Vaccine xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

A novel live attenuated anthrax spore vaccine based on an acapsular *Bacillus anthracis* Sterne strain with mutations in the *htrA*, *lef* and *cya* genes

Theodor Chitlaru *, Ma'ayan Israeli, Shahar Rotem, Uri Elia, Erez Bar-Haim, Sharon Ehrlich, Ofer Cohen, Avigdor Shafferman

Department of Biochemistry and Molecular Genetics, Israel Institute for Biological Research, Ness-Ziona, Israel

ARTICLE INFO

Article history:
Available online xxxx

Keywords:
Bacillus anthracis
Anthrax
Live attenuated vaccine
HtrA
Anthrax toxins

ABSTRACT

We recently reported the development of a novel, next-generation, live attenuated anthrax spore vaccine based on disruption of the *htrA* (High Temperature Requirement A) gene in the *Bacillus anthracis* Sterne veterinary vaccine strain. This vaccine exhibited a highly significant decrease in virulence in murine, guinea pig and rabbit animal models yet preserved the protective value of the parental Sterne strain. Here, we report the evaluation of additional mutations in the *lef* and *cya* genes, encoding for the toxin components lethal factor (LF) and edema factor (EF), to further attenuate the SterneΔ*htrA* strain and improve its compatibility for human use. Accordingly, we constructed seven *B. anthracis* Sterne-derived strains exhibiting different combinations of mutations in the *htrA*, *cya* and *lef* genes. The various strains were indistinguishable in growth *in vitro* and in their ability to synthesise the protective antigen (PA, necessary for the elicitation of protection). In the sensitive murine model, we observed a gradual increase (Δ*htr*A < Δ*htr*AΔ*cya* < Δ*htr*AΔ*lef* < Δ*htr*AΔ*lef*Δ*cya*) in attenuation – up to 10^8-fold relative to the parental Sterne vaccine strain. Most importantly, all various SterneΔ*htr*A derivative strains did not differ in their ability to elicit protective immunity in guinea pigs. Immunisation of guinea pigs with a single dose (10^9 spores) or double doses (>10^7 spores) of the most attenuated triple mutant strain SterneΔ*htrAlef*^{MUT}Δ*cya* induced a robust immune response, providing complete protection against a subsequent respiratory lethal challenge. Partial protection was observed in animals vaccinated with a double dose of as few as 10^5 spores. Furthermore, protective immune status was maintained in all vaccinated guinea pigs and rabbits for at least 40 and 30 weeks, respectively.

1. Introduction

The gram-positive spore-forming *Bacillus anthracis* is the aetiological agent of anthrax, a currently rare disease in humans that is however potentially associated with intentional bio-terror use and, accordingly, considered a top select agent bio-weapon [1–4].

1.1. Bacillus anthracis pathogenesis

Bacillus anthracis is an obligate pathogen in that bacterial reproductive cycles occur only in a suitable host; in nature, it exists in the form of spores that are metabolically inert and resistant to environmental challenges. Anthrax is mainly a disease of herbivores that inhale or ingest spores while grazing. Human cutaneous and gastro-intestinal forms of anthrax are caused by the penetration of spores via skin abrasions or the ingestion of spores present in contaminated animal products. Inhalation anthrax, the most severe manifestation of the disease, is caused by inhalation of *B. anthracis* spores, which are taken up by alveolar macrophages and transported to the lymph nodes. Spores germinate into fast-dividing vegetative cells that secrete toxins and virulence factors (for a review, see [2,5,6]), resulting in massive bacteraemia and, consequently, generalised systemic failure and death. On an historical note, live attenuated vaccines against anthrax were developed early in modern medicine through the pioneering work of Louis Pasteur (see the republished classic Pasteur article in [7]). The virtual eradication of anthrax as a major agricultural-occupational concern highlights the outstanding contribution to public health of an extensive campaign of vaccination [2,8].

* Corresponding author at: Israel Institute for Biological Research, PO Box 19, Ness-Ziona 74100, Israel.
E-mail address: theodorc@iibr.gov.il (T. Chitlaru).

http://dx.doi.org/10.1016/j.vaccine.2017.03.033
0264-410X/

The lethality of anthrax involves the *B. anthracis* exotoxins and the remarkable proliferation ability of the bacteria in the host which indicates that *B. anthracis* is optimally equipped with physiological functions required for resilience to stress insults encountered during infection. *B. anthracis* secretes lethal toxin (LTx) and edema toxin (ETx), composed of binary combinations of the three proteins protective antigen (PA), lethal factor (LF) and edema factor (EF). PA, the non-harmful common subunit of both toxins, plays the essential role of mediating the intracellular translocation of the lethal subunits of the toxin complex LF (a zinc protease, [9]) and EF (an adenylate cyclase, [10–12]). PA, LF and EF are encoded by the *pag*, *lef* and *cya* genes, respectively, located on pXO1, one of the two virulence plasmids naturally harboured by *B. anthracis*. A polyglutamate anti-phagocytic capsule is synthesised by enzymes encoded by genes located on the second native plasmid, pXO2. The contribution of each of the toxins to *B. anthracis* pathogenesis may differ among various animal genera [13–15]; however, anthrax is considered a toxinogenic disease because of the lethality of pure toxin preparations and because mutational abrogation of toxin function results in significant virulence attenuation. In addition to the toxins, during infection, *B. anthracis* secretes a large number of proteins, many of which have biological functions indicative of a role in the onset and progression of the disease [16–18].

The protein HtrA (high temperature requirement A), which emerged as an important *B. anthracis* virulence factor from genomic-proteomic-serological global surveys [16–23], is a chromosomally encoded bifunctional chaperone/protease abundantly expressed during infection [24]. Disruption of the *htrA* gene in the virulent Vollum strain demonstrated that HtrA is necessary for tolerance to a variety of stress stimuli and resulted in dramatic attenuation in guinea pig, rabbit and murine models, although the mutated bacteria were able to express the PA, LF and EF toxin subunits and the capsule [25,26]. The virulence attenuation associated with *htrA* disruption was significantly higher than that promoted by the disruption of other *B. anthracis* virulence determinants (other than the LT and ET toxins and the capsule biosynthetic operon themselves), which suggests a role in bacterial pathogenesis [25].

1.2. Anthrax vaccines

PA, the non-detrimental toxin subunit, elicits a protective immune response and therefore represents the basis for all preventive anthrax countermeasures. Accordingly, anthrax protein vaccines that are licensed for human use include partially purified PA preparations potentiated by various adjuvants [2,27–30].

Live attenuated toxin-producing bacteria, such as the nonencapsulated Sterne strain ($pXO1^+$; $pXO2^-$) are currently suitable in the Western world, for veterinary purposes only. However, the protection conferred by the Sterne live vaccine to experimental animals may be superior to that provided by vaccination with PA regarding the longevity of the protective response, efficacy against a broad spectrum of strains and the need for fewer administrations (1–2 injections, compared to the initial cumbersome 3–6-dose vaccination schedule with the existing licensed subunit native-PA vaccine or novel recombinant PA-based vaccines [31,32]). Furthermore, non-toxinogenic *B. anthracis* cells can induce partial immune protection, suggesting that other essential unknown antigens in addition to the "classical" toxins contribute to protection against anthrax [33,34]. This notion is substantiated by the observation that many *B. anthracis* proteins, other than the LT and ET toxins, are secreted or exposed during infection and are potent immunogens [20–22,35,36], strongly suggesting that live attenuated vaccines may be superior to the subunit PA vaccine.

Further attenuation of a Sterne vaccine strain may likely be a beneficial step in the development of next-generation live anthrax vaccines for human use. Indeed, we recently documented that disruption of the *htrA* gene in the *B. anthracis* Sterne-vaccine platform is sufficient to generate a highly attenuated strain that preserves its ability to induce a protective immune response [25]. In the present study, this novel live Sterne*ΔhtrA* vaccine was further attenuated through the inclusion of additional mutations in the toxin genes. A multiple mutated acapsular Sterne*ΔhtrA/Δcya/lef*^{MUT} vaccine exhibiting disruption of the *htrA* and *cya* genes and mutation of the Zn-binding domain of LF, which abrogates its proteolytic activity, is shown to be highly attenuated while preserving the ability to confer robust protective immunity in guinea pigs and rabbits.

2. Materials and methods

2.1. Bacterial strains, media, and growth conditions

The *B. anthracis* strains used in this study are listed in the online Supplementary Table S1. The cells were cultured either in brain-heart infusion (BHI, Difco/Becton Dickinson, MD, USA), Terrific broth (TB, 1.2% tryptone, 2.4% yeast extract, 0.5% glycerol, 17 mM KH_2PO_4, and 72 mM K_2HPO_4) or Dulbecco's modified Eagle medium (DMEM, Biological Industries, Beit Haemek, Israel) at 37 °C with vigorous agitation. For expression of CO_2-induced toxins, the cells were grown at 37 °C in DMEM supplemented with 10% foetal calf serum (FCS, Biological Industries, Beit Haemek, Israel) without agitation under 10% CO_2. The spores were prepared in Schaeffer's sporulation media (SSM) at 30 °C for 72 h with vigorous shaking. In all cases, the cultures were initiated with a starter inoculum diluted to a final OD (660 nm) of 0.1. *Escherichia coli* strains (Supplementary Table S1, online) were used for plasmid construction. The antibiotic concentrations used for selection in Luria-Bertani (LB, Difco) agar/broth were as follows: for *E. coli* strains, ampicillin (Ap, 100 μg ml^{-1}); for *B. anthracis* strains, kanamycin (km, 10 μg ml^{-1}), chloramphenicol (Cm, 5 μg ml^{-1}) and erythromycin (Em, 5 μg ml^{-1}).

2.2. Plasmid and strain construction

The plasmids and oligonucleotide primers used in this study are summarised in Supplementary Table S1. The oligonucleotide primers were designed according to the genomic sequence of *B. anthracis* "Ames ancestor" strain (accession number AE017334, GI 50082967). Genomic DNA was extracted as previously described [37,38]. PCR amplifications were performed using the *Taq* (Qiagen) or Expand High Fidelity (Roche) systems. The DNA sequences were determined using the ABI rhodamine termination reaction (ABI310 Genetic Analyzer, Applied Biosystems). The plasmids used for *cya* and *lef* disruption and the I-sceI expression vector have been previously described [13,39]. pEGS-LF^{Mut} was used for the construction of strains harbouring mutations in the Zn-binding domain of LF. The H686A and H690A [9,40] mutations were introduced by PCR using specific primers (Supplementary Table S1). A silent T/C conversion, which generated a diagnostic EcoRI site, was tailored into the primer that generated the DNA replacement segment (see Fig. 1). pEGS-LF^{Mut} was constructed in five steps as follows: (i) the vector pEGS-EF (Table X) was digested with NotI and AscI and was gel purified; (ii) a NotI-EcoRI 526 bp restriction fragment of the *lef* gene was derived by PCR using BA771 (forward) and BA772 (reverse) primers; (iii): an EcoRI-AscI 525 bp restriction fragment of the *lef* coding region and a 3′ UTR was derived by PCR using BA773 (forward) and BA774 (reverse) primers; (iv) a NotI-AscI LFMut cassette was generated by PCR using BA771 (for-

Please cite this article in press as: Chitlaru T et al. A novel live attenuated anthrax spore vaccine based on an acapsular *Bacillus anthracis* Sterne strain with mutations in the *htrA*, *lef* and *cya* genes. Vaccine (2017), http://dx.doi.org/10.1016/j.vaccine.2017.03.033