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Yersinia pestis Live Vaccine with Improved Characteristics

V.A. Feodorova[a,*], L.N. Pan'kina[a], E.P. Savostina[a], O.S. Kuznetsov[a], N.P. Konnov[a], L.V. Sayapina[b], S.V. Dentovskaya[c], R.Z. Shaikhutdinova[c], S.A. Ageev[c], B. Lindner[d], A.N. Kondakova[e], N.A. Kocharova[e], S.N. Senchenkova[e], O. Holst[d], G.B. Pier[f], Y.A. Knirel[e], A.P. Anisimov[c], and V.L. Motin[g]

[a] *Russian State Anti-Plague Research Institute "Microbe", 46, Universitetskaya Street, 410005 Saratov, Russia*
[b] *Tarasevich State Institute for Standardisation and Control of Biomedical Preparations, 41, Sivtsev Vrazhek, 121002 Moscow, Russia*
[c] *State Research Center for Applied Microbiology and Biotechnology, 142279 Obolensk, Moscow Region, Russia*
[d] *Research Center Borstel, Leibniz Center for Medicine and Biosciences, D-23845, Borstel, Germany*
[e] *N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, 47, Leninskii Prospect, 11999, Moscow, Russia*
[f] *Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA*
[g] *Departments of Pathology/Microbiology & Immunology, University of Texas Medical Branch, Galveston, TX 77555, USA*

Abstract

The live plague vaccine strain *Y. pestis* EV line NIIEG widely used for human and animal immunisation proves a high level of specific immunity against both bubonic and pneumonic plague. We constructed an EV NIIEG mutant in the acyltransferase gene *lpxM* that resulted in the production of a less toxic penta-acylated lipid A of lipopolysaccharide (LPS) of *Y. pestis*, tested the synthesis and immunoreactivity of the major known antigens involved in virulence and eliciting immunity against plague, and also evaluated the protective properties of the *lpxM* derivative.

The expression of antigens was determined by dot-ELISA, immunoblotting, the indirect hemagglutination test, electron microscopy (capsule), and fibrinolytic and plasma-coagulase activities (plasminogen activator Pla). Stumulation of TNF-alpha production with different types of LPS was revealed by using the J774 macrophage-like cell line. The antibody immune response, followed by immunisation, was estimated by ELISA. Protective efficacy was evaluated in mice and guinea pigs by subcutaneous challenge with the wild-type *Y. pestis* 231.

In comparison with the parental strain, the *lpxM* mutant was found to produce a reduced amount of capsular antigen F1, multifunctional virulence factor LcrV and murine toxin Ymt as well as had an altered activity of Pla. Moreover, this mutant possessed a modified cell surface as jusjed by the immunoreactivity with monoclonal antibodies to different carbohydrate epitopes and by the resistant phenotype to the plague diagnostic bacteriophage L-413C. The penta-acylated LPS of *Y. pestis* revealed a reduced capacity of stimulating TNF-alpha *in vitro*. Immunisation with the *lpxM* mutant provided an enhanced protection over the original *Y. pestis* EV NIIEG in both mice and guinea pigs. Thus, the pleiotropic effect of the *lpxM* mutation led to the construction of the live plague candidate vaccine strain with improved characteristics toward safety, reactogenicity and protective properties.

* Corresponding author. Tel.: +7 8452 296 967; fax: +7 8452 200 830.
E-mail address: feodorovav@mail.ru, feodorova@san.ru (V.A. Feodorova).

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1. Main text

Yersinia pestis, the plague pathogen, is a potential agent for both bioweapons and bioterrorism. Since the end of 19th century, when the plague bacillus was discovered by Alexander Yersin, a number of vaccines against plague have been developed. However, only one of them, a live plague vaccine based on the *Y. pestis* strain EV76, was found to be able to induce a marked level of protection against both bubonic and pneumonic plague. The virulent parental strain was initially isolated in 1926 by Girard and Robic from a human case of bubonic plague in Madagascar, attenuated by multiple passages *in vitro* and designated as EV by the initials of the plague patient. The *Y. pestis* strain EV76 is the most frequently used derivative of the EV strain. The strain was transferred to Russia by Girard in 1936 from the Pasteur Institute, Tananarive. Since that, its derivative, the *Y. pestis* EV NIIEG, was used as a live plague vaccine in the USSR and has continued to be used in the countries of the Former Soviet Union (FSU) and in some former French colonies. From the time of the first human vaccination trials in 1932, different lines or subcultures of the EV76 strain including the *Y. pestis* EV NIIEG, were inoculated into more than 10 million people worldwide, having resulted in a considerable decrease of cases of fatal human plague [1-5].

The main advantage of the *Y. pestis* EV NIIEG is its ability to produce a high level of plague immunity in vaccinees after a single injection and to provide a relatively prompt (day 7 post-vaccination) and pronounced immunity in vaccinees lasting, as documented, for 10–12 months against both bubonic and pneumonic plague. The vaccine was found to be effective after administration by different routes, including subcutaneous (*s.c.*), cutaneous, inhalation, and oral applications. The highest level of specific immunity against plague was achieved either by scarifying the dermis followed by injection or after vaccination by inhalation [3-5].

The EV NIIEG strain has been reported to be relatively avirulent among the majority of mammalian species, including baboons (*Papio hamadryas*), and showed a relatively low frequency of adverse effects in humans [3]. For more than 70 years, there has been no evidence of its reversion to virulence in humans or development of any detectable persistence within inoculated individuals [2,3,5]. However, other *Y. pestis* EV76 derivatives were reported to yield severe local and systemic reactions and gross tissue changes in some other species of nonhuman primates independent of the route of inoculation [4]. It has been suggested that the majority of the adverse reactions are likely to be mediated by the endotoxic activity of the *Y. pestis* lipopolysaccharide (LPS), due to a capability of the plague bacteria to produce a highly toxic hexa-acylated lipid A at ambient temperature, although it was found that *in vitro* at 37°C *Y. pestis* produced a less toxic tetra-acylated LPS [6-8]. The toxic highly acylated lipid A phenotype is provided by the *lpxM* gene encoding lauroyltransferase. To develop the EV NIIEG derivative with a reduced toxicity, a *Y. pestis* EV NIIEG mutant was constructed by the allelic exchange resulting in deletion of the *lpxM* gene that was demonstrated by PCR using primers flanking the targeted region [8, 9]. The mutant lipid A composition was confirmed by structural analysis using high-resolution mass-spectroscopy. As expected, the *lpxM*-mutant synthesised at most penta-acylated lipid A which made the strain less toxic for outbred mice than the parent one [8]. When the protective activity and residual virulence (innocuity test) of the *lpxM* mutant and the parental vaccine strain were compared, the mutant was found to be completely avirulent and safe in three animal models used (outbred mice, guinea pigs, inbred BALB/c mice). In this test, outbred mice were inoculated with 1×10^5-1×10^9 CFU of either mutant or EV NIIEG strain, BALB/c mice received 2×10^2-2.5×10^4 CFU, and guinea pigs were vaccinated with 1×10^7, 2×10^9 or 1.5×10^{10} CFU of either strain. There was no systemic spread or deaths among outbred mice and guinea pigs inoculated with the *Y. pestis* EVΔ*lpxM* mutant. At the same time, among the Balb/c mice challenged with the parental *Y. pestis* EV strain NIIEG, there was at least one vaccine-related death in each of the groups given different doses and overall 8 of 28 mice tested died from infection caused by this strain. The *Y. pestis* EV NIIEG bacteria could be found by plating tissue specimens from all dead mice, including those which died 20 days post-vaccination. The results obtained with the blood smears confirmed the presence of the *Y. pestis*

EVΔ*lpxM* mutant survived and no tissue specimens positive for *Y. pestis* were recovered from these animals after they were sacrificed at day 21. Moreover, among the guinea pigs vaccinated with the mutant, a two- to threefold swelling of the lymph nodes were registered at all immunisation doses given. Notably, less swelling of the lymph nodes was found in animals challenged with the *Y. pestis* EV NIIEG, a characteristic feature of this live vaccine strain [9,10].

The parent EV NIIEG strain typically provides protection in the range of 50% of mice and guinea pigs against 200 MLD (2,000 CFU) of a virulent *Y. pestis* strain [10]. Therefore, to document any potential superiority of the *lpxM* mutant over the current vaccine strain, the challenge dose with virulent *Y. pestis* was increased by 6-10 fold, representing 1,200-2,000 MLD (12,000-20,000 CFU). The animals were immunised by *s.c.* injection either with the mutant or the parental strain, followed by challenge with 12,000-20,000 CFU (1,200-2,000 MLD) of the virulent *Y. pestis* 231 strain (lethal dose$_{50}$ (LD_{50}) ≤ 10 CFU). The efficacy of vaccination was estimated by the number of surviving animals. The mutant strain protected both mice and guinea pigs from the high challenge dose of the fully virulent *Y. pestis* strain (85.7% outbred mice against 2,000 MLD, and 42.8% BALB/c mice and 100% guinea pigs against 1,200 MLD, respectively) while all animals vaccinated with the parent strain died. These results clearly demonstrated a superior efficacy of the Δ*lpxM* strain in eliciting a protective immunity and demonstrated a significant improvement of its immunogenic properties in comparison with the parental *Y. pestis* EV NIIEG strain.

Likely, the decreased adverse effects of the mutant can be directly attributed to the production of a modified less toxic LPS. However, it is not apparent why the mutant possessed improved protective properties. One of the possible explanations for the increased protective immunity of the *lpxM* mutant could be an altered expression of major immunoreactive antigens that might result in a modification of their presentation to the host immune system. Indeed, deleting the *lpxM* gene in other bacterial pathogens often leads to pleiotropic effects, resulting in membrane alterations and attenuation in virulence [11,12]. To test this possibility, the *lpxM* mutant of the EV NIIEG was carefully investigated in respect to the synthesis and immunoreactivity of the known major antigens involved in virulence of *Y. pestis* and development of the immunity against plague. The mutant showed a marked reduction in the expression and immunoreactivity as well as changes in the epitope specificity of the major surface proteins, which are involved in formation of resistance to phagocytosis, namely the capsule-like envelope antigen “fraction I” (F1), Pla protease with versatile virulence-associated functions and V antigen, a multifunctional virulence factor and protective antigen of *Y. pestis*. A marked modification was observed in the biosynthesis or epitope specificity of either LPS or galactolipid but not in Enterobacterial Common Antigen (ECA) in the *Y. pestis lpxM* mutant. These findings were confirmed by analysis of protein profiles using SDS-PAGE. The production of capsular antigen F1, Pla protease, V antigen, murine toxin Ymt as well as a few thermoregulated proteins was significantly reduced in the mutant in comparison with the parental EV NIIEG strain cultured either at 37°C or at 28°C. These changes were accompanied by alterations in the corresponding phenotypic activities. Moreover, the *Y. pestis* EVΔ*lpx*M bacteria had a reduced growth rate and demonstrated an altered resistance to the plague diagnostic bacteriophage L-413C. Transmission electron microscopy revealed that a capsule-like substance was produced by the *Y. pestis* EV NIIEG at 37°C but not at 28°C, whereas the *Y. pestis* EVΔ*lpx*M bacteria synthesised little capsule substance at both temperatures.

Although the production of capsule was reduced in the *lpxM* mutant, to our surprise, we found that immunisation of mice with the mutant strain resulted in better antibody titer to this antigen. Typically, *Y. pestis* strains produce a massive amount of the capsule which may provide an immuno-paralitic effect in high doses, particularly in a guinea pig animal model [13]. Therefore, a reduction in the capsule production can be in fact beneficial, perhaps, providing a more optimal dose-effect induction of the humoral immune response.

Finally, we tested directly a stimulating potency of differently acylated *Y. pestis* LPS using macrophage-like cell line J774. The ability of the hexa-acylated LPS to induce production of TNF-α was high and similar to that of control LPS of *E. coli* O55:B5. The penta-acylated *Y. pestis* LPS had a decreased ability to induce the production of TNF-α although it was higher than that of *F. tularensis* LPS, known for its week endotoxic activity. The tetra-acylated LPS demonstrated the reduced stimulating activity in comparison with the penta-acylated LPS.

In conclusion, we found that: (i) the *lpxM* mutant of the live plague vaccine strain EV NIIEG displayed improved characteristics, such as decreased endotoxic activity and overall reactogenicity, and an enhanced protective immunity in comparison with the parental vaccine strain; (ii) the *lpxM* mutation in *Y. pestis* caused pleiotropic effects resulting in modifications of the biosynthesis and epitope specificity of the major surface proteins and carbohydrate antigens accompanied by alterations in the corresponding phenotypic activities; (iii) the *Y. pestis*

more efficient and less toxic live plague vaccines.

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