Chapter 10. Live vaccines against plague and pseudotuberculosis

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Abstract

The reemergence of plague in the world, the appearance of antibiotic-resistant strains and the risk that genetically modified *Y. pestis* could serve as a bioterrorist weapon have fostered a renewed interest for vaccination. Currently, researchers mainly follow two distinct vaccinal strategies: one is the development of acellular sub-unit vaccines based on two recombinant targets (F1 and V), and the other is the development of improved live vaccines. Live plague vaccines have been previously largely used in humans and their efficiency against bubonic plague is not disputed. Rather, critics pointed to a limited duration of protection, an unverified protection against pneumonic plague, instability of vaccine seed strain characteristics and the fact that vaccination could induce severe local and systemic reactions. New live vaccine candidates should combine both the known advantages of replicating vaccines: humoral and cell-mediated immune responses, robustness against mutant microorganisms, easiness of mass production and use, limited cost, etc. whilst providing guarantees in terms of security, stability and efficiency against pneumonic plague. They are based not only on attenuated *Y. pestis* strains, but also on other *Yersiniae* and live vectors (*Salmonella*, viruses) expressing *Y. pestis* antigens. Vaccines against enteropathogenic *Yersiniae* are also developed.

***Yersinia pestis* and plague**

Plague has affected mankind along at least three pandemics which killed millions of people. Originating from Asia, these pandemics first affected the ancient Mediterranean world, then devastated all of Europe as the "black death" of the middle ages, to finally spread all over the world at the end of the XIXth century following commercial steamboat routes. In addition to ancient foci of the disease, such as those of China, this last pandemic allowed plague to develop new foci into previously virgin territories such as Madagascar, Southern Africa, Australia and the Americas. Currently active plague foci result from these great pandemics. Although man has largely contributed to disseminate the disease all over the world, he is not the main reservoir of the disease, which is a zoonosis primarily affecting rodents. The pathogen is transmitted between animals via the bite of an infected flea, and man can become an accidental host when this epidemiological cycle is disturbed. Thus, plague persists in rodents occupying large areas from which it may spread after extended periods of silence. During the last twenty years, plague outbreaks were, for example, observed in countries such as Algeria, Libya, Zambia and India after decades without any reported human case. Therefore, WHO declared that plague is now a re-emerging disease.

Improvement of living conditions and the availability of efficient therapeutic strategies in developed countries make it unlikely that plague might cause pandemics as it did in the past. However, plague has already been used several times in human history as a warfare agent, and experts agree that the plague pathogen, *Yersinia pestis,* could be aerosolized to cause the very contagious and very lethal pneumonic form of the disease in a great number of persons. *Y. pestis* is available around the world and is easy to produce in large amounts, so the possibility that it could be used as a bioterrorist weapon is now considered as a serious threat (Dennis, 2005). Therefore, *Y. pestis* has been included in the short list of critical biological agents developed by the US Centers for Disease Control (CDC).

Plague is most often acquired via the bite of an infected flea. Bacteria colonize the draining lymph node, giving rise to the "bubonic" form of the disease, a severe infectious syndrome with high fever. Infection generally reaches the liver and spleen, ultimately entering the blood stream to cause an often-lethal septicemia. Unless adequately treated, bubonic plague is fatal in 40 to

70% of cases. Pneumonic plague is a possible complication of bubonic plague. In this very contagious and lethal form of the disease, the patient develops a lung infection and a bloody cough, producing infective aerosols which can transmit the pathogen through direct inhalation, causing another pneumonic plague generally fatal in less than three days.

The prophylaxis of plague is mostly based on antibiotic treatment of patients and the plague bacillus is usually susceptible to most antibiotics. In 1995, two different strains of *Y. pestis* showing high-level resistance to antibiotics, currently used to treat patients, were identified in Madagascar (Guiyoule et al., 1997). Both had acquired stable conjugative plasmids carrying resistance determinants, and it was shown that one of these plasmids was present in numerous enterobacteria widespread in food. The fact that multidrug-resistant *Y. pestis* strains might cause new pandemics represents a major threat for public health, and vaccination is now required more than ever.

The plague bacillus, *Yersinia pestis,* is a Gram-negative enterobacteria recently evolved from *Y. pseudotuberculosis,* with which it shares strong sequence homology (Chain et al., 2002). How *Y. pestis* evolved from this much less virulent enteropathogen to become the dreadful agent of plague is still not fully understood. Its evolution involves the acquisition of two plasmids named pPla (pPCP1) and pFra (pMT1) together with the acquisition or deletion of few chromosomal genes, and multiple single-nucleotide differences. The pPla plasmid encodes a limited number of genes including the bacteriocin pesticin, and the plasminogen activator Pla required for the development of pneumonic plague and for bacterial dissemination from the dermal site of entry (Lathem et al., 2007). The large pFra plasmid encodes numerous genes of unknown function and the caf operon, which produces the pseudocapsular antigen F1. Whereas bacteria cured of pPla are strongly attenuated in mice, the absence of F1 or the whole pFra does not reduce virulence. Other virulence factors essential to *Y. pestis* pathogenicity include the chromosomal High Pathogenicity Island, encoding an extremely efficient iron-capture system, and the pYV (pCD1) virulence plasmid encoding a Type Three Secretion System (TTSS) common to all pathogenic *Yersinia*. The TTSS consists of the Ysc (Yop secretion) injectisome or "needle", and the secreted Yops (Yersinia outer protein) effector toxins, which are injected into the cytoplasm of host cells

to neutralize phagocytosis and inflammatory cytokine production, and to induce death by apoptosis.

Due to its very recent emergence, *Y. pestis* presents little variability among isolates found around the world. Differences in the metabolism led to define 3 biovars, but these differences are thought to have no impact on immunogenicity. Mutations in genes involved in the glycosylation of membrane lipopolysaccharide (LPS) cause the absence of O-antigens, and thus *Y. pestis* has no serotype variation. Therefore, *Y. pestis* is very homogenous, a characteristic that facilitates vaccination. Exceptionally, pseudocapsule F1-negative strains have been observed and were fully virulent (Friedlander et al., 1995; Winter et al., 1960).

History of plague vaccines

Although the history of the disease is long, the history of plague vaccines starts only after Alexandre Yersin discovered the plague bacillus *Yersinia pestis* in 1894. A few years later (1897) Haffkine started the first large-scale vaccination of humans during an outbreak in Bombay, demonstrating that patients could be protected by vaccination. Several vaccination campaigns followed during the first half of the XXth century, the most famous being held by Girard and Robic in Madagascar (1932 - 1959) and by Otten in Java (1934 - 1939).

Each of these investigators developed his own vaccine recipe. Haffkine's "lymph" was a dense suspension of virulent *Y. pestis* killed by heat and injected subcutaneously (sc). Although no reliable monitoring of vaccination efficiency was performed, evidence is convincing that Haffkine's vaccine was incompletely protective against bubonic plague. This vaccine was however unacceptably reactogenic. Due to unstandardized production methods, the volume injected was determined according to fever induced in vaccinees, and reactions varied from insignificant to very severe (Girard, 1963; Meyer, 1970). As a result, vaccination was refused by certain populations, and the heat-killed whole-cell vaccine was logically stopped.

Both Girard and Robic at the Pasteur Institute of Madagascar and Otten in Java followed the idea of a live attenuated strain, following the concept exemplified shortly before by Calmette and Guérin with their BCG vaccine against tuberculosis. The vaccine of Girard and Robic, EV76, was

obtained by experimentally attenuating the virulent EV strain. EV76 substantially reduces plague occurrence and mortality (Girard, 1963) and was given to millions of people between 1933 and 1959. In Java, Otten vaccinated thousands of persons with a naturally attenuated strain named Tjiwidej, which appeared less efficient than EV76, although less reactogenic as well.

During the second half of the XXth century, the reactogenicity of existing live vaccines, the development of antibiotics together with new methods of breaking the rodent-flea-rodent cycle using insecticides and rodenticides caused a progressive loss of interest for vaccines. Research however continued with distinct strategies in the two political blocks of the cold war. Russians pursued with live vaccines, while on the other side of the iron curtain, Meyer and colleagues developed a formalin-killed whole cell vaccine (Army-type vaccine, later USP vaccine). Now that the risk of terrorist attacks on the territories of occidental countries is considered serious, the current status of plague vaccines in those countries is however directly inherited from the past political situation: live vaccines have poorly evolved, whereas non-replicating sub-unit vaccines being developed in the USA and the UK are supplanting the killed vaccine.

Lessons from live EV76 and Tjiwidej vaccines

The non-pigmented strain EV76 was obtained by Girard and Robic after numerous sub-cultures of the virulent isolate EV collected from a child who died of plague. Its strongly reduced virulence is due to the deletion of the unstable *pgm* locus, initially detected by the loss of its hemin storage operon (*hms*). *Y. pestis* virulence in fact is not due to the *hms*, but to another operon of the *pgm* region, named the High Pathogenicity Island (HPI). The HPI encodes a system which mediates biosynthesis and uptake of the siderophore yersiniabactin (Ybt), allowing bacteria to acquire free iron with high efficiency from the iron-deprived environment of the host (Carniel, 2001). Attenuation in the Tjiwidej strain resulted from the loss of the virulence pYV/pCD1 plasmid. Whereas the two strains displayed the F1 pseudocapsule considered as important for vaccination, Tjiwidej lacked the V antigen and this was considered as the explanation for its lesser efficiency. With the recent understanding of the role played by the pYV-encoded TTSS in virulence, it is obvious that Tjiwidej could not persist or cause severe reactions, whereas EV76 could persist and thus trigger a more sustained immune response. Therefore, reactogenicity in great part results from an incomplete attenuation.

EV76 was clearly reactogenic, causing local swelling and erythema plus sometimes high fever, lymphadenopathy, malaise and headache. EV76 was considered by Girard as attenuated, not avirulent (Girard, 1963), and could be virulent in non-human primates (Meyer et al., 1974a) and in mice injected with iron. An additional criticism raised against EV76 was that it failed to protect against pneumonic plague. In fact, the ability of EV76 to protect humans against pneumonic plague is unclear. Records of human plague cases in Madagascar show that both bubonic and pneumonic plagues were decreased after vaccination. However, because pneumonic plague outbreaks start with a bubonic plague causing a secondary pneumonic infection, vaccination may have only protected against the bubonic form. Animal studies actually argue in favor of protection. Girard quotes Russian experiments in the Guinea pig exposed to aerosols of *Y. pestis* which demonstrated a protective effect (Girard, 1963). Protection was also excellent in mice (Russel et al., 1995). Finally, Meyer *et al.* reported that the antibody titer against F1 declines two to three months after vaccination in humans, and this led him to conclude that the protection was short-lived (Meyer et al., 1974a) and required recall injections every year. This conclusion is however based on the assumption that only humoral immunity is able to confer protection. As we will see in the section about protective immunity below, this view is no longer valid and live vaccines are potent inducers of cell-mediated immunity.

Maintenance of the seed strain characteristics during the large-scale production process is clearly a difficulty encountered with the EV76 vaccine. Recent microarray analysis of the genome of EV76 preparations used for vaccine production in France, Vietnam and the USSR revealed that some of them had lost plasmids and chromosomal loci, generating genetic variability (Zhou et al., 2004) and the strains also varied widely in terms of vaccinal potency in mice and guinea pigs (Meyer et al., 1974a). The presence of abundant IS (insertion sequences) in the *Y. pestis* genome certainly accounts for this plasticity, and this criticism therefore applies to any *Y. pestis* strain used as vaccine.

A sub strain of the initial EV76, named EV NIIEG, was derived in the former Soviet Union and has been used from 1940 on to vaccinate humans. It has been up to now given to more than 100 millions of persons. Used during outbreaks in Manchuria (1945-46) and Mongolia, high efficacy

was reported (Feodorova et al., 2007). This derivative of the EV76 vaccine had retained its initial characteristics: presence of the three plasmids and absence of the pgm locus. Whereas the EV76 could recover its virulence during repetitive passages in animals, the EV NIIEG did not, suggesting that the sub-culture process could have eliminated residual virulent bacteria present in the original EV76. These virulent bacteria could then have contributed to the reported reactogenicity of the EV76 vaccine, whereas the EV NIIEG was weakly reactogenic (Feodorova and Corbel, 2009). However, it was recently reported that the EV NIIEG (up to 10^9 cfu via the sc route) conferred little protection to inbred or outbred mice or guinea pigs against a sc plague challenge (Feodorova et al., 2007), suggesting that it may also have varied.

Killed whole-cell vaccine

The formalin-killed whole cell vaccine developed by Meyer and colleagues was experimented in humans in 1939 and tests were continued on thousands of patients during the war in military hospitals (Meyer, 1970). The vaccine commercialized up to the end of the XXth century by Cutter Biologicals and later Greer Laboratories consisted of preparations of virulent *Y. pestis* (Indian Isolate 195/P) grown at 37°C and then killed using formalin. Human vaccination consisted of two intramuscular injections and the vaccine had to be given twice because the first injection conferred little or no active immunity, with antibody titers declining rapidly. Although bacteria were killed, local and systemic reactions were noted, essentially as a response to the second injection, leading to temporary incapacities to work. The principal indications of protection for humans result from tests of human serum passively transferred to mice, and protection obtained was variable. US soldiers serving in Vietnam were vaccinated and reports conclude that almost no case of bubonic plague was recorded, suggesting immunity. Immunity against pneumonic plague was probably low because human cases were observed in vaccinated subjects (Meyer, 1970).

Therefore, the conclusion from the use of this killed vaccine was that it provided immunity against bubonic plague that was short-lived and had to be renewed. Upon recall vaccination, reactogenicity was increased and could become severe. Thus, the formalin-killed vaccine had unsatisfactory performances, and much recent research was performed in order to define candidate vaccines able to induce a long-lasting immunity without reactogenicity.

F1-V sub-unit plague vaccines

These vaccines are the topic of chapter 2-2-1 and will only be briefly discussed here. Identification of the F1 and V fractions of *Y. pestis* as the most important for immunity led to propose the F1-V sub-unit vaccines currently under development. They are produced as recombinant F1 and V mixed together (rYp vaccine, Dstl Porton Down, UK (Williamson et al., 2005) or a F1-V fusion protein (rF1V vaccine, USAMRIID, USA (Heath et al., 1997)). Recent reports are very conclusive that such vaccines are not very reactogenic and can provide protection to mice against bubonic and pneumonic plague (Heath et al., 1997; Williamson et al., 2005). Phase I and II trials in humans have been performed. The main limitation of such vaccines is the fact that they would be unable to protect against virulent strains purposely deleted of F1 and exhibiting modified V antigens.

New attenuated *Yersinia pestis* candidate vaccines

In spite of their potential side effects, there has been a sustained interest in developing these vaccines because of their indisputable qualities. They are made of multiple antigenic targets including the F1 and V antigens, so they should be efficient against variants of the pathogen. They induce a strong immune response without requiring an adjuvant. Because they are easily modifiable by genetic engineering, virulence factors can be removed or antigens added according to necessities. Their capacity to replicate at least several times in vivo also guarantees a sustained stimulation. Finally, they are easy and cheap to produce, so that large amounts can be rapidly obtained in response to an emergency need.

A sufficiently low virulence is the first and inescapable requirement for a live *Y. pestis* vaccine, and most recently described attenuated strains result from studies on the determinants of *Y. pestis* virulence rather than from a deliberate strategy to create a live vaccine (Table 1). Reasons to lose virulence can be classified in at least three categories: auxotrophy, weakening of bacteria's armamentarium against the host, and loss of the ability to disseminate and stay invisible to host innate immune system. Auxotrophy -the incapacity to grow in the absence of a given molecular substrate- has been widely used to control the replication of live bacteria in vivo, for example in the Δ*aroA, B* or *C Salmonella* strains requiring aromatic amino acids (Calhoun and Kwon, 2006;

Oyston et al., 1996; Tacket et al., 1997; Titball et al., 1997). Auxotrophies for several amino-acids including the aromatic ones, however, did not result in attenuation of *Y. pestis* in vivo, except for purines (Burrows, 1963) and guanine as recently shown with Δ*guaBA* mutants ((Oyston et al., 2010) and Table 1) which provided protection against bubonic plague. Availability of amino acids may however increase in case of host tissue damage linked to the infection, or due to any other cause, and unwanted residual virulence could then be observed.

Perturbation of the Type Three Secretion System is an obvious way to attenuate *Y. pestis*. To avoid curing the pYV plasmid as in the Tjiwidej strain, selective deletion of TTSS components can be used. Among effector Yops injected into host cells, YopH is a recognized virulence factor and YopH deletants are attenuated by the sc or intranasal (in) routes and provide a high degree of protection from bubonic and pneumonic plague (Bubeck and Dube, 2007). More surprisingly, replacement of the effector YopJ in *Y. pestis* KIM by its YopP equivalent of *Y. enterocolitica* resulted in an increased cytotoxicity for macrophages and a strongly reduced virulence. As a consequence, the YopP-expressing *Y. pestis* was able to rapidly induce protection against bubonic and pneumonic plague (Zauberman et al., 2009). Deletion of the YopK effector causes strong attenuation by inducing ectopic deregulating the secretion of other Yops (Holmstrom et al., 1997) and allowing their recognition by the inflammasome of host cells (Brodsky et al., 2010). Mutations outside of the pYV plasmid were also shown to alter TTSS functions and damper virulence. DNA adenine methylase (dam) mutation causes attenuation by inducing ectopic secretion of Yops (Julio et al., 2001; Robinson et al., 2005). A Δ*dam Y. pestis*, although not completely avirulent, protected mice from plague (Robinson et al., 2005). Similarly, an *smpB-ssrA* mutant affected in its translational quality control does not secrete YopB, YopD and V, is severely attenuated and efficiently prevents pneumonic plague (Okan et al., 2010). Only mutants which were evaluated for vaccination are mentioned here, and several other attenuating mutations are potentially interesting. Last but not least, the capacity of *Y. pestis* to disseminate or escape the host innate immune system was targeted. *pcm* and *nlpD* mutants have altered cell separation functions and are filamenting, their dissemination in host's organs is impaired, and mice injected with these strongly attenuated mutants are immune for pneumonic and bubonic plague (Flashner et al., 2004; Tidhar et al., 2009). One way for *Y. pestis* to escape killing is the weak recognition of its LPS by the TLR4 receptors of host immune system cells. By introducing the *E. coli* LpxL

acyl-transferase into *Y. pestis*, Montminy *et al.* enhanced the acylation level of the LPS, which consequently became a strong inducer of TLR4-mediated responses. This modification transformed the pathogen into a completely avirulent bacterium with excellent vaccinal performances against bubonic or pneumonic plague (Montminy et al., 2006). Analysis of the immune response triggered by this strain, additionally deleted of its *pgm* locus, demonstrated its ability to trigger a protective T cell response (Szaba et al., 2009). Deletion of the *Y. pestis* LpxM acyl-transferase also reduces virulence, although the effects of the mutations were more diverse (Feodorova et al., 2009; Feodorova et al., 2007). Enhancing recognition by the innate immune system is a way to facilitate the establishment of an adaptive immune response and thus works as an adjuvant.

Protective target antigens of live attenuated *Y. pestis* vaccine strains

The F1 pseudocapsular antigen

The pseudocapsule is a major component of the bacillus grown at 37°C but not at 20°C. Upon fractionation, the major "fraction 1" composed essentially of protein, was shown to contain an important part of the vaccinal potential of the bacteria (Meyer et al., 1974a). Analysis revealed that its most abundant component was a polymer of a single subunit, thereafter named F1, product of the *caf* operon located on the pFra plasmid.

Although the F1 antigen confers anti-phagocytic properties to the bacteria in vitro (Carr et al., 1997), most (Davis et al., 1996; Friedlander et al., 1995; Quenee et al., 2008; Welkos et al., 1995)-but not all- (Weening et al., 2011) observers concluded that it is not required for virulence. In fact, absence of F1 might even enhance virulence in mammals, as it facilitates the degradation of anti-bactericidal peptides such as cathelicidin by the bacterial surface-anchored Pla protease (Galvan et al., 2008). Also, absence of F1 uncovers the presence of abundant PsaA/pH6 antigen (Liu et al., 2006), which is a recognized virulence factor. Nonetheless, immunization with recombinant F1 is protective (Simpson et al., 1990), and monoclonal antibodies against F1 can be used to treat *Y. pestis*-infected mice (Hill et al., 2003). Interestingly, the protective immune response induced by attenuated *Y. pestis* strains appears focused against this capsular material (Burrows and Bacon, 1958; Quenee et al., 2008), as demonstrated by the inability of the attenuated Δ*pgm Y. pestis* strain to protect against a challenge with an F1-negative *Y. pestis*

(Quenee et al., 2008). This is also supported by the poor protective potential of an attenuated Δ*pgm* F1-negative strain against pneumonic or bubonic plague (Quenee et al., 2008). This central role of F1 in protective immunity induced by attenuated *Y. pestis* would probably result from its abundance and surface localization. Supporting this view, Girard reported in 1963 that the dose of vaccinal live bacteria to protect 50% of mice varied widely among 15 strains tested and could be directly related to the percentage of F1 found in the capsular material (Girard, 1963). Therefore, and most worryingly, naturally F1-negative *Y. pestis* virulent strains can be more resistant to immunity (Cornelius et al., 2009; Quenee et al., 2008). Moreover, an immunity focused on F1 favors the selection of F1-negative mutants (Anderson et al., 1997). Although such F1-negative mutants are infrequent in nature, their engineering for bioterrorist purposes would only require standard and widely available laboratory methods.

The V antigen

The V antigen (formerly designated LcrV) is among the targets best recognized by sera of mice and rabbits vaccinated with a live *Yersinia* EV76 (Benner et al., 1999; Li et al., 2005) and former plague patients (Li et al., 2009). Burrows first identified the V antigen as a virulence-determining antigen able to induce a humoral immune response protective against plague (Burrows, 1956). It was later understood that V is a component of the Type Three Secretion System localized as a pentamer at the tip of the injectisome. It is absolutely required to generate the pore in the membrane of the target cell through which effector Yops will be translocated into the cytoplasm.

In addition to its activity in TTSS-mediated inhibition of inflammatory cytokine production, the V from *Y. pestis* can perform immunomodulatory functions by inducing the release of the anti-inflammatory cytokine IL-10 (Nedialkov et al., 1997). This effect appears to result from the interaction of V with the TLR-2 receptors (Sing et al., 2005) for *Y. enterocolitica* and TLR6 receptors for *Y. pestis* (DePaolo et al., 2008; Sing et al., 2005). Indeed TLR-2 KO mice are normally susceptible to infection by *Y. pseudotuberculosis* (Auerbuch and Isberg, 2007) and *Y. pestis* (DePaolo et al., 2008; Pouliot et al., 2007), whereas TLR-6 KO mice are less susceptible to plague (DePaolo et al., 2008). To prevent a potential systemic suppression of innate immunity by V, thus prohibiting the use of such a plague vaccine in humans, it has been modified to reduce its ability to induce IL-10 and to inhibit pro-inflammatory cytokines (Overheim et al., 2005). A

recombinant form of the V of *Y. pestis* is able to bias the immune response against the model antigen ovalbumin toward IL-10-producing CD4 T cells (DePaolo et al., 2008), and V-induced IL-10 is thought to contribute to plague pathogenesis. However, conclusions obtained with IL-10 KO mice have been recently contested (Turner et al., 2009), and the point awaits clarification. Finally, there are currently no elements available indicating that V-induced IL-10 could affect the efficiency of avirulent live vaccines or cause immunosuppression. IL-10 is known to favor the humoral part of the immune response, and therefore its induction could be useful to antibody production against the vaccine.

As V plays an essential role in virulence and is exposed at the surface of the bacteria, it is an interesting target for a vaccine. Vaccination with recombinant V protects mice against both bubonic and pneumonic plague caused by F1-positive or negative *Y. pestis* strains (Anderson et al., 1996). Antibodies appear able to play a major role, because a monoclonal antibody directed against V passively protects against bubonic and pneumonic plague. (Hill et al., 2003; Philipovskiy et al., 2005). This activity may result from different effects: first, impairment of the TTSS pore formation and Yop translocation; second, opsonization of the bacteria, facilitating their phagocytosis and destruction by phagocytes (Cowan et al., 2005); and third, inhibition of IL-10 induction.

Although overall conserved in Yersiniae, the V antigen contains two hypervariable regions which determine two main variant forms of the molecule. One, termed LcrV-Yps or V-O:3, is found in a group comprising *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* serotypes O:3, O:9 and O:5,27 (Roggenkamp et al., 1997), whereas the other form, termed LcrV-Yen O8 or V-O:8, is found mainly in pathogenic *Y. enterocolitica* serotype O:8. The first hypervariable region at N-terminal amino acids 31-47 of V-O:8 includes the TLR2-binding site able to trigger the release of IL-10 by macrophages (Sing et al., 2005), whereas V-0:3 poorly engages TLR-2. The second hypervariable region, at amino acids 225-232 of *Y. enterocolitica,* corresponds to a major protective epitope of the V antigen, so antisera raised against the V-O:3 or V-O:8 forms are able to passively transfer protection strictly against enteropathogenic strains bearing the same V (Roggenkamp et al., 1997). Additional differences between the V of *Y. pestis* or *Y. pseudotuberculosis* affect the immune response and vaccination against plague is only efficient if

the *Y. pestis* V is used and not the V-O:3 or V-O:8 forms (Daniel et al., 2009; Griffin et al., 1998). Moreover, a distinct V antigen has been observed in a *Y. pestis* subspecies named pestoides (Worsham and Hunter, 1998). Whether immunization with the conventional V from *Y. pestis* protects against such strains would be interesting to know. Indeed, a virulent *Y. pestis* strain having a different V antigen and lacking the dispensable F1 antigen could completely escape the immunity raised by the F1-V based molecular vaccines.

Influence of the Type Three Secretion System on immunization with a live Yersinia

The possibility to use antibodies against the V antigen as plague treatment illustrates the effectiveness of antibodies when they neutralize an essential virulence factor. It leads to the view that the ideal vaccine should include all the pathogen's essential virulence factors so that the immune response could neutralize them all. Unfortunately, the usual way to attenuate a pathogenic strain is to delete virulence factors, with the regret that the immune response will not be directed against them. The TTSS is a typical example of this dilemma.

The main reason to remove the TTSS is that Yersiniae lacking the pYV plasmid which encodes it (like Tjiwidej strain) are completely avirulent. The effects of the TTSS are twofold. First, it enhances the virulence by suppressing the innate immune response (reviewed in (Viboud and Bliska, 2005)). The EV76 strain, which has a functional TTSS and is not completely avirulent, was reported by Payne *et al.* in 1955 to induce an elevated mortality when associated with cortisone or iron (Payne et al., 1955). In contrast, pYV-cured strains did not become virulent in cortisone or iron-treated mice and were thus truly avirulent. Second, the TTSS also impairs the adaptive immune response by its effects on dendritic cells (DC), the main cells presenting antigen to naive T cells (Heesemann et al., 2006). DCs, with macrophages and neutrophils, are the preferred targets of Yop injection by pathogenic *Yersinia* (Marketon et al., 2005). Several aspects of the DC response to stimulation, like cytokine production, functional maturation and migration to lymph nodes, can be impaired by Yops, which, in addition, eventually trigger DC apoptosis (Erfurth et al., 2004; Robinson et al., 2008; Velan et al., 2006). As a result, both the T4 and T8 responses are impaired. The TTSS, therefore, has a negative impact on the immunogenic potential of a live vaccine.

The absence of the TTSS reduces but does not abrogate the efficiency of vaccines. The avirulent Tjiwidej vaccine strain (cured of its virulence plasmid) was less efficient than the more immunogenic pYV-positive strain EV76, and this was ascribed to the absence of V antigen (Girard, 1963). A pYV-cured *Y. pseudotuberculosis* YPIII strain injected intravenously (iv) provided partial protection against bubonic plague (Simonet et al., 1985), and against pseudotuberculosis when given orally (Balada-Llasat et al., 2007). More recently, a pYV-cured Δ*dam Y. pseudotuberculosis* IP32953 strain given orally fully protected mice against bubonic plague (Robinson et al., 2005).

On the other hand, immunity against the TTSS is desirable. Many molecules of the TTSS machinery (in addition to V), including Yops and Ysc elements of the injectisome, are immunogenic (Benner et al., 1999). Although several Yops do not induce a protective immune response when used alone as vaccine (Andrews et al., 1999; Leary et al., 1999), protection was obtained by vaccinating with structural elements of the injectisome, such as the V antigen, the YopBDE translocon complex (Ivanov et al., 2008) and the abundant needle component YscF (Matson et al., 2005; Swietnicki et al., 2005).

To obtain both immunity against the TTSS and attenuation, a functional inactivation of the TTSS can be performed. Deletion of some structural/regulatory Yscs in *Y. pseudotuberculosis* resulted in attenuation, and the mutants induced protection against pseudotuberculosis (Balada-Llasat et al., 2007). As already mentioned, deletion of the YopH effector in *Y. pestis* (Bubeck and Dube, 2007) or replacement of the YopJ effector by its more active YopP equivalent of *Y. enterocolitica* (Zauberman et al., 2009) both strongly reduced virulence and such *Y. pestis* mutants were able to induce protection against bubonic and pneumonic plague.

Target antigens other than F1 and TTSS components

By testing the vaccinal value of several mutants of the *Y. pestis* strain KIM lacking either F1, V, the *pgm* locus or combinations of these, Quenee *et al*. reached the conclusion that live attenuated Δ*pgm Y. pestis* vaccines harbor only two main protective antigens, F1 and V, in agreement with the opinion of Burrows and Girard more than 50 years earlier (Burrows and Bacon, 1958; Girard, 1963). They however point to additional undefined protective targets which would be located in

the *pgm* locus (Quenee et al., 2008), as demonstrated by the ability of an attenuated Δ*lcrV* CO92 but not a Δ*pgm* Δ*V* KIM to protect against a Δ*caf1* CO92. These targets could be the High Pathogenicity Island (HPI), a fimbrial operon and the *hms* gene involved in PNAG exopolysaccharide synthesis. The iron capture system encoded in the HPI includes the secreted siderophore yersiniabactin and its membrane receptor named Psn. As the HPI is required for virulence, Psn is an interesting vaccine target. Its contribution to immunity has however been little evaluated because it is absent in *Y. pestis* vaccine strains lacking the *pgm* locus. Recently reported work showed that vaccination with a Psn-expressing *Salmonella* induced a partial protection against plague (Branger et al., 2007; Branger et al., 2010), suggesting that Psn could be the target antigen postulated by Quenee *et al*. Because HPI-deleted strains induce no immunity against Psn, a deletion inactivating the iron capture system without deleting Psn could be interesting.

Whereas F1 and V are certainly the key players of protective immunity induced by attenuated *Y. pestis*, other antigens most probably also play a role, although to a lesser extent, because they are less abundant, or less immunogenic. Antibodies from mice, rabbits and humans having recovered from plague or vaccinated with the EV76 vaccine recognize many *Y. pestis* antigens in addition to F1 and V (Li et al., 2008a; Li et al., 2008b; Li et al., 2009). Several antigens also stimulate the T cell compartment. Using a high-throughput screening of *Y. pestis* antigens able to stimulate T cells from mice vaccinated with EV76, Li *et al*. observed that among 101 ORFs encoding surface-associated antigens that could be produced as recombinant molecules, 36 stimulated memory T cell proliferation and at least ten provided a protective immunity when used as molecular vaccines (Li et al., 2009). These include the V antigen, but also molecules exerting various functions, such as an ABC transporter, a putative sugar binding protein, a heat shock protein, a sulfatase and an hemolysin activator. These molecules are also present in *Y. pseudotuberculosis*, suggesting that they may contribute to the ability of attenuated *Y. pseudotuberculosis* strains to induce protective immunity against *Y. pestis* infection.

Certain *Y. pestis* molecules could be interesting targets of immunity, although evidence for that is still lacking. They are 1) important for the bacteria because their deletion or neutralization by antibodies affects virulence, and 2) exposed at the surface of the bacteria so antibodies can reach

them. Among others, at least two characterized factors correspond to this definition: Pla, and PsaA. The Plasminogen activator Pla is a protease exposed at the bacterial surface, and is essential to *Y. pestis* virulence during both bubonic and pneumonic plague (Lathem et al., 2007; Sebbane et al., 2006). Among multiple activities, it favors *Y. pestis* dissemination by cleaving fibrin and reduces chemoattraction of inflammatory cells (Sodeinde et al., 1992). Pla is well recognized by the immune system (Benner et al., 1999; Easterbrook et al., 1995), and should be a good target of vaccination. As far as we know, the vaccinal value of Pla has only been tested as a DNA vaccine, which failure to protect may have had various causes (Wang et al., 2008). Pla is a membrane-anchored molecule which is difficult to produce in a recombinant form due to its numerous hydrophobic regions. Its presence in a live vaccine is however possible and Pla-expressing *E. coli* have been constructed (Kukkonen et al., 2001). The pH6 antigen (PsaA) is a fibrillar antigen which, like F1, homopolymerizes to form a fimbrial structure at the surface of the bacteria. In *Y. pestis*, it is part of the pseudocapsule (Liu et al., 2006) and has adhesin properties to epithelial cells (Liu et al., 2006) whereas, like F1, it inhibits phagocytosis by macrophages. Its role in virulence could depend on the route of infection, for example inhibition of its expression in *Y. pestis* KIM5 induced a strong decrease of virulence in mice by the iv route (Lindler et al., 1990), whereas virulence of other mutants injected by in or sc routes was unaffected (Anisimov et al., 2009; Cathelyn et al., 2006). Via the oral route, Δ*psaA Y. pseudotuberculosis* mutants are attenuated (personal communication). The PsaA antigen induces a T cell response in mice (Li et al., 2009) and induces an antibody response in plague patients (Li et al., 2008b) and EV76–vaccinated rabbits (Li et al., 2005).

Yersinia live candidate vaccines other than *Y. pestis*

With his *vaccinia* vaccine against smallpox, Edward Jenner successfully pioneered an approach of vaccination consisting in the use of a microorganism both different from the pathogen of interest (so it is avirulent to humans), and sufficiently related to it to induce a cross-species immunity. Thus, *vaccinia*, closely related to the smallpox agent, is a natural pathogen for cows harmless to humans. The genus *Yersinia* is composed of 14 species, among which only three are pathogenic for humans and other mammals: *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*. Most *Yersinia* species are therefore avirulent for man and could have been considered as candidate vaccines against plague. However, to our knowledge, this approach has

not been tried, possibly because of the quite recent identification of these species. However, several live candidate vaccines against plague based on *Y. pseudotuberculosis* and *Y. enterocolitica* have been proposed. These candidate vaccines strains were either naturally attenuated or attenuated by genetic engineering (Table 1).

Y. pestis is a clone emerged from *Y. pseudotuberculosis* only 1500-20000 years ago, and is therefore genetically very closely related to its ancestor (Achtman et al., 1999). *Y. enterocolitica* is more distantly related to them: its separation from *Y. pseudotuberculosis* is thought to have occurred 24-187 million years ago. Therefore, *Y. pseudotuberculosis* shares more antigenic homology with *Y. pestis* than *Y. enterocolitica* (for example for the V antigen, see above). Whereas the genome of *Y. pestis* possesses numerous insertion sequences (IS) which considerably facilitate sequences rearrangements and loss of genetic material, *Y. pseudotuberculosis* has only few IS, and so is much more stable (Chain et al., 2002). As a consequence, the problems of genome variation and deletions observed for the EV76 strain are unlikely to occur with a *Y. pseudotuberculosis* vaccine.

In the first half of the XXth century, Wake obtained 50% protection against plague by sc injection of a low dose of virulent *Y. pseudotuberculosis* (Wake et al., 1978), demonstrating that there was protective cross-species immunity. Devignat and others proposed that the mild natural infections by enteropathogenic Yersiniae could provide immunity against plague. Devignat had observed that regions of plague and enteropathogenic Yersiniae were almost non-overlapping in the world, and proposed that plague epidemics could not develop in countries where enteropathogenic Yersiniae were present (Mollaret, 1995). According to him, the diffusion of enteropathogenic Yersinia in Europe during the last centuries could explain why Europe was not affected by the third plague pandemic as severely as during the previous ones. Indeed, a partial immunity against bubonic plague has been provided to mice with a pYV-cured *Y. pseudotuberculosis* YPIII strain injected iv or a *Y. enterocolitica* O:3 injected sc (Alonso et al., 1980; Simonet et al., 1985).

In our laboratory, the oral route was favored as the most natural to induce mucosal immunity using live enterobacteria. Using a naturally attenuated *Y. pseudotuberculosis* strain (IP32680) we

observed that such a strain colonizes the gut for more than two months upon oral vaccination, with only a very transient penetration to deep organs, and induces a strong systemic humoral immune response. Mice receiving two oral doses were then protected almost completely against bubonic plague, but only weakly against pneumonic plague. Similarly, a Δ*dam* *Y. pseudotuberculosis* was previously shown by others to protect against bubonic plague (Taylor et al., 2005), although the strain was pYV-cured, so immunity against the TTSS was not induced. Recently, we generated a well defined and irreversibly attenuated strain by deleting genes encoding three essential virulence factors (HPI, the pH6 Ag and YopK) from the sequenced *Y. pseudotuberculosis* IP32953. To increase efficiency, production of the *Y. pestis* F1 capsule was additionally induced (Derbise A. *et al*. unpublished). F1 production did not increase virulence and oral inoculation of this new, attenuated and capsulated V674pF1 strain induced both humoral and cellular components of immunity, at the systemic and mucosal levels. A single oral dose was sufficient to confer 100% protection against a lethal pneumonic plague challenge (33xLD_{50} of the fully virulent *Y. pestis* CO92 strain) and 94% against a high challenge dose (3,300xLD_{50}). Most interestingly, V674pF1 also fully protected against a F1-negative *Y. pestis* (CO92 Δ*caf*), a protection that a live attenuated *Y. pestis* (KIM D27: Δ*pgm*) live candidate vaccine failed to provide (Cornelius et al., 2009; Quenee et al., 2008).

Vaccination against the enteropathogenic Yersiniae

Human infections by *Y. pseudotuberculosis* and *Y. enterocolitica* are usually self-limiting, so vaccination is generally not considered as a public health priority. Attempts to vaccinate have been mainly motivated by veterinary needs, because infection of livestock animals (cattle, buffaloes, deer and sheep) by *Y. pseudotuberculosis* has a non-negligible economic impact, and pseudotuberculosis also recurrently affects zoological gardens and wildlife parks.

Y. pseudotuberculosis vaccine against pseudotuberculosis

Most investigators used the oral route of vaccination to obtain a protective mucosal immunity at the gastrointestinal interface (Table 2). Hypo-virulent *Y. pseudotuberculosis* strains given via the oral route are able to induce protective immunity against highly pathogenic serotype Ia strains (Quintard et al., 2009; Thornton and Smith, 1996). To obtain the low oral virulence, the group of J. Mecsas used deletion of certain Yops, resulting in an impairment of the TTSS, and protection

was efficient against intraperitoneal (ip), intranasal or oral challenge (Balada-Llasat et al., 2007). The same strain cured of the whole pYV also protected against oral infection, showing that immunity against the TTSS machinery may not be required in this case.

One theoretical difficulty of vaccination against enteropathogenic Yersiniae is that the surface of different strains may be covered by different O-antigens (the molecular basis of serotypes) due to differences in the glycosylation machineries. Antibodies against O-antigens are abundant after infection or vaccination against a given strain, but might not work against a strain of a different serotype. This idea had led to mixing strains of different serotypes in a killed vaccine to obtain a broad spectrum immunity (Poelma and de Voogt, 1969). However, the use of the avirulent *Y. pseudotuberculosis* strain IP32680 (serotype II, described above) as an oral vaccine against pseudotuberculosis in the guinea pig, an animal very susceptible to this infection, conferred protection against a virulent strain of a different serotype (IP32953, serotype Ia, (Quintard et al., 2009). Moreover, IgG induced by vaccination recognized the five most frequent serotypes (I to V) equally well by ELISA, strengthening the view that a protective cross-serotype immunity could be raised.

Y. enterocolitica vaccine against Yersinia enterocolitis

Up to now, efforts to vaccinate against enterocolitis caused by *Y. enterocolitica* have been infrequent, and live vaccines have been often unsuccessful. Attenuated Δ*aroA* or Δ*ompR* mutants conferred only partial protection against the homologous virulent strain (Bowe et al., 1989; Dorrell et al., 1998). Δ*yadA*, Δ*sodA* and ΔHMW P1 mutants were also well attenuated and upon challenge no bacteria was detected in organs of vaccinated animals (Igwe et al., 1999), however long-term survival was not assessed.

In devising a live *Yersinia* vaccine against *Y. enterocolitica*, the variability of the V antigen may have to be taken into account. For example, serotype O:3 and O:8 strains, which have two different forms of V (V-O:3 and V-O:8), are both often pathogenic. The *Y. pseudotuberculosis* V-O:3 antigen, expressed by a *Salmonella* vector, was able to protect against a pathogenic *Y. pseudotuberculosis* expressing this form of V, whereas it failed to protect against another pathogenic strain expressing the divergent (V-O:8) form (Branger et al., 2009). Therefore, a mix

of *Yersinia* strains expressing the two forms of V, or the construction of a strain co-expressing the two forms might be required to protect against the whole array of enteropathogenic *Yersinia* strains.

Live vector plague vaccines expressing *Yersinia* antigens

Several microorganisms have been extensively studied as potential vectors able to transfer vaccinal antigens. They were generally selected for their absence of virulence and their ability to induce an immune response presenting the desired characteristics, such as mucosal, cytotoxic, memory, etc.

Salmonella vector plague vaccines

Because most of the recent work to develop plague vaccines aims at protecting against the pneumonic form of plague, vaccines able to induce a protective mucosal immune response have been searched for. Several *Salmonella enterica* strains, serovar Typhi or Typhimurium, have thus been tested as vectors for heterologous delivery of *Y. pestis* antigens (Table 1). *Salmonella* are Gram-negative enterobacteria which can colonize the gut after oral ingestion. They reach and penetrate the Peyer's patches via M cells, triggering a local immune response of this gut-associated lymphoid tissue. In addition, they traffic to the spleen and liver where they are found in macrophages. Strains presenting several mutations including *aroA*, *aroC*, *aroD*, PhoP/*PhoQ* and *htrA* are efficiently attenuated by the oral route and establish limited infections within their host. *aro* genes are involved in the synthesis of aromatic metabolites, so mutants are auxotrophic for these compounds and fail to grow longer than a few days in vivo. As live vaccine vectors, these attenuated strains persist adequately in the gut and lymphoid organs, with minor lesions, and are therefore fully capable of eliciting both humoral and cell-mediated, mucosal and systemic immune responses (Calhoun and Kwon, 2006; Titball et al., 1997; Titball and Williamson, 2001; Yang et al., 2007). Most of them have been found to be well tolerated and safe in Phase I clinical trials in humans (Dilts et al., 2000; Hohmann et al., 1996; Tacket et al., 1997). Protection of mice against bubonic plague was obtained by oral immunization with *Salmonella* vectors expressing the two well-studied *Y. pestis* antigens, F1 (Oyston et al., 1995; Titball et al., 1997) and V (Branger et al., 2007; Branger et al., 2009; Garmory et al., 2003) (Table 1) or both (Yang et al., 2007). A nearly complete protection against bubonic but not pneumonic plague was also obtained

with the Psn antigen (Branger et al., 2007). As expected, both humoral (IgA and IgG antibodies) (Yang et al., 2007) and cellular responses (Titball et al., 1997) directed against target antigens were observed when such *Salmonella* vectors expressing *Y. pestis* antigens were used.

In order to obtain an efficient immune response, multicopy plasmids inducing a high level of antigen production are generally used in *Salmonella*, although this may lead to a metabolic burden favoring the loss of the plasmid in vivo or during vaccine preparation. Low-copy-number plasmids appear be more stable and high expression of the F1 antigen was observed in in-vivo conditions (Titball et al., 1997). As an alternative, a clever regulation of plasmid copy number controlled by a promoter repressed by arabinose has been proposed, in which replication to the high-copy number state is induced by the absence of arabinose in vivo (Torres-Escobar et al., 2010). Also, to prevent plasmid loss, a selective pressure is usually maintained via the antibiotic resistance genes included in the plasmids. These antibiotic resistance genes raise serious security concerns for use in humans considering that they might be transferred to other bacteria, and methods are currently investigated to avoid such cassettes (Calhoun and Kwon, 2006). For example, Yang *et al.* inserted a plasmid carrying the F1 and V antigens plus a functional *asd* gene into an auxotroph *asd- Salmonella* unable to grow in the absence of diaminopimelic acid (DAP) (Yang et al., 2007). Mutants were then complemented for the *asd* function and could be selected on a culture medium lacking DAP. This live oral vaccine successfully protected mice against both bubonic and pneumonic forms of plague. *Salmonella* vaccines have been tested mostly via the oral route which is natural to them, however protection against plague has also been obtained following intranasal immunization with a *S. enterica* serovar Typhi expressing the F1 or V antigens (Liu et al., 2007; Morton et al., 2004) and immunization through the intranasal route with *Salmonella* vaccines against other pathogens has been found to be as efficient as and often superior to the oral route in inducing mucosal and systemic responses (Pasetti et al., 2000).

Finally, a *Salmonella* vector (*S. enterica* serovar Typhimurium) expressing the V antigen of *Y. pestis*, has been evaluated as vaccine against infection by *Y. pseudotuberculosis* and *Y. enterocolitica*. Consistent with the polymorphism of the V antigen of *Yersiniae,* the vaccine induced complete protection against *Y. pseudotuberculosis*, and 60% against *Y. enterocolitica* 0:3

which have V antigens related to that of *Y. pestis*, but failed to protect against a *Y. enterocolitica* O:8 which have a more divergent V antigen (Branger et al., 2009; Julio et al., 2001).

Virus vector plague vaccines

Virus vectors are also potentially very interesting for heterologous expression of target antigens due to their high immunogenicity, their ability to replicate in vivo and to induce antigen expression by host cells. Virus-induced immunity is generally long-lasting and is mainly T cell - mediated, although antibody production is also involved. Although there is to date no viral-vector vaccine licensed for human use, they are considered as most promising for use against complex diseases such as HIV or Malaria. On the other hand, viral vaccines replicating strongly in vivo carry a risk of unwanted reactogenicity and may undergo recombination with loss of antigens or reversion to virulence. An attenuated Vesicular Stomatitis virus (VSV), a pathogen of livestock harmless to humans, was recently engineered to induce expression of the V antigen. This vector was suitable for intranasal vaccination, but this route required a prime-boost regimen of vaccination to provide effective protection to mice against pneumonic plague (Palin et al., 2007). However, protection against pneumonic plague was obtained with a single high dose given via the intramuscular route, and this protective systemic response was found to involve the recruitment of CD4+ T cells (Chattopadhyay et al., 2008).

Another approach made use of a replication-defective Adenovirus expressing the V antigen, and later the V or F1 antigens fused to the pIX capsid protein (Boyer et al., 2010; Chiuchiolo et al., 2006). Adenoviruses are attractive for vaccination because they are stable and easy to manipulate, produce and purify. In vivo, they infect various cell types and act as adjuvants by causing inflammation and rapidly promoting an adaptive immune response (reviewed by (Lasaro and Ertl, 2009)). One dose of virus expressing V given intramuscularly induced both T cell requirement and antibody production, and protected mice against pneumonic plague (Chiuchiolo et al., 2006). Two doses were required when F1 or V were incorporated in the capsid (Boyer et al., 2010). Further genetic engineering of the vector and selection of infrequent serotypes were subsequently carried out to overcome innate or pre-existing immunity against Ad vectors that could constitute a serious obstacle to the application of this technology by causing the fast destruction of infected cells (Lasaro and Ertl, 2009). Of note, an Adenovirus vector was also used to produce a

functional anti-V antibody, which successfully protected against pneumonic plague (Sofer-Podesta et al., 2009) and could thus represent an interesting therapeutic alternative to antibiotics treatment.

Very recently, the vaccinia virus serving as vaccine against smallpox was also used as vector producing the F1 and/or V antigens. Two intramuscular injections of virus were required to induce protection against pneumonic plague when the vaccinia produced a truncated form of V(Brewoo et al., 2010), whereas a single dose was required when vaccinia producing V fused with a surface protein was laid on abrased skin (Embry et al., 2011) or when vaccinia produced V fused with the F1 antigen was given orally (Bhattacharya et al., 2010).

Vector vaccines against pseudotuberculosis

A non-invasive and non-colonizing *Lactococcus lactis* modified to express the *Y. pseudotuberculosis* V antigen was tested as vaccine, and only the intranasal route but not the intragastric (ig) route of immunization was effective to protect against oral or systemic *Y. pseudotuberculosis* infection (Daniel et al., 2009). This route was also the only one to result in the production of IgA and to a mixed Th1-Th2 cell response, indicating that the induction of such a mucosal response may be important for the protection against *Y. pseudotuberculosis.*

Recombinant plant vectors expressing *Y. pestis* antigens

A recent and important development of recombinant vaccines research is to produce so-called edible vaccines, i.e. vaccines made of transgenic plants expressing vaccinal antigens and delivered orally. Edible vaccines hold great promise because this vaccine delivery system appears highly cost-effective, easy-to-administer, easy-to-store and psychologically acceptable. Plant vaccines could allow to considerably upscale production and simplify vaccination procedures for mass-vaccination, for example in developing countries. The technology offers two principal axes of application which might ideally be combined: first using plants as a bio-reactor for mass production of vaccine antigens, and second using raw transgenic plant tissue as edible vaccine material. The technology for the first step is now largely available, and several molecules like albumin, human alpha interferon, human erythropoetin, and murine IgG and IgA immunoglobulins have been produced in an active form. The feasibility of a plant-derived plague

vaccine has been evaluated, and several groups reported the generation of tobacco and tomato plants expressing the two main *Y. pestis* antigens F1 and V, and also the F1-V fusion protein (reviewed by Alvarez (Alvarez and Cardineau)). These antigens purified from plants were as effective as recombinant antigens obtained using classical techniques such as production in *E. coli* bacteria.

To our knowledge, oral vaccination against plague with an edible vaccine has not yet been obtained, although tomatoes expressing the F1-V fusion protein have been produced. In fact, edible vaccines would be intermediates between sub-unit vaccines and live vaccines: antigens would be delivered encapsulated in live plant cells which would break open and release their content in the intestines, but these vegetal cells would not replicate in the host. Oral vaccination with a plant vaccine will require addressing questions related to oral immune tolerance, toxicity of the genetically modified organism, effective vaccine doses and immunogenicity of the preparation. Whereas it is expected that these vaccines should be well tolerated, the question of their immunogenicity is crucial. Indeed, in the absence of an adjuvant, it is not guaranteed that such vaccines can induce an immune response of sufficient intensity to be protective.

An upstream prophylactic approach: vaccination of reservoir animals

Because plague is mostly a zoonosis affecting rodents which constitute the reservoir of the disease, researchers have considered the possibility of vaccinating wild reservoir animals against plague. A wide scale animal vaccination effort is expected not only to limit the number of reservoir or sick animals, but also to prevent transmission and this way to exponentially reduce the occurrence of the disease in wildlife, and hence in man. The previous wide scale rabies vaccination of wild foxes in Europe and USA have been spectacular successes and demonstrated the validity of this approach (Pastoret and Brochier, 1999). The strategy relies on the vaccination of animals by volunteer consumption of recombinant vector-virus vaccine-laden baits. Following the same idea, it was proposed to control plague in North America by vaccinating one of its main wild reservoirs, the prairie dog, using a recombinant Raccoon Pox Virus (RCN). RCN infects mucosal tissues where it induces an immune response without being lethal, and hence is well suited for use as an oral vaccine vector. Moreover, human infections by this virus generally cause only limited lesions, limiting the consequences of an accidental contamination. First expressing

F1 (Mencher et al., 2004), then F1 plus V (Rocke et al., 2010), this vaccine was given orally to prairie dogs on a volunteer basis, and induced their almost complete protection against plague. Recently, the vaccinia virus expressing the rabies virus glycoprotein, that presently gives the best results against rabies on the field (Pastoret and Brochier, 1996), was induced to produce the F1-V fusion protein, and a single dose of oral vaccine fully protected mice against pneumonic plague (Bhattacharya et al., 2010). These reservoir-targeted vaccines therefore represent a promising means of controlling plague persistence and diffusion in wildlife.

The protective immune response against plague brought about by vaccination

The protective value of vaccines was historically evaluated as their ability to induce antibodies able to limit disease by neutralizing a toxin or blocking the spread of the infectious agent, so vaccinal formulations were generally chosen according to the resulting antibody titer. Because it was (and still is) technically more difficult to evaluate the intensity and more importantly the protective value of the cell-mediated immune response (CMI), this parameter has often been overlooked. Recent evidence however shows that both antibodies and cells can confer immunity.

That efficient antibodies can be sufficient to prevent plague is a long-held and well-established view supported by several lines of evidence. The ability of serum from immunized animals to passively transfer protection to naïve animals has been used for a long time to evaluate the level of protection obtained and this “mouse protection assay“ (MPI) has been routinely used for decades to verify the quality of the formalin-killed plague vaccine (Meyer et al., 1974b). Definitive evidence came from the ability of immune serum to transfer protection to severely immunodeficient SCID/*Beige* mice which lack lymphocytes (Green et al., 1999). Monoclonal antibodies directed to V or F1 are now considered as potential therapeutic tools against plague (Philipovskiy et al., 2005). In addition to the previously mentioned ability of antibodies to neutralize specific molecular targets, they also induce complement activation and promote phagocytosis of bacteria by opsonization, resulting in the activation of phagocytes and enhancement of their bacteriolytic ability. An anti-V antibody was indeed shown to contain bacterial growth only in neutrophil-competent mice (Cowan et al., 2005).

The importance of CMI to protect against plague was first revealed by observations on the role of cytokines. TNFα and IFNγ, classical mediators of CMI, were shown to protect macrophages from lysis by *Y. pestis*, and injection of these cytokines rescued mice from plague (Nakajima and Brubaker, 1993). TNFα and IFNγ, together with Nitric Oxide Synthase (NOs), support the action of protective humoral immunity during lung infection (Parent et al., 2006), and can even be central to protection in the absence of antibodies. Indeed, antibody-deficient μMT mice can be vaccinated against a pneumonic challenge (Parent et al., 2005) and protection requires TNFα and IFNγ (Kummer et al., 2008). Mouse vaccination with a live attenuated *Y. pestis* strain primed CD4 and CD8 T cells which provide immunity to naïve mice upon adoptive transfer (Philipovskiy and Smiley, 2007) demonstrating that CMI conferred protection by itself. Finally, the link between T cells and cytokines was made when it was demonstrated that STAT4-deficient mice, which are impaired for IFNγ production by T cells, failed to be protected by F1-V vaccination in spite of an adequate antibody response to the vaccine (Elvin and Williamson, 2004).

The cytokines released by T cells determine the effector mechanisms of immunity. Th1 cells activate macrophages by producing IFNγ and other pro-inflammatory cytokines, and as mentioned above, experiments with STAT4-deficient mice indicated that IFNγ production by T cells is required for anti-plague immunity. On the opposite, IL-4-producing Th2 cells support the humoral compartment of the immune response and exert anti-inflammatory functions by producing also IL-10. As shown by experiments with IL-4 knockout mice and STAT6-deficient mice (which do not develop Th2 cells), the Th2 type of response is dispensable to protection (Elvin and Williamson, 2000, 2004). Because the protective value of well-targeted antibodies is not questioned by these experiments, a reasonable conclusion is that a combination of cell-mediated and humoral immune responses should be the most protective.

Additional types of effector T cells were recently characterized, and it was observed that those producing IL-17 (called Th17 cells) are key players of vaccine-induced protection against mucosal infections by pathogenic bacteria, parasites, viruses and fungi (Khader et al., 2009; Lin et al., 2010). Indeed, IL-17 is a powerful inducer of PMN recruitment and release of antimicrobial peptides, and IL-17-producing T cells contribute to the immunity induced against

pneumonic plague by an attenuated *Y. pestis* candidate vaccine, and collaborate with Th1 lymphocytes (Lin et al., 2011). Unexpectedly, not only CD4 T cells but also CD8 T cells, classically endowed with cytotoxic functions, contribute to anti-plague immunity (Philipovskiy and Smiley, 2007). A mechanism involving the killing by CD8 T cells of bacteria-associated antigen-presenting cells has recently been analyzed in *Y. pseudotuberculosis* infection (Bergman et al., 2009) and could explain this observation. Also unexpectedly, a T cell subset exhibiting a gamma-delta T-cell receptor has been shown to prevent lesions to lung tissue during pneumonic plague (Huang et al., 2009).

Adjuvanticity of live vaccines

The role of vaccine adjuvants is to trigger cytokine production and cell maturation processes required to prime the adaptive immune response. Because they replicate, live attenuated bacteria typically achieve sufficient antigen expression and immune stimulation to elicit a long-lasting immune response in the absence of adjuvants. This helps explain why non-replicating (killed) live-vector vaccines and purified antigens generally evoke weak specific immune responses and often require the use of high doses, booster immunizations and the addition of adjuvants. Aluminum salts (Alum) remain the sole adjuvant approved for human use in the majority of countries worldwide. Aluminum can enhance specific antibody responses but has little capacity to stimulate cell-mediated immunity. This contributes to explain why high antibody titers could be observed in spite of a lack of protection (Anderson et al., 1996; Bashaw et al., 2007). Because the safety of Aluminum salts has recently been questioned, new adjuvant formulations are actively searched for.

Gram-negative bacteria generally trigger a potent innate immune response resulting from the recognition of their conserved molecular signatures, such as LPS, flagellin or peptidoglycan, by Toll-like receptors (TLRs) and intracellular NODs (van Duin et al., 2006). *Y. pestis* however has a poorly stimulatory LPS and no flagellin, characteristics which contribute to its escape from immunity. Thus, modification of *Y. pestis* LPS can improve its adjuvant properties, and LPS-modified candidate live vaccines have both a reduced virulence and an increased immunogenicity (Feodorova et al., 2009; Feodorova et al., 2007; Montminy et al., 2006).

Correlates of the protective immunity induced by live vaccines

EV76 owes its reputation to the successful control of plague by mass vaccination of Malagasy people (Girard, 1963). At present, with low plague incidence in the world and more severe ethical demands, such an evaluation of vaccine efficacy on the field can no longer be performed. Predictions about the vaccine's efficacy in humans can now only be drawn from comparisons of the immune response of vaccinated laboratory animals surviving a plague challenge with that of vaccinated human volunteers, without challenge tests. This has led to search for reliable correlates of protective immunity in animals that could serve as surrogate markers in humans (Plotkin, 2008). In some cases, this scientific necessity has now become an administrative requirement. For example, USA agencies such as the NIAID or the DOD request them as part of their sponsoring of research on vaccines for biodefense purposes. As summarized by Stanley Plotkin, "definition of a correlate is often the first step in the development of strategies of vaccination against a disease, it provides an objective criterion for protection of individual vaccinees, and even more practically, it permits the licensure of a vaccine without demonstration of field efficacy" (Plotkin, 2008).

There is, as far as we know, no correlate of immunity established for a live vaccine against plague (Welkos et al., 2008; Zauberman et al., 2008), probably because no live vaccine was entered into industrial development since the EV76 strain. The question is however important to address for the future. Correlates of immunity are generally found among humoral or cell-mediated mechanisms playing a key role against the pathogen. Historically, serum antibodies - easy to obtain and to test- caught most of the attention. The protective value of a given antibody depends not only on the recognized target but also on its capacity to participate in various protective mechanisms (neutralization of target antigen activity, opsonization of the bacteria, activation of complement, presence at mucosal surfaces etc.). Antigen-specific antibody titers are good correlates in humans for certain vaccines (tetanus, typhoid fever). However, in studies using F1 / V as vaccine targets, the antibody titers were often indicative but not statistically significant predictors of immunity because of a high variability of values (Anderson et al., 1996; Bashaw et al., 2007; Chattopadhyay et al., 2008; Garmory et al., 2004; Garmory et al., 2003; Mencher et al., 2004; Morton et al., 2004). Although extensive studies are missing, a similar lack of correlation was observed for example with anti-V or F1 antibodies induced by a whole-cell *Y. pestis*

(Flashner et al., 2004) used as plague vaccine, or with antibodies directed against an unfractionated bacterial lysate upon vaccination with a whole-cell *Y. pseudotuberculosis* (Quintard et al., 2009) against pseudotuberculosis. As an alternative to Ig titers, a correlate to predict immunity induced by V or F1-V subunit vaccines is the inhibition by serum antibodies of bacteria-induced cytotoxicity for macrophages (Welkos et al., 2008; Zauberman et al., 2008). Thus, what is specifically tested in this kind of assay is probably the inhibition of the TTSS by anti-V antibodies and opsonization of the bacteria by anti-F1 antibodies, facilitating phagocytosis. Antibodies directed against other surface molecules present in attenuated *Yersinia* vaccines could also contribute to opsonization. The use of this assay has not yet been reported for whole-cell vaccines. Finally, it must be noted that the prediction can be restricted to the microorganism tested. For example an assay using a F1-positive *Y. pestis* strain will only predict protection provided by a F1-based vaccine against a wild-type strain, and not against an engineered F1-negative strain.

Monitoring T cells is very challenging due to numerous limitations, both logistic and technical (reviewed by (Salerno-Goncalves and Sztein, 2006)). They include the complexity of the assays, the difficulties resulting from the fragility and diversity of living cells, and the absence of standardized quantitative assays of T cell functions. Measurement of soluble cytokines produced by cell populations is not adequate, and the frequency of cells producing cytokines (for example IFNγ) in response to antigens may correlate more efficiently with protection. This information can be obtained by ELISPOT or flow-cytometry assays in which multiple intracellular cytokines (IFNγ and others) are labeled for single-cell detection.

Perspectives

Which lessons can be drawn from all these efforts? Live plague vaccines have progressed towards less residual virulence due to a better controlled attenuation. Their efficient stimulation of both humoral and cell-mediated immune responses and their capacity to protect from pneumonic plague is more and more often demonstrated. The next steps may now be careful elimination of antibiotic-resistance cassettes, evaluation of reactogenicity and efficiency in non-human primates, and ultimately the start of tests in humans.

Plague is still a serious health problem for several countries in the world, and live vaccines may represent an efficient and available solution for them. Because most naturally arising pathogenic strains exhibit the standard F1 and V antigens, F1-V sub-unit vaccines would be generally efficient. However strains with different V or lacking F1 have been observed, or can be generated by evil-minded bioterrorists. In such cases, whole-cell vaccines, with their antigenic diversity and stronger ability to induce a cell-mediated immune response, would be more efficient than sub-unit vaccines. Their availability, in addition to molecular vaccines, would contribute to narrow the spectrum of plague risks vaccination could prevent.

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Table 1: *Yersinia* strains and heterologous live vectors proposed for vaccination against plague [a]

Strain background [b]	Characteristics	Attenuation [c]	Animal	Route of vaccination[c]	Protection conferred against		References
					Bubonic plague [d]	Pneumonic plague [e]	
Y.p. EV76	Δ*pgm*	LD50>10^9 cfu, severely reactogenic	guinea pig, mouse	im	100% @ various doses	100% @ 9,5x10^5 cfu	(Girard, 1963; Meyer et al., 1974a; Russel et al., 1995)
Y.p. CO92	Δ*pgm*	LD50>10^7 cfu, severely reactogenic	mouse	in	nt	100% @ 10^5 cfu	(Bubeck and Dube, 2007)
Y.p. GB	*Δdam*	LD50 = 2.3x10^3 cfu	mouse	sc	100% @ 7500 cfu	nt	(Taylor et al., 2005)
Y.p. EV NIIEG	Δ*lpxM*	sc avirulent to mice & guinea pigs	mouse & guinea pig	sc	40-80% @ 2x10^4 cfu (mice), 100% @ 1.2x10^4 cfu (G.P.)	nt	(Feodorova et al., 2007)
Y.p. CO92	Δ*yopH*	strongly attenuated via sc or in: LD50>10^7 cfu	mouse	sc / in	100% @ 10^5 cfu	80% @ 10^5 cfu	(Bubeck and Dube, 2007)
Y.p. Kimberley 53	Δ*pcm*	strongly attenuated via sc: LD50>10^7 cfu	mouse	sc	100% @ 10^5 cfu	nt	(Flashner et al., 2004; Tidhar et al., 2009)
Y.p. CO92 Δ*pgm*	Δ*smpB* Δ*ssrA*	strongly attenuated: LD50> 10^6 cfu iv, 10^8cfu in	mouse	in	nt	100% @ 2x10^5 cfu of *Y.p*. Δ*pgm*	(Okan et al., 2010)
Y.p. KIM 1001	+*lpxL* of *E. coli*	strongly attenuated via sc: LD50>10^7 cfu	mouse	sc	100% @ 10^6 cfu	100% @ 5x10^4 cfu	(Montminy et al., 2006; Szaba et al., 2009)
Y. p. GB	Δ*guaBA*	LD50>7x10^4 cfu by sc or iv, auxotroph	mouse	iv / sc	iv: 100%, sc : 20% @ 7x10^4 cfu	nt	(Oyston et al., 2010)
Y.p. Kimberley 53 Δ*yopJ*	+*yopP* of *Y.e.*	LD50>10^6 cfu by sc, virulent by iv or in	mouse	sc	100% @ 10^3 cfu	70% @ 8x10^3 cfu	(Zauberman et al., 2009)

Y.e. 4052 or 4246 (O:III)	wt	naturally avirulent	mouse	iv, sc, oral	iv: up to 100% @ 200 cfu	nt	(Alonso et al., 1978)
Y.ptb. IP32680 (O:II)	wt	naturally avirulent	mouse	oral	88% @ 10^3 cfu	30% @ $8x10^3$ cfu	(Blisnick et al., 2008)
Y. *ptb.* V674pF1 (O:I)	ΔHPI Δ*psaA* Δ*yopK* pGEN-*caf*	avirulent by oral route	mouse	oral	81% @ 10^3 cfu	100%@ 10^5 cfu CO92 94% @ 10^7 cfu CO92 100% @ 10^7 cfu CO92Δ*caf*	Derbise A. *et al*. submitted.
Y.ptb. IP32953 (O:I)	*Δdam* pYV-cured	LD50 oral >10^9 cfu, iv >$2x10^7$ cfu	mouse	iv / oral	iv : 100% @ 10^2 cfu oral : 70% @ 10^2 cfu	nt	(Taylor et al., 2005)
S.enterica Typhimurium Δ*aroA*	+ *caf1* operon	avirulent and auxotroph	mouse	oral	100% @ $1.2x10^7$ cfu	nt	(Titball et al., 1997)
S. enterica Typhi Δ*aroA, ΔaroC, ΔhtrA*	+ *caf1* operon	avirulent and auxotroph	mouse	in	65% @ 100 cfu	nt	(Liu et al., 2007; Morton et al., 2004)
S. enterica Typhimurium Δ*crp, Δasd*	+ caf1 operon or *lcrV* or *Psn* of *Y.p*.	avirulent and auxotroph	mouse	oral	80%@$2x10^3$ cfu with F1, 60-100% with V, 81% with *Psn*	37% @ $4.4x10^4$ cfu with *Psn*, 50% with V	(Branger et al., 2010; Liu et al., 2007)
S. enterica Typhimurium Δ*aroA*	+ *lcrV* of *Y.p.*	avirulent and auxotroph	mouse	oral	30% @ 100 cfu	nt	(Garmory et al., 2003)
S. Typhimurium Δ*aroA* Δ*asdS*	+ *caf1* operon + *lcrV* of *Y.p.*	avirulent and auxotroph	mouse	oral	87% @ 10^4 cfu	87% @ 10^4 cfu	(Yang et al., 2007)
Vesicular Stomatitis Virus	+ *lcrV* of *Y.p.*	species specific	mouse	im / in	nt	90% @ 10^4 cfu	(Chattopadhyay et al., 2008; Palin et al., 2007)
Replication-defective Adenovirus	+ *lcrV* of *Y.p.*	species specific	mouse	im	nt	100% @ $3x10^3$ cfu	(Boyer et al., 2010; Chiuchiolo et al., 2006)
Raccoon Poxvirus	+ *caf1*	species specific	mouse	id	100% @ 10^5 cfu 75% @ $1.5x10^6$ cfu	nt	(Osorio et al., 2003)

Raccoon Poxvirus	+ *caf1*	species specific	prairie dog	oral	56% @ $1.3x10^5$ cfu	nt	(Mencher et al., 2004)
Raccoon Poxvirus	+ *caf1* + *lcrV of Y.p.*	species specific	mouse	im	100% @ $7x10^4$ cfu 75% @ $7x10^5$ cfu	nt	(Rocke et al., 2009)

a: The list is not exhaustive

b: *Y.p.*: *Y. pestis*; *Y .ptb.*: *Y. pseudotuberculosis*; *Y.e.*: *Y. enterocolitica*. The serotype of *Y. e.* and *Y. ptb.* strains is precised between brackets.

c: in : intranasal; sc : subcutaneous; id : intradermic; iv : intravenous; im : intramuscular.

d: Challenge: sc inoculation of virulent *Y. pestis* (various strains *pgm*+ pYV+ pPla+ pFra+). The challenge dose is indicated after the @ symbol.

e: Challenge: in instillation or exposure to aerosols of virulent *Y. pestis* (various strains all *pgm*+ pYV+ pPla+ pFra+) except when indicated.

Table 2: Candidate vaccines against enteropathogenic *Yersinia* [a]

Strain background [b]	Characteristics	Attenuation [c]	Animal	Route of vaccination [c]	Protection against [d]	References
Y. ptb. M5 (O:Ia)	wt	$3.6x10^6$ cfu avirulent orally, virulent by iv	mouse	oral	100% @ 10^8 cfu *Y.ptb.*	(Thornton and Smith, 1996)
Y. *ptb.* IP32680 (O:II)	wt	avirulent by oral route	guinea pig	oral	80% @ 10^9 cfu *Y.ptb.*	(Quintard et al., 2009)
Y. ptb. YPIII (O:III)	overexpressing *dam*	LD50 oral $>10^{11}$ cfu	mouse	oral	100% @ $2x10^{10}$ cfu *Y.ptb.*	(Julio et al., 2001)
Y.ptb. IP32953 (O:I)	*Δdam pYV-cured*	LD50 oral $>10^9$ cfu, iv $>2x10^7$ cfu	mouse	iv / oral	100% @ 300 cfu *Y.ptb.*	(Taylor et al., 2005)
Lactococcus lactis	+ *lcrV* of *Y.pestis*	avirulent (probiotic)	mouse	oral	100% @ $4x10^8$ cfu *Y. ptb.* oral 90% @ 10^8 cfu *Y. ptb.* iv	(Daniel et al., 2009)
S. enterica Typhimurium Δcrp, Δasd	+ *lcrV* of *Y.pestis*	avirulent and auxotroph	mouse	oral	100% @ $2x10^9$ cfu *Y.ptb* 60% @ $1x10^9$ cfu *Y.e.*	(Branger et al., 2009)

a: The list is not exhaustive

b: *Y .ptb.*: *Y. pseudotuberculosis*; *Y.e.*: *Y. enterocolitica*.

c: iv : intravenous; ig: intragastric.

d : Challenge : ig inoculation of virulent *Y. pseudotuberculosis* (various strains all HPI+ pYV+) except when indicated