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Vaccination against bubonic and pneumonic plague

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Abstract

Yersinia pestis is the etiological agent of bubonic and pneumonic plague, diseases which have caused over 200 milllion human deaths in the past. Plague still occurs throughout the world today, though for reasons that are not fully understood pandemics of disease do not develop from these outbreaks. Antibiotic treatment of bubonic plague is usually effective, but pneumonic plague is difficult to treat and even with antibiotic therapy death often results. A killed whole cell plague vaccine has been used in the past, but recent studies in animals have shown that this vaccine offers poor protection against pneumonic disease. A live attenuated vaccine is also available. Whilst this vaccine is effective, it retains some virulence and in most countries it is not considered to be suitable for use in humans. We review here work to develop improved sub-unit and live attenuated vaccines against plague. A sub-unit vaccine based on the F1- and V-antigens is highly effective against both bubonic and pneumonic plague, when tested in animal models of disease. This vaccine has been used to explore the utility of different intranasal and oral delivery systems, based on the microencapsulation or Salmonella delivery of sub-units.

Keywords: Plague vaccine; Yersinia pestis; Microencapsulation; Salmonella vaccine vector

1. Plague — the disease

1.1. Yersinia pestis

The etiological agent of plague is *Yersinia pestis*, a Gram-negative bacterium which is a member of the enterobacteriacae family. *Y. pestis* is closely related to the other human pathogenic *Yersiniae*. However, unlike *Y. enterocolitica* and *Y. pseudotuberculosis*, *Y. pestis* does not infect the host by the enteric route. Some of the genes which are required for invasion by this route in *Y. pseudotuberculosis* and *Y. enterocolitica*, such as the *inv* and *yadA*, are present in *Y. pestis* but are not expressed as a consequence of mutations [1,2]. Another major difference is that *Y. pestis* is unable to survive outside of an animal host, whereas *Y. enterocolitica* and *Y. pseudotuberculosis* can survive in the environment. These findings suggest that *Y. pestis* might have evolved from the other human pathogenic *Yersiniae*. This suggestion is supported by a comparison of the genetic diversity of several housekeeping genes in *Y. pestis* and *Y. pseudotuberculosis* and suggest that *Y. pestis* evolved from *Y. pseudotuberculosis* 1500–20000 years ago [3].

The life cycle of *Y. pestis* differs from the other human pathogenic *Yersiniae* because the bacterium is transmitted from one animal host to another either directly or via a flea vector (often *Xenopsylla cheopis*). In areas of the world where plague is endemic the bacterium appears to survive by causing chronic disease in animal reservoirs, such as rats, ground squirrels or marmots. The occasional transfer of the bacteria to other mammalian hosts can result in acute disease, which is recognised as plague. Therefore, outbreaks of plague in man are often associated with close contact with animal reservoirs. Such events are often the consequence of natural disasters such as earthquakes or as a result of the man travelling into areas where the disease is endemic. In the USA the changing pattern of disease appears to be related to the residential encroachment on former rural areas which contain enzootic foci of plague [2]. More recently, *Y. pestis* has been of concern as one of the microorganisms which might be used illegitimately against civilian or military communities.

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1.2. Bubonic, septicaemic and pneumonic plague

There are three recognised forms of plague in man. Bubonic plague is the most common form of disease and arises following a bite from a flea which has fed previously on an infected animal [4]. The bacteria are disseminated from the initial site of infection to the draining lymph nodes, which become swollen and tender forming a bubo. The bubo can reach the size of a hen's egg and is the classical feature of bubonic plague. A bacteraemia may develop [2] with blood culture counts in the range $< 10–4 \times 10^7$ cfu/ml. Almost all of the plague which now occurs in the world is the bubonic form of the disease. Septicaemic plague occurs when there is a bacteremia without the development of buboes and is characterised by an elevated temperature, chills, headache, malaise and gastrointestinal disturbances [4]. Because of the generalised nature of these symptoms a diagnosis of plague is often delayed, and even with medical intervention 50% of patients die, probably as a result of the induction of the systemic inflammatory response syndrome [2]. The most feared form of plague arises when there is colonisation of the alveolar spaces leading to a pneumonia [4]. Pneumonic plague results in the production of a highly infectious bloody sputum. Coughing results in the production of airborne droplets containing bacteria, which can be inhaled by susceptible individuals [4,5]. The pneumonic form of the disease is feared because of the rapidity with which the disease develops (1–3 days), the high mortality rate in infected individuals (approaching 100%) and the rapid spread of disease from man to man [4]. In the context of the illegitimate use of *Y. pestis* as a weapon pneumonic plague is the likely outcome.

1.3. Incidence of disease

Yersinia pestis is generally recognised to have caused three major pandemics of disease in the 1st, 14th–17th and 19th centuries. Credible estimates indicate that together these resulted in 200 million deaths [2]. It is likely that both bubonic and pneumonic forms of plague occurred during the past pandemics. During the second pandemic of plague (the Black Death) it is estimated that over 30% of the population of Europe died from plague. Although *Y. pestis* no longer causes disease on this scale there is still a public health problem from plague (Fig. 1), especially in Africa, Asia and South America [6,7]. During the period 1967–1993 the average worldwide incidence of plague worldwide was 1666 cases [6,7]. Although the incidence trend was downwards until 1981 there has been an apparent increase in the incidence of disease over the last decade [2,6,7], possibly because of more efficient diagnosis and reporting of cases. However, many cases of plague are not diagnosed and it is likely that the true worldwide incidence of disease is several times the WHO figures. The Surat outbreak of plague in 1994 reminded the world that plague was still a potential problem. Although the extent of the disease was probably overstated, there were at least 876 presumptive cases of plague and 54 fatalities [8]. The potential for the rapid spread of the disease throughout the world by air transport systems was of particular concern during the Indian outbreak of plague. This concern was related especially to the pneumonic form of the disease, because asymptomatic individuals who boarded a flight could become infectious during the flight [9].

1.4. Use of vaccines and antibiotics

Both vaccines and antibiotics are used to prevent or treat the disease. However, the killed whole cells plague vaccine requires a course of injections over a period of 6 months [10]. Therefore, this vaccine is used mainly in those individuals who might be exposed to the pathogen, for example veterinarians, those engaged in research with the bacterium and individuals who are travelling to parts of the world where the disease is endemic. Cases of bubonic plague are often successfully treated with antibiotics, and streptomycin is the drug of choice [2]. However, the successful treatment of septicaemic and pneumonic plague with antibiotics is much less likely because the disease develops rapidly and treatment must commence during the early stages of the infection. The treatment of fulminant plague is especially difficult because of the possibility of bacteriolysis, with the subsequent release of large amounts of endotoxin. The recent isolation of a multiple antibiotic resistant strain of *Y. pestis* [11] indicates that the longer term potential for the use of antibiotics to treat plague is less certain.

Fig. 1. Incidence of plague worldwide, during the period 1970–1995. —, Cases of plague reported, · · · · deaths from plague.

2. Existing vaccines against plague

Both live attenuated and killed whole cells vaccines have been used in man. Killed whole cells vaccines are used throughout the Western World, whilst live attenuated vaccines have been used especially in the former USSR and in the former French colonies. Although there is circumstantial evidence for the efficacy of these vaccines, none have been subjected to a controlled and randomised clinical trials [12].

2.1. The EV76 live attenuated vaccine

The live attenuated vaccine (EV76 strain [10,13]) is a pigmentation negative mutant of *Y. pestis* which was derived from a fully virulent strain. The vaccine has been in use since 1908 and is given as a single dose of 5.8×10^6 cfu. Immunisation of mice with the EV76 vaccine induces an immune response which provides protection against subcutaneous and inhalation challenges with *Y. pestis* [14]. These findings suggest that immunisation with the EV76 vaccine will provide protection against both bubonic and pneumonic plague in man. However, the safety of this vaccine in man is questionable, because the EV76 strain is not avirulent. In studies with mice, a fatality rate of approximately 1% of vacinees has been reported [14].

2.2. Killed whole cell vaccines

The earliest report of a killed whole cell vaccine against plague was in 1897. However, it was not until 1946 that a killed whole cells vaccine was developed for use in man. Various methods of killing the bacterial cells have been used, including formaldehyde and heat treatment [10]. The vaccine is currently produced by the Commonwealth Serum Laboratories and is usually given as a course of three doses over a period of two months. Side effects, such as malaise, headaches elevated temperature and lymphadenopathy occur in approximately 10% of those immunised with killed whole cells vaccines. There are no definitive clinical trials which demonstrate the efficacy of killed whole cell vaccines [12]. However, studies in several animal species have demonstrated protection against bubonic plague. Also there is circumstantial evidence for the efficacy of the vaccine in humans derived from data on the incidence of bubonic plague in immunised US servicemen serving in Vietnam during the period 1961–1971. During this period there were many thousands of cases of plague in Vietnamese civilians (equating to 333 cases/10^6 person years of exposure). In immunised servicemen there were eight cases of plague (equating to an incidence of 1 case/10^6 person years of exposure) even though *Y. pestis* infection was commonly found in rodent populations surrounding US military installations [7,15]. It is possible that differences in the lifestyles of servicemen and Vietnamese civilians were responsible for the reduced incidence of plague in the former group. However, the incidence of murine typhus, which like *Y. pestis* is spread by *X. cheopis* in Vietnam, was reportedly similar in Vietnamese civilians and US servicemen [7,15]. Evidence for the efficacy of killed whole cells vaccines for the prevention of pneumonic plague is less conclusive. Cases of pneumonic plague have been reported in individuals immunised with this vaccine [16,17]. More recently it has been shown that mice immunised with this vaccine are protected against subcutaneous challenge, but not against inhalation challenge with *Y. pestis* [14]. Together, these findings suggest that killed whole cell vaccines do not induce a response which provides protection against pneumonic plague.

3. Improved vaccines against plague

In view of the continuing worldwide incidence of plague and the increased likelihood of illegitimate use of *Y. pestis*, there is a requirement for a vaccine which protects against both bubonic and pneumonic plague. Ideally this should be a reduced dose vaccine (two doses or ideally a single dose) which is free of any adverse side effects. Essentially there have been two approaches to the development of such a vaccine; the identification of a rationally attenuated mutant strain of *Y. pestis* and the identification of sub-units of the bacterium which could be formulated for single dose delivery.

3.1. Live attenuated mutants

The finding that the immunisation with the EV76 strain of *Y. pestis* induced protection against bubonic and pneumonic plague, but that this strain was not suitably attenuated for use in man, suggested that a rationally attenuated mutant of *Y. pestis* might be exploited as a vaccine. A variety of genes have been disrupted in other pathogens to yield defined live vaccines. In various pathogens of man and animals, including *Salmonella typhimurium*, *Salmonella typhi*, *Shigella flexneri*, *Pasteurella multocida* and *Aeromonas salmonicida*, inactivation of genes encoding enzymes involved in the shikimate pathway yields attenuated strains which can be exploited as live vaccines [18–25]. Other workers have shown that inactivation of the PhoP/PhoQ regulatory system in *S. typhimurium* or in *S. typhi* results in strains which are suitably attenuated for use as vaccines [26–28]. Inactivation of the *htrA* gene in *S. typhimurium*, *S. typhi* or *Brucella abortus* also results in attenuation [24,25,29,30]. None of these mutations resulted in a suitable level of attenuation in *Y. pestis*

Table 1
Virulence of defined mutants of *Y. pestis* strain GB

Genotype	Mouse[a]		Guinea pig[b]	
	MLD[c]	ttd[d]	MLD[c]	ttd[d]
Wild type	1	108 ± 7.75	2	ND
Δ*aroA* [31]	2	159 ± 8.6	>26	ND
Δ*phoP* [32]	75	221 ± 31.3	ND	ND
Δ*htrA* [33]	30	197 ± 6.12	ND	ND

[a] Balb/c mouse, s.c. challenge.
[b] Dunkin Hartley guinea pigs, s.c. challenge.
[c] Median lethal dose.
[d] Mean time to death (h) ± S.E.M.

(Table 1). An *aroA* mutant of *Y. pestis* was fully virulent in the murine model of disease but attenuated in guinea pigs [31]. A *phoP* mutant was 75-fold attenuated in the murine model of disease [32] whilst a *htrA* mutant was 30-fold attenuated [33]. The reasons why mutations which markedly attenuate *S. typhimurium* have little effect on *Y. pestis* are not clear. Clearly the development of a live attenuated mutant of *Y. pestis* would require additional studies with other mutants and might ultimately be dependent on the construction of a strain containing multiple mutations. However, the finding that immunisation of guinea pigs with sub-lethal doses of the *aroA* mutant or immunisation of mice with sub-lethal doses the *phoP* mutant did induce protective responses indicates the potential for these approaches

3.2. Subunit vaccines

In recent years, effort has focused on the development of a sub-unit vaccine for plague, based on virulence factors which might be located on the surface of the bacterium (Table 2). Immunisation with all of these components induced good circulating antibody responses. However, only the fraction 1 (F1) and virulence (V) antigens induced responses which consistently provided protection against challenge with *Y. pestis*. The F1 antigen is a capsular protein, located on the surface of the bacterium, which is thought to have anti-phagocytic properties [45]. The V antigen is a protein secreted by *Y. pestis* under low calcium growth conditions [46] and it is thought to have a key structural and regulatory role in the type III system. The recent finding that V-antigen can be detected on the surface of the bacterium suggests that it may form part of the injectosome [47]. Mutants of *Y. pestis* which do not produce V antigen are unable to deliver other Yops, which would have an array of anti-host activities, into the eukaryotic host cell [48].

The F1 and V antigens have been produced as recombinant proteins [42,49] and have been demonstrated to induce protective responses when used individually. However, a combination or fusion of these proteins had an additive protective effect when used to immunise mice against plague [37,50–52] (Fig. 2).

The immunogenicity of the proteins is dependent on their conformations and is maximal if they are in a similar conformation to the native proteins. The monomeric unit of F1 antigen has a molecular mass of 15.5 kDa. Aggregation to a large molecular mass complex (in excess of 3 MDa) occurs spontaneously in solution. Dissociation into the monomer by boiling in SDS reduced, but not abrogated, the protective efficacy of the protein in the mouse model of disease. The monomeric protein re-assembled into a high molecular complex in a time-dependent manner to regain the native conformation and protective efficacy [49]. This data is consistent with the identification of linear B-cell epitopes in F1 antigen [53]. Similarly the V antigen, which has a monomeric mass of 37 kDa, is thought to exist as a mixture of oligomers in a physiological medium (Miller, Personal communication). There is evidence from a number of studies that B-cell epitopes

Table 2
Immunogenicity and protective efficacy of sub-unit antigens of *Y. pestis*

Sub-unit	Function	Immunogenic	Protective efficacy (bubonic/peumonic model)
Pla	Surface plasminogen activator protease	Y[a]	Not tested [34]
pH6 antigen	Putative surface adhesin	Y	Bubonic — not protective [35]
LPS	Lipopolysaccharide	Y	Bubonic — not protective [36]
F1 antigen	Surface capsule	Y	Bubonic & pneumonic — protective [37,38]
YopD	Type III system — translocation Yop	Y	Bubonic —partially protective [39]
YopH	Type III system — PTPase effector Yop	Y	Bubonic — not protective [39]
YopE	Type III system — cytotoxin effector Yop	Y	Bubonic — not protective [39,40]
YopN	Type III system — regulates Yop release?	Y	Bubonic — not protective [39,40]
YopK	Type III system — regulates Yop release?	Y	Bubonic — not protective [39,40]
YopM	Type III system — effector Yop	Y	Bubonic — not protective [39,41]
Ypk A	Type III system — Ser/Thr kinase effector Yop	Y	Bubonic — delayed time to death [39]
V antigen	Type III system — part of the injectosome?	Y	Bubonic & pneumonic — protective [37,42–44]

[a] Y = detection of circulating antibody after immunisation of mice.

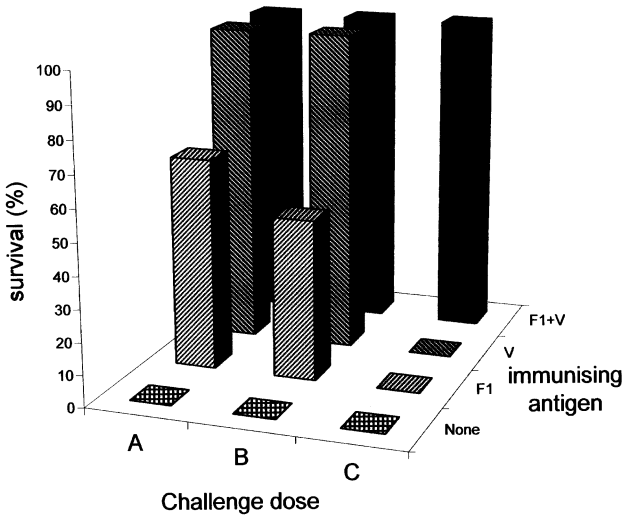

Fig. 2. Protection afforded against plague in mice immunised with F1 antigen, V antigen or a mixture of F1 and V antigens. Sub-cutaneous challenge with 10^5 (A), 10^7 (B) or 10^9 (C) cfu of *Y. pestis* strain GB.

in the V protein are conformational [43,54] and thus its tendency to oligomerise may have functional significance. The optimum molar ratio of F1 to V, for induction of a protective immune response has been shown to be 2:1 and this combination maximises the IgG titre which develops in the first 6 weeks post-immunisation [55]. These observations on the interdependence of conformation and immunogenicity would suggest that a vaccine comprising the combined free sub-units generally has some advantage over that based on a genetic fusion of the two.

Much work has been carried out on the efficacy of the combination of F1 and V antigens, delivered parenterally in a number of adjuvants including incomplete Freunds adjuvant [37], alhydrogel [52,55,56] and the Ribi adjuvant system [57]. In each case the protective capacity of the F1 + V combination vaccine against subcutaneous challenge with *Y. pestis* has been demonstrated in the mouse model. However, of these adjuvants, only alhydrogel is currently approved for human use and it is the alhydrogel formulation of the F1 + V vaccine which will be taken forward into clinical trials.

Protection against aerosol challenge in the mouse model has also been achieved by alhydrogel-adsorbed parenteral delivery of the sub-unit proteins [52,56]. Protection was attributed primarily to the induction of systemic IgG, which transudated into the lung, to protect against inhaled *Y. pestis* [52]. The added presence of F1 + V-specific IgA, particularly in the upper respiratory tract, may also confer protection against inhalational exposure to *Y. pestis* and this type of response could be induced by the mucosal delivery of the sub-unit proteins. This route of vaccine delivery would also be advantageous in terms of logistics (reduced need for intervention by medical personnel) and acceptability (to the vaccinee) to deliver this vaccine without the need for needles. Consequently, a considerable body of work has now been carried out on a means of polymeric microencapsulation of the sub-units which would permit mucosal delivery.

The routes which have been used to access the mucosal immune system include nasal, oral or inhalational delivery. The working principle is that the priming of immune effector cells at any one of these mucosal immune surfaces is followed by the migration of primed cells via the lymphatic system to seed a secondary mucosal surface where they can induce a protective immune response. The secondary mucosal site may be quite distal from the primary site. Seeding of primed immune effector cells into the systemic immune system or direct priming of systemic immune effector cells by entry of mucosally-delivered antigen into the blood stream is also likely [58].

3.3. Microencapsulated sub-unit vaccines

The first report of the successful individual encapsulation of the F1 and V antigens into microspheres was made by Williamson et al. [51]. A low molecular weight poly-L-lactide (PLLA) was used to encapsulate the individual sub-units into microspheres and the latter were demonstrated to be immunogenic in mice by the intraperitoneal route, inducing both systemic and mucosal immunity. By combining the individual microsphere batches, it was demonstrated that an additive protective effect could be achieved and this was further enhanced by the addition of the mucosal adjuvant cholera toxin B sub-unit (CTB) to give 100% protection against 2×10^5 MLD (median lethal doses).

Subsequently, many refinements have been made to the formulation the principle of which was the successful co-encapsulation of the sub-units such that both retained immunogenicity [59] which led to additive protective efficacy [60,61]. Further refinements included the substitution of many different polymer types depending on the application required. For example, block co-polymers afforded increased stability to the microspheres [62] whilst the substitution of a high molecular weight form of PLLA (≥ 100 kDa) permitted the generation of 5–6 μm spheres which were particularly effective at inducing protective immunity following nasal dosing [63]. Microsphere size has been varied from 1 to 10 μm down to spheres which are 150–300 nanometres in diameter [64]. A large mass of spheres of nanometre diameter appears to be effective for oral immunisation applications [65]. Similarly, the hydrophobicity of the polymeric spheres is critical in determining their immunogenicity and half-life in vivo. Optimum immunogenicity and sustained release has been achieved for microspheres which are strongly hydrophobic [59].

Other physico-chemical characteristics of microspheres determine their half-life in vivo as well as their release characteristics. Three key parameters determine the release rate of protein from polymeric spheres in vivo: ratio of protein:polymer, degradation of the polymer and distribution of protein in the polymer. Initially, protein near the surface of microspheres will be released by diffusion through water-filled pores and fissures. The appropriate addition of excipients during formulation can enhance this first phase of release. Subsequently, swelling and degradation of the polymer occurs. The rate of degradation of PLLA polymers can be increased by co-polymerisation of esters of lactic acid with glycolide. Low crystalline polymers prepared for example with equal content of PLLA and poly-L-lacticglycollicacid (PLGA) will degrade more rapidly than more crystalline polymers, e.g. the homopolymer PLLA [64]. Thus, batches of microspheres can be prepared according to the requirement for either burst or sustained release, or a sequential burst plus sustained release.

To enhance mucosal immunity, CTB has been used regularly as an adjuvant with encapsulated F1 + V antigens and has been demonstrated to enhance the immunogenicity of these preparations [51]. Indeed, the co-administration of CTB by the intra-nasal route with the unencapsulated F1 and V antigens has been demonstrated sufficient to convert a non-protective response to a protective response [61]. The form of CTB used was native CTB which may contain traces of holotoxin. Clearly, although a potent adjuvant, it is difficult to envisage that native CTB would be approved for long-term use in man. More recently, a microsphere preparation suitable for nasal administration has been derived which does not incorporate CTB and which is fully protective against an inhalation challenge after only two immunising doses in the mouse model [63].

At the mucosal surface, polymeric micro- or nanospheres can access and cross the mucosal epithelium by both active sampling and passive diffusion. The polymeric spheres form particulate antigen at the mucosal surface, which will be sampled by M-cells in the nasal and gut mucosa and thence have access to local lymph nodes. Soluble antigens in the gut/bronchial or nasal lumen will diffuse across the respective epithelial surface, where they will be phagocytosed by intra-epithelial dendritic cells and transported to draining lymph nodes to induce an immune response. The influence of surface ligands or excipients on mucosal absorption can be significant. Carbohydrate biopolymers such as chitosan and gellan are known to increase mucosal permeability by opening up tight junctions and have been used to enhance the absorption of drugs [65] and sub-unit vaccines across mucosal surfaces [66]. A chitosan derivative, *N*-trimethyl chitosan chloride, has been demonstrated to enhance the immune response to nasally-administered F1 and V antigens [67] and chitosan microspheres have been demonstrated to have enhanced immunogenicity nasally and orally [64].

Currently, efforts are directed towards developing a formulation which can be used in single dose mucosal immunisation to achieve protective immunity. A summary of progress to date in mucosal immunisation with microsphere preparations to protect against plague is presented in Table 3.

3.4. *Salmonella* based orally delivered vaccines

Live attenuated mutants of *Salmonella* have attracted considerable attention as vectors for the delivery of a variety of heterologous vaccine antigens. After delivery by the oral route the bacteria enter the intestinal subepithelium via M-cells and are trafficked via mesenteric lymph nodes to fixed macrophages in the spleen and liver. This colonisation pathway results in the induction of mucosal and systemic immune responses. Attenuated *Salmonella typhi*, such as *aroA*, *aroD*, *htrA* or *phoP*/*phoQ* or *cya*, *crp*, *cdt* mutants are the likely delivery system for heterologous antigens in humans. In small-scale human trials these strains have been shown to be well tolerated and safe [24,25,28,69]. Further, derivatives of these strains expressing antigens such as tetanus toxin fragment C [70], *Helicobacter pylori* urease [71] or hepatitis B antigen [69] have been evaluated in humans. Murine models of *S. typhi* infection which mimic the infection in man are difficult to establish [72]. In contrast, *Salmonella typhimurium* has been shown to cause a typhoid-like illness after oral administration into mice. Therefore, much of the preliminary work, which might lead to *Salmonella*-based vaccines for use in humans, has been carried using attenuated *Salmonella typhimurium* (frequently *aroA* mutants) in murine models.

There have been a number of reports of the expression of the F1 antigen in *S. typhimurium*. Early studies involved the cytoplasmic expression of the gene encoding the F1 antigen (*caf1*) from the constitutive *lac* promoter [73], Table 4. Whilst oral delivery of this recombinant organism into mice resulted in the induction of a protective immune response, the plasmid was unstable and dosing of mice with ampicillin was necessary to ensure plasmid maintenance. An improvement in plasmid stability was achieved by the expression of *caf1* from promoters which were induced *in vivo* and oral immunisation of mice with these recombinant *Salmonella* resulted in the induction of protective levels of antibody [74]. An alternative approach to stable expression involved the cloning of the entire *caf* operon into a low copy number vector and expression in *S. typhimurium aroA* [75]. In *Y. pestis* expression of the F1 antigen is induced when bacteria are cultured at 37°C and repressed at 28°C. The similar pattern of expres-

sion in recombinant *Salmonella* indicated that the *caf* operon regulatory protein (Caf1R) was functional in *S. typhimurium*. The other encoded proteins of the *caf* operon also appeared to be expressed and to be functional; the F1 antigen was exported and appeared to be assembled into a capsule-like structure on the cell surface [75]. Oral immunisation of mice with this recombinant *Salmonella* resulted in high level protection against a subcutaneous challenge with *Y. pestis* [75]. Stable expression of the V-antigen in *Salmonella* has been more difficult to achieve. However, one approach which has shown promise involved the expression of an F1 antigen/V antigen fusion protein [76]. Oral Immunisation of mice with each of these recombinants induced serum antibody, which was predominantly of the $IgG2_a$ subclass. In addition, splenic T-cells showed proliferative responses to purified F1 antigen, indicating the induction of CMI responses. None of these recombinant *Salmonella* have been evaluated for their abilities to induce responses which protect against pneumonic plague. However, the finding that IgA could be detected in the lung and gut of mice which had been orally immunised with *S. typhimurium* expressing F1 antigen on the surface [75] suggests that this recombinant would provide protection against inhalation challenge with *Y. pestis*.

Whilst this work shows great promise, the development of an orally-delivered plague vaccine for use in humans will now require the generation of attenuated *Salmonella typhi* strains expressing *Y. pestis* F1 and V-antigens. Work is currently underway to develop and evaluate such strains.

4. Correlates and mechanisms of protection

Evidence has accumulated from a number of studies that antibody plays a key role in protection against plague [55,77]. Circulating antibody specific for the F1 and V antigens would be able to access the bacterium in its predominantly extracellular existence and bind to surface exposed protein. The observation that a neutralising monoclonal antibody raised to the V antigen could alone protect mice against live organism challenge [43], underlined the critical role of this virulence factor in the pathogenesis of plague infection.

That antibody to F1 + V antigens could be protective against injected whole organism challenge was demonstrated by the passive transfer of F1 + V immune serum from immunised parent strain mice into severe combined immunodeficient (SCID) recipient strain mice [77]. Having no inherent functional immune system, the observed protection in the SCID mice could be attributed only to the donated antibody and indicated the importance of neutralising these two key virulence factors to protect against plague infection.

A study designed to identify the minimum protective immunising dose in the mouse, demonstrated that the

Table 3
Protection of mice immunised with encapsulated F1+V antigens against *Y. pestis*

Formulation[a]	Route of delivery[b]	Immunogenic[c]	Protective efficacy[d]
2 μm diameter, PLLA 2 kDa, 25 μg F1+25 μg V+10 μg CTB; days 1, 14, 28 [51]	i.p.	Y	100% against 2×10^5 MLD[e] subcutaneous challenge
Or 2 μm diameter, PLLA 2 kDa, 25 μg F1+25 μg V; days 1, 14, 28 [51]	i.p.	Y	80% against 2×10^5 MLD subcutaneous challenge
150 nm diameter, L101/poly DL-lactide, 100 μg F1; days 1, 3 [62]	p.o.	Y	Not done
Or 800 nm diameter, L121/poly DL-lactide, 100 μg F1; days 1, 3 [62]	p.o.	N	Not done
1 μm diameter, PLGA, 10 μg F1; day 0 [68]	i.p.	Y	100% against 10^3 MLD subcutaneous challenge
Or 8 μm diameter PLGA; 10 μg F1; day 0 [68]	i.p.	Y	71% against 10^3 MLD subcutaneous challenge
5.8 μm diameter; 100 kDa PLLA, 3 μg V+1 μg F1+10 μg CTB; days 1, 7 [60]	i.n. ×2	Y	80% against 10^3 MLD aerosol challenge
6 μm diameter, 100 kDa PLLA, 3 μg V+0.5 μg F1; day 1, 63 [61]	i.n. ×2 Or i.m.+i.n. Or i.t.+i.n.	Y Y Y	Not done
6 μm diameter; 100 kDa PLLA, 30 μg V+5 μg F1; days 1, 67 [63]	i.n.x2	Y	100% protection against 10^2 MLD aerosol challenge

[a] Microsphere mean diameter, matrix, loaded antigen; dosing schedule.
[b] i.p., intraperitoneal; p.o., per os; i.n., intranasal; i.m., intramuscular; i.t., intratracheal.
[c] Y/N = detection/failure to detect circulating antibody after immunisation.
[d] % of mice which survive following subcutaneous challenge (bubonic model of plague) or inhalation challenge (pneumonic model of plague) with *Y. pestis* strain GB or strain CO92.
[e] MLD = median lethal dose.

Table 4
Expression of *Y. pestis* F1 and V antigens in *S. typhimurium aroA*

Antigen expressed	Level of expression[a]	Site of expression	*Y. pestis* challenge[b]	Survivors[c]
None[d]	NA	NA	1.2×10^5	0/6
			1.2×10^7	0/6
F1 antigen [73]	12.2 g/10^8 cells	Cytoplasmic	1.2×10^5	3/6
			1.2×10^7	2/6
F1 antigen [75]	12.8 g/10^8 cells	Cell surface	1.2×10^5	6/6
			1.2×10^7	6/6
F1-V antigen fusion protein [76]	ND	Cytoplasmic	7.4×10^2	6/7
			7.4×10^4	6/7

[a] F1 antigen in cell sonicates, measured using an ELISA.
[b] Subcutaneous challenge with *Y. pestis* strain GB.
[c] After oral immunisation of BALB/c mice on days 1 and 21 (1 and 14 for F1-V-fusion protein) with 2-5 $\times 10^8$ cfu (5×10^6 cfu for F1-V-fusion protein) of recombinant *Salmonella*.
[d] NA = not applicable; ND = not determined.

antibody titre declined with a reduction in the immunising dose and that the combined anti-F1 + V antigen titre (specifically of the IgG1 isotype) significantly correlated with protection against live organism challenge [55]. The ability to enrich the sub-unit vaccine with optimum levels of the combined immunogens confers a significant advantage over use of the killed whole cell vaccine included in this study which was estimated to contain approximately a one hundredth of the optimum immunising dose of the F1 antigen and no V antigen.

There is no doubt that the mechanism of protection following immunisation with the F1 + V antigens also involves T-cell memory and this has been demonstrated in the mouse model [51,55]. In the mouse model, the T-cell response to the alhydrogel-adsorbed formulation is biased towards Th2 and this response is highly protective. However, recent work has illustrated that although delivery of the F1 + V proteins formulated in the Ribi adjuvant system to IL4T mice (genetic knock-outs for the IL4 receptor) induced predominantly a Th1 response, the passive transfer of their antiserum into B-cell deficient knock-out mice, with no intrinsic antibody, protected the latter fully against live organism challenge [57]. Subsequently, actively immunised IL4T mice have been demonstrated to be protected against 10^6 mouse lethal doses of *Y. pestis*. Such experimental data suggests that the F1 + V combination is sufficiently potent and that there is sufficient plasticity in the immune response induced to them, to confer robust protective immunity in a wide range of genetic backgrounds.

5. Conclusions

Y. pestis remains a significant cause of disease in humans, which has the potential to spread in an epidemic manner. The efficacy of existing vaccines is not proven, and the use of these vaccines is known to be associated with side effects. Against this background there is a need for an efficacious plague vaccine which is well tolerated. A recombinant sub-unit vaccine containing the F1- and V-antigens, adjuvanted with alhydrogel, offers such potential and is currently being developed for use in humans. It is unlikely that randomised clinical trials to demonstrate the efficacy of this vaccine will be possible. In this respect, the licensing of this plague vaccine presents similar problems to those associated with the licensing of other vaccines against diseases which occur infrequently and unpredictably. The licensing of such vaccines may require a greater emphasis on an understanding of the mechanisms of protection against disease, and the demonstration that these mechanisms are evoked in immunised humans. Thus the identification of immune correlates of protection which can be used as surrogate markers of vaccine efficacy in humans is a key objective in the development of these vaccines. In this respect, the lessons learnt from the work reported here may provide useful insights into the likely problems and solutions associated with the licensing of other orphan vaccines.

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