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REVIEW

Molecular Bases of Vaccine-Prevention of Plague

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Abstract—The molecular mechanisms of pathogenicity and properties of the formation of specific plague immunity are reviewed. The history and modern state of vaccine-prevention of plague are described. Special attention is focused on the prospects in the plague vaccine development, and possible approaches to the improvement of vaccine preparations are considered.

Keywords: *Yersinia pestis*, pathogenicity, immunity, prevention

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INTRODUCTION

Plague is the most dangerous of bacterial infections. The Justinian Plague (531–580 A.D.), Black Death (1347–1407 A.D.), and third plague pandemic (1894 to the present period), which have carried away more than 200 million human lives [112], were caused by plague pathogens [54]. Plague epidemics have often led to the fall of states and destruction of ancient civilizations. In some countries, up to 90% of the population perished [14, 66, 112].

A pure culture of the plague microbe, *Yersinia pestis*, was isolated by A. Yersin in 1894 during a plague epidemic in Hong Kong. This microorganism belongs to the *Yersinia* genus of the Enterobacteiacea family, which includes 17 species at present [79, 103, 104]. Two other human-pathogenic species (*Y. pseudotuberculosis* and *Y. enterocolitica*) cause alimentally transmitted intestinal diseases that are usually of low- and medium-severity and have an inclination toward a subacute course. The disease caused by *Y. pestis* significantly differs from pseudotuberculosis and intestinal yersiniosis in epidemiological properties and pathogenicity. The major pathways of *Y. pestis* are transmission by infected fleas and aerosol route. If there is no treatment, lethality reaches 60% in the case of bubonic plague and 100% in the case of septicemic and pneumonic plague. Usually, the period from the moment of infection to the death of a plague-sensitive warm-blooded animal is no more than 1 week [43, 112].

MOLECULAR MECHANISMS OF PLAGUE PATHOGENICITY

In order to constantly circulate in ecosystems of natural plague foci, *Y. pestis* bacteria [158] must penetrate into a host's organism, successfully counteract the innate immunity of the rodent, and propagate to induce bacteremia needed for subsequent transmission by fleas to a new host [76, 155]. These stages of cyclical existence are each ensured by many pathogenicity factors and "housekeeping" genes of *Y. pestis*, which can act jointly and individually. Each of these factors can, in turn, take part in different stages of the infectious process or pathogen transmission. The microorganism's biomolecules and organelles that ensure the pathogenicity of the infection are customarily included among pathogenicity factors [2, 3, 31, 33].

In the middle of the last century, T. Burrows [47–49] determined a set of signs that were present in all the virulent *Y. pestis* strains studied by him—the classical "virulence determinants." He considered the capability of cells to sorb exogenous dyes and hemin (Pgm^+); dependence of growth at 37°C on the presence of Ca^{2+} ions in a medium (Ca^-); synthesis of V and W-antigens, "murine" toxin (Tox^+), and FI capsule antigen (Fra^+ and F1); combined synthesis of pesticin (Pst^+), fibrinolysin (Fb^+), and plasmocoagulase (Cg^+); and purine independence or capability to synthesize endogenous purines (Pur^+) as such determinants. After the five decades of studies and discussions have passed since the moment of postulating the "virulence determinants," some of them, such as the W-antigen, pesticin, and capability to synthesize endogenous purines, ceased to be considered pathogenicity factors [2, 31, 112].

The main pathogenicity factor in yersiniae is a complex of properties coded by the pCad plasmid, which is compulsory for the manifestation of virulence by the plague pathogen. This complex of signs is the Yop virulon system, which permits extracellularly located bacteria to neutralize cells taking part in a

host's immune response, destroys the links between them, and causes apoptosis by injecting bacterial effector proteins. This system consists of Yop proteins and a third-type apparatus for their secretion called Ysc. The Ysc apparatus consists of 25 proteins. The majority of Yop-proteins can be divided into two groups according to their functions. Some of them are intracellular effectors (YopE, YopH, YpkA/YopO, YopP/YopJ, YopM, YopT); meanwhile, others (YopB, YopD, LcrV) form a translocation apparatus that develops on the surface of the bacterium to deliver effectors through a plasmatic membrane into eukaryotic cells. Secretion of the Yop-proteins starts in contact with eukaryotic cells and is controlled by virulon proteins, including YopN, TyeA, and LcrG, that supposedly close a bacterial secretory channel as a lock. The precise operation of the system also requires chaperons called Syc proteins, which are in a bacterial cytosol. Gene transcription is controlled by temperature and the activity of the secretion apparatus [147]. In recent years, a number of new pathogenicity factors have been revealed, some of which are presented in the table along with the classical ones.

As has been noted above, the plague pathogen circulates in populations of rodents and/or lagomorphs and is transmitted through bites of fleas parasitizing on them [17, 76, 155]. Less than ten *Y. pestis* bacteria are needed for terminal infection in rodents and primates in the case of intracutaneous, subcutaneous, or intravenous infection [3, 17, 31, 112]. In addition, infection is possible in the case of eating dead bodies/meat of animals that have died from plague [2, 17, 50, 112] and/or inhaling aerosolized respiratory discharge from patients with a pneumonic type of infection [2, 17, 64, 112]. LD_{50} values grow up to 10^2–10^4 and 10^5–10^9 bacterial cells in the case of aerosol and alimentary infection, respectively [3, 17, 33, 50, 56].

Depending on the stage of existence in an organism of a poikilothermic flea or warm-blooded mammal, *Y. pestis* is affected by external signals, primarily by the temperature of an ecological niche [2], which cause a range of genes expressed at this instant and, correspondingly, the antigenic structure that is typical for the vectorial and/or hostal phase of existence in the bacterium [72, 102]. Thus, at the temperature of 21–28°C that is inherent to the conditions of *Y. pestis* in the alimentary tract of a flea, cells of the plague microbe synthesize a hexa-acylated lipopolysaccharide (LPS) with six fatty-acid residues [86], which strongly triggers the innate immune system in mammals [12, 82] owing to recognizing and binding by a Toll-like receptor 4 (TLR-4)-MD2-CD14 [100, 101].

Just after a bite by an infected flea and penetration of *Y. pestis* into an organism of a warm-blooded host, bacteria with the hexa-acylated LPS are recognized by a TLR4-MD2-CD14 receptor complex [100, 101], easily ingested, and die in neutrophiles [52] (in $D11c^+$ cells of the lungs in the case of primary pneumonic plague [40]), but survive and propagate in macrophages [52]. The plague microbe propagates in macrophage phagolysosomes [134], the content of which is characterized by low pH values, as well as by a significant content of reactive oxygen and nitrogen forms and antimicrobial peptides and proteases [115], probably due to the capability of *Y. pestis* to neutralize the low pH values of a phagolysosome [116]. *Y. pestis* were also shown to be able to inhibit the production of nitric oxide [115]. The survival of yersiniae in macrophages was ascertained to require the functioning of a PhoP/PhoQ pleiotropic regulatory two-component system of gram-negative bacteria. Experiments using the *phoP*-mutants of *Y. pestis* in vitro showed the PhoP/PhoQ system to be necessary for existence under low pH values, oxidative stress, and low content of Mg^{2+} [109]. The knock-out *Y. pestis* mutants by the *phoP* gene [77] that regulates the *arn* operon (*pm-rHFIJKLM*) charged with joining cationic monosaccharide 4-amino-4-desoxy-L-arabinose (Ara4N) to the phosphate groups of the LPS lipid A or by the *arnT* gene (*pmrK*) that codes Ara4N-transferase proved to be sensitive to cationic antimicrobial peptides.

Y. pestis was later shown to propagate not only in macrophages ($CD11b^+/CD11c^-$), but also in dendritic cells ($Cd11c^+/Cd11b^-$), as well as in monocytes ($Gr\text{-}1^+$) [95]. The plasminogen activator of *Y. pestis*, Pla, proved to interact with the C-DEC-205 type lectin receptor (CD-205) that is present on the surface of macrophages and dendritic cells. Any disorders in the formation of a Pla-DEC-205 complex decreased the degree of dissemination of *Y. pestis* cells in mice organisms [162]. Consequently, being higher at the temperature of a warm-blooded host, the production and specific activity of the plasminogen activator Pla (the major factor of the plague microbe propagation) promote the penetration of the bacteria located inside the antigen-presenting cells of a host through the lymphatic flow from the place of a flea bite to regional lymph nodes. In addition, Pla, hydrolyzing the plasminogen of a host, transfers it into plasmin, which results in uncontrolled proteolysis and damage to mammal tissues, which promotes subsequent dissemination of *Y. pestis* [88, 89]. Having penetrated into a regional lymph node, the microbe continues to propagate, provoking an inflammatory process that covers all the neighboring lymph nodes and adjacent subcutaneous cellular tissue.

For the next stage of dissemination, which is already extracellular, the plague pathogen must counteract the components of the immune system, such as capture by dedicated phagocytes, secretion of cytokines, and complementarily sequential lysis of bacteria. During dissemination in macrophages at a temperature of 37°C and more, the Yop virulon starts to function in *Y. pestis* and then F1 and pH6 antigens (see table) are synthesized that give the bacterium resistance to absorption by any types of phagocytic cells after exiting destroyed macrophages [52, 55, 78, 147].

Some factors that ensure the vital activity of *Y. pestis* in an organism of a warm-blooded host (modified from article [31] with allowance for the materials [32, 41, 57, 67, 129, 142, 160])

Bacterial factor	Function or activity	Reported decrease of virulence in knock-outs (by order of magnitude)		
		>4	4–2	2–0
Stimulon coded by the pCad plasmid and reacting to the low concentration of Ca^{2+} (low-calcium response—LCR) and including the type III secretion system (T3SS) associated with the virulence of the bacterium as well as *Yersinia* outer membrane proteins (Yop), namely	System that permits extracellularly located yersiniae to counteract a nonspecific immune response by the counteraction to phagocytosis, signal activity of macrophages, and induction of apoptosis of phagocytic cells (translocation of toxic bacterial Yop proteins from extracellularly located bacteria into the cytosol of an eukaryotic cell)	+	–	–
V-antigen (LerV)	Yop protein translocation; inhibition of neutrophile chemotaxis; suppression of the synthesis of γ-interferon and tumor necrosis factor of α–cytokines needed for nonspecific activation of professional phagocytes and formation of productive granulomas owing to the stimulation of the production of the IL-10 repressor of the above-indicated cytokines that is mediated by the interaction with the Toll-like receptor 2 (TLR2); inhibition of the 1β interleukin production in macrophages induced by the LPS	+	–	–
YopD	Translocation of Yop proteins	ND*	ND	ND
YopE	Counteraction to phagocytosis	+	–	–
YopH	Counteraction to phagocytosis; protein-tyrosine-phosphatase; apoptosis inducer; inhibition of lymphocyte proliferation	+	–	–
YopM	Disorder in the interaction of thrombin with thrombocytes and suppression of their aggregation needed for the formation of grumes	+	–	–
YopJ/YopP	Decrease in the inflammatory response of macrophages and epithelial and endothelial cells owing to blocking the activation of mitogen-activated protein kinase and nuclear factor kB	–/+**	–	+
YopT	Counteraction to phagocytosis; inactivation of Rho-proteins; depolymerization of actinic stress fibers	ND	ND	ND
YpkA/YopO	Counteraction to phagocytosis; inactivation of Rho-proteins; autophosphorylating serine/threonine kinase	ND	ND	ND
YscF	Formation of T3SS outer "needle"; participation in the secretion of effector virulence proteins, their translocations through eukaryotic membranes, attachment of a bacterium to an eukaryotic cell and regulation of the work of T3SS depending on a concentration of Ca^{2+}	ND	ND	ND
Plasminogen activator (Pla) (coded by the pPst plasmid)	Propagation factor ensuring the generalization of the infection; protease determining the fibrinolytic (37°C) and plasmocoagulase (28°C) activity of the plague microbe; post-translation hydrolysis of Yop proteins; hydrolysis of cationic antimicrobial peptides of the respiratory tract; adhesive and invasive activity	+	+	+***
Braun murein lipoprotein (Lpp)	Induces endotoxic shock jointly with the LPS	–	–	+
NlpD lipoprotein	Propagation factor ensuring generalization of the infection	+	–	–
pH6-antigen (PsaA)	Counteraction to phagocytosis; adhesive activity	+	+	+
Fraction I capsule antigen (FI, F1; Cafl) (coded by the pFra plasmid)	Counteraction to phagocytosis; adhesive activity; protection from cationic antimicrobial peptides of the respiratory tract	+	+	+

Table. (Contd.)

Bacterial factor	Function or activity	Reported decrease of virulence in knock-outs (by order of magnitude)		
		>4	4–2	2–0
Ail	Adhesive activity; resistance to the bactericide action of the serum complement	–	+	–
Murine toxin (Tox, Ymt) (coded by the pFra plasmid)	Development of the toxic shock (in mice and rats); potentiation of endotoxic shock in mammals	–	–	+
LPS	Development of endotoxic shock; resistance to bactericidal action of antimicrobial cationic peptides and serum complement; adhesive activity; support of the enzymatically active folding of the Pla molecule	+	–	–
Factors responsible for the feeding of the bacterium and "housekeeping" genes				
Synthesis and transport of siderophore—yersiniabactin	Siderophore-dependent system for transport of iron into a bacterial cell	+	–	–
Hemin storage (Hms)	Hemin storage on the surface of bacterial cells	–	–	+
Enzymes of purine biosynthesis	De novo purine synthesis	+	–	–
Dam	Methylation of the DNA adenine	–	+	–
PhoP/Q two-component regulatory system	Activation of bacterial resistance to innate immunity factors	–	–	±

* ND—no data;

** When the proper YopJ effector protein characterized by the decreased capability of translocation into eukaryotic cells is replaced in *Y. pestis* cells by the functionally full-value Yop homolog from *Y. enterocolitica*, this results in the formation of plague microbe cells that are cytotoxic with respect to macrophages and decrease virulence in case of subcutaneous infection by seven orders of magnitude;

*** Several signs + or the sign ± indicate contradictory data obtained by different groups of researchers.

The synthesis of the V-antigen suppresses the innate immunity owing to stimulation of the synthesis of the interleukin 10 (IL-10), anti-inflammatory cytokine inhibiting the synthesis of γ-interferon, and tumor necrosis factor α that takes part in inducing the inflammatory process [45]. Complete removal of IL-10 from organisms of laboratory animals (owing to the mutation or application of monoclonal antibodies to IL-10) infected with *Y pestis* was shown to lead to increased resistance to infection [113].

In addition, the synthesis of a tetra-acylated LPS [86] that is not recognized by the TLR4-MD2-CD14 receptor complex, in contrast to the hexa-acylated LPS, takes place in *Y. pestis* at a temperature of 37°C or more [101]; as a result, cells of the plague pathogen start the propagation that is already not controlled by a host's organism [52]. If the infectious process does not stop at the stage of the bubonic form of the disease, secondary septicemia develops, which is accompanied by penetration of the plague microbe into other organs [31, 112].

In the primary septicemic form of the disease, bacteria enter the blood after a flea bite, escaping the lymphatic system and passing through the stage of primary reproduction in macrophages in the blood channel itself [128]. Being higher at a temperature of 37°C and more, the synthesis of the Ail adhesin from the Ail/Lom family of outer-membrane proteins gives *Y. pestis* cells resistance to the action of a serum complement and, correspondingly, the possibility of extracellular propagation [38, 57]. In addition, the Pla hydrolyzes the components of the complement system, also increasing the resistance of *Y. pestis* to the bactericidal action of the serum [87].

In addition to sepsis, generalization can result in secondary pneumonia, which represents the greatest danger alongside primary pneumonic plague, since a patient becomes a source of infection for other people [17, 31, 112]. The resistance of *Y. pestis* to the action of cationic antimicrobial peptides of the respiratory tract is ensured by a capsule that consists of F1, but not of pH6-antigen, and screens bacterial cells; in bacteria deprived of the F1 capsule, this resistance is ensured by the Pla that in the absence of the capsule hydrolyzes human cathelicidin LL-37 and cationic antimicrobial peptides from bronchoalveolar lavage fluid of rats [67].

Against the background of bacteremia, the LPS initiates the development of an endotoxic (septic) shock typical for infections caused by other gram-negative bacteria and, then, the death of a warm-blooded animal [12, 28, 50, 58, 146].

The data presented in the table indicate that the mutations of genes that are responsible for the synthesis of separate pathogenicity factors or "housekeeping"

genes can decrease the virulence of *Y. pestis* to a different extent. As early as 1957, T. Burrows [48] concluded that an optimal plague vaccine must include several protective antigens that are compulsory pathogenicity factors of *Y. pestis*, making allowance for the data on the mutation of the "virulence determinants" he had discovered. Subsequent experiments performed using a site-directed mutagenesis, specific inhibitors of the pathogenicity factors, and/or an estimate of the protective activity of individual purified antigens (or even separate epitopes) showed [31] the pathogenetic approach to selecting plague vaccine components to be correct, although the number of antigens that actually have protective activity is very limited.

PECULIARITIES OF FORMATION OF SPECIFIC PLAGUE IMMUNITY

Since plague is a zoonosis circulating in populations of rodents and lagomorphs [14, 33, 66, 112], it is evident that laboratory animals relating to these taxonomic groups (mice, rats, cavies, and rabbits) must be adequate models for the study on the infectious process in wild carriers, and the results from studying the plague immunogenesis based on these models can lead to understanding how plague immunity is formed in people [92, 97, 118]. Before clinical tests, candidates for plague vaccines are assessed in experiments on monkeys, which are phylogenetically closer to people than rodents [5, 10, 97, 119, 126, 127].

As early as the 1930s, studies of the protective antigens of the plague pathogen were started by H. Schutze [126, 127], who was the first to show that the antigen from bacteria grown on nutrient media at 37°C or in organisms of warm-blooded animals infected with *Y. pestis* and contained in their capsule was immune-dominant for mice, rats, and monkeys. Later, it was renamed the "fraction I" capsular antigen (F1) [36, 97]. When the immunizing activity of F1 was studied on guinea pigs, the preparation was revealed as able to provoke a state of immune paralysis when introduced in milligram doses; however, when introduced with an adjuvant in microgram amounts, F1 preparations stimulated a protective response [91, 133, 150]. Meanwhile, according to the data of other authors [27], double immunization by small doses at both a short and long interval between injections resulted not only in total death of guinea pigs infected with the virulent *Y. pestis* strain, but also in a significant decrease in average lifespan in vaccinated animals in comparison with control. Later, the *Y. pestis* strains F1$^-$ were shown to have selective advantages in organisms of white mice preliminarily immunized by wild strains or the classical capsule antigen [15, 63] and in organisms of guinea pigs that had been ill with the experimental plague caused by the wild strains but not in guinea pigs vaccinated once with an living plague vaccine [1].

A water-insoluble "residual" R antigen that is one more immune-dominant substance with a high protective activity was discovered in cells of *Y. pestis* and *Y. pseudotuberculosis* [126]. It had a high protective activity for guinea pigs, inducing long-term protection from plague [44, 47, 97, 127], but did not protect other species of laboratory animals. The chemical characteristic of this immunogen is complicated owing to its being associated with insoluble membranous components. The partial purification of the preparation was achieved by insonation and soft alkaline hydrolysis [83]. The subsequent purification was complicated by the presence of the large amount of the LPS [90]. According to data of Russian researchers, the "residual" antigen in the structure of a basic somatic antigen (BSA) or B antigen protected not only guinea pigs, but also monkeys from death from an experimental plague [6, 10, 33].

T. Burrows and G. Bacon [49] showed that the rabbit serum to the V (virulence) antigen they had discovered ensured the passive protection of mice from the infection by *Y. pestis*. The V-antigen was more immunogenic for rabbits than for mice and guinea pigs [92]. Meanwhile, both active immunization of guinea pigs by the V-antigen and passive immunization of mice with rabbit anti-V-serum protected from infection by the virulent *Y. pestis* strain.

A humoral immune response to no less than 50 antigens has recently been ascertained to develop in rabbits in the case of introducing a *Y. pestis* vaccine strain EV. In addition to the F1 and V-antigens, 11 more proteins are immune-dominant for this animal species (YscB, LcrG, YopD, VirG, PsaA, OmpA, MlpA, and KatY, as well as bacteriophage proteins coded by YPO2093, YPO2113, and YPO2118 genes [93]); however, a significant protectivity was only shown for the F1 and V-antigens.

Consequently, the responses of different animal species to the same antigen/vaccine preparation are not analogous [48, 81, 148]. The differences uncovered in the results of experiments on determining the protectivity of vaccine preparations with respect to monkeys are also likely to be due to using different species of these animals in the experiments: rhesus monkeys (*Macaca mulatta*), hamadryads (*Papio hamadryas*), vervet monkeys (*Cercopithecus pygerythrus*), or green monkeys (*Chlorocebus aethiops*) [5, 71, 98, 152]. Separate mice lines also differ in sensitivity to *Y. pestis* and properties of immunogenesis [20, 53, 125, 144, 149, 151]. Consequently, not all of the data obtained using laboratory animals may be used unconditionally to explain the processes taking part in a human organism. Moreover, it is customary to think that the degree of the sensitivity to plague may differ in various races and even ethnic groups [25, 26, 37, 51]. The opinion exists that people with blood type B(II) are less liable to the infection by the plague pathogen and survive this disease more easily [26]. Based on analysis of historical descriptions of plague epidemics, M.V. Supotnitskii [25] suggested that there is a predisposition in the

Europeoid and Mongoloid races for the bubonic and pneumonic form of the infection, respectively.

As has been noted above, *Y. pestis* combines the stages of intracellular and extracellular parasitism at the hostal phase of its vital cycle [2, 52, 95, 162]; therefore, it is not amazing that the most effective possible protection from the plague infection can only be ensured by combining T-cellular and humoral immunity [7, 20, 97, 111, 131, 138]. Many experiments have showed that specific antibodies to plague microbes [159], namely, to the F1 and V-antigens, ensured the expressed protection of mice from an experimental plague [44, 74, 75, 81, 145, 154]. In 1896, A. Yersin managed to cure several patients with plague using the horse hyperimmune serum [159], but the numerous attempts of his followers to use seroprophylaxis and serotherapy of plague in people using the hyperimmune sera of horses, mules, and buffaloes did not prove to be a success [16, 22]. In addition, the immunization of mice deprived of the thymus by the vaccine strain EV or F1 capsular antigen did not result in the development of an immunity, although such mice had normal B-cells [139].

The phagocytosis of the plague microbe in vitro was ascertained to strongly increase in the presence of sera of plague-resistant animals [16, 80] and to acquire a finished character, as immunity develops. T-lymphocytes isolated from spleens of the BALB/c immune mice, being cultivated jointly with *Y. pestis* and spleen cells obtained from intact animals, significantly inhibited the proliferation of the pathogen in comparison with a control group [156]. The immunization of mice deprived of B-cells with an attenuated Pgm^- strain KIM5 (2×10^5 CFUs against the background of serotherapy) ensured protection in the case of aerosol infection with 2×10^5 CFUs of the same strain. Mice that underwent only serotherapy died from an analogous aerosol infection [111].

A BRIEF HISTORY OF PLAGUE VACCINE PREVENTION

In 1781, the Russian scientist Danilo Samoilovich Sushkovskii (Samoilovich) (1744–1805) suggested that people be protected from plague using pus from buboes of patients with plague as an inoculative material, since, when he had been in contact with this pus and was infected with plague, he and another doctor, Pogoretskii, who was worked with him became ill with its mild form [24]. However, of the six people inoculated with this method at the end of the 19th century by Dr. Cerutti, five died from plague [35].

Scientifically grounded methods of plague immunization date back to 1895, since A. Yersin, A. Calmette, and A. Borrel showed that it is possible to protect rabbits from infection with plague by immunization with killed bacteria of the virulent strain [159]. Since this time, methods for disinfecting killed plague vaccines have been repeatedly varied by different researchers. Suspensions of *Y. pestis* have been disinfected by heating and/or adding phenol, formalin, ether, ethanol, glycerin, sucrose, etc. [16, 18, 19, 97, 163]. The greatest practical application has been received by the Hawkins vaccine, which was first tested in 1897 on prisoners at Bikul during a plague epidemic and then used to immunize several tens of millions of citizens in India [73, 140] and the USP plague vaccine killed by formalin that was developed in the early 1940s at the University of California under the direction of K. Meyer and became the most widespread for vaccination of US army soldiers in the period of the Vietnam War (1966–1972) [96, 99]. The USD vaccine was used in the United States, Canada, United Kingdom, and a number of other countries until 1998 in order to immunize laboratory staff working with plague pathogens; however, owing to the low efficiency with respect to the pneumonic form of the infection and postvaccine complications in inoculated people, as well as the need for repeated introduction with a view to developing an expressed immunity, the vaccine was taken out of production in the United States, but it was produced in the Commonwealth Serum Laboratories (CSL) Australian company until 2005 [112, 124, 153]. At present, the list of products on the official website of the CSL (http://www.csl.com.au/products/complete-product-list.htm) does not include the plague vaccine.

The first experiments on people with living plague vaccines based on attenuated *Y. pestis* strains were carried out in 1906–1907. A. Yersin was the first, testing monkey-avirulent cultures on himself [16, 18]. R. Strong repeated Yersin's experiment on himself and then on prisoners who had been condemned to death (an immunizing dose of about 2×10^{10} CFUs per person), and all survived. These experiments served as a foundation for introducing the "Strong criterion"—one loop of an agar culture of a vaccine strain that does not kill a guinea pig is not dangerous for a human being [135]. Later, the following attenuated *Y. pestis* strains were used to immunize people: EV, Tjiwidej, K-120, no. 2, MP-40 (M211-40), 1, 17, K-1, etc. [16, 18, 19]. Two of them (EV and Tjiwidej) showed a high efficiency in decreasing plague morbidity in Madagascar and Java, respectively; the strain MP-40 showed its efficiency in Manchuria [18]. However, all of them, with the exception of the strain EV, are only of historical interest at present.

The *Y. pestis* strain EV has been used as a living plague vaccine for people for already about 80 years. It effectively wards off death from the bubonic and pneumonic form of the infection [18, 23, 119, 124]. The initial virulent strain was isolated in 1926 by G. Girard and J. Robic in Madagascar from the corpse of a girl who had died from bubonic plague and then was attenuated by serial reinoculations of artificial nutrient media at 18–25°C for 6 years. The loss of virulence by the vaccine strain EV was much later shown to be due

to spontaneous deletion of the *pgm*-locus—the chromosome fragment with a length of 102 kb that includes the *hms*-locus (responsible for hemin storage) and a cluster of genes ensuring the biosynthesis and transport of siderophore (yersiniabactin) [112]; the probability of its "direct reversion" (if there is no possibility of horizontal gene transfer from other microorganisms that may not be present in the mother culture of the vaccine strain by definition) is close to zero. In 1932, the creators of the vaccine strain started its large-scale testing on people [69]. After the first test vaccination in 1932, the strain EV was passed on to the laboratories of many countries to study and make a live plague vaccine, and it came to the Soviet Union in 1936. After the Second World War, the high epidemiological efficiency of the EV strain-based vaccine was proven by Soviet physicians in China. Different lines of the *Y. pestis* strain EV were introduced into more than 10 million people [21, 23].

Most countries do not use the dry live plague vaccine for reasons of safety, since the reversion of the vaccine strain EV to virulence was not excluded before the moment of determining the cause of its attenuation. One more reason for the refusal of using the EV stain-based plague vaccine is the capability to cause heavy local and system reactions and even a generalized infectious process in people with a depressed immune status [20, 98, 112], as well as lethal infection in some species of non-human primates [98] independent of introduction modes.

COMMERCIAL PLAGUE VACCINES

Until recently, health protection was practiced using two plague vaccines—the live vaccine based on the attenuated *Y. pestis* strain EV and killed USP vaccine based on the virulent *Y. pestis* strain 195/P [20, 112].

The dry live plague vaccine produced at present in Russia (*vaccinum pestosum vivum siccum*) is a suspension of living bacteria of the plague microbe vaccine strain EV of the NIIEG line that is freeze-dried in a sucrose-gelatin medium with thiourea or in a sucrose-gelatin medium with glutamin-acid sodium, thiourea, and peptone or in a lactose-dextrin medium with thiourea and ascorbic acid. Plague epizootics in rodents and the possibility of bringing in an infection with a sick person, as well as works with the virulent *Y. pestis* strains at a laboratory are indications for vaccination. The vaccine can be introduced by the cutaneous, intracutaneous, subcutaneous, intramuscular, and inhalation ways [8]. The same series of the vaccine can be used for any introduction mode depending on dissolving the initial preparation. In addition, there is a tablet form of the vaccine for oral use. The vaccine is inoculated once. It ensures immunity to both bubonic and pneumonic plague with a duration of up to 1 year. Revaccination is implemented in 12 months [20].

After the live plague vaccine is introduced, total and local postvaccine reactions can arise, the intensities of which depend on the individual properties of inoculated people and vaccination method. In the case of the cutaneous mode (scarification), edemas, hyperemia, vesicular rash along incisions, and sometimes infiltration can appear; lymphangitises and regional lymphadenitises develop less often. A local reaction starts to manifest itself in 8–10 h and reaches its maximum 24–48 h after vaccination. In 1% of vaccinated people, a total reaction can be accompanied by a rise in temperature up to 37.6–38.5°C over 1 day. A local reaction to subcutaneous and intracutaneous inoculations is characterized by extended hyperemia, swelling, tenderness of soft tissues in the place of introduction. Regional lymphadenitises develop less often. In the case of these introduction modes, a local reaction starts to develop in 6–10 h, reaches its maximum in 24–48 h, and disappears 4–5 days after vaccination. A total reaction manifests itself in indisposition, headache, and rise in a body temperature up to 37.5 (a weak reaction), 37.6–38.5 (a medium reaction), and 38.6°C and more (a strong reaction). Medium and strong reactions are observed in 29 and 5% of vaccinated people, respectively. A total reaction takes place on the first to second day and usually disappears 1–3 days after vaccination [20].

The vaccine is kept in a dark dry place at a temperature of from –20 to 6°C. At room temperature (20–25°C), the vaccine can be kept for up to 2 months, including the transportation period. The application time of the vaccine produced in a sucrose-gelatin medium with thiourea is 2 years with subsequent recontrol and prolongation for 1 more year; the application time of the vaccine in a sucrose-gelatin medium with glutamin-acid sodium, thiourea, and peptone is 3 years from the moment of drying and recontrol in 3 and 5 years and its prolongation for 2 more years each time; a storage time of up to 5 years has been established for the vaccine in a lactose-dextrin medium with thiourea and ascorbic acid after the control of its vital capacity 6 months after the moment of production with subsequent recontrol and prolongation for 2 more years [20].

The USP vaccine was made in the United States (Cutter Biological) from cells of the virulent strain 195/P inactivated by formaldehyde and conserved by 0.5% phenol [65, 157]. The killed vaccine efficiently protects from bubonic plague, but not from pneumonic plague. Revaccination was performed every year. The major disadvantages of the killed vaccine are a need to work with the virulent strain up to the moment of its inactivation under the conditions of a BSL-3 laboratory, high cost of preparation, need for repeated introduction with the purpose of developing immunity, and presence of local and total reactions in 11–24 and 4–10% of vaccinated people, respectively.

MAJOR AREAS OF CONSTRUCTING NEW-GENERATION PLAGUE VACCINES

Analysis of the contribution made by separate pathogenicity factors to the virulence of *Y. pestis* and protective activity with respect to plague in combination with developing a genetic-engineering technology and available information on the full-genome sequences of a whole series of plague microbe strains has enabled the formation of several areas in producing modern plague vaccines.

The greatest success has been achieved in constructing subunit (chemical, molecular) vaccines. British scientists from the Defense Science and Technology Laboratory (Porton Down) have carried out the two first phases of clinical tests of a subunit vaccine that includes a mixture of recombinant *Y. pestis* antigens—F1 and LcrV [59, 120, 131, 154]. Researchers at the US Army Medical Research Institute of Infectious Diseases (Fort Detrick, United States) are testing a F1-LcrV fusion protein coded by a chimeric gene that includes the nucleotide sequences of two initial genes in one open reading frame [60, 114, 120, 131]. Protective antigens can be developed in transgenic plants (tomatoes, potatoes), which significantly decreases the cost of the final product. These chimeric vegetables with bacterial antigens hold promise for use as the most conveniently applied "edible" vaccine [30]. Subunit vaccines based on the F1 and BSA or F1 and B antigen that have been suggested by Russian researchers are also undergoing restricted clinical tests [6, 10]. Meanwhile, the F1 and LcrV-based vaccines have been discovered to perfectly protect mice and cynomolgus macaques (*Cynomolgus macaques*) from plague but to be insufficiently efficient in experiments on green monkeys (*Chlorocebus aethiops*) [131].

LcrV has been shown to suppress synthesis of cytokines, such as γ-interferon and tumor necrosis factor α [105], owing to stimulating the production of IL-10, which is a repressor of the above-indicated cytokines [106]. These properties of the V-antigen revealed in experiments on mice give grounds to suppose that an expressed immune-suppressive effect that is analogous to that in the case of immunizing guinea pigs with microgram amounts of F1 can be obtained using a model of other animals [133]. However, since the immune-dominant epitopes of the LcrV [70, 75, 84, 117] and F1 [70, 143, 163] antigens are known at present, construction of proteins that have lost their pathogenetic properties but retained protective properties does not present significant difficulties. Thus, a group of researchers from the University of Chicago has shown that a short deletion from the 271-th to the 300-th amino-acid residue results in the formation of LcrV that has a decreased capability to induce production of IL-10 but can induce protective immunity [108, 121]. A protective polypeptide with analogous properties has been obtained by Z. Qi et al. [118] by completely removing the C-terminal section of LcrV starting from the 271st amino-acid residue.

Indian researchers from the laboratory of D.N. Rao have constructed a whole series of chimeric proteins, having included only the protective B and T-cellular epitopes of F1 and LcrV among them [70]. At the present moment, the chimeric proteins are undergoing comprehensive assessment of their capability to stimulate different parts of immune system in the case of varied introduction and presentation modes. Experiments on protection of animals immunized by the B-T-chimeras of F1 and LcrV from death in the case of infection with virulent *Y. pestis* strains have not yet been described.

The living analogs of the subunit vaccines are recombinant vaccines based on heterological human-avirulent microbes, such as *Salmonella* spp., *Lactococcus* spp., adenoviruses, stomatitis, and raccoon pox viruses, in which *lcrV* and *lcrV-caf1* genes and a whole *caf*-operon are cloned. They also induce an immune response to one or two antigens and simultaneously stimulate not only the production of antibodies, but also T-cellular immunity, in contrast to the subunit vaccines [59, 131].

Concurrently, research aimed at developing a new generation of live plague vaccines [107, 110] that stimulate not only humoral, but also cellular, immunity are growing in number. The interest in live vaccines is largely due to the fact that the F1 and V-antigen based subunit vaccines are insufficiently efficient for immunization of guinea pigs and African green monkeys (*Cerpithecus aethiops*) [131].

Potential target genes are chosen to attenuate virulent strains using random mutagenesis by individually labeled transposons [62] with application of reverse vaccinology methods [68, 122, 136], or analogs of genes of other bacterial pathogens are studied in which mutations result in a decreased virulence [110]. In order to construct vaccine strains of the plague microbe, W. Sun et al. [137] have developed an efficient two-stage method of recombination for deletion and/or embedding of DNA fragments without incorporating additional nucleotide sequences into the *Y. pestis* genome that can be used for repeated genetic manipulations. The method combines λ Red recombination and counterselective screening using the *sacB* gene.

Different laboratories have preliminarily assessed the vaccine properties of the mutant *Y. pestis* strains with deletions in the *relA* and *spoT* [130], *dam* [123], *yopH* [46], *smpB-ssrA* [107], and *guaBA* [110] genes. For most of the investigated mutants, the loss of virulence was combined with a high immunogenicity that often surpassed the analogous indicator of the vaccine strain EV76. The possibility of precision attenuation of pathogens has also revived the interest in living plague vaccines based on *Yersinia pseudotuberculosis* [6, 39, 141] and *Salmonella enterica* [42].

One more variant of constructing precisely attenuated strains is to introduce avirulence genes into their genome and stabilize them in it. Thus, when a plasmid with the expressing *Escherichia coli* gene that codes an LpxL acyltransferase is introduced into *Y. pestis* cells, the plague microbe forms LPS molecules with six rather than four fatty-acid residues at 37°C, which results in rapid recognition and destruction of bacterial cells by the innate immune system [138]. When the proper YopJ effector protein characterized by the decreased capability of translocation into eukaryotic cells is replaced in *Y. pestis* cells by the functionally full-value Yop homolog from *Y. enterocolitica*, this results in the formation of plague microbe cells that are cytotoxic with respect to macrophages and decrease virulence in case of subcutaneous infection by seven orders of magnitude [160]. Moreover, the simultaneous subcutaneous infection of animals with the initial virulent $YopJ^+YopP^-$ and attenuated $YopJ^-YopP^+$ cultures did not lead to the death of animals [160], just as in case of the survival phenomenon of N.N. Ginsburg [4, 9]. The protective action of the $YopJ^-YopP^+$ variant of the plague microbe that is cytotoxic with respect to macrophages was noted at an $\geq 10:1$ ratio to the virulent strain (when introducing 10^4 LD_{50} virulent strain).

One more approach to improving living plague vaccine is to decrease its reactogenicity induced by the LPS with six fatty-acid residues by deletion of the *lpxM* gene coding the acyltransferase that is responsible for the embedding of the sixth fatty-acid residue into the lipid A [13, 29, 34]. A series of comparative experiments with Δ*lpxM*:*kan* (the *lpxM* gene was replaced by the gene ensuring resistance to kanamycin) derived from the vaccine strain EV of the line NIIEG and initial strain have shown that the decrease in reactogenicity is accompanied by the reliable growth in the protective activity of the mutant strain [29, 34, 59–61].

CONCLUSIONS

Despite the significant number of publications devoted to improving the plague vaccine prevention, an ideal vaccine has not yet been created. Each of the reviewed vaccine preparations (or candidates for vaccine preparations) has both advantages and disadvantages. At present, it is already not disputed that, compared to the chemical and subunit vaccines, the living attenuated strains of pathogenic microorganisms can stimulate a much more efficient immunity that approaches in expression to the postinfection immunity and protects from infection with pathogen variants with different antigen structures. However, revaccination with living plague vaccines may be performed not earlier than in 12 months [20]. In earlier terms, a sufficiently expressed immunity persists that does not permit cells of a vaccine strain to propagate and persist in an immunized organism for a limited period sufficient for revaccination. The immunization scheme suggested by S.M. Dalvadyants et al. [10] that includes the immunization with a living vaccine and then the revaccination by a subunit vaccine is evident to be optimal for the urgent revaccination in early periods of time after vaccination with the living vaccine.

Searches for an optimal attenuation method during constructing a vaccine strain or determination of the antigen/epitope and adjuvant structures of a molecular vaccine, as well as methods for its presentation, are continuing.

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