops occurs at 37^0C in the absence of Ca^{2+}. Perry *et al.* (1986), using insertion mutagenesis, identified the *lcr*GVH operon encoding the V antigen and is regulated at the transcriptional level by temperature and Ca^{2+}. The genes encoding the Yops proteins are arranged in multiple operons dispersed over the Lcr plasmid. The arrangement of V and Yops genes on the Lcr plasmid raises the possibility of co-ordinate regulation which was suggested by comparison of V antigen activity with expression of beta-galactosidase from the operon fusion phage Mu dI (Ap lac) inserted in Yop operons (Straley and Bowmer, 1986). The maximal expression of V at 37^0C in the absence of Ca^{2+} suggests that during infection V is synthesised when *Yersiniae* are resident within a macrophage which would provide such an environment (Pollack *et al.*, 1986; Straley and Brubaker, 1981). Yops are also expressed under these conditions, some Yops are expressed within the phagosome and others by adherent cells (section 1.3.4). Therefore, the low Ca^{2+} response (Lcr) of *Yersinia* includes a regulatory cascade and a set of

virulence-related proteins. The regulatory genes modulate both bacterial growth and expression of the virulence-related proteins in response to temperature and the presence of Ca^{2+}.

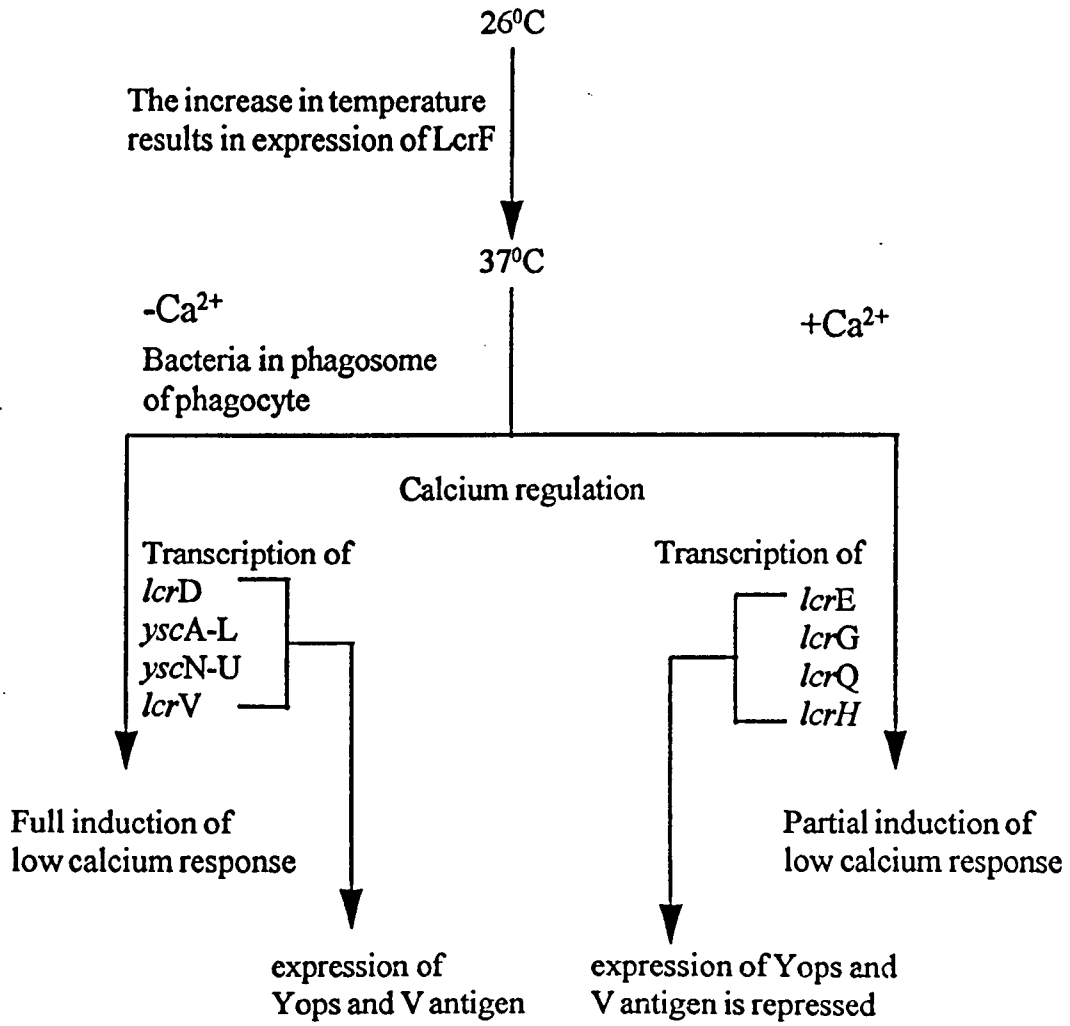
As a consequence of the Lcr, *Y. pestis*, when shifted from 26 to 37^{0}C, growth ceased within two generations, indicating growth restriction (Zahorchak and Brubaker, 1982). Reinitiation of growth occurred when the cultures were returned to 26^{0}C; at 37^{0}C, growth restriction was prevented if Ca^{2+} was present in the growth medium. Certain nucleotides, such as ATP, were also found to have a similar effect. However, the effects of Ca^{2+} and ATP on growth were not identical, as ATP, unlike Ca^{2+}, never fully restored maximal growth at 37^{0}C. Barve and Straley (1990) found that Ca^{2+}-independent mutants were unable to induce strong expression of V antigen, therefore, Ca^{2+} depletion was the primary factor in V antigen expression. The genes involved in the regulation by Ca^{2+} are found on a region of the Lcr plasmid, called the Ca^{2+}- dependence region. In *Y. pestis* this region contains at least five *lcr* genes that are thermally induced to different extents and function in the regulation of Yop and Vantigen expression. Fig. 1.5 shows the regulation of the Lcr and where the genetic loci are believed to participate. However, there is no proof that this phenomenon actually occurs *in vivo*, calcium-dependent growth restriction may be an *in vitro* artifact. It is possible that the true signal recognised by the *lcr* genes is not calcium but some other signal that is mimicked by calcium. Another possibility is that interaction with phagocytic cells is the trigger for V antigen and Yops expression.

1.5 Plague Vaccines

1.5.1 The history of plague vaccination

Research into producing an effective plague vaccine has a long history. In 1897, Haffkine developed a killed plague vaccine, which was very crude and caused severe side-effects. During World War II, servicemen were certain to be deployed to areas in which they would be exposed to plague. At this time, before the use of antibiotics, an effective plague vaccine was urgently required. The problem was approached by studying the metabolism and physiology of the bacillus to ascertain the best method for obtaining an adequate yield of micro-organisms for a vaccine. The main approach was to disrupt the plague bacillus and investigate the component antigens. Animal models were designed for reliable potency testing. Serological tests were developed that would enable evaluation of the antibody response to vaccination with various preparations and promising vaccines were studied in

Fig. 1.5 Regulation of the low-calcium response in *Y. pestis*



volunteers. In the meantime killed vaccines were prepared for immediate requirements. The approach remains much the same to date.

Live attenuated and killed vaccines were studied with equal intensity and during the 1940's and 50's more was probably discovered about plague and the plague bacillus than had been known previously. Most important, perhaps from the vaccination standpoint, was the isolation and characterisation of a crystalline protein antigen from the capsular material of the plague bacillus.

This antigen, the fraction 1 or F1 antigen, was shown to be the specific major antigen of *Y. pestis*. The F1 antigen could be used in several serological tests that were invaluable in evaluation of antibody responses to vaccination, for diagnosis of plague and for use in serum surveys to detect plague in wild rodent populations. This still remains the case today and Meyer's work led to the development of the plague vaccine in use today (Meyer *et al.*, 1974a).

1.5.2 Types of Plague Vaccine

1.5.2.1 Killed plague vaccines

Killed vaccines comprising inactivated micro-organisms have certain advantages in that they can be inexpensive to manufacture and require only cultivation and recovery of the infectious agent, followed by simple chemical or thermal inactivation. However, in the case of plague vaccines procedures would have to be carried out in containment, increasing the production costs. Because they are made from whole bacteria or viruses, killed vaccines are composed of more than one immunogen. However, adverse side-effects may be more common with this type of vaccine. Furthermore, inactivated micro-organisms may not induce cell-mediated responses and will induce mucosal responses only when delivered in sufficient quantity through multiple doses (Clemens *et al.*, 1990).

Haffkine (1898) initially established the dosage of his killed plague vaccine which needed to be administered in a single inoculation as "that amount of a particular brew necessary to produce a temperature reaction up to 102^0F in the majority of those inoculated". The Haffkine vaccine was injected sub-cutaneously and administration of this early vaccine was associated with localised pain, swelling and erythema, with peak intensity at 24-48hr; regional lymphadenopathy was also frequent. Systemic responses were also recorded manifesting as generalised weakness, headache and anorexia. These severe side-effects led to

the value of the Haffkine vaccine being seriously questioned.

During the decade preceding World War II, research on killed plague vaccines was limited until the work by K.F. Meyer. During 1941 the Subcommittee on Tropical Diseases National Research Council Committee on Medical Research passed the following resolution :- " Even though the available knowledge does not seem to afford definite evidence of the benefits from the use of plague vaccine, it is considered advisable to vaccinate with plague bacillus of an approved strain, all military or naval personnel under serious threat of exposure to bubonic plague". On the basis of this recommendation, vaccination with killed plague vaccine was initiated in the forces. This formalin-killed *Y. pestis* plague vaccine and the way in which it is inoculated produces less frequent systemic and milder local reactions than the earlier killed vaccines.

There is little evidence in humans that vaccination with Plague Vaccine USP elicits an antibody response which is similar to that observed in animals proven by challenge to be immune. However, no military personnel in the armed forces vaccinated with killed vaccine contracted plague during World War II, although many personnel were located in areas where plague was occurring (Meyer *et al.*, 1974). Similar observations were made in Vietnam where the potential for exposure to plague was great (Butler *et al.* 1974; Marshall, 1967).

1.5.2.2 Live attenuated vaccines

Live attenuated vaccines are composed of living viruses or bacteria that are avirulent either because of attenuating mutations or because of replication restrictions associated with their range of hosts. In theory, a single inoculation with a small dose of a live vaccine can expand by replication to a larger (and therefore more immunogenic) dose of vaccine within the recipient. During the course of this replication, the live vaccine must express all or most of the important target immunogens that ordinarily lead to protection after the natural infection. Live vaccination in this way more closely mimics natural infection. The infection with live vaccine can induce the production of cytokines which in turn can recruit elements of the immune system, such as macrophage and other antigen-presenting cells, that may not respond to inactivated or sub-unit vaccines. When the live vaccine is a virus or intracellular micro-organism, it is capable of inducing both humoral and cell-mediated responses e.g. cytotoxic T lymphocytes. Because the live vaccine replicates in a manner analogous to the target agent, it promotes the processing and presentation of antigens in a way that is most

similar to the natural infection. For a live vaccine to induce a mucosal response, it must be administered to a mucosal surface and adhere to antigen sampling cells, such as M cells in the gut (Owen, 1977). The fact that some viruses and bacteria naturally target the lymphoid tissue of the mucosal epithelium suggests that attenuated mutants of these agents might make excellent mucosal vaccine carriers.

Live attenuated vaccines for plague were first developed and introduced by Girard and Robic in 1933 and by Otten in 1934. Both the E.V. (Girard) and the Tjiwidej (Otten) vaccines were considered to have been highly effective in producing immunity to plague. The unpleasant side-effects of live plague vaccines were first reported by Rotman in 1945 as severe systemic and local reactions. In the USSR in the 1940's adverse reactions were also reported and the Russians tried many different routes of administration in attempt to overcome these adverse side-effects. By the 1970's these problems had still to be overcome and Meyer confirmed that mass vaccinations with the E.V. strain 76 was inadvisable because of the adverse side-effects (Reisman, 1970).

Other factors influence the efficacy of attenuated plague vaccines. Great difficulties have been encountered in maintaining seed material for these vaccines. When several strains of E.V. used for vaccine production in France, Vietnam and the USSR were studied by the criteria of Burrows (1963) and Surgalla (1960) to ascertain virulence determinants, great variation was observed. The E.V. strains varied as to presence or absence of fibrinolytic activity, pigment and dependence on an exogenous source of uracil. In terms of vaccine potency, the E.V. strains vary from poor to fair in mice and from fair to good in guinea pigs. It was also noted that the "residual" virulence of the standard Girard E.V. 76 isolate could be enhanced by passage through rats and guinea pigs, making it a potentially dangerous vaccine candidate.

Therefore, mass inoculations against plague present serious dangers in terms of adverse side-effects and in the case of attenuated vaccines, a potential return to full virulence. Measures to eliminate these complications are therefore required, but attempts to avoid them have so far proved unsuccessful. Although mass immunisations with plague vaccines are often required to prevent epidemics, little information has been assembled on which to base an evaluation of the relative merits of the plague vaccines in current use. Although both live attenuated and killed vaccines have been developed, these carry with them the dangers of adverse side-effects. The live EV76 vaccine induces good protection against *Y. pestis* infection but the

vaccine still has the inherent problem of adverse side-effects. EV76 grown at 37^{0}C to induce expression of the F1 antigen appears to induce a greater protective response in mice than when grown at 28^{0}C, in that time to death is greatly extended (Russell *et al.*, 1995), presumably because there is an enhanced anti-F1 reaction induced as part of the primary response. A comparison of the immune responses induced by these vaccines, and the effect of the presence of F1 antigen in the administered dose, could indicate how a subunit vaccine could be developed.

Oyston *et al.*, (1995) produced a live attenuated recombinant AroA mutation of *Salmonella thypimurium* expressing F1 antigen and found that it gave protection against challenge with virulent plague. However, there were problems in that the organisms were not cleared efficiently from the tissues of the mouse model.

1.5.2.3 Subunit vaccines

Subunit vaccines are composed of purified proteins, carbohydrates or peptides and are generally considered to be the safest form of vaccine because of their well-defined biochemical makeup. The ability to manipulate these vaccines chemically also provides a means of altering the immune response in specific ways.

It was generally considered in areas where the risk of contracting plague was high, that the benefits of the whole cell vaccine outweighed the risks. However, there are a number of reasons for the development of an acellular vaccine; (i) to reduce the adverse side-effects of the killed whole cell and live attenuated vaccine, (ii) to improve and prolong the degree of protection against plague, especially compared to whole cell vaccines, and (iii) to improve vaccine safety, as there is a risk of a return to full virulence of live attenuated vaccines.

Ideally, an acellular vaccine should aim to inhibit more than one stage of the disease process. Candidate antigens for inclusion in an acellular vaccine must meet with certain criteria. They must elicit a protective immune response in the host, have a well-defined, demonstrable role in the infection process and be non-toxic. Other characteristics to consider are the properties that make a vaccine practical to deliver, particularly in developing countries where use of a plague vaccine might be more important. There is no effective plague subunit vaccine to date. The first attempt at producing a sub-unit vaccine was by Meyer in 1974, who immunised mice with the F1 antigen of *Y. pestis* (Meyer *et al.*, 1974c).

More recent research into an acellular vaccine has demonstrated that recombinant F1 antigen (Simpson *et al.*, 1990) and and a recombinant V antigen (Leary *et al.*, 1995) are protective in mice. In addition to this, Williamson *et al.* (1995) used a combination of native F1 and recombinant V antigens as an acellular vaccine in mice. F1 or V antigen alone gave protection against challenge with virulent plague; however, this protection was increased considerably when the antigens were administered as a combination vaccine. Why V should be such a protective antigen is unclear as it is expressed as a cytoplasmic protein, although it is secreted after the bacteria have been phagocytosed.

Other antigens that might elicit a protective immune response are outer membrane proteins, such as plasminogen activator (Pla), Yops and the surface protein, pH6 antigen. Native Pla would present problems, as it is a fibrinolytic enzyme and might damage the host, therefore outweighing any advantage it might have as a protective antigen. The pH6 antigen, on the other hand, might be an effective component in an acellular vaccine. It is a protein expressed on the surface of the bacteria and is thought to have a role in adhesion. Antibodies against this antigen might prove effective in protecting against pneumonic plague. To assess the use of Yops in a sub-unit vaccine, the protective efficacy of the individual Yops would need to be studied. Studies have shown that YopE and H might be protected from neutralisation by antibody and therefore it is reasonable to predict that these Yops would not be protective antigens. In contrast, Yops such as YopM and N, have extracellularly exposed domains and so might be suitable components of a subunit vaccine (Straley *et al.*, 1993). Therefore, a combination of purified protective antigens either in the native or recombinant form, might prove to be an effective acellular vaccine protecting against bubonic and pneumonic plague.

1.5.3 Vaccine development

An important development in plague vaccination would be to improve the existing vaccines with respect to adverse side-effects and batch-to-batch variation. A major development would also be to produce a vaccine which was effective after only one dose against both systemic and aerosol infection, and immunised preferably via a non-invasive route, for example, oral or intranasal.

There are a number of new approaches to vaccine development:

a) The isolation of relevant protective antigens.

b) The use of anti-idiotype antibody preparations to mimic B cell epitopes.

c) The synthesis of oligo- or polypeptides which may reflect naturally occurring amino-acid

sequences in proteins of the pathogen.

d) The use of recombinant DNA technology to obtain DNA coding for antigens of pathogens or other factors, such as cytokines, which could be used: i) To transfect cells *in vitro* so that the inserted DNA is translocated and expressed to produce antigen. (ii) To directly inject into the cells *in vivo*, where it is translated and expressed and an immune response initiated. (Ada 1996). (iii) The use of live vaccine vectors to deliver protective immunogens, or the DNA encoding these proteins, to host target tissues (Schodel, 1994).

However, of the above approaches, sub-unit peptide or protein vaccines are likely to be important in the development of a new generation of plague vaccines. Developments may include vaccine formulations, such as combination vaccines where more than one vaccine is administered at the same time. This may not be such an important development for plague vaccination as its use will probably be limited to people travelling or living in areas where plague is endemic. Another possibility is the use of mixed vaccine formulations, where different methods of presenting the antigen are used at the same time. This may favour priming or boosting an immune response if the antigen is presented in a different way at each immunisation and more than one antigen could be given. Another important advancement in plague vaccination would be the development of effective vaccine delivery systems that could be tailored to meet the requirements of a particular vaccine. For example, a combination or mixed vaccine where the antigen is delivered and presented in such a way as to optimise the immune response, provide longer-lived protection and ideally be given as a single dose vaccination (Ellis and Douglas, 1994). The earlier work on plague vaccination is still valid today and to build upon this the use of combination vaccines and novel adjuvants may be important in the future development of plague vaccination.

1.6 Immunity to Plague Vaccination

In the development of any vaccine, it is important to use relevant animal models as well as *in vitro* models for the evaluation of pathogenicity and protection. Each model should be carefully characterised and used to explore only the aspects of infection for which it has been proven valid. Historically, numerous animal models have been used to study plague infection and its prevention (Burrows, 1963).

1.6.1 Immunity in mice

Chen and Meyer (1955) studied the immunogenicity in mice of avirulent variants selected

from virulent strains of *Y. pestis* and were able to demonstrate that, in general, strains producing large amounts of F1 conferred immunity in smaller doses than those producing lesser amounts of this antigen. This was confirmed by Burrows and Bacon (1956) who compared the vaccine potential of various live mutant strains of plague derived by single-step mutations from a fully virulent strain with killed whole cell vaccines. In all cases $F1^+$ vaccines immunised more effectively than the $F1^-$ strains in mice. However, they also found that $F1^- / VW^+$ vaccines gave effective immunity, that VW^+ strains were more protective than VW^- and in mice the most protective vaccine formulations contained both F1 and VW.

Infection of a murine model with plague has shown that the LD_{50} dose of the bacterium was lowest when the bacteria were delivered by the subcutaneous route (s.c.), which is not suprising since this route most accurately mimics the natural route of infection from a flea bite. However, the time to death was significantly greater for bacteria delivered by this s.c. route rather than by the i.p. or i.n. routes, possibly because the bacteria require an initial phase of multiplication in the dermal tissues before dissemination throughout the body when administered s.c. Immunised animals showed side-effects of varying severity and the killed vaccine was less effective in terms of dose-protection than the live attenuated vaccine. The murine model may not accurately reflect the disease process in man since a murine-specific toxin is produced by *Y. pestis*. However, plague vaccines have been successfully evaluated in the mouse model (Burrows, 1963).

Some of these early studies with mice (Burrows, 1963), found in experimental plague infections that the immunity developed depended on the level of F1 antibody in immunised animals; animals having high immunity to challenge infection tended to have high levels of F1 antibody. Therefore, F1 is important but may not be the only antigen to produce immunity to plague in mice. A recombinant F1 (Simpson *et al*, 1990), native F1 and recombinant V antigen (Leary *et al.*, 1995; Williamson *et al.*, 1995) have been found to be protective in mice and more recently, research has been done to improve the murine model (Russell *et al*, 1996). A recent study (Russell *et al.*, 1995) has compared protection given by the formaldehyde-killed vaccine (Plague vaccine USP) with that given by the live attenuated vaccine strain of *Y. pestis* (EV76). The infectious dose of a virulent strain of *Y. pestis*, delivered to mice by several routes mimicking the natural routes of infection, was determined for the challenge of immunised mice.

1.6.2 Immunity in guinea pigs

Historically, immunisation of guinea pigs using killed cells or proteins derived from them, has proved more difficult than the immunisation of mice using similar materials. In particular, purified F1, effective in mice, proved ineffective in guinea pigs even in large quantities. However, live vaccines did confer immunity (Spivack *et al.*, 1958). Various researchers tried administering vaccine formulations in different adjuvants and found that these formulations increased immunity and protection in guinea pigs. Tests on the efficacy of plague vaccines administered in combination with vaccines against other diseases have been conducted with guinea pigs (Korobkova *et al.*, 1958). Dried live plague vaccine was given combined with killed cholera vaccine and found to protect better than plague vaccine alone, which may have been due to the adjuvant effect of cholera toxin. These findings could still be of use in today's plague vaccine research; an acellular vaccine might be more effective when administered with material of known adjuvant effect and comparison in mice and guinea pigs would provide useful information as to the efficacy of the vaccine.

1.6.3 Immunity in non-human primates

Most of the data for immunity to experimental plague has been accumulated mainly from studies with mice and guinea pigs and most of the research with extensive use of animal models took place between the 1940s and 1970s, particularly the 1950s. However, it is questionable as to whether this data would be a true assessment of what would happen in man. Most of the research on experimental plague in monkeys again took place during the 1950s. Ehrenkranz and Meyer (1955) compared three types of vaccine; live avirulent EV76, formalin-killed virulent organisms and purified, crystalline F1 antigen. They tested the ability of these vaccines to protect against intratracheal challenge in monkeys and doses recommended for man were used. Multiple single doses were given at intervals over 6-12 weeks and full protection was afforded; the proper spacing of vaccine doses was as important as the total dose given. Resistance to subsequent challenge was associated with detectable F1 antibody; however, live EV76 vaccine was also effective in monkeys, once again suggesting that F1 may not be the only protective antigen in animal models.

The challenge route was different to that used in mice and guinea pigs, therefore comparison of results may be misleading. The intratracheal challenge route may be a better model for pneumonic plague than the bubonic disease.

Sub-cutaneous challenge in monkeys was investigated by Meyer (1961) and this work

suggested that vaccine formulations effective against bubonic plague may not be protective against the pneumonic disease. Burrows (1963) compared intratracheal, sub-cutaneous and intra-muscular immunisation of plague vaccines in monkeys. He concluded that immunisation via the respiratory route may provide the best means of protecting against the pneumonic form of the disease. Therefore, it might be possible to develop a plague vaccine that can be given by inhalation and by using recently developed adjuvants and novel delivery systems it might be possible to protect against bubonic and pneumonic plague.

1.6.4. Immunity in man

In past epidemics, vaccination with living, avirulent plague vaccines produced reduction in infection rate and in the mortality of vaccinated subjects (Burrows, 1963). Immunisations with the Tjiwidej (VW^-) strain did not decrease the incidence of pneumonic plague, whereas no cases of pneumonic plague occurred in people immunised with live EV vaccine (VW^+) in contrast to non-vaccinated cases. However, in the absence of recent comparisons of vaccination against epidemic plague, it is difficult to determine whether advances have been made and the strategy of plague vaccination has remained largely unchanged.

Circulating antibodies can be assessed in animal models but these may or may not be protective in man. In addition, humoral antibodies may not be of sole importance in immunity to plague, which is transmitted by the sub-cutaneous and respiratory routes.

Meyer *et al.* (1974b; 1974c) compared the USA Army killed whole-cell, live avirulent A1122 and Tjiwidej vaccines and F1 antigen. The serological response was assessed and it was found that the concentration of serum antibodies resulting from vaccination declined within a few weeks, but was rapidly restored or exceeded by revaccination. Some individuals, however, failed to produce detectable antibody, even after repeated inoculations. Meyer concluded that even in individuals that responded well to vaccination, booster doses at intervals of three to six months were essential to protect against plague infection. This, essentially, remains the case today and clearly there is a need for the development of an improved plague vaccine.

1.7 Properties and Function of the Different Components of the Immune System

The immune system is, by nature, very complex in order to be able to deal with many

different types of infection. Many organisms gain access across the epithelia of the gastrointestinal or urogenital tracts. In the case of *Y. pestis*, it is injected directly into the bloodstream via an insect bite, although in the pneumonic form gains access via the nasopharynx and lung. The site of infection and type of pathogen largely determines which immune responses will be effective. The success of a vaccine depends upon its safety and efficacy and on generating immunological memory.

1.7.1 Adaptive and Innate Immunity

The innate response in host tissues is mediated by activation of neutrophils and macrophages, they recognise the many common constituents of micro-organisms, internalise the pathogens and destroy them. Since these cells use non-specific recognition systems which allow them to bind to a variety of antigens and do not rely on the antigen-specific receptors of either B cells or T cells, they result in innate immune responses. Innate immunity, therefore, acts as a first line of defense against infection.

The adaptive immune response is highly specific for a particular pathogen. Moreover, the response improves with each successive encounter with the same pathogen and results in immunity from infection. The key factors of the adaptive immune response are specificity and memory.

1.7.2 Humoral and cell-mediated immunity

Immunity to infectious diseases involves a complex interplay between humoral and cell-mediated immune responses against antigens on the foreign micro-organism. Humoral immunity, either in the form of local IgA antibodies at the site of infection or neutralising antibodies in the serum, provides the first line of defense against invading micro-organisms. Cellular immunity, mediated by T-cells, also plays a major role in protection against foreign pathogens. CD8+ CTLs kill cells infected with viruses or bacteria. CD4+ T helper (Th) cells provide "help" for B-cells in antibody production and secrete a range of cytokines that are involved in a variety of immunoregulatory functions or have a direct effect on invading pathogens.

Th1 cells secrete interleukin 2 (IL-2), γ-interferon (IFN-γ) and tumour necrosis factor-β (TNF-β) and are involved in delayed hypersensitivity and inflammatory responses. This population of cells stimulates B-cells to produce IgG subclass immunoglobulin which

opsonise invading bacteria, making them more susceptible to killing by activated macrophages. Th2 cells secrete interleukins IL-4, IL-5, IL-6 and IL-10, which play a crucial role in immunoglobulin (Ig) class switching and B-cell differentiation, in particular for IgE, IgA and IgG antibody production.

Therefore, Th1 cells and CD8+ CTLs mediate cellular immunity against intracellular pathogens, whereas Th2 cells stimulate humoral immunity against extracellular pathogens. The repertoire of T- and B-lymphocytes in the immune system is vast with an almost infinite capacity to respond to foreign antigens. In a naive individual, the frequency of lymphocytes specific for an individual epitope on a foreign antigen is very low. However, following exposure to a micro-organism or antigen expressing that epitope, clonal expansion of precursor lymphocytes occurs and memory T- and B- cells are generated. Therefore, primary infection with a micro-organism, or vaccination by exposure to an attenuated or killed virus or bacterium, or to a purified native or recombinant antigen, results in the stimulation of a small, but specific population of lymphocytes *in vivo*. In a subsequent encounter with the antigen, as through infection with the pathogen, the memory B- and T-cells proliferate rapidly and allow the immune system to deal effectively with the invading micro-organism. The mechanisms of B- and T-cell responses are described in sections 1.7.2.1 and 1.7.2.2. For vaccine design it is important to induce distinct T-cell responses required for effective immunity to different pathogens. (Mills 1996).

1.7.2.1 B cells

The role of the antibody produced by mature, differentiated B cells is as a first line of specific defense in neutralizing the infectious agent, by opsonisation and subsequent phagocytosis or by the production of bactericidal antibody. B cells produce specific antibody that recognise specific epitopes. These epitopes may be either continuous or discontinuous and this is dependent on the conformation of the antigen. A second role of B cells is to facilitate lysis of cells that display foreign antigens on their surface. A specific antibody that recognises these antigens may lyse the cells by one of two mechanisms, antibody-dependent cellular cytotoxicity and/or bactericidal antibody, which is mediated by complement. The third role of B lymphocytes is to facilitate phagocytosis of cellular debris resulting from the lysis of the infecting organism and cytotoxicity during infection.

1.7.2.2 T cells

T cells are needed to control intracellular pathogens and to activate B cell responses to most

antigens. Cytotoxic T lymphocytes (CTLs) are almost exclusively class I MHC-restricted $CD8^+$ T cells. These lymphocytes are important in viral infections or those involving intracellular pathogens as they recognise and are responsible for the lysis of infected cells. They also secrete a specific pattern of cytokines, for example, IFN-γ which may facilitate the clearance of viral infection.

T lymphocytes with helper activity are class II MHC-restricted $CD4^+$ cells. They interact with B cells and help in the process of producing different immunoglobulin isotypes (G, A or E) by B cells and in the production of B memory cells. The exact signals promoting these activities are not fully understood. T cell-independent antigens do not induce the switch to G, A or E immunoglobulin isotypes and do not generate B cell memory. Another group of T helper cells interact with phagocytic cells, helping them to destroy pathogens.

Both $CD4^+$ and $CD8^+$ T-cells recognise a complex between the MHC molecule and a peptide from the foreign protein. In the case of $CD4^+$, the peptide derived from the antigen is degraded in the lysosomes, and associates with class II MHC antigens. In the case of $CD8^+$, the peptide is derived from newly synthesised antigen in the cytoplasm and associates with class I MHC antigen. Since nearly all cell types in the body express class I MHC molecules, the role of $CD8^+$ CTLs has been described as performing a continuous molecular audit of the body (Mills, 1996). T cells generate their effects either by releasing cytokines or by direct cell-to-cell interaction. (Clark and Ledbetter, 1994).

1.7.3 Mucosal immunity

Many pathogens gain entry to the host via a mucosal surface. The response to such infections is to produce localised antibodies of the secretory IgA (s-IgA) isotype. This isotype constitutes the majority of all antibodies produced at mucosa-associated tissues and results in local immune protection.

It is now established that antigens which are most often encountered by inhalation or ingestion, can be taken up into specialised lymphoreticular tissues in the upper respiratory tract and gastro-intestinal tract, referred to as BALT (bronchus-associated) and GALT (gut-associated) lymphoid tissue. GALT is represented by tissues such as Peyer's patches (PPs) and appendix. PPs contain a dome region which is enriched for lymphocytes, macrophages and some plasma cells. This is covered by a unique epithelium enriched for specialised antigen-sampling cells, referred to as microfold (M) cells. M cells are responsible for the

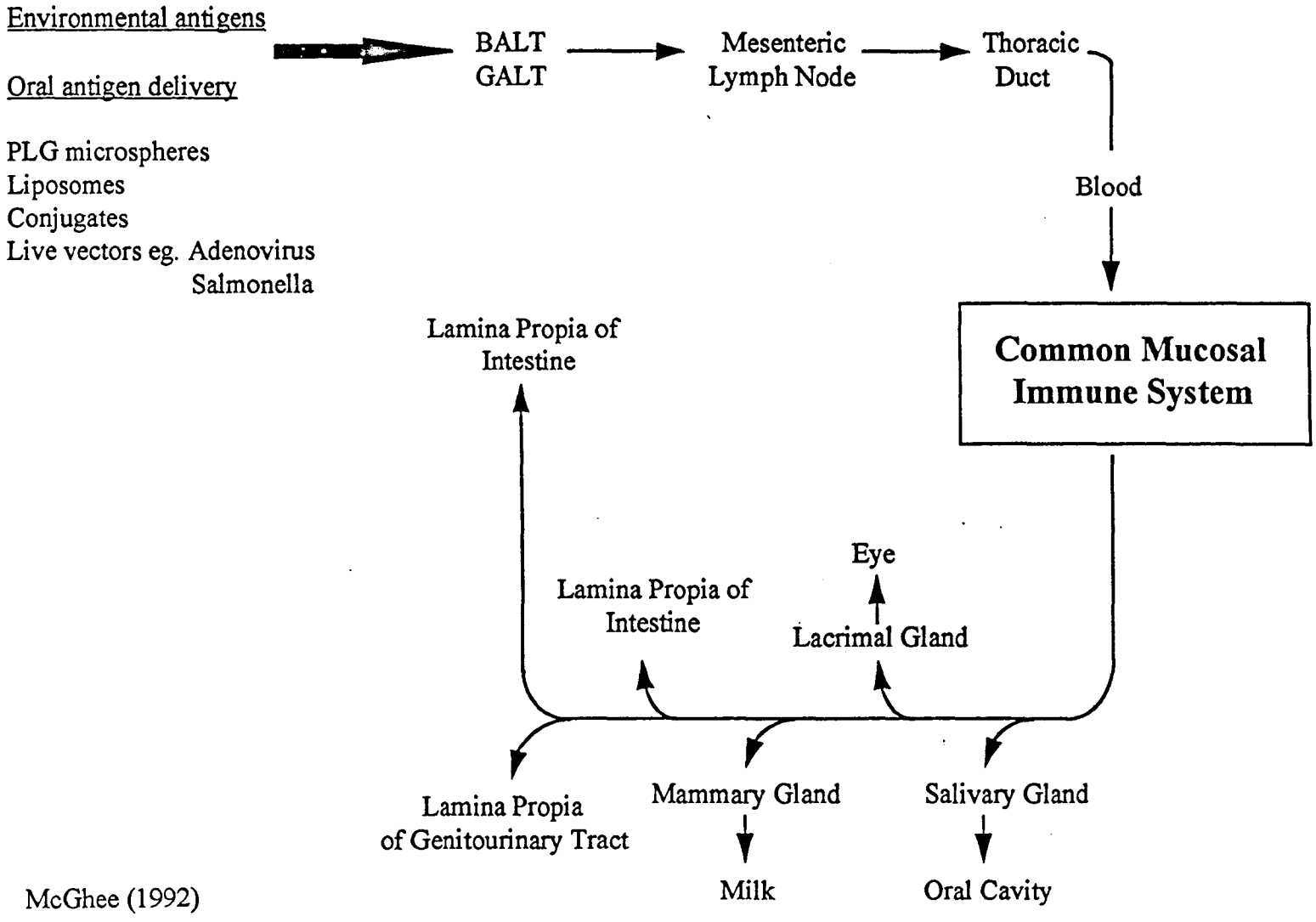
uptake and transport of lumenal antigens, which are not degraded but delivered intact to underlying lymphoid tissue. All the necessary immunocompetent cells, T-cells (CD4+ Th cells and CD8+ CTLs), B-cells and others such as macrophages are present in these IgA inductive sites (McGhee, 1992).

Following stimulation with antigen in the PPs and its presentation to B- and T-cells, the antigen-induced B- and T-cells leave the PPs via the efferent lymphatics and reach the systemic circulation through the thoracic duct and in this way reach distant mucosal tissue, where circulating B-cells are selectively retained. The B-cells clonally expand under the influence of antigen, T-cells and cytokines becoming mature IgA-secreting plasma cells (Mestecky and McGhee, 1987; McGhee *et al.*, 1989). This cell distribution pathway from IgA inductive tissues to IgA effector sites (bronchi, genitourinary tract and secretory glands) is termed the "common mucosal immune system" (Mestecky *et al.*, 1994) (Figure 1.6). However, this is still a subject of some debate and some researchers argue that the mucosal immune system is compartmentalised with distinct regional immune tissue (Lewis and Hayward, 1996).One of the most effective ways to stimulate PPs to produce effective mucosal immunity is by oral delivery of antigens. However, oral administration of proteins, including subunit vaccines, may induce a state of unresponsiveness, termed oral tolerance (Tomasi, 1980). Oral tolerance may have been a factor when Thomas *et al.* (1992) immunised mice by the intragastric route with F1 antigen and found that it failed to stimulate a protective immune response against challenge with *Y. pestis*. The development of a plague vaccine that could induce mucosal immunity as well as systemic immunity would be of great advantage in prevention of pneumonic plague infection, but still give protection against the bubonic form of the disease.

Mucosal antibodies have been shown to inhibit microbial adherence and prevent absorption of antigen from mucosal surfaces (Abraham *et al.*, 1985). However, when specific IgA reacts with the corresponding antigen in the mucosal surface, the absorption of the antigen is blocked, but it does not result in complement activation followed by the generation of cleavage products of C3 and C5 components (McGhee *et al.*, 1992). These fragments would induce local inflammatory reactions including the influx of polymorphonuclear leukocytes and release of substances that enhance the permeability of mucosal membranes due to tissue damage.

The inability of human secretory IgA or serum IgA complexed to antigens to activate

Fig. 1.6 The Common Mucosal Immune System



McGhee (1992)

Induction of antigen-specific mucosal immune responses by the common mucosal immune system and possible antigen delivery systems for the priming of IgA-committed B cells in IgA inductive sites (eg. GALT)

complement, by either pathway, may be of paramount importance in the maintenance of integrity of mucosal membranes. This is important in vaccine development because it is unacceptable to give a vaccine that would lead to damage of host tissues and the natural role of s-IgA at mucosal sites might be to minimise the inflammatory side-effects produced by IgE and IgG isotypes, leading to a balance of the immune response.

1.8 Vaccine Delivery and Enhancement of the Immune Response

There are three main natural routes of infection ; a mucosal surface, an abrasion in the skin or a biting vector. The immune system must be able to respond effectively regardless of how a pathogen enters the host. Different responses would generate a different type of immunity, either humoral, mucosal or cell-mediated immunity. It should be possible to deliver a vaccine formulation in such a way as to provide the best form of immunity for a particular infection, in other words, to have selective induction of different immune responses.

To achieve high efficacy, a vaccine should ideally fulfill a number of immunological requirements. Vaccination should lead to activation and differentiation of the antigen presenting cells (APCs), synthesis and secretion of cytokines and processing of antigens entering the cell to form appropriate peptides. In addition, activation and differentiation of specific T and B cells is required resulting in a high yield of memory cells and generation of T helper cells and CTLs to several peptide determinants. Long-term persistence of antigen in its native conformation is essential to allow continued formation and recruitment of memory B cells to form antibody-secreting cells (ASCs), thus ensuring the steady production of antibody (Ada, 1996). Many vaccines, including the present plague vaccines do not fulfill all of these requirements. A vaccine is required that will provide long term immunity against *Y. pestis* infection both via a flea bite and across a mucosal surface.

1.8.1 Adjuvants

Generally, soluble proteins and peptides are poorly immunogenic and must be administered with preparations that potentiate the immune response. Adjuvant design evolved from the widely held view that the activity of adjuvants is attributable partly to their "depot" effect, whereby an antigen is absorbed and slowly released to the surrounding medium over a certain period of time. Adjuvants can be grouped as follows : (i) those causing depot formation at the site of injection, e.g. mineral compounds, oil-based adjuvants, liposomes, biodegradable polymer microspheres, (ii) those acting as delivery vehicles for the antigens

which may help in targeting antigens to immune competent cells, e.g. liposomes, oil adjuvants, biodegradable polymer microspheres, and (iii) those acting as immunostimulators, e.g. Freunds complete adjuvant (FCA), muramyl dipeptide (MDP), lipopolysaccharide (LPS), lipid A, monophosphoryl lipid A (MPL), pertussis toxin, cytokines (Gupta and Siber, 1995).

Alum, aluminium hydroxide or Alhydrogel, first used more than 50 years ago, in addition to aluminium phosphate, is still the only adjuvant preparation licensed for general medical use in humans. Recent studies have demonstrated the importance of electrostatic attractive forces in the adsorption of antigens by aluminium-containing adjuvants (Gupta and Siber, 1995). Alhydrogel is a better adsorbent of acidic proteins than basic ones, because it has an isoelectric point (IEP) of ca. 11 and is positively charged at pH7.4. Consequently, it is a good adsorbent for negatively charged proteins. The surface charge characteristics can be modified by adding phosphate ions which are substituted for the hydroxyl ions in the adjuvant. Therefore, to some extent, it is possible to optimise the surface charge of aluminium hydroxide adjuvant in relation to the antigen by pre-treating with phosphate ion (Gupta and Siber, 1995). The mechanism of adjuvanticity of aluminium compounds includes depot formation at the site of injection and slow release of the antigen. This stimulates immune competent cells through activation of complement, activation of macrophages and efficient uptake of aluminium adsorbed antigen particles by APCs due to their particulate nature and optimal size of less than 10μm.

With so much interest in subunit and peptide-based vaccines, many new adjuvant preparations are being investigated. An ideal adjuvant would be chemically defined so that it can be manufactured consistently. Adjuvant preparations should enhance a protective immune response with a weak antigen. The adjuvant should be stable with regard to adjuvanticity and toxicity and without any interaction with the antigen. It should be biodegradable and non-immunogenic by itself. In recent years, adjuvants have received much attention due to their ability to selectively modulate the immune response to elicit humoral and/or cellular immune responses. Humoral response may be further modulated to select antibody isotype, IgG subclasses, avidity and affinity of antibodies. Antigen processing can be modulated with adjuvants leading to vaccines which can elicit both T-helper cells and CTLs (Mills, 1996).

In addition to aluminium hydroxide, adjuvants currently used include oil emulsions, LPS and

immunostimulatory complexes.(i) Oil emulsions, such as Freund's adjuvant have a long history of use for increasing the immune response in animals. The oil emulsions as adjuvants did not receive much attention until Freund, in 1937, demonstrated the adjuvant effect of mineral oil mixed with killed *Mycobacteria,* referred to as Freund's complete adjuvant (FCA). The water-in-oil emulsion without *Mycobacteria*, known as Freund's incomplete adjuvant (FIA), has been used in a number of veterinary vaccines. The mode of action of Freund's adjuvant is attributed to depot formation at the site of injection and slow release of the antigen with stimulation of ASCs. FCA is too toxic to be used as an adjuvant for human vaccines. FIA is not currently used in humans because of the side-effects such as local reactions at the site of injection, oil-induced neoplasms in mice and Arlacel-A induced carcinogenicity in mice. In the 1970s, the minimal structure needed for adjuvanticity of FCA was defined as N-acetyl muramyl-alanyl-D-isoglutamine (MDP). However, the most promising derivatives of MDP were not pursued as vaccine adjuvants due to lack of advantage over aluminium adjuvants. (ii)The adjuvant effect of LPS was described in 1956 and most of the adjuvanticity and toxicity of LPS are due to the lipid A region of the molecule. However, LPS and lipid A are too toxic to use for human vaccines. Incorporation into liposomes reduced the toxicity of LPS or lipid A while retaining or even increasing the adjuvant effect. (iii) Immunostimulating complexes (ISCOMs) are non-covalently bound complexes of Quil-A adjuvant, cholesterol and amphipathic antigen. ISCOMs have been shown to stimulate both humoral and cell-mediated immune response particularly $CD8^+$ CTL response. The adjuvant properties of ISCOMs have been demonstrated with a variety of viral capsid antigens and non-viral antigens of relevance for human vaccination (Gupta and Siber, 1995). ISCOMs are used in veterinary vaccines and have not been approved for human vaccines mainly due to side-effects of Quil-A, although these side-effects were almost absent when Quil-A was incorporated into ISCOMs (Morein *et al.*, 1989). (iv) Biodegradable microspheres such as liposomes and PLGs (section 1.8.2.1 and 1.8.2.2).

The basic knowledge of adjuvant action is very important for developing vaccines and for subunit or synthetic vaccine antigens, potent adjuvants may be necessary. The practical application of peptide-based vaccines depends on their ability to successfully mimic naturally occurring antigenic determinants present in the infectious agent and on their capacity to stimulate the appropriate response to induce immunity to the natural infection. Unfortunately, most of these synthetic peptides are poor immunogens and require the use of adjuvants to increase their immunogenicity. Therefore the prospect of using synthetic peptide antigens in vaccines will become a reality only if safe and effective adjuvants are developed.

1.8.2 Delivery systems

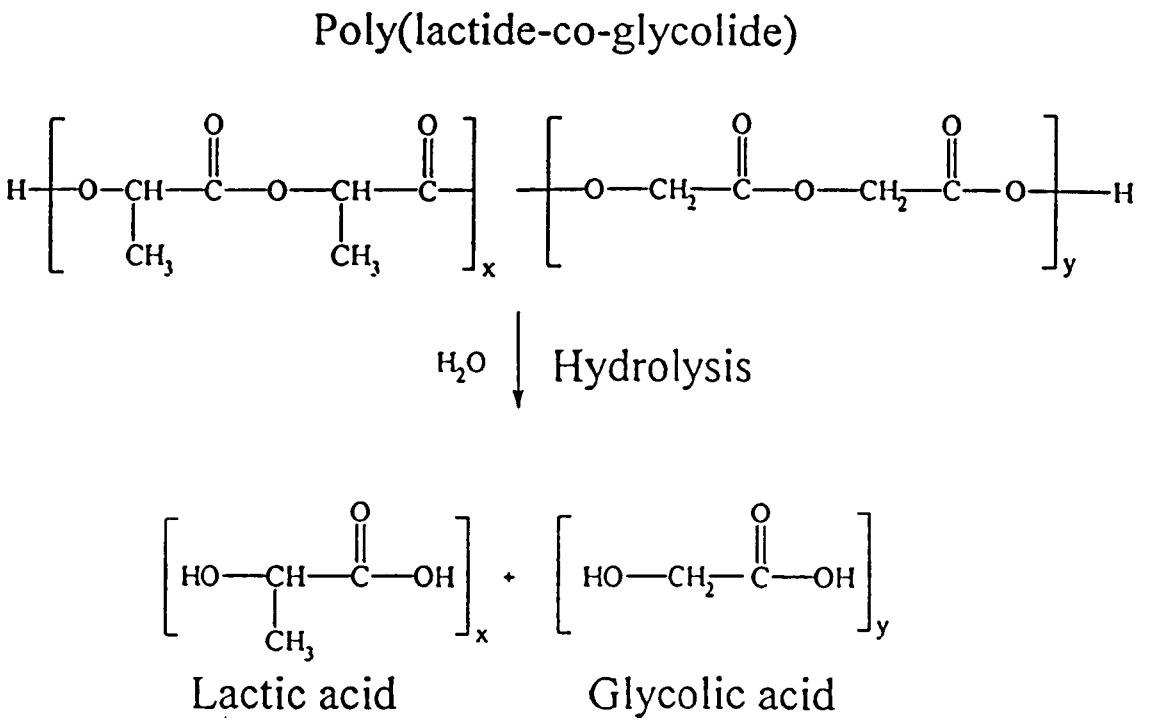
One approach to adjuvant design incorporates concepts of controlled-release technology, in which therapeutic molecules are delivered from vesicles or polymeric systems at a pre-determined rate for a definite time. In addition, the immune response can be enhanced and targeted by appropriate packaging of antigens and use of adjuvants. There have been advances in the packaging of antigens to emulate some of the advantages of live agents, such as targeting to particular cells and constructing a mosaic pattern or specific aggregates of protein molecules. Three delivery systems are of particular interest; liposomes, ISCOMs and biodegradable microspheres. Liposomes and microspheres were studied during the course of this work and are of special interest because they may offer the prospect of reducing the size of the antigen dose and the number of administrations of some vaccines, whilst still giving long-term immunity. Liposomes and microspheres also give the ability to target the vaccine to a particular site in the body and to stimulate a particular response, for example, enhancing the immune response via both parenteral and oral vaccination routes.

1.8.2.1 Biodegradable microspheres

Spherical, sustained-release polymer particles can be either monolithic, with the antigen interspersed throughout the particle (microspheres) or consist of true core-wall capsule structures with a core reservoir of antigen surrounded by an outer polymer shell (reservoir microcapsules). The most studied polymers for controlled-release delivery are poly (lactide-co-glycolide) (PLG) polymers. The lack of toxicity in these polymers has been established and PLG has been used for years as absorbable suture material.

Scanning electron microscopy (SEM) and gel permeation chromatography (Cohen *et al.*, 1991) have confirmed that antigen is released from the microspheres by diffusion through matrix pores and by matrix degradation. Monolithic microspheres release an initial surge of antigen from surface pores and then continue to release antigen at a nearly constant rate as the matrix of the microsphere degrades (Nuwayser *et al.*, 1984). Reservoir microcapsules will release steady low levels of antigen by diffusion until degradation disrupts the shell integrity and the core antigen is released (Morris *et al.*, 1994). In addition to spontaneous degradation of the copolymer matrix, ultrasound, light, magnetism and other external factors have been investigated for their potential in influencing polymer breakdown and controlling the rate of antigen release (Cohen *et al.*, 1991). Fig. 1.7 shows poly (DL-lactide-co-glycolide) structure and degradation.

Fig.1.7 Poly (DL-lactide-co-glycolide) structure and degradation



Chemical structure of PLG and its hydrolysis to yield lactic and glycolic acids. The rate of decay of PLG microparticles is dependent on the size of the microparticles, the molecular weight of the polymer ($x + y$), and the ratio of lactic:glycolic acids (x:y).

The possible mechanisms for enhancement of the immune response by sustained release of antigen include a depot effect, analogous to that of alum adjuvants and the delivery of antigen directly to antigen-presenting cells such as macrophages. Direct uptake by macrophage is possible with microspheres of up to 10μm which are taken up by gut-associated lymphoid tissue making successful oral immunisation with microspheres a possibility (Jones *et al.*, 1996).

An ideal vaccine delivery pattern should mimic the profile of antigen concentrations that are seen in the course of a natural infection i.e. a high dose of antigen within a few days of the injection followed by a period of decreasing amounts of antigen. The initial high load of antigen will influence the extent of memory T-cell formation, whereas the subsequent steady decrease in antigen load will enhance the development of antibody affinity maturation. PLG microsphere technology has the potential benefits of reducing the number of inoculations, enhancing the immune response and in reducing the total antigen dose required for effective immunisation. In addition, PLG microspheres have the potential to protect antigen from the harsh gastric environment and in delivering antigen to lymphatic tissues, permitting effective oral primary or booster immunisations.

To overcome problems encountered using organic solvents during the encapsulation of antigens, the multiple-emulsion method was developed (O'Hagan *et al.*, 1991; Jones *et al.*,1995). This method combines the use of water and organic phases in which protein is dissolved in the aqueous solution and is dispersed in the PLG solution by homogenisation to create the first inner emulsion of water and oil. This emulsion is then emulsified again in a second water phase containing the surface-active material, polyvinyl alcohol (PVA). Upon contact with the second water phase PLG precipitates to create embryonic microspheres that are further solidified when the organic solvent is removed by evaporation.

The multiple emulsion method produces spherical PLG organic microspheres with diameters between 1 and 100μm, a parameter easily controlled by the conditions of formulation. Microspheres can be injected through a 25-gauge needle, therefore no surgical implantation is required. Protein encapsulation yields may be greater than 90% and most importantly, several vaccine candidates, e.g. tetanus toxoid, pertussis fimbriae and malaria

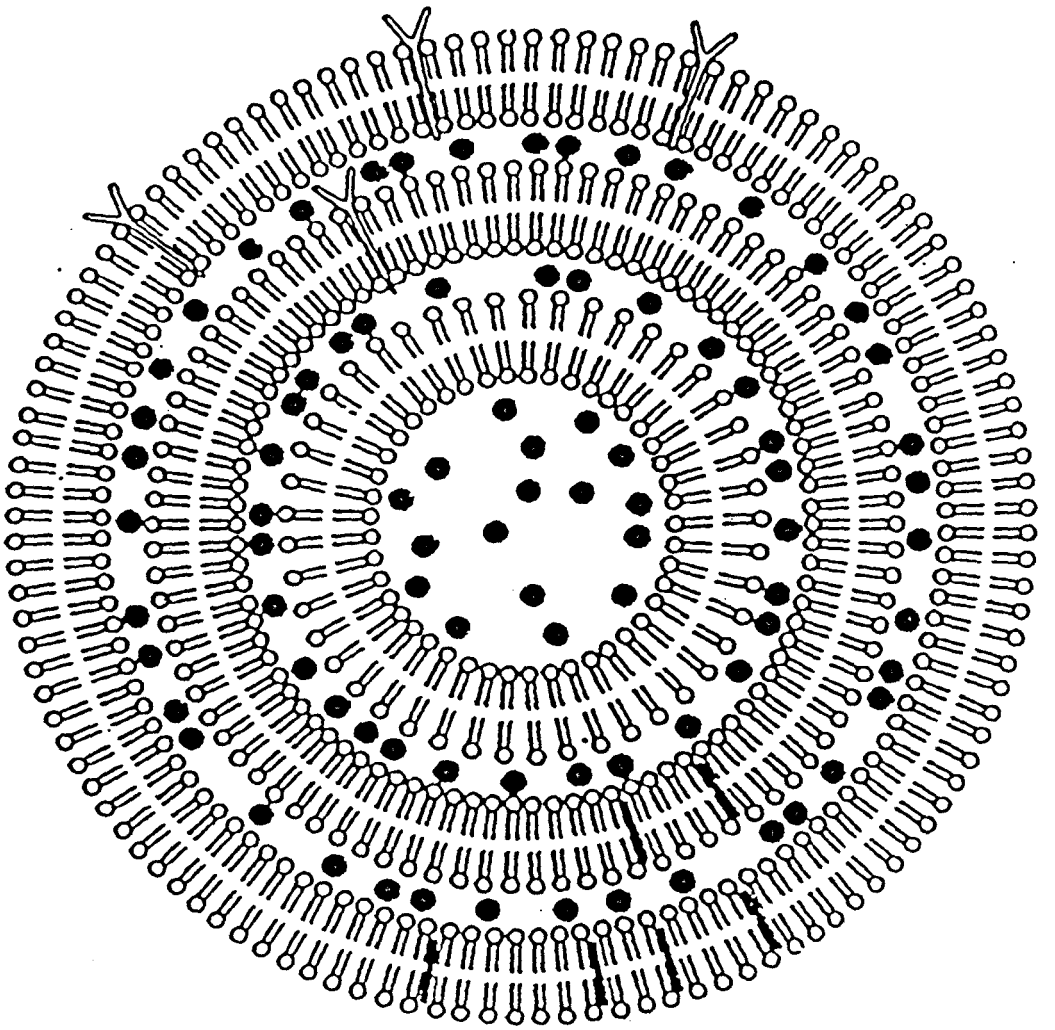
synthetic peptides retain solubility, activity and immunogenicity following this microencapsulation procedure (Jones *et al.*, 1995). Other advantages include simplicity, speed, ability to scale-up the procedure and there is special interest in using PLGs for delivery of oral vaccines, for which they are considered to be especially suited.

1.8.2.2 Liposomes

One approach to increase the immunogenicity of purified microbial antigens and to potentiate the therapeutic action of antimicrobial agents is to employ liposomes as vehicles for their delivery. Liposomes are membrane-like vesicles consisting of one or more phospholipid bilayers surrounding aqueous compartments.(Figure 1.8) and can be multilamellar or unilamellar. Hydrophobic agents can be embedded in the lipid bilayers and hydrophilic agents can be entrapped in the internal aqueous space of the liposomes. The charge, rigidity, size and surface properties of liposomes can be varied and controlled by incorporating different types of lipids and by varying the preparation method. Therefore liposomes are considered as versatile delivery systems and have an advantage in that the lipids most often used for liposome preparation are those commonly found in cell membranes and so are biodegradable, relatively non-toxic and non-antigenic or weakly antigenic. They can also be tailored to avoid the mononuclear phagocyte system, to deliver immunomodulators (cytokines, such as IFN γ) and liposome-mediated delivery of microbial antigens in vaccines.

The immunological adjuvant action of liposomes was demonstrated by Allison and Gregoriadis (1974) and this has been followed by numerous studies on their use for the immunopotentiation of a large variety of whole cell, protein and peptide vaccines (Gregoriadis, 1991; Alving, 1991). The structural versatility of liposomes has permitted tailoring of the system towards optimal adjuvanticity and the appropriate choice of vesicle composition with respect to surface charge and phospholipid to antigen mass ratio. It is also possible to achieve ligand-mediated targeting of liposomes to immunocompetent cells (Garcon *et al.*, 1989; Mbawuike *et al.*, 1990) to enhance the immune response and to produce cell-mediated and humoral immunity. Small, submicron vesicles can be used as carriers for peptides and proteins. Antimisiaris *et al.* (1993) produced giant vesicles, 5.5μm in diameter, to carry attenuated killed viruses and bacteria in an attempt to produce more effective live-attenuated vaccines.

Fig. 1.8 Schematic diagram of a multilamellar liposome vesicle



Bergers (1995)

The rationale of using liposomes to carry antigens for immunisation is based on the known preferential uptake by macrophage of parenterally injected liposomes (Wassef *et al.*, 1994). In vaccine preparations tried to date, the immune response elicited by the liposomal vaccines generally has been higher than those obtained with fluid vaccines, but lower than those obtained with peptides immunised in Freund's complete adjuvant(Cohen *et al.*, 1991). Moreover, at least two injections of antigens in liposomes have been needed to elicit an adequate immune response (Davis and Gregoriadis, 1987), which may be attributable in part to the inherent instability of liposomes in the host, as they are rapidly destroyed by high-density lipoproteins and destructive cells (Cohen *et al.*, 1991). The action of liposomes is attributed to depot formation, hydrophobicity, rendering soluble antigens particulate and efficient targeting of the vesicles to antigen-presenting cells of the immune system (Clarke and Stokes, 1992). One influential factor concerns transition temperature (Tc) of the lipids used; above the Tc, the major phospholipid molecules that form the lipid bilayers become less rigid allowing leakage of contents. Orally administered liposomes used as carriers have reportedly increased the intestinal absorption or efficacy of entrapped agents such as insulin (Patel and Ryman, 1976), glucose oxidase (Depergolas *et al.*, 1976), and coagulation factor VIII (Hemker *et al.*, 1980). Liposomes have also been investigated as delivery systems in DNA therapy for conditions such as, cystic fibrosis (McLachlan *et al.*, 1995).

Few studies have investigated the immune response to relevant antigens fed in liposomes. Liposome entrapment of streptococcal antigens increased salivary antibody responses in rats (Wachsmann, 1985, 1986; Gregory *et al.*, 1986). Many future vaccine preparations are likely to be based on relatively pure, low MW proteins. Therefore liposomes may be a good vaccine delivery system for such antigens. Aramaki *et al.* (1994) demonstrated that liposomes consisting of distearolphosphatidylcholine (DSPC): phosphatidylserine (Pser) : cholesterol (CH) are negatively charged and stable in the gastrointestinal tract and are preferentially taken up by PPs following oral administration to rats (Tomizawa *et al.*, 1993). Other liposomal vaccines include *Streptococcus pneumoniae* type 3 and 14 (Snippe *et al.*, 1989). Oligosaccharides from *S. pneumoniae* type 3 and 14 polysaccharides were linked to liposomal membranes which resulted in a T-cell independent response. Therefore, to elicit a T-cell dependent response it may be necessary to use additional adjuvants to change the immunological characteristics of liposomes. Alving *et al.* (1989) investigated an experimental malaria vaccine, using liposomes as a carrier for synthetic

antigens derived from the structure of the CS protein on the surface of *Plasmodium falciparum* sporozoite. Liposomes were found to be an effective carrier and could be used to stimulate higher antibody titres than those elicited by non-encapsulated antigen.

Liposomes might be especially suited to intranasal delivery of vaccines because the lipid membrane will fuse with cells lining the mucosal epithelium, therefore facilitating rapid uptake of antigen. However, for effective protection, liposomes given intranasally needed to reach the lower respiratory tract and this was done effectively by anaethestising mice prior to administration of the liposomes (Aramaki *et al.*, 1994).

1.8.3 Enhancement of the immune response using delivery systems

Using adjuvants and vaccine delivery methods alone or in combination, it is possible to tailor the immune response to a particular disease by inducing a stronger cell mediated or mucosal response depending on which of these would provide the best protection. However, in the case of plague, where infection can be via a flea bite or across a mucosal surface, enhancement of the overall response to give a balanced immune response to infection would be the most desired effect of administering vaccine antigens using delivery systems. A balanced immune response would be one which would induce humoral immunity to give a B-cell response and the production of opsonising and bactericidal antibodies as a first line of defence. This would be improved if at this stage mucosal immunity could be enhanced to protect mucosal surfaces from invasion by the pathogen, if it could also induce a cell-mediated response, it would be some way towards an ideal vaccine. Therefore, in vaccine development, it is important to study the type of immune response that the vaccine formulation produces as well as its protective efficacy.

1.9 Study Aims

1.9.1 Identification, isolation and characterisation of the native F1 antigen of *Y. pestis*

The F1 antigen has been shown to provide protection against plague. In recent years a recombinant F1 has been shown to be protective in mice (Simpson *et al.*, 1990). F1 antigen has also been described as having a major subunit F1 (fraction 1A) as a 17.5kda glycoprotein. This study aimed at purifying native F1 antigen, characterising the major subunit and determining the glycosylated state of the protein. The study also attempted to determine whether any of these factors are important for immunogenicity and protection against plague. The study also addressed scaling up the purification of native F1 for vaccine

production.

1.9.2 Production and characterisation of rF1 antigen

The nF1 antigen has inherent problems in that large volumes of *Y. pestis* have to be handled in order to purify sufficient quantities for immunogenicity and protection studies. In addition, problems may arise in proving that it is completely safe for inclusion in an acellular vaccine. Development of a suitable rF1 antigen would overcome these difficulties and may prove to be a more suitable alternative candidate antigen. This study aimed at developing an effective process for the production of a rF1 and the scaling-up of this process.

1.9.3 Immunogenicity and protective potency of the nF1 and rF1

The immunogenicity of nF1 and rF1 will be assessed and their ability to provide protection against challenge with virulent *Y. pestis* in an experimental animal model determined. The aim of this study was directed towards assessing which of these antigens would be most suitable for use as an acellular vaccine antigen.

1.9.4 Evaluation of F1 and V antigens of *Y. pestis* as protective antigens

This study also aimed at assessing the V antigen as a protective antigen and as a candidate for an acellular vaccine. The protective potency of F1 and V, administered separately and as a combined subunit vaccine was examined.

1.9.5 Development of vaccine delivery systems for the F1 and V antigens of *Y. pestis.*

PLG microspheres and liposomes have been shown to be suitable vaccine delivery vehicles for protein subunits and peptides. One of the objectives of this project was to produce PLG microspheres and liposomes for delivery of the F1 and V antigens as protective antigens administered separately and as a combined vaccine. The novel delivery systems will be compared with antigens administered in aluminium hydroxide as adjuvant.

Chapter 2

Materials and Methods

2.1 Bacterial strains and growth conditions

2.1.1 *Yersinia pestis* strains

Yersinia strains were stored either as freeze-dried cultures at 4^0C or as glycerol stocks at -70^0C. *Y. pestis* strains were grown either in chemically defined medium (YPMH) (Straley and Bowmer, 1986), the composition of this medium is detailed in table 2.1, or blood agar base (Oxoid) for 48h at 37^0C or 28^0C as required.

2.1.2 Recombinant *E. coli* strains

Recombinant *E. coli* JM109 strains were stored at -70^0C in 15% glycerol. These strains containing pGEX plasmids expressing a recombinant F1-glutathione-S-transferase (GST) fusion protein (produced as a collaboration between CAMR and CBD, DERA) or a recombinant V-GST fusion protein (produced at CBD, DERA) were grown in L-broth (table 2.2) or Terrific broth (Sigma Chemical Co., made according to manufacturers instructions), both containing 100μg/ml of ampicillin.

2.2 Expression of *Y. pestis* antigens

2.2.1 Expression of native F1 antigen

Freeze-dried or frozen stocks of *Y. pestis* MRE 1447 or 1176 were cultured on blood agar plates. After 48h incubation at 37^0C, single colonies were inoculated into 10ml volumes of YPMH in McCartney bottles. These primary cultures were incubated for 24-48h at 37^0C and each 10ml volume used to inoculate 1L of YPMH in a polypropylene conical flask (Nalgene). After incubation at 37^0C for 48h in an Orbital shaking incubator (180rpm), the culture was centrifuged (6000 x g for 30min) and the F1 was purified from the culture supernatant.

For larger-scale expression of F1 antigen, two 1L YPMH cultures were used to inoculate a 35L fermenter (Chemap fermenter with a CBC50 controller) with a running volume of 25L. *Y. pestis* was cultured for 48h at 37^0C (stirrer speed 400rpm; air, 2L/min; pH maintained at 7.4), cells were then harvested by continuous flow centrifugation and the supernatant retained.

Table 2.1 Components of YPMH medium

Component	Amount (mM)
HEPES	25.0
K2HPO4	2.5
MgCl2	20.0
MnCl2	0.01
Na2S2O3	2.5
NH4Cl	10.0
D-Biotin	0.002
Calcium pantothenate	0.004
Thiamine	0.003
Glucose	10.0
DL-Alanine	2.5
L-Arginine hydrochloride	1.0
L-Asparagine	2.5
L-Aspartate	1.0
L-Glutamate	5.0
L-Glutamine	1.0
Glycine	5.0
L-Histidine	1.0
L-Isoleucine	1.0
L-Leucine	1.0
L-Lysine	1.0
L-Methionine	1.0
L-Phenylalanine	1.0
L-Proline	5.0
L-Serine	5.0
L-Threonine	2.5
L-Tryptophan	0.1
L-Tyrosine	1.0
L-Valine	1.0

The above ingredients were added in the appropriate amounts to water, the pH was adjusted to 7.4 with 0.1M NaOH and the media was filter-sterilised. (Straley and Bowmer, 1986).

Table 2.2 Components of L-Broth

Component	Amount per Litre
Difco Bacto tryptone	10g
Difco Bacto yeast extract	5g
NaCl	10g

The above ingredients were dissolved in water, the pH adjusted to 7.5 with 0.1M NaOH and sterilised by autoclaving at 15psi for 15min.

For L-agar, the above method was used with the addition of 15g per Litre of Difco Bacto agar.

2.2.2 Expression of recombinant F1 antigen

A recombinant F1-GST fusion protein was cloned using the pGEX plasmid (Pharmacia). A positive clone expressing the fusion protein (*E. coli* JM109 + pKR4) was selected for purification of recombinant F1 (rF1) antigen.

All media contained ampicillin (100μg ml^{-1}) to stabilise the recombinant plasmid within the *E. coli* strain. A stock culture of *E. coli* JM109 + pKR4 was used to inoculate L-agar plates (table 2.2). After 18h incubation at 37^0C, single colonies were used to inoculate 10ml volumes of L-broth in McCartney bottles, which were incubated for 18h at 37^0C. These broth cultures were used to inoculate 1L amounts of the same medium in 2L conical flasks, which were incubated in an Orbital shaking incubator (180rpm) until the absorbance of the cultures at 600nm reached 0.3 to 0.6. Expression of the recombinant antigen was then induced by the addition of isopropylthiogalactopyranoside solution (IPTG, manufactured by BCL, final concentration of 0.1 mM in PBS). Incubation was continued for a further 5h before the cells were harvested by centrifugation (10000 x g, 30min, 4^0C). All centrifuge pots and buffers were sterilised prior to use to avoid contamination with extraneous proteases.

For larger-scale production of recombinant protein, 2 x 1L L-broth cultures (without IPTG) were used to inoculate a 25L fermenter (stirrer speed, 400rpm; air 2L/min; pH maintained at approximately 7.5, 5 - 10ml antifoam, PPG 2000, was added after 4h), the expression of rF1 was induced using IPTG as described for small-scale cultures.

2.3 Protein purification

2.3.1 Purification of native F1 antigen

Culture supernatant obtained in section 2.2.1 was filtered through a Sartobran capsule containing a 0.2μm membrane with a 0.45μm prefilter. F1 antigen was extracted from this cell-free supernatant by precipitation with 40%(w/v) ammonium sulphate. The resulting precipitate was sedimented by centrifugation (34000 x g, 30min, 4^0C) and resuspended in 10mM phosphate buffer, pH7.4. Repeated resuspension and centrifugation steps were necessary to extract the majority of F1 from the precipitated pellet and reduce the size of the pellet.

Final supernatants were pooled and concentrated using centrifugal concentrators (Filtron) or solid polyethylene glycol (PEG, MW 20000) before being dialysed against 4L 10mM phosphate buffer, pH7.4 (3 changes). This material was examined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and by Limulus Amoebocyte Lysate (LAL) assay (192 Test Kit), according to manufacturer's instructions, for lipopolysaccharide content. The concentrated antigen was stored at -20^{0}C. A flow diagram representing the F1 purification process was prepared (Fig. 3.10).

2.3.1.1 Size exclusion chromatography

Sephacryl S200 pre-swollen gel (Pharmacia) was used to prepare a gel filtration column (16mm x 100mm) with a packed bed volume of 177ml. The void volume of the column was determined using a solution of blue dextran (3mg ml^{-1}) and found to be 78ml. The column was calibrated using the protein molecular weight standards, bovine serum albumin (BSA) (66kDa), carbonic anhydrase (29kDa) and cytochrome c (12.4kDa). F1 antigen (5mg) was applied to the column in 100mM Tris-HCl, pH8.0 at a flow rate of 0.3ml per min. Fractions (2ml) were collected and elution monitored by absorbance at 280nm. Fractions under the absorbance peak were examined by SDS-PAGE.

2.3.1.2 Ion exchange chromatography

A 1ml sample of F1 antigen (approximately 200μg) in 20mM imidazole, pH7.0 (column buffer A) was injected onto a MA 7Q anion exchange HPLC column (Pharmacia). Protein was eluted using a sodium chloride gradient applied by adding increasing amounts of 20mM imidazole, 1M NaCl (column buffer B). Fractions (1ml) were collected and elution was monitored by absorbance at 280nm by the UV monitor on the HPLC system (Biorad). Fractions under the absorbance peak were examined by SDS-PAGE.

2.3.2 Purification of rF1 from recombinant *E. coli*

Recombinant *E. coli* strain JM109 + pKR4 was grown under conditions described in section 2.2.2. Cells were harvested by centrifugation (10000 x g, 30min, 4^{0}C) and resuspended in phosphate buffered saline (PBS) (2g wet weight of cells in 10ml PBS). Lysozyme (final concentration, 250μg ml^{-1}) was added to the cell suspension and incubated at 37^{0}C for 30min. Triton X-100 was then added (1% (v/v) final concentration), this suspension was mixed gently and placed on ice for 10min. The cold suspension was

sonicated (MSE soniprep, 100mm probe) in 50ml amounts for 10 x 30second bursts with a 1min pause for cooling between each burst. The sonicate was centrifuged (25000 x g, 30min at 4^0C) to remove cell debris.

The rF1 was purified from the sonicate supernatant by affinity chromatography using glutathione 4B Sepharose (Pharmacia) after equilibration of the affinity column (5ml bed volume) with PBS (5 column volumes). The sonicate supernatant was incubated with the gel at room temperature for 1h. Unbound fusion protein was then removed by washing the column (x4) with PBS containing 0.1% (v/v) Triton X-100. The rF1 antigen was separated from bound GST by cleavage with bovine Factor Xa (Enzyme Research Laboratories). Factor Xa (5ml) was added to the affinity column (1mg Factor Xa to 200mg fusion protein) and incubated for 18h at 4^0C. Cleaved rF1 antigen was eluted from the column with 10ml PBS and 2-5ml fractions collected. The protein concentration in each fraction was estimated by the Pierce Bicinchoninic acid (BCA) assay according to manufacturers instructions, fractions were then pooled and concentrated (section 2.3.1) before dialysis against three changes of 4L PBS, samples were stored at -20^0C until required.
Samples of rF1 were examined for purity by SDS-PAGE.

2.3.3 G25 Sephadex size exclusion chromatography after solubilisation of sonicate

Bacterial paste (from recombinant *E. coli* strain JM109 + pKR4) was dispersed in approximately 10 volumes of 50 mM Tris/HCl, 10% (w/v) sucrose, 5mM EDTA, pH7.5, lysozyme (final concentration 200μg ml^{-1}) was added and the cell suspension frozen at -20^0C. The solution was thawed at 20^0C and diluted with 9 volumes of ice-cold lysis buffer which contained 150mM diethanolamine, 50mM NaCl and 5mM dithioerythritol, pH7.5. N-laurylsarcosine (sarcosyl) was added to 2% (w/v) and the cell suspension sonicated on ice for 7 x 30 seconds with 15 second intervals to allow for cooling. The sonicate was centrifuged at 27000 x g for 30min to remove bacterial debris and the supernatant applied to a Sephadex G25 gel filtration column to remove sarcosyl and exchange the buffer to PBS (required for affinity chromatography, section 2.3.2) (Frankel *et al.*,1991). The sephadex G25 column was prepared by swelling enough Sephadex G25-M to make a column of approximately 130ml, packed bed volume, in PBS (column buffer). The gel was packed into a column (2.5cm x 30cm) and washed with 100ml column buffer. The void volume of the column was calculated using blue dextran and found to be 45ml. Cell sonicate supernatant was applied to the column at a flow rate of 1ml per minute and

protein eluted with column buffer. Fractions (2ml) were collected and protein was detected by absorbance at 280nm on a Pharmacia single path UV monitor and recorded using a Pharmacia chart recorder. Fractions corresponding to the absorbance peak were analysed by SDS-PAGE and those containing protein were pooled. Fusion protein from pooled fractions was finally purified by affinity chromatography on glutathione sepharose (section 2.3.2).

2.3.4 Purification of rV antigen

A recombinant V-GST fusion protein was cloned using the pGEX plasmid (Pharmacia) by colleagues at CBD, DERA, Porton Down, Salisbury. The recombinant strain of *E. coli* expressing this fusion protein was grown as described in section 2.2.2 and purified (section 2.3.2).

2.4 Molecular genetics procedures

2.4.1 Isolation of DNA from *Y. pestis* strains

DNA was isolated from *Y. pestis* GB strain at CBD, DERA, Salisbury, using Marmur's modified method (1961), which extracts all, but mainly chromosomal DNA. The *caf1* gene encoding the F1 protein is carried on a plasmid, but sufficient plasmid DNA was extracted using this method to proceed with the cloning strategy.

2.4.2 Cloning of the *caf1* gene

A GST-F1 sequence map was used to develop a cloning strategy for the production of an F1-GST fusion protein using pGEX plasmids (Pharmacia).

The cloning strategy was to digest the PCR product of F1/5'D and F1/3'C primers (fig. 2.1) and the pGEX-5x-2 plasmid vector (Pharmacia) with restriction enzymes, EcoR1 and BamH1 as a forced cloning. Primers were also used to check the clones produced.

2.4.2.1 PCR

PCR was used to amplify the *caf1* gene from *Y. pestis* DNA, the predicted size of the PCR product was 450 base pairs. The amplified product was suitable for cloning into the pGEX-5x-2 plasmid vector.

Fig. 2.1 PCR primers used for cloning the *caf1* gene

F1/5'D and F1/3'C primers were constructed to give a predicted PCR product of450bp.

5' Primer:

BamH1 digest site

F1/5'D ACGCGGATCCCCGCAGATTTAACTGCAAGCACC 33-mer

----- (12-mer tail)

3' Primer:

EcoR1 digest site

F1/3'C GATCGAATTCTATATGGATTATTGGTTAGATAC 33-mer

stop stop (10-mer tail)

Using *Y. pestis* GB strain DNA as template (approx. 2ng μl^{-1}) and F1/5'D and F1/3'C primers at 125pmol μl^{-1} of each primer; 2µl GB DNA, 1µl each primer were added to the PCR mix containing 20µl of PCR buffer (x10 concentration, Boehringer Manheim), 2µl dNTPs (10mM each, Pharmacia), 0.5µl Taq polymerase (Boehringer Mannheim) and made up to 96µl with distilled water giving a total PCR volume of 100µl.

The PCR cycle used involved 1min at 96^0C for initial denaturation of DNA; 20sec at 96^0C for further denaturation; 20sec at 50^0C to allow annealing of DNA ; 30sec at 72^0C for final extension. The PCR product was checked by agarose gel electrophoresis.

2.4.2.2 Agarose gel electrophoresis

This was performed using a horizontal slab gel electrophoresis system (Biorad miniprotean II). Gels were formed using a gel casting tray (7 x 10cm tray for single gels, 15 x 15cm tray for double gels). Agarose gels were prepared by mixing the appropriate amounts of 1 x TAE buffer (appendix 1) with agarose to give 0.7% (w/v) gels for standard gels and 1% (w/v) gels for analysis of PCR products; this was then heated in a microwave oven at low power until the agarose had completely melted. The molten gel was allowed to cool until comfortable to the touch before adding ethidium bromide to a final concentration of 0.5µg ml^{-1} and pouring into the casting tray.

The gel was placed in the electrophoresis tank and covered with TBE buffer (appendix 1) containing 0.5µg ml^{-1} ethidium bromide. Samples were mixed with one sixth volume of sample buffer containing 0.25% (w/v) bromophenol blue, 40% (w/v) sucrose, 50mM Tris/HCl, 5mM EDTA, pH7.5, and loaded into the wells. DNA was separated by electrophoresis using a voltage across the gel of 80V until the dye front had migrated an appropriate distance through the gel. After electrophoresis, DNA was visualised by medium wavelength UV transillumination and photographed with a Polaroid camera.

2.4.2.3 Concentration of the PCR product

The product of the PCR reaction was concentrated using microconcentrators (Amicon). The sample reservoir in the microconcentrator was pre-washed with 0.5ml 0.1M NaOH. After adding 0.5ml sterile distilled water (dH_2O), the microconcentrator was centrifuged

(3000 x g for 3min) and reduced the volume by 50%, 100µl PCR product was added to the sample reservoir and 0.4ml dH_2O was added. This solution was centrifuged (3000 x g for 5min at 25^{0}C), reducing the sample volume to less than 100µl. This procedure was repeated until the sample volume had reduced to 10-20µl.

2.4.2.4 Ethanol precipitation of DNA

DNA was precipitated from solution by the addition of one tenth volume of 3M sodium acetate, pH5.2 and two volumes of ice-cold absolute ethanol. The solution was mixed and incubated at -20^{0}C for 15mins. After precipitation, DNA was recovered by centrifugation at 14000rpm for 15min at 4^{0}C, the supernatant was carefully removed and discarded. The DNA pellet was washed with 1ml of 70% (v/v) ethanol and, after carefully discarding the supernatant, the precipitate was allowed to air-dry for 10-15 min. The dried DNA pellet was finally resuspended in an appropriate volume of TE buffer (appendix 1).

2.4.2.5 Preparation of restriction digests

Restriction digests of the *caf1* PCR product and pGEX vector were prepared using EcoR1 and BamH1 restriction enzymes. Appropriate amounts of the restriction enzymes (Boehringer Manheim), purified DNA, incubation buffer (Boehringer Manheim) and dH_2O were mixed according to manufacturers instructions. These restriction enzymes gave a double digest and produced cohesive ends in both *caf1* DNA and the pGEX vector, digests were purified as described in section 2.4.2.3 and 2.4.2.4.

2.4.2.6 Ligation of restriction digests

A typical ligation reaction consisted of the pGEX and *caf1* restriction digests, ligation buffer, T4 DNA ligase (1 unit per ml) and dH_2O using a 1:3 vector:insert ratio. A ligation control was prepared including DNA molecular weight markers but without the restriction digest. Also, a vector self-ligation control was prepared where the pGEX restriction digest was used but not *caf1*. The ligation reaction was checked by agarose gel electrophoresis in order to visualise the increase in the molecular weight of the ligated DNA.

2.4.2.7 Transformation of *E. coli*

Ligations and controls were electroporated into *E. coli* JM109. Cells were grown to mid-log phase (A_{600} = 0.5-1.0) and harvested by centrifugation (10000 x g, 30min at 4^{0}C)

followed by washing the pellet with 500ml cold 10% glycerol, this was repeated using 250ml 10% glycerol. The final pellet was resuspended in 1-1.5ml of the glycerol solution. This suspension was divided into aliqouts of 50µl and stored at -70^{0}C until required.

Cells were thawed on ice, 40µl was mixed with 1-2µl DNA and incubated on ice for approximately 1min; the mixture was then transferred to a cooled electroporation cuvette (Biorad) and the solution pulsed 2.5kV, 25uF and 200ohms. The cuvette was removed and immediately 1ml SOC broth (table 2.3) was added and the suspension incubated at 37^{0}C for 1h in a polypropylene tube. The suspensions were diluted and plated onto L-ampicillin agar plates to select for transformed cells. After incubation at 37^{0}C for 18h, 30 recombinant colonies were picked across onto L-ampicillin plates, marked with a grid, using a disposable needle (Nunc), taking care to transfer only one colony at a time.

Colony lysates were prepared by boiling the inoculated dH_2O in a boiling water bath for 5min. The lysates were cooled and PCR reactions were prepared using F1/5'D and F1/3'C primers. PCR products were examined by agarose electrophoresis and positive clones identified by a 450bp product. Two positive clones identified in this way, JM109 + pKR4 and JM109 + pKR10, were selected and stock cultures of these prepared.

2.4.2.8 Culture lysates of the recombinant strains

Culture lysates of induced and non-induced cultures (section 2.3.2) were prepared by microfuging a small amount of culture for 4mins at high speed, the pellet was washed in 0.5ml PBS and the final pellet was resuspended in 50µl dH_2O. One fifth volume of SDS-PAGE sample buffer was added to give final concentration of 1%(w/v) SDS, 0.1%(v/v) mercaptoethanol, 10%(v/v) glycerol and 1mg ml^{-1} bromophenol blue. The suspension was heated in a boiling waterbath for 5min to lyse the cells and debris was sedimented by microfuging at high speed for 4min. Samples of the supernatant were then examined by SDS-PAGE and Western blot for the presence of F1-GST fusion protein.

2.4.3 Purification of plasmid DNA

Plasmid DNA was extracted and purified from uninduced recombinant *E. coli* using the Qiagen Plasmid Kit. The maxi-prep protocol was used according to the manufacturers instructions. The final pellet of plasmid DNA was gently resuspended in 200µl sterile

Table 2.3 Composition of SOC Broth

Component	Amount
Bacto tryptone	20g per litre
Bacto yeast extract	5g per litre
NaCl	10mM
KCl	2.5mM

The above components were added to 1L of Ultrapure water (Elgastat) and allowed to dissolve. The media was then autoclaved at 15psi for 15min.

Prior to use, the following filter-sterilised solutions at pH7 were added to give a final concentration of:

10mM MgCl2.6H2O
10mM MgSO4.7H2O
2mM glucose

dH2O and stored at -20^{0}C until required. The plasmid preparation was examined by agarose gel electrophoresis for the presence of covalently closed circular plasmid DNA.

2.4.3.1 Determination of DNA concentration in plasmid preparations

The optical density of diluted DNA samples were measured at 280nm and 260nm using paired quartz cuvettes, typical dilutions were 1:100 to 1:500. To calculate the DNA concentration; for short single stranded oligos an OD260nm of 1.0 is equivalent to 20μg ml^{-1} and for double stranded chromosomal and plasmid DNA, an OD260nm of 1.0 is equivalent to 50μg ml^{-1} . The ratio of OD260 : OD280 is then determined, the optimal ratio of 1.8:1 indicates DNA is relatively free of contaminating protein.

2.4.4 Automated cycle sequencing of DNA

2.4.4.1 Preparation of DNA template and primer

Template and primer DNA were added to a screw-capped eppendorf in the following quantities: 0.2pmol plasmid template, 10pmol primer, 1μl dimethyl sulphoxide (DMSO), this was made up to 16μl with dH2O. Dyedeoxy terminator sequencing chemistry was performed at CBD, DERA, using an annealing temperature of 50^{0}C in a Molecular Biology Lab. Station (Catalyst, Applied Biosystems). DNA sequencing samples were purified by phenol extraction to remove excess Dyedeoxy terminators section (2.4.4.2).

2.4.4.2 Purification of DNA sequencing samples

The volume of sample was made up to 100μl with dH_2O. A mixture of phenol, water and chloroform (ABI) (100μl) was mixed thoroughly with the sample (vortexed 2-4mins), the upper aqueous layer was retained and this process was repeated. The sequencing ladder was then precipitated by adding 20ml 10M ammonium acetate and 300μl ethanol for 10-15min at -20^{0}C. The mixture was centrifuged for 15min at maximum speed in a refrigerated microfuge, the ethanol was then removed and the pellet washed with 300μl ice-cold 70% ethanol. The solution was then centrifuged as before and all traces of ethanol removed and the pellet allowed to dry. Samples were then sequenced on a TAQ sequencer by dideoxy sequencing chemistry by CBD, DERA.

2.5 SDS-PAGE

2.5.1 Polyacrylamide gels

SDS-PAGE of proteins was essentially performed by the method of Laemmli (1970). Resolving gels contained 15% (w/v) acrylamide, 0.4% (w/v) bis-acrylamide in 0.375M Tris-HCl, 0.1%(w/v) SDS, pH8.8, and were prepared by diluting a 30% (w/v) acrylamide/0.8% (w/v) bis-acrylamide stock solution (Protogel) 1:1 in the appropriate buffer. Polymerisation of the gel was induced by the addition of 450µl of a 10% (w/v) ammonium persulphate solution and 45µl of N,N,N',N'-tetramethylethylenediamine (TEMED) to 60ml of the above solution prior to pouring into a gel casting chamber.

Stacking gels contained 4% (w/v) acrylamide, 1% (w/v) bis-acrylamide and 20% sucrose in 0.125M Tris-HCl, 0.1% (w/v) SDS, pH6.8. Polymerisation was induced by the addition of ammonium persulphate and TEMED in the same proportions as for the resolving gel. Prior to pouring the stacking gel, a comb of the appropriate size was placed on top of the resolving gel in the casting chamber, loading wells were formed by pouring the stacking gel with the comb in place.

2.5.2 Preparation of samples

Samples were diluted to the appropriate concentration in PBS, then diluted 4:1 in SDS-PAGE sample buffer to give a final concentration of 1% (w/v) SDS, 0.1% (v/v) mercaptoethanol, 10% (v/v) glycerol and 1mg ml^{-1} bromophenol blue. Samples were then heated at 100°C for 5min and allowed to cool before loading onto the gel. Up to 100µl or 40µl of sample were loaded onto 16cm or 6cm gels respectively.

2.5.3 Electrophoresis

Slab gels of either 16cm (Biorad, Protean II system) or 6cm (Hoeffer, Mighty Small II system) were used and electrophoresis carried out using a constant current of 50mA for 6h or 25mA for 90min respectively. The buffer used for electrophoresis contained 25mM Tris-HCl, 0.2M glycine and 0.1%(w/v) SDS, pH8.3.

2.5.4 Molecular weight determination

The molecular weight of proteins was determined using low range molecular weight markers (Pharmacia). The molecular weight of proteins was determined by comparing their electrophoretic mobilities with those of the molecular weight markers. Molecular weight was confirmed by image analysis (Millipore Bio Image System), the relative proportions of proteins in samples was also quantified in this manner.

2.5.5 Coomassie blue staining

Proteins were visualised by staining with 0.1%(w/v) Coomassie brilliant blue R-250, prepared by dissolving in 10% (v/v) glacial acetic acid and 25% (v/v) isopropanol. Gels were incubated for 4-5h in the above solution and destained by incubating with shaking in 40% (v/v) methanol, with several changes of destaining solution.

2.5.6 Image analysis of SDS-PAGE gels

Image analysis was carried out using either a Millipore Bio Image system or Bio-Rad Imaging Densitometer (model GS-690) to quantify the relative proportions of proteins in samples after SDS-PAGE and Coomassie blue staining.

2.5.7 Periodic acid Schiffs (PAS) stain for glycoproteins

Polyacrylamide gels were stained using a PAS stain protocol developed from published protocols (Matthieu and Quarles, 1973; Segrest and Jackson, 1971). Purified native F1 antigen was loaded onto a 15% polyacrylamide gel (30μg protein). After electrophoresis (2.5.3), proteins were fixed by immersing the gel in a mixture of ethanol, glacial acetic acid and distilled water in a ratio of 8:1:11 respectively, for 1h at room temperature. The gel was then incubated in 0.7%(v/v) periodic acid for 2-3h at room temperature with gentle agitation, followed by 2-3h incubation in 0.2%(w/v) sodium metabisulphite. Finally, the gel was incubated in Schiff's reagent (Sigma) for 12-18h at room temperature, excluded from light. Glycosylated proteins were detected as intense pink bands.

2.5.8 Determination of isoelectric point (pI)

The Pharmacia Phast Gel system, designed for the rapid electrophoresis of proteins, was used to determine the (pI) of F1 antigen. Pharmacia pre-packed IEF 4-6.5 gels, which contained carrier ampholytes to generate a stable linear pH gradient across the gel were

used. Purifed F1 antigen (section 2.3.1) was loaded onto gels and electrophoresed at 250V and 10mA for approximately 20min. Proteins were visualised by Coomassie blue staining, carried out automatically in the Phast system, using a pre-programmed method. The gel was calibrated using Pharmacia low pI calibration kit containing human carbonic anhydrase B (pI 6.6), bovine carbonic anhydrase B (pI 5.9), β-lactoglobulin A (pI 5.1), soyabean trypsin inhibitor (pI 4.6), glucose oxidase (pI 4.2) and amylglucosidase(pI 3.6). The pI point of F1 antigen was determined by comparing its electrophoretic mobility with those of the standards.

2.6 Structural studies on the F1 antigen of *Y. pestis*

2.6.1 N-terminal sequencing

Edmans reagent (phenyl isothiocyanate) is coupled to the free N-terminus under basic conditions, for example, aqueous trimethylamine supplied as a vapour. This produces the phenylthiocarbamyl (PTC) protein. The derivatized N-terminal amino-acid is cleaved using a strong, usually volatile acid, for example, trifluoroacetic acid. This produces a free unit, the anilinothiozolinone (ATZ) amino-acid and leaves the protein with a fresh N-terminus, which can be derivatized in turn. The ATZ amino-acid is unstable and needs to undergo a further reaction before analysis. The ATZ amino-acid is converted to the more stable PTH (phenylthiohydantoin) formed by reaction with aqueous acetonitrile is then injected onto the HPLC system for identification and quantitation. Identification of PTH amino-acids is by the retention time on the HPLC column.

Purified F1 antigen at varying concentrations was applied to SDS-PAGE gels (section 2.5), typically 2-20μg of protein was loaded. Following SDS-PAGE, proteins were transferred to nitrocellulose membranes by Western blot (section 2.7.2). Membranes were then stained with Amido black stain containing 0.1%(w/v) amido black, 25%(w/v) isopropanol and 10%(v/v) acetic acid to visualise the proteins. This was followed by destaining with a mixture of 25%(v/v) propanol and 10%(v/v) acetic acid. The F1 band of the appropriate intensity was selected for N-terminal sequencing using the method detailed above. Native and recombinant F1 were sequenced by the Protein and Nucleotide Chemistry section at CAMR.

2.6.2 Fast Atom Bombardment Mass Spectroscopy (FAB-MS)

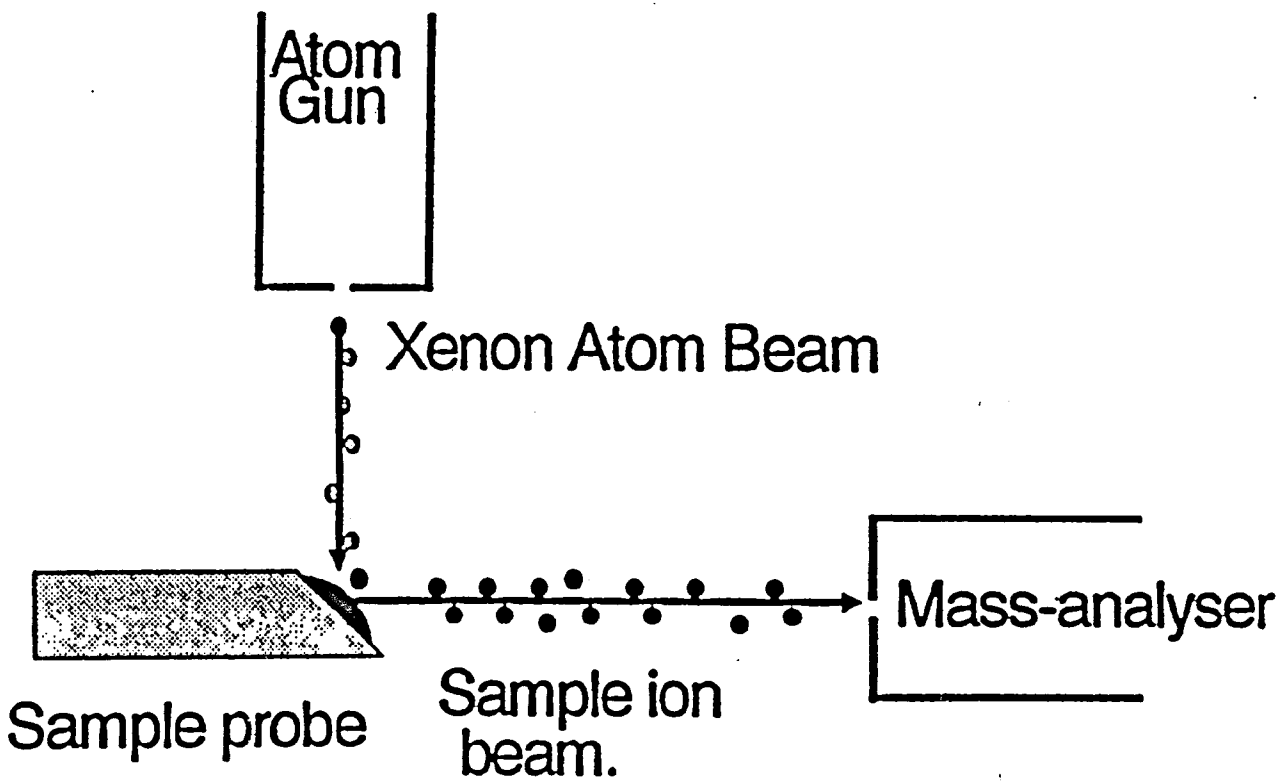
FAB-MS was used to determine whether any post-translational modification, such as glycosylation, was detectable by comparing the actual and calculated mass of peptide fragments from F1 antigen by FAB-MS.

In FAB-MS the sample is dissolved in a liquid of low vapour pressure, often glycerol, thioglycerol or diethrythrietol (this is referred to as the matrix), and is bombarded by a beam of energetic particles, such as xenon atoms, that sputter sample molecules from the surface layers of the matrix into the mass spectrometer vacuum. Proton or other cation attachment produces abundant positively charged ions characteristic of the molecular mass of the sample. Negatively charged ions are also generated and spectra may be recorded in either mode by selection of the polarity of the ion extraction voltages.

At low mass FAB, spectra are generally dominated by signals attributable to ionisation of the matrix. The background of "chemical noise" extending to high mass, which gives FAB spectra their characteristic peak-at-every-mass appearance, is probably attributable to direct hits on sample and matrix molecules by the bombarding species.

The technique is most effectively deployed in conjunction with conventional strategies for protein characterisation. There are, however, a number of considerations when using this technique. The purity and quality of the sample can determine the success of an FAB-MS experiment. During preparation of the sample, detergent-solubilisation steps should be avoided. Ionic materials employed in sample preparation, such as, ammonium sulphate, denaturing reagents, like urea and guanidium HCl and ion-pairing reagents can also seriously interfere with FAB-MS and need to be removed. Similarly, sodium and potassium salts, common in samples of biological origin, cause problems (Wait, 1993). Figure 2.2 shows a typical FAB-MS experiment. A number of different approaches were investigated.

Figure 2.2 Schematic diagram of a typical FAB-MS experiment



The sample, dissolved in a liquid of low vapour pressure on the probe tip, is bombarded with a beam of energetic particles, such as, xenon atoms, which sputters ionised sample molecules into the mass spectrometer vacuum. The resulting ions are then mass-analysed.

Wait (1993)

2.6.2.1 Acetolysis

Purifed native F1 antigen was examined by acetolysis (Naik *et al.*, 1985). The procedure is used as a preliminary to a more detailed structural study and is based on acetolysis of intact glycoconjugate generating peracetylated carbohydrate groups from oligosaccharides and proteins. Chloroform-extracted carbohydrate fragments are then analysed by FAB-MS.Any peptide fragments generated by acetolysis in addition to any salt, remain in the water layer and do not interfere with the subsequent mass spectrometric analysis. In this way, the presence of hexoses, aminohexoses and sialic acid can be determined. The aim of this was to determine whether acetolysis and subsequent FAB-MS can confirm the presence of carbohydrate.

F1 antigen (1mg) and ribonuclease A (positive control) were dissolved in a mixture of glacial acetic acid, acetic anhydride and sulphuric acid in a ratio of 10:1:1 respectively. The mixtures were heated to 60^0C, 0.2ml aliqouts were removed at 0.5, 2, 5, 8 and 24h and the reaction quenched by the addition of 1ml of distilled water. An equal volume of chloroform was added to extract the peracetylated carbohydrate fragments. The chloroform was allowed to evaporate and the resulting material examined by FAB-MS.

2.6.2.2 Tryptic digestion of F1 antigen

This series of experiments aimed to digest F1 antigen with trypsin, followed by separation of the resulting peptide fragments by HPLC and subsequent analysis of the fragments by FAB-MS.

F1 was digested using trypsin (bovine pancreas from Boehringer Manmheim) using a ratio of 50:1 respectively, digestion took place at 37^0C and samples were removed at 4, 6 and 18h; digestion was terminated by freezing at -20^0C. The resulting peptide fragments were examined by FAB-MS and compared with the calculated mass of fragments that should be produced by tryptic digestion. F1 antigen in its native state proved to be resistant to digestion and to overcome this problem, two approaches were used:

a) F1 in 10mM Tris/HCl pH8 was denatured by heating at 100^0C for 5min

b) Native F1 antigen was diluted in 8M urea and subsequently digested in 2M urea (Allen, 1983).

For both (a) and (b), tryptic digests were sampled at 4, 6 and 18h, digestion was terminated by freezing the samples at -20^{0}C. Samples were thawed and desalted on a PD10 G25 Sephadex column (Pharmacia) and the resulting protein solution was freeze-dried to concentrate the protein prior to HPLC.

In preparation for HPLC, samples were resuspended in 0.1% trifluoroacetic acid (10μg ml^{-1}). HPLC was carried out using a Browning Spheri-5 RP8 column, 200μg of tryptic digest was injected onto the column for each run and peptide fragments eluted using an acetonitrile gradient. Alpha-melanocyte Stimulating Hormone (α-MSH) was used as a positive control, this protein has two tryptic digest sites.

2.6.3 Electrospray ionisation mass spectroscopy (ESI-MS)

Electrospray ion production requires two steps; dispersal of highly charged droplets at near atmospheric pressure, followed by droplet evaporation. An electrospray is generally produced by application of a high electric field to a small flow of liquid (typically, 1 - 40μl/min) from a capillary tube. For effective ESI-MS, an appropriate liquid flow rate results in dispersion of the liquid as a fine mist, both thermally assisted or gas-nebulisation assisted electrosprays allow higher flow rates to be used, but decrease the extent of droplet charging.

The electric field results in charge accumulation on the surface of the liquid at the capillary terminus; therefore, the flow rate, resistance and surface tension are important factors in droplet production. The high electric field results in disruption of the liquid surface and formation of highly charged liquid droplets, positively or negatively charged droplets can be produced depending on capillary bias. The electrospray "source" is typically a metal or glass capillary incorporating a method for electrically biasing the liquid solution relative to a counter electrode. Solutions, typically water-methanol mixtures, containing analyte and often other additives, such as, acetic acid, flow to the capillary terminus. The principle requirement of an ESI-MS interface is to transport ions from the high-pressure region into the mass spectrometer as efficiently as possible (Geisow, 1991; Fenn *et al.*, 1990).

The components of ESI-MS instrumentation are; i)the electrospray source, or "tip", where electrostatic nebulisation of the liquid stream occurs, ii)the atmospheric/vacuum interface

where droplets and resulting ions are desolvated and transferred from near-atmospheric pressure to the vacuum environment of the mass spectrometer and iii) the mass spectrometer, typically of conventional design, where analysis occurs. In recent years ESI-MS has become an important tool in the analysis of peptides and proteins. Figure 2.3 is a schematic of a typical ESI-MS experiment.

Native F1 antigen in distilled water (1mg ml^{-1}) was examined by ESI-MS at CBD, DERA. The molecular weight obtained by ESI-MS was compared to the molecular weight as predicted from the nucleotide and deduced amino-acid sequence.

2.7 Vaccine Delivery

2.7.1 Preparation of PLG microspheres

The F1 antigen was microencapsulated in PLG microspheres by an emulsion-based process (O'Hagan *et al.*, 1994; Jones *et al.*, 1996).

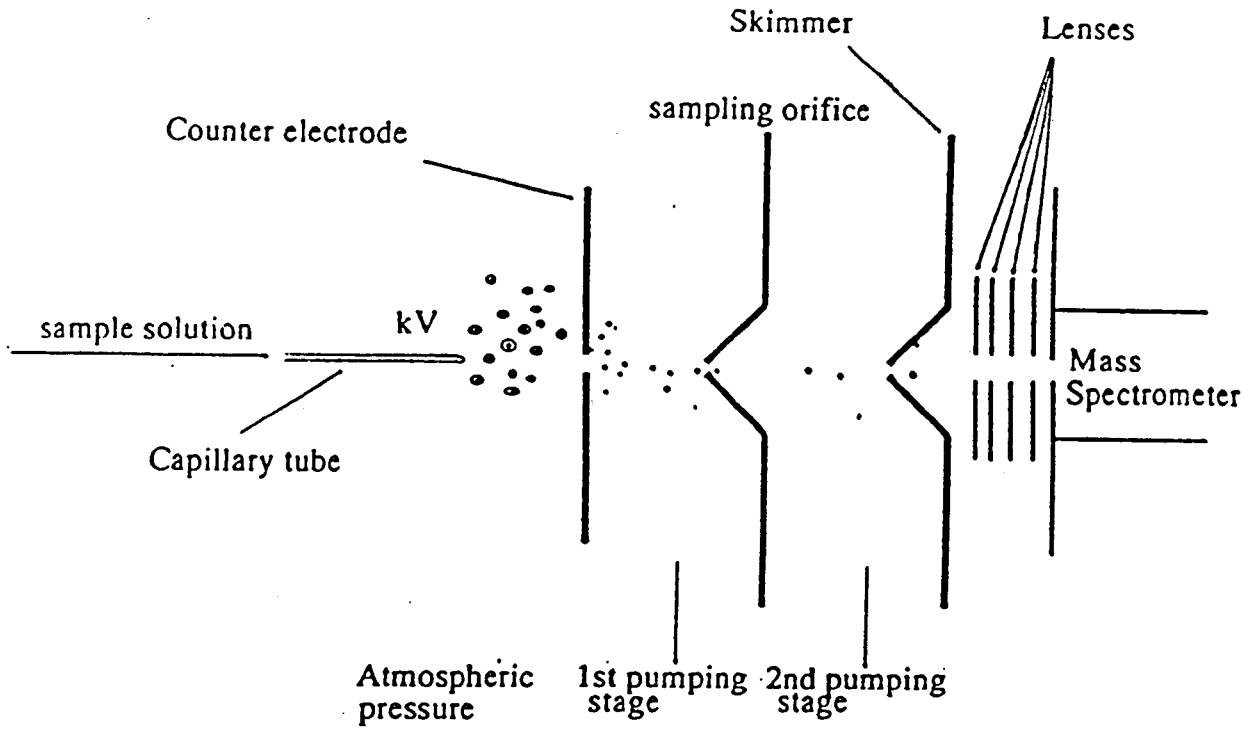
2.7.1.1 8μm microspheres

500mg of Poly (D,L-lactide-co-glycolide 50:50) PLG (Resomer RG506, Alpha Chemicals Ltd) was dissolved in 3ml dichloromethane and mixed with 0.3ml F1 (20mg ml^{-1}). This suspension was emulsified using a Silverson homogeniser for 2.5min at half maximum speed. The emulsion was then added to 100ml 8%(v/v) polyvinylalcohol (PVA) and emulsification continued for a further 2.5min. This suspension was immediately added to 1L distilled water, stirring continuously. The resulting microspheres were washed and centrifuged at 5500 x g for 33min, followed by two washes at 5500 x g for 18min. The final pellet of microspheres was resuspended in a small amount of distilled water and gently homogenised using a hand held homogeniser to ensure an even suspension. The PLG microspheres were then freeze-dried and stored under vacuum over dessicant at 4^0C until required.

2.7.1.2 1μm microspheres

1μm microspheres were prepared in the same way as the 8μm spheres, except 1.54g PLG was dissolved in 14.2ml of dichloromethane; 4.6ml of this solution was mixed with 0.4ml

Fig. 2.3 Schematic diagram of ESI-MS



A fine spray of charged droplets emerges from the capillary; as the droplets evaporate, multiply charged sample ions are ejected and pass through the sample orifices into the mass spectrometer.

Wait, 1993

protein solution (0.3ml F1 at 20mg ml^{-1} in 0.1ml distilled water). The emulsion was then added to 50ml 8%(v/v) PVA and treated as described 2.7.1.1

2.7.2 Sizing of microspheres

2.7.2.1 Coulter counter

After calibration with latex beads of the appropriate size, microspheres were sized using a Coulter counter ZM with a 30μM aperture orifice tube, the preset gain was set at 1.0, attenuation at 16.0 and a current of 1000mA was used.

2.7.2.2 Preparation and examination of microspheres by SEM

Lyophilised microsphere samples were dusted onto double-sided sticky carbon tabs mounted on 1/2 inch aluminium SEM specimen tabs. Specimens were then coated with approximately 10nm of gold-palladium (Au/Pd, 60:40) by ion-beam sputtering prior to examination in a Philips XL30 FEG scanning electron microscope operated at 4kV. Micrographs were recorded on Kodak T-max 100 film.

2.7.3 Quantification of antigen in microspheres

A 5mg sample of microspheres were resuspended in 50μl 100mM NaOH containing 1%SDS. Samples were then heated at 100^0C for 4min in a boiling waterbath to disrupt the microspheres. After cooling, samples were microfuged at 13000rpm for 10min and the supernatant retained. The protein concentration in each sample was estimated using the BCA protein assay. From this, the entrapment efficiency of the microspheres was calaculated:

$$\frac{\text{μg protein per mg microspheres}}{\text{μg protein per mg PLG polymer used}} \times 100\% = \text{entrapment efficiency}$$

2.7.4 *In vitro* release of antigen from microspheres

A 50mg sample of microspheres were resuspended in 500μl PBS containing 0.05%(w/v) sodium azide. Microspheres were then incubated at 37^0C on a Rotamix revolving eppendorf rack in eppendorfs sealed by "O-rings". Samples (50μl) were removed at intervals up to 42 days, then microfuged at 13000rpm for 10min and the supernatant stored at -20^0C until required.

The amount of protein in each sample was estimated using the Pierce BCA protein assay by either the standard or enhanced protocol as appropriate according to manufacturers instructions.

2.7.5 Preparation of liposomes

Liposomes were prepared using the dehydration-rehydration method of Gregoriadis (1991, 1989). Liposomes encapsulating antigen were composed of dipalmitoylphosphatidylcholine (DPPC),CH and dicetyl phosphate (DCP) in the molar ratio 7:2:1 respectively. Lipids were dissolved in chloroform prior to evaporating the solvent under reduced pressure to leave a film of lipid which was then lyophilised.

Liposomes surface-labeled with antigen were made up of distearoylphosphatidylcholine (DSPC), hydrogenated phosphatidylserine (DPPS), CH and the N-hydroxysuccinimide ester of palmitic acid (NHSP) in the molar ratio of 1:1:2:0.56 respectively. The lipids were dissolved in a mixture of chloroform and methanol (9:1, v/v). The lipid film was produced by evaporation as described above.

For liposomes encapsulating antigen, the lipid film was rehydrated in 4ml of solution containing 4mg ml^{-1} of antigen in PBS, pH7.2 and then incubated in at 58^0C for 15mins. However, for surface-labeled liposomes, the first step is to produce "ghost" liposomes by adding 2ml PBS, pH7.2 to the lipid film and incubating as above.

The lipid mixture was then freeze-thawed several times at 58^0C to ensure that the lipid film was completely dissolved. The liposomes were formed by extrusion through 0.4μm Nucleopore polycarbonate (PC) membranes (Costar) for 2 cycles followed by extrusion through a 0.2μm PC membranes for 8 cycles.

The "ghost" liposomes produced in this way were then surface-labeled with the F1 antigen by adding 2ml of antigen solution (8mg ml^{-1} in PBS, pH7.2) to the empty liposome vesicles. This mixture was then incubated at 37^0C for 18h sealed under nitrogen to allow the condensation reaction of antigen onto NHSP on the liposomal surface.

The liposomes were separated from free antigen by centrifugation at 100000 x g for

60min, followed by two washes in PBS, pH7.2. The final liposome pellet was resuspended in the same buffer.

2.7.6 Sizing of liposomes

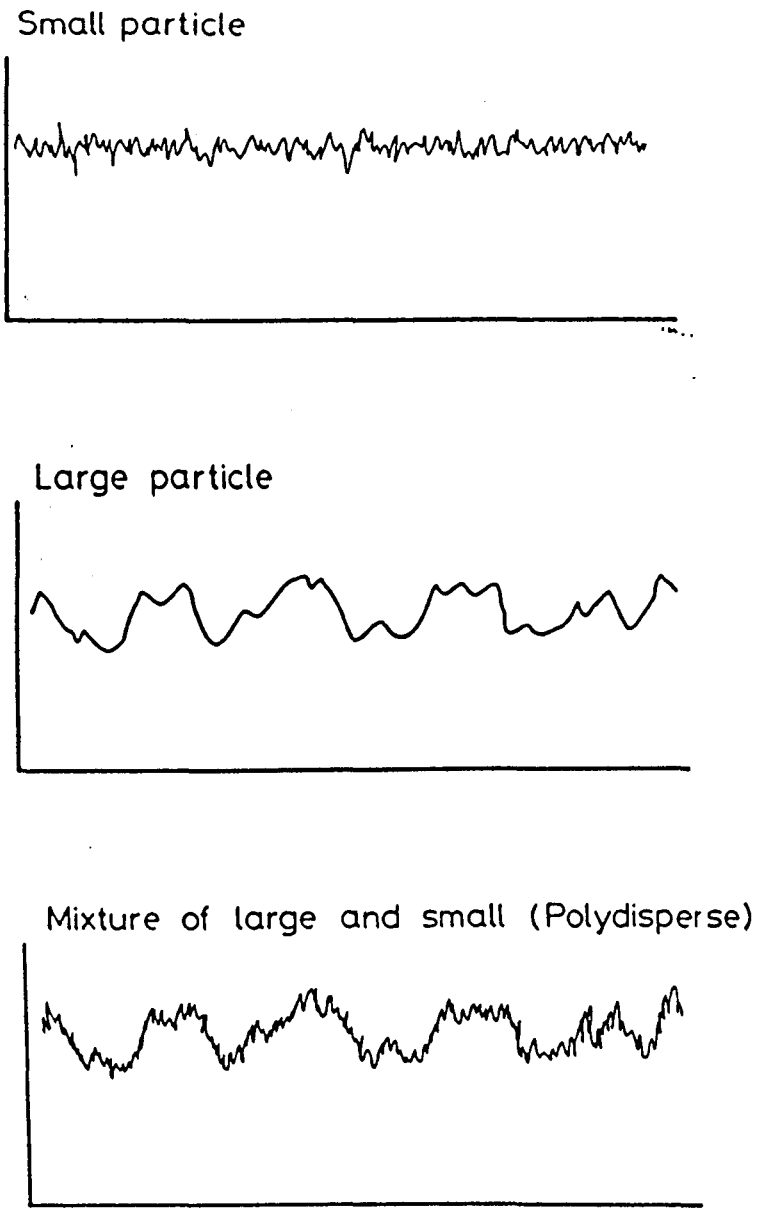
Two techniques were used, the first based on laser light-scattering by particles in solution and the second invoving direct observation of the liposomes by electron microscopy.

2.7.6.1 Photocorrelation spectroscopy (PCS)

The size and size-distribution of liposomes was determined by PCS using a BI-90 particle sizer (Brookhaven Instruments). PCS is the analysis of the time dependence of intensity fluctuations in scattered laser light due to Brownian motion of particles in solution or suspension. Since small particles diffuse more rapidly than large particles, the rate of fluctuation of scattered light intensity varies accordingly; the movement of large particles in a fluid medium is slower than for small particles, and the fluctuations in light intensity are therefore, correpondingly slower, large particles produce scattered light of a greater intensity than smaller particles (fig.2.4). Using the BI-90 it was possible to measure particles in the range of about 5nm to 5μm. A laser light source (helium, neon or argon) is focused on the contents of a cuvette containing the sample. The cuvette is housed within a thermostatically controlled cell. The temperature of this compartment should preferably be within +/- 0.1^{0}C of that required. This minimises any potential errors due to variation in fluid viscosity, but more importantly, will minimise random convection currents superimposed onto Brownian movement which could lead to substantial errors in particle size measurement.

The temperature at which PCS was performed was 25^{0}C, the machine equilibrated to this temperature automatically before commencing measurments. Scattered laser light from the sample was detected by a photomultiplier assembly situated at an angle of 90^{0} relative to the laser beam. Once the signal was recorded as a series of photomultiplier bursts over a period of time the mathematical process of correlation was carried out automatically. Data was obtained as mean particle size, these measurements were amplified and measurements at different time points were analysed to give a correlation function.

Fig. 2.4 Fluctuations in light intensity in relation to particle size.



Fluctuations in light intensity of a beam of laser light passing through a suspension of particles occur because of movement of the particles in the beam as a result of Brownian motion. The movement of large particles in a fluid medium is slower than for small particles, and fluctuations in light intensity are therefore correspondingly slower.

All buffers used for suspension of liposomes were filtered through a 0.2μm minisart filter before PCS to eliminate problems caused by dust. The samples were diluted to give a suitable count rate of between 30000-150000, typically 15-25μl sample was added to 3ml of filtered PBS and then measured.

2.7.6.2 Preparation and examination of liposomes by transmission electron microscopy (TEM)

Approximately 3μl of liposome suspension (sufficient to form a shallow meniscus) was applied to a 400 mesh copper electron microscopy specimen grid coated with a formvar/carbon film. After approximately 30sec. the suspension was removed by touching the edge of the grid with a piece of moist filter paper.

A similar volume of negative stain (1% sodium silicotungstate, pH6.8) was then applied to the grid and after 10sec removed in a similar way. The grid was then allowed to air dry (approx. 5min) prior to examination in a Philips CM100 transmission electron microscope operated at 80kV. Micrographs were recorded on Ilford EM technical film. Fig. 2.5 shows a schematic diagram of negative staining for TEM.

2.7.7 Quantification of antigen in liposomes

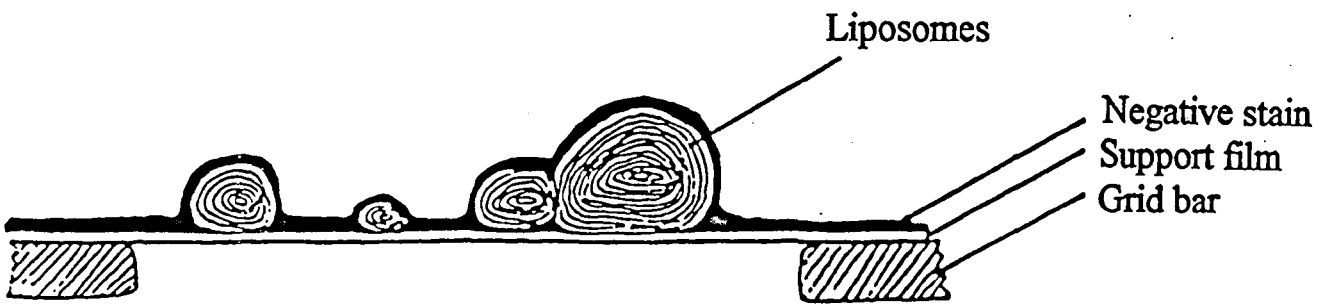
The enhanced BCA protein assay (Pierce) was used to estimate the amount of protein in liposome preparations. Fifty microlitres of liposome solution was mixed with an equal amount of PBS and to this 10μl of 10%(v/v) Triton X-100 was added to lyse the liposomes. The mixture was then assayed using the BCA enhanced protein assay protocol according to the manufacturers instructions. For surface-labeled liposomes, the above method was used but the addition of Triton X-100 was omitted because the liposomes needed to be intact.

2.8 Immunological techniques

2.8.1 Enzyme linked immuno-absorbent assay (ELISA)

ELISA was used for the detection of specific antibodies in murine and rabbit sera. The appropriate purified antigen was coated onto 96well plates (Nunc maxisorb immuno-plate) by placing 100μl of a 10μgml-1 solution diluted in carbonate coating buffer (0.05M

Fig. 2.5 Negative staining of liposomes for electron microscopy



Liposomes are embedded in a thin film of negative stain, electron-transparent material shows up as bright areas and electron-dense material as dark areas on the electron micrograph.

New (1994)

sodium carbonate, pH 9.6) into each well. Following overnight incubation at 4^0C, the plates were washed 3 times with PBS containing 0.01% (v/v) Tween 20, after which 250µl of PBS containing 0.01% (v/v) Tween 20 and 4% normal horse serum (Gibco) were added to each well and incubated for 1h at room temperature to block any unoccupied sites in the wells.

Serial dilutions of test sera were prepared in blocking buffer in the blocked ELISA plates to produce a final volume of 100µl per well, plates were then incubated at room temperature for 2h. After further washing 100µl of an appropriate dilution of anti-Ig class-specific and species specific antibody conjugated to HRP was added to each well and incubated for a further 1h. Finally, the plates were washed and colour development carried out using N,N,N',N' - tetramethylbenzidene (TMB) substrate solution prepared using TMB tablets (Sigma) and prepared according to manufacturers instructions. To each well, 100µl of TMB solution was added and incubated, with shaking, for 10min at room temperature. The reaction was stopped by the addition of 25µl of 2M sulphuric acid to each well and absorbances measured at 450nm on a Titretek (MCC 340) ELISA reader. All plates contained wells from which the primary antibody had been omitted to allow non-specific background levels of absorbance due to conjugate binding to be determined. ELISA titres were expressed as the reciprocal of the serum dilution required to give 50% endpoint absorbance.

2.8.2 Analysis of spleens and Peyer's patches (PPs)

Spleens were removed from animals into RPMI tissue culture media (ICN Flow laboratories) and pooled according to treatment group. A crude suspension of spleen cells was prepared by sieving through a narrow gauge wire mesh. Lymphocyte suspensions were then prepared by density gradient separation using Hyacolin (Sigma). PPs were treated in the same way and in both cases lymphocytes were used in a modified ELISA for the detection of antibody-secreting cells (ASCs) (Czerinsky *et al.* 1988).

2.8.2.1 Assay of ASCs in spleen and PPs *in vitro*

A modifed ELISA was used in which sterile microtitre plates (Nunc maxi-sorp) were coated with F1 in PBS (1µg ml^{-1}) overnight at 4^0C under aseptic conditions. The plates were blocked with RPMI containing 10% (v/v) foetal calf serum (FCS) for 1h at 37^0C.

Samples (200µl) of lymphocytes from spleen or PP's (2 x 10^7 cells) were added to the first well of the appropriate row on the assay plate, doubling dilutions were then prepared for each sample. Plates were covered and incubated at 37^{0}C, 5% CO_2 for 20h. The plate were then gently washed (x3) in PBS containing 0.01% (v/v) Tween 20, prior to the addition of peroxidase-labeled anti-mouse immunoglobulin conjugate (IgG, A or M from Jackson). Plates were incubated for 1h at 37^{0}C and washed as before. Soluble TMB substrate (100µl per well) was added and the colour reaction allowed to develop for up to 20min at 20^{0}C, the reaction was stopped by the addition of 50µl per well of 2M sulphuric acid. Colour development was measured at 450nm (Titertek Multiscan MCC, ICN Flow). Lymphocytes from spleens and PP's from unimmunised mice were used as controls.

2.8.3 Western blotting

Proteins separated by SDS-PAGE were electrophoretically transferred onto nitrocellulose membrane (Schleicher and Schuell) with a pore size of 0.2µm. SDS-PAGE gels were soaked prior to blotting in renaturation buffer (appendix 1) as this enhanced the detection of F1 antigen by Western blot. Membranes were wetted with transfer buffer containing 25mM Tris/HCl, 192mM glycine, pH8.3. Transfer was carried out in a Hoeffer TE-22 Transphor unit (6cm gels) or a Bio-rad Transblot cell (16cm gels) containing transfer buffer at 30mA, 4^{0}C overnight.

After transfer, membranes were incubated for 2h in PBS containing 0.01% (v/v) Tween 20 and 1% (w/v) skimmed milk powder to block unoccupied sites on the membrane. Following this blocking step, the membrane was washed three times for 10min periods in PBS containing 0.01%(v/v) Tween 20 and then probed by incubation for 1-2h with an appropriate dilution of test antibody or sera in PBS containing 0.01% (v/v) Tween 20. After further washing, the membrane was incubated for 1h with an appropriate dilution of an anti-Ig class-specific and species-specific antibody conjugated to horseradish peroxidase (HRP). After further washing the membrane, but this time in PBS alone, colour development was carried out using the substrate 4-chloro-1-napthol were dissolved in 1ml methanol and 200µl of this solution was added dropwise, with stirring, to 20ml of PBS, followed by the addition of 10µl of hydrogen peroxide. Membranes were incubated in this solution and colour development was stopped, when appropriate, by rinsing in water and allowing the membrane to air-dry.

2.8.3.1 Molecular weight determination

Membranes were calibrated by loading biotinylated molecular weight markers (Pharmacia, low range) onto the gel to be transferred. Development was carried out as described in 2.6.2, with the addition of streptavidin (Amersham) diluted 1:1000 in PBS for 1h as an additional incubation step. Prestained molecular weight markers (Bio-Rad, low range) were also loaded onto the gel to be transferred. These were used only to confirm the efficient transfer of proteins and the positions of relevant proteins on membranes and not for precise molecular weight determination.

2.8.3.2 Detection of glycoprotein by enzyme immunoassay

To confirm the PAS stain (2.5.6), F1 antigen was examined using a dioxygenin (DIG) glycan detection kit (Boehringer Mannheim). In this assay, adjacent hydroxyl groups in sugars are oxidised to aldehyde groups by mild periodate treatment. The spacer-linked steroid hapten, dioxygenin (DIG), is then covalently attached to these aldehydes via a hydrazide group. DIG-labelled glycoconjugates are subsequently detected in enzyme immunoassay using a DIG-specific antibody conjugated to alkaline phosphatase.

Purified F1 was electrophoresed on a 12% polyacrylamide gel, together with glycoprotein positive (transferrin) and negative (creatinase) controls and proteins transferred to a 2μm nitrocellulose membrane. The membrane was then stained with the Ponceau S stain supplied in the kit and the positions of the molecular weight markers, F1 and controls marked. The membrane was then probed using DIG glycan detection kit reagents to detect glycoproteins bound to the membrane, a positive result was recorded if a dark grey/black band developed.

2.8.4 Immunisations

2.8.4.1 Rabbit serum

A New Zealand White rabbit was immunised with purified native F1 antigen for the production of anti-F1 polyclonal serum. For the primary immunisation, the rabbit was given a total of 350μg of antigen, mixed with Alhydrogel as adjuvant, using both intramuscular and subcutaneous injections. Approximately 3weeks later the rabbit was boosted with the same dose of antigen mixed with Alhydrogel. The rabbit was then bled from a superficial vein and the serum antibody titre determined. On the basis of this assay,

a further booster immunisation of the same dose as above was administered. Two weeks later the rabbit was bled as before and, after overnight incubation at 4^0C, the serum was separated from the whole clotted blood. The serum was centrifuged at 6000 x g for 5min to remove any remaining red blood cells, then aliquoted and stored at -20^0C.

2.8.3.2 Immunisation of mice

For serological and mucosal antibody response studies, groups of 5 mice were immunised with either antigen adsorbed to Alhydrogel, 1μm or 8μm sized PLG microspheres or with liposome formulations. Single or multiple doses were given i.p., intranasally or by gavage and sera collected, separated and stored as above.

Intraperitoneal immunisation

Mice were immunised i.p with purified antigen mixed with Alhydrogel to give a final concentration of 50μg ml^{-1}, each animal received a 10μg dose i.p. Alternatively, the appropriate dose of PLG microspheres or liposomes was injected i.p. in PBS. Mice were immunised with one dose or three doses given at two week intervals.

Oral immunisation

Sufficient freeze-dried PLG microspheres were resuspended in 0.1M sodium bicarbonate to give a solution of 50μg ml^{-1}. Aliquots (0.2ml) of this solution were given to individual mice by oral gavage. Control groups received 0.2ml of non-encapsulated antigen (50μg ml^{-1}) in PBS. Sufficient liposome suspension was given by oral gavage so that each animal received 6–10μg antigen as required.

Intranasal immunisation

For immunisations given i.n., mice were lightly anaethestised with diethyl ether, turned ventral side uppermost and the appropriate dose of non-encapsulated antigen, PLG microspheres or liposomes was administered to the nostrils in PBS. The vaccine formulation was allowed to be completely inhaled by the mouse by slowly releasing it into the nostrils using an automatic displacement pipette (Finnpipette).

2.8.4 Collection and preparation of murine samples

Sera

Six to eight week old female Balb/c mice (Charles River Laboratories) were immunised with various antigen formulations for the production of polyclonal sera as described above. Serial bleeds were taken from the major tail vein and mice were terminally bled by cardiac puncture, whilst under anaesthesia, the sera were separated and stored as in section 2.8.3.1.

Faecal pellets

Freshly voided faecal pellets were collected from animals and stored at -70^0C until required. Prior to assays, pellets were resuspended in PBS (50µl per pellet), vigorously vortexed and incubated at 20^0C for 15min. Samples were again vortexed and centrifuged at 1000 x g for 5min. The supernatants were collected and used immediately for ELISA (section 2.8.1).

Saliva

Saliva samples (approximately 50µl) were collected in capillary tubes following i.p. injection of mice with 0.1ml of a 1mg ml^{-1} aqueous solution of pilocarpine. Samples were transferred to Eppendorf tubes and stored at -70^0C until required for assay.

2.8.5 Production of monoclonal antibody

A hybridoma cell line secreting an IgA anti-F1 monoclonal antibody was obtained from NCTC. The hybridoma cell line was cultured by ECACC, CAMR and tissue culture supernatant collected. This tissue culture fluid was used in the immunological methods described above.

2.9 Protection studies using a mouse sub-cutaneous challenge model

2.9.1 Immunisation of mice

In the protection experiments, a single 10µg dose or multiple doses of F1 antigen encapsulated in 1µm or 8µm PLGmicrospheres were given i.p., orally or i.n. to groups of 8 to 10 mice on day 0. The same doses of antigen in Alhydrogel was also administered to other groups.

Mice were immunised with single or multiple doses of liposome-antigen formulations, given either i.p., orally or i.n. All immunisations were given in doses of 6 to 10μg of antigen.

Whole cell vaccine (Greer Laboratories) was given i.m. in two doses, on day0 and day16 of the immunisation schedule. Each dose consisted of 0.1ml of vaccine (representing one fifth of the human dose) and contained approximately 2 x 10^8 formaldehyde-killed plague bacilli.

2.9.2 Sub-cutaneous challenge

Challenge inocula were prepared and infection carried out at CBD, DERA. Mice were challenged with 10^3, 10^5 or 10^7 colony forming units (cfu) of *Y. pestis* GB strain. This strain was isolated from a fatal case of plague and has an LD_{50} of <1 cfu in Balb/c mice by the sub-cutaneous route (Russell *et al.*, 1995). Survivors were monitored up to 14 days after challenge for development of symptoms. Animals which succumbed to challenge were autopsied, livers and spleens were scored for enlargement and any evidence of abnormality was noted. Samples of blood, sections of liver and spleen and swabs from the challenge site were smeared onto Congo Red agar or Yersinia selective agar (YSA, Oxoid). The plates were incubated at 28^0C and observed for growth after 48h. At the end of the 14day observation period, survivors were killed and tissues removed for bacteriological and gross morphological analysis as above.

Chapter 3

Purification of F1 antigen

3.1 Introduction

The aim of this study was to purify and characterise the native F1 antigen from *Y. pestis* as well as a recombinant F1 protein, to determine the contribution of these antigens to the protective immune response and to assess their suitability as component antigens in an acellular vaccine. In order to do this, an earlier extraction method (Baker *et al.*, 1952) was modified and used to optimise the conditions for the production of large quantities of highly purified F1 antigen.

The F1 antigen of *Y. pestis* was first extracted by Baker *et al.* (1952) from acetone-dried cells grown on agar plates using a solution of toluene-saturated sodium chloride. The antigen was partially purified by differential ammonium sulphate precipitations, which yielded two F1-containing fractions, 1A and 1B. Fraction 1A consisted of protein and carbohydrate, whereas fraction 1B was composed of protein only. In Baker's extraction method, F1 was the primary component of both fraction 1A and fraction 1B, however, other *Y. pestis*-specific antigens were most likely present in these early preparations and these were used for the early plague protection studies in animal models (Burrows and Bacon, 1956). Early purification studies suggested that F1 is released from the bacterial cell and is present in the culture supernatant (Englesberg and Levy, 1954; Baker *et al.*, 1952).

The rF1 antigen in this study was produced as a GST-fusion protein in *E. coli*. Gene fusion systems have been successfully used to facilitate the expression of proteins and peptides. A number of systems have been developed for this purpose. Most commonly, peptides have been fused to β-galactosidase and expressed in *E. coli* using the ribosome binding site and translation start of the *lac Z* gene (Casadaban *et al.*, 1980; Koenen *et al.*, 1982). Such hybrid proteins can be purified by affinity chromatography (Ullman, 1984). Other vectors direct the synthesis of peptides as fusions with staphylococcal protein A (Nakajima *et al.*, 1995), which can be purified by affinity chromatography on IgG Sepharose (Uhlen *et al.*, 1983; Lowenadler *et al.*, 1986). Disadvantages of such methods include the possibility that denaturing reagents used during purification may alter the antigenic and functional properties of the purified product. An additional problem with the protein A system is that the binding of fusion proteins to IgG complicates the immunological analysis. Other

expression strategies involve the synthesis of polypeptides containing polyarginine at their C-terminus which can be purified by cation-exchange chromatography (Sassenfeld and Brewer, 1984) or the production of peptides or proteins from recombinant bacterial strains that secrete the peptide or protein into the culture medium (Andrews *et al.*, 1996). An alternative approach, which may avoid several of the difficulties described, is to fuse the protein of interest to a specific binding protein, such as GST or maltose binding protein. Maltose binding protein (MBP) is the product of the *mal E* gene from *E. coli* K12 (Maina *et al.*, 1988). A MBP fusion protein can be affinity purified by binding to amylose resin. The fusion protein is then eluted with free maltose and the protein of interest is then cleaved using factor Xa protease. The MBP is then separated from the free protein of interest by repeating the affinity chromatography with amylose resin. GST is a protein purified from *Schistosoma japonicum* and binds glutathione. The GST fused to the protein of interest can be purified by affinity chromatography on Glutathione B sepharose. The protein can then be cleaved from the GST using factor Xa, which can be done while the fusion protein is bound to the column or after eluting the fusion protein with free glutathione, followed by further affinity chromatography. The GST method has been used to produce a recombinant V antigen (Leary *et al.*, 1995) and was the method employed here for the production of a recombinant F1.

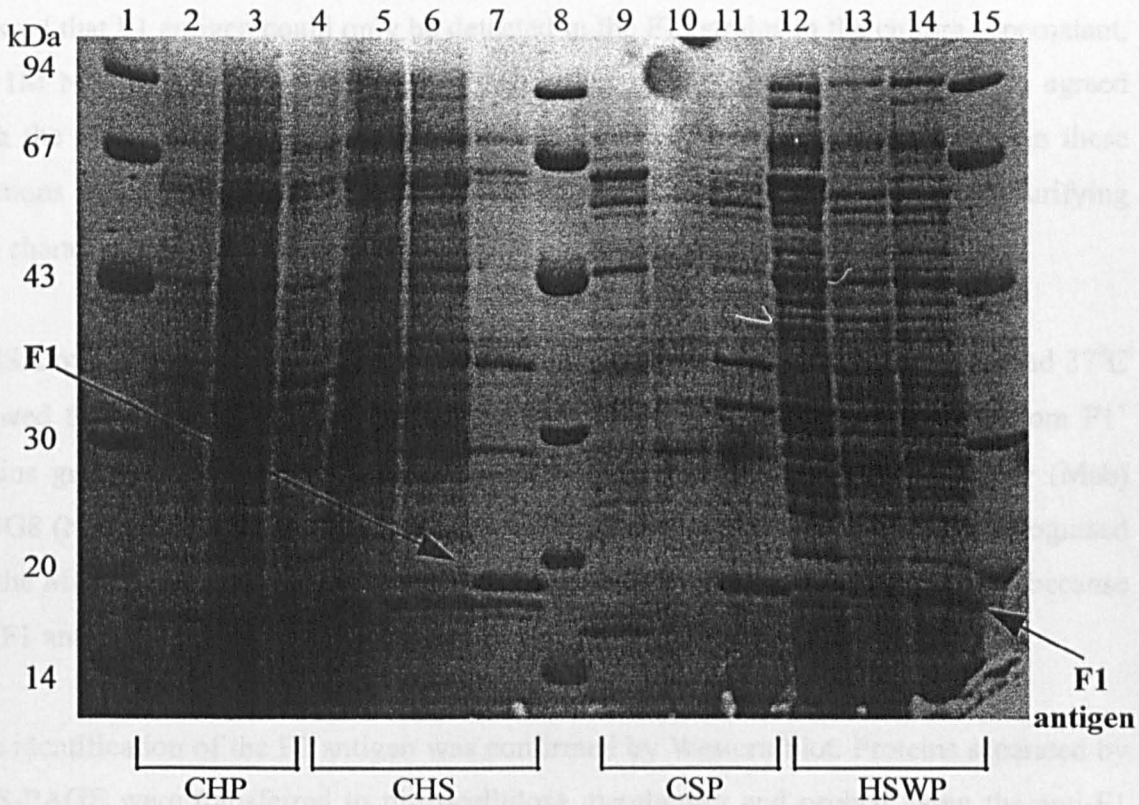
3.2 Purification of Native F1 Antigen

3.2.1 Detection and identification of the F1 antigen

Two $F1^+$ strains of *Y. pestis* were examined, MRE 1176 and MRE 1447.The $F1^-$ strain MRE 2486 was also examined as a negative control. The strains were grown at 37°C in chemically defined medium (Straley and Bowmer, 1986). Cells were harvested and then homogenised using glass beads in a bead beater (Stratech Ltd). Proteins in the culture supernatant were precipitated by the addition of acetone cooled to -20°C (Glosnicka and Gruszkiewicz, 1980). Proteins were similarly precipitated from homogenised whole cells and from the cell homogenate supernatant.

A sample of whole cells from the cell pellet was washed with a solution of 1M NaCl. The supernatant from this salt wash was retained and the proteins precipitated using cold acetone as above. The acetone precipitates were examined by SDS-PAGE (fig. 3.1). A 17.5kDa band was seen in the $F1^+$ strains but not in the $F1^-$ strain. This band was especially

Fig. 3.1 Detection of the Fraction 1 (F1) antigen from *Y. pestis* by SDS PAGE



Lane 1, 8 and 15 , molecular weight markers; Lane 2, 5, 9 and 12, MRE 2486 (F1-); Lane 3, 6, 10 and 13, MRE 1176 (F1+); Lane 4, 7, 11 and 14, MRE 1447 (F1+)

CHP :	Pellet from homogenised cells
CHS :	Supernatant from homogenised cells
CSP :	Proteins in precipitate of culture supernatant
HSWP :	Proteins in precipitate of 1M NaCl wash

evident in MRE1447 and was tentatively identified as the F1 antigen. The F1 antigen band was evident in the salt wash, culture supernatant and the supernatant from homogenised cells; however, it was not clear in the pellet fraction of homogenised cells.

Protein precipitated from the cytoplasm, cell homogenate, culture supernatant and 1M NaCl wash were tested by ELISA using monoclonal antibody, MAb F13G8, an IgA monoclonal antibody from the NCTC which is specific for the F1 antigen. The results showed that F1 antigen could only be detected in the $F1^+$ strains in the culture supernatant, the 1M NaCl wash and the cell homogenate supernatant (fig. 3.2). These findings agreed with the SDS-PAGE results and confirmed that MRE 1447 produced more F1 in these fractions than MRE 1176. Therefore, future experimental work concentrated on purifying and characterising the F1 antigen from MRE 1447.

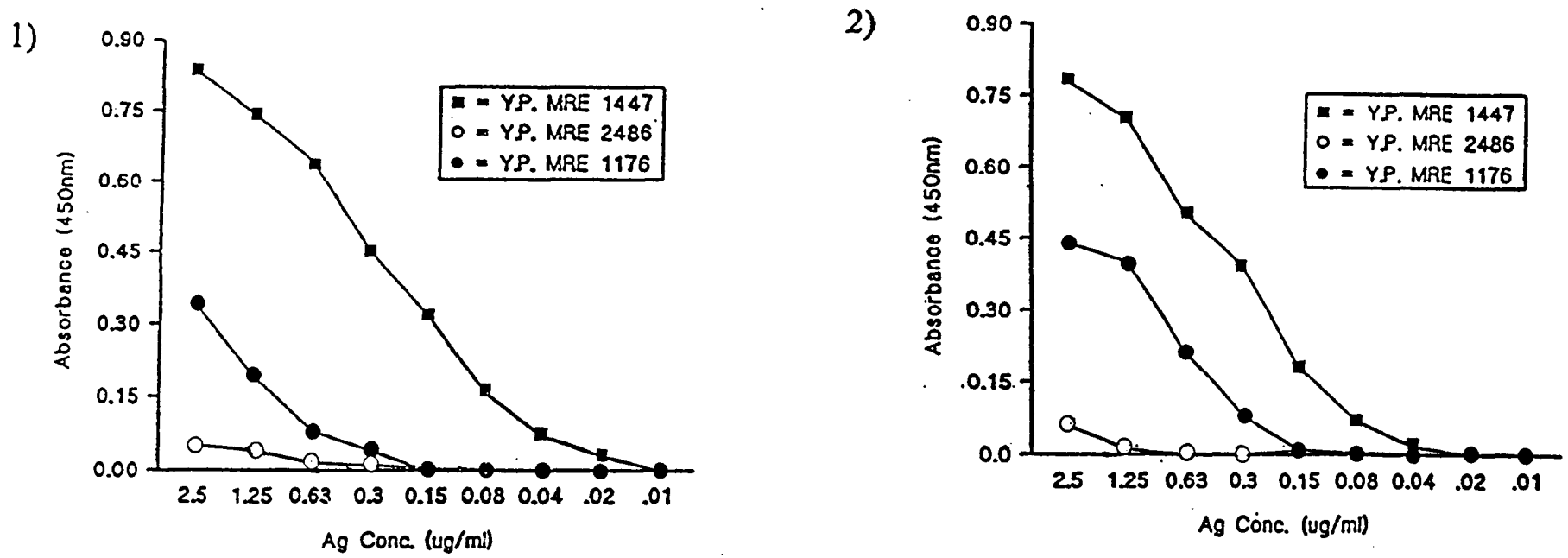
ELISA of culture supernatants prepared from $F1^+$ and $F1^-$ strains grown at 26°C and 37°C showed that samples from the $F1^-$ strain grown at 26^0C or 37^0C and samples from $F1^+$ strains grown at 26°C were not recognised by the anti-F1 monoclonal antibody (Mab) F13G8 (NCTC). The samples prepared from the $F1^+$ strain grown at 37°C were recognised by the Mab (fig. 3.3). These assays further identified the protein as the F1 antigen because the F1 antigen is produced at 37°C but not at 26°C (Bennet and Tornabene, 1974).

The identification of the F1 antigen was confirmed by Western blot. Proteins separated by SDS-PAGE were transferred to nitro-cellulose membranes and probed using the anti-F1 MAb F13G8. Fig. 3.4 shows that the Mab reacted only with the 17.5kDa protein in the $F1^+$ samples. The Western blot procedure included a renaturation step prior to blotting (section 2.8.2) which enhanced the detection of the 17.5kDa protein (Dunn, 1986).

3.2.2 Growth of *Y. pestis* to determine the most suitable starting material for purification.

The $F1^+$ strain of *Y. pestis*, MRE1447, was examined in addition to the $F1^-$ strain, MRE2486, under different culture conditions and extraction methods to determine the most appropriate initial source of the F1 antigen (refer to fig 3.1). As a result of these investigations, *Y.pestis* MRE1447 grown in YPMH medium at 37°C for 48h, was found to produce substantial amounts of the F1 antigen in the culture supernatant. Washing the

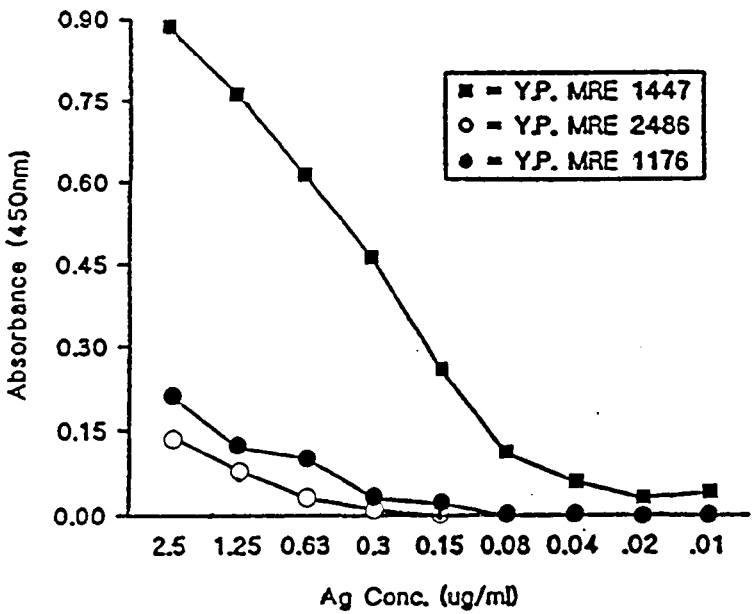
Fig. 3.2 **ELISA of cell fractions from the *Y. pestis* F1$^+$ strains MRE 1447 and MRE 1176 and the F1$^-$ strain MRE 2486 using Mab F13G8.**



1) F1 in culture supernatant; 2) 1M NaCl wash of whole cells; 3) supernatant from homogenised whole cells; 4) pellet from homogenised whole cells.

Fig. 3.2 continued.

3)



4)

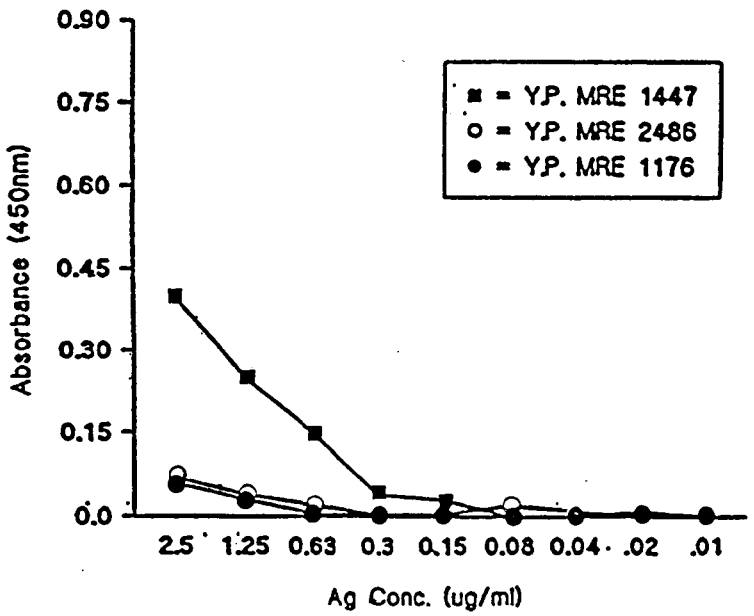
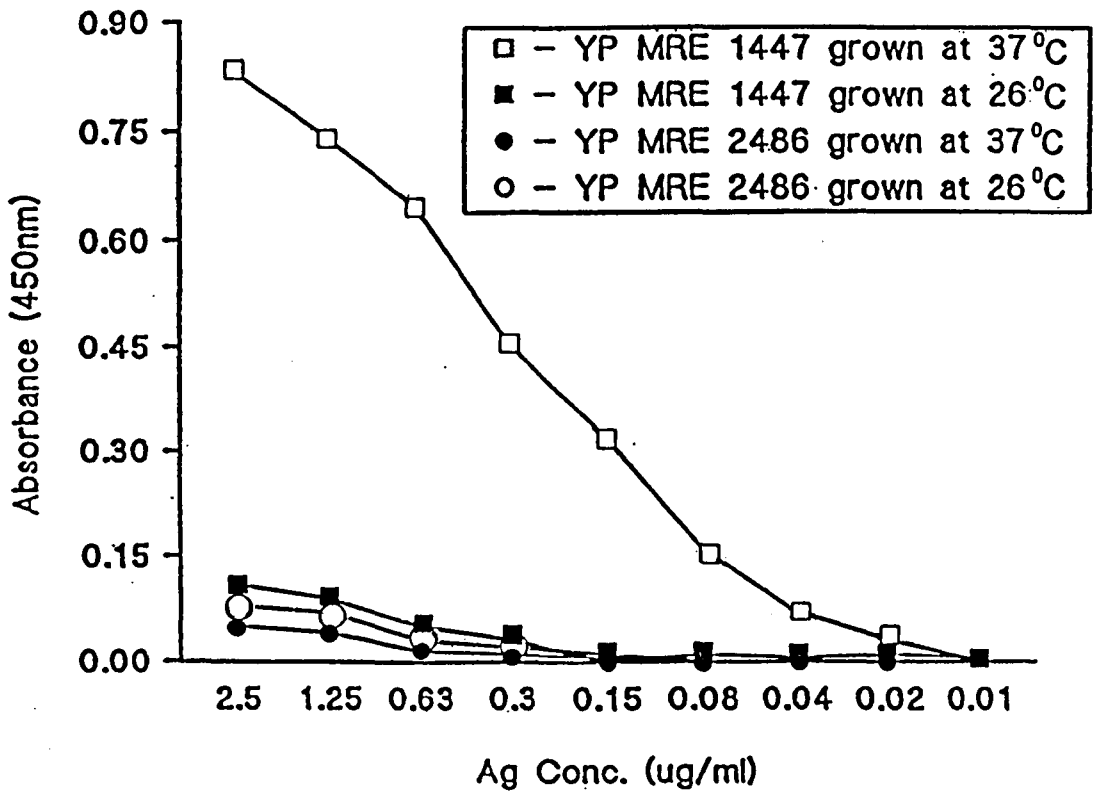
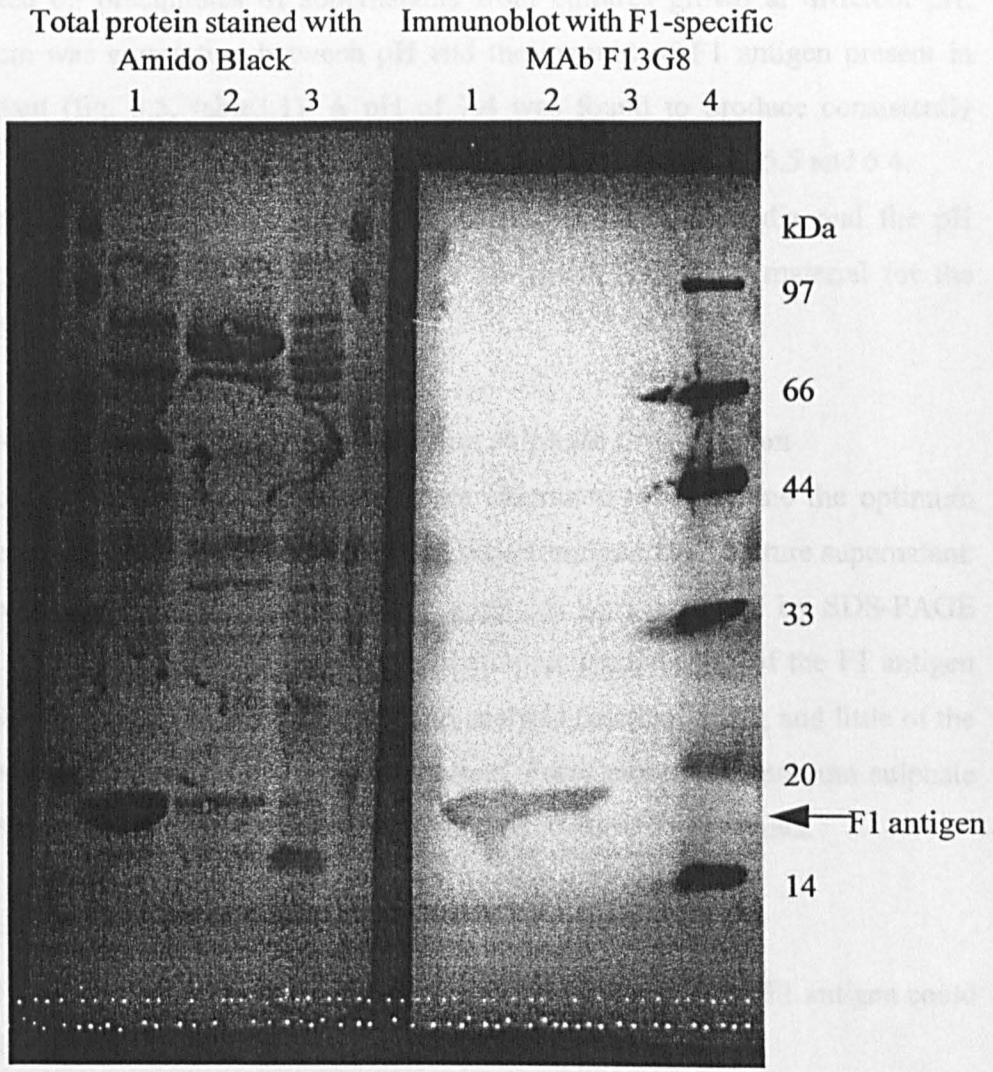


Fig. 3.3 ELISA to demonstrate the effect of temperature on F1 antigen in culture supernatant



The graph shows F1 precipitated from culture supernatant after growing the $F1^+$ strain, MRE 1447, at 26°C and 37°C and similar precipitates from the $F1^-$ strain, MRE 2486.

Fig. 3.4 Identification of the Fraction 1 antigen of *Y. pestis* by Western Blotting using MAb F13G8



Lane:

1. 1M NaCl wash of *Y. pestis* MRE 1447 (F1+)
2. Culture supernatant from *Y. pestis* MRE 1447
3. Culture supernatant from *Y. pestis* MRE 2486 (F1-)
4. Biotinylated molecular weight markers

whole cells with 1M NaCl removed the F1 antigen from the cell surface, but the resulting sample was more heavily contaminated with other proteins than YPMH culture supernatant. Culture supernatants were therefore selected as the most appropriate starting material.

The effect of pH on the amount of F1 produced in culture supernatant was also examined. ELISA performed on precipitates of supernatants from cultures grown at different pH, showed that there was correlation between pH and the amount of F1 antigen present in culture supernatant (fig. 3.5, table3.1). A pH of 7.4 was found to produce consistently higher yields of F1 antigen in culture supernatant than lower culture pH of 5.5 and 6.4. Therefore, *Y. pestis* MRE1447 ($F1^+$) was grown in YPMH liquid media and the pH maintained at pH7.4. The culture supernatant was retained as starting material for the purification of the F1 antigen.

3.2.3 Purification of the F1 antigen by ammonium sulphate precipitation

Different ammonium sulphate concentrations were examined to determine the optimum concentration for the extraction and purification of the F1 antigen from culture supernatant. Aliquots of supernatants and pellets from the precipitates were analysed by SDS-PAGE (fig.3.6) which showed that 40% ammonium sulphate precipitated most of the F1 antigen at greater than 90% purity as determined by image analysis (section 2.5.6), and little of the protein was precipitated by 30% ammonium sulphate. Forty percent ammonium sulphate was therefore used for purification of the F1 antigen from culture supernatants.

3.2.4 Gel filtration chromatography to further purify F1 antigen

Sephacryl S200 chromatography was carried out to determine whether the F1 antigen could be further purified according to the molecular weight of the protein.

A Sephacryl S200 HR column (Pharmacia), with a protein fractionation range of 5-250kDa and a bed height of 80cm was set up and washed with 0.1M Tris HCl, 0.1M NaCl, pH 8.0. The void volume was calculated by the elution of a blue dextran solution and the column was calibrated using standard proteins, bovine serum albumin, carbonic anhydrase and cytochrome c. Twenty milligrams of F1 precipitated from culture supernatant was

Fig. 3.5 ELISA showing the effect of pH on F1 antigen in culture supernatant.

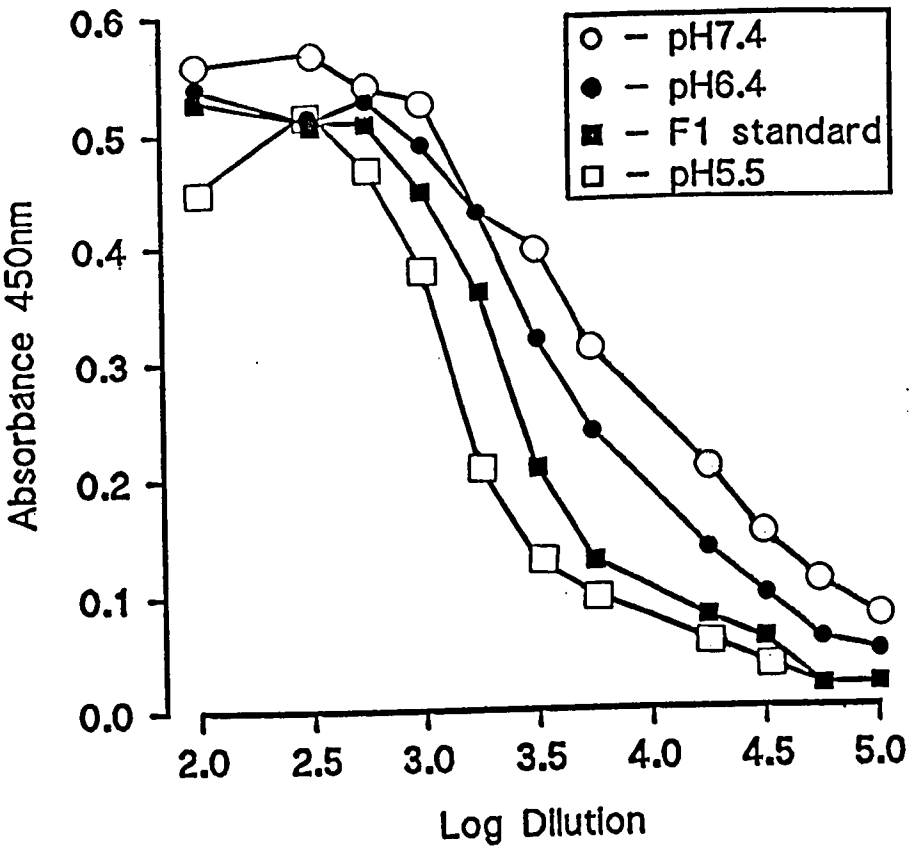
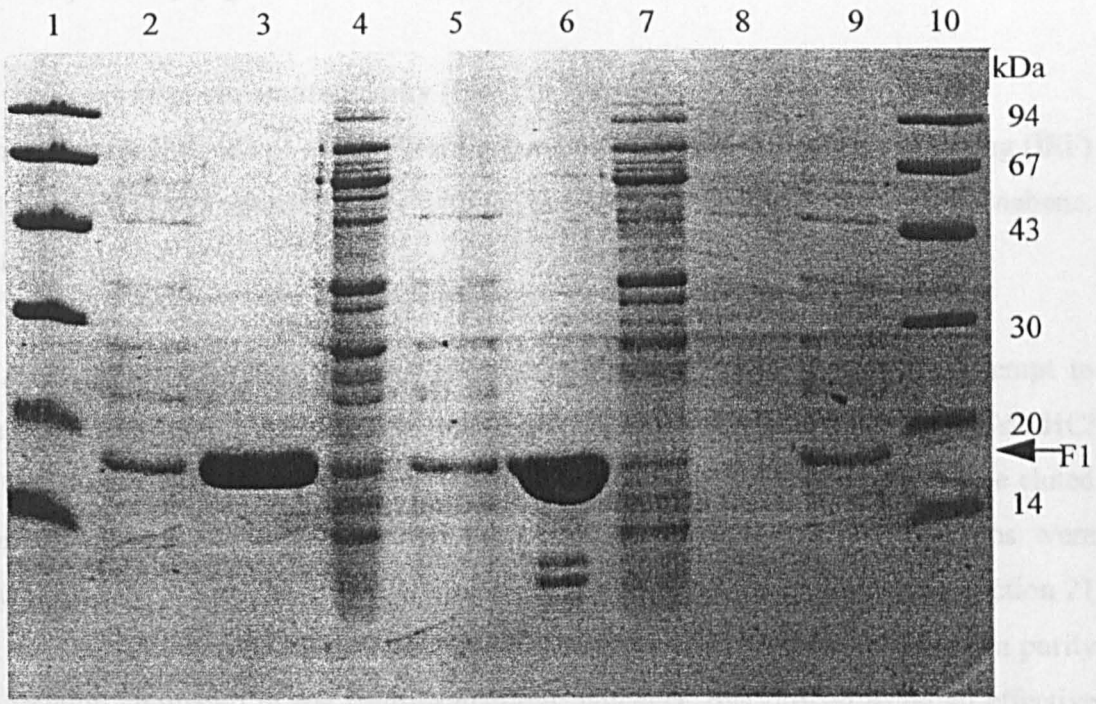


Table 3.1

pH of culture	Concentration of purified F1 ($\mu g\ ml^{-1}$)
5.5	6.5
6.4	22.5
7.4	42.5
F1 standard	20.0

The graph shows F1 precipitated from culture supernatant after growing the $F1^+$ strain, MRE 1447 at 37^0C at culture pH of 5.5, 6.0 and 7.4. An F1 standard was also included so that the amount of F1 produced at each pH could be quantified, table 3.1.

Fig. 3.6 SDS PAGE showing purification of the F1 antigen using different concentrations of ammonium sulphate



Lane:

1. Molecular weight markers
2. *Y. pestis* MRE 1447 culture supernatant (starting material)
3. Proteins precipitated by 40% ammonium sulphate
4. Proteins precipitated by 60% ammonium sulphate after 40% precipitation
5. As lane 2
6. Proteins precipitated by 50% ammonium sulphate after 30% precipitation
7. Proteins precipitated by 60% ammonium sulphate after 50% precipitation
8. Proteins precipitated by 30% ammonium sulphate
9. As lane 2
10. As lane 1

applied to the surface of the column, eluted at a flow rate of 0.3ml/min and fractions (3ml) were collected and tested for the presence of F1 by SDS PAGE; only the fractions eluted at the void volume (1 to 6) contained F1. The F1 peak was found to elute very close to the void volume giving an estimated molecular weight of 60-300kDa (fig. 3.7). This result indicates that the small 17.5kDa F1 subunit forms high molecular weight aggregates. This was confirmed by running supernatant precipitates from $F1^+$ and $F1^-$ strains on a non-denaturing polyacrylamide gel and observation of a protein present in the stacking gel in the $F1^+$ samples only (figure not shown).

3.2.5 Ion exchange chromatography (IEC) to further purify F1 antigen

In preparation for IEC, the pI of the F1 antigen was determined by isoelectrofocusing (IEF) to be 4.5, which agrees approximately with the published pI of 4.8 (Bennet and Tornabene, 1974) (fig.3.8).

An MA7Q anionic exchange column (Biorad Laboratories) was used in an attempt to further purify the ammonium sulphate precipitated F1 antigen. Protein in 10mM Tris HCl pH 8.0 was bound to the column and eluted using a 0 to 1M NaCl gradient. F1 in the eluted fractions was detected by immunoblotting with MAb F13G8. The F1 fractions were examined by SDS-PAGE, which showed that the majority of F1 was present in fraction 21 and in agreement with the HPLC trace (fig. 3.9). There was no observed increase in purity of the fraction compared to the starting material; however, this proved to be an effective method for concentrating the F1 antigen.

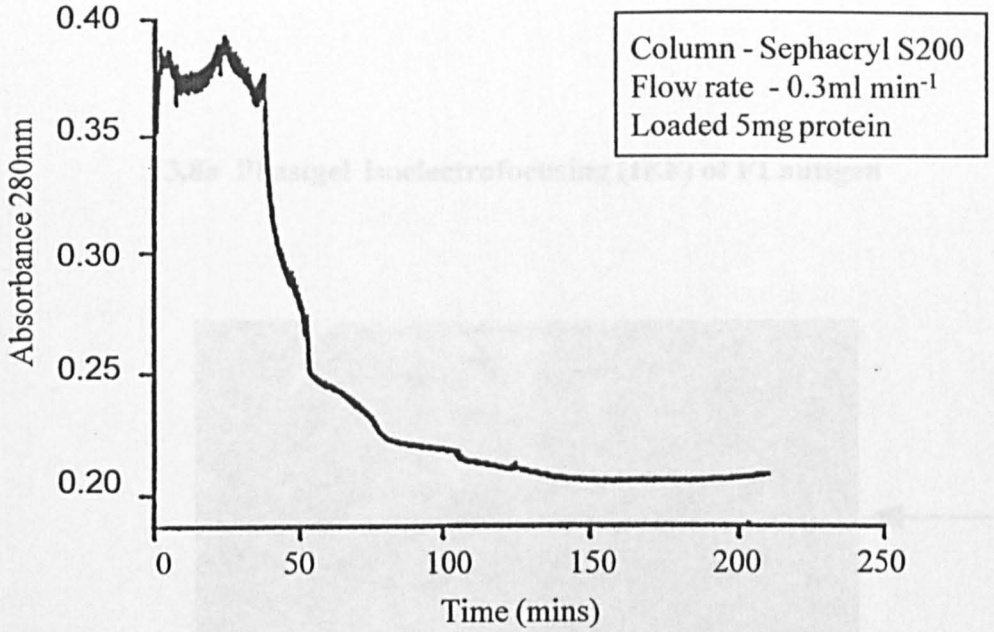
3.2.6 Larger-scale production and purification of the F1 antigen

Large-scale purification of the F1 antigen was achieved by growing Y. pestis MRE 1447 in a 25litre fermenter in the chemically defined medium YPMH (Straley and Bowmer, 1986) with the pH maintained at 7.4. The F1 antigen was precipitated from the culture supernatant by the addition of 40% ammonium sulphate.

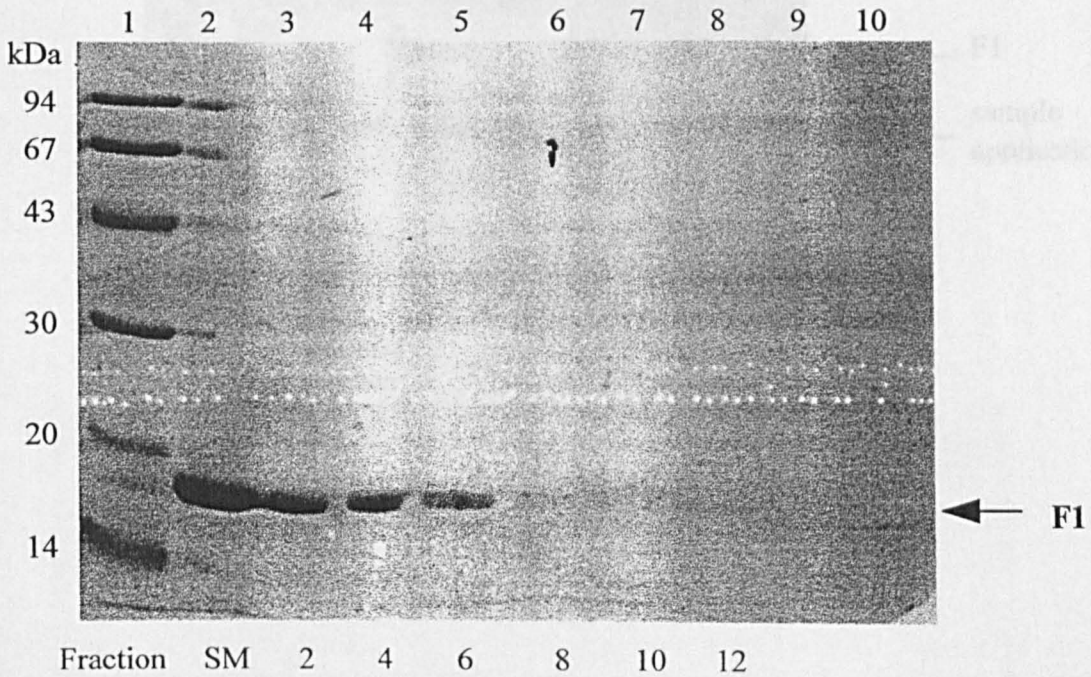
Repeated resuspension and centrifugation of the precipitated pellet in a low salt buffer produced a solution of the antigen. This process yielded up to 45mg of soluble purified F1 antigen per litre of culture supernatant and with consistent purity of >90%. Fig. 3.10 shows a purification scheme for *Y. pestis* F1 antigen.

Fig. 3.7 Sephacryl S200 gel filtration chromatography of F1 antigen.

3.7a Typical elution profile



3.7b SDS PAGE of eluted fractions



Lane:
1. Molecular weight markers
2. Starting material (SM)
3 - 8. Fractions 2 - 12

Fig. 3.8 Isoelectrofocusing to determine the isoelectric point (pI) of the F1 antigen

3.8a Phastgel isoelectrofocusing (IEF) of F1 antigen

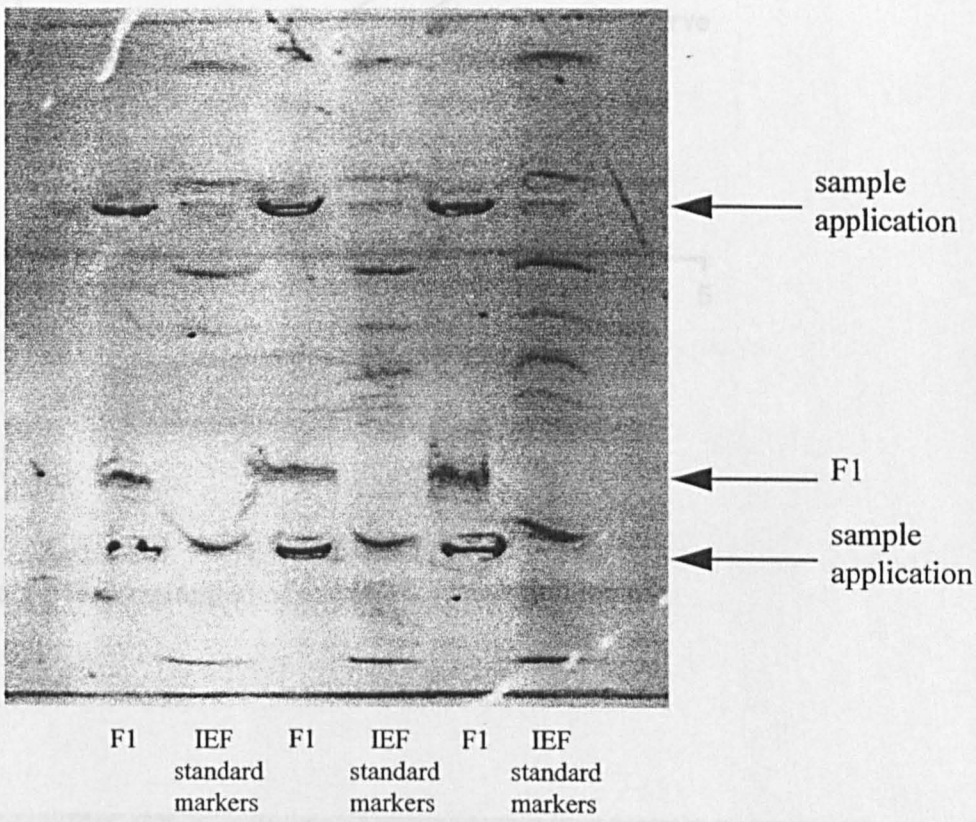


Fig. 3.8 continued.

3.8b Graph to determine pI of F1 antigen

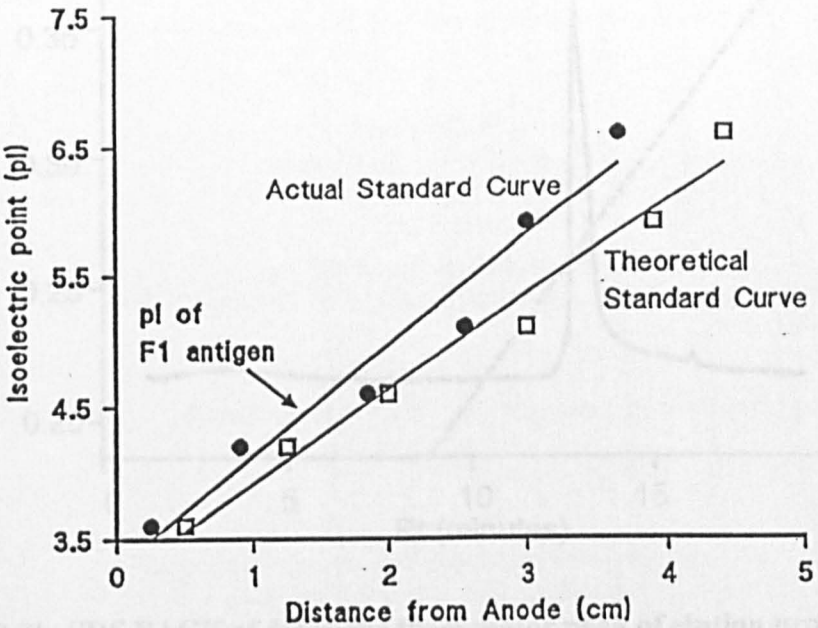
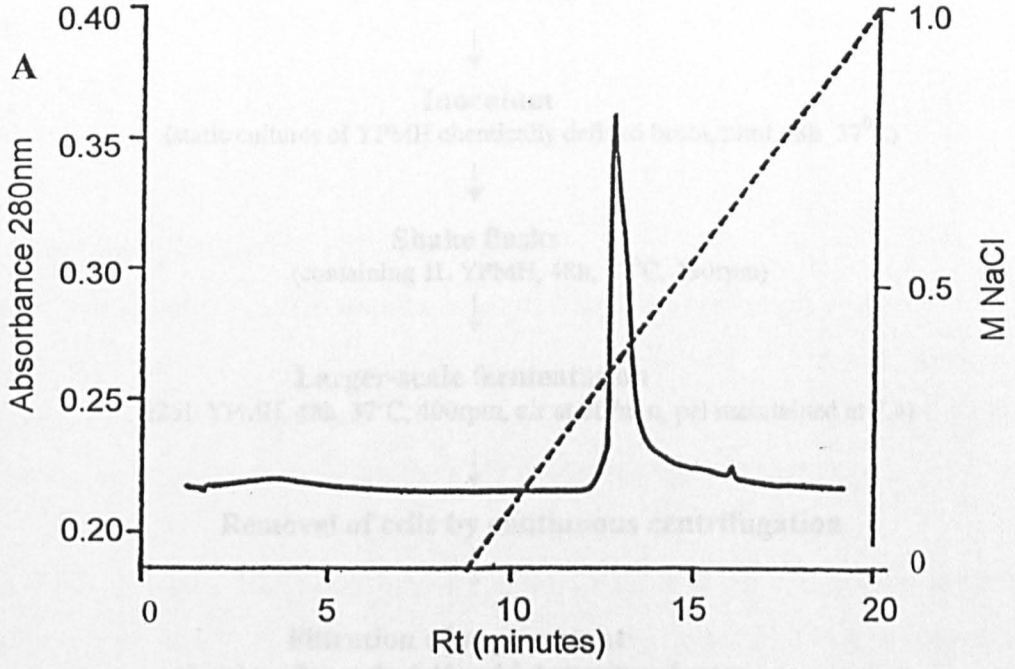


Table 3.2 Determination of pI of F1

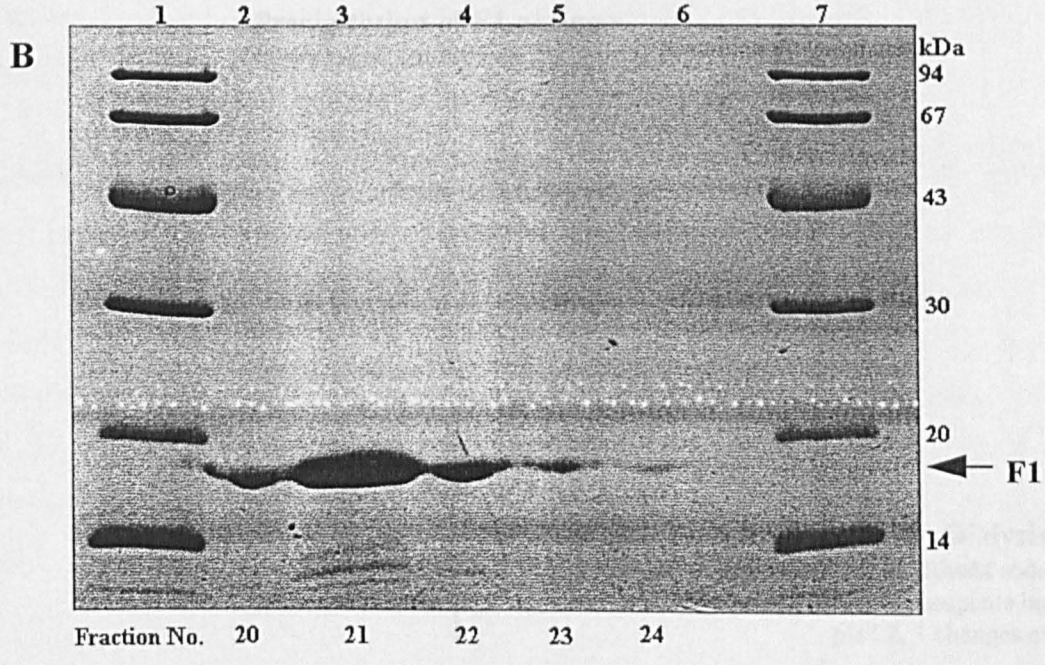
Sample	pI	Distance from anode (cm)	
		Theoretical	Actual
Sandard IEF markers	3.6	0.5	0.25
	4.2	1.25	0.9
	4.6	2.0	1.85
	5.1	3.0	2.55
	5.9	3.9	3.0
	6.6	4.5	3.65
F1 antgen samples	4.5		1.35
	4.5		1.35
	4.5		1.35

Fig. 3.9 Ion Exchange Chromatography of F1 antigen of *Y. pestis*

3.9a. Typical elution profile



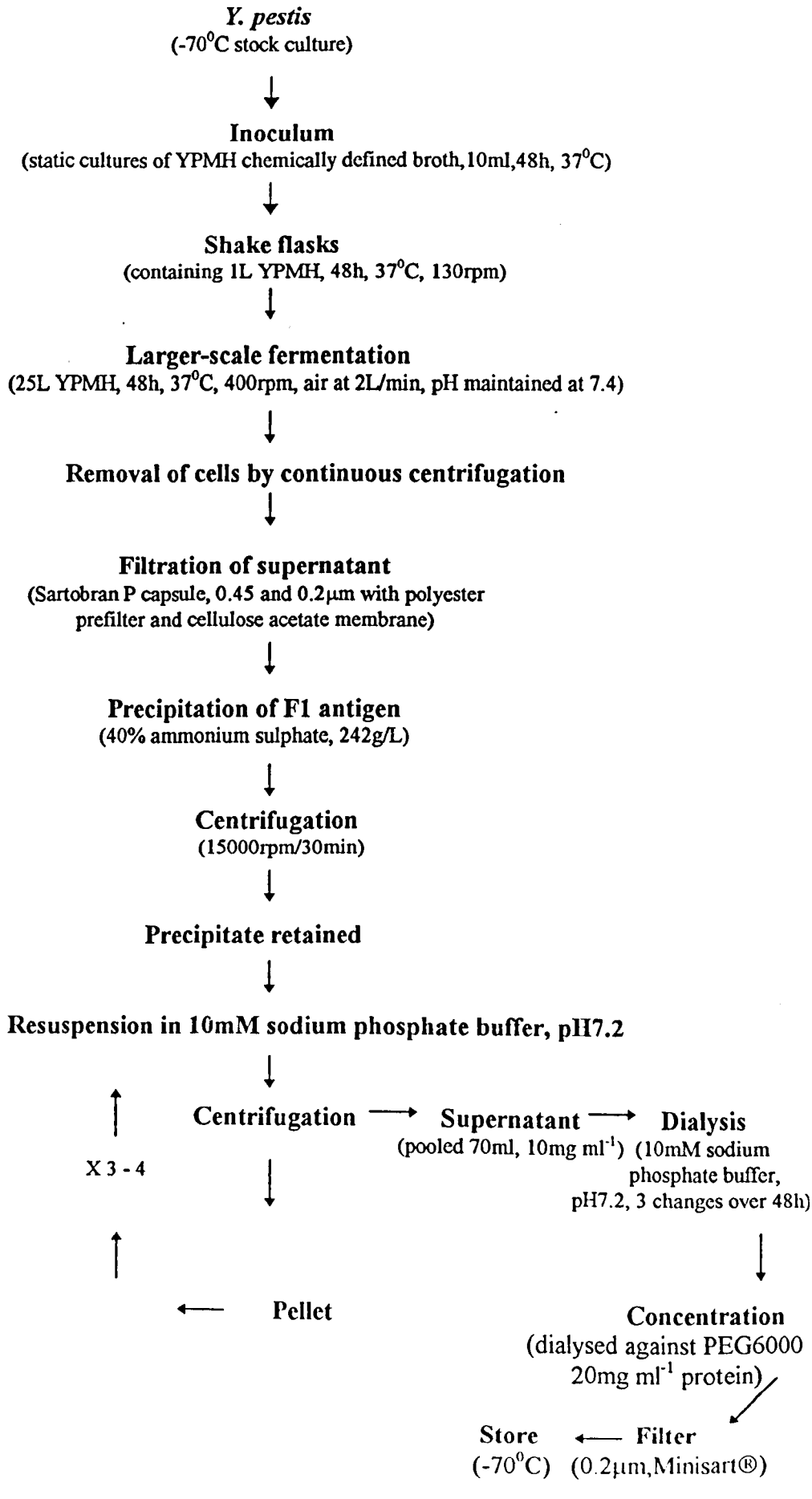
3.9b. SDS PAGE of fractions from major peak of elution profile



Lane: 1 and 7, molecular weight markers
2 to 6, fractions eluted from column

A, shows the elution profile of the F1 antigen and B, SDS PAGE of the eluted fractions

Fig. 3.10 Schematic representation of the purification of the *Y. pestis* F1 antigen



3.2.7 Endotoxin levels in purified native F1

Endotoxin levels in the purified F1 antigen were tested using the Limulus Amebocyte Lysate (LAL) assay, according to manufacturers instructions. Samples typically contained 500ng endotoxin per mg of F1; therefore, mice upon immunisation would receive 2ng endotoxin per 10μg dose F1. No attempt was made to remove the endotoxin for the purposes of this study.

3.3 Recombinant F1 antigen (rF1)

3.3.1 Cloning of the *caf*1 sequence into pGEX-5x-2 expression vector

The approach used for the production of rF1 antigen was as a fusion protein, using a method similar to that described by Leary *et al*, 1995. The vector used was the pGEX-5x-2 expression vector, which codes for the GST carrier protein (fig. 3.11).

DNA from *Y. pestis* GB strain was extracted using Marmur's modified method (1961) which extracts mainly chromosomal DNA but enough plasmid DNA is also extracted to enable cloning of the *caf*1 gene. The *caf*1 gene was amplified by means of the polymerase chain reaction (PCR) using F1/5'D and F1/3'C primers. The PCR reaction was analysed by electrophoresis on a 1% agarose gel and a PCR product of the predicted size (450bp) was observed (fig 3.12), this was used to proceed with the cloning strategy.

The next stage of the cloning strategy was to prepare restriction digests of the amplified *caf*1 gene and the pGEX-5x-2 vector using the restriction enzymes EcoR1 and BamH1, in order to obtain a double digest which produces cohesive ends on the *caf*1 PCR product and the pGEX vector. The resulting restriction digests were purified and the DNA extracted by ethanol precipitation. The concentration of DNA in the pGEX and *caf*1 restriction digests was estimated and a ligation reaction was set up using a 1:3 vector:insert ratio. The ligation reaction was examined on a 0.7% agarose gel which showed that the molecular weight of the DNA had increased indicating that the ligation reaction was successful (fig. 3.13).

Purified, ligated DNA was electroporated into *E. coli* JM109 and the transformed cells were grown up on L-ampicillin (L-amp) plates overnight at 37^0C. Thirty recombinant colonies were picked across onto L-amp plates marked with a grid and were again incubated overnight at 37^0C. The remaining cells were used to inoculate 50μl of sterile

Fig. 3.11 Diagrammatic representation of the pGEX-5x-2 cloning vector.

3.11a Structural elements on pGEX-5x-2

Glutathione S-transferase gene region: *tac* promoter: -10: 205 - 211; -35: 183 - 188; *lac* operator: 217 - 237; Ribosome binding site for GST: 244; Start codon (ATG) for GST: 258; Coding region for factor Xa cleavage: 921 - 932; Primer region for double-stranded sequencing: 874 - 890.

Main cloning site (MCS): 934 - 970.

β-lactamase gene region: Promoter: -10: 1334 - 1339; -35: 1311 - 1316; Start codon (ATG): 1381; Stop codon (TAA): 2239.

lac I^q gene region: Start codon (GTG): 3322; Stop codon (TGA): 4402.

Plasmid replication region: Site of replication initiation: 2999; Region necessary for replication: 2306 - 3002.

3.11b Restriction map of pGEX-5x-2

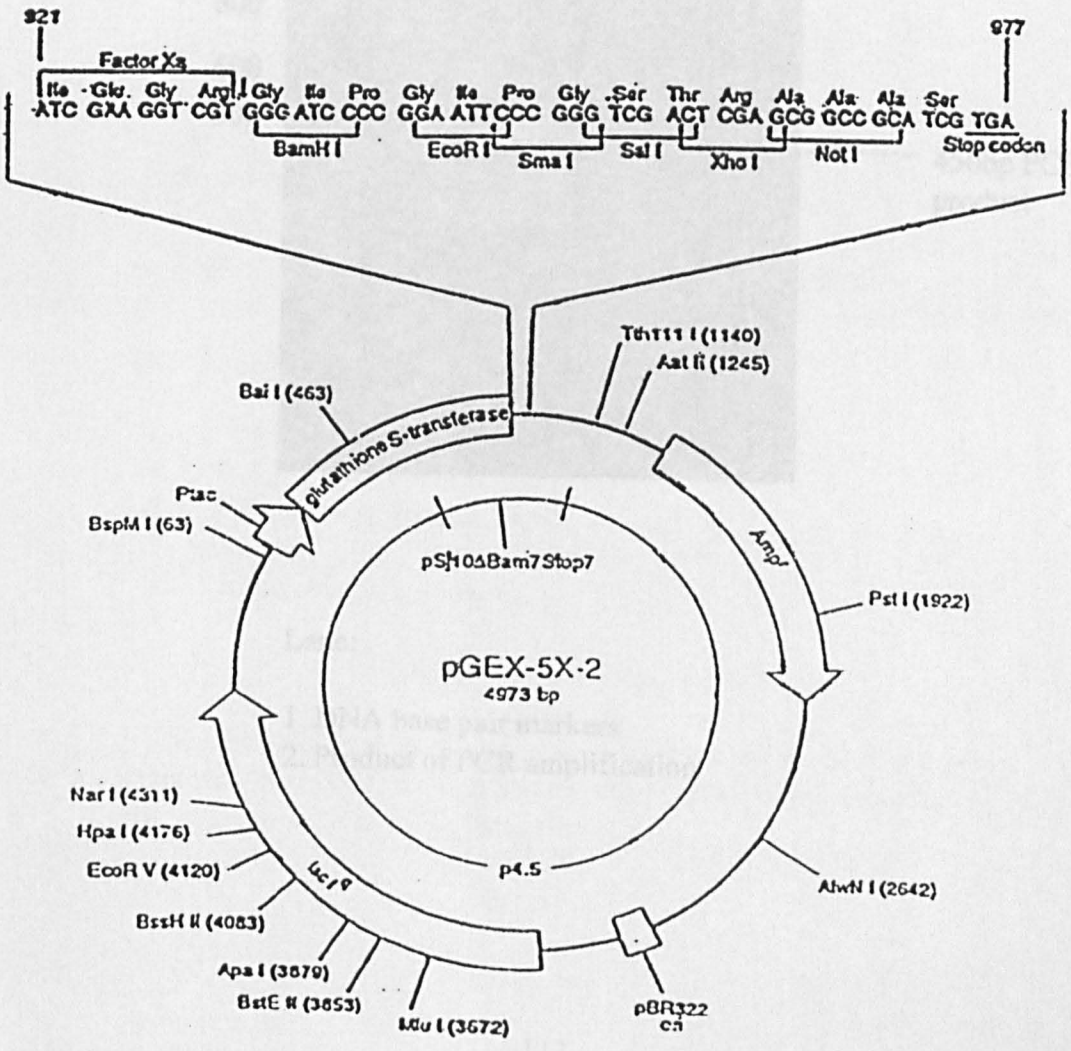
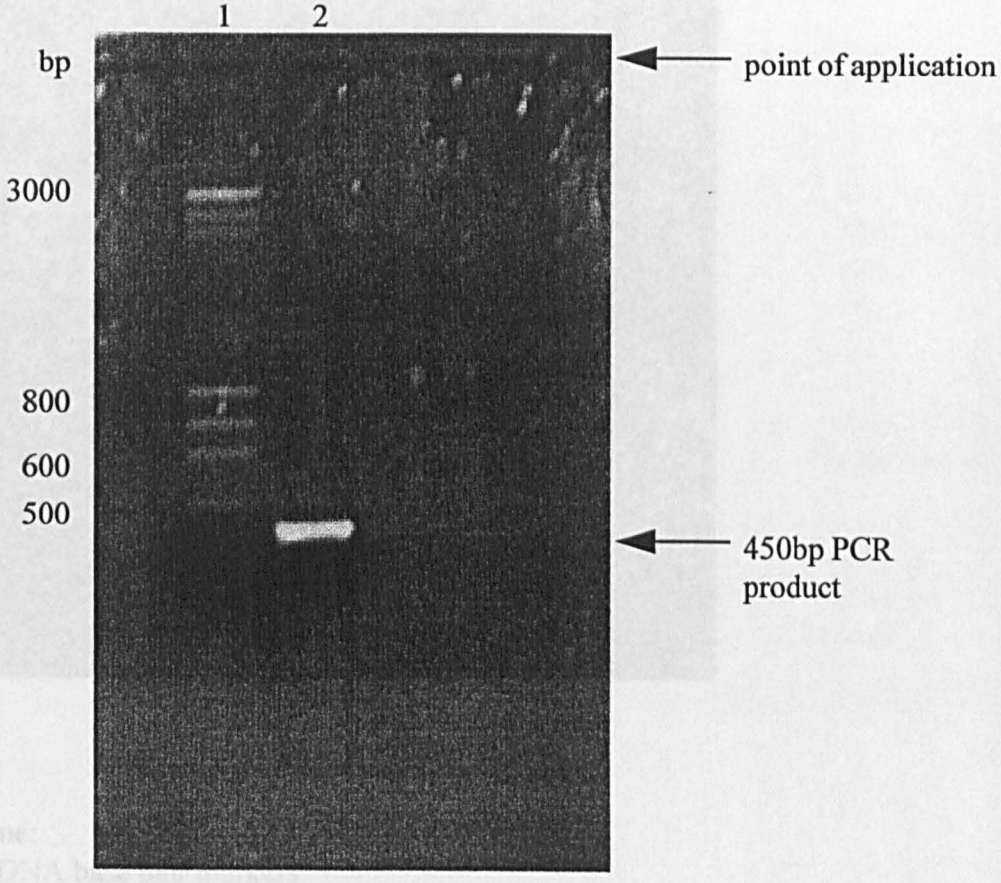


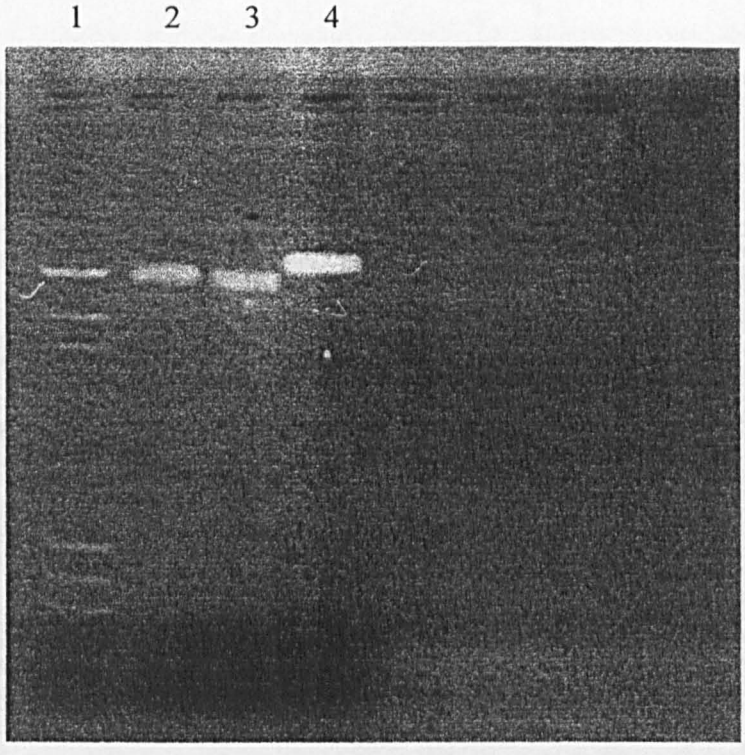
Fig.3.12 Agarose gel electrophoresis of PCR reaction of *caf*1 DNA using F1/5'D and F1/3'C primers.



Lane:

1. DNA base pair markers
2. Product of PCR amplification

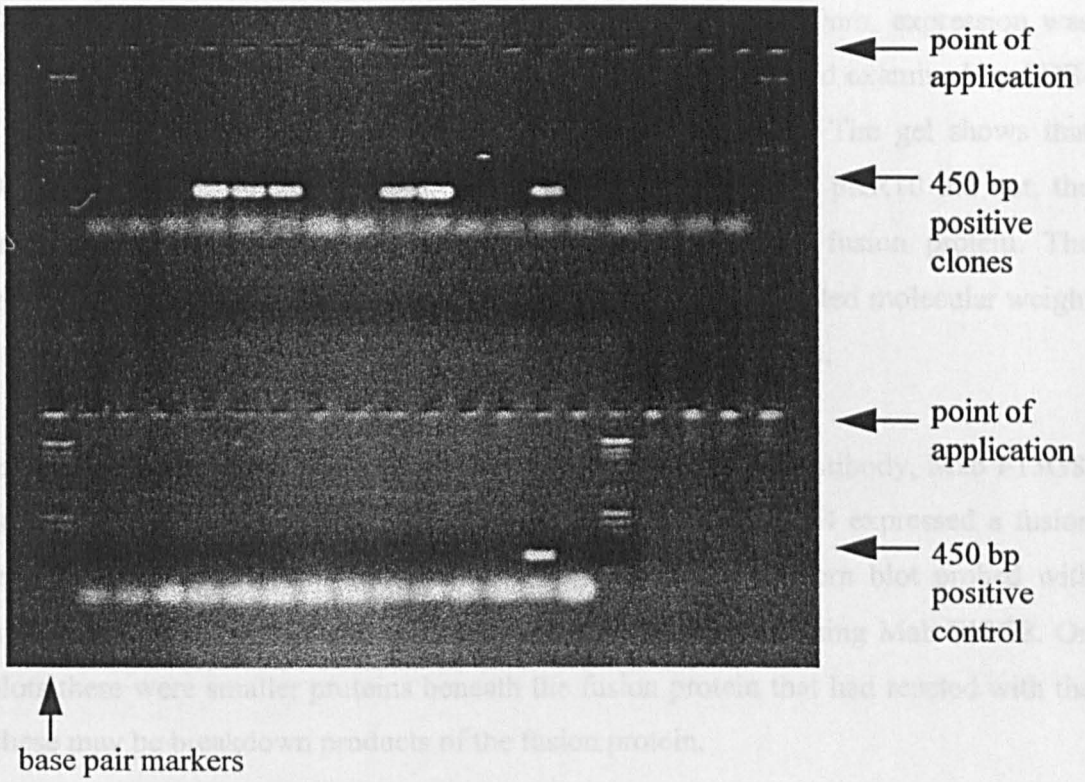
Fig. 3.13 Agarose gel electrophoresis of ligated *caf*1 DNA and pGEX vector.



Lane:
1. DNA base pair markers
2. pGEX vector restriction digest
3. As lane 2.
4. Ligation reaction product

The DNA had increased in size after ligation when compared with the restriction digests. Therefore, the ligation reaction was successful and the *caf*1 had been inserted into the pGEX expression vector.

Fig. 3.14 Agarose gel electrophoresis of recombinant *E. coli* JM109 colony lysate polymerase chain reaction (PCR) products.



On the double gel six clones showed to be positive, indicated by a caf1 PCR product of 450bp. The positve control used DNA of approximately 450bp from *Y. pestis* GB strain.

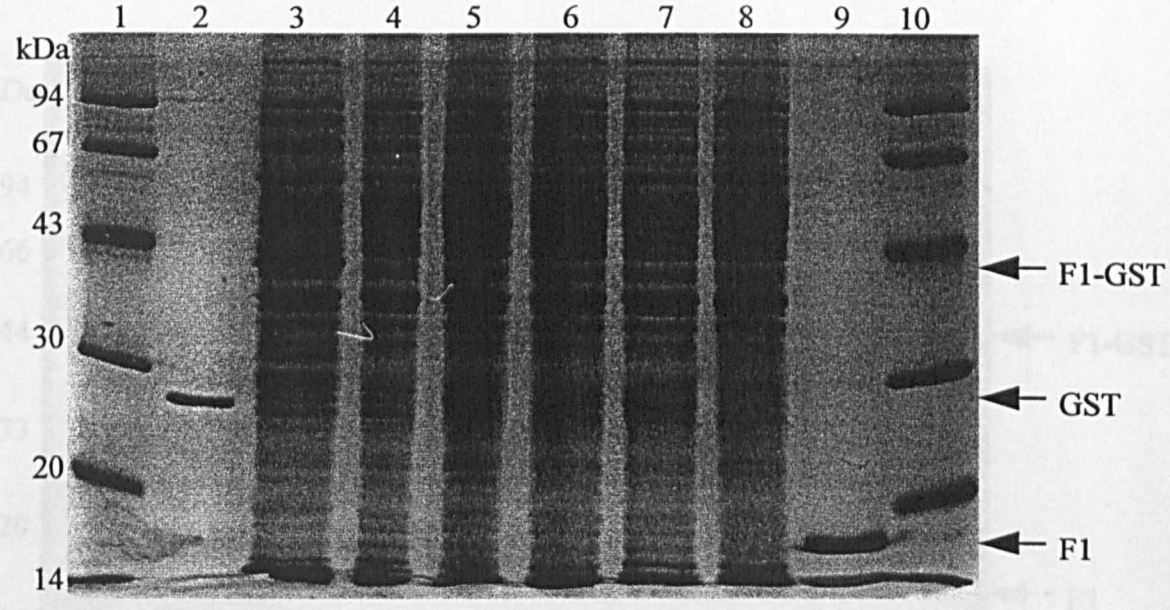
distilled water per colony and these suspensions were used to prepare colony lysates followed by PCR reactions to screen for positive clones. PCR reactions of colony lysates were prepared using the same F1 5' and 3' primers as before, a *caf*1 PCR product of 450bp would indicate a positive clone; examination of the colony lysate PCR reactions on 1% agarose gel showed six positive clones (fig. 3.14). Two of these clones were randomly selected and referred to as JM109 + pKR4 and JM109 + pKR10. L-amp broth cultures (100ml) were set up to test these positive clones for expression of the F1-GST fusion protein and a culture of JM109 + pGEX-5x-2 (i.e. no insert) was included as a negative control. After cultures reached an appropriate optical density at 600nm, expression was induced by the addition of IPTG and culture lysates were prepared and examined by SDS-PAGE for expression of the F1-GST fusion protein (figure 3.15). The gel shows that JM109 + pKR4 expressed the F1-GST fusion protein but JM109 + pKR10 did not; the uninduced culture and the negative control did not express the fusion protein. The molecular weight of the fusion protein, 43kDa, agreed with the predicted molecular weight of a fusion protein between F1 (17.5kDa) and GST (26kDa) (fig. 3.15).

Immunoblotting of the culture lysates with an anti-F1 monoclonal antibody, Mab F13G8, and mouse polyclonal anti-F1 antibody, showed that JM109 + pKR4 expressed a fusion protein that reacted with both antibodies. Fig. 3.16 shows a Western blot probed with mouse polyclonal anti-F1 antibody, an identical blot was obtained using Mab F13G8. On both blots there were smaller proteins beneath the fusion protein that had reacted with the Mab, these may be breakdown products of the fusion protein.

The pKR4 plasmid DNA was purified with the Qiagen Plasmid Kit, using the maxi-prep protocol. The DNA was sequenced on a TAQ sequencer and the resulting sequence confirmed the cloning site (results not shown).

The strain expressing the fusion protein was used to evaluate the amount of fusion protein produced during the time course of induction with IPTG. The strain was grown as described in section 2.1.2 and 500µl samples were taken and prepared for SDS-PAGE, immediately prior to induction and at 30min or 1h intervals after induction. The cultures were induced during the exponential phase of growth and the production of fusion protein did not appear to affect subsequent growth. Increasing amounts of fusion protein were observed up to 5h after induction (figure 3.17) indicating that the lac I repressor protein

Fig. 3.15 Expression of F1-GST fusion protein in culture lysates in selected strains of recombinant *E. coli* JM109.

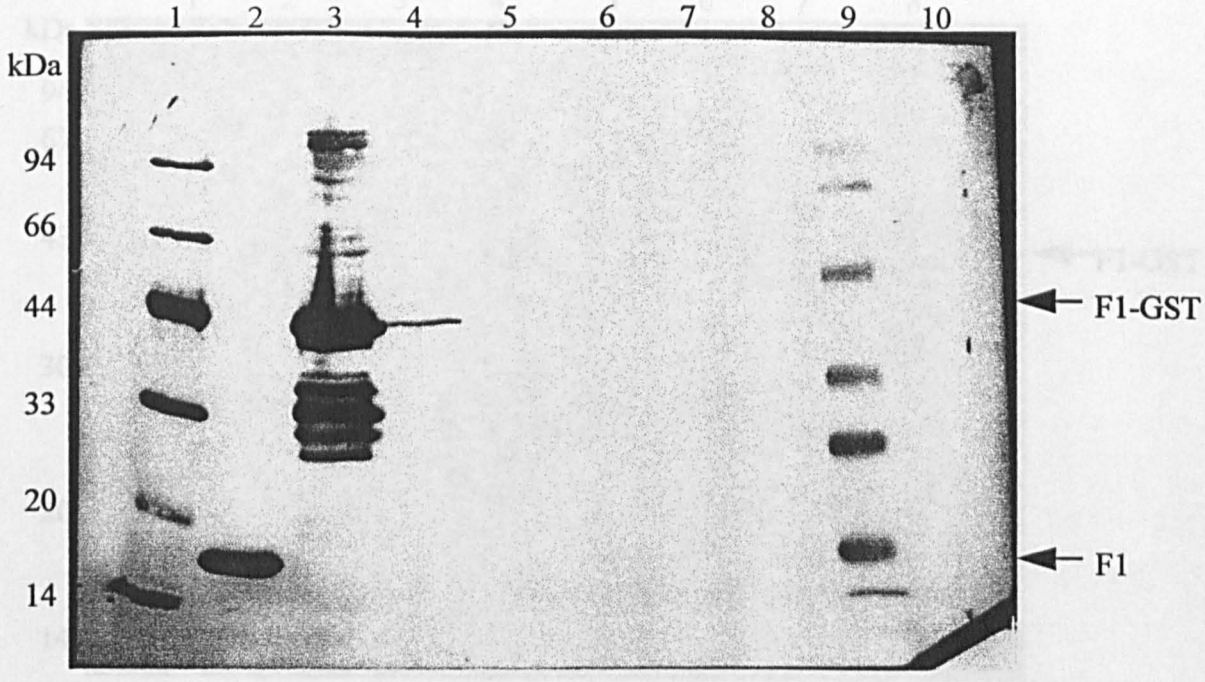


Lane:

1. Molecular weight markers
2. Purified GST
3. IPTG induced strain JM109 + pKR4
4. Strain JM109 + pKR4, no induction
5. IPTG induced strain JM109 + pKR10
6. Strain JM109 + pKR10, no induction
7. IPTG induced pGEX
8. pGEX, no induction
9. Purified F1 antigen
10. As lane 1.

Only the culture lysate from JM109 + pKR4 induced with IPTG expressed the F1-GST fusion protein. The JM109 + pGEX induced and non-induced cultures were included as controls, the induced culture expressed only GST.

Fig. 3.16 Western blot of culture lysates from recombinant *E. coli* JM109 strains.

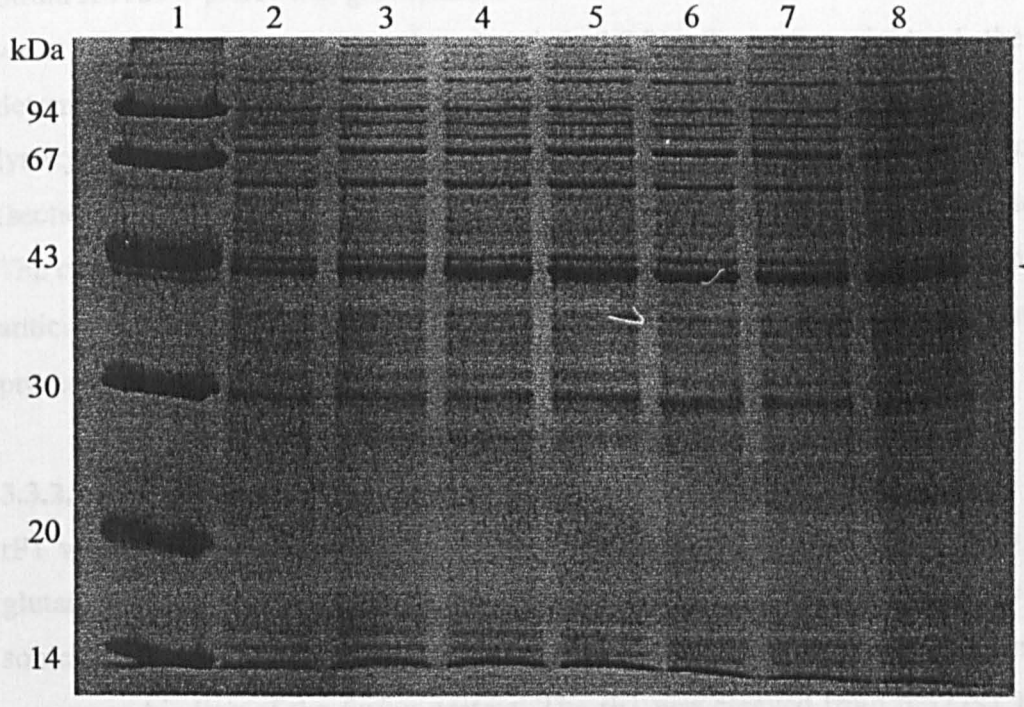


Lane:

1. Biotinylated molecular weight markers
2. Purified F1antigen
3. IPTG induced strain JM109 + pKR4
4. Strain JM109 + pKR4, no induction
5. IPTG induced strain JM109 + pKR10
6. Strain JM109 + pKR10, no induction
7. IPTG induced strain JM109 + pGEX
8. Strain JM109 + pGEX, no induction
9. Prestained molecular weight markers

The Western blot was probed with murine anti-F1 polyclonal antibody

Fig. 3.17 Time course of appearance of F1-GST fusion protein in culture lysate of *E. coli* JM109 + pKR4 after induction with IPTG



Time:	1h	2h	2.5h	3h	3.5h	4h	5h
% fusion protein:	21%	24%	36%	34%	35%	37%	43%

The induced culture was sampled at timed intervals and culture lysates were examined by SDS PAGE. The amount of fusion protein increased with time and after 5h incubation with IPTG the cultures lysate contained 43% F1-GST fusion protein when examined by image analysis.

prevented expression in the absence of IPTG, as confirmed by the results shown in figures 3.15 and 3.16. After 5h induction time, the F1-GST fusion protein constituted approximately 43% of the total cellular protein as determined by image analysis (fig. 3.17).

3.3.2 Purification of rF1

3.3.2.1 Preparation of cell extracts of strain JM109 + pKR4

Strain JM109 + pKR4 was grown and induced as described in section 2.1.2 and 2.2.2. Cells were harvested by centrifugation (section 2.2.2); the wet weight of the cells was determined and found to be approximately 4g per litre of culture. After the addition of lysozyme and Triton X-100, a freeze-thaw sonication method was used to lyse cells (section 2.3.2) with 10 x 30sec bursts of sonication, according to a predetermined protocol. The cell sonicate was centrifuged and both the supernatant and pellet were retained. It was anticipated that the fusion protein would be expressed in the periplasm and therefore present in the sonicate supernatant (Leary *et al.*, 1995).

3.3.2.2 Affinity chromatography on glutatione Sepharose

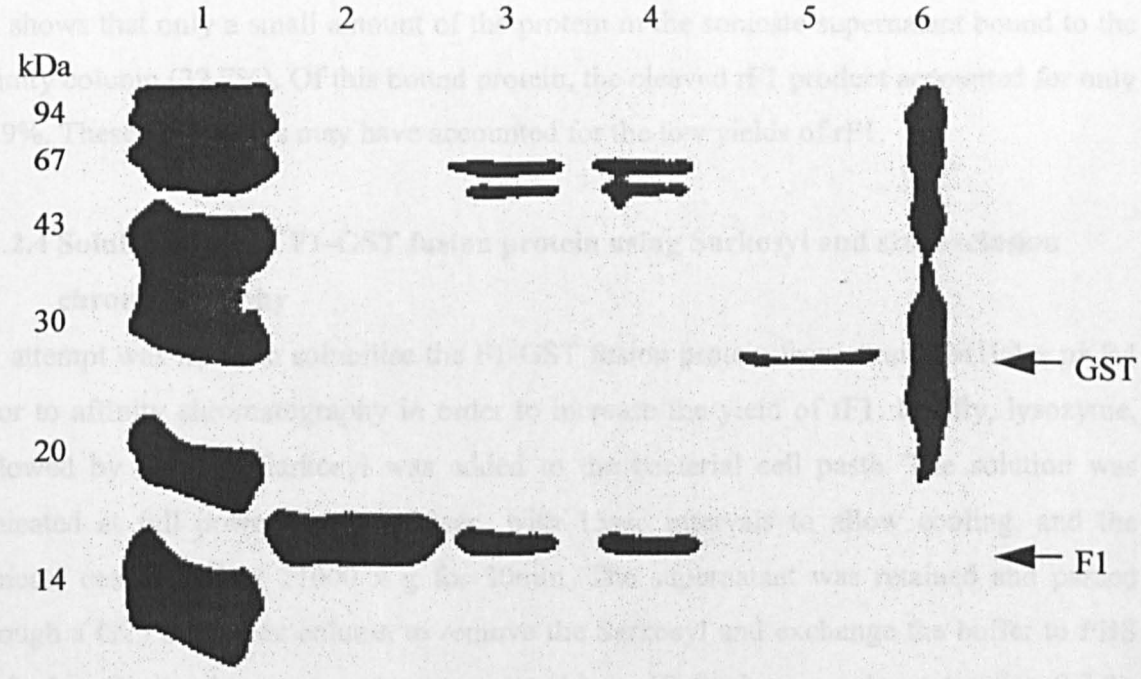
rF1 was purified using the affinity of the GST part of the fusion protein to bind to the glutathione on the Sepharose in the affinity column. Supernatant from the centrifuged sonicate was passed three times down the glutathione Sepharose affinity column to ensure maximum binding of the fusion protein. The rF1 was cleaved from the GST bound to the matrix using overnight incubation at 4^0C with 60 units of factor Xa. The rF1 was then eluted in 4ml PBS and the concentration of protein was estimated and found to be approximately 0.35mg ml^{-1} from 20ml sonicate.

The recombinant protein was examined by SDS-PAGE and compared with the purified native F1 (fig. 3.18). The rF1 was not as pure as native F1 and was contaminated with some high molecular weight proteins. However, no GST had leached from the column indicating that cleavage was successful.

3.3.2.3 Yield of rF1 obtained from strain JM109 + pKR4

In a typical growth experiment, approximately 4g (wet weight) of cells were obtained from 1L of Terrific Broth culture of strain JM109 + pKR4 resulting in 20ml of cell sonicate. From this sonicate, approximately 1.4mg of cleaved protein was obtained from 1L of the original JM109 + pKR4 culture. This, however, was a low yield when compared to native

Fig. 3.18 SDS PAGE of recombinant F1 after affinity chromatography and cleavage from GST with factor Xa



Lane:

1. Molecular weight markers
2. Purified F1 from *Y. pestis*
3. Purified recombinant F1 after cleavage with factor Xa
4. As lane 3.
5. Purified GST
6. Prestained molecular weight markers

F1, and this low yield of rF1 was consistent from batch-to-batch.

A BCA protein assay was carried out on the different fractions, including the sonicate before and after centrifugation, and the post-column eluate to determine the potential loss of fusion protein. These sonicate and eluate fractions were analysed by SDS-PAGE (fig. 3.19) which indicated that most of the fusion protein remained in the sonicate pellet. Table 3.3 shows that only a small amount of the protein in the sonicate supernatant bound to the affinity column (32.7%). Of this bound protein, the cleaved rF1 product accounted for only 6.19%. These two factors may have accounted for the low yields of rF1.

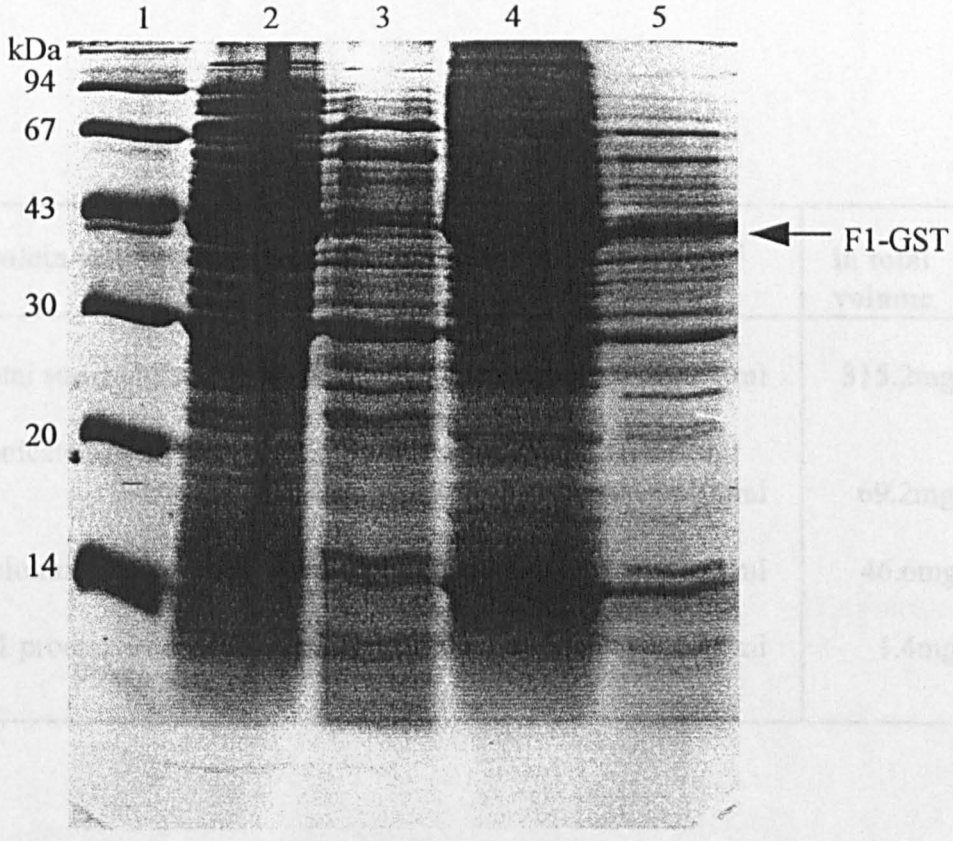
3.3.2.4 Solubilisation of F1-GST fusion protein using Sarkosyl and size exclusion chromatography

An attempt was made to solubilise the F1-GST fusion protein from strain JM109 + pKR4 prior to affinity chromatography in order to increase the yield of rF1. Briefly, lysozyme, followed by 2% w/v Sarkosyl was added to the bacterial cell paste. The solution was sonicated at full power for 7 x 30sec, with 15sec intervals to allow cooling, and the sonicate centrifuged at 27000 x g for 30min. The supernatant was retained and passed through a G25 Sephadex column to remove the Sarkosyl and exchange the buffer to PBS ready for affinity chromatography on a glutathione 4B Sepharose column (section 2.3.2). The elution profile from the G25 Sephadex column (fig 3.20) shows that most of the protein was present in fractions 15 to 23. Fractions were collected from the peak and examined by SDS-PAGE (fig 3.20).

The sonicate supernatant starting material (100ml) contained approximately 200mg of protein, estimated by BCA assay. After 10 runs (10ml of sample per run) on the Sephadex G25 column, approximately 157mg protein was obtained. Therefore, 78.5% of the total protein was recovered.

This material was then applied to an affinity chromatography column of Glutathione Sepharose 4B and cleavage was carried out using 60 units of factor Xa in 4ml PBS. Fractions from the affinity column were examined by SDS-PAGE (fig. 3.21) and this showed that the unbound fraction contained a large amount of fusion protein (43kDa). These are similar to the results observed before solubilisation of the F1-GST fusion protein (section 3.3.2.3). Although factor Xa cleavage was successful, fusion protein was eluted

Fig. 3.19 SDS PAGE of sonicate fractions from *E. coli* JM109 + pKR4



Lane :
1. Molecular weight markers
2. Culture lysate
3. Total sonicate before centrifugation
4. Sonicate pellet
5. Sonicate supernatant

Table 3.3 Amount of F1-GST bound to glutathione affinity column

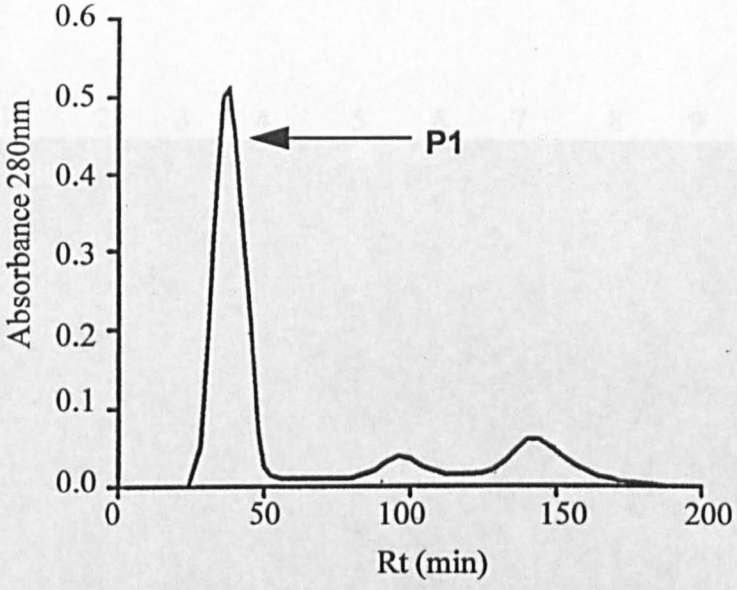
Protein estimation in fractions :	**mg ml^{-1}**	**in total volume**
Total sonicate (before centrifugation)	15.76mg/ml	315.2mg
Sonicate supernatant (starting material before affinity column)	3.46mg/ml	69.2mg
Column eluent (post affinity column)	2.33mg/ml	46.6mg
rF1 product after cleavage from GST	0.35mg/ml	1.4mg

% protein bound to affinity column = 32.7%

rF1 product as a percentage of bound protein = 6.19%

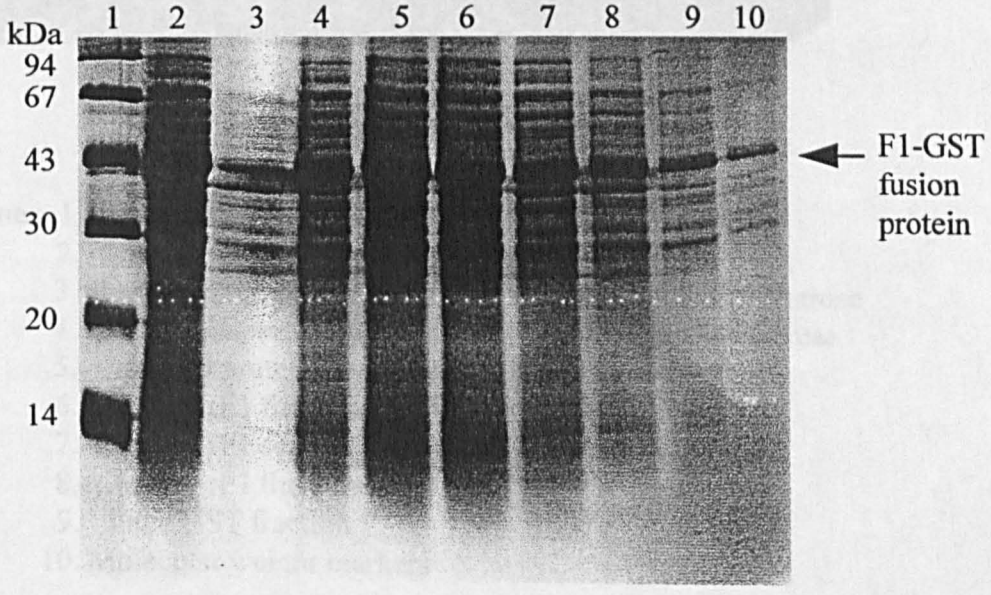
Fig. 3.20 G25 Sepharose size exclusion chromatography of rF1 antigen after solubilisation with Sarkosyl

3.20a Typical elution profile



Chromatography was carried out as described in section2.3.3. The column was eluted with column buffer and 2ml fractions collected.

3.20b SDS PAGE analysis of fractions from major peak of the elution profile

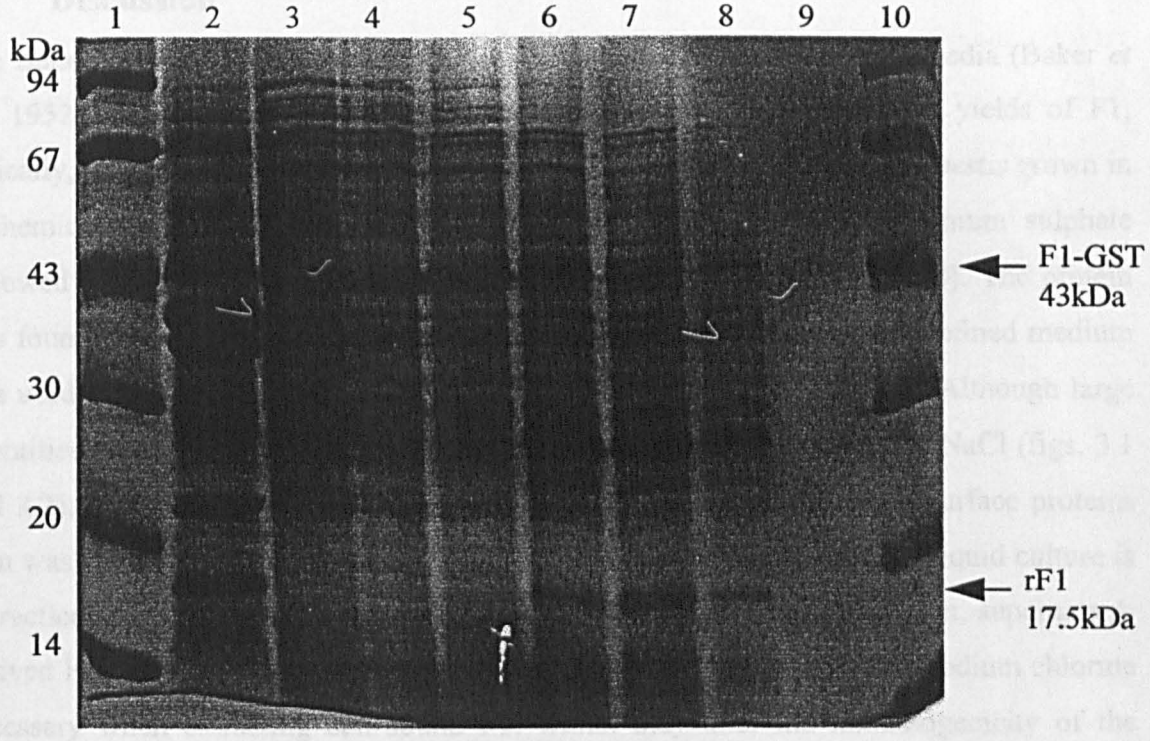


Fraction
Number: SM 13 15 17 19 21 23 24 25

Virtually all of the protein was eluted in peak 1(P1). Fractions 15 - 23 corresponding to P1 were pooled for further purification.

SM : starting material; Rt : retention time

Fig. 3.21 SDS PAGE of fractions after solubilisation of F1-GST fusion protein followed by affinity chromatography and cleavage with factor Xa.



Lane : 1. Molecular weight markers
2. Sonicate pellet
3. Sonicate supernatant before gel filtration on G25 sepharose
4. Sonicate supernatant after gel filtration on G25 sepharose
5. Unbound protein from affinity chromatography
6. Cleaved rF1 first fraction from affinity column
7. Cleaved rF1 second fraction from affinity column
8. Cleaved rF1 third fraction from affinity column
9. Eluted GST fraction
10. Molecular weight markers

and only a small amount of cleaved rF1 was obtained. Therefore, solubilisation of the fusion protein in Sarkosyl prior to affinity chromatography did not increase yields of cleaved rF1. There may have been problems with the binding of the GST moiety of the fusion protein to the glutathione affinity column and this may have been why no GST was observed in the eluted GST fraction after affinity chromatography.

3.4 Discussion

The Baker extraction method for F1 from *Y. pestis* cells grown on solid media (Baker *et al.*, 1952) has been used extensively over the years. In this study large yields of F1, typically, up to 45mg per litre were obtained from culture precipitates of *Y. pestis* grown in a chemically defined liquid medium by precipitation with 40% ammonium sulphate followed by resuspension and centrifugation in a low salt buffer (fig. 3.10). The protein was found to be >90% pure using this method and because a chemically defined medium was used there were no contaminating proteins from the culture medium. Although large quantities of cell-bound F1 were released by washing whole cells with1M NaCl (figs. 3.1 and 3.2), these extracts were more heavily contaminated with other cell-surface proteins than was culture supernatant-derived F1. Therefore, growth of *Y. pestis* in liquid culture is a practical approach to scaling-up the production of F1 antigen. In addition, supernatant-derived F1 was not exposed to organic solvents or high concentrations of sodium chloride necessary when extracting cell-bound F1, which may alter the immunogenicity of the protein.

The findings that F1 was released from the cell surface and could be purified from culture supernatant agree with previous reports (Andrews *et al.*, 1996; Englesberg and Levy, 1954). This phenomenon was exploited for the development of this extraction and purification scheme, cell-free F1 was isolated and purified more easily than cell-bound antigen and simplified the procedure when scaled-up.

Endotoxin levels in the purified antigen were tested using the LAL assay. The levels present were thought to be acceptable and no attempt was made to remove it. However, it is unclear as to how endotoxin might interact with protective antigens and how their immunogenicity and protective potency might be affected by the presence of this immunomodulatory outer membrane component. This question could be addressed in any future studies. For example, LPS could be removed using Polymyxin B as described by

Andrews *et al.*, 1996.

The rF1, however, was more difficult to purify than native F1. The *caf*1 sequence was cloned successfully into the pGEX vector and the GST-F1 fusion protein expressed in adequate quantities. However, the fusion protein was found to be insoluble and the majority remained in the sonicate pellet. Attempts were made to solubilise the fusion protein in the sonicate using sarkosyl and purification of the fusion protein by gel-filtration chromatography (fig. 3.20). This was successful but problems were then encountered with the fusion protein not binding efficiently with the glutathione S sepharose column. This may have been caused by conformational changes in the GST part of the fusion protein because of the *caf*1 insert and, therefore, only low yields of recombinant F1 were obtained. However, sufficient rF1 was obtained to carry out some characterisation studies and to examine the protective efficacy of the antigen to a limited extent.

Chapter 4

Characterisation of F1 antigen

4.1 Introduction

The extracellular material surrounding *Y. pestis* has been described by terms such as, "capsule", "envelope" and "slime layer". Englesberg and Levy (1954) found that F1 antigen, the main component of this layer, went into solution prior to extraction, suggesting that the antigenic material was derived from the envelope layer. This material was shed into the culture medium and could be extracted from cell-free culture supernatant (Englesberg and Levy, 1954; Baker *et al.*, 1952), suggesting that it is a soluble envelope or slime layer, rather than a distinct capsule.

Crocker *et al.* (1956) compared the extracellular substance of virulent and avirulent strains, using electron and light microscopy and found that the extracellular substance was more abundant in virulent strains and was easily removed by washing in distilled water.

The ability to synthesise this capsule is characteristic of all fully virulent strains (Burrows and Bacon, 1956); there has been only one recorded human case of plague caused by an $F1^-$ strain and very few naturally occurring virulent strains without the capsule have been isolated from non-human sources (Friedlander *et al.*, 1995; Winter *et al.* 1960). Therefore, F1 is an important virulence determinant and more detailed characterisation of the chemical and physical properties of this antigen might explain why this is so.

The F1 antigen has been reported to be glycosylated, bearing galactose and fucose residues as determined by gas-liquid chromatographic analyses performed on acid hydrolysates of the antigen. Protein and peptide fragments were removed from the hydrolysates with Dowex 1-X8 ion exchange resin. Sugar derivatives were analysed by gas liquid chromatography (GLC) and the main carbohydrate component was identified as galactose (Bennet, and Tornabene 1974; Glosnicka and Gruszkiwicz, 1980). It was also found that F1 formed large aggregates and eluted close to the void volume of a gel filtration column and this was also observed in the present study (section 3.2.4). Aggregated F1 has low mobility when applied to a native polyacrylamide gel, but may be disaggregated by boiling in SDS/mercaptoethanol for 5 to 10min, as demonstrated by increased mobility in polyacrylamide gels under denaturing conditions (section 3.2.3). Disaggregated F1 will reaggregate if the denaturing reagent is removed (Bennet, and Tornabene 1974). F1 aggregates of large molecular weight dissociate into sub-units of between 15-17kDa under

denaturing conditions (Glosnicka and Gruskiwicz, 1980).

The size of the F1 monomer, predicted by the coding sequence of the structural gene, agreed with previous studies which reported it to be 15.0-17.5kDa polypeptide with an isoelectric point of 4.5 in denaturing and IEF gels (section 3.2.5, 3.2.1). The amino acid sequence predicts a hydrophobic protein β-sheet secondary structure (Galyov *et al.*, 1990), and this hydrophobicity may account for the aggregation observed under physiological conditions.

4.2 N-terminal sequencing of the F1 antigen

The primary structure of F1 antigen has been deduced from the nucleotide sequence (Galyov *et al.*, 1990). The amino acid sequences of the native and recombinant F1 were determined in order to make a comparison with the deduced sequence. In these experiments, the Pehr Edman reaction process was used (Tschesche and Dietl, 1975), involving the sequential degradation of proteins and peptides to determine the sequence of the constituent amino acids. The process allows repeated cycles of degradation of the N-terminal amino acid and subsequent identification.

Samples of purified native F1 or recombinant F1 were applied to a polyacrylamide gel using 2μg, 6μg, 12μg, 14μg and 20μg of protein. The proteins were then transferred to nitro-cellulose membrane (0.2μm pore size) by Western blot. The proteins were stained using Amido Black total protein stain and the F1 band of an appropriate intensity was selected for sequencing. The first ten amino acids were successfully sequenced (fig. 4.1) and agreed with the published sequence (Galyov *et al.*, 1990). The only exception is that in the rF1, the first three amino acids are from the pGEX plasmid, indicating that the cleavage site is a few amino acids before the *caf*1 sequence begins. In addition, less rF1 was loaded onto gels and hence fewer amino acids were sequenced.

4.3 Investigation of the glycosylation of the F1 antigen

Attempts were made during the course of this study to confirm the report that the native F1 is glycosylated (Bennet and Tornabene, 1974; Glosnicka and Gruszkiwicz, 1980).

Fig. 4.1 N-terminal amino-acid sequence of F1 antigen determined by Edman degradation

4.1a Native F1

Residue 1 5 10 15

A - D - L - T - X - X - T - T - A - - - - - - -

4.1b Recombinant F1

Residue 1 5 10 15

G - I - P - A - D - L - T - A - - - - - - - -

Key : G = glycine
I = isoleucine
P = proline
A = alanine
D = aspartic acid
L = leucine
T = threonine
X = undetermined

4.3.1 PAS stain for glycoproteins

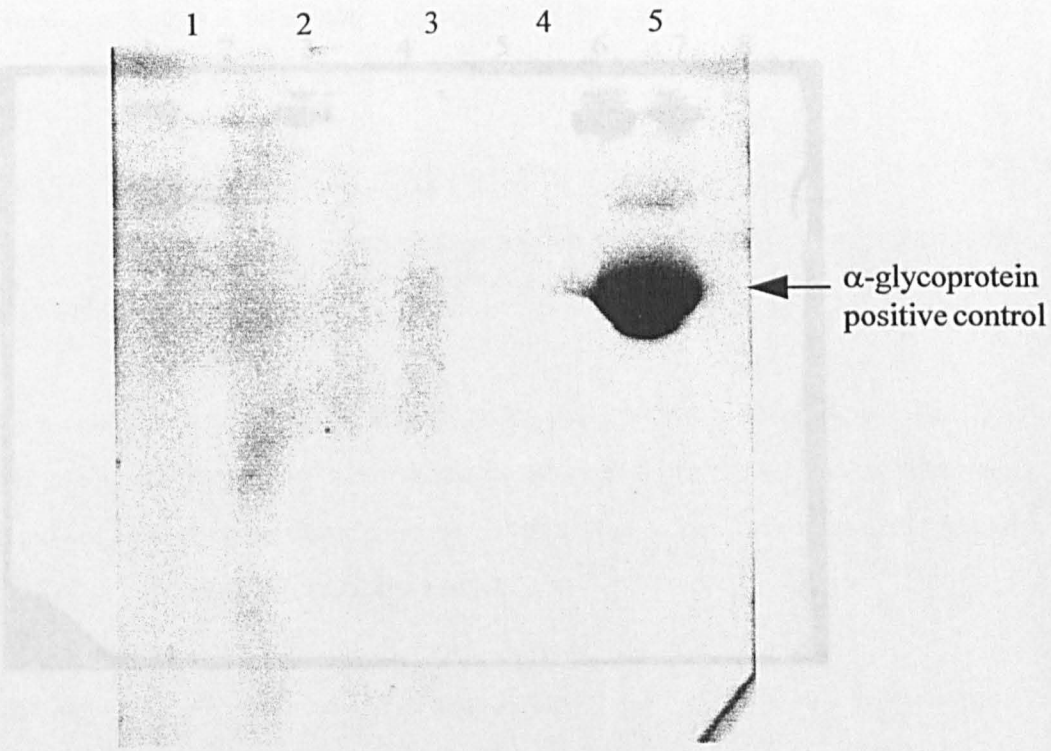
Polyacrylamide gels were stained using a PAS stain procedure (section 2.5.6) developed from published methods (Matthieu and Quarles, 1973: Segrest and Jackson, 1971). Purified F1 antigen was run on polyacrylamide gels together with a glycosylated (α-glycoprotein) and a non-glycosylated protein (lysozyme) as positive and negative controls respectively. The glycosylated α-glycoprotein stained as a pink band, whereas the F1 and the non-glycosylated lysozyme did not stain, indicating that F1 is not sufficiently glycosylated to be detected by this method (fig 4.2). This procedure was repeated several times using increasing concentrations of protein, but staining of the F1 antigen was not observed.

4.3.2 Detection of glycosylation by enzyme immunoassay

To confirm the PAS stain results, the F1 antigen was tested using a DIG glycan detection kit (Boehringer Manheim). In this assay, adjacent hydroxyl groups in sugars are oxidised to aldehyde groups by mild periodate treatment. The spacer-linked steroid hapten, DIG, is then covalently attached to these aldehydes via a hydrazide group. DIG-labelled glycoconjugates are subsequently detected in enzyme immunoassay using a DIG-specific antibody conjugated to alkaline phosphatase. Purified F1 was run on polyacrylamide gels together with glycoprotein positive and negative controls. Proteins were then transferred onto a 2μm nitro-cellulose membrane, and stained with Ponceau S stain supplied in the kit. The position of the molecular weight markers, F1 and controls were marked (this is demonstrated in fig. 4.4b). The membrane was then probed using DIG glycan detection kit reagents to detect glycoproteins bound to the membrane, a positive result being indicated by development of a dark band. In figure 4.3, only the positive control glycoprotein produced a dark band, again suggesting that F1 is not glycosylated to a level detectable by this method. The positive and negative controls were transferrin and creatinase respectively and were supplied in the glycan detection kit.

Since the previous experiments suggested that the purified material was not glycosylated, a further study was carried out using F1 samples extracted from *Y. pestis* culture supernatants derived from cells grown in YPMH and a complex medium (blood agar base broth). Cultures (100ml) in both media were grown at 37^{0}C for 48h. F1 antigen was

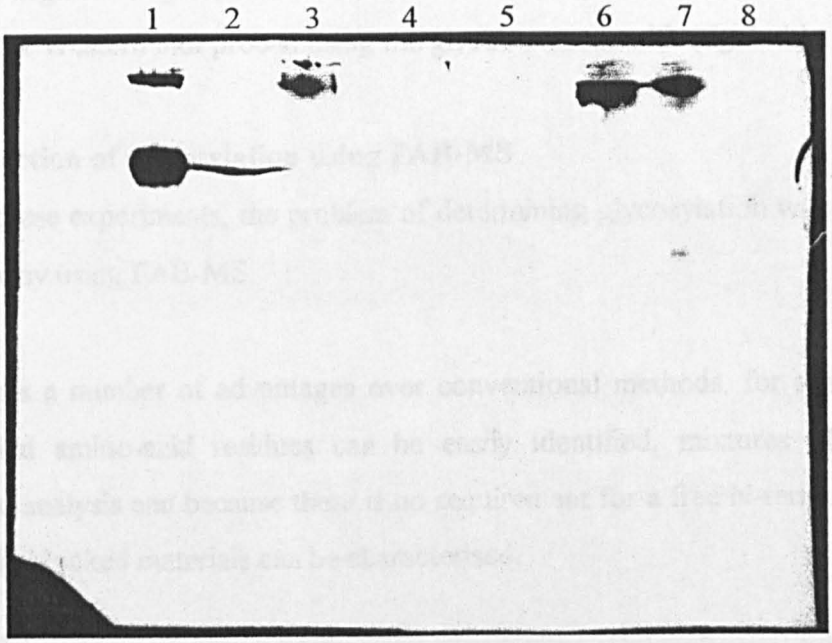
Fig. 4.2 Periodic Acid Schiffs (PAS) Stain for glycoproteins



Lane : 1. Molecular weight markers
2. Lysozyme (negative control)
3. F1 antigen
4. F1 antigen
5. α-glycoprotein (positive control)

Only the positive control glycoprotein has stained with PAS stain.
Glycoproteins show up as an intense pink band when stained using this method.

Fig. 4.3 Detection of glycoproteins using dioxygenin (DIG) glycan detection system



Lane : 1. Transferrin (positive control glycoprotein)
2. no sample
3 - 6. F1 antigen
7. Creatinase (negative control)
8. Molecular weight markers

The dioxygenin probe reacts with glycosylated proteins producing an intense, dark band. The transferrin positive control was detected in this way, there was some reaction with the negative control but the band was not as intense.There was some reaction with material at the top of the gel where the material had not run into the SDS-page gel used for this Western blot, also some of the material from lane 1 had leaked into lane2. F1 antigen was not detected.

purified from the culture supernatant directly or from a 1M NaCl wash of the cells in an attempt to determine whether the F1 antigen requires a more complex media in order to be glycosylated or that the F1 more closely associated with the cell is glycosylated. Proteins bound to a nitro-cellulose membrane were again stained with Ponceau S and marked (fig.4.4b), the membrane was then probed using the DIG glycan detection kit. The results showed that only the positive control glycoprotein produced a dark band, all F1 samples tested were negative. Figure 4.4a shows a Coomassie Blue stained polyacrylamide gel and the equivalent Western blot probed using the glycan detection kit (fig 4.4b).

4.3.3. Detection of glycosylation using FAB-MS

Following these experiments, the problem of determining glycosylation was approached in a different way using FAB-MS.

FAB-MS has a number of advantages over conventional methods, for example, unusual and modified amino-acid residues can be easily identified, mixtures of peptides are amenable to analysis and because there is no requirement for a free N-terminus, cyclic and N-terminally blocked materials can be characterised.

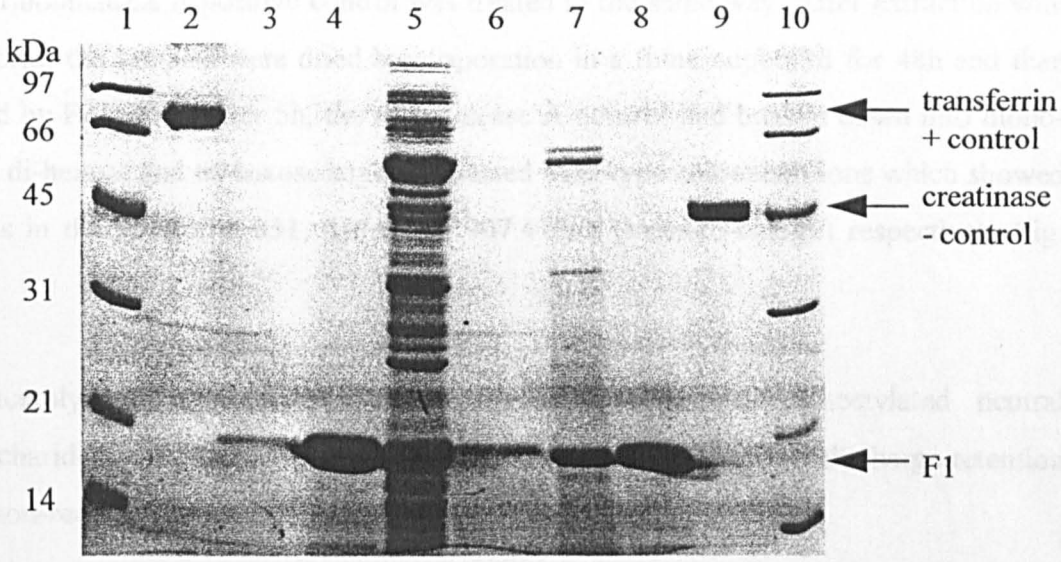
The technique is particularly useful in the identification of post-translational modifications including glycosylation. FAB-MS can also be used to check the correctness of chromosomal DNA-derived protein sequences.

4.3.3.1 Acetolysis

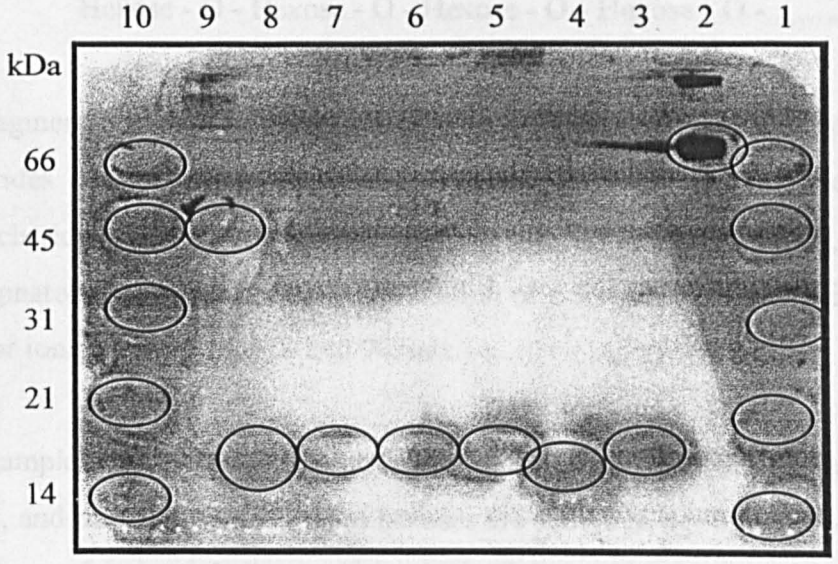
Purified F1 was examined by FAB-MS after acetolysis of the native protein (Naik *et al.*, 1985). The procedure is a useful preliminary to a more detailed structural study. The method is based on acetolysis of an intact glycoconjugate generating a mixture of peracetylated carbohydrate groups from oligosaccharides and glycoproteins which are then extracted with chloroform and analysed by FAB-MS. Contaminating salt and peptide fragments generated by acetolysis remain in the water layer and do not interfere with the subsequent mass spectrometric analysis. In this way, the presence of hexoses, aminohexoses and sialic acid can be determined. The aim of these experiments was to determine whether acetolysis and subsequent FAB-MS of F1 could detect the presence of carbohydrate; the time course used, in principle, would permit the detection of fragments from labile and resistant glycosidic bonds.

Fig. 4.4 Comparison of F1 antigen grown in different culture media for detection of glycosylation using DIG kit

4.4a SDS PAGE



4.4b Western blot and detection of glycosylation by dioxygenin conjugate

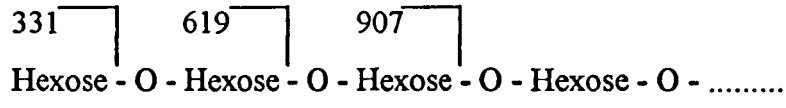


For both gels:

Lane: 1. Molecular weight markers
2. Transferrin (+ control)
3. F1 in YPMH culture supernatant
4. F1 purifed from YPMH culture supernatant
5. F1 in 1M NaCl wash of cells grown in YPMH
6 -8. As lanes 3 to 4 except the samples are from cells grown in Blood Agar Base Broth
7. Creatinase (- control)
8. Molecular weight markers

One milligram of purified F1 antigen was used and samples taken at 0.5h, 2h, 5h, 8h and 24h. A ribonuclease A positive control was treated in the same way. After extraction with chloroform, the samples were dried by evaporation in a fume cupboard for 48h and then analysed by FAB-MS. After 5h, the ribonuclease A control had broken down into mono-hexose, di-hexose and tri-hexose sugars, detected as B-type carbenium ions which showed as peaks in the spectra at 331, 619.2 and 907.41m/z (mass-to-charge) respectively (fig. 4.5a).

With acetolysis, the dominant pattern of cleavage of the peracetylated neutral polysaccharides is on the non-reducing side of the glycosidic oxygen, with charge retention on the non-reducing fragment, giving B-type carbenium ions:



Such fragments would however, also arise from peracetylated monosaccharides, disaccharides and trisaccharides generated by acetolysis. The presence of free monosaccharide cannot be inferred from the presence of the ion at 331m/z, it could equally have originated from (gas-phase) fragmentation of a longer oligosaccharide and this also applies for ions generated at 619 and 907m/z.

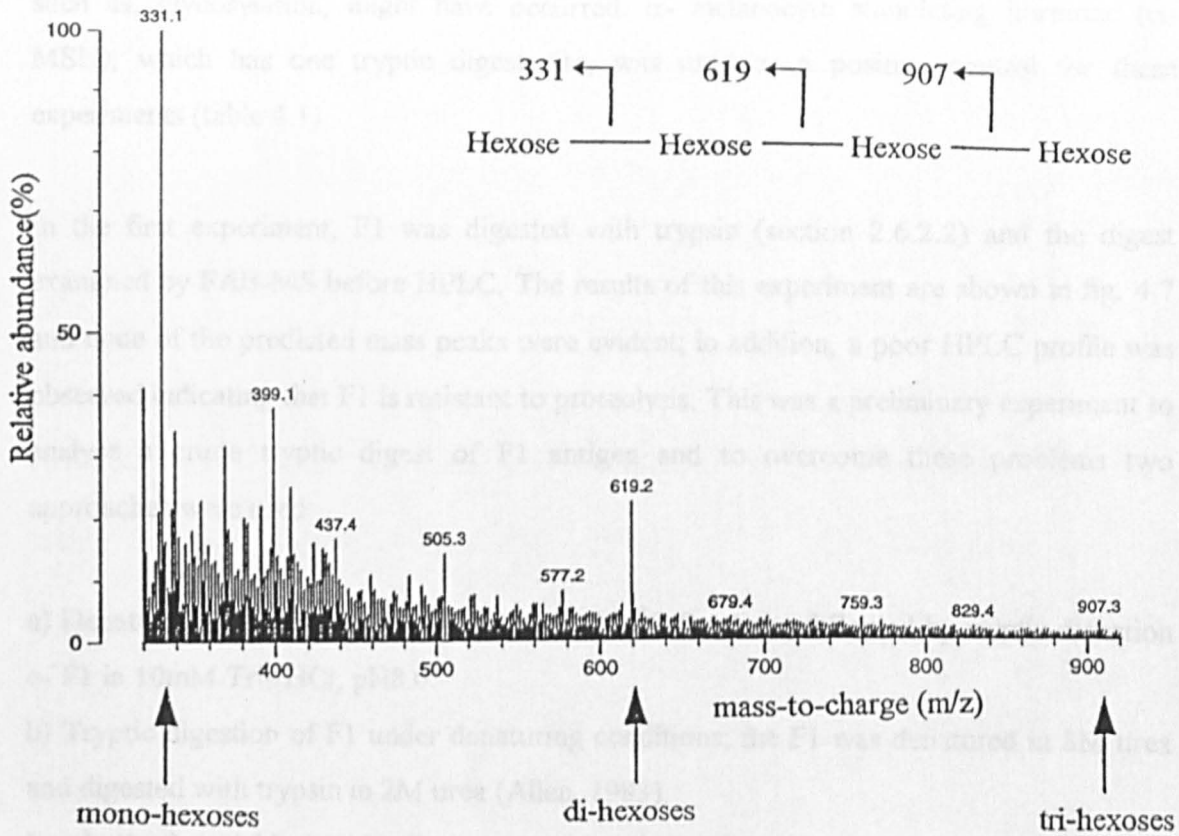
The F1 samples collected at different time points during acetolysis were also analysed by FAB-MS, and peaks corresponding to hexoses are shown in fig. 4.5b. The peaks produced by the F1 samples were similar to those obtained from the positive control, indicating the presence of carbohydrate in the purified antigen. However, this technique was only an indication of glycosylation and does not establish that a protein is a glycoprotein; a sample contaminated with polysaccharide would give similar results to a bonafide glycoprotein.

4.3.3.2 Tryptic digestion of the F1 antigen

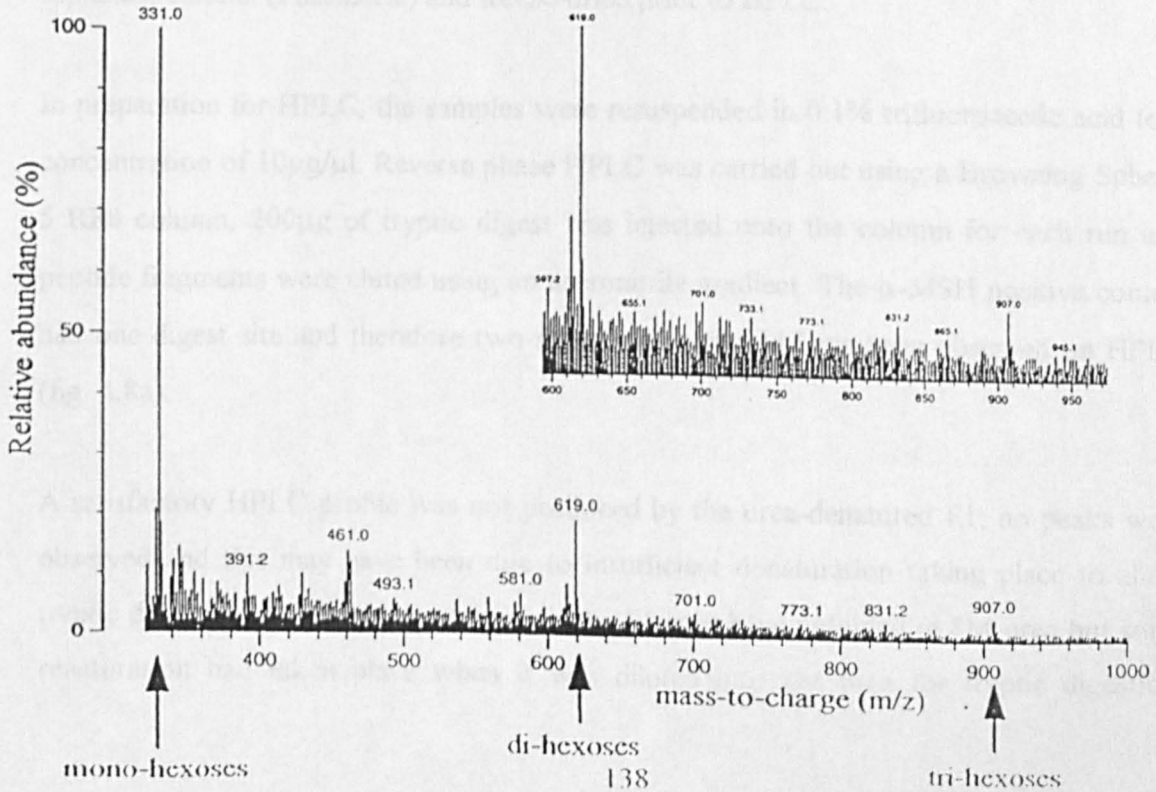
This series of experiments aimed to digest the F1 with trypsin, followed by separation of the resulting peptide fragments by Reverse Phase HPLC (Mant and Hodges, 1991) and subsequent analysis of the fragments by FAB-MS. The predicted fragments produced by

Fig. 4.5 FAB-MS of positive control glycoprotein (ribonuclease A) after 5h of acetolysis showing the signals corresponding to hexose polymers and comparison with the F1 antigen.

4.5a. Ribonuclease A



4.5b. F1 antigen



digestion with trypsin for both F1 and the control protein, and the calculated masses of these fragments, are shown in tables 4.1 and 4.2 and fig.4.6. If actual mass of fragments does not agree with the calculated mass, post-translational modification of the protein, such as, glycosylation, might have occurred. α- melanocyte stimulating hormone (α-MSH), which has one tryptic digest site, was used as a positive control for these experiments (table 4.1).

In the first experiment, F1 was digested with trypsin (section 2.6.2.2) and the digest examined by FAB-MS before HPLC. The results of this experiment are shown in fig. 4.7 and none of the predicted mass peaks were evident; in addition, a poor HPLC profile was observed indicating that F1 is resistant to proteolysis. This was a preliminary experiment to analyse a crude tryptic digest of F1 antigen and to overcome these problems two approaches were used:

a) Denaturation of F1 antigen by heating at 100^0C for 5min, followed by tryptic digestion of F1 in 10mM Tris/HCl, pH8.0.
b) Tryptic digestion of F1 under denaturing conditions; the F1 was denatured in 8M urea and digested with trypsin in 2M urea (Allen, 1983).
For both a) and b), tryptic digests were sampled at 4h, 6h and 16h and freezing the samples at -20^0C terminated digestion. The samples were desalted using a PD10 G25 sephadex column (Pharmacia) and freeze-dried prior to HPLC.

In preparation for HPLC, the samples were resuspended in 0.1% trifluoroacetic acid to a concentration of 10μg/μl. Reverse phase HPLC was carried out using a Browning Spheri-5 RP8 column, 200μg of tryptic digest was injected onto the column for each run and peptide fragments were eluted using an acetonitrile gradient. The α-MSH positive control had one digest site and therefore two major peaks should have been observed on HPLC (fig. 4.8a).

A satisfactory HPLC profile was not produced by the urea-denatured F1; no peaks were observed and this may have been due to insufficient denaturation taking place to allow tryptic digestion of the protein. Alternatively, F1 may have unfolded in 8M urea but some renaturation had taken place when it was diluted into 2M urea for tryptic digestion,

Table 4.1 Amino acid sequence of α-MSH showing the tryptic digest site, predicted mass of intact peptide and peptide fragments after tryptic cleavage are also shown.

Peptide	Sequence	Calculated Mass[M+H]$^{++}$
Intact	SYSMEHFR↑WGKPV	1664.8
T1	SYSMEHFR	1098.483
T2	WGKPV	585.3274

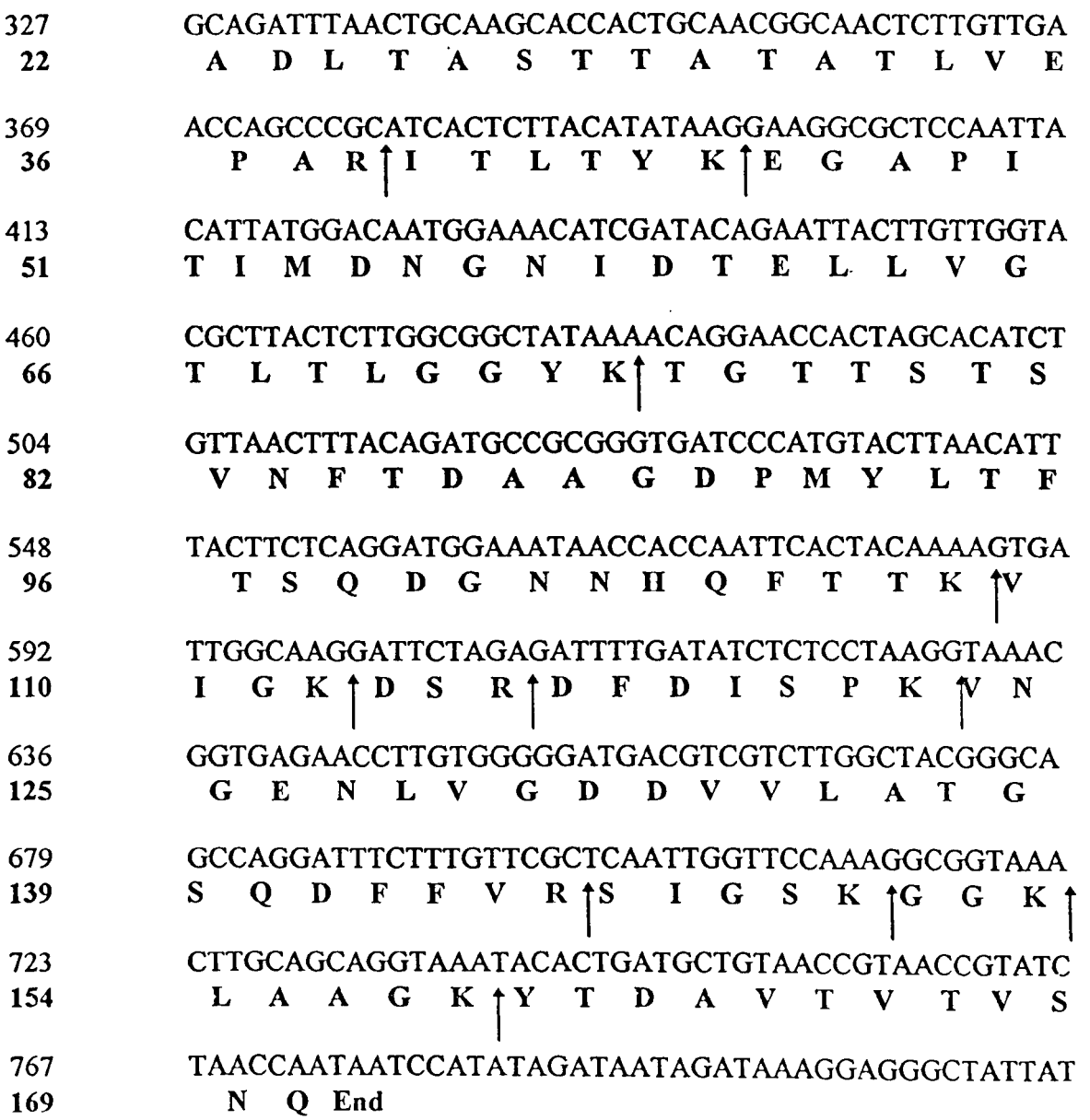
Arrow indicates tryptic digest site.
Peaks on the FAB-MS spectra should be obtained at the predicted mass values

Table 4.2 Predicted peptide fragments from the tryptic cleavage of the product of *Y. pestis caf*1 gene.

Peptide	Sequence	Calculated Mass[M+H]$^+$
TI	I_3 - R_{39}	3504.9
T2	I_{40} - K_{45}	738.87
T3	E_{46} - K_{73}	2907.282
T4	T_{74} - K_{108}	3757.95
T5	V_{109} - K_{112}	416.5
T6	D_{113} - R_{115}	377.35
T7	D_{116} - K_{122}	821.88
T8	V_{123} - R_{145}	2452.65
T9	S_{146} - K_{150}	491.54
T10	G_{151} - K_{153}	261.277
T11	L_{154} - K_{158}	459.54
T12	K_{159} - Q_{170}	1498.606

Peaks on the FAB-MS spectra should be obtained at the predicted mass values. However, some of the low mass fragments may not be detected.

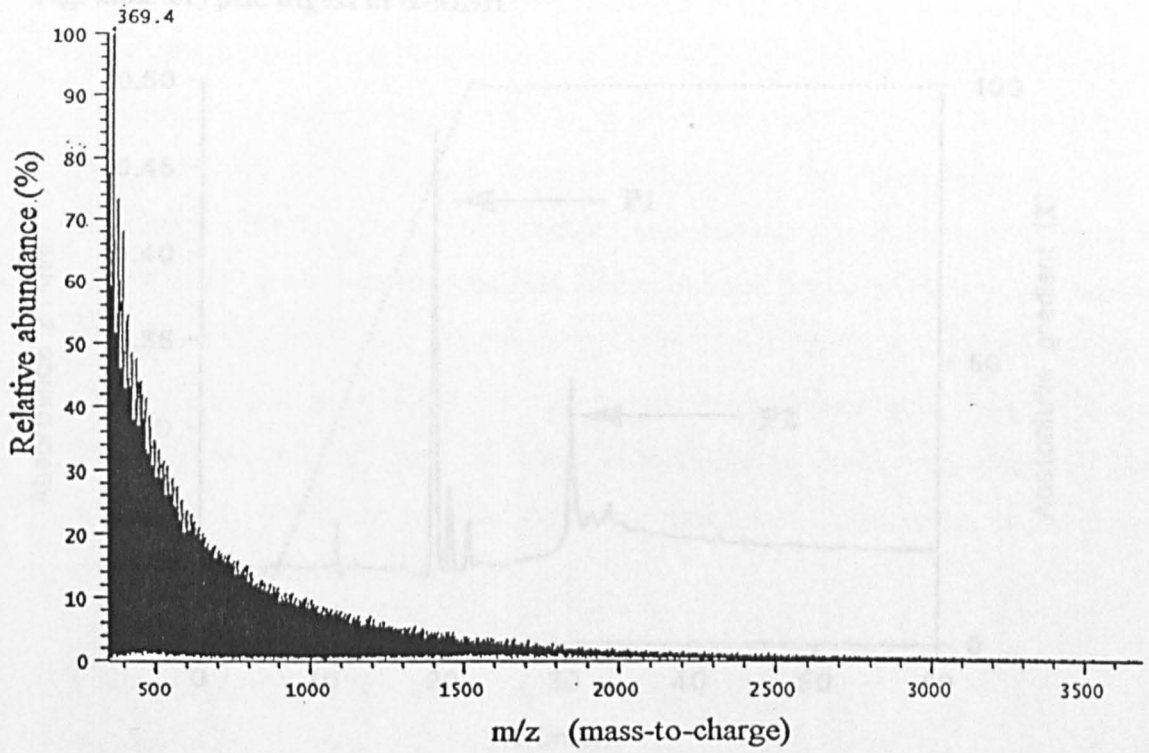
Fig. 4.6 **Predicted peptides produced by the tryptic cleavage of the product of *Y. pestis caf*1 gene**



Nucleotide and deduced amino acid sequence of the *Y. pestis caf*1 gene (Galyov *et al.*, 1990). Arrows indicate tryptic digestion sites.

Fig. 4.7 FAB-MS of tryptic digest of F1 antigen

4.7a. Scans 3 -9



4.7b. Scans 4 - 10

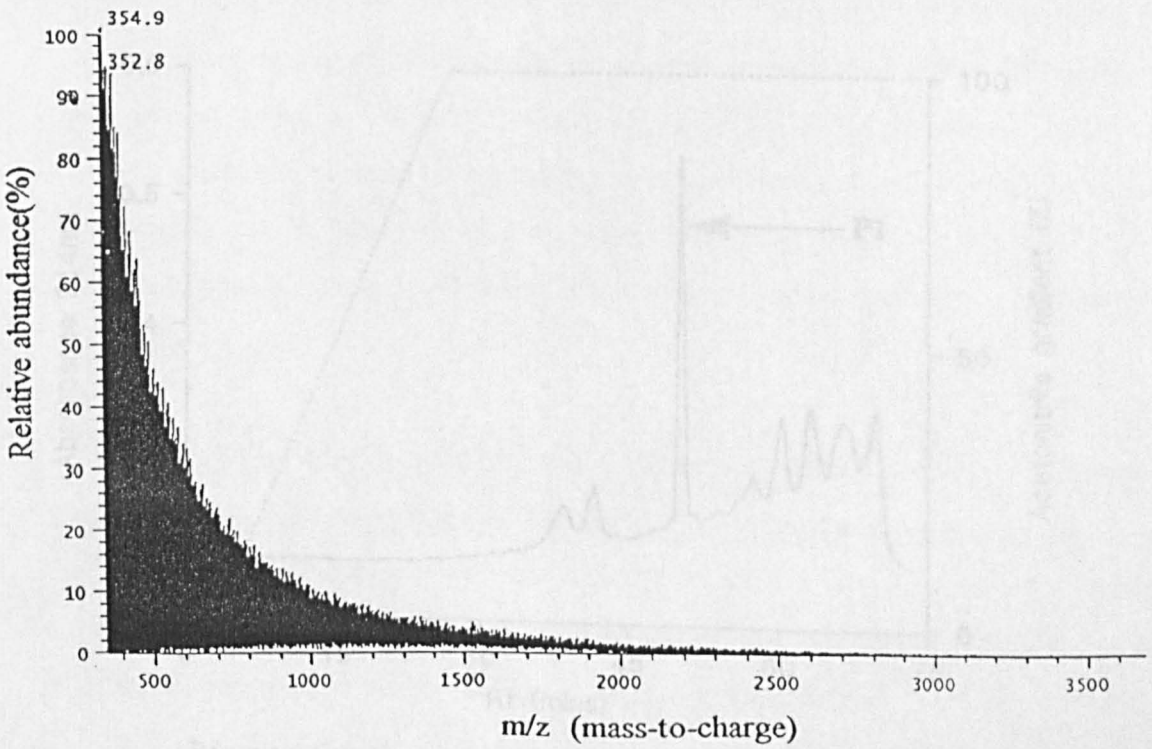


Fig. 4.8 HPLC of tryptic digests of α-MSH and F1 antigen using a Spheri-5 RP8 column

Fig. 4.8a Tryptic digest of α-MSH

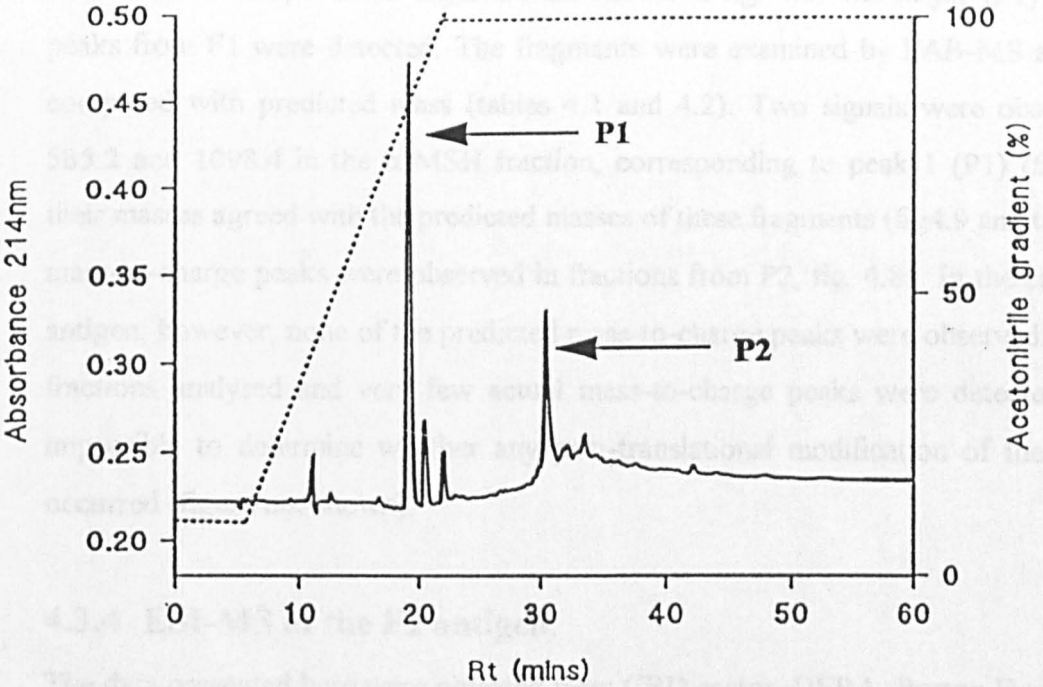
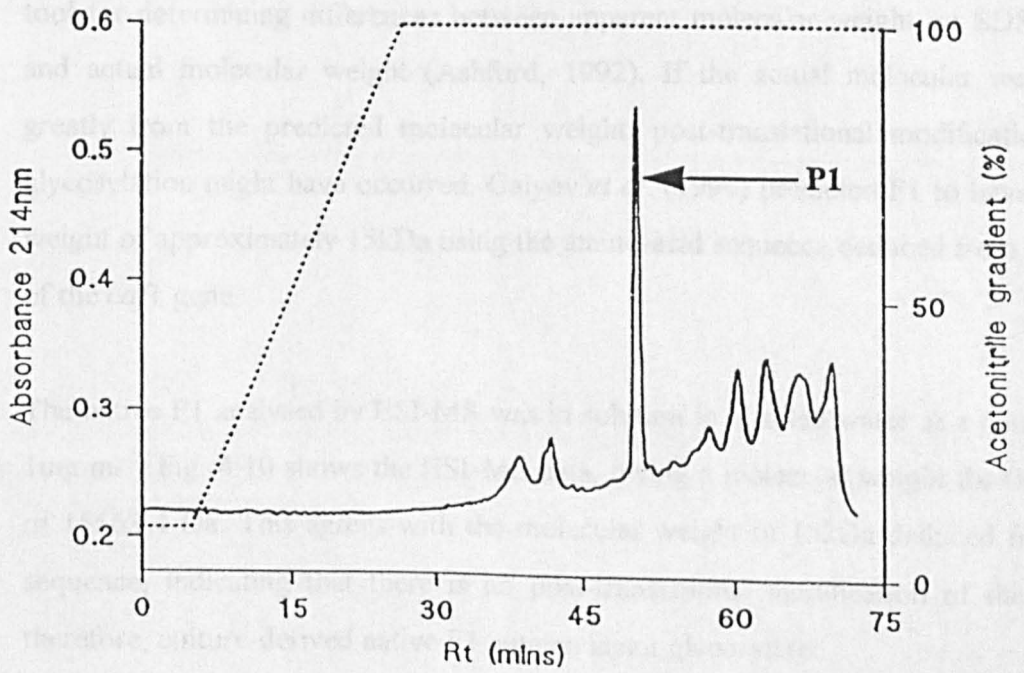


Fig. 4.8b Tryptic digest of heat - denatured F1 after 6h incubation with Trypsin



Rt : retention time

thereby masking the tryptic digest sites. Tryptic digestion takes place in 2M urea because above this concentration trypsin is inactive.

The HPLC profiles of peptide fragments produced from the positive control and the heat-denatured F1 sample at 6h digestion are shown in fig. 4.8, one major (P1) and 8 minor peaks from F1 were detected. The fragments were examined by FAB-MS and their size compared with predicted mass (tables 4.1 and 4.2). Two signals were observed at m/z 585.2 and 1098.4 in the α-MSH fraction, corresponding to peak 1 (P1) (fig. 4.8a) and their masses agreed with the predicted masses of these fragments (fig4.9 and table 4.1). No mass-to-charge peaks were observed in fractions from P2, fig. 4.8a. In the case of the F1 antigen, however, none of the predicted mass-to-charge peaks were observed in any of the fractions analysed and very few actual mass-to-charge peaks were detected, making it impossible to determine whether any post-translational modification of the protein had occurred (figure not shown).

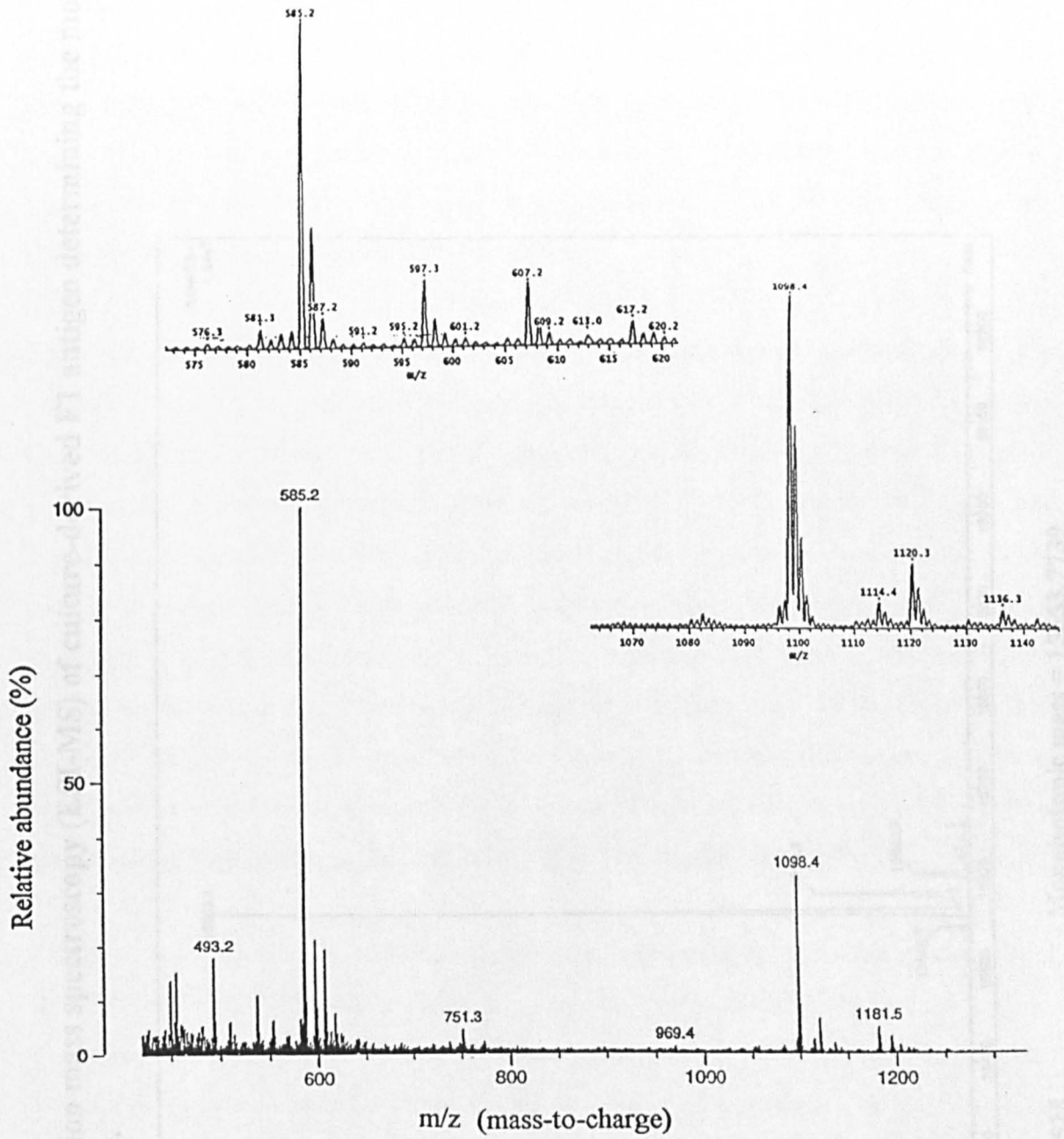
4.3.4 ESI-MS of the F1 antigen.

The data presented here were obtained from CBD sector, DERA, Porton Down, Salisbury and ESI-MS was carried out using the method described in section 2.6.3.

ESI-MS enables an accurate determination of molecular weight and is therefore a useful tool for determining differences between apparent molecular weight on SDS-PAGE gels and actual molecular weight (Ashford, 1992). If the actual molecular weight differed greatly from the predicted molecular weight, post-translational modifications, such as glycosylation might have occurred. Galyov *et al.* (1990) predicted F1 to have a molecular weight of approximately 15kDa using the amino-acid sequence deduced from the sequence of the *caf*1 gene.

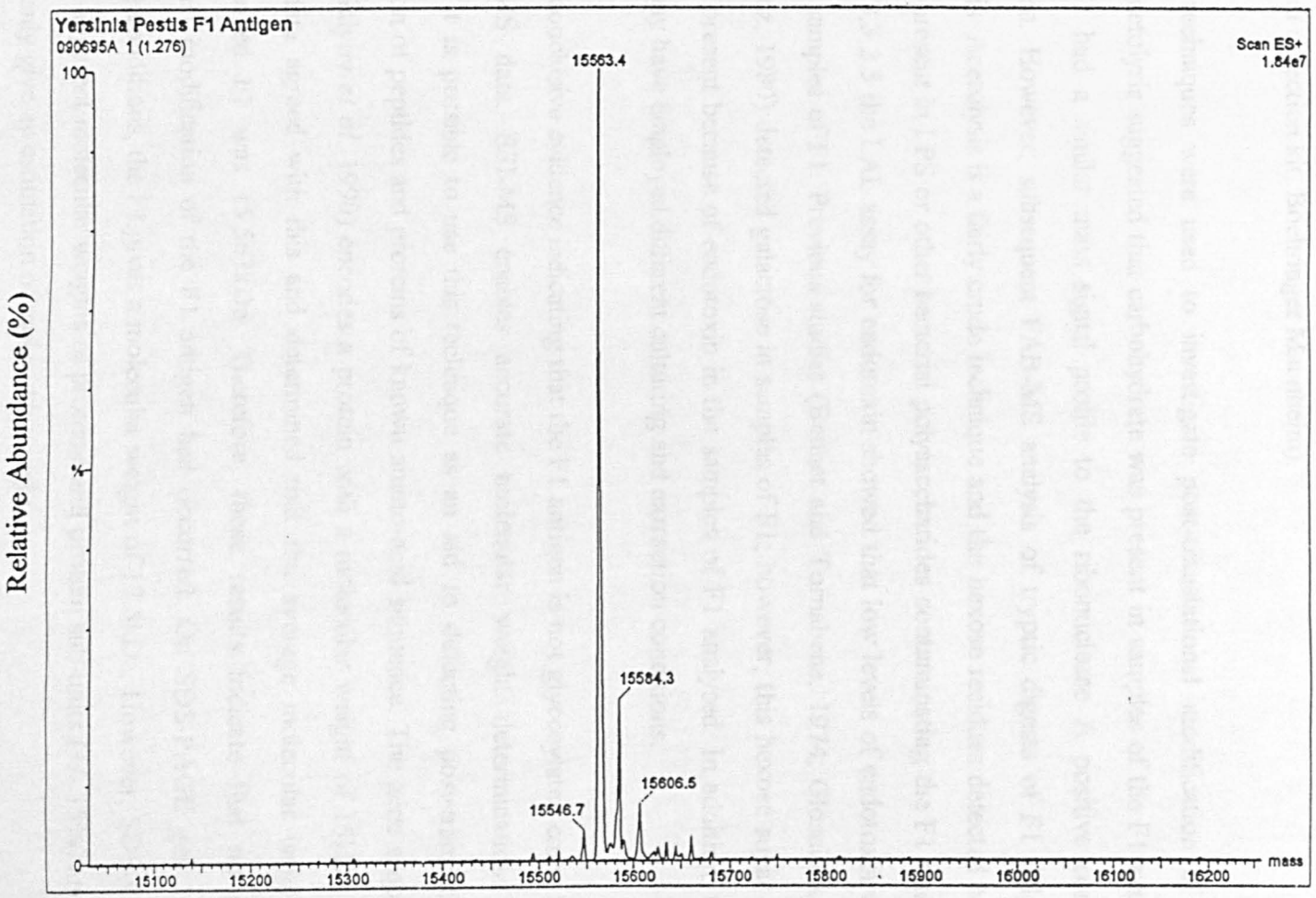
The native F1 analysed by ESI-MS was in solution in distilled water at a concentration of 1mg ml^{-1}. Fig. 4.10 shows the ESI-MS data, giving a molecular weight for the F1 antigen of 15563.4 Da. This agrees with the molecular weight of 15kDa deduced from the gene sequence, indicating that there is no post-translational modification of the protein and therefore, culture-derived native F1 antigen is not glycosylated.

Fig. 4.9 FAB-MS of tryptic digest of α-MSH



This mass spectra was obtained from the fraction corresponding to P1 (fig 4.8a), two major mass peaks were detected corresponding to the expected mass of the predicted peptide fragments (table 4.1).

Fig. 4.10 **Electrospray-ionisation mass spectroscopy (ESI-MS) of culture-derived F1 antigen determining the molecular weight of the protein.**



Average mass = 15563.2528 Monoisotopic mass = 15553.7739

4.4 Discussion

This series of experiments were designed to further characterise the F1 antigen, especially with respect to the glycosylation of the antigen. A number of techniques were used to explore the glycosylated state of the antigen. The classic stain for carbohydrates, PAS stain, only showed the presence of carbohydrate in the glycosylated control protein. Similar results were obtained when proteins were analysed using an enzyme immunoassay (DIG glycan detection kit, Boehringer Mannheim).

FAB-MS techniques were used to investigate post-translational modification of the antigen. Acetolysis suggested that carbohydrate was present in samples of the F1 antigen because F1 had a similar mass signal profile to the ribonuclease A positive control glycoprotein. However, subsequent FAB-MS analysis of tryptic digests of F1 did not confirm this. Acetolysis is a fairly crude technique and the hexose residues detected could have been present in LPS or other bacterial polysaccharides contaminating the F1 sample. In section 3.3.2.5 the LAL assay for endotoxin showed that low levels of endotoxin were present in samples of F1. Previous studies (Bennet and Tornabene, 1974; Glosnicka and Gruszkiwicz, 1980) detected galactose in samples of F1; however, this hexose sugar may have been present because of endotoxin in the samples of F1 analysed. In addition, their research may have employed different culturing and extraction conditions.

The most conclusive evidence indicating that the F1 antigen is not glycosylated came from the ESI-MS data. ESI-MS enables accurate molecular weight determinations and therefore, it is possible to use this technique as an aid to detecting post-translational modification of peptides and proteins of known amino-acid sequence. The gene sequence of *caf*1 (Galyov *et al.* 1990) encodes a protein with a molecular weight of 15kDa. The ESI-MS data agreed with this and determined that the average molecular weight of culture-derived F1 was 15.563kDa. Therefore, these results indicate that no post-translational modification of the F1 antigen had occurred. On SDS-PAGE gels under denaturing conditions, the F1 gives a molecular weight of 17.5kDa. However, SDS-PAGE gives only apparent molecular weights of proteins and protein sub-units (+/- 15%) and can therefore only give an estimation of molecular weight.

Chapter 5

Encapsulation of F1 antigen

5.1 Introduction

A licensed formaldehyde-killed whole cell *Y. pestis* vaccine developed in the 1930's is still in use today despite its known limitations. One major limitation is that cases of pneumonic plague have been reported in vaccinees and the vaccine can induce adverse clinical side effects. Subunit vaccines using protective components from *Y. pestis* may avoid adverse side effects, but will require adjuvants or delivery vehicles to boost immunity. Appropriate delivery formulations may induce both systemic and mucosal immunity thereby increasing the protection against both bubonic and pneumonic plague.

Currently, the only adjuvant licensed for clinical use in humans is Alhydrogel. The sustained release of antigens from depots by formulation of vaccines with adjuvants, such as, Alhydrogel and oil-based adjuvants, is a prerequisite for the generation of potent immune responses. However, the release of antigens from these systems is short-lived compared with the escape of some slow release drugs used for contraception or cancer therapy, which are microencapsulated in biodegradable matrices (Beck *et al.*, 1983; Verrijk *et al.*, 1992). The use of a biodegradable matrix for the encapsulation of antigens may give an extra degree of control in the delivery of vaccines (Kohn *et al.*, 1986; Morris *et al.*, 1994; Walker, 1994), with particular emphasis on mucosal immunisation. Orally administered particulate materials of diameter <10μm are selectively taken up by the specialised M-cells in the gastrointestinal tract (GIT) and accumulate in the immune inductive tissues in the GIT, the Peyer's patches (PPs) (Bockman and Cooper, 1973; O'Hagan *et al.*, 1990). Thus, the formulations of antigens into microparticles within this size range should facilitate their uptake into the PPs and initiate a mucosal immune response. Subsequently, microparticles may pass into the mesenteric lymph nodes and spleen, to generate a systemic immune response.

The use of artificial lipid bilayers in the form of vesicles is another efficient means of presenting antigens to stimulate a potent immune response (Gregoriadis, 1988; Rooijen and Su, 1989). A large variety of phospholipids can be used in an aqueous solution of antigen to form either unilamellar or multilamellar vesicles. Antigens may be lipid soluble and insert into the artificial lipid bilayer, bind to the bilayer or become entrapped inside the liposome vesicle. In multilamellar vesicles, antigen may also become entrapped in the aqueous compartments between the bilayers. The surface may also have a net positive or

negative charge, a feature that can modulate the immune response (Stewart-Tull *et al.*, 1978; Davies and Gregoriadis, 1987). By using phospholipids with a high transition temperature (Tc), or with an excess of cholesterol (CH), the lipid bilayers become rigid at 37^0C and this results in a slower release of antigen.

In this study the F1 antigen was entrapped in PLG microspheres and in liposomes. Additionally, F1was used to label the surface of liposomes. The aim was to produce PLG microspheres of different sizes (1μm and 8μm) and characterise these biochemically and immunologically. The intended size of liposomes was 200nm; phospholipids with high Tc values were used in addition to CH so that the liposomes would release antigen more slowly and as for PLG microspheres, the liposomes were characterised biochemically and immunologically.

5.2 Biochemical characterisation of PLG microspheres containing F1 antigen

5.2.1 Entrapment efficiency

PLG microspheres encapsulating F1 antigen were produced under various conditions. The F1 antigen was dialysed into different buffers in order to determine whether one type of buffer might have an advantage over another with respect to encapsulation efficiency and the amount of F1 entrapped. The solvents used were distilled water, 10mM phosphate buffer, pH7.2 and a pI buffer with a pH at the isoelectric point of the antigen. In this study the pI buffer used was citrate buffer, pH 4.8.

Using the three different buffer systems described above, F1 antigen was microencapsulated into 1μm and 8μm microspheres (Morris *et al.* 1994; Jones *et al.*, 1995). Varying the ratio of polymer used during the encapsulation process (section 2.7.1) controlled the size of the microspheres.

The method for determining the amount of protein encapsulated is described in detail in section 2.7.3. Briefly, microspheres were resuspended in 50μl of 100mM NaOH containing 1% (w/v) SDS and then heated at 100^0C for 4min to disrupt the microspheres and release the entrapped antigen. Samples were then allowed to cool and then centrifuged at 13000rpm for 10min. A BCA reagent protein assay was performed on the supernatant

from each sample; a bovine serum albumin (BSA) protein standard was included in the assay. The BSA standard (2mg ml^{-1}) was treated in the same manner as the microsphere samples, after which it was diluted to a working solution of 1mg ml^{-1}.

The efficiency of entrapment and percent entrapment was calculated for each formulation (Fig. 5.1). The efficiency of entrapment showed that the protein present in distilled water gave the lowest levels of entrapment (approx. 40%), while antigen in phosphate and pI buffers overall had higher entrapment efficiencies (60-80%).

5.2.2 Determination of the size of PLG microspheres encapsulating F1 antigen

The size of the microparticles produced for each formulation was checked using a Coulter counter after calibration with latex beads. For the target size of 8μm PLG microspheres, the average particle size was found to be 7.35μm for all three formulations. However, for the target size of 1μm microspheres the average size was different for each formulation. Microparticles encapsulating F1 antigen in distilled water were found to have an average particle size of 1.72μm; in phosphate buffer, the average was 1.65μm and in pI buffer the average size of the microspheres was 1.99μm. Due to the limitations of the probe used it was not possible to measure the spread of particle size and to overcome this scanning electron microscopy was performed on samples of PLG microspheres (section 5.2.4).

5.2.3 Electron microscopy of PLG microspheres

Microspheres were examined using scanning electron microscopy to determine the spherical nature of the particles, confirm their size and the homogeneity of the PLG preparation (fig. 5.3 and 5.4).

5.2.4 *In vitro* release of the F1 antigen from PLG microspheres

An *in vitro* release study was carried out for each formulation to determine the amount of antigen released over a period of several weeks. Examination of the release profiles of the antigen also enables determination of whether the F1 is internalised or coating the microparticles; if the antigen is coating the microspheres a much more rapid release profile would be expected. Freeze-dried microspheres (50μg) were resuspended in 50μl PBS containing 0.05%(w/v) sodium azide and incubated at 37^0C on a Rotamix eppendorf rack. Samples (50μl) were removed at timed intervals over a period of six weeks. The

Fig. 5.1 **Entrapment efficiencies and percent entrapment in different formulations of microencapsulated F1 antigen**

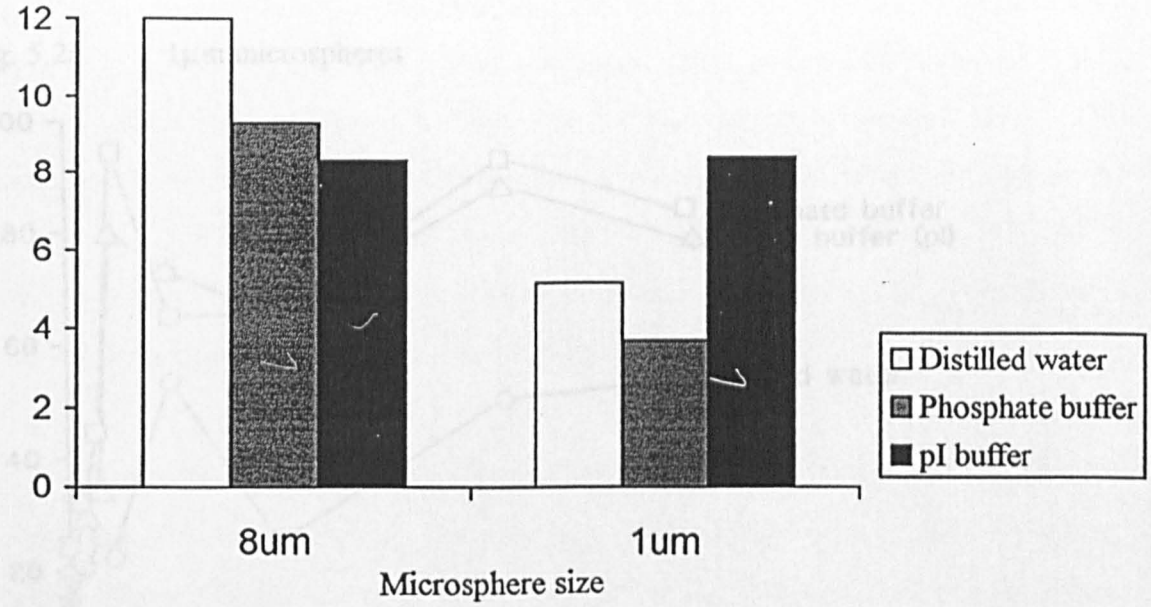


Table 5.1

Microsphere size (μm)	Buffer used	Protein conc in microspheres (μg ml^{-1})	% Protein entrapped
8	dH_2O	12	42
8	10mM phosphate	9.3	77.5
8	10mM citrate (pI)	8.3	69.2
1	dH_2O	5.2	43
1	10mM phosphate	3.7	31
1	10mM citrate (pI)	8.4	70

Fig. 5.2 ***In vitro*** **release profiles of microencapsulated F1 antigen in 1μm and 8μm microspheres using three different buffer systems.**

Fig. 5.2a 1μm microspheres

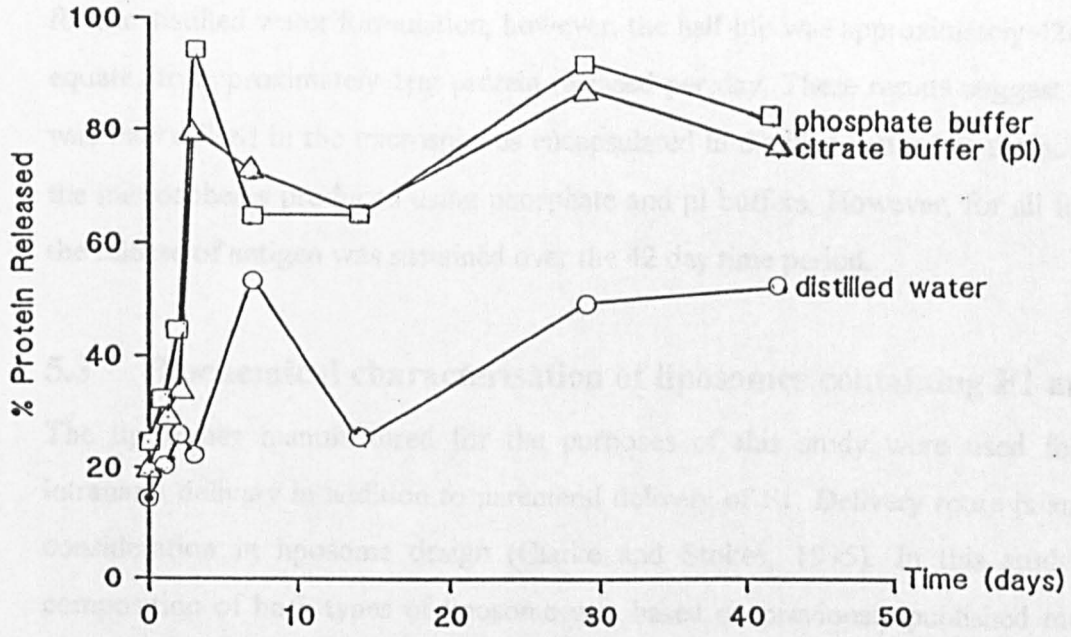
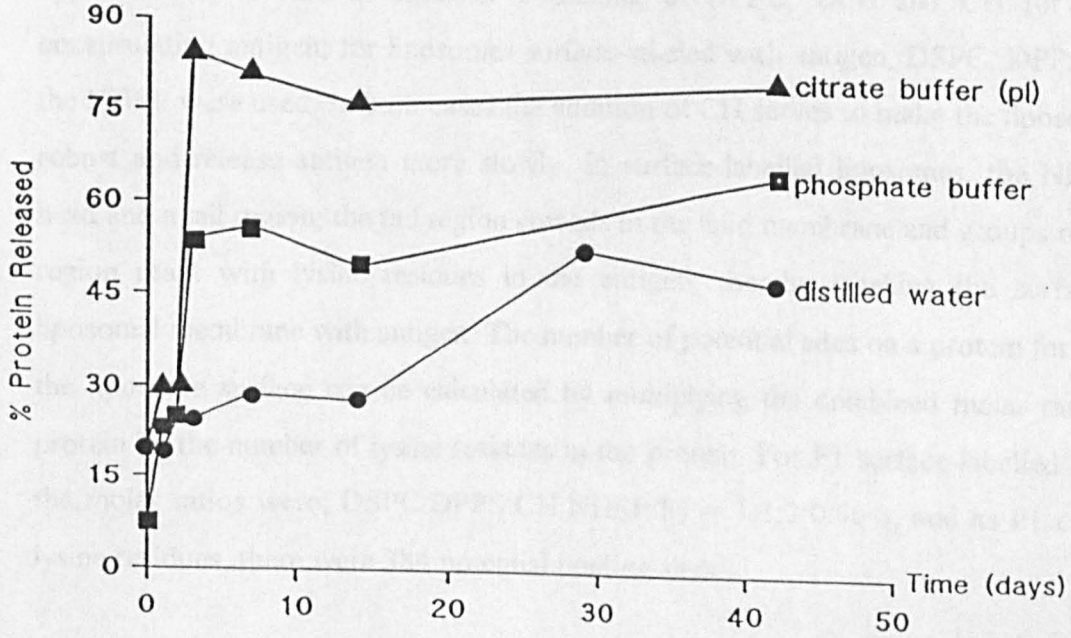


Fig. 5.2b 8μm microspheres



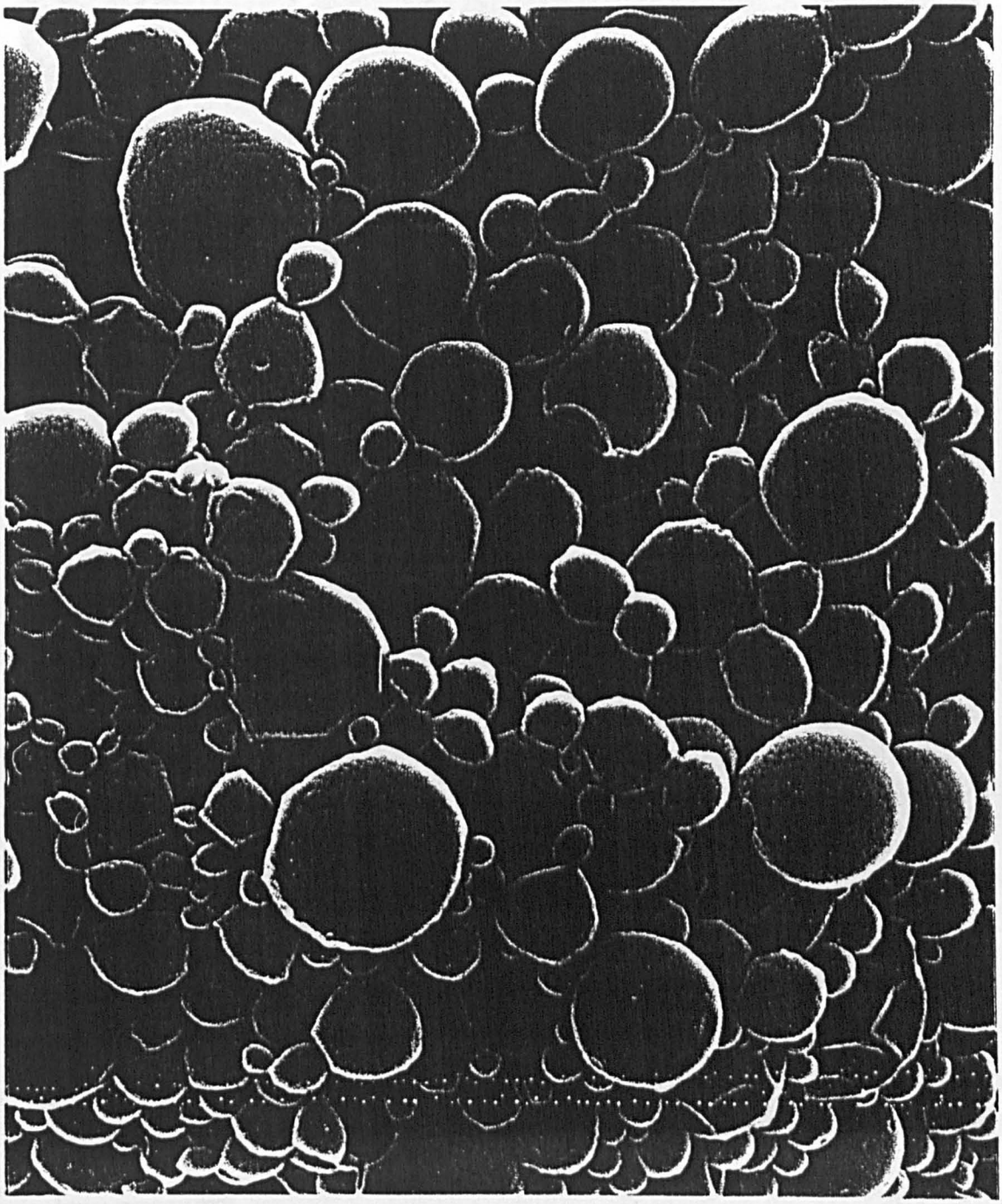
supernatant from these samples was assayed using BCA reagent to determine the protein concentration and the percent protein released was plotted against time.

Fig. 5.2 shows the *in vitro* release profile for each formulation. 1μm and 8μm microspheres with F1 microencapsulated in phosphate and citrate buffers released the antigen much more rapidly than the distilled water formulation. The time taken to release 50% of the total protein (half-life) for phosphate and citrate buffer was less than 10 days; for the distilled water formulation, however, the half-life was approximately 42days, which equates to approximately 1μg protein released per day. These results suggest that the F1 was internalised in the microspheres encapsulated in distilled water but surface bound on the microspheres produced using phosphate and pI buffers. However, for all formulations the release of antigen was sustained over the 42 day time period.

5.3 Biochemical characterisation of liposomes containing F1 antigen

The liposomes manufactured for the purposes of this study were used for oral and intranasal delivery in addition to parenteral delivery of F1. Delivery route is an important consideration in liposome design (Clarke and Stokes, 1995). In this study, the lipid composition of both types of liposome was based on previously published methods and formulated for oral delivery (Childers *et al.*, 1987), achieving as high a value as possible for entrapped volume:lipid ratio. The aim was to produce unilamellar vesicles of approximately 200nm in diameter consisting of DPPC, DCP and CH for liposomes encapsulating antigen; for liposomes surface-labeled with antigen, DSPC, DPPS, CH and the NHSP were used. In both cases the addition of CH serves to make the liposomes more robust and release antigen more slowly. In surface-labelled liposomes, the NHSP has a head and a tail region; the tail region embeds in the lipid membrane and groups on the head region react with lysine residues in the antigen, thereby labelling the surface of the liposomal membrane with antigen. The number of potential sites on a protein for binding to the liposome surface can be calculated by multiplying the combined molar ratios of the protein by the number of lysine residues in the protein. For F1 surface-labelled liposomes, the molar ratios were; DSPC:DPPS:CH:NHSP:F1 = 1:1:2:0.56:8, and as F1 contains 48 lysine residues, there were 384 potential binding sites.

Fig. 5.3 Scanning electron microscopy of 1μm PLG microspheres encapsulating F1 antigen

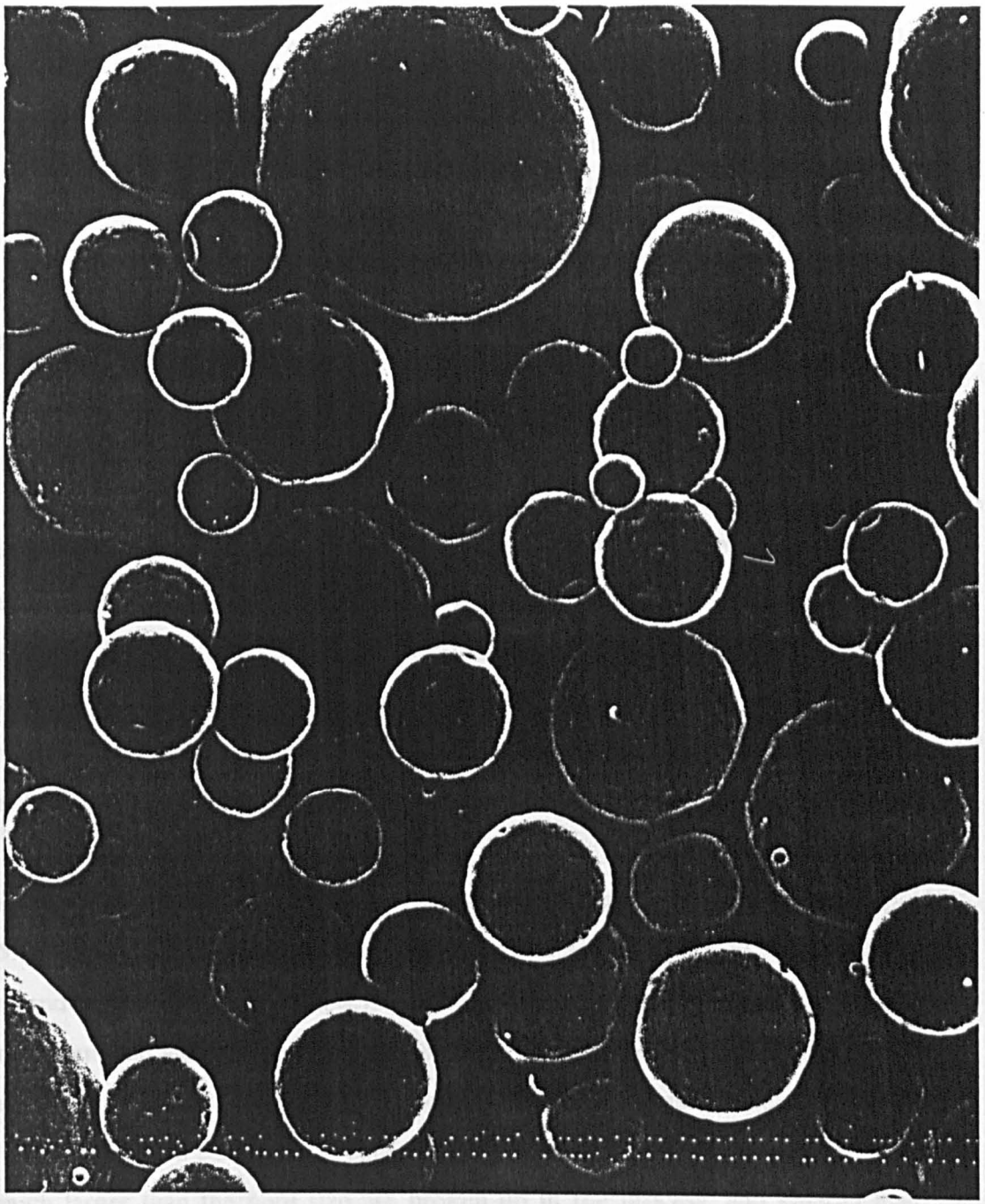


2.0μm

Magnification x 6239

In a sample of 30 randomly selected microspheres : smallest = 0.276μm
largest = 1.86μm
mean diameter = 0.88μm
standard deviation = 0.47

Fig. 5.4 Scanning electron microscopy of 8μm PLG microspheres encapsulating F1 antigen



20.0μm

Magnification x 1612

In a sample of 30 randomly selected microspheres : smallest = 2.90μm
largest = 24.1μm
mean diameter = 9.82μm
standard deviation = 5.64

Surface-labeled liposomes were produced for comparison with liposomes encapsulating antigen to determine whether any advantage might be gained from F1 being presented on the external surface. The hypothesis was that surface-labeled liposomes might mimic the *in vivo* situation where F1 is presented on the surface of *Y. pestis*.

The liposomes encapsulating the F1 antigen had a net negative charge because DCP was included in their formulation, whereas for surface bound F1, the net charge of the liposomes was dependent on the net charge on the protein.

5.3.1 Sizing of liposomes

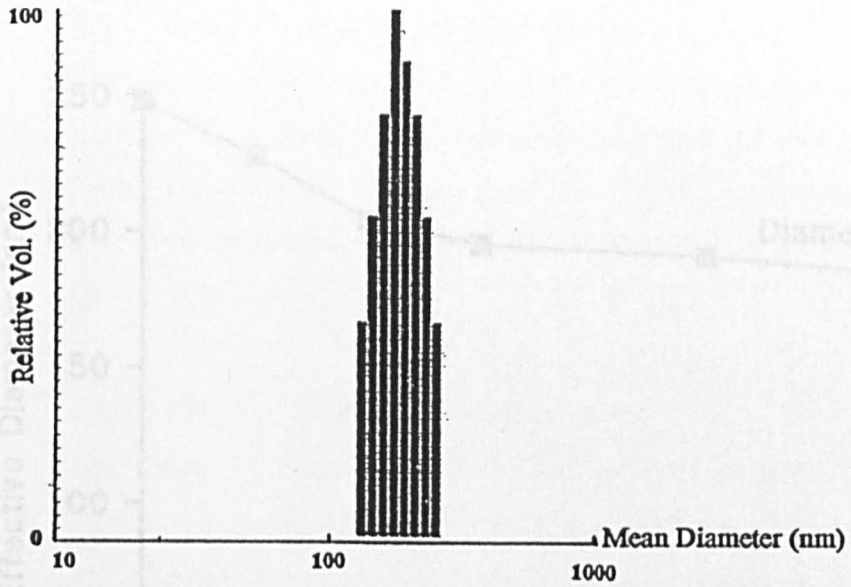
Liposomes were sized using PCS; a description of this method is given in section 2.7.6.1. The size of liposomes encapsulating F1 antigen was consistent from batch-to-batch, ranging from 181nm to 220nm; for surface-labeled liposomes the average diameter was approximately 200nm with polydispersity values of approximately 0.1, which indicated that the samples were homogenous suspensions of liposomes of uniform size. Fig. 5.5 shows a typical PCS profile for both encapsulating and surface-labeled liposomes.

In one study, the size of the liposomes was monitored throughout the extrusion process (fig. 5.6). Extrusion of liposomes was carried out using Nucleopore PC membrane filters, 0.4μm and 0.2μm in size (section 2.7.5). The pores in this type of membrane go straight through from one side to the other and so offer minimal resistance to material passing through it. The inherent flexibility of phosphlipid lamellae enables liposomes to change their conformation so that they can squeeze through the pores. However, liposomes which are much larger than the pore size are broken up in the process, and emerge from the membrane pore smaller than before. After several extrusion cycles through the membrane, a population of liposomes will be reduced in size to an average diameter somewhat smaller than the diameter of the membrane pore, with a small proportion still larger than the pore-size, having squeezed through without breaking up. This was evident from the size distribution analysis (fig. 5.5).

After extruding through two 0.4μm membranes, the liposomes had an effective diameter of 250nm. As the liposomes were extruded through 0.2μm membrane filters for eight

Fig. 5.5 Typical PCS profiles of encapsulating and surface-labelled liposomes

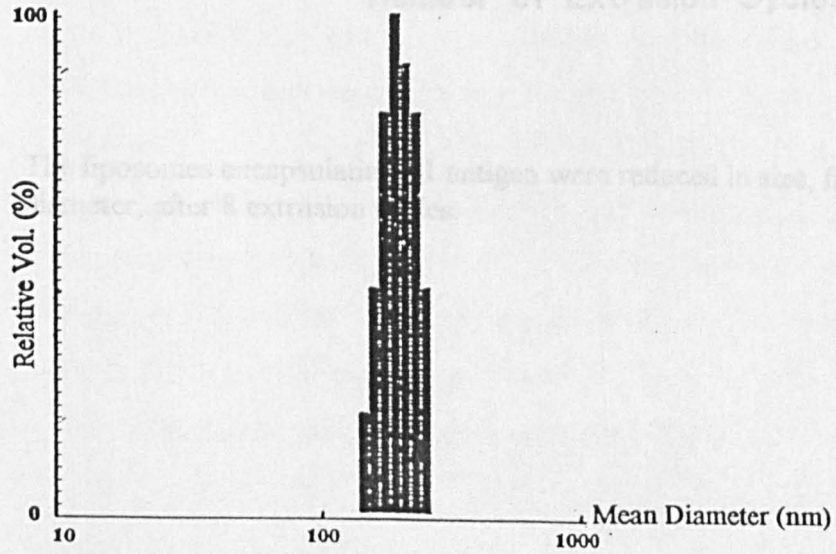
Fig. 5.5a. Liposomes encapsulating F1 antigen



Effective diameter = 181nm

Polydispersity = 0.042

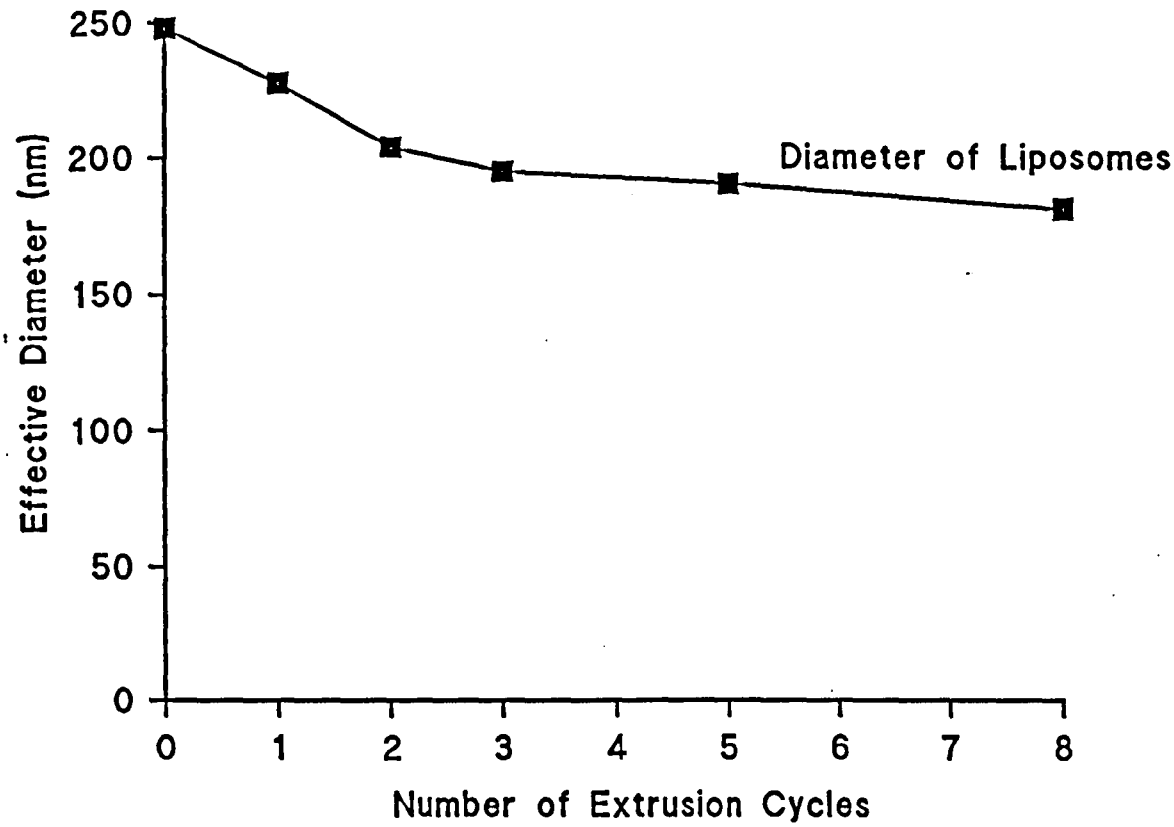
Fig. 5.5b. Liposomes surface-labelled with F1 antigen



Effective diameter = 206nm

Polydispersity = 0.103

Fig. 5.6 Sizing of liposome vesicles during the extrusion process



The liposomes encapsulating F1 antigen were reduced in size, from 250nm to 181nm in diameter, after 8 extrusion cycles.

cycles the size of the liposomes was reduced to 181nm as the large liposomes were broken up to form smaller vesicles during the extrusion process. Overall, the polydispersity remained the same over eight cycles; the value was less than 0.1, suggesting that a homogenous suspension of liposomes was obtained (fig. 5.6).

5.3.2 Quantification of antigen in liposomes

The amount of antigen in both liposome formulations was determined using the enhanced BCA protein assay protocol (section 2.8). Table 5.2 shows the protein assay results giving the amount of protein entrapped and indicates that only a small percentage (2.3 - 3.3%) of protein was entrapped or surface-bound. However, a large excess of protein was used in the starting material.

5.3.3 Electron microscopy of liposomes

Liposomes were examined by TEM using a negative stain (section 2.7.6.2). The micrographs showed the liposomes to be membranous structures of uniform size (fig. 5.7 and 5.8); the liposomes encapsulating antigen collapsed as they dried onto the grid and folds in the lipid membrane were observed (fig. 5.7). Fig 5.8 shows surface-labelled liposomes; overall they are of a uniform size of approximately 200nm and in this micrograph the lipid membrane has not dried onto the grid to give a folded appearance.

5.4 Examination of F1 antigen after encapsulation

5.4.1 SDS-PAGE of PLG microspheres and liposomes

PLG microspheres encapsulating F1 antigen and liposomes encapsulating or surface-labelled with antigen were examined by SDS-PAGE (section 2.5). The gel showed that the 17.5kDa F1 antigen was present in all samples (fig. 5.9). F1 encapsulated in 8μm microspheres and both liposome formulations had the same molecular weight as non-encapsulated antigen; however, the F1 bands in the liposome formulations were very faint. The 1μm PLG particles produced two bands on the SDS-PAGE gel. The upper band had a molecular weight of approximately 17.5kDa; the molecular weight of the lower band was approximately 15kDa, determined by image analysis (section 2.5.6).

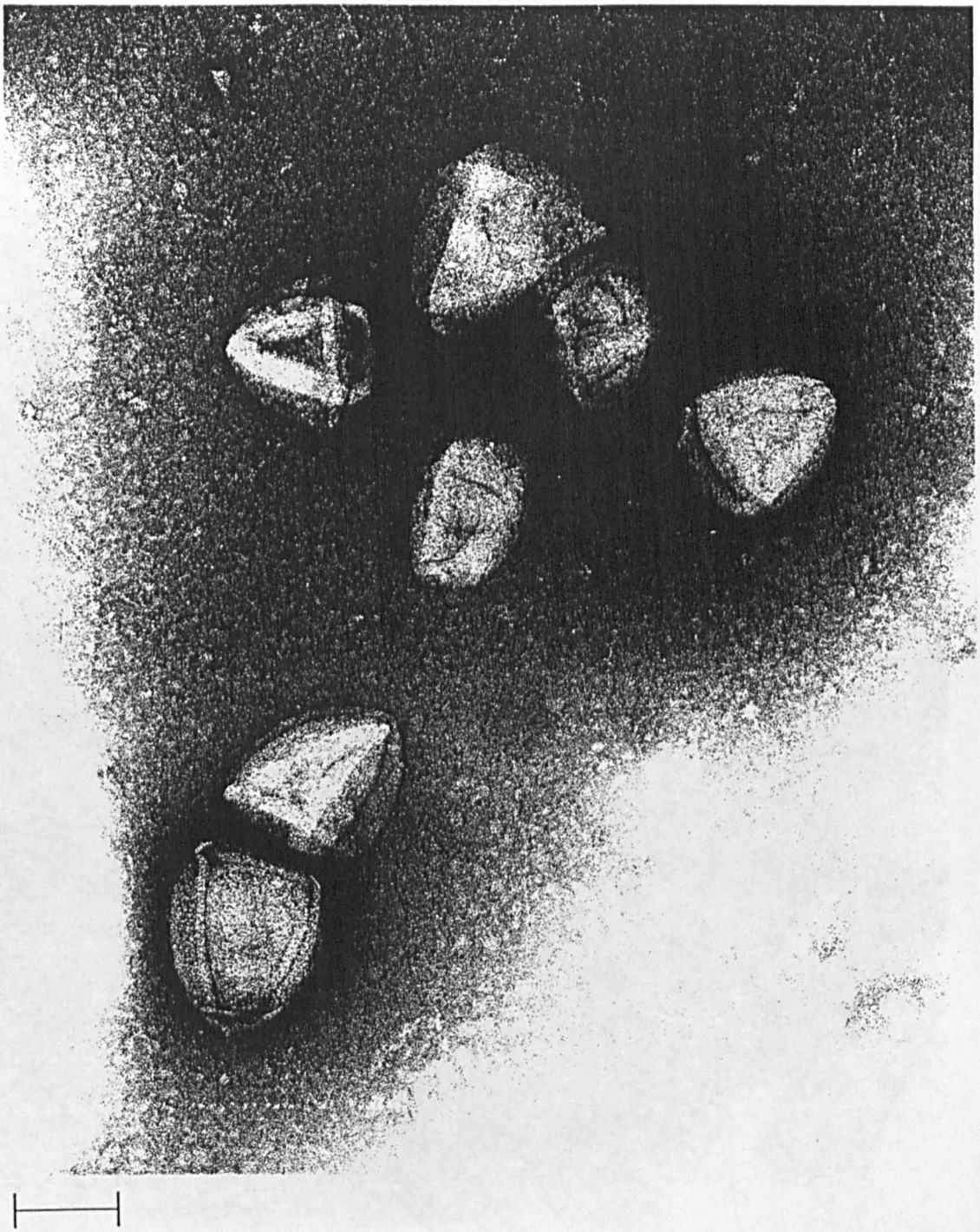
5.4.2 Immunological examination of encapsulated antigen

Samples were run on an SDS-PAGE gel as described in section 5.4.1, proteins were then

Table 5.2 BCA protein assay of liposome formulations

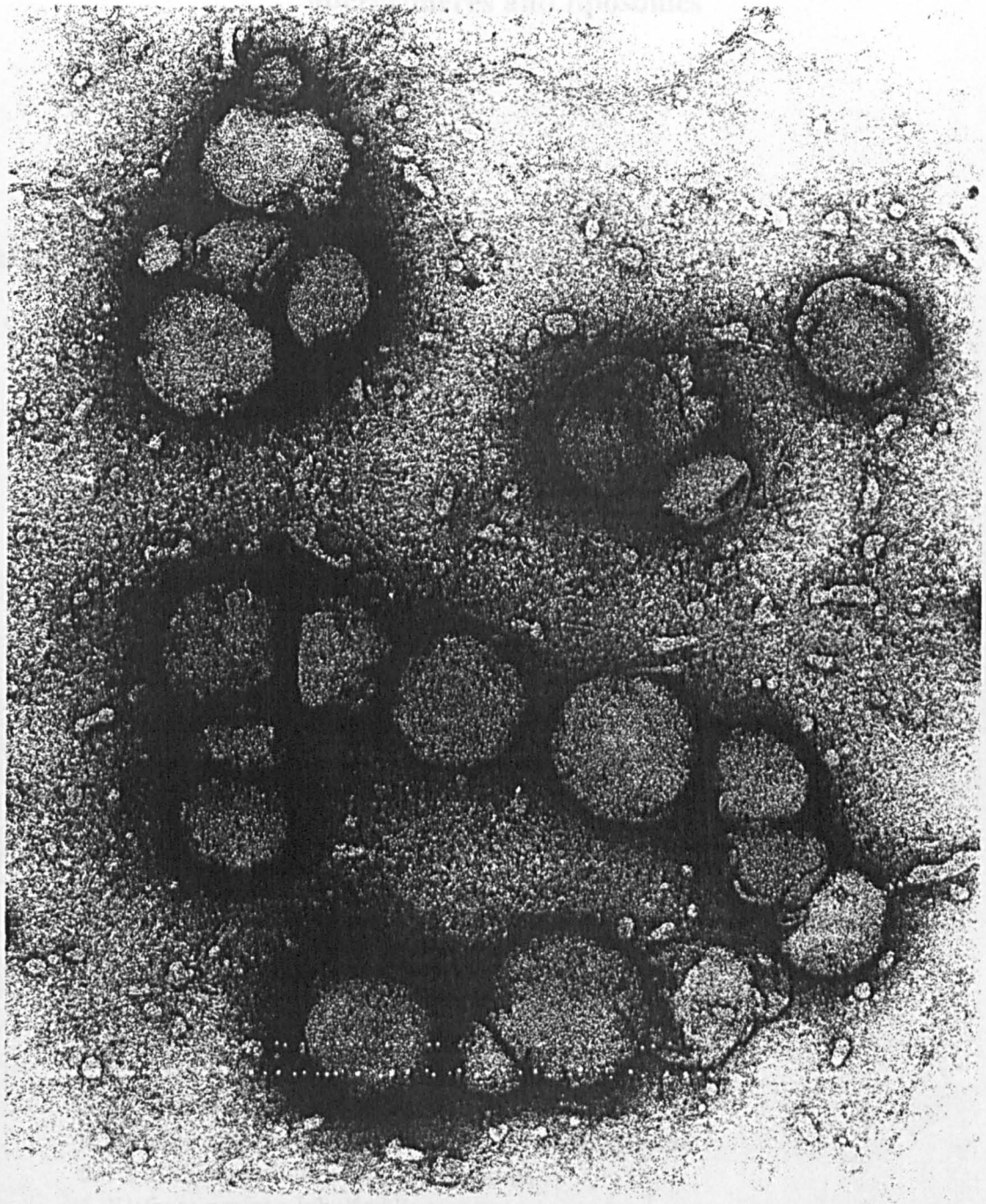
Liposome	Extrusion Temperature	Protein in starting material (mg/ml)	Protein in liposome (μg/ml)	% Protein Encapsulated
Encapsulating F1 antigen	56^0C	4	130.95	3.27
Surface-labelled with F1 antigen	56^0C	8	189.8	2.33

Fig. 5.7 Transmission electron microscopy of liposomes encapsulating F1 antigen



Magnification x 126,000

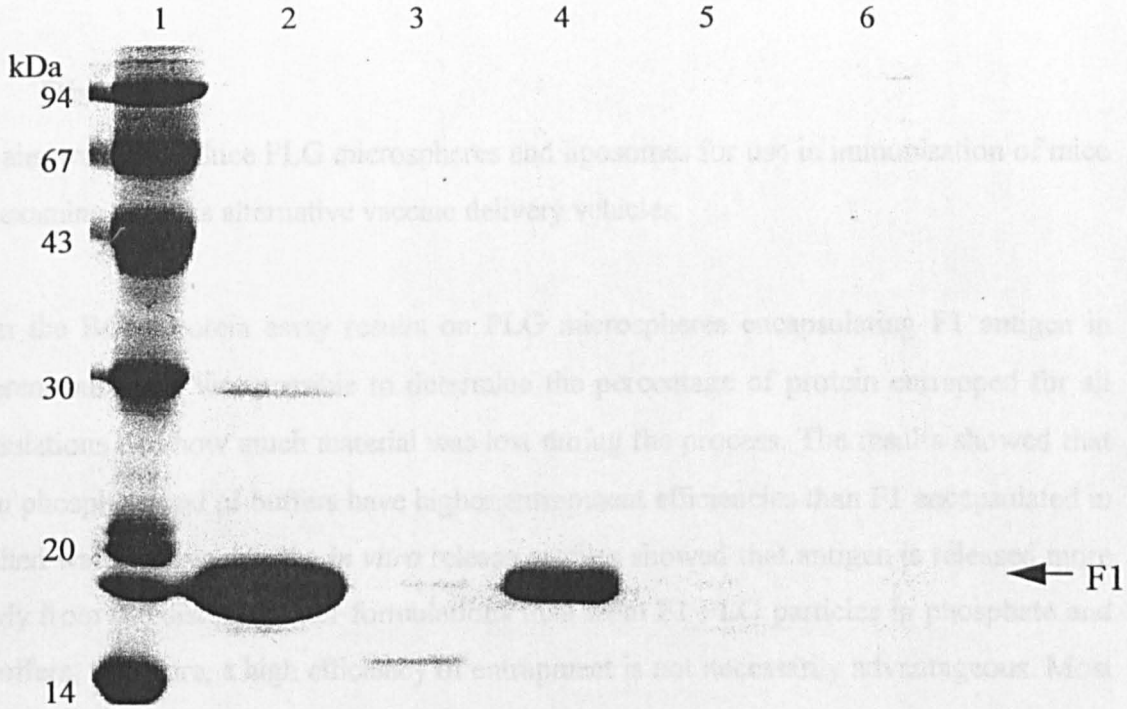
Fig. 5.8 Transmission electron microscopy of liposomes surface-labeled with F1 antigen



100nm

Magnification x 88,000

Fig. 5.9 SDS-PAGE of F1 after encapsulation in PLG microspheres and liposomes



Lane:
1. Molecular weight markers
2. Native F1 antigen
3. F1 after encapsulation into 1μm PLG microspheres
4. F1 after encapsulation into 8μm PLG microspheres
5. F1 after encapsulation into liposomes
6. F1 from surface-labelled liposomes

There was a faint band at approximately 17kDa visible in both liposome preparations.

transferred to nitro-cellulose membrane (section 2.8.3) and probed with murine anti-F1 polyclonal antibody. Fig. 5.10 showed that the 17.5kDa protein in the 1μm and 8μm microspheres remained antigenic; the lower protein band in the 1μm formulation did not react with the antibody. In the liposome formulations, the 17.5kDa protein also remained antigenic but produced only a faint reaction, probably because only a small amount of protein was present in the sample.

5.5 Discussion

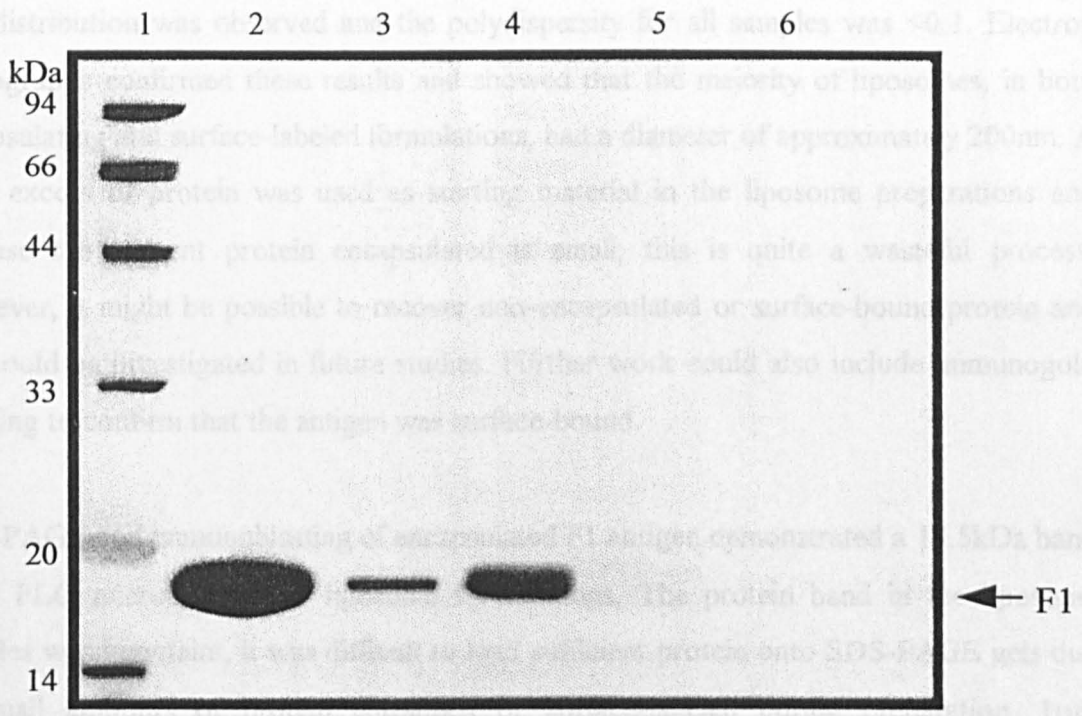
The aim was to produce PLG microspheres and liposomes for use in immunisation of mice and examine these as alternative vaccine delivery vehicles.

From the BCA protein assay results on PLG microspheres encapsulating F1 antigen in different buffers it was possible to determine the percentage of protein entrapped for all formulations and how much material was lost during the process. The results showed that F1 in phosphate and pI buffers have higher entrapment efficiencies than F1 encapsulated in distilled water. However, the *in vitro* release profiles showed that antigen is released more slowly from the distilled water formulations than from F1 PLG particles in phosphate and pI buffers, therefore, a high efficiency of entrapment is not necessarily advantageous. Most of the antigen in phosphate and pI buffer formulations is released over a short time (< 10 days) after which the release was sustained over a period of 42 days.

The PLG microspheres were sized using a Coulter counter and the average size of the microspheres was found to be as expected. However, electron micrographs for both 8μm and 1μm microspheres showed a considerable variation in size, although the average size was 0.88μm for the 1μm PLG microparticles and 9.82μm for the 8μm microspheres. SEM would be the preferred method in future studies. In addition to this, future studies would include modifications of the manufacturing process or inclusion of a filtration step to attempt to produce a more homogeneous preparation.

However, this variation in particle size might not be disadvantageous because particles <10μm in size are taken up by the specialised M-cells in the PPs and may result in a potent mucosal and serological immune response. All of the 1μm PLG microspheres were

Fig. 5.10 Western blot of F1 after encapsulation in PLG microspheres and liposomes



Lane:
1. Biotinylated molecular weight markers
2. Native F1 antigen
3. F1 after encapsulation into 1μm PLG microspheres
4. F1 after encapsulation into 8μm PLG microspheres
5. F1 after encapsulation into liposomes
6. F1 from surface-labelled liposomes

There was insufficient antigen in the liposome preparations to produce an intense band in an immunoblot

<10μm in diameter. The 8μm microspheres, however, may be disadvantaged by having a broad size range as evidence suggests that particles >10μm will not be taken up by the PPs in the GIT.

Liposome preparations were more homogenous as indicated by sizing using PCS; a narrow size distribution was observed and the polydispersity for all samples was <0.1. Electron micrographs confirmed these results and showed that the majority of liposomes, in both encapsulating and surface-labeled formulations, had a diameter of approximately 200nm. A large excess of protein was used as starting material in the liposome preparations and because the percent protein encapsulated is small, this is quite a wasteful process. However, it might be possible to recover non-encapsulated or surface-bound protein and this could be investigated in future studies. Further work could also include immunogold labelling to confirm that the antigen was surface-bound.

SDS-PAGE and immunoblotting of encapsulated F1 antigen demonstrated a 17.5kDa band in all PLG microsphere and liposome formulations. The protein band in the liposome samples was very faint, it was difficult to load sufficient protein onto SDS-PAGE gels due to small amounts of protein entrapped or surface-labeled during preparation. 1μm microspheres produced two protein bands, the lower band proved to be non-antigenic when probed with anti-F1 polyclonal antibody. The identity of this protein is unclear; the protein could be a breakdown product of F1 produced during the encapsulation process, however, some reaction with the anti-F1 antibody would be expected if this were the case.

Chapter 6

Immunogenicity and protective efficacy of encapsulated antigen

6.1 Introduction

The F1 antigen confers protection against *Y. pestis* infection in a number of different animals species (Rust *et al.*, 1971; Simpson *et al.*, 1990), and high anti-F1 titres detected in individuals surviving plague infection (Shepherd *et al.*, 1986; Williams *et al.*, 1982) indicate that the F1 antigen may be a major protective antigen in humans.

To overcome the limitations of the current vaccine, a new subunit vaccine should ideally induce prolonged protective systemic and mucosal immunity against *Y. pestis*, giving protection irrespective of the transmission route. Most of the work to date on the protective potency of the F1 antigen has been done with the protein subunit in appropriate adjuvants.

Recent studies have highlighted the adjuvant properties of PLG microspheres (Jones *et al.*, 1995; Eldridge *et al.*, 1991a) and liposomes (Gregoriadis *et al.*, 1989; Aramaki *et al.*, 1994) for use as vaccine delivery systems. In these cases, induction of both serological and mucosal responses has been observed following appropriate delivery. These two strategies for vaccine delivery both rely on concepts of controlled-release technology. Both are aimed at delivering an immunogen to immune effector cells (targeting), protecting vaccine antigens from harsh host environments, such as the gut, and to some extent timed-release delivery to mimic booster immunisations; short-term or long-term depot effect may be achieved, the latter giving either a continuous or pulsed release (Cox *et al.*, 1997). In addition, liposomes can be used to fuse with the host cell membrane to facilitate intracellular delivery. Therefore, controlled-release systems may provide advantages over conventional adjuvant systems, such as antigens adsorbed onto Alhydrogel, by targeting antigens to sites where they will be more effective at inducing an immune response. Formulation of antigens into vaccine delivery systems may also improve the stability of vaccines, making them more useful in under-developed countries with respect to cost and shelf life. In addition, administration of vaccines orally or intranasally removes the trauma of injection and the reliance on medical staff to perform injections.

Research has shown that oral vaccination of antigens incorporated into liposomes induces a secretory immune response (Childers *et al.*, 1987). However, there is little information

concerning size, concentration and form of liposomes that potentiate maximum induction of mucosal immune responses.

In this study, F1 antigen was incorporated into both PLG microspheres (Jones *et al*, 1995) and liposomes (Gregoriadis *et al.*, 1988; Aramaki *et al.*, 1994) and the immunogenicity of all formulations assessed. The immune responses to the antigen delivered in this way were characterised and compared with those induced by Alhydrogel-adsorbed antigens. The comparative efficacy of liposomes and PLG microspheres in delivering the F1 antigen by the i.p., i.n. and oral routes was also assessed in this study. Protective potency was assessed by challenging groups of immunised mice sub-cutaneously with virulent *Y. pestis* GB strain.

The protection afforded by F1 antigen against *Y. pestis* challenge can be augmented by co-immunisation with the V antigen of *Y. pestis* (Williamson *et al.*, 1995). A recombinant V antigen (rV) was produced as a fusion protein with GST (CBD sector, DERA, Porton Down, Salisbury) and purified by affinity chromatography. The antigen was found to be protective against sub-cutaneous challenge with *Y. pestis* (Leary *et al.*, 1995). Williamson *et al.* (1995) found that a combination vaccine of F1 and rV antigens gave increased protection up to a challenge dose of 10^9 cfu *Y. pestis* GB strain. An rF1 antigen expressed in *E. coli* protected mice from bubonic and pneumonic plague after challenge with *Y. pestis* by the s.c.route or by aerosol respectively (Andrews *et al.*, 1996).

Therefore, further studies were performed in which F1 was co-encapsulated with rV antigen in liposomes and in PLG microspheres; the protective efficacy of the combinations was compared with that afforded by the F1 alone after s.c. challenge with *Y. pestis*.

6.2 Immune response to F1 in PLG microspheres and liposomes

In all graphs, results are expressed as geometric means of ELISA titres observed from treatment groups of mice at each sample point. The Student's *t* test was used to determine whether observed differences in group mean titres were significant in samples taken at either day 28 or day 42.

6.2.1 Antibody titre in mice immunised i.p. with F1 antigen in PLG microspheres

Initial experiments were designed to compare the immunogenicity of F1 in different encapsulation formulations with respect to encapsulation buffer and size. Mice were sampled at day 28 to assess the immune response after one i.p. dose of encapsulated antigen and to determine the influence of the different buffers used to prepare microspheres on the response. PLG microspheres prepared with distilled water and phosphate buffer induced the highest serum IgG responses, especially for 1μm microspheres (fig. 6.1) and the titres were significantly higher than titres produced by F1 in alhydrogel ($P<0.02$). Of the formulations tested, 1μm PLG microspheres produced in phosphate buffer elicited the highest serum IgA response. An IgA response in stools was observed in all formulations after i.p. immunisation (fig. 6.2).

From this preliminary comparison, there was little difference in antibody titre between the different formulations. However, PLG microspheres produced with F1 encapsulated in phosphate buffer were selected for further study to compare the effectiveness and influence of different delivery routes; making a direct comparison with the native F1 antigen and liposomes which were prepared in phosphate buffer.

6.2.2 Immunogenicity of native and recombinant F1 antigen

Serological and mucosal antibody responses in mice immunised i.p. with native or rF1 antigen in Alhydrogel or the Greer vaccine given as multiple doses were compared to those induced by native F1 antigen encapsulated into PLG microspheres administered in a single i.p. dose.

6.2.2.1 Serum antibody response

F1 antigen entrapped in PLG microspheres stimulated high serum IgG antibody titres when given i.p. (fig. 6.3a), similar to the titre induced in mice immunised with Alhydrogel-adsorbed antigens. Serum IgG titres did not vary significantly between the different formulations ($P > 0.1$). However, after day 28 the antibody titre had started to fall in mice immunised with Greer vaccine and PLG microspheres, whereas the titre was sustained in groups immunised with native and rF1. It must be stressed, however, that mice vaccinated with PLG microspheres received only one immunisation, whereas other groups were give three doses (fig 6.3a), in order to determine whether the depot release effect of PLG

Fig. 6.1 Serum anti-F1 antibody titres 28 days after immunisation for mice immunised IP with F1 PLG formulations

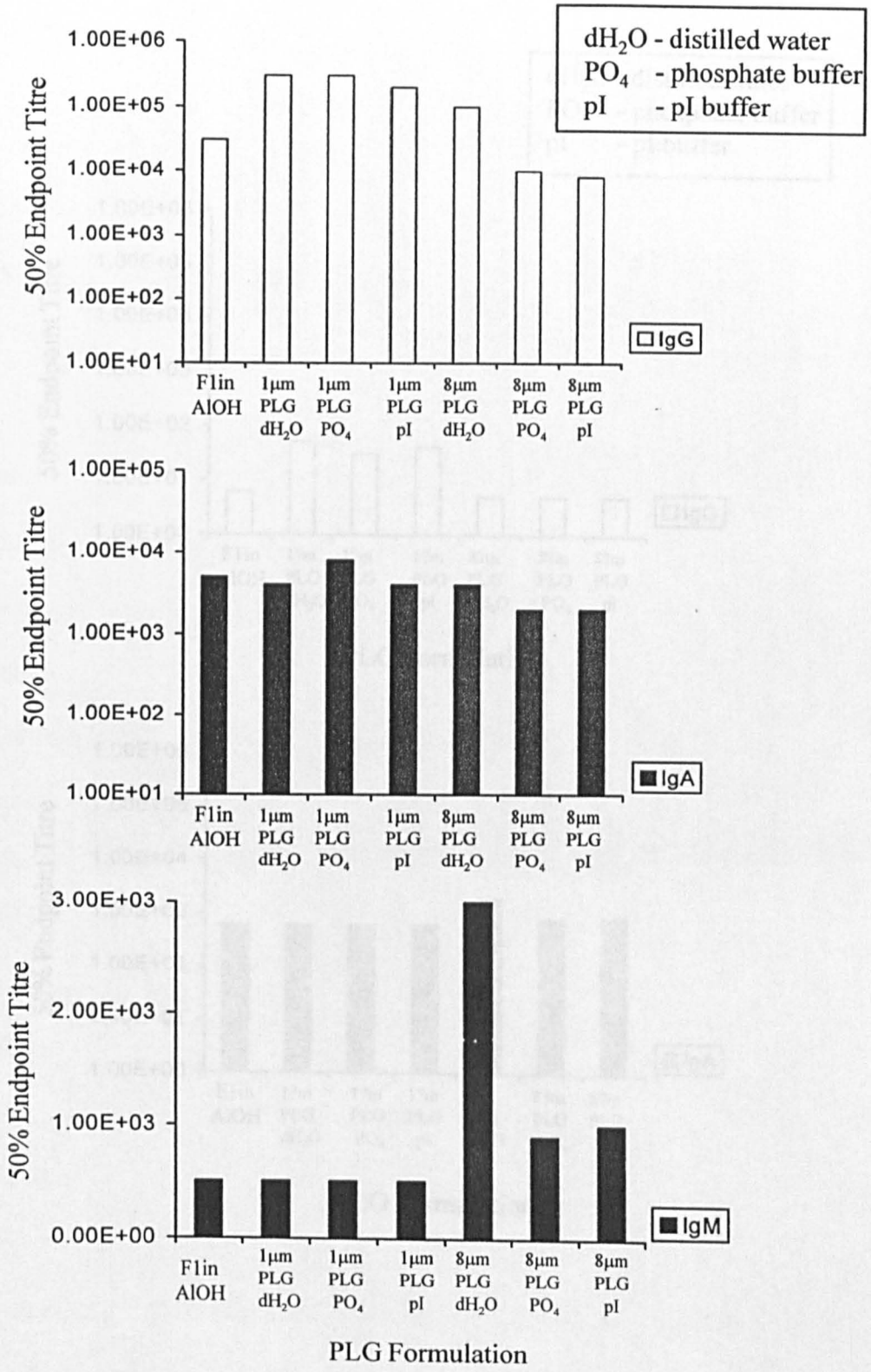


Fig. 6.2 Stool anti-F1 antibody titres 28 days after immunisation for mice immunised IP with F1 PLG formulations

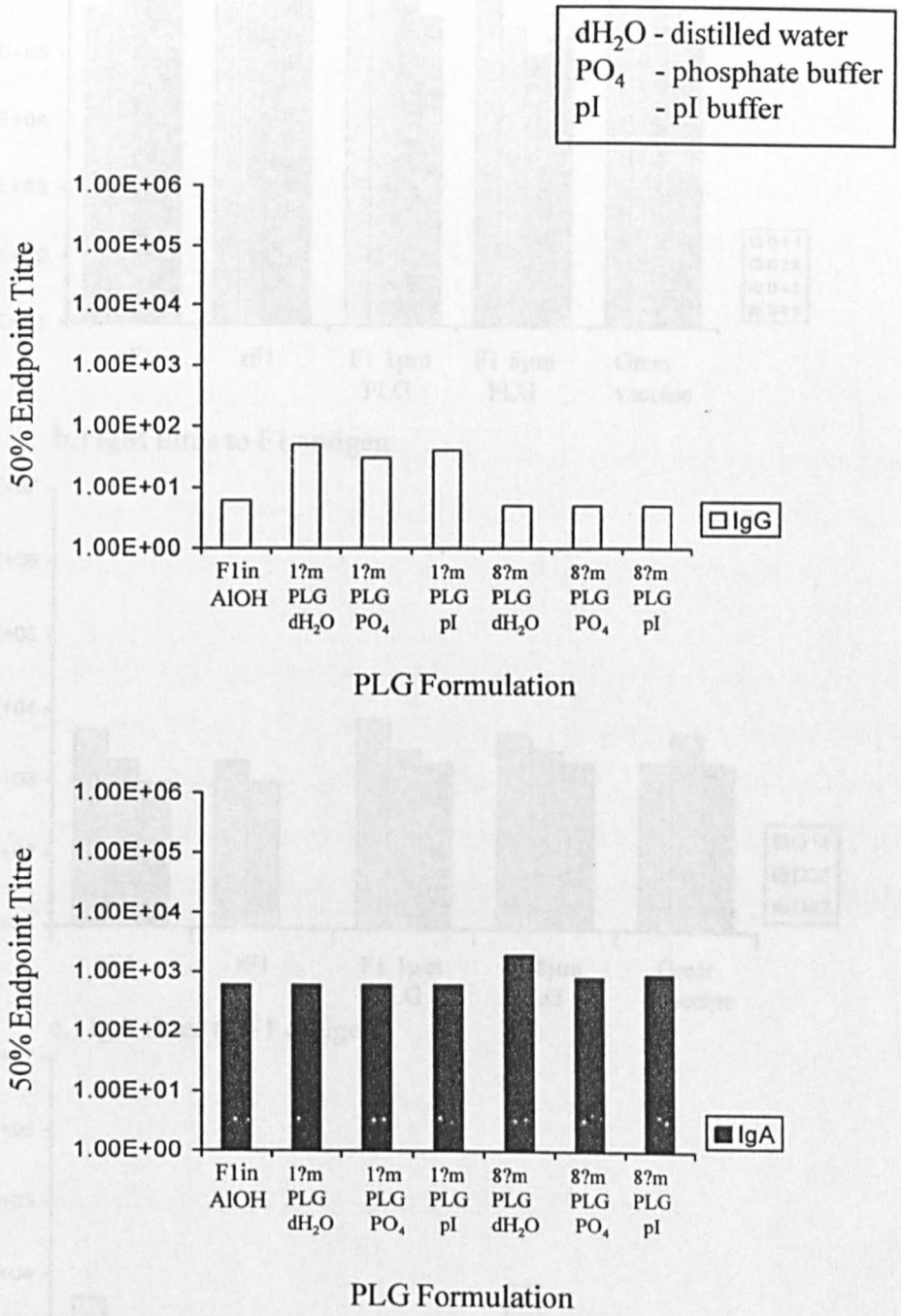
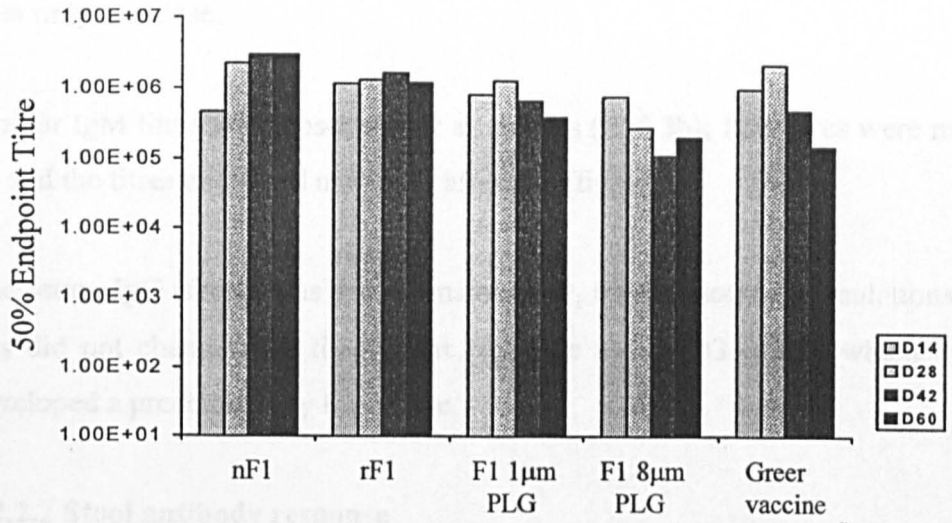
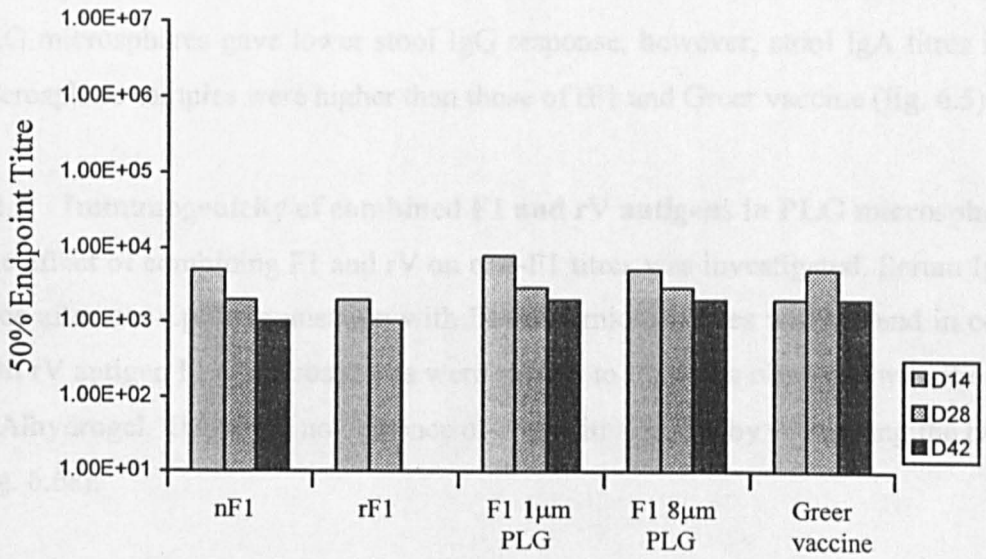


Fig. 6.3 Serum antibody response to F1 antigen after immunisation of mice with F1 vaccine formulations

a.) IgG titres to F1 antigen



b.) IgM titres to F1 antigen



c.) IgA titres to F1 antigen

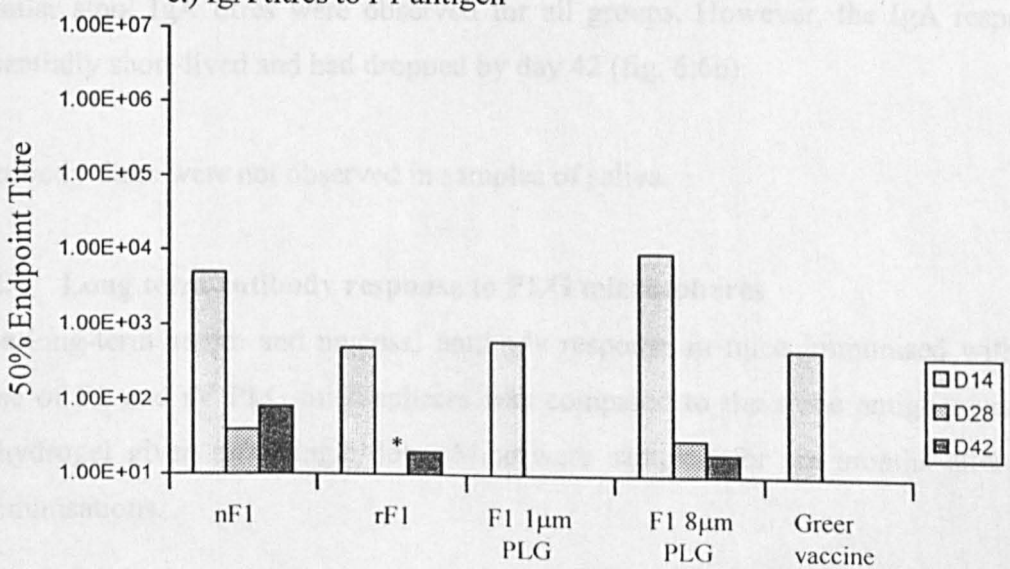


Fig. 6.3a). Serum IgG titres were also assessed at day 60 prior to s.c. challenge of mice with *Y. pestis*. nF1 and rF1 were given i .p. in Alhydrogel, PLG formulations were administered i.p. in PBS. Greer vaccine was given i.m. * No response to rF1 at day 28.

microparticles could sustain a similar immune response to Alhydrogel-adsorbed antigens after only one dose.

Similar IgM titres were observed for all groups (fig6.3b); IgA titres were maximal at day 14 and the titres decreased markedly after this (fig.6.3c).

The serum IgG isotype was predominately IgG_1 for all vaccine formulations (fig. 6.4) and this did not change with time, apart from the 8μm PLG group, which by day 42 had developed a predominately IgG_{2a} titre.

6.2.2.2 Stool antibody response

Stool IgG titres were similar for mice immunised with nF1, rF1 and Greer vaccine. F1 in PLG microspheres gave lower stool IgG response; however, stool IgA titres in the PLG microsphere samples were higher than those of rF1 and Greer vaccine (fig. 6.5).

6.2.3 Immunogenicity of combined F1 and rV antigens in PLG microspheres

The effect of combining F1 and rV on anti-F1 titres was investigated. Serum IgG titres in mice after one i.p. immunisation with F1 PLG microspheres without and in combination with rV antigen PLG microspheres were similar to the titres observed with the F1 antigen in Alhydrogel. There was no evidence of an additive effect by combining the two antigens (fig. 6.6a).

Similar stool IgA titres were observed for all groups. However, the IgA response was essentially short-lived and had dropped by day 42 (fig. 6.6b).

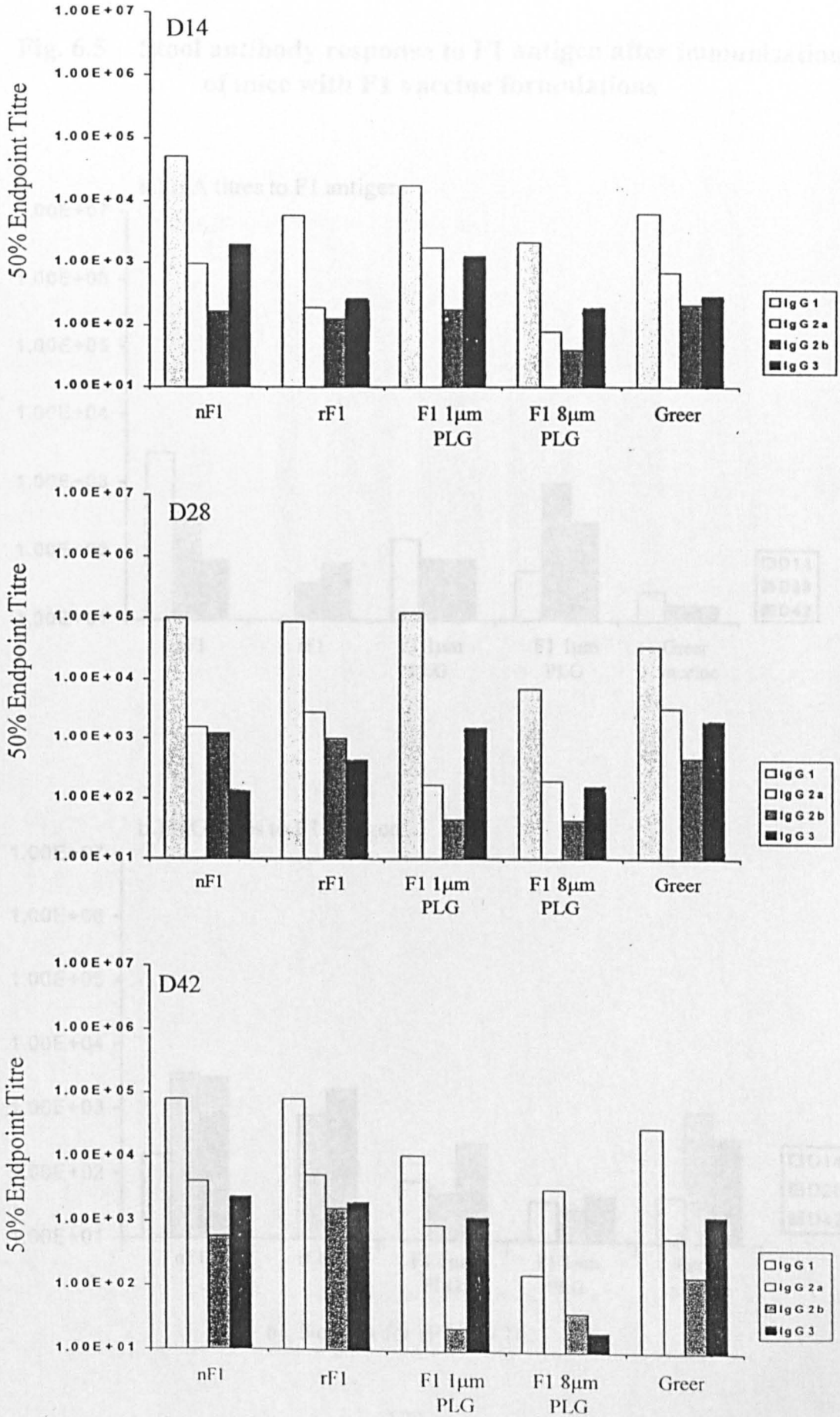
Antibody titres were not observed in samples of saliva.

6.2.4 Long term antibody response to PLG microspheres

The long-term serum and mucosal antibody response in mice immunised with one i.p. dose of F1 and rV PLG microspheres was compared to the same antigens adsorbed to Alhydrogel given as a single dose. Mice were sampled for six months after the final immunisations.

Anti-F1 serum IgG titres were similar for different formulations and titres were virtually constant throughout the experiment (fig. 6.7). IgA and IgG titres were determined in

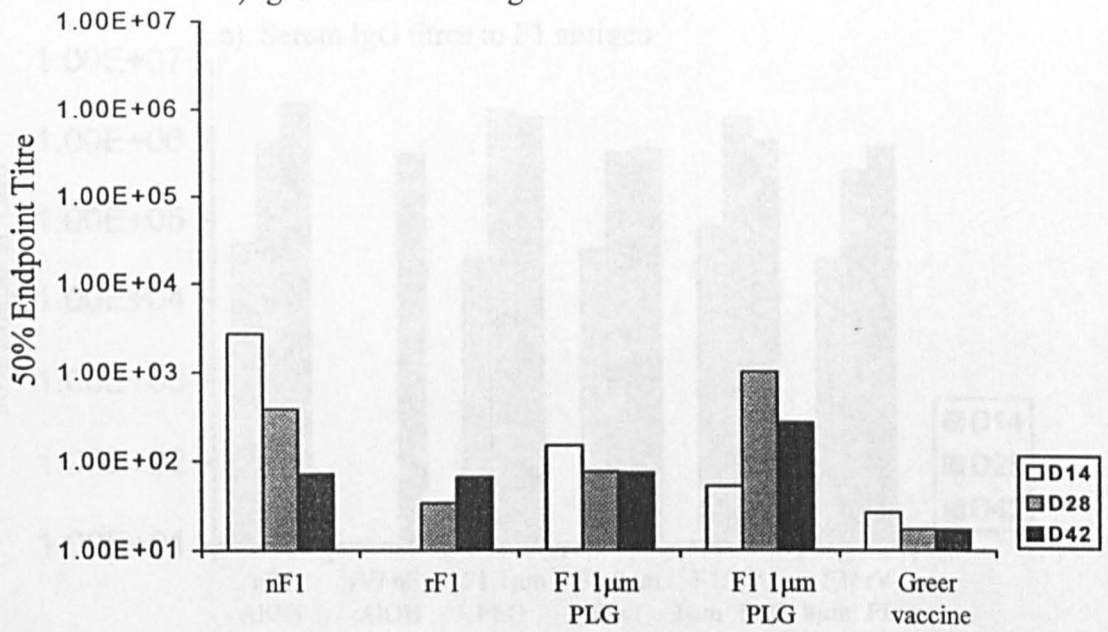
Fig.6.4 Serum IgG isotype to F1 antigen after immunisation of mice with F1 vaccine formulations



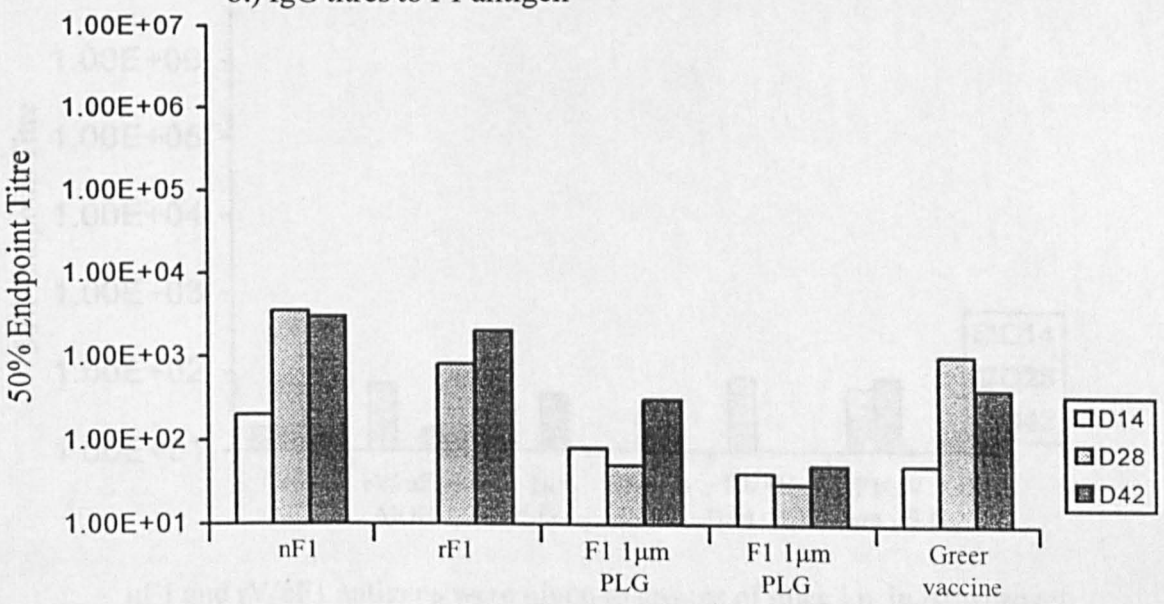
All immunisations were i.p. in either Alhydrogel or PBS except Greer vaccine which was given i.m.

Fig. 6.5 Stool antibody response to F1 antigen after immunisation of mice with F1 vaccine formulations

a.) IgA titres to F1 antigen

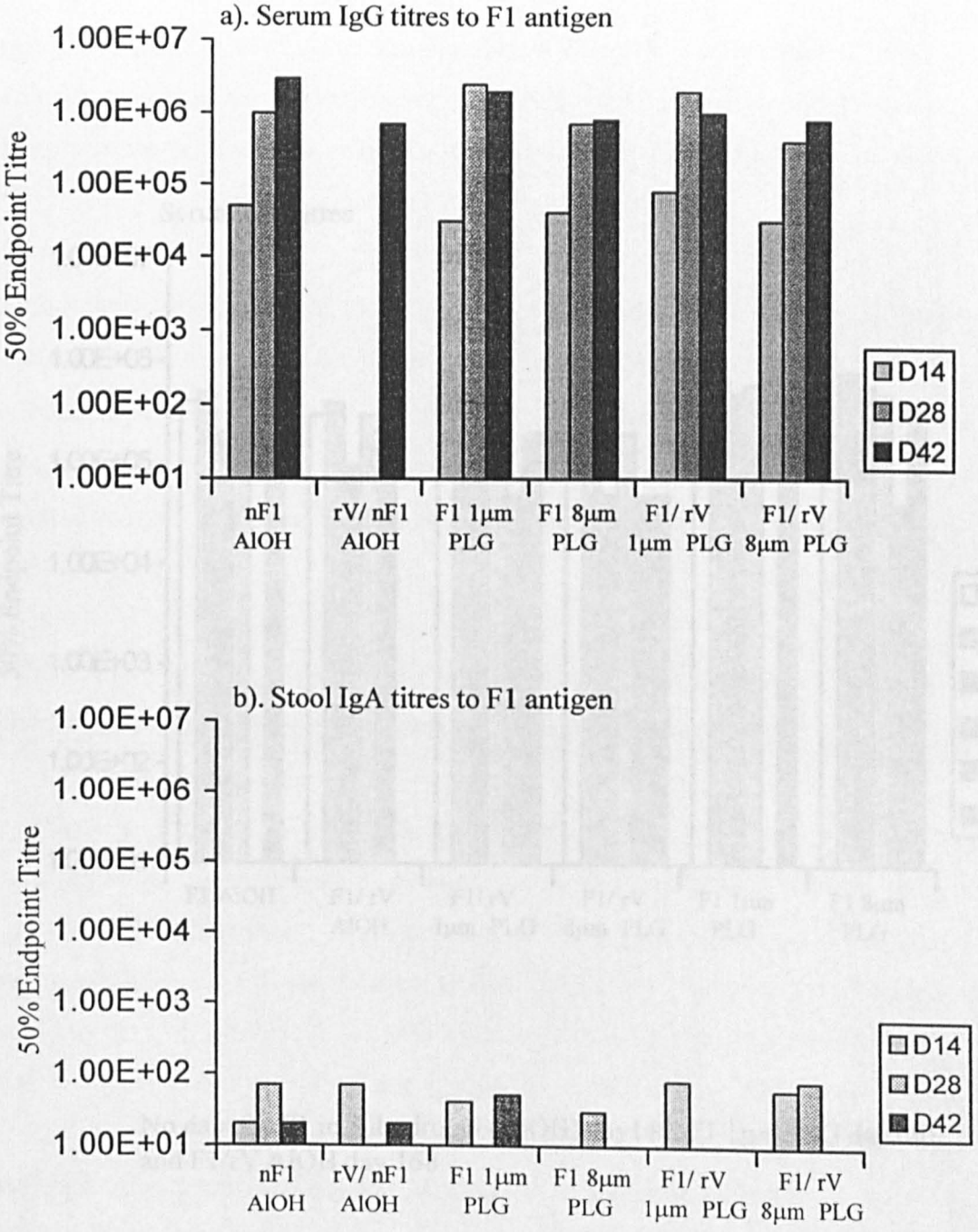


b.) IgG titres to F1 antigen



a). No data for rF1 day 14.

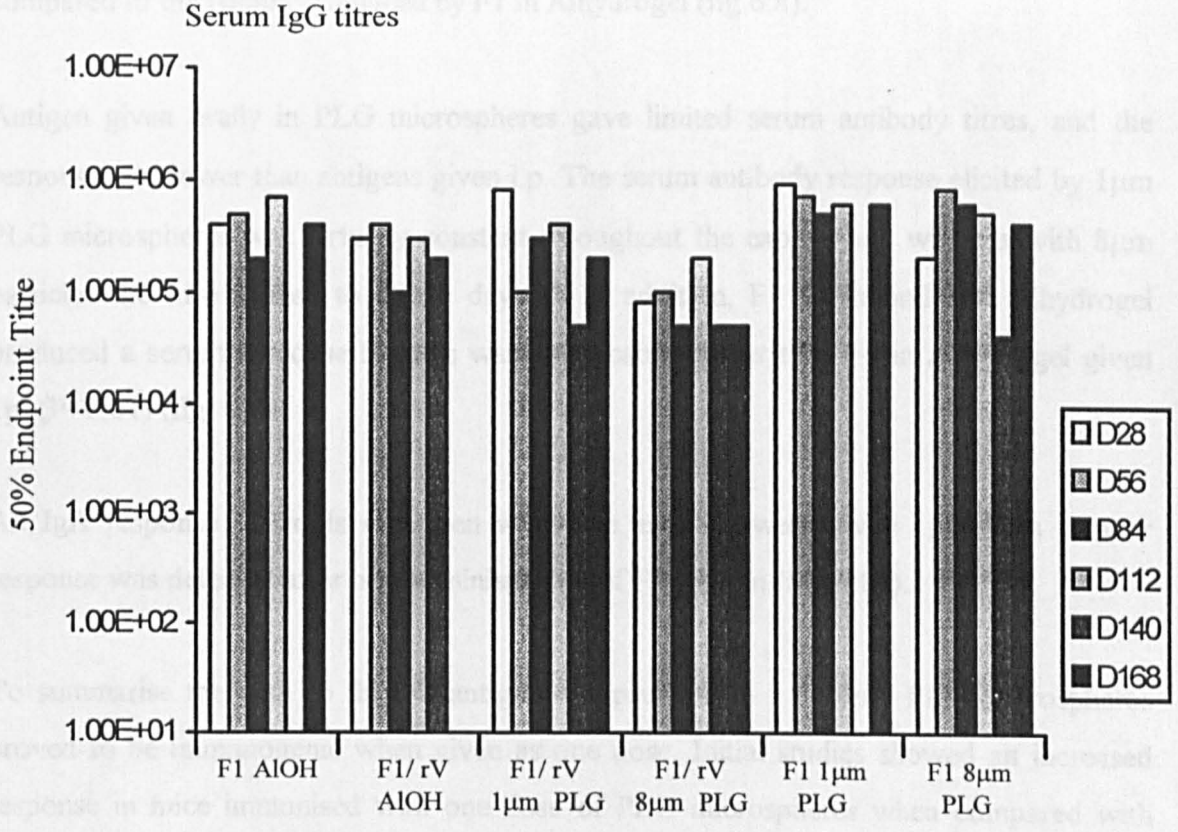
Fig. 6.6 Immunogenicity of combined F1 and rV antigens in PLG microspheres to native F1 antigen after i.p. immunisation



nF1 and rV/nF1 antigens were given to groups of mice i.p. in Alhydrogel, PLG microsphere formulations were administered in PBS

a). No data for rV/nF1 in Alhydrogel (AlOH) at day14 and day28

Fig. 6.7 Anti-F1 long-term antibody response to F1 and rV antigens encapsulated in PLG microspheres



No data for F1 in Alhydrogel (AlOH) day140, F1 1µm PLG day140 and F1/rV AlOH day 168

stools and saliva. Antibodies were not detected in saliva. In stools, IgA was detected but the titres were more variable than those for serum IgG and titres were not sustained throughout the sampling period; a similar result was obtained for IgG titres in stools (results not shown).

6.2.5 Comparison of oral and i.p. delivery of F1 in PLG microspheres

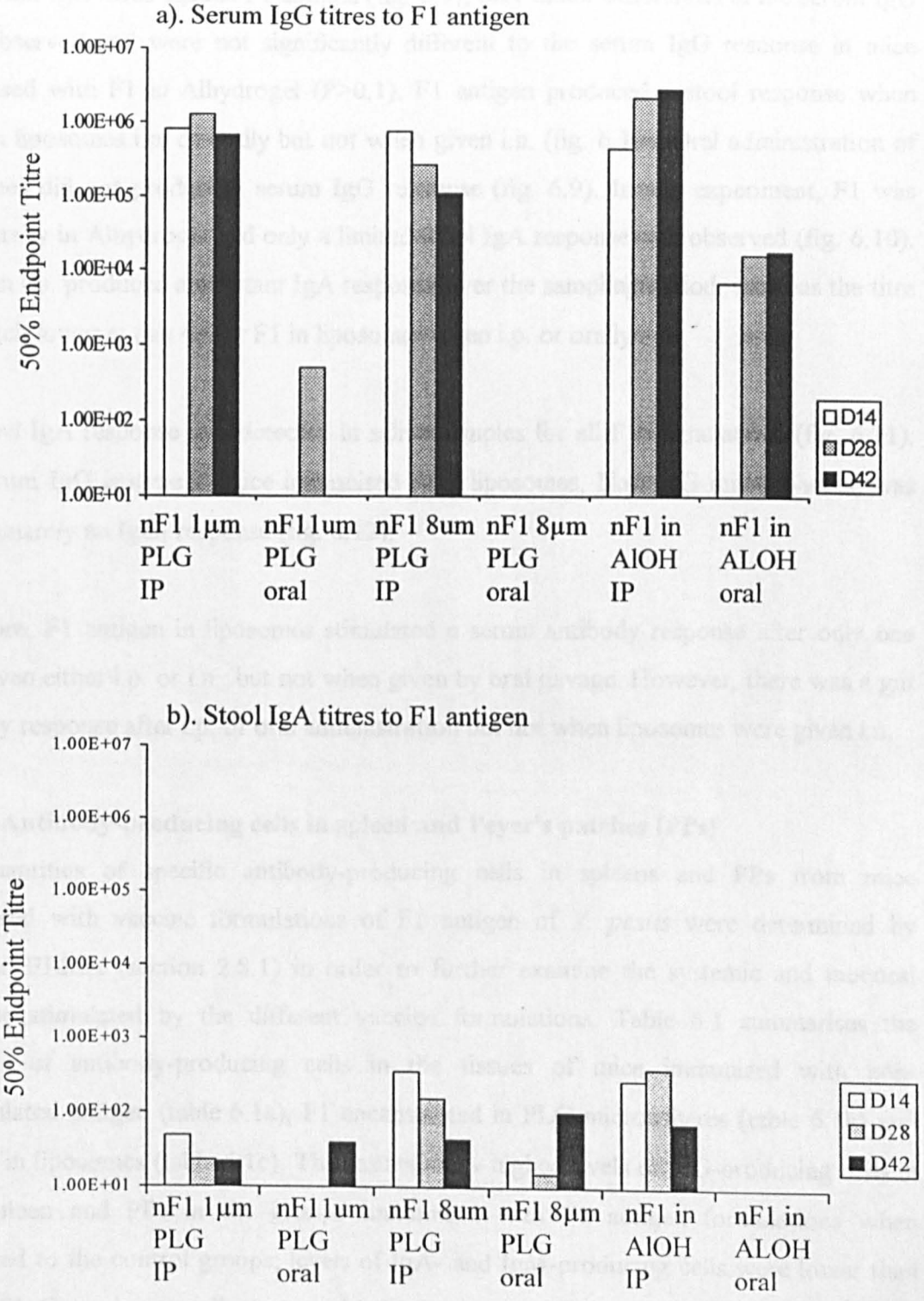
F1 in 1μm and 8μm microspheres was given i.p. or orally to groups of 6-8 week old mice, each group being given one dose. Antibody response was determined over 42 days and compared to the response induced by F1 in Alhydrogel (fig 6.8).

Antigen given orally in PLG microspheres gave limited serum antibody titres, and the response was lower than antigens given i.p. The serum antibody response elicited by 1μm PLG microspheres was virtually constant throughout the experiment, whereas with 8μm particles the titre started to fall at day 28. In addition, F1 given orally in Alhydrogel produced a serum response but this was significantly lower than F1 in Alhydrogel given i.p. ($P<0.01$) (fig. 6.8a).

An IgA response in stools was seen when the antigens were given i.p. and a weaker response was detected after oral administration of F1 antigen (fig. 6.8b).

To summarise the data so far, F1 antigen encapsulated in 1 or 8μm PLG microspheres proved to be immunogenic when given as one dose. Initial studies showed an increased response in mice immunised with one dose of PLG microspheres when compared with non-encapsulated F1 antigen. Further studies comparing nF1 or rF1 in Alhydrogel and Greer vaccine given in three doses with F1 in PLG microspheres administered as one dose, showed that serum IgG titres did not vary significantly ($P>0.1$), therefore antigen encapsulated in PLG microparticles can elicit a sustained response after only one dose. Stool IgA titres gave an indication of mucosal response and titres were higher in mice immunised with encapsulated antigen when given i.p.compared to oral administration.

Fig. 6.8 Comparison of oral and i.p. delivery of F1 in PLG microspheres



6.2.6 Antibody titre in mice immunised with F1 antigen in liposomes

Mice immunised with one dose, i.p. or i.n., with F1 surface-labeled liposomes produced high serum IgG titres against F1 antigen (fig. 6.9); only minor differences in the serum IgG were observed and were not significantly different to the serum IgG response in mice immunised with F1 in Alhydrogel ($P>0.1$). F1 antigen produced a stool response when given in liposomes i.p. or orally but not when given i.n. (fig. 6.10). Oral administration of liposomes did not produce a serum IgG response (fig. 6.9). In this experiment, F1 was given orally in Alhydrogel and only a limited stool IgA response was observed (fig. 6.10). F1 given i.p. produced a constant IgA response over the sampling period, whereas the titre was much lower at day 42 for F1 in liposomes given i.p. or orally.

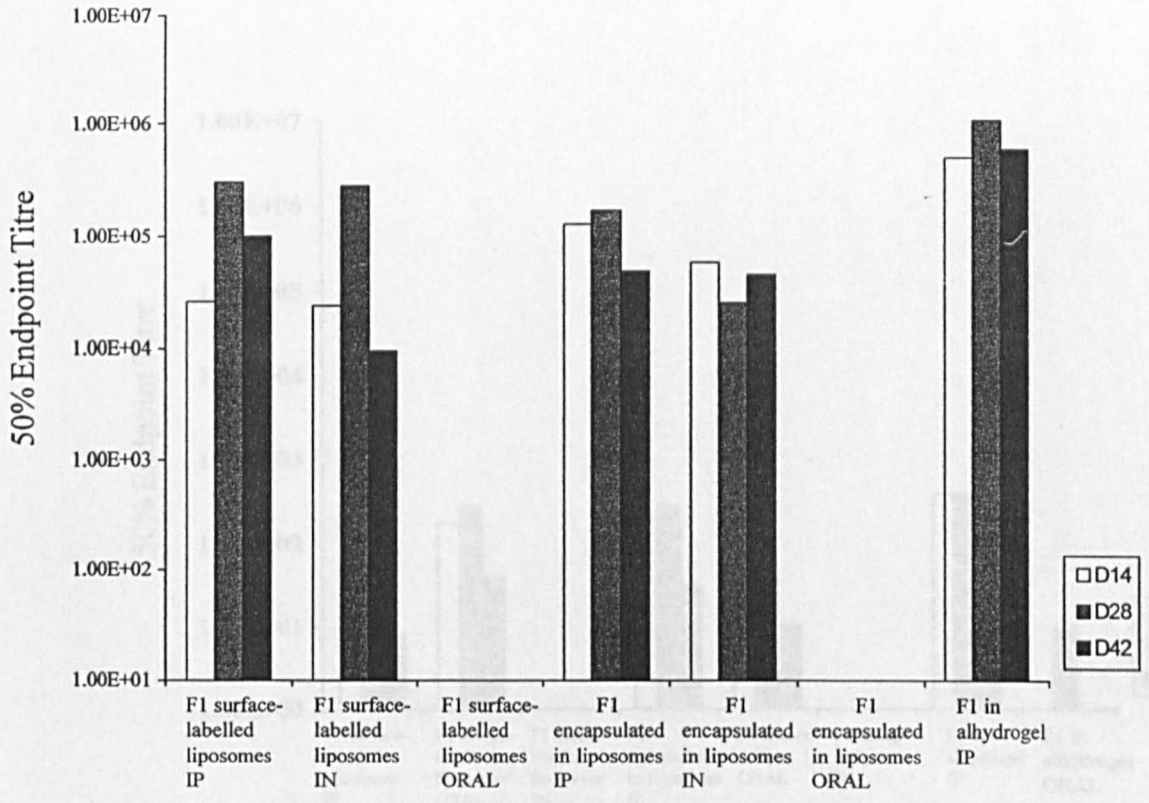
A limited IgA response was detected in saliva samples for all F1 formulations (fig. 6.11). The serum IgG isotype in mice immunised with liposomes, like PLG microspheres, was predominantly an IgG_1 response (fig. 6.12).

Therefore, F1 antigen in liposomes stimulated a serum antibody response after only one dose given either i.p. or i.n., but not when given by oral gavage. However, there was a gut antibody response after i.p. or oral administration but not when liposomes were given i.n.

6.3 Antibody-producing cells in spleen and Peyer's patches (PPs)

The quantities of specific antibody-producing cells in spleens and PPs from mice immunised with vaccine formulations of F1 antigen of *Y. pestis* were determined by modified ELISA (section 2.8.1) in order to further examine the systemic and mucosal response stimulated by the different vaccine formulations. Table 6.1 summarises the amounts of antibody-producing cells in the tissues of mice immunised with non-encapsulated antigen (table 6.1a), F1 encapsulated in PLG microspheres (table 6.1b) and antigen in liposomes (table 6.1c). The results show higher levels of IgG-producing cells in both spleen and PPs in the groups immunised with F1 antigen formulations when compared to the control groups; levels of IgA- and IgM-producing cells were lower than those of IgG-producing cells. IgA- and IgM-producing cells were also detected in some of the control groups, indicating either non-specific binding or a limited immune response to empty ("ghost") liposomes and PLG microspheres.

Fig. 6.9 Anti-F1 serum IgG titres in mice immunised parenterally, intranasally or by gavage with liposomes



Groups of mice were immunised with liposomes in PBS. F1 encapsulated in or surface-labeled onto liposomes produced a serum antibody response when delivered parenterally or intranasally, however, liposomes administered by oral gavage did not produce an antibody response in mice.

Fig. 6.10 Anti-F1 stool IgA titres in mice immunised parenterally, intranasally or by gavage with liposomes

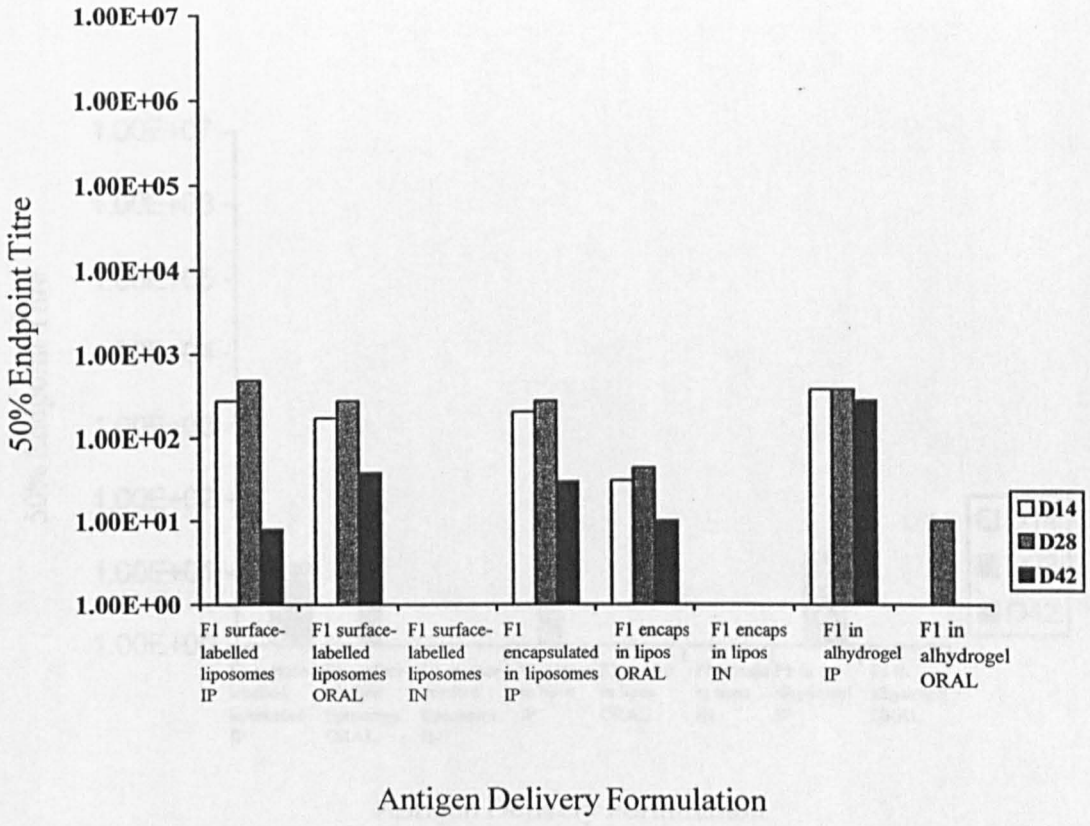


Fig. 6.11 Anti-F1 IgA titres in saliva from mice immunised parenterally, intranasally or by gavage with liposomes

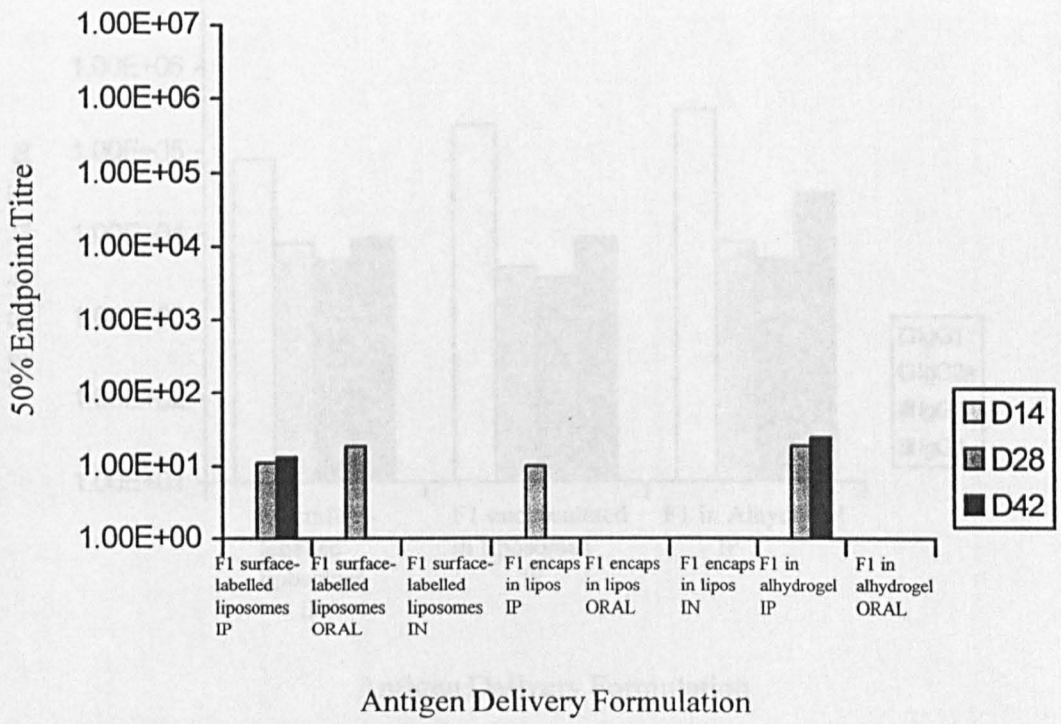


Fig. 6.12 Serum anti-F1 IgG isotype distribution in mice immunised with liposomes

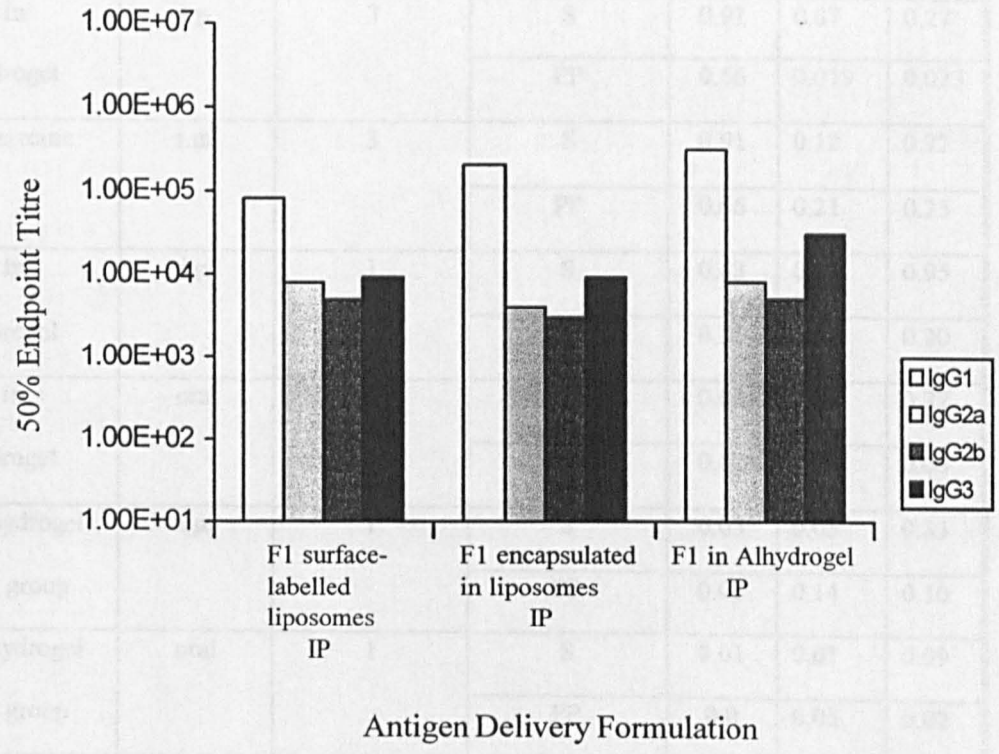


Table 6.1 Antibody producing cells detected in spleen and Peyer's patches from mice immunised with vaccine formulations of F1 and V antigens of *Y. pestis*.

Table 6.1a. Response to antigen in Alhydrogel or PBS

Antigen Delivery Formulation	Route	No. of Doses	Immune inductive site	OD_{450nm} at 2×10^7 lymphocytes using immunoglobulin isotype conjugate		
				IgG	IgA	IgM
F1 in Alhydrogel	i.p.	3	S	0.91	0.07	0.27
			PP	0.66	0.019	0.023
Greer vaccine	i.m.	3	S	0.91	0.12	0.72
			PP	0.66	0.21	0.25
F1 in Alhydrogel	i.p.	1	S	0.63	0.17	0.95
			PP	0.35	0.25	0.20
F1 in Alhydrogel	oral	1	S	0.03	0.04	0.37
			PP	0.02	0.11	0.06
PBS/Alhydrogel control group	i.p.	1	S	0.03	0.05	0.33
			PP	0.03	0.14	0.10
PBS/Alhydrogel control group	oral	1	S	0.01	0.01	0.09
			PP	0.0	0.05	0.02

S spleen
PP Peyer's patches

Table 6.1b. Response to antigen in PLG microspheres

Antigen Delivery Formulation	Route	No. of Doses	Immune inductive site	OD_{450nm} at 2×10^7 lymphocytes using immunoglobulin isotype conjugate		
				IgG	IgA	IgM
F1 in 1μm PLG microspheres	i.p.	1	S	0.98	0.07	0.35
			PP	0.82	0.19	0.13
F1 in 8μm PLG microspheres	i.p.	1	S	0.55	0.03	0.21
			PP	0.36	0.15	0.15
Ghost PLGs	i.p.	1	S	0.0	0.01	0.31
			PP	0.0	0.01	0.13

S spleen
PP Peyer's patches

Table 6.1c. Response to antigen in liposomes

Antigen Delivery Formulation	Route	No. of Doses	Immune inductive site	OD_{450nm} at 2×10^7 lymphocytes using immunoglobulin isotype conjugate		
				IgG	IgA	IgM
F1 surface-labelled liposomes	i.p.	1	S	0.82	0.15	0.82
			PP	0.22	0.28	0.27
F1 surface-labelled liposomes	oral	1	S	0.02	0.10	0.62
			PP	0.07	0.0	0.23
Liposomes encapsulating F1 antigen	i.p.	1	S	0.22	0.01	0.24
			PP	0.17	0.13	0.09
Liposomes encapsulating antigen F1 antigen	oral	1	S	0.02	0.07	0.43
			PP	0.04	0.24	0.13
Ghost liposomes (a)	i.p.	1	S	0.0	0.02	0.21
			PP	0.15	0.15	0.13
Ghost liposomes (a)	oral	1	S	0.01	0.03	0.36
			PP	0.02	0.24	0.18
Ghost liposomes (b)	i.p.	1	S	0.03	0.0	0.42
			PP	0.03	0.27	0.21
Ghost liposomes (b)	oral	1	S	0.0	0.02	0.09
			PP	0.01	0.06	0.02

S spleen
PP Peyer's patches

6.4 Multiple immunisations with F1 and rV antigens

Research has shown a combination of F1 and rV antigen to be protective in mice when given s.c. in Freund's adjuvant (Williamson *et al.*, 1995) and there was evidence of an additive effect.

Previous experiments in this study have involved immunising mice with a single 6 - 10μg dose of encapsulated antigen or multiple doses of antigen in Alhydrogel. Therefore, the immunostimulatory effect of giving three doses of encapsulated antigen formulations by different delivery routes was assessed and the protective efficacy of these combination vaccine formulations was examined (Section 6.7).

6.4.1 Serum antibody response to F1 antigen

Consistently high levels of circulating antibody to F1 antigen were detected in groups given antigens i.n. or i.p. in liposomes and PLG microspheres; the IgG titres were similar to titres observed in mice given F1 in Alhydrogel. Response to PLG microspheres given orally was poor. However, the formulation incorporating F1 on the surface of liposomes and encapsulated rV antigen produced higher titres when given i.p. or i.n. than the liposome formulations in which both antigens were encapsulated. The titres elicited by liposomes given i.n. were higher than titres produced by antigens in PBS using the same immunisation route. These results suggest that multiple immunisations with antigens formulated in liposomes or PLG microspheres produced sustained levels of circulating antibody (fig. 6.13). In addition, encapsulation of subunit antigens in PLG microspheres or liposomes has an adjuvant effect when given i.n. compared to non-encapsulated antigens.

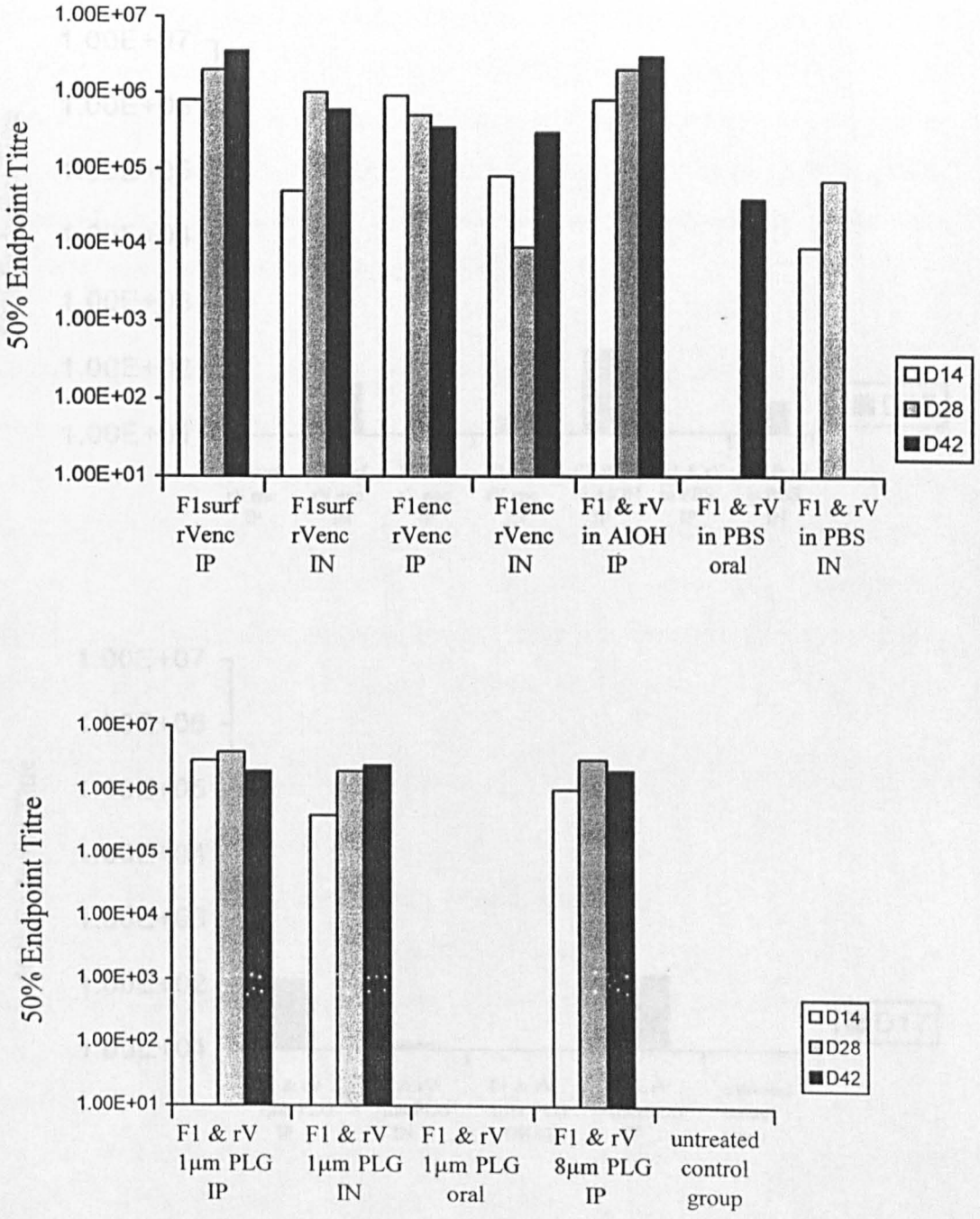
6.4.2 Mucosal antibody response

Only low levels of IgA antibodies were detected in stools (fig. 6.14). Antibodies were not detected in saliva.

6.5 Summary of the immunogenicity of vaccine formulations

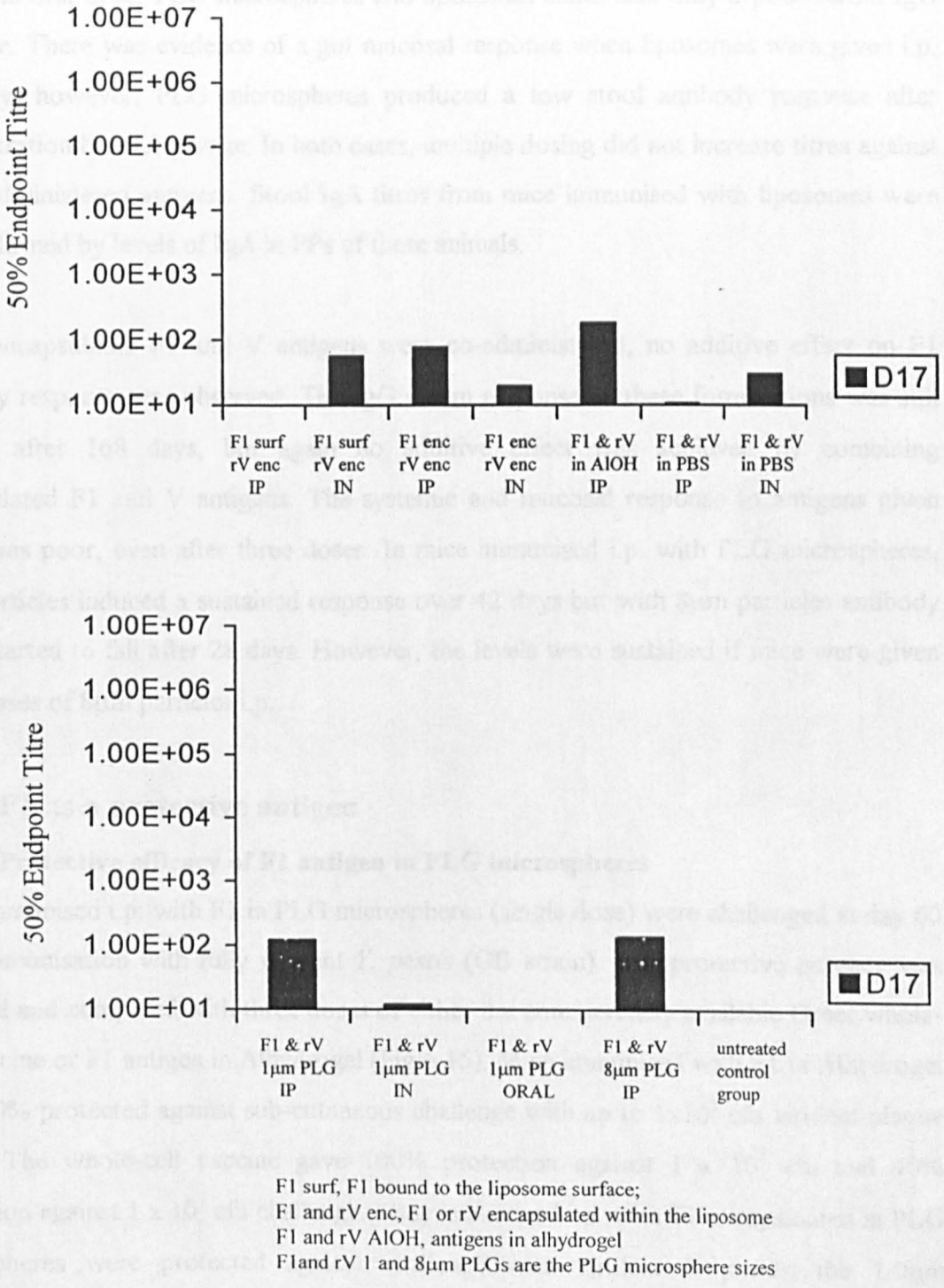
F1 antigen was immunogenic in PLG microspheres and liposomes; high circulating levels of antibody were observed after only one dose i.p. Antibody titres were similar to those obtained for F1 in Alhydrogel and the commercial Greer vaccine where three doses were

Fig. 6.13 Anti-F1 serum IgG response to multiple immunisations with F1 and rV antigens



F1 surf, F1 bound to the liposome surface;
F1 and rV enc, F1 or rV encapsulated within the liposome
F1 and rV AlOH, antigens in alhydrogel
F1and rV 1 and 8µm PLGs are the PLG microsphere sizes

Fig. 6.14 Anti-F1 stool IgA response to multiple immunisations with F1 and rV antigens



F1 surf, F1 bound to the liposome surface;
F1 and rV enc, F1 or rV encapsulated within the liposome
F1 and rV AlOH, antigens in alhydrogel
F1and rV 1 and 8μm PLGs are the PLG microsphere sizes

given. Liposomes also produced a serum response after one dose by the i.n. route or i.p. IgG-producing cells were present in the spleens and PPs of animals immunised i.p. with all of the vaccine formulations examined.

After one oral dose, PLG microspheres and liposomes stimulated only a poor serum IgG response. There was evidence of a gut mucosal response when liposomes were given i.p. or orally, however, PLG microspheres produced a low stool antibody response after administration by oral gavage. In both cases, multiple dosing did not increase titres against orally administered antigens. Stool IgA titres from mice immunised with liposomes were not confirmed by levels of IgA in PPs of these animals.

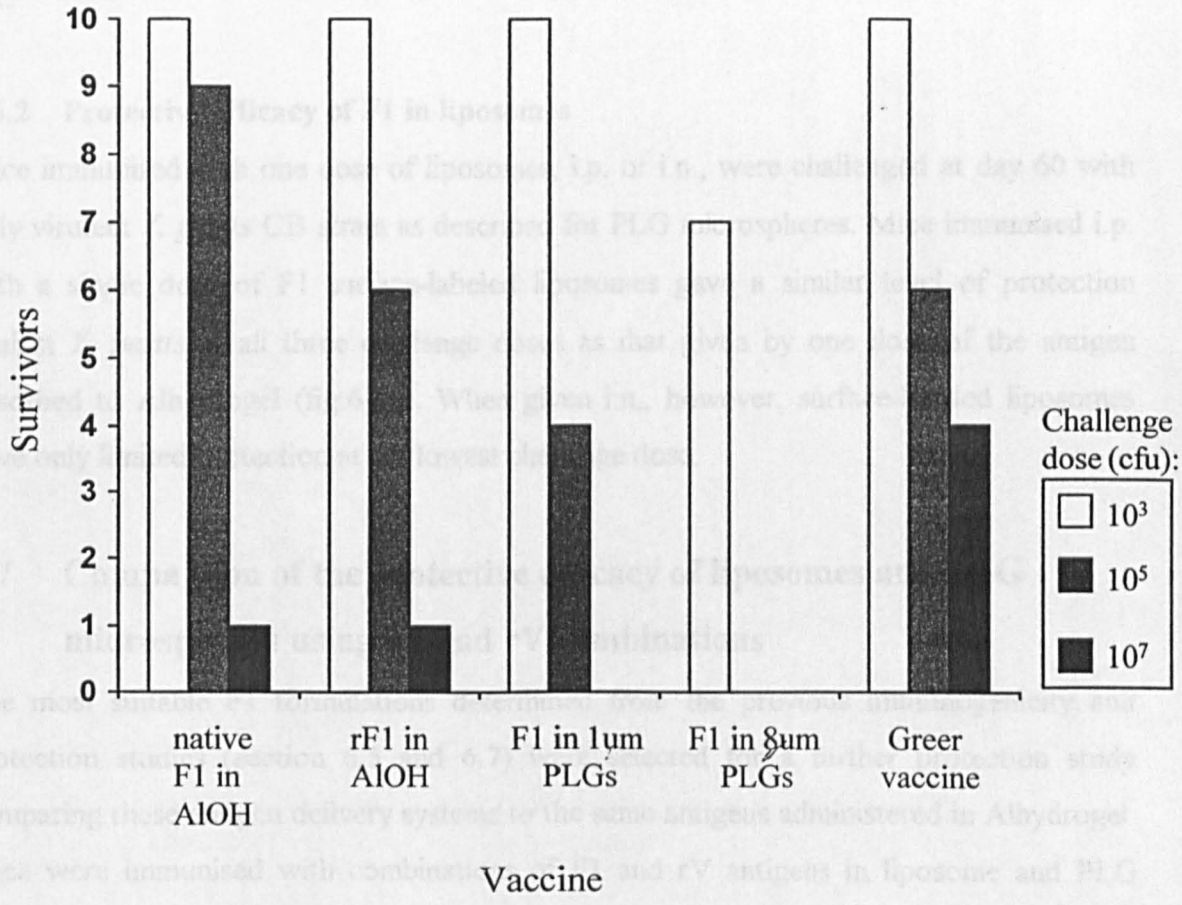
When encapsulated F1 and V antigens were co-administered, no additive effect on F1 antibody response was observed. The IgG serum response to these formulations was still evident after 168 days, but again no additive effect was achieved by combining encapsulated F1 and V antigens. The systemic and mucosal response to antigens given orally was poor, even after three doses. In mice immunised i.p. with PLG microspheres, 1μm particles induced a sustained response over 42 days but with 8μm particles antibody levels started to fall after 28 days. However, the levels were sustained if mice were given three doses of 8μm particles i.p.

6.6 F1 as a protective antigen

6.6.1 Protective efficacy of F1 antigen in PLG microspheres

Mice immunised i.p. with F1 in PLG microspheres (single dose) were challenged at day 60 after immunisation with fully virulent *Y. pestis* (GB strain). The protective potency was assessed and compared with three doses of either the commercially available Greer whole-cell vaccine or F1 antigen in Alhydrogel (Fig.6.15). Mice immunised with F1 in Alhydrogel were 90% protected against sub-cutaneous challenge with up to $1x10^5$ cfu virulent plague bacilli. The whole-cell vaccine gave 100% protection against 1×10^3 cfu and 40% protection against 1×10^7 cfu challenge. Mice immunised i.p. with F1 encapsulated in PLG microspheres were protected against challenge with virulent *Y. pestis*; the 1.0μm microspheres were more protective than the 8.0μm PLG microparticles, giving 100% protection at a challenge dose of 1×10^3 cfu, 40% protection against $1x10^5$ cfu, but no protection with 1×10^7 cfu virulent plague bacilli. F1 in Alhydrogel and the Greer whole-cell vaccine were more protective at the higher challenge doses than the 1.0μm PLG

Fig. 6.15 Protective efficacy of F1 antigen in PLG microspheres



Groups of mice immunised with nF1 or rF1 in Alhydrogel (AlOH) or Greer vaccine received three doses, whereas groups of mice receiving 1 or 8µm PLG microspheres were given only one dose.

microspheres. However, the mice immunised with F1 in Alhydrogel and Greer whole-cell vaccine were given 2 to 3 doses of antigen before challenge, whereas mice receiving PLG microspheres were only immunised at day 0. A single dose of 1.0μm or 8.0μm PLG microspheres gave protection against sub-cutaneous challenge with $1x10^3$ cfu plague bacilli; the 1.0μm PLG microspheres gave full protection whereas the 8.0μm microspheres gave partial (70%) protection. A single dose of 1.0μm PLG microspheres was as effective as three doses of F1 in Alhydrogel or the Greer whole-cell vaccine at the lowest challenge dose, but protection was not as effective at the higher challenge doses of $1x10^5$ and $1x10^7$ cfu.

6.6.2 Protective efficacy of F1 in liposomes

Mice immunised with one dose of liposomes, i.p. or i.n., were challenged at day 60 with fully virulent *Y. pestis* GB strain as described for PLG microspheres. Mice immunised i.p. with a single dose of F1 surface-labeled liposomes gave a similar level of protection against *Y. pestis* at all three challenge doses as that given by one dose of the antigen adsorbed to Alhydrogel (fig.6.16). When given i.n., however, surface-labeled liposomes gave only limited protection at the lowest challenge dose.

6.7 Comparison of the protective efficacy of liposomes and PLG microspheres using F1 and rV combinations

The most suitable F1 formulations determined from the previous immunogenicity and protection studies (section 6.5 and 6.7) were selected for a further protection study comparing these antigen delivery systems to the same antigens administered in Alhydrogel. Mice were immunised with combinations of F1 and rV antigens in liposome and PLG formulations to assess the protection given by co-immunisation and to determine the effect of giving multiple immunisations by different routes. In this study, all mice were given three doses of antigen. Mice receiving liposomes or PLG microspheres were given 5μg of antigen per dose (there was insufficient antigen to give 10μg). Animals receiving antigens in Alhydrogel were immunised with three, 10μg doses. The results of this protection study are illustrated in fig. 6.17.

Fig. 6.16 Protective efficacy of F1 in liposomes

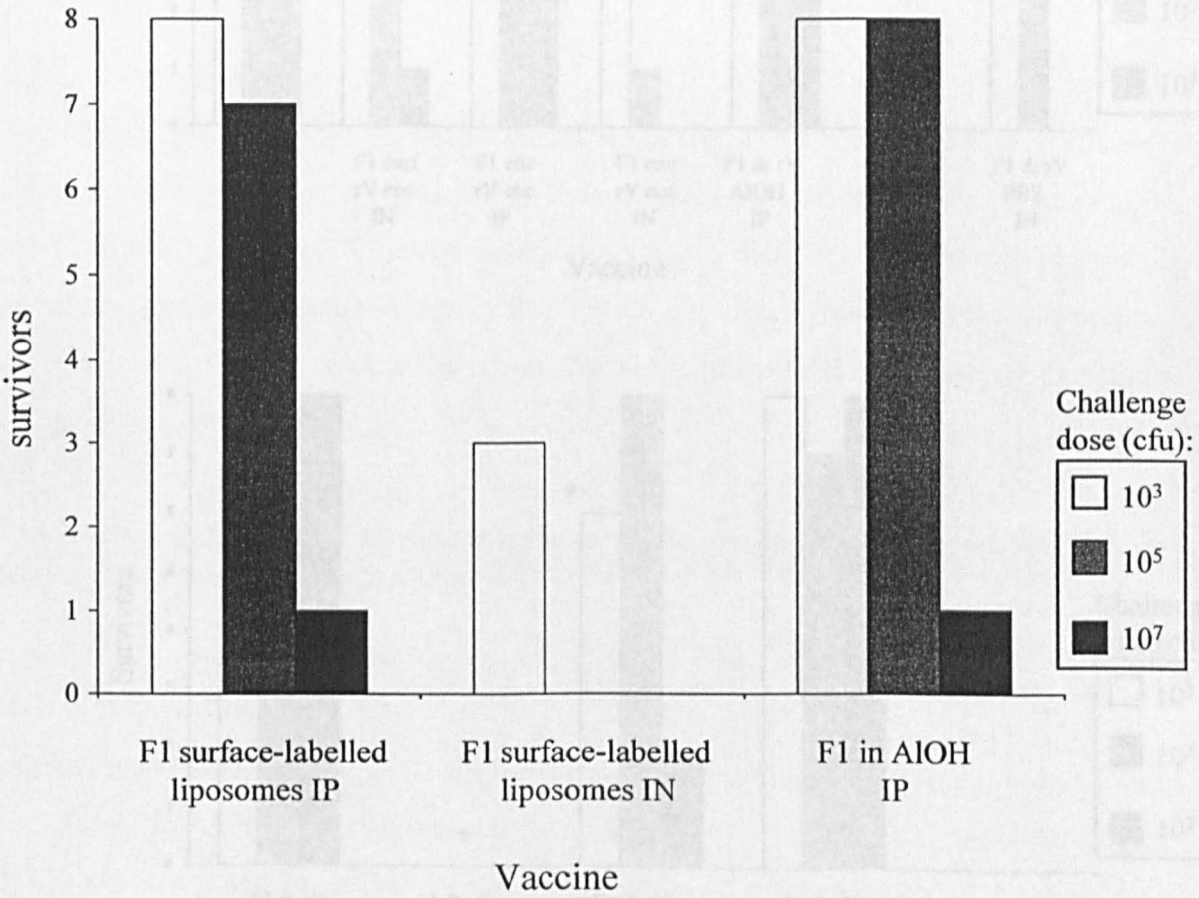
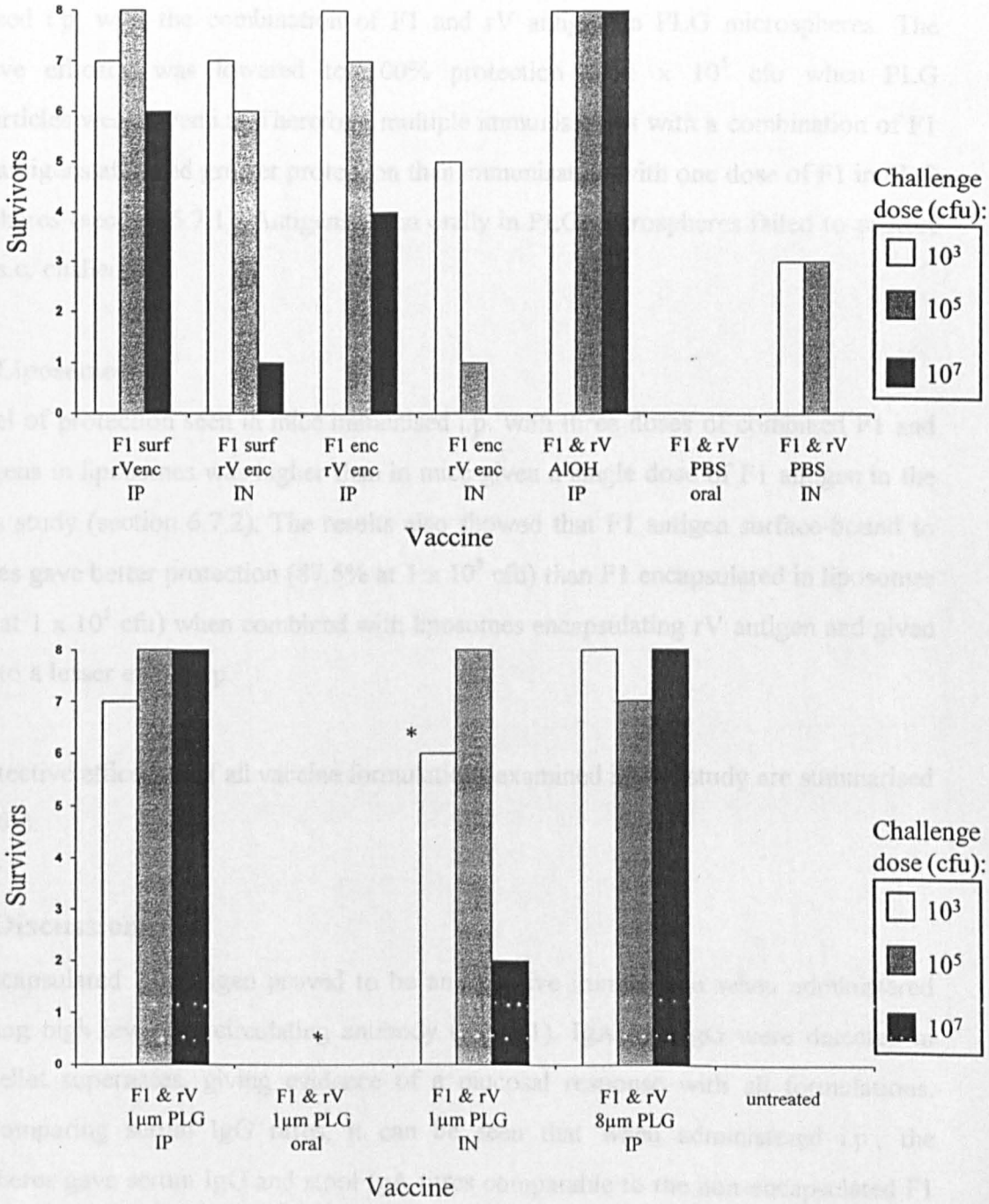


Fig. 6.17 Protective efficacy of F1 and rV in liposomes and PLG microspheres



8 mice per group except for those marked * where 7 mice were used
untreated control consisted of 5 mice per group.

F1 surf, F1 bound to the liposome surface;
F1 and rV enc, F1 or rV encapsulated within the liposome
F1 and rV AlOH, antigens in alhydrogel
F1and rV 1 and 8µm PLGs are the PLG microsphere sizes

6.7.1 PLG microspheres

There was 100% protection against the highest challenge dose of 1 x 10^7 cfu when immunised i.p. with the combination of F1 and rV antigen in PLG microspheres. The protective efficacy was lowered to 100% protection at 1 x 10^5 cfu when PLG microparticles were given i.n. Therefore, multiple immunisations with a combination of F1 and rV antigens afforded greater protection than immunisation with one dose of F1 in PLG microspheres (section 6.7.1). Antigens given orally in PLG microspheres failed to protect against s.c. challenge.

6.7.2 Liposomes

The level of protection seen in mice immunised i.p. with three doses of combined F1 and rV antigens in liposomes was higher than in mice given a single dose of F1 antigen in the previous study (section 6.7.2). The results also showed that F1 antigen surface-bound to liposomes gave better protection (87.5% at 1 x 10^5 cfu) than F1 encapsulated in liposomes (12.5% at 1 x 10^5 cfu) when combined with liposomes encapsulating rV antigen and given i.n. and to a lesser extent i.p.

The protective efficacies of all vaccine formulations examined in this study are summarised in table 6.2.

6.8 Discussion

Microencapsulated F1 antigen proved to be an effective immunogen when administered i.p., giving high levels of circulating antibody (fig. 6.1). IgA and IgG were detected in faecal pellet supernates, giving evidence of a mucosal response with all formulations. When comparing serum IgG titres, it can be seen that when administered i.p., the microspheres gave serum IgG and stool IgA titres comparable to the non-encapsulated F1 antigen (fig. 6.3 and 6.5).

The immunogenicity elicited by immunising mice with various vaccine formulations of the F1 antigen of *Y. pestis* and combinations of nF1 and rV antigens was compared. Mice immunised with F1 in PLG microspheres or liposomes developed high titres of circulating IgG when the antigens were given as one dose i.p. However, F1 given orally as one dose

Table 6.2 A summary of the protective efficacy of vaccine formulations of F1 and V antigens of *Y. pestis*

Table 6.2a The protective efficacy of vaccine formulations in Alhydrogel or PBS comparing one dose with three doses of antigen(s)

Antigen Delivery Formulation	Route	No. of Doses	% Survivors at day 14 after challenge		
			10^3	10^5	10^7
nF1 in Alhydrogel	i.p.	3	100%	90%	10%
rF1 in Alhydrogel	i.p.	3	100%	60%	10%
Greer vaccine	i.m.	3	100%	60%	40%
nF1 in Alhydrogel	i.p.	1	100%	100%	12.5%
nF1 and rV in Alhydrogel	i.p.	1	100%	100%	100%
nF1 and rV in Alhydrogel	i.p.	3	100%	100%	100%
nF1 and rV in PBS	oral	3	0%	0%	0%
nF1 and rV in PBS	i.n.	3	37.5%	37.5%	0%
Untreated control group	-	-	0%	0%	0%

Table 6.2b The protective efficacy of PLG microsphere vaccine formulations comparing one dose with three doses of antigen(s)

Antigen Delivery Formulation	Route	No. of Doses	% Survivors at day 14 after challenge		
			10^3	10^5	10^7
nF1 in 1μm PLG microspheres	i.p.	1	100%	40%	0%
nF1 in 8μm PLG microspheres	i.p.	1	70%	0%	0%
nF1 and rV in 1μm PLG microspheres	i.p.	3	87.5%	100%	100%
nF1 and rV in 1μm PLG microspheres	oral	3	0%	0%	0%
nF1 and rV in 1μm PLG microspheres	i.n.	3	87.5%	100%	25%
nF1 and rV in 8μm PLG microspheres	i.p.	3	100%	87.5%	100%
Untreated control group	-	-	0%	0%	0%

Table 6.2c The protective efficacy of liposome vaccine formulations comparing one dose with three doses of antigen(s)

Antigen Delivery Formulation	Route	No. of Doses	% Survivors at day 14 after challenge		
			10^3	10^5	10^7
nF1 surface-labelled liposomes	i.p.	1	100%	87.5%	12.5%
Liposomes surface-labelled with nF1 and encapsulating rV	i.p.	1	62.5%	50%	12.5%
nF1 surface-labelled liposomes	i.n.	1	37.5%	0%	0%
Liposomes surface-labelled with nF1 and encapsulating rV	i.p.	3	87.5%	100%	75%
Liposomes surface-labelled with nF1 and encapsulating rV	i.n.	3	87.5%	75%	12.5%
Liposomes encapsulating nF1 and rV	i.p.	3	100%	87.5%	50%
Liposomes encapsulating nF1 and rV	i.n.	3	62.5%	12.5%	0%
Ghost liposomes	i.p.	1	0%	0%	0%
Untreated control group	-	-	0%	0%	0%

in PLG microspheres did not elicit such an immune response in serum (fig. 6.8a). There was evidence of a gut mucosal response in faecal pellets when antigen was given i.p. but only a limited mucosal response was observed when the antigen was given by oral gavage (fig.6.8b). The presence of IgA in faecal pellets indicates the involvement of the gut associated lymphoid tissue (GALT) in the immune response to microparticles. Therefore, i.p. immunisation with microencapsulated subunit antigens effectively induced both systemic and mucosal immunity. Although the normal route by which antigen is taken up by the GALT is via the epithelial surface of the gut lumen, Peyer's patch tissue in the GALT can also be accessed via the serosal surface if antigen is administered i.p. (Williamson *et al.*, 1996). However, the lack of IgA in faecal pellets after oral immunisation suggests that microencapsulated antigen was not processed in the normal way.

When F1 antigen in liposomes were used to immunise mice, i.n. or i.p., high circulating antibody titres developed (fig. 6.9). The supernates from homogenised faecal pellets were assayed to determine whether a gut mucosal response had been produced and F1 in liposomes given i.p. or by oral gavage produced IgA titres indicative of a mucosal response. However, when given i.n. liposomes did not produce IgA titres in faecal pellet supernates (fig. 6.10). Therefore, like PLG microspheres, liposomes can access immune inductive sites in the gut after i.p. immunisation. In addition, a gut mucosal response was induced after oral administration indicating liposome-entrapped antigens are processed in the normal way. However, immunisation via the oral route did not induce a serum response. F1 surface-labeled liposomes also gave higher titres of gut mucosal antibody when given i.p. compared to i.n., indicating that systemic immunity but not gut mucosal immunity was activated following a single i.n. dose of liposomes. Therefore, the delivery route and antigen localisation in liposomes, in addition to the number of immunisations, may affect the immune adjuvant effect.

Multiple oral immunisations with F1 alone or F1 and V antigens combined did not overcome the poor antibody response observed after one dose of encapsulated antigen. The lack of response following oral immunisation of PLG microspheres may have been due to either the induction of oral tolerance, or destruction of antigen by stomach acid or digestion. Further doses of vaccine may be required to compensate for the loss of vaccine during passage through the GIT if oral delivery is to be effective. Enough vaccine needs to

be administered so that sufficient particulate antigen can be taken up by the PPs in the small intestine. In addition to this, other mucosal surfaces, such as lung and vaginal washings, would need to be sampled to provide evidence of a disseminated mucosal immune response. However, the gut IgA response in mice fed antigen entrapped in liposomes suggested that unlike PLG particles, liposome-antigen formulations could be processed in the GIT via the epithelial surface in the gut lumen.

The serum IgG antibody for all vaccine formulations examined was predominantly a Th_2 (IgG_1) response indicating that the subunit antigens presented in these formulations were similarly processed by APCs.

The protection results showed that F1 in PLG microspheres when given i.p. gave effective protection against challenge with *Y. pestis* after only one dose, especially if the size of the PLG microspheres was restricted to 1.0μm. The protection was consistent with the F1-specific antibody response developed in mice. Previous studies found that the strong adjuvant activity of PLG microspheres was dependent on the microspheres being no more than 10μm in diameter (Eldridge *et al.*, 1991a; 1990). The protection given by 1μm PLG microspheres after only one dose may be a direct result of this as all of the microparticles were <10μm in diameter (fig. 5.7). In addition to this, the depot release properties of the microspheres mean that less antigen and so fewer doses may be necessary to give protection (Eldridge *et al.*, 1991a; Cohen *et al.*, 1994), even though in this study there was evidence that some of the F1 antigen had broken down and a smaller protein was present in addition to F1 (section 5.4).

When F1 antigen in liposomes were used to immunise mice, high circulating antibody titres developed when liposome formulations were given i.p. and i.n. The protective efficacy of F1 in liposomes given i.p. and i.n. was compared with the antigen given i.p. in Alhydrogel. F1, when surface-labeled onto liposomes and given i.p, protected against challenge with up to $1x10^5$ cfu plague bacilli and this was similar to the protective efficacy of F1 given i.p. in Alhydrogel. The same liposomes given i.n., however, only gave 37.5% protection at the lowest challenge dose of $1x10^3$cfu. Therefore, although the serum titres were comparable to the liposomes given i.p., liposomes given i.n. are not as protective as liposomes given i.p. after one dose. More doses may be required for i.n. administered liposomes to give

protection against s.c. challenge.

The protection results from multiple immunisations with a combination of F1 and rV antigens showed that the greatest protection was afforded by antigens administered i.p., whether they were adsorbed to Alhydrogel or in PLG microspheres and liposomes. The most effective liposome formulation was with F1 antigen bound to the vesicle surface and rV encapsulated in the lipid vesicle. When this formulation was given i.n., it was more effective than Alhydrogel-adsorbed antigens given in the same way. This was possibly due to the way in which the antigen was presented on the surface of the liposome vesicle, which may have been of a similar conformation to F1 and V antigens *in vivo*.

The results showed that multiple immunisations of a combination of F1 and rV in liposomes or PLG microspheres gave greater protection than F1 antigen given alone as a single dose in the same vaccine delivery formulations. The most effective vaccine formulation overall against sub-cutaneous challenge was a combination of F1 and V antigens administered i.p. as three doses in Alhydrogel. However, this study has shown the vaccine potential of encapsulated F1 and V antigens given intra-nasally. A vaccine for use in humans that can be administered by a less invasive route would be a significant advance in vaccine development. Additionally, the aerosol challenge route should be investigated to determine protection against pneumonic plague infection.

Chapter 7

General Discussion

7.1 Introduction

The F1 antigen of *Yersinia pestis* has been used in the diagnosis of plague over many years (Chen and Meyer, 1966; Balmanyar and Cavanaugh, 1976; Williams *et al.*, 1988) and identified as a protective antigen (Meyer *et al.*, 1974; Simpson *et al.*, 1990). However, it is of value to fully characterise the antigen, to understand how the antigen affords protection against plague and to examine methods that might be used to increase the protective efficacy against plague infection.

7.2 Identification and purification of F1

This study succeeded in purifying large quantities of native F1 by modification of an earlier extraction method (Baker *et al.*, 1952). Although the extraction method used by Baker, where cultures were grown on agar medium, has been used for many years for the preparation of F1 antigen from *Y. pestis*, other studies found that bacteria grown in liquid culture can yield substantial quantities of F1 comparable to *Y. pestis* grown on solid culture media (Englesberg and Levy, 1954). In addition to this, growth of *Y. pestis* in liquid culture proved to be a practical approach in developing a scaled-up method of producing large quantities of antigen (fig. 3.10). A recombinant F1 antigen was produced in an attempt to overcome the problems of having to culture large quantities of *Y. pestis* under category 3 containment, which would increase the cost of any resulting vaccine.

Abath *et al.* (1990) examined the immunochemical localisation of F1 antigen and found it to be localised on the cell surface. In this study, SDS-PAGE and Western blot analysis (section 3.2.1) identified a 17.5kDa protein as the F1 antigen. The antigen was found to be secreted into the culture supernatant or loosely associated with the outer membrane, but was not present in membrane fractions after homogenisation (section 3.2.2). As expected for F1 antigen, it was produced only when cultures were incubated at 37^{0}C. The pH of larger-scale cultures was maintained at 7.4 as this was found to produce consistently higher yields of F1 antigen from culture supernatants.

Andrews *et al.* (1996) used a modification of Baker's method to purify F1; the method employed in this study was similar to that of Andrews *et al.*, except that culture supernatants were always used as starting material for purification of native F1. The yield

of F1 antigen was maximised by repeated washing and centrifugation of the ammonium sulphate precipitated pellet. The resulting F1 antigen was >90% pure and free of any major contaminating proteins. Attempts to further purify the F1 antigen did not produce a purer product. However, ion exchange chromatography was found to be an effective way of concentrating the purified product.

Lipopolysaccharide (LPS) was detected in purified protein samples (section 3.3.2.5) and, although removal of this potentially immunomodulatory component was not addressed in this study, it might be possible to remove endotoxin using a number of methods. On a gel filtration column, LPS from *Y. pestis* forms large aggregates of high molecular weight (Hartley *et al.*, 1974) and would therefore be difficult to separate from native F1 antigen, which also forms large aggregates in solution and eluted close to the void volume of the gel filtration column used during the course of this study. Andrews *et al.* (1996) reduced levels of endotoxin in purified F1 using immobilised polymyxin B that bound LPS. However, LPS levels present in this study did not have an observed toxic effect in mice, therefore further reduction in LPS content may not be required.

To improve the safety of an antigen for use as a vaccine component, further considerations would be to show that successive batches of antigen contained only pure product and any compounds used during purification and concentration are removed to acceptable limits. Standard procedures that exist for vaccine testing, include; (i) protein assay, SDS-PAGE and an antigenicity test, such as Western blot, to test protein purity and identity; (ii) sterility tests in appropriate media and at appropriate temperatures; (iii) the general safety of a vaccine component can be assessed by observation of immunised animals after administration of excessive doses; (iv) pyrogen testing by LAL assay for quantitation of endotoxin; and (v) assessment of potency by *in vitro* tests such as bactericidal assay and/or *in vivo* protection tests. These criteria can be specified for each vaccine component and must always fall within prescribed limits (Parkman and Hardegree, 1994).

To overcome problems of growing large quantities of *Y. pestis*, recent studies have examined the production of a recombinant F1 antigen. Oysten *et al.* (1995) produced a *Salmonella aro*A mutant expressing F1 antigen, but the safety of such live vaccine vectors is questionable and enhancement of virulence in the vector by expression of foreign proteins needs to be addressed. A recombinant *E. coli* strain expressing F1 as a surface

antigen has been used as a source of F1 antigen and purified using methods similar to those used for native F1 (Andrews *et al.*, 1996) but sequence analysis of the recombinant protein were not reported. In the present study, the *caf*1 gene was successfully amplified and cloned into the GST vector. The recombinant strain of *E. coli* produced adequate amounts of fusion protein, but problems were encountered when trying to cleave rF1 in large enough quantities for vaccine and production studies. The F1-GST fusion protein constituted 43% of the total protein after 5h of induction but was found to be insoluble and only 32.7% of the protein in the sonicate supernatant starting material bound to the affinity column. The rF1 product accounted for only 6.19% of the bound protein(section 3.3.2.3). Attempts to solubilise the protein using Sarkosyl (section 3.3.2.4) resulted in recovery of 75% of the protein; however, when this material was applied to the glutathione affinity column, again low efficiency of binding was observed resulting in low yields of rF1 (section 3.3.2.4), possibly due to the conformation of the rF1 masking the glutathione binding site on the GST moiety of the fusion protein. Therefore, native F1 antigen was purified for vaccine and production studies but further study of a recombinant F1 antigen is required to produce an antigen that is easily purified in large quantities. An alternative expression vector could be used or a recombinant *E. coli* capable of expressing the antigen *in vitro* (Andrews *et al.*, 1996; Titball *et al.*, 1997). Currently, there is no advantage in using a recombinant F1 instead of the native antigen which was purified in large quantities during the course of this study.

7.3 Characterisation of F1 antigen

The study was also successful in characterising the F1 antigen using novel methods to those previously used (Bennet and Tornabene, 1974; Glosnicka and Gruszkiewicz, 1980). The amino-acid sequence of F1 was predicted from the DNA sequence by Galyov *et al.*(1990). N-terminal sequencing of the first ten amino acids of both the native and recombinant antigens agreed with the predicted amino-acid sequence (section 4.2).

When Baker *et al.* (1952) first purified the F1 antigen of *Y. pestis*, it was found to consist of a 15kDa subunit (protein only) and a 17kDa subunit (containing both protein and carbohydrate). Other researchers have supported this finding (Bennet and Tornabene, 1974; Glosnicka and Gruszkiewicz, 1980) and therefore, F1 has been referred to as a 17.5kDa glycosylated protein.

A number of approaches were investigated during this study to determine whether purified native F1 was glycosylated, as this might have an immunomodulatory effect on a subunit vaccine containing this protein. PAS-stained polyacrylamide gels did not show F1 to bear carbohydrate residues even when gels were heavily loaded with protein (section 4.3.1). Similarly, F1 was not detected by enzyme immunoassay (section 4.3.2) designed to detect glycan residues in proteins. In addition to different growth media, high salt washes of whole cells and F1 derived from culture supernatant were examined to determine whether growth conditions or close association of F1 with the outer membrane affected the glycosylated state of the antigen. In all cases, glycosylation was not detected.

Samples of purified F1 were also analysed by mass spectroscopy using a number of approaches. The first of these methods, acetolysis, is a fairly crude method for detecting carbohydrate and is a useful first step before continuing with a more detailed mass spectroscopy study. Acetolysis provided evidence of hexose residues in F1 antigen, but this was not confirmed by further investigation involving tryptic digestion of the antigen prior to FAB-MS; F1 proved to be resistant to denaturation with urea and subsequent digestion with trypsin (section 4.3.3.2). This series of experiments provided no evidence of post-translational modification of F1 antigen. This result was confirmed by ESI-MS which showed purified culture-derived F1 to have a molecular weight of 15.363kDa (section 4.3.4). This agreed with the molecular weight predicted from the gene sequence (Galyov *et al.*, 1990) and confirmed, therefore, that no post-translational modification of the antigen had occurred indicating that culture-derived F1 antigen from *Y. pestis* was not glycosylated. This is beneficial for vaccine production in that the native F1, as purified in this study and shown to be protective, was not glycosylated which will simplfy the quality control of the antigen considerably.

The hexose residues detected by acetolysis may have arisen from contaminating endotoxin. LPS contamination might also explain why carbohydrate moieties were detected in earlier studies. However, earlier studies also employed different growth media, *Y. pestis* strains and methods of purification that may have contributed to the detection of carbohydrates or glycoproteins in these earlier studies.

7.4 Vaccine development

The problem with using synthetic peptides or purified antigens as vaccine candidates is that, compared with the intact organism, the level of induced antibody can be much lower, unless the method of presentation is correct. When proteins are released from their natural environment conformational changes may diminish their immunogenic activity, or at least modify it. Therefore, in addition to immunising with antigen adsorbed onto Alhydrogel (at present the only adjuvant registered for use in humans), F1 was successfully encapsulated into PLG microspheres and liposomes to achieve higher levels of protective antibody. Using vaccine delivery vehicles such as these can modulate antigen processing, possibly leading to the development of vaccines which can elicit both systemic and secretory antibody responses. F1 was also surface-labeled onto liposomes to assess whether this might mimic the *in vivo* conformation of the antigen and optimise the immune response. Liposomes and PLG microspheres have adjuvant properties, therefore less antigen may be required to stimulate a potent immune response, thus potentially reducing the cost. In addition, immunisation via a mucosal surface, possible with both PLG microspheres and liposomes, is much less invasive and easier to administer.

7.4.1 PLG microspheres

7.4.1.1 Microsphere formulation

The model of antigen release from microspheres can be divided into three phases; an initial "burst" release of surface-located and poorly entrapped protein, followed by a period of minimal release while polymer degradation starts, and a third phase of degradation-dependent release, which continues until the microparticles are depleted of protein (O'Hagan *et al.*, 1991). The immune response might be dependent on the physiological site at which protein is released and the rate of release. The study of microparticles with different release rates has shown that the more rapidly degrading polymer, Resomer RG506, was most effective for induction of high levels of salivary IgA, while the more slowly degrading polymer, RG 755, was most effective for the induction of serum IgG antibody (O'Hagan *et al.*, 1994). In this study, microspheres were produced using RG506 in an attempt to demonstrate a mucosal antibody response to determine whether this would result in increased protection against s.c. *Y. pestis* challenge.

7.4.1.2 Size-effect of PLG microspheres

Tabata *et al.* (1996) examined the size-effect of PLG microspheres on mucosal response using ovalbumin as a model antigen. After oral administration, the amount of microspheres taken up by PPs increased with increasing size up to11μm, uptake decreased thereafter and finally became zero when their diameter reached >21μm. After being taken up into PPs, microspheres <5μm in diameter seemed to be translocated to the spleen, where released antigen produced a serum antibody response. However, larger microspheres probably remained in the PPs leading to induction of IgA secretion. Therefore, size distribution of microspheres could be a key factor in regulating the induction of a systemic and mucosal response. Tabata *et al.*, (1996) determined the number of microspheres in tissue sections by fluorescent microscopy and microspheres were separated according to size into fractions by counterflow centrifugal elutriation (CCE).

Eldridge *et al.*, (1990,1991b) found that oral administration of staphylococcal enterotoxin B in microspheres with diameter of 1-10μm stimulated a higher serum antibody response than that of free antigen; secretion of IgA in mucosal tisssues was also enhanced by microsphere administration if the size was restricted to <10μm. Therefore, IgA secretion may not be simply a case of larger microspheres being retained by PPs and stimulating production of IgA.

In this study, examination of microspheres by SEM showed a marked variation in size, but size variation was less in the 1μm sample than in the 8μm microsphere sample. In the 1μm microspheres all particles sampled measured <2μm in diameter; the mean diameter for the 8μm microspheres was <10μm but some microspheres in the sample were as large as 20μm in diameter. This may have disadvantaged the 8μm microsphere in that immunisation with these particles might not lead to a disseminated immune response because larger particles would not be taken up as efficiently by the PPs with subsequent translocation to the spleen. Antibody-secreting cells (ASCs) in spleens and PPs of mice immunised with PLG formulations were examined. When mice were given one dose i.p. of 1μm microspheres, increased levels of ASCs in both immune inductive sites were observed compared with one dose of F1 in Alhydrogel given i.p. Larger microspheres found in the 8μm sample would be expected to remain in PPs leading to IgA secretion,

however, increased levels of IgA-secreting cells were not observed (section 6.4).

7.4.2 Liposomes

7.4.2.1 Liposome formulation

Although the basic model of a liposomal vesicle comprises uni- or multi-lamellar bilayers of phospholipid, the possibilities for variations of this basic model are many. Clarke and Stokes (1992) investigated liposome stability *in vitro* with liposomes composed either of PC, CH and DCP or DPPC, CH and DCP. Both formulations demonstrated negligible release at 4^0C, similar amounts of protein were released at 37^0C and showed substantial resistance to disruption in the presence of acidic stomach contents. However, DPPC-containing liposomes were more resistant to disruption in the presence of bile. Mori *et al.* (1992) found that inclusion of DSPC and CH in the phospholipid bilayer produced more rigid vesicles and increased the circulation time of liposomes in body fluids, such as serum. Phillips *et al.* (1996) examined the influence of phospholipid composition on antibody response to protein and peptide antigens encapsulated in liposomes. Vesicles composed of DPPC and DMPG were found to be the only effective adjuvant for the induction of s-IgA and circulating IgG antibody against *Schistosoma mansoni* glutathione-s-transferase after oral administration.

Two liposome formulations were examined in this study, the first was similar in composition to vesicles used by Michalek *et al.*, (1992) for oral immunisation; a homogeneous suspension of liposomes (ca. 200nm) consisting of DPPC, CH and DCP. In the second formulation, NHSP was added producing liposomes surface-labeled with F1 antigen; these vesicles also contained CH to make them more rigid and therefore more likely to withstand the harsh environment of the gut. Empty or "ghost" liposomes were produced as negative controls for immunogenicity and protection experiments. Both liposome-antigen formulations induced a serum IgG response after i.p. and i.n. dosing but not after oral immunisation. Liposomes given orally or i.p induced a limited IgA response in stools but i.n. immunisation did not induce a stool IgA response. However, surface-labeled liposomes were found to be more effective at inducing a protective immune response to *Y. pestis* challenge. Therefore, although optimal antigen presentation in liposomes is unknown, whether they should consist of free antigen in liposome suspension (unincorporated), totally encapsulated or surface-labeled with antigen, the results of this study indicated that presentation of the antigen would seem to be an important factor in liposome design.

7.4.2.2 Size-effect of liposomes

Michalek *et al.* (1992) examined the effect of liposome size on the secretory IgA response in rats and found that unilamellar vesicles of approximately 100nm in size induced higher levels of secretory IgA than a non-homogeneous suspension of multi-lamellar vesicles after gastric intubation of antigen.

As with PLG microspheres, the size of the vesicles and release of antigen may affect the adjuvanticity of liposomes. Some researchers have incorporated soluble and particulate antigens in giant liposome vesicles with a mean diameter of 5.5μm and successfully encapsulated whole cells (Antimisiaris *et al.*, 1993) in such liposomes. Michalek *et al* (1992) fed rats with either a non-homogeneous suspension of liposomes ranging from 150nm to 600nm in diameter or an homogeneous suspension of liposomes encapsulating antigen of approximately 100nm and found that the smaller liposomes induced higher levels of s-IgA. In this study, size variation was not studied in detail. Liposomes designed to withstand the harsh conditions of the gut and stimulation of a mucosal response were used for oral, i.n. and i.p. immunisation, based on the findings of previous studies (Clarke and Stokes, 1992). Homogeneous suspensions of liposomes encapsulating or surface-labeled with antigen of approximately 200nm in diameter were examined.

7.5 Immune response and protective efficacy in mice

The immunogenicity and protective potency of F1 antigen, alone or in combination with rV antigen were assessed using different vaccine delivery methods and were compared with these antigens given to Balb/c mice in Alhydrogel or PBS depending on the delivery route.The ability of these vaccination regimens to elicit mucosal and systemic responses was assessed and protective potency was determined by challenging mice by the sub-cutaneous route with virulent *Y. pestis* GB strain.

Long term mucosal immunity would be advantageous in protecting against pneumonic plague. However, long-term systemic immunity would also be needed to prevent bubonic plague and reduce the number of vaccinations required. Essentially, mucosal antibodies are short-lived, whereas systemic antibody response is long-lived and this has implications for immunity to mucosal infection. Delivery systems such as PLG microspheres and liposomes have the potential to produce a more long-term mucosal and

systemic response because of their depot-release properties and can be administered by various, less invasive routes.

7.5.1 PLG microspheres

7.5.1.1 Intraperitoneal (i.p.) immunisation

The adjuvant effect of PLG microspheres following i.p. injection of mice was examined by Nakaoka *et al.*, (1996) using ovalbumin (OVA) as a model system. A strong antibody response was induced compared with free OVA and the response was maintained over a period of 16weeks. Jones *et al.*, (1995) encapsulated *Bordetella pertussis* fimbriae in microspheres of approximately 24μm in diameter, protein was released *in vitro* over 42days, and mice immunised i.p. were protected against challenge with *B. pertussis*. Antibodies to encapsulated fimbriae were slightly lower than non-encapsulated fimbriae in Alhydrogel but both formulations protected against *B. pertussis* challenge.

In the present study, the *in vitro* release of antigen from PLG microspheres was sustained for a period of 42 days. There was an initial burst-release of antigen during the first five days, followed by a sustained level of release over the following 37 days (section 5.2.4). The immune response to F1 antigen released from PLG microspheres *in vivo* was assessed; a systemic response was observed up to 168 days after one dose i.p. immunisation, although in encapsulated formulations a decline in titre was observed. However, a sustained response was also observed in mice given one dose of F1 in Alhydrogel i.p. Therefore, in addition to the amount of antigen released over a period of time, the immune response might also depend on the physiological site at which the antigen is released and the results indicated that the *in vivo* release of antigen was slower than the rate of release *in vitro* (42 days, section 5.2.4). Other researchers have demonstrated that PLG microspheres form a depot at the injection site from which antigen is released over prolonged periods of time (Gupta *et al.*, 1996) and this would also explain why in this study a sustained response was observed after only one dose.

When F1 was given i.p. either Alhydrogel-absorbed or encapsulated in PLG microspheres, high circulating levels of IgG were observed which compared well with the commercial Greer vaccine. Titres were consistent over the 60day sampling period for F1 in Alhydrogel (3 doses) but in mice given one dose of PLG particles i.p. or three doses of Greer vaccine i.m., titres were lower after 42 days for 1μm microspheres, 14 days for 8μm

microspheres and after 28 days for Greer vaccine. Therefore, although three i.p. doses of non-encapsulated F1 gave the most sustained serum IgG response, the 1μm PLG microspheres gave a sustained response after only one dose. The number of IgG-producing cells in spleens and PPs in mice given one dose of 1μm microspheres i.p. was similar to that observed in mice given three doses of F1 in Alhydrogel i.p.. Therefore, antigen in 1μm PLG microspheres induced a response equal to that induced by non-encapsulated antigen and only one dose is required if encapsulated antigen is administered i.p. Elevated stool IgA titres were used as a marker for mucosal response; however, increased stool IgA titres were not observed in mice given one dose of PLG microspheres i.p. and combining microspheres containing F1 and V antigens did not have a synergistic effect.

Immunisation of mice i.p. with one dose of 1 or 8μm formulations of PLG microspheres containing F1 protected against *Y. pestis* challenge at 10^3 cfu, at higher challenge doses the 8μm microspheres did not protect, while 1μm PLG microspheres afforded some protection. After three doses i.p., both 1 or 8μm microspheres provided 100% protection against 10^7cfu. Therefore, although the presence of much larger microspheres in the 8μm formulation may have disadvantaged their uptake by PPs and subsequent translocation to the spleen and other immune inductive sites, this was apparently overcome by giving three doses of the 8μm PLG microsphere vaccine i.p..

7.5.1.2 Oral immunisation

Oral delivery of 1 or 8μm PLG microspheres did not augment the mucosal response; serum IgG and stool IgA titres were lower than those observed with antigens given i.p.. Additionally, elevated levels of IgG and IgA secretion by ASCs from spleen and PPs were not observed.

Oral immunisation of PLG encapsulated F1 antigen or a combination of F1 and V antigens resulted in at best only poor systemic and gut mucosal responses, irrespective of the number of doses given. By giving antigen in PLG microspheres of <10μm orally it should be possible to elicit a mucosal response by delivery of antigen to ASCs by M cells in the GALT followed by a disseminated response via the common mucosal immune system to distant mucosal tissues (section 1.7.5).

O'Hagan *et al.* (1994) found salivary and serum IgA responses in mice dosed orally with OVA in PLG microspheres made with the RG506 resomer after two doses of encapsulated OVA. Jones *et al.* (1996) found that orally administered microencapsulated *B. pertussis* (2μm diameter) protected mice from respiratory infection and mounted an increased mucosal response compared with antigen adsorbed onto Alhydrogel given i.p. Although the same PLG resomer was used in this study as O'Hagan *et al.* (1994) and a similar microsphere formulation was used by Jones *et al.* (1996), the antibody response and protection afforded by oral administration of PLG formulations was poor and multiple doses or co-administration with V antigen did not improve the outcome (section 6.8.1).

7.5.1.3 Intranasal (i.n.)immunisation

Almeida *et al.* (1993) examined nasal delivery of tetanus toxoid associated with PLG microspheres in rats, rabbits and guinea pigs. They observed that microspheres with a mean diameter of 510-930nm entered the blood circulation from the rat nasal cavity.

In the present study, F1 in 1μm PLG microspheres were co-administered with microspheres containing V antigen in three doses. The antibody response and protective efficacy was higher than free antigens in PBS given i.n.. Even at the highest challenge dose, PLG microspheres gave 25% protection compared with no protection afforded by the non-encapsulated antigens. It is possible that i.n. administration of PLG microspheres resulted in a protective immune response because antigen was delivered directly to lung macrophage and therefore to the BALT. This can then act as an inductive site for migration of IgA-committed B-cells to other mucosal tissues and at the same time stimulation of a systemic IgG response (de Haan *et al.*, 1995). In addition, nasal administration of PLG microspheres resulted in sustained levels of anti-F1 serum IgG.

7.5.2 Liposomes

7.5.2.1 I.P. immunisation

I.P. immunisation with liposomes has not been extensively studied by other researchers as the main routes of administration are considered to be oral and i.n. However, this study found that i.p. immunisation stimulated high serum antibody titres, which were sustained over the 42day sampling period. There was also evidence of a gut mucosal response, demonstrated by stool IgA titres but titres declined after day28. Salivary IgA was also

measured but only a low response was observed that was not sustained. IgG and IgM-producing cells were detected in spleen and PPs from mice immunised with surface-labeled liposomes. Lower levels of ASCs were observed with liposomes encapsulating antigen. When mice were given three doses of F1 and rV liposomes co-administered, serum IgG titres were at a similar level to those observed in mice given antigens in Alhydrogel. Protection was afforded against *Y. pestis* infection, especially when liposomes were surface-labeled with F1 antigen. After one dose of surface-labeled liposomes given i.p., 70% protection was observed with a challenge dose of 10^5cfu and when the number of immunisations was increased to three doses given i.p. before challenge, 100% protection was observed at 10^5cfu.

7.5.2.2 Oral immunisation

Clarke and Stokes (1992) examined intestinal and serum humoral response of mice to systemically and orally administered antigens in liposomes. They failed to demonstrate improved levels of intestinal IgA levels after feeding antigen in liposomes. The results obtained in this study confirmed this. Therefore, feeding antigen in liposomes does not enhance the serum humoral response or s-IgA response in the gut or saliva.

The results for both PLG microspheres and liposomes administered orally indicated that there was low or no intestinal absorption of liposomes or PLG microspheres. Therefore, the properties responsible for the adjuvant action of liposomes and PLG microspheres administered parenterally may be inapplicable to the induction of an immune response in the intestine. Repeated doses of PLG microspheres and liposomes failed to overcome low immunogenicity and protection afforded by oral administration of liposomes and PLG microparticles; no protection against *Y. pestis* infection was observed even after three doses. A reason for the low response could be induction of oral tolerance to F1 antigen. Thomas *et al.* (1992) evaluated the F1 antigen as a potential oral immunogen in mice and found that intragastric intubation of F1 antigen failed to show an elevated immune response and protection against *Y. pestis* infection. The observations made in this study agreed with these findings even when the antigen is protected by encapsulation in PLG microspheres or liposomes. Either the antigen did not survive proteolysis or the quantity absorbed was too low to stimulate antibody production and therefore induced oral tolerance.

7.5.2.3 I.N. immunisation

Abraham (1992) demonstrated that i.n. administration of bacterial polysaccharide in liposomes enhanced the antigen-specific pulmonary secretory response compared with non-encapsulated polysaccharide , therefore acting as an adjuvant for i.n. administration of a poorly immunogenic antigen. Aramaki *et al* (1994) investigated the effects of liposomes on the systemic and mucosal immune response following nasal administration in Balb/c mice using BSA-associated liposomes as a model and determined BSA-specific serum and salivary IgA levels. Levels were found to be significantly elevated after two doses of i.n administered liposomes. Antigen localisation was found to be important; mucosal response was activated by liposomes surface-labeled with BSA and a systemic response was activated by liposomes surface-labeled with or encapsulating BSA. High salivary IgA levels were observed when BSA was coupled to the outer surface of liposomes and no increase in this parameter was observed in liposomes encapsulating BSA.

This study showed that after i.n. administration of liposomes, a sustained serum IgG response was induced but no s-IgA was observed in stools or saliva. Liposomes surface-labeled with F1 antigen induced the highest titres and also gave enhanced protection against *Y. pestis* challenge compared with liposomes encapsulating antigen; this did not change when three doses of antigen were given and increased protection was observed in groups receiving liposomes encapsulating rV and surface-labeled with F1 antigens. Therefore, antigen localisation would seem to be an important factor following nasal administration of liposomes. The protective efficacy was lower when this formulation was administered i.n. compared with antigens adsorbed to Alhydrogel given i.p. However, i.n. administered liposomes were more protective than F1 and V antigens given i.n. in PBS, giving 75% compared with 37.5% protection at 10^5cfu *Y. pestis* (tables 6.2a and 6.2c).

In future studies of both liposomes and PLG microspheres, other mucosal sites, for example, lung and vaginal washes, could be sampled to assess a number of distant mucosal sites and to determine the s-IgA response, thereby further characterising the mucosal response to encapsulated antigens administered parenterally or to a mucosal surface.

7.6 Implications of the present study for vaccine development

The killed whole cell plague vaccine is, at present, the only widely used vaccine that protects against plague infection. However, protection is limited and it is questionable whether it will protect against respiratory infection. Therefore, an improved vaccine giving protection against plague infection by the s.c. and respiratory route is required.

In the present study, F1 antigen protected against infection with virulent *Y. pestis*, but a combination vaccine of F1 and rV antigens in Alhydrogel afforded greater protection. Anderson *et al.* (1996) found that rV gave protection against infection with both capsule positive and negative strains of *Y. pestis*. Although naturally occuring F1-negative strains are rare, some cases have been reported (Friedlander *et al.*, 1995; Winter, 1960). The present study succeeded in evaluating F1 antigen alone or co-administered with rV antigen as a potential vaccine against *Y. pestis* infection.

Comparison of i.p., i.n. and oral delivery showed that i.p. administered antigens induced high levels of circulating antibody and provided effective protection against s.c. challenge. The most effective formulation was F1 and rV co-administered in three doses i.p. in Alhydrogel, which gave full protection at the highest challenge dose. Antigens delivered intra-nasally in liposomes or PLG microspheres induced high serum IgG titres and protected against s.c. *Y. pestis* challenge but the gut mucosal response was poor. Encapsulated antigens afforded increased protection against s.c. challenge compared with F1 and rV antigens delivered intranasally in PBS. Orally immunised mice mounted only a poor systemic and mucosal response and this was reflected in the protective efficacy of oral formulations.

Therefore, i.n. administration of encapsulated antigens by delivering antigen to the BALT stimulated a protective response, but a gut mucosal response was not observed. The current hypothesis is that IgA committed B cells migrate to distant mucosal sites via the common mucosal immune system. However, there is some evidence of subcompartmentalisation of the common mucosal immune system (Mestecky *et al.* 1994) (fig. 7.1) where the highest levels of IgA secretion would occur locally at the site of immunisation. IgA-secreting B cells could then disseminate into the different compartments of the mucosal immune system and the site more distant from the site of

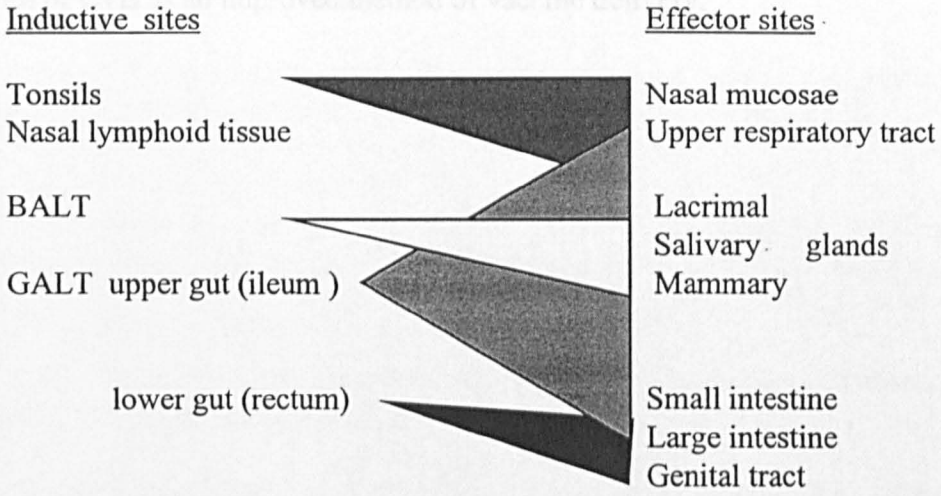
immunisation would have diminished amounts or no antibody response to the antigen. This may be the reason why a poor gut response to i.n. administered antigens was observed. If higher levels of antibody were present at the immunisation site, it follows that better protection would be afforded at the natural site of infection. Therefore, with pneumonic plague i.n. immunisation may be the preferred route of immunisation.

PLG microspheres are considered suitable for oral delivery of peptides (O'Hagan *et al.*, 1994) and the reasons for a poor oral response, possibly due to oral tolerance, should be investigated in future studies. Previous studies have shown that parenteral priming followed by oral boosting can produce increased antibody levels in animal models (Keren *et al.*, 1988). This sort of immunisation schedule may also overcome the problem of oral tolerance. Similarly, parenteral priming followed by i.n. administration of encapsulated antigens may increase protection against *Y. pestis* infection.

This study showed that there was no advantage in using encapsulated F1 and rV antigens compared with antigens injected i.p. in Alhydrogel against sub-cutaneous *Y. pestis* infection. However, encapsulation of antigenic peptides or proteins can be used for vaccine development and may be more effective when infection is by other routes, such as, i.n. or by aerosol. The immune responses induced to PLG microspheres and liposomes were similar to that induced by non-encapsulated antigens especially after three doses and protection was afforded by both methods. However, in liposome formulations antigen presentation would seem to be important. Further work should include an aerosol infection model to determine whether encapsulated antigens give increased protection over injection of non-encapsulated F1and rV antigens against respiratory infection. However, the results of this study did show that i.n. administration of liposomes and PLG microspheres were more protective against *Y. pestis* s.c. challenge than F1 and rV in PBS administered by the same route and so might be a possible vaccine to protect against both s.c. and aerosol challenge. Additionally, i.p. immunisation could not be transferred directly to use in humans and other routes , such as i.m. or s.c. need to be investigated.

Additionally, to further augment the protective immune response with encapsulated antigens, especially with respect to increased s-IgA, further work could include cytokines or the cholera toxin B subunit (CTB) as adjuvants. Cytokines have been shown to be involved in many aspects of the immune response including activation, proliferation and differentiation of B and T lymphocytes, the activation of mononuclear phagocytes and

Fig. 7.1 Subcompartmentalisation of the common mucosal immune system



Higher levels of mucosal antibody are achieved locally at the site of immunisation and a reduced response at distant mucosal effector sites

attraction of cells such as macrophage to the site of infection (Taylor, 1995). Certain cytokines have the ability to stimulate the production of a particular antibody isotype, for example, interleukin-5 (IL-5) can augment IgA secretion. Williamson *et al.*, 1996 demonstrated increased protection against *Y. pestis* infection when microencapsulated F1 and rV antigens were co-administered i.p. with CTB. Therefore, it might be possible to modify the immune response using encapsulated antigen combined with adjuvants such as cytokines or CTB as an improved method of vaccine delivery.

REFERENCES

Abath, F.G., Almeida, A.M., Ferreira, L.C. 1990. Immunochemical localisation of the fraction 1 antigen, a virulence determinant of *Yersinia pestis*. An. Acad. Bras. Cienc. **62** : 291-297.

Abraham, E. 1992. Intranasal immunisation with bacterial polysaccharide containing liposomes enhances antigen-specific pulmonary secretory antibody response. Vaccine. **10** : 461-468.

Abraham, S.N. and Beachey, E.H. 1985. Host defenses against adhesion of bacteria to mucosal surfaces. In Advances in Host Defense Mechanisms. **4** : 63. (Eds. Gallin, J.F. and Fauci, A.S.).

Ada,G. 1996. Overview of vaccines. Ch1, 1-16. Methods in Molecular Medicine : Vaccine Protocols, edited by A. Robinson, G. Farrar and C. Wiblin. Humana Press Inc., Totowa, NJ.

Allen, G. 1983. Sequencing of proteins and peptides. Laboratory techniques in biochemistry and molecular biology. Ch3, 51-59.

Allison, A.C. and Gregoriadis, G. 1974. Liposomes as immunological adjuvants. Nature: **252** : 252-53.

Almeida, A.J., Alpar, H.O. and Brown, M.R.W. 1993. Immune response to nasal delivery of antigenically intact tetanus toxoid associated with poly(L-lactic acid) microspheres in rats and guinea pigs. J. Pharm. Pharmacol. **45** : 198-203.

Alving, C.R. 1991. Liposomes as carriers of antigens and adjuvants. J. Immunol. Methods. **140** : 1 - 13.

Alving, C.R., Richards, R.L., Hayre, M.D., Hockmeyer, W.T. and Wirtz, R.A. 1989. Liposomes as carriers of vaccines: Development of a liposomal malaria vaccine. Immunological Adjuvants and Vaccines, edited by Gregoriadis, G., Allison, A.C., Poste, G. Plenum Press, New York and London. Page 123-131.

Amirmozafari, N. and Robertson, D.C. 1993. Nutritional requirements for synthesis of heat-stable enterotoxin by *Yersinia enterocolitica*. Appl. Environ. Microbiol. **59** : 3314-3320.

Ampel, N.M. 1991. Plagues - what's past is present : thoughts on the origin and history of new infectious diseases. Rev. Inf. Dis. **13** : 658 - 665.

Anderson,G.W., Leary, S.E.C., Williamson, E.D., Titball, R.W., Welkos, S.L., Worsham, P.L., Friedlander, A.M. 1996. Recombinant V antigen protects mice against pneumonic plague caused by F1-capsule positive and -negative strains of *Yersinia pestis*. Infect. Immun. **64** : 4580-4585.

Andrews, G.P., Heath, D.G., Anderson, G.W., Welkos, S.L. and Friedlander, A.M. 1996. Fraction 1 capsular antigen (F1). Purification from *Yersinia pestis* CO92 and from an *Escherichia coli* recombinant strain and efficacy against lethal plague challenge.

Infect. Immun. 64 : 2180 - 2187.

Antimisiaris, S.G., Jayasekera, P and Gregoriadis, G. 1993. Liposomes as vaccine carriers. Incorporation of soluble and particulate antigens in giant vesicles. J. Immunol. Methods. **166** : 271 - 280.

Aramaki, Y., Fujii, Y., Yachi, K., Kikuchi, H. and Tsuchiya, S. 1994. Activation of systemic and mucosal immune response following nasal administration of liposomes. Vaccine. **12** : 1241-1244.

Ashford, D.A. 1992. Carbohydrate analysis. Current opinion in biotechnology. **3** : 45-48.

Baker, E.E., Sommer, H., Foster, L.E., Meyer, E., Meyer, K.F. 1952. Studies on immunisation against plague I. The isolation and characterisation of the soluble antigen of *Pastuerella pestis*. J. Immunol. **68** : 131-145.

Balmanyar, M. and Cavanaugh, D.C. 1976. Plague Manual, 25-28. WHO Geneva.

Bartelloni, P.J, Marshall, J.D. and Cavanaugh, D.C. 1973. Clinical and serological responses to plague vaccine USP. Milit. Med. **138** : 720-722.

Barve, S.S., Straley, S.C. 1990. lcrR, a low-Ca^{2+} -response locus with dual Ca^{2+} - dependent functions in *Yersinia pestis*. J. Bact. **172** : 4661 - 4671.

Beck, L. E., Pope, V. Z., Flowers, C. E., Cowsar, D. R, Tice, T. R., Lewis, D. H., Dun, R.L., Moore, A. B. and Gilley, R. M. 1983. Poly (DL-lactide-co glycolide)/norethisterone microcapsules: an injectable biodegradable contraceptive. Biol. Reprod. **28** : 186 - 195.

Ben-Efraim, S., Aronson, M. and Bichowsky-Slomnicki, L. 1961. A new antigenic component of *Pastuerella pestis* formed under specified conditions of pH and temperature. J. Bact. **81** : 704.

Bendiner, E. 1989. Alexandre Yersin:Pursuer of Plague. Hosp. Pract Off. Ed. **24** : 121-148.

Ben-Gurion, R. and Shafferman, A. 1981. Essential virulence determinants of different *Yersinia* species are carried on a common plasmid. Plasmid. **5** : 183-187.

Bennet, L.G. and Tornabene, T.G. 1974. Characterisation of the antigenic subunits of the envelope protein of *Yersinia pestis*. J. Bact. **117** : 48-55.

Bercouvier, H., Mollaret, H.H., Alonso, J.M., Brault, G.R., Fanning, G.R., Steigerwalt, A.G. and Brenner, D.J. 1980. Intra- and interspecies relatedness of *Yersinia pestis* by DNA hybridisation and its relationship to *Yersinia psuedotuberculosis*. Curr. Microbiol. **4** : 225-229.

Bergers, J.J., ten Hagen, T.L.M., van Etten, E.W.M., Bakker-Woudenberg, I.A.J.M. 1995. Liposomes as delivery systems in the prevention and treatment of infectious diseases. Pharm. World. Sci. **17** : 1-11.

Bliska, J.B., Copass, M.C. and Falkow, S. 1993. The *Yersinia pseudotuberculosis* adhesin YadA mediates intimate bacterial attachment to and entry into Hep-2 cells. Infect. Immun. **61** : 3914-3921.

Bockman, D.E. and Cooper, M.D. 1973. Pinocytosis by epithelium associated with lymphoid follicles in the bursa of Fabricus, appendix and Peyer's patches. An electron microscope study. Am. J. Anat. **136** : 455-477.

Bolin, I., Portnay, D.A., Wolf-Watz, H. 1985. Expression of temperature-inducible outer membrane proteins of *Yersiniae*. Infect. Immun. **48** : 234-240.

Bolin. I., Wolf-Watz, H. 1984. Molecular cloning of the temperature-inducible outer membrane protein 1 of *Yersinia pseudotuberculosis* Infect. Immun. **43** : 72-78.

Brenner, D.J., Steigerwalt, A.G., Falcao, D.P., Weaver, R.E. and Fanning, G.R. 1976. Characterisation of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* by deoxyribonucleic acid hybridisation and by biochemical reactions. J. Sys. Bacteriol. **26** : 180-194.

Brubaker, R.R. 1991. Factors promoting acute and chronic diseases caused by *Yersiniae.* Clin. Micro. Rev. **4** : 309-324.

Brubaker, R.R. 1991b. The V antigen of *Yersinia* - an overview. Contrib. Microbiol. Immunol. **12** : 127-133.

Brubaker, R.R. 1972. The genus *Yersinia* : biochemistry and genetics of virulence. Curr. Top. Microbiol. **57** : 111-158.

Brubaker, R.R. 1970. Mutation rate to non-pigmentation in *Pasteurella pestis.* J. Bact. 98 : 1404-1406.

Bullen, J.J, Rogers, H.J. and Griffiths, E. 1978. Role of iron in bacterial infection. Curr. Top. Microbiol. Immunol. **80** : 1-35.

Burrows, T.W. 1958. The effects of loss of different virulence determinants on virulence and immunogenicity of strains of *Pastuerella pestis*. Brit. J. Exp. Path. **39** : 278.

Burrows, T.W. and Bacon, G. A. 1956. The basis of virulence in *Pasteurella pestis*; an antigen determining virulence. Brit. J. Exp. Path. **37** : 481.

Burrows, T.W. 1963. Virulence of *Pastuerella pestis* and immunity to plague. Erg. Der. Mikro. Imm. Expt. Therapie. **37** : 59-113.

Burrows, T.W. 1957. Virulence of *Yersinia pestis*. Nature. **179** : 1246-1247.

Butler, T., Bell, W., Link, N. *et al.* 1974. *Yersinia pestis* infection in Vietnam : Clinical and hematologic aspects. J. Inf. Dis. **129** : 578-584.

Carniel, E. and Mollaret, H.H. 1990. Yersiniosis. Comp. Immun. Microbiol. Infect. Dis.

13 : 51 - 58.

Casadaban, M.J., Chou, J. and Cohen, S.N. 1980. *In vitro* gene fusions that join an enzymatically active β-galactosidase segment to amino-terminal fragments of exogenous proteins : *Escherichia coli* plasmid vectors for the detection and cloning of translational initiation signals. J. Bact. **143** : 971-980.

Cavanaugh, D.C. and Randall, R. 1959. The role of multiplication of *Pasteurella pestis* in mononuclear phagocytes in the pathogenesis of flea-borne plague. J. Immunol. : **83** : 348-363.

Chand, V.K. 1994. Plague in India. Lancet. **344**: 1298.

Chart, H., Cheasty, T. and Rowe, B. **1995. Differentiation of *Y. pestis* and *Y. pseudotuberculosis* by SDS-PAGE analysis of lipopolysaccharide. Letts. App. Micro.** 20 : 369-370.

Childers, N.K., Michalek, S.M., Denys, F. and McGhee, J.R. 1987. Characterisation of liposomes for oral vaccines. Adv. Exp. Med. Biol. **216** : 1771-1780.

Chen, T.H. and Meyer, K.F. 1966. An evaluation of *Pasteurella pestis* fraction-1 specific antibody for the conformation of plague infections. Bull. WHO. **34** : 911-918.

Chen, T.H. and Meyer, K.F. 1955. Studies on immunisation against plague XI. A study of the immunogenicity and toxicity of eleven avirulent variants of virulent strains of *Pasteurella pestis*. J. Inf. Dis. **96** : 145.

Clarke, C.J. and Stokes, C.R. 1992. The intestinal and serum humoral immune response of mice to systemically and orally administered antigens in liposomes : I The response to liposome-entrapped soluble proteins. Veterinary Immunology and Immunopathology. **32** : 125-138.

Clark, E.A. and Ledbetter, J.A. 1994. How B and T cells talk to each other. Nature. **367** : 425-428.

Clemens, J.D., Sack, D.A., Harris, J.R., Chakraborty, J., Khan, M.R., Stanton, B.F., Kay, B.A., Khan, M.U., Yunus, A.M., Atkinson, W., Svennerholm, A.M. and Holgren, J. 1990. Field trial of oral cholera vaccines in Bangladesh. Results from three-year follow-up. Lancet. **335** : 270-273.

Cohen, S., Alonso, M. J., Langer, R. 1994. Novel approaches to controlled-release antigen delivery. Int. J. Tech. Ass. Health Care. : **10** : 121-130.

Cohen, S., Yoshioka, T., Lucarelli, M., Hwang, L.H. and Langer, R. 1991. Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres. Pharm. Res. **8** : 713-720.

Cornelis, G. 1992. *Yersiniae*, finely tuned pathogens. Molecular Biology of Bacterial Infection. Hormaeche, C.E., Penn, C.W., Smyth, C.J. (eds). Cambridge University Press, symp. 49, 231-265.

Cornelis, G, Laroche, Y., Balligard, G., Sory, M.P. and Walker, G. 1987. *Yersinia enterocolitica*, a primary model for bacterial invasiveness. Rev. Inf. Dis. **9** : 64-87.

Cox, J.C. and Coulter, A.R. 1997. Adjuvants-a classification and review of their modes of action. Vaccine. **15** : 248-256.

Crocker, T.T., Chen, T.H., Meyer, K.F. 1956. Electron microscopic study of the extracellular materials of *Pasteurella pestis*. J. Bact. **72** : 851-857.

Czerkinsky, C., Nilsson, L.A., Tarkowski, A., Koopman, W.J., Mestecky, J. and Ouchterlony, O. 1988. The enzyme-linked immunospot assay for detection of specific antibody-secreting cells. Methodology and applicability. In Theoretical and Technical Aspects of ELISA and Other Solid Phase Immunoassays. (D.M. Kemeny and R.J. Challacombe, eds.) p217-239. John Wiley and Sons, Chichester and New York.

Davies, D.A.L. 1956. A specific polysaccharide of *Pasteurella pestis*. Biochem. J. **63** : 105-116.

Davis, D. and Gregoriadis, G. 1987. Liposomes as adjuvants with immunopurified tetanus toxoid : Influence of liposomal characteristics. Immunology. **61** : 229-234.

de Haan, A., Renegar, K.B., Small, P.A. and Wilschut, J. 1995. Induction of a secretory IgA response in the murine female urogenital tract by immunisation of the lungs with liposome-supplemented viral subunit antigen. Vaccine. **13** : 613-616.

Depergolas, G., Neeruryun, E.D. and Gregoriadis, G. 1976. Penetration of target areas in the rat by liposome-associated bleomycin, glucose oxidase and insulin. FEBS Lett.**63** : 235-239.

Dunn, S.D. 1986. Effects of modification of transfer buffer composition and the renaturation of proteins in gels on the recognition of proteins on Western blots by monoclonal antibodies. Anal. Biochem. **157** : 144-153.

Ehrenkranz, N.J. and Meyer, K.F. 1955. Studies on immunisation against plague. VIII. Study of three immunising preparations in protecting primates against pneumonic plague. J. Inf. Dis. **96** : 138.

Eldridge, J.H., Staas, J.K., Meulbrock, J.A., McGhee, J.R., Tice, T.R. and Gilley, R.M. 1991a. Biodegradable microspheres as vaccine delivery system. Mol. Immunol. **28** : 287-294.

Eldridge, J.H., Staas, J.K., Meulbrock, J.A., Tice, T.R. and Gilley, R.M. 1991b. Biodegradable and biocompatible poly(DL-lactide-co-glycolide) microspheres as an adjuvant for Staphylococcal enterotoxin B toxoid which enhances the level of toxin-neutralising antibodies. Inf. Imm. **59** : 2978-2986.

Eldridge, J.H., Hammond, C.J., Meulbrock, J.A., Staas, J.K., Gilley, R.M. and Tice, T. M. 1990. Contolled vaccine release in the gut-associated lymphoid tissue. 1. Orally administered biodegradable microspheres target the Peyer's patches. J. Control. Rel. **11** :

205-214.

Ellis, R.W. and Douglas, R.G. 1994. Combination vaccines. Int. J. Tech. Assess. Health Care. **10** : 185-192.

Englesberg, E. and Levy, J.B. 1954. Studies on the immunisation against plague VI. Growth of *Pasteurella pestis* and the production of the envelope and other soluble antigens in a casein hydrolysate mineral glucose medium. J. Bact. **67** : 438-449.

Fenn, J.B., Mann, M., Meng, C.K., Wong, S.F. 1990. Electrospray ionisation-principles and practice. Mass Spectrometry Reviews. **9** : 37-70.

Frankel, S., Sohn, R.and Leinward,L. 1991. The use of sarkosyl in generating soluble protein after bacterial expression. Proc. Natl. Acad. Sci. USA. **88** : 1192-1196.

Friedlander, A.M., Welkos, S.L., Worsham, P.L., Andrews, G.P., Heath, D.G., Anderson, G.W., Pitt, M.L.M., Estep, J. and Davis, K. 1995. Relationship between virulence and immunity as revealed in recent studies of the F1 capsule of *Yersinia pestis*. Clin. Inf. Dis. **21**: S178-S181.

Galyov, E.E., Karlishev, A.V., Chernoskaya, T.V., Dolgikh, D.A., Smirnov, O.Y., Volkvoy, K.I., Abramov, V.M., Zav'yalov, V.P. 1992. Expression of the envelope antigen F1 of *Yersinia pestis* is mediated by the product of the *caf*1 gene having homology with the chaperone protein PapD of *Escherichiae coli*. FEBS Lett. **286** : 79-82.

Galyov, E.E., Smirnov, O.Y., Karlishev, A.V., Volkovoy, I.K., Deesyuk, A.I., Nazimov, I.V., Rustov, K.S., Abramov, V.M., Dalvadynoz, S.M., Zav'yalov, V.P. 1990. Nucleotide sequence of the *Yersinia pestis* gene encoding the F1 antigen and the primary structure of the protein. FEBS Lett. **227** : 230-232.

Ganem, D.E. 1986. Plasmids and pestilence - Biological and clinical aspects of bubonic plague. Med. Staff. Conf., Univ. Calif., San Fransisco. West. J. Med. **144** : 447 - 451.

Garcon, N.M., Six, H.R., Frazer, J., Hazelwood, C., Gilbert, B.E. and Knight, V. 1989. Liposomes of enviroxime and phosphatidylcholine : definition of the drug-phospholipid interactions. Antiviral Res. **11** : 89-98.

Geisow, M. 1991. Electrospray mass spectrometry of proteins. The Biochemist. **13** : 36-38.

Glosnicka, R. and Gruszkiewicz,E. 1980. Chemical composition and biological activity of the *Yersinia pestis* envelope substance. Infect. Immun. **30** : 506-512.

Goguen, J.D., Yother, J. and Straley, S.C. 1984. Genetic analysis of the low calcium response in *Yersinia pestis* Mu dl (Ap lac) insertion mutants. J. Bact. **160** : 842-848.

Gregoriadis, G. 1990. Immunological adjuvants : a role for liposomes. Immunol. Today. **11** : 89-93.

Gregoriadis, G. 1991. Overview of liposomes. J. Antimicrobial Chemo. **28** : 39-48.

Gregoriadis,G. and Panagiotidi, C. 1989. Immunoadjuvant action of liposomes : comparison with other adjuvants. Immunol. Lett. **20** : 237-240.

Gregoriadis, G. 1988. Fate of injected liposomes : observations on entrapped solute retention, vesicle clearance and tissue distribution in vivo. Liposomes as Drug Carriers : Recent Trends and Progress. Gregoriadis, G., ed. Wiley. Chichester and N.Y. pp 3-18.

Gregory, R.L, Michalek, S.M., Richardson, G., Harman, C., Hilton, T. and McGhee, J.R. 1986. Characterisation of immune response to oral administration of *Streptococcus sobrinus* ribosomal preparations in liposomes. Infect. Immun. **54** : 780-786.

Gupta, R.K., Chang, A.C., Griffin, P., Rivera, R. and Siber, G.R. 1996. *In vivo* distribution of radioactivity in mice after injection of biodegradable polymer microspheres containing ^{14}C-labeled tetanus toxoid. Vaccine. **14** : 1412-1416.

Gupta, R.K. and Siber, G. R. 1995. Adjuvants for human vaccines - current status, problems and future prospects. Vaccine. **13** : 1263-1276.

Hall, P.J. and Brubaker, R.R.1978. Pesticin-dependent generation of osmotically stable sphaeroplast-like structures. J. Bact. : **136** : 786-789.

Hartley, J.L., Adams, G.A. and Tornabene, T.G. 1974. Chemical and physical properties of lipopolysaccharide of *Yersinia pestis*. J. Bact. **118** : 848-854.

Hemker, H.C., Hermans, W. Th., Muller, A.D. and Zwaal, R.F.A. 1980. Oral treatment of haemophilia A by gastroabsorbtion of factor VIII entrapped in liposomes. Lancet. **1** : 70-71.

Hu, P.C. and Brubaker, R.R. 1974. Characterisation of pesticin. J. Biol. Chem. **249** : 4749-4753.

Isberg, R.R. and Leong. 1990. Multiple β1-chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. Cell. **60** : 861-871.

Isberg, R.R. 1989. Mammalian cell adhesion of enteropathogenic *Yersinia* species. Mol. Microbiol. **3** : 1449-1453.

Jacob-John, T. 1994. Plague in India. The Lancet. **344** : 1359-1360.

Jackson, S., Mestecky, J., Childers, N.K., Michalek, S.M. 1990. Liposomes containing anti-idiotypic antibodies : an oral vaccine to induce protective secretory immune responses specific for pathogens of mucosal surfaces. Inf. Imm. **58** : 1932-1936.

Jackson, S. and Burrows, T.W. 1956. The virulence enhancing effect of iron on non-pigmented mutants of virulent strains of *Pasteurella pestis*. Brit. J. Exp. Path. **37** : 577-583.

Janssen, W.A., Fukui, G.M, and Surgalla, M.J. 1958. A study of the fate of *Pasteurella pestis* following intracardial injection into guinea pigs. J. Inf.Dis. **103** : 183.

Jones, D.H., McBride, B.W., Thornton, C., O'Hagan, D.T., Robinson, A. and Farrar, G.H. 1996. Orally administered microencapsulated *Bordetella pertussis* fimbriae protect mice from *B. pertussis* respiratory infection. Infect. Immun. **64** : 489-494.

Jones, D.H., McBride, B.W., Jeffery, H., O'Hagan, D.T., Robinson, A. and Farrar, G.H. 1995. Protection of mice from *Bordetella pertussis* respiratory infection using microencapsulated pertussis fimbriae. Vaccine. **13** : 675-681.

Karlyshev, A.V., Galyov, E.E., Abramov, V.M. and Zav'yalov, V.P. 1992. *Caf*1M gene and it's role in the regulation of capsule formation of *Yersinia pestis*. FEBS. **305** : 37-40.

Karlyshev, A.V., Galyov, O.Y., Smirnov, O.Y., Guzayev, A.P., Abramov, V.M. and Zav'yalov, V.P. 1991. A new gene of the f1 operon of *Yersinia pestis* involved in capsule biogenesis. FEBS Lett. **297** : 77-80.

Keren, D.F., McDonald, R.A. and Carey, J.L. 1988. Combined parenteral and oral immunisation results in enhanced mucosal immunoglobulin A response to *Shigella flexneri*. Infect. Immun. **56** : 910-915.

Koenen, M, Ruther, U. and Muller-Hill, B. 1982. Immunoenzymatic detection of expressed gene fragments in the lacZ gene of *Escherichia coli*. EMBO. J. **1** : 509-512.

Kohn, J., Niemi, S.M., Albert, E.C., Murphy, J.C., Langer, R. and Fox, J. 1986. Single-step immunisation using controlled release biodegradable polymer with sustained adjuvant activity. J. Immunol. Methods. **95** : 31-38.

Korobkova, E.I., Vereninova, N.K., Kalacheva, N.F., Petrova, B.I. and Krainova, A.N. 1958. A study of a combined vaccine from dead *Vibrio comma* and live *Pasteurella pestis*. J. Micro. Epidem. Immunobiol. **29** : 1745.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophages T4. Nature. **227** : 680-685.

Lawton, W.D., Erdman, R.L. and Surgalla, M.J. 1963. Biosynthesis and purification of V and W antigen in *Pastuerella pestis*. J. Immunol. **91** : 179-184.

Leary, S.E., Williamson, E.D., Griffin, K.F., Russell, P., Eley, S.M., Titball, R.W. 1995. Active immunisation with recombinant V antigen from *Yersinia pestis* protects mice against plague. Inf. Imm. **63** : 2854-2858.

Lewis, D.J.M., Hayward, C.M.M. 1996. Stimulation of mucosal immunity. Methods in Molecular Medicine. Vaccine Protocols. Chapter 15, 187-195.

Lindler, L.E. and Tall, B.D. 1993. *Yersinia pestis* pH6 antigen forms fimbriae and is induced by intracellular association with macrophages. Mol. Microbiol. **8** : 311-324.

Lindler, L.E., Klempner,M.S. and Straley, S.C. 1990. *Yersinia pestis* pH6 antigen : genetic, biochemical and virulence characterisation of a protein involved in the

pathogenesis of bubonic plague. Infect. Immun. 58 : 2569-2577.

Lowenadler, Nilsson, B., Abrahamsen, L., Moks, T., Ljunqvist, L., Holmgren, E., Paleus, S., Josephson, S., Philipson, L. and Uhlen, M. 1986. Production of specific antibodies against protein A fusion proteins. EMBO. J. 5 : 2393-2398.

Lucky, M., Pollack, J.R., Wayne, R., Ames, B.N. and Neilands, J.B. 1972. Iron uptake in *Salmonella typhimurium* : utilisation of exogenous siderochromes as iron carriers. J. Bact. **111** : 731-738.

Luderitz, O. 1977. Endotoxins and other cell wall components of gram-negative bacteria and their biological activities. Schlessinger, D. (ed.). ASM, Washington D.C. Microbiology 1977, 239.

Maina, C.V., Riggs, P.D., Grandea, A.G., Slatko, B.E., Moran, L.S., Tagliamonte, J.A., McReynolds, L.A. and Guan, C.D. 1988. An *Escherichia coli* vector to express and purify foreign proteins by fusion to and separation from maltose-binding protein. Gene. 74 : 365-373.

Mant, C.T. and Hodges, R.S. 1991. High-performance liquid chromatography of peptides and proteins. Separation, analysis and conformation, 69-213. CRC Press. London.

Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. 3 : 208-218.

Marshall, J.D. 1967. Plague in Vietnam 1965-1966. Am. J. Epidemiol. 86 : 603-616.

Matthieu, J. M., Quarles, R.H. 1973. Quantitative scanning of glycoproteins on polyacrylamide gels with periodic acid-Schiff reagent (PAS) : Anal. Biochem. 55 : 313-316.

Mbawuike, I.N., Wyde, P.R. and Anderson, P.M. 1990. Enhancement of the protective efficacy of inactivated influenza A virus vaccine in aged mice by IL-2 liposomes. Vaccine. 8 : 347-352.

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.

McGhee, J.R., Mestecky, J., Elson, C.O. and Kiyono, H. 1989. Regulation of IgA synthesis and immune response by T cells and interleukins. J. Clin. Immunol. 9 : 175-199.

McEvedy, C. 1988. The bubonic plague. Sci. Am. 258 : 118-123.

McLachlan, G., Davidson, D.J., Stevenson, B.J., Dickinson, P., Davidson-Smith, H., Dorin, J.R. and Porteous, D.J. 1995. Evalution in in vitro and in vivo of cationic liposome-expression construct complexes for cystic fibrosis gene therapy. Gene Therapy. : 2 : 614-622.

Mestecky J., Moldoveanu, Z., Novak, M. and Compans, R.W. 1994. Mucosal immunity and strategies for novel microbial vaccines. Acta. Ped. Jap. **36** : 537-544.

Mestecky, J. and McGhee, J.R. 1987. Immunoglobulin A (IgA) : molecular and cellular interactions involved in IgA biosynthesis. Adv. Immunol. **40** : 153.

Meyer, K.F., Cavanaugh, J.D., Bartelloni, P.J. and Marshall, J.D. 1974a. Plague immunisation I : Past and present trends. J. Inf. Dis. **29** : S13-S18.

Meyer, K.F., Smith, G., Foster, L.E., Marshall, J.D. and Cavanaugh, D.C. 1974b. Plague immunisation IV.: Clinical reactions and serological response to inoculation of Haffkine and freeze-dried plague vaccine. J. Inf. Dis. **129** : S30-S36.

Meyer, K.F., Hightower, J.A. and McCrumb, F.R. 1974c. Plague immunisation VI. Vaccination with the fraction 1 antigen of *Yersinia pestis*. J. Inf. Dis. **129** : 541-545.

Meyer, K.F. 1961. Pneumonic plague. Bact. Rev. **25** : 249

Michalek, S.M., Childers, N.K., Katz, J. *et al.* 1992. Liposomes and conjugate vaccines for antigen delivery and induction of mucosal immune responses. Adv. Exp. Med. Biol. **327** : 191-198.

Michalek, S.M., Kiyono, H. Wannemuehler, M.J., Mosteller, L.M. and McGhee, J.R. 1982. Lipopolysaccharide (LPS) regulation of the immune response : influence on oral tolerance induction. J. Immunol. **128** : 1992-1998.

Mikulskis, A.V., Delor, I., Ha Thi, V., Cornelis, G.R. 1994. Regulation of the *Yersinia enterocolitica* enterotoxin *Yst* gene. Influence of growth phase, temperature, osmolarity, pH and bacterial host factors. Molec. Microbiol. : **14** : 905-915.

Miller, V.L. 1992. *Yersinia* invasion genes and their products. ASM News. **58** : 26-33.

Miller, V.L. 1989. The *ail* locus is found uniquely in *Yersinia enterocolitica* serotypes commonly associated with disease. Inf. Imm. **57** : 121-131.

Mills, K.H.G. 1996. Induction and detection of T-cell responses. Methods in Molecular Medicine. Vaccine Protocols. Humana Press Inc. Chapter 16, 197-221.

Morein, B., Lovgren, K., and Hoglund, S. 1989. Immunostimulating complex (ISCOM) by G. Gregoriadis, A.C. Allison, G. Poste (eds), Immunol. Adj. and Vaccines, Plenum Press, New York and London, 153-161.

Morris, W., Steinhoff, M.C. and Russell, P.K. 1994. Potential of polymer microencapsulation technology for vaccine innovation. Vaccine. **12** : 5-11.

Mosmann, T.R and Coffman, R.L. 1987. Two types of mouse helper T cell clones : Implications for immune regulation. Immunol. Today. **8** : 227-233.

Naik, S., Oates, J.E., Dell, A., Taylor, G.W., Dey, P.M. and Pridham, J.B. 1985. A novel mass spectrometric procedure for the rapid determination of the types of

carbohydrate chains present in glycoproteins : Application to α-galactosidase 1 from *Vicia faba* seeds. Biochem. Biophys. Res. Comm. **132** : 1-7.

Nakajima, R., Motin, V., Brubaker, R.R. 1995. Supression of cytokines in mice by protein A-V antigen fusion peptide and restoration of synthesis by active immunisation. Infect. Immun. **63** : 3021-3029.

Nakaoka, R., Tabata, Y. and Ikada, Y. 1996. Adjuvant effect of biodegradable poly(DL-lactic acid) granules capable for antigen release following intraperitoneal injection. Vaccine. **14**: 1671-1676.

Neilands, J.B. 1982. Microbial iron compounds. Ann. Rev. Biochem. **50** : 715-731.

New, R.R.C. 1994. Liposomes : a practical approach. IRL Press. Oxford. NY. Tokyo.

Nuwayser, E.S., Williams, D.L., Kerrigan, J.H., Nucefora, W.A. and Armstrong, J.C. 1984. Microencapsulation of contraceptive steroids. In: Long-acting contraceptive delivery systems (Eds. Zatuchi, G.I., Goldsmith, A. Shelton, J.D. and Sciarra, J.J.) Harper and Row, Philadelphia. p64-73.

O'Hagan, D.T., Rahman, D., Jeffery, H. Sharif, S. and Challacombe, S.J. 1994. Controlled release microparticles for oral immunisation. Int. J. Pharmaceutics. **108** : 133-139.

O'Hagan, D.T., Rahman, D., McGhee, J.P. et al. 1991. Biodegradable microparticles as controlled-release antigen delivery systems. Immunol. **73** : 239-242.

O'Hagan, D.T. 1990. Intestinal translocation of particulates - implications for drug and antigen delivery. Adv. Drug Delivery Rev. **5** : 265-285.

Owen, R.L. 1977. Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine. An ultrastructural study. Gastroenterology. **72** : 440–451.

Oyston, P.C.F., Williamson, E.D., Leary, S.E.C., Eley, S.M., Griffin, K.F. and Titball,R.W. 1995. Immunisation with live recombinant aroA *Salmonella typhimurium* producing F1 antigen protects against plague. Infect. Immun. **63** : 563-568.

Parkman, P.D. and Hardegree, M.C. 1994. Regulation and testing of vaccines. In: Vaccines second edition (Eds. Plotkin, S.A. and Mortimer, E.A.) Philadelphia. p889-901.

Patel, H.M. and Ryman, B.E. 1976. Oral administration of insulin by encapsulation within liposomes. FEBS Lett. **62** : 60-63.

Perry,R.D., Pendrak, M.L. and Schuetze, P. 1990. Identification and cloning of a haemin storage locus involved in the pigmentation phenotype of *Yersinia pestis*. J. Bact. **172**: 5929-5937.

Perry, R.D., Haddix, P., Atkins, E.B., Soughers, T.K. and Straley, S.C. 1987. Regulation of expression of V antigen and outer membrane proteins in *Yersinia pestis*.

Contr. Microbiol. Immunol. 9 : 173-178.

Perry, R.D., Harmon, P.A., Bowmer, W.S. and Straley, S.C. 1986. A low Ca^{2+} response operon encodes the V antigen of *Yersinia pestis*. Infect. Immun. **54** : 428-434.

Perry, R. D. and Brubaker, R.R. 1979. Accumulation of iron by Yersiniae. J. Bact. **137** : 1290-1298.

Phillips, N.C., Gagné, L., Ivanhoff, N. and Riveau, G. 1996. Influence of phospholipid composition on antibody response to liposome-encapsulated protein and peptide antigens. Vaccine. **14** : 898-904.

Pollack, C., Straley, S.C. and Klempner, M.S. 1986. Probing the phagosomal environment of human macrophages with a Ca^{2+}-responsive operon fusion in *Yersinia pestis*. Nature. **322** : 834-837.

Pollack, J.R., Ames, B.N. and Neilands, J.B. 1970. Iron transport in *Salmonella typhimurium* : mutants blocked in the biosynthesis of enterobactin. J. Bacteriol. **104** : 635-639.

Pollitzer, R. 1954. Plague. WHO Monograph series No 22. Geneva.

Porat, R., Mosseri, R., Kaplan, E., Johns, M.A. and Shibolet, S. 1992. Distribution of polysaccharide side chains of lipopolysaccharide determine resistance of *Escherichia coli* to the bactericidal activity of serum. J. Inf. Dis. **165** : 953-956.

Portnoy, D.A., Martinez, R.J. 1985. Role of a plasmid in the pathogenicity of *Yersinia* species. Curr. Topics in Microbiol. and Immunol. **118** : 29-51.

Portnoy, D.A., Wolf-Watz, H., Bolin, I., Beeder, A.B. and Falkows. 1984. Characterisation of common virulence plasmids in *Yersinia* species and their role in the expression of outer membrane proteins. Inf. Imm. **43** : 108-114.

Price, S.B., Freeman, M.D. and Kuang-Sheng Yeh. 1995. Transcriptional analysis of the *Yersinia pestis* pH6 antigen gene.J. Bact. **177** : 5997-6000.

Prostenko, O.A., Filippov, A.A. and Kutyev, V.V. 1991. Integration of the plasmid encoding the synthesis of capsular antigen and murine toxin into *Yersinia pestis* chromosome. Microbial Pathogenesis. **11** : 123-128.

Reisman, R.E. 1970. Allergic reactions due to plague vaccine. J. Allergy : **46** : 49-55.

Rodrigues, C.G., Carneiro, C.M.M., Barbosa, C.F.T. and Nogueira, R.A. 1992. Antigen F1 from *Yersinia pestis* forms aqueous channels in lipid bilayer membranes. Braz. J. Med. Biol. Res. **25** : 75-79.

Rooijen, N. and Su, D. 1989. Immunoadjuvant action of liposomes : mechanisms in Immunological Adjuvants and Vaccines. (Gregoriadis, G, Allison, A.C. and Poste, G., eds.). Plenum, N.Y. pp95-106.

Russell, P., Eley, S.M., Hibbs, S.E., Manchee, R.J., Staggs, A.J. and Titball, R.W. 1995. A comparison of plague vaccine, USP and EV76 vaccine induced protection against *Yersinia pestis* in a murine model. Vaccine. **13** : 1551-1556.

Russell, P., Eley, S.M., Bell, D.L., Manchee, R.J. and Titball R.W. 1996. Doxycycline or ciprofloxacin prophylaxis and therapy against experimental *Yersinia pestis* infection in mice. J. Antimicrob. Chemother. **37** : 769-774.

Rust, J.H., Cavanaugh, D.C., O'Shita, R. and Marshall, J.D. 1971. The role of domestic animals in the epidemiology of plague. I. Experimental infection of dogs and cats. J. Inf. Dis. **124** : 522.

Salyers, A.A. and Whitt, D.D. 1994. *Yersinia* infections. Bacterial pathogenisis, a molecular approach. Washington ASM Press, 213-228.

Sassenfeld, H.M. and Brewer, S.J. 1984. A polypeptide fusion designed for the purification of recombinant proteins. Bio/Tech. **2** : 76-81.

Schodel, F. 1994. Introduction : nucleic acid vaccines. Vaccine : **12** : 1491-1550.

Segrest, J.P. and Jackson, R.L. 1971. Molecular weight determination of glycoproteins by polyacrylamide gel electrophoresis in sodium dodecyl sulphate. Anal. Methods. Ch5, 54-63.

Shepherd, A.J., Hummitzsch, D.E., Leman, P.A., Swanepoel, R. and Searle, L.A. 1986. Comparative tests for detection of plague antigen and antibody in experimentally infected wild rodents. J. Clin. Microbiol. **24** : 1075 1078.

Sikkema, D.J. and Brubaker, R.R. 1989. Outer membrane peptides of *Yersinia pestis* mediating siderophore-independant assimilation of iron. Bio. Metals. **2** : 174-184.

Simpson, W.J., Thomas, R.E. and Schwan, T.G. 1990. Recombinant capsular antigen (fraction 1) from *Yersinia pestis* induces a protective antibody response in Balb/c mice. Am. J. Trop. Med. Hyg. **43** : 389-396.

Skurnik, M. and Toivanen, P. 1993. *Yersinia enterocolitica* lipopolysaccharide : genetics and virulence. Trends Microbiol. **1** : 148-151.

Smith, M.D, Vinh, D.X., Hoa, N.T.T., Wain, J., Thung, D. and White, N.J. 1995. In vitro antimicrobial susceptibilities of strains of *Yersinia pestis*. Antimicrobial Agents and Chemotherapy. **39** : 2153-2154.

Snippe, H., Verheul, A.F.M. and Van Dam, J.E.G. 1989. Liposomal vaccine to *Streptococcus pneumoniae* Type 3 and 14. In Immunological. Adjuvants and Vaccines. p107-122 (eds. Gregoriadis, G., Allison, A.C. and Poste, G.).

Sodeinde, O.A., Subrahmanyam, Y.V., Stark, K., Quan, T. and Goguen, J.D. 1992. A surface protease and the invasive character of plague. Science. **258** : 1004-1007.

Sodeinde, O.A. and Goguen, J.D. 1989. Nucleotide sequence of the plasminogen

activator gene of *Yersinia pestis*.:relationship to ompT of *Escherichia coli* and gene E of *Salmonella typhimurium*. Inf. Imm. 57 : 1517-1523.

Sodeinde, O.A., Sample, A.K., Brubaker, R.R. and Goguen, J.D. 1988. Plasminogen activator/coagulase gene of *Yersinia pestis* is responsible for degradation of plasmid-encoded. outer-membrane proteins. Inf. Imm. **56** : 2749-2752.

Spivack, M.L., Foster, L., Larson, A.,Chen, T.H., Baker, E.E. and Meyer, K.F. 1958. The immune response of the guinea pig to the antigens of *Pasteurella pestis*. J. Immunol. **80** : 132.

Stewart-Tull, D.E.S., Davies, M. and Jackson, D.M. 1978. The binding of adjuvant-active mycobacterial peptidoglycolipids and glycopeptides to mammalian membranes and their effect on artificial lipid bilayers. Immunology. **34** : 57-67.

Straley, S.C. and Perry, R.D. 1995. Environmental modulation of gene expression and pathogenesis in *Yersinia*. Trends Micribiol. **3** : 310-317.

Straley, S.C., Skrzypek, E., Plano, G.V. and Bliska, J.B. 1993. Yops of *Yersinia* spp. pathogenic for humans. Inf. Imm. : **61** : 3105-3110.

Straley, S.C. 1993. Adhesins in *Yersinia pestis*. Trends in Microbiol. **1** : 285-286.

Straley, S.C. and Cibull, M.L. 1989. Differential clearance and host-pathogen interactions of YopE- and YopK-YopL- *Yersinia pestis* in BALB/c mice. Inf. Imm. **57** : 1200-1210.

Straley, S.C. and Bowmer, W.S. 1986. Virulence genes regulated at the transcriptional level by Ca^{2+} in *Yersinia pestis* include structural genes for outer membrane proteins. Inf. Imm. **51** : 445-454.

Straley, S.C. and Brubaker, R.R. 1981. Cytoplasmic and membrane proteins of *Yersiniae* cultivated under conditions stimulating mammalian intracellular environment. Proc. Natl. Acad. Sci. USA. : **78** : 1224-1228.

Surgalla, M.J. 1960. Properties of virulent and avirulent strains of *Pasteurella pestis*. Ann. NY Acad. Sci. **88** : 1136.

Tabata, Y., Inoue, Y. and Ikada, Y. 1996. Size effect on systemic and mucosal immune responses induced by oral administration of biodegradable microspheres. Vaccine. **14** : 1677-1685.

Tauxe, R.V.,Vandepitle, J., Wanters, G. *et al.* 1987. *Yersinia enterocolitica* infections and pork : the missing link. Lancet. ii. 1129-1132.

Taylor, C.E. 1995. Cytokines as adjuvants for vaccines: antigen-specific responses differ from polyclonal responses. Infect. Immun. **63** : 3241-3244.

Thomas, R.E., Simpson, W.J., Perry, L.L. and Schwan, T.G. 1992. Failure of intragastrically administered *Yersinia pestis* capsular antigen to protect mice against

challenge with virulent plague:suppresion of fraction1-specific antibody response. Am. J. Trop. Med. Hyg. **47** : 92-97.

Tipple, M.A., Bland, L.A., Murphy, J.J. et al 1990. Sepsis associated with transfusion of red blood cells contaminated with *Yersinia enterocolitica*. Transfusion. **30** : 207-213.

Titball, R.W., Howells, A.M., Oyston, P.C.F. and Williamson, E.D. 1997. Expression of the *Yersinia pestis* capsular antigen (F1 antigen) on the surface of an *aro*A mutant of *Salmonella typhimurium* induces high levels of protection against plague. Infect. Immun. **65** : 1926-1930.

Tomasi, T.B. 1980. Oral tolerance. Transplantation. **29** : 353-356.

Tomizawa, H., Aramaki, Y. Fujii, Y, Hara, T., Suzuki, N., Yachi, K. *et al.* 1993. Uptake of phosphatidylserine liposomes by rat Peyer's patches following intraluminal administration. Pharm. Res. **10** : 549-552.

Tschesche, H. and Dietl, T. 1975. The amino-acid sequence of isoinhibitor K form snails (Helix pomatia). A sequence determination by automated Edman degradation and mass-spectral identification of the phenylthiohydantoins. Eur. J. Biochem. **58** : 439-451.

Uhlen, M., Nilson, B., Guss, B., Lindberg, M., Gatenbeck, S. and Lennart, P. 1983. Gene fusion vectors based on the gene for *Staphylococcal protein* A. Gene. **23** : 369-378.

Ullman, A. 1984. One-step purification of hybrid proteins which have β-galactosidase activity. Gene. **29** : 27-31.

Une, T. and Brubaker, R.R. 1984. Roles of V antigen in promoting virulence and immunity in *Yersiniae*. J. Immunol. **133** : 2226-2230.

Verrijk, R., Smolders, I. J. H., Bosnie, N. and Begg, A.C. 1992. Reduction of systemic exposure and toxicity of cisplatin by encapsulation in poly-lactose-co-glycolide. Cancer Res. **52** : 6653 - 6656.

Wachsmann, D., Klein, J.P., Scholler, M., Ogier, J., Ackermans, F. and Frank,R.M. 1986. Serum and salivary antibody orally immunised with *Streptococcus mutans* carbohydrate protein conjugate associated with liposomes. Inf. Imm. **52** : 408-413.

Wachsmann, D., Klein, J.P., Scholler, M., Frank, R.M. 1985. Local and systemic immune response to orally administered liposome-associated soluble *Streptococcus mutans* cell wall antigens. Immunology. **54** : 189-193.

Wait, R. 1993.Introduction to Mass Spectrometry. Methods in Molecular Biology. **17** : 204-207. Eds. C. Jones, B. Mullay and A.H. Thomas. Humana Press Inc. Totowa, N.J.

Wait, R. 1993. Fast Atom Bombardment Mass Spectrometry of peptides. Methods in Molecular Biology. **17** : 237-283. Eds. C. Jones, B. Mullay and A.H. Thomas. Humana Press Inc. Totowa, N.J.

Wake, A., Misawa, M. and Matsui, A. 1975. Siderochrome production by *Yersinia*

pestis and its relation to virulence. Inf. Imm. **12** : 1211-1213.

Walker, R.I. 1994. New strategies for using mucosal vaccination to acheive more effective immunisation. Vaccine : **12** : 387-400.

Wardlaw, A.C. 1993. Practical Statistics for Experimental Biologists. Ch 3, page 42-66. Pub. John Wiley and Sons. Chichester. NY. Brisbane. Toronto. Singapore.

Wassef, N.M., Alving, C.R. and Richards, R.L. 1994. Liposomes as carriers for vaccines. Immunomethods. : **4** : 217-222.

Wauters, G., Kandolo, K. Janssens, M. 1987. Revised biogrouping scheme of *Yersinia enterocolitica*. Contrib. Microbiol. Immunol. **9** : 14-21.

Williams. J.E., Gentry, M.K., Braden, C.A., Tyndal, G.L., Altieri, P.L., Berman, S. and Robinson, D.M. 1988. A monoclonal antibody for the specific diagnosis of plague. Bull. WHO. **66** : 77-82.

Williams, J.E. and Cavanaugh, D.C. 1979. Measuring the efficacy of vaccination in affording protection against plague. Bull. WHO. **57** : 309-313.

Williams, R.C., Gewurz, H. and Quie, P.G. 1972. Effects of fraction I from *Yersinia pestis* on phagocytosis in vitro. J. Inf. Dis. **126** : 235-241.

Williamson, E.D., Sharp, G.J.E., Eley, S.M., Vessey, P.M., Pepper, T.C., Titball, R.W. and Alpar, H.O. 1996. Local and systemic immune response to a micro-encapsulated sub-unit vaccine for plague. Vaccine. **14** : 1613-1619.

Williamson, E.D., Eley, S.M., Griffin, K.F., Green, M., Russell, P., Leary, S.E.C., Oysten, P.C.F., Easterbrook, T., Reddin, K.M., Robinson, A and Titball, R.W. 1995. Anew improved sub-unit vaccine for plague:the basis for protection. FEMS Immunology and Medical Microbiology. **12**: 223-230.

Wolf-Watz, H., Portnoy, D.A., Bolin, I. and Falkow, S. 1985. Transfer of the virulence plasmid of *Yersinia pestis* to *Yersinia pseudotuberculosis*. Infect. Immun. **48** : 241-243.

Winter , C.C., Cherry, W.B. and Moody, M.D. 1960. An unusual strain of *Pasteurella pestis* isolated from a fatal human case of plague. Bull. WHO. **23** : 408-409.

Wong, J.F. and Elberg, S.S. 1977. Cellular immune responses to *Yersinia pestis* modulated by products from thymus-derived lymphocytes. J. Inf. Dis. **135** : 67-78.

Worsham, P. L., Stein, M.P. and Welkos, S.L. 1994. Construction of F1-negative mutants of virulent *Yersinia pestis*. Poster presentation at the 6th international symposium on Yersinia, Rome, Italy.

Zahorchak, R.J. and Brubaker, R.R. 1982. Effect of exogenous nucleotides on Ca^{2+} dependence and V antigen sysnthesis in *Yersinia pestis*. Inf. Imm. : **38** : 953-959.

Zav'yalov, V.P., Abramov, V.M., Cherepanov, P.G., Spirina, G.V., Chernovskaya,

T.V., Vasiliev, A.M. and Zav'yalova, G.A. 1996. pH6 antigen (PsaA protein) of *Yersinia pestis*, a novel bacterial Fc-receptor. FEMS Immunol. and Med. Microbiol **14** : 53-57.

Zav'yalov, V.P., Chernovskaya, T.V., Navolotskaya, E.V., Karlyshev, A.V., MacIntyre, S., Vasiliev, A.M. and Abramov, V.M. 1995. Specific high affinity binding of human interleukin1β by Caf1A usher protein of *Yersinis pestis*. FEBS Letters : **371** : 65-68.

Appendix 1

Buffers used

Renaturation buffer

50mM Tris

20% Glycerol

pH adjusted to 7.4 with 6M HCl.

TAE buffer

121g Tris/HCl

28.6ml glacial acetic acid

50ml 0.5M EDTA

This was made up in 500ml distilled water and the pH adjusted to 8.0

TBE buffer

89mM Tris

89mM boric acid

2.5mM EDTA

pH 8.2

TE buffer

10mM Tris/HCl

1mM EDTA

pH8.0

Appendix 2

Published work