Construction of A Bivalent Vaccine Against Anthrax and Smallpox Using The Attenuated Vaccinia Virus KVAC103

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Abstract

Background: Anthrax and smallpox are high-risk infectious diseases, and considered as potential agents for bioterrorism. To develop an effective countermeasure for these diseases, we constructed a bivalent vaccine against both anthrax and smallpox by integrating a gene encoding protective antigen (PA) of *Bacillus anthracis* to the genome of the attenuated vaccinia virus strain, KVAC103.

Results: Immunization with this bivalent vaccine induced antibodies against both PA and vaccinia virus in a mouse model. We also observed that the efficacy of this vaccine can be enhanced by combined immunization with immunoadjuvant-expressing KVAC103. Mice groups co-immunized with PA-expressing KVAC103 and either interleukin-15 (IL-15) or cholera toxin subunit A (CTA1)-expressing KVAC103 showed increased anti-PA IgG titer and survival rate against *B. anthracis* spore challenge compared to the group immunized with PA-expressing KVAC103 alone.

Conclusions: We demonstrated that the attenuated smallpox vaccine KVAC103 is an available platform for a multivalent vaccine and co-immunization of immunoadjuvants can improve vaccine performance.

Background

Bacillus anthracis and *Variola* virus are causative agents of anthrax and smallpox, respectively, and representative pathogens that can be possibly utilized as bioterrorism or biological weapons. Development of effective medical countermeasures against these pathogens is a national task of high priority [1, 2].

The biological attack in 2001 by *B. anthracis* spores via the US postal system has prompted the need to develop vaccines and therapeutics against anthrax [1]. Protective antigen (PA) is one of the major component of anthrax toxin, and also a principal ingredient of two licensed anthrax vaccines, Anthrax Vaccine Adsorbed (AVA) and Anthrax Vaccine Precipitated (AVP) [3]. Recently, a recombinant PA protein vaccine is being developed by Korea Centers for Disease Control (KCDC), and clinical trials are in progress [4, 5].

Although endemic smallpox was declared eradicated since the last case observed in 1977, *Variolar* virus still remains a potential biological weapon [2], and smallpox vaccines have been stockpiled for strategic use in some nations. To reduce side effects of conventional smallpox vaccines, attenuated vaccinia virus strains have been investigated in various ways [6]. KVAC103 is an attenuated vaccinia virus developed by KCDC [7].

Interleukin-15 (IL-15) is a cytokine involved in the proliferation and maintenance of $CD8^+$ memory T cells, and has been suggested as an effective vaccine adjuvant [8, 9]. Previous studies on HIV-1 vaccine demonstrated that co-immunization of IL-15 strongly increased antigen-specific memory T cells and long-term immunity [10, 11]. Smallpox vaccines with integrated IL-15, tested in a mouse model, showed increased and prolonged cellular and humoral immunity [12]. This IL-15-containing smallpox vaccine also

has been applied in a multivalent influenza vaccine [13]. Co-administration of IL-15 with staphylococcal enterotoxin B vaccine increased the number of dendritic cells in a mouse model [14].

Cholera toxin (CT) also has long been investigated as an efficient immunoadjuvant. The toxin is composed subunit A and B, and subunit A contains two fragments, A1 and A2 [15]. The ADP-ribosyltransferase activity of cholera toxin subunit A1 (CTA1) is known to be important for enhancing immune responses [16]. The effect of CTA1 as an immunoadjuvant has been demonstrated against numerous pathogens, such as influenza A virus, HIV, *Helicobacter pylori*, and *Mycobacterium tuberculosis* [17–20].

Vaccinia virus is a popular platform for gene transfer and multivalent vaccine against various diseases [21, 22]. In a previous study, a dual vaccine for smallpox and anthrax has been developed by inserting PA gene of *B. anthracis* into Wyeth or modified vaccinia Ankara (MVA) strain [23]. A viral vector system that utilizes KVAC103 as a gene delivery system and a multivalent vaccine has been previously invented [7, 24]. In this study, we constructed a bivalent vaccine candidate against both smallpox and anthrax, by integrating a recombinant anthrax PA-encoding gene into KVAC103, using a viral vector pVVT1-EGFP-C7L. We examined the protective efficacy of KVAC103-derived bivalent vaccine in a mouse model. In addition, we observed that the vaccine supplemented with immunoadjuvant such as IL-15 or CTA1 can increase immune response against anthrax.

Results

A human codon-optimized PA was cloned into viral vector pVVT1 to generate smallpox/anthrax dual vaccine candidate. A signal peptide derived from the tissue plasminogen activator was attached to the N-terminal of PA (thPA). We also constructed viral vector clones encoding a human IL-15 (hIL15) or a human codon-optimized CTA1 (hCTA1) gene. The viral vectors were integrated into the KVAC103 genome by homologous recombination at the thymidine kinase (TK) gene site (Fig. 1).

Protein expression of PA and CTA1 in the dual vaccine candidate viruses were confirmed by immunoblot assay (Fig. 2A). PA and CTA1 were detected in virus-infected cell lysates. This indicates that cells infected by KVAC-thPA-C7L or KVAC-hCTA1-C7L viruses properly express PA or CTA1, respectively. The IL-15 ELISA result shows that cells infected by KVAC-hIL15-C7L also secreted IL-15 *in vitro* (Fig. 2B).

The *in vivo* efficacy of the dual vaccine candidate virus with or without adjuvant-expressing viruses was estimated in a mouse model (Fig. 3). We immunized A/J mice ($n = 8$) with our vaccine candidate KVAC-thPA-C7L with or without adjuvant expressing viruses 2 times with a 3-week interval. The anit-PA antibody levels of all groups immunized with KVAC-thPA-C7L were increased compared to the groups immunized with the adjuvant only (KVAC-hIL15-C7L or KVAC-hCTA1-C7L). Mice groups vaccinated with KVAC-thPA-C7L plus an immunoadjuvant-containing strain (KVAC-hIL15-C7L or KVAC-CTA1-C7L) exhibited higher median values of antibody titers compared to the group immunized with KVAC-thPA-C7L only (Fig. 3A). The result indicates that co-expression of immunoadjuvant stimulated the production of antibodies

Neutralizing antibodies against vaccinia virus in mice sera were measured by PRNT assay. Unlike the anti-PA antibodies, production of neutralizing antibodies against vaccinia virus does not appear to be significantly affected by the presence of immunoadjuvant (Fig. 3B). In a previous study, IL-15 expressing vaccinia virus induced increased neutralizing antibodies compared to the control vaccinia virus in a mouse model [12]. We suppose that the little difference of neutralizing antibody titers between sera immunized with and without adjuvant expressing virus in this study was because the amount of antibodies induced by KVAC-thPA-C7L without adjuvant was large enough to reach to the saturation level and the adjuvant effect could not be observed.

Immunized mice were challenged with *B. anthracis* Sterne spores 3 weeks after the final vaccination. Survival rates were monitored for 2 weeks. All mice immunized with adjuvant only were dead within a week. In the group immunized with KVAC-thPA-C7L only, 62.5% of mice survived, while groups immunized with both KVAC-thPA-C7L and immunoadjuvant expressing virus (KVAC-hIL15-C7L or KVAC-CTA1-C7L) were fully protected from the challenge (Fig. 3C). The result indicates that enhanced immunity achieved by co-expression of adjuvant can protect the mice more effectively.

Discussion

Poxviruses have been often used as a vector system for vaccines because of their large DNA genome and convenience in manipulation [21, 22]. In a previous study, engineered vaccinia strains expressing both PA and IL-15 showed enhanced immunogenicity against *B. anthracis* compared to the conventional anthrax vaccine AVA in animal test [23]. Our result presented that the co-expression of IL-15 in KVAC103 also enhanced protective efficacy of our bivalent vaccine. Co-expression of CTA1 induced immune response against the PA-expressing vaccine in the similar level to IL-15. Our result demonstrated that co-immunization of CTA1, as well as IL-15, was effective enough to enhance the immune responses against PA and reconfirmed that CTA1 is a suitable adjuvant for multivalent vaccines derived from KVAC103. This result is the first observation of the effect of CTA1 as an immunoadjuvant in a viral vaccine system.

Conclusion

In summary, we explored the possibility of developing a bivalent vaccine using KVAC103, an attenuated vaccinia virus strain. Like other vaccinia virus strains previously utilized, it is confirmed that KVAC103 also can serve as a useful platform for multivalent vaccines. In addition, the vaccine can be further effective with the supplement of cytokines or adjuvants.

Methods

Cell and virus

Vero cell (African green monkey kidney cell) was purchased from the American Type Culture Collection (ATCC, USA). Cells were grown in Opti-Eagle`s Minimum Essential Medium (Opti-MEM, Invitrogen)

supplemented with 2% heat-inactivated fetal bovine serum (FBS, Invitrogen), incubated at 37°C, and humidified with 5% CO_2. The attenuated vaccinia virus strain KVAC103 and the viral vector pVVT1-EGFP-C7L were provided by Korea National Institute of Health (KNIH). This vector contains the vaccine virus C7L gene which encodes interferon antagonist, and this is one of the 26 genes defective in KVAC103 compared to its ancestor strain. This gene is required for enhanced viral reproduction [24].

Construction of anthrax/smallpox dual vaccine candidate vectors

Viral vector constructs were generated using the pVVT1-EGFP-C7L vector [24] as a template (Fig. 1). Human IL-15 gene and human codon-optimized *B. anthracis* PA and CTA1 genes were synthesized (Bioneer). The synthesized genes were cloned into the vector using *Sfi*I restriction enzyme site. The constructed vectors were mixed with Lipofectamin (Invitrogen) and transfected into KVAC103-infected Vero cells. Single plaques were isolated from the original infected cells and verified using PCR.

Virus preparation

Viruses were infected to mono-layered Vero cells with 0.01 MOI. The virus-infected cell media were harvested when more than 80% of total cells showed cytopathic effect. From the harvested culture supernatant, viruses were collected by ultra-centrifugation. The pellet was resuspended in 1× PBS, pH 7.0 (Gibco). The concentration of viral particles was determined by the standard plaque assay.

Western blot analysis

Virus-infected Vero cells or their culture supernatants were lysed in 1× RIPA buffer (G-Bioscience) containing 1% PMSF (Theromfisher Scientific) at 4°C. Proteins were resolved on denaturing polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane (Amersham). Expression levels of PA and CTA1 proteins were detected using monoclonal antibodies against PA and cholera toxin, respectively (Abcam).

Mouse immunization and serum collection

Female A/J mice (5-week old) were purchased from SLC, Inc (Japan) and housed in an animal biosafety level 2 (ABL2) facility in KCDC. Mice were immunized with the vaccine candidate virus (5×10^7 pfu/mouse) with or without the adjuvant-expressing virus (5×10^7 pfu/mouse) 2-times at 3-week intervals subcutaneously (*s.c.*). Mice sera were collected 20 days after final immunization to measure anti-PA IgG and vaccinia virus plaque reduction neutralizing antibody titers.

Enzyme-linked immunosorbent assay (ELISA)

The anti-PA IgG titers of mice sera were determined by ELISA as previously described with some modifications [25]. Briefly, 96 well plates were coated with 1μg/ml of recombinant PA (Green Cross, Korea). Serially diluted mice sera were loaded to each well and incubated for 1 h at 37°C. Horseradish [redacted] antibody (Invitrogen) and 3,3, 5, 5-tetramethylbenzidine

(TMB) substrate were used for detection. The optical density of each well was measured at 450 nm and the half maximal effective concentration (EC_{50}) was calculated by 4-parameter logistic equation regression using SoftMaxPro5.3 (Molecular Device, USA).

The IL-15 expression level of KVAC103 derivatives were determined by the IL-15 ELISA kit (Biolegend) according to the manufacturer's protocol.

Plaque reduction neutralization test (PRNT)

Serial two-fold dilutions of heat-inactivated mouse sera were mixed with vaccinia virus Lister strain of approximately 50 plaque forming units (PFU). After 2 h incubation at 37°C, the serum and virus mixtures were inoculated onto monolayered Vero cells. After two days incubation at 37°C with 5% CO_2, cells were fixed and stained using a mixture of crystal violet and formalin for 10 minutes. Stained plates were dried in air at room temperature and the plaque numbers were counted. The neutralizing antibody titer of serum was defined as the reciprocal of dilution fold that reduced plaque number in half (50%) compared to a control.

B. anthracis spore challenge

Immunized mice were challenged with 50-fold of lethal dose 50 (LD_{50}) of *B. anthracis* Sterne spore by *s.c.* injections. Survival of the mice was monitored for 14 days. Spores were prepared according to a previous study [26]. The LD_{50} determined by Reed-Muench method [27] in A/J mice model via *s.c.* route was 1794 spores. Survived animals were euthanized using CO_2 gas. Animal study protocols (KCDC-102-16-2A and KCDC-039-17-2A) were approved by the Institutional Animal Care and Use Committee (IACUC) of Korea Centers for Disease Control and Prevention (KCDC). All procedures involved in the housing and care of animal strictly followed guidelines and requirements of the IACUC.

Abbreviations

AVA: Anthrax Vaccine Adsorbed

AVP: Anthrax Vaccine Precipitated

CTA1: Cholera Toxin subunit A1

ELISA: Enzyme-Linked Immunosorbent Assay

IL: Interleukin

KCDC: Korea Centers for Disease Control and Prevention

LD_{50}: Lethal Dose 50

PRNT: Plaque Reduction Neutralization Test

Declarations

Ethics approval and consent to participate

Animal study protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Korea Centers for Disease Control and Prevention (KCDC). All experimental procedures were approved by Institutional Review Board (IRB) of KCDC.

Consent to publish

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

Experiments were performed by DBP, HSS, YRL, YRK, and SKJ. Data analysis were performed by DBP and BEA. The study was supervised by JHC, JYY, MMC, and GER. The original draft was written by BEA and DBP. The manuscript was reviewed and edited by BEA and GER.

Acknowledgement

Not applicable

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Figures

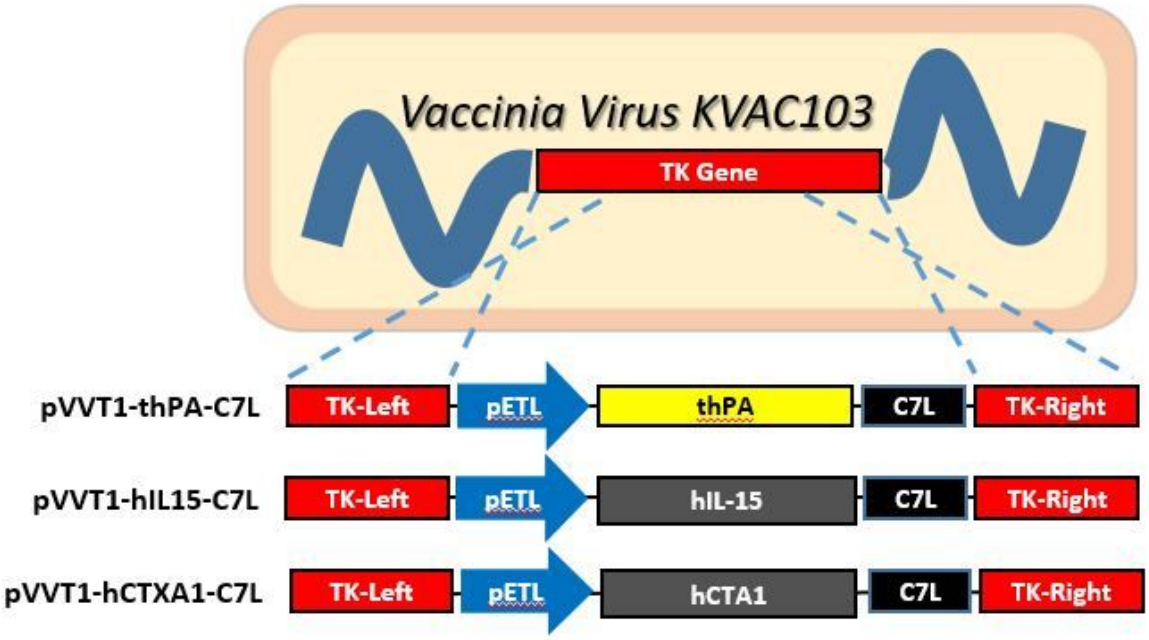

Figure 1

A diagram of viral vector construction Human codon-optimized genes encoding PA with a signal peptide derived from the tissue plasminogen activator polypeptide (thPA), IL-15 (hIL15), or CTA1 (hCTA1) were cloned into pVVT1-EGFP-C7L. Viral vector constructs are integrated into KVAC103 genome by homologous recombination at TK gene site.

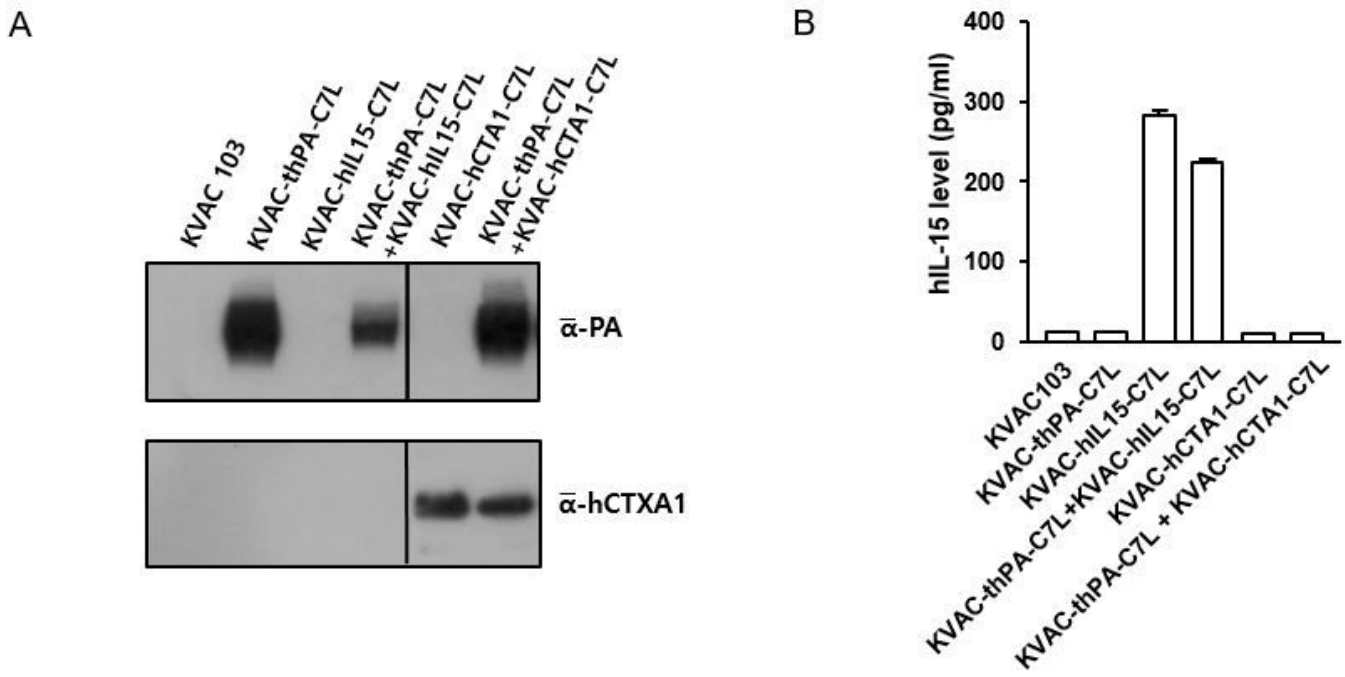

Figure 2

Expression of integrated proteins in vitro (A) The expression of PA and CTA1 was detected by immunoblot assay in cell lysates. (B) The expression level of IL-15 was detected by ELISA.

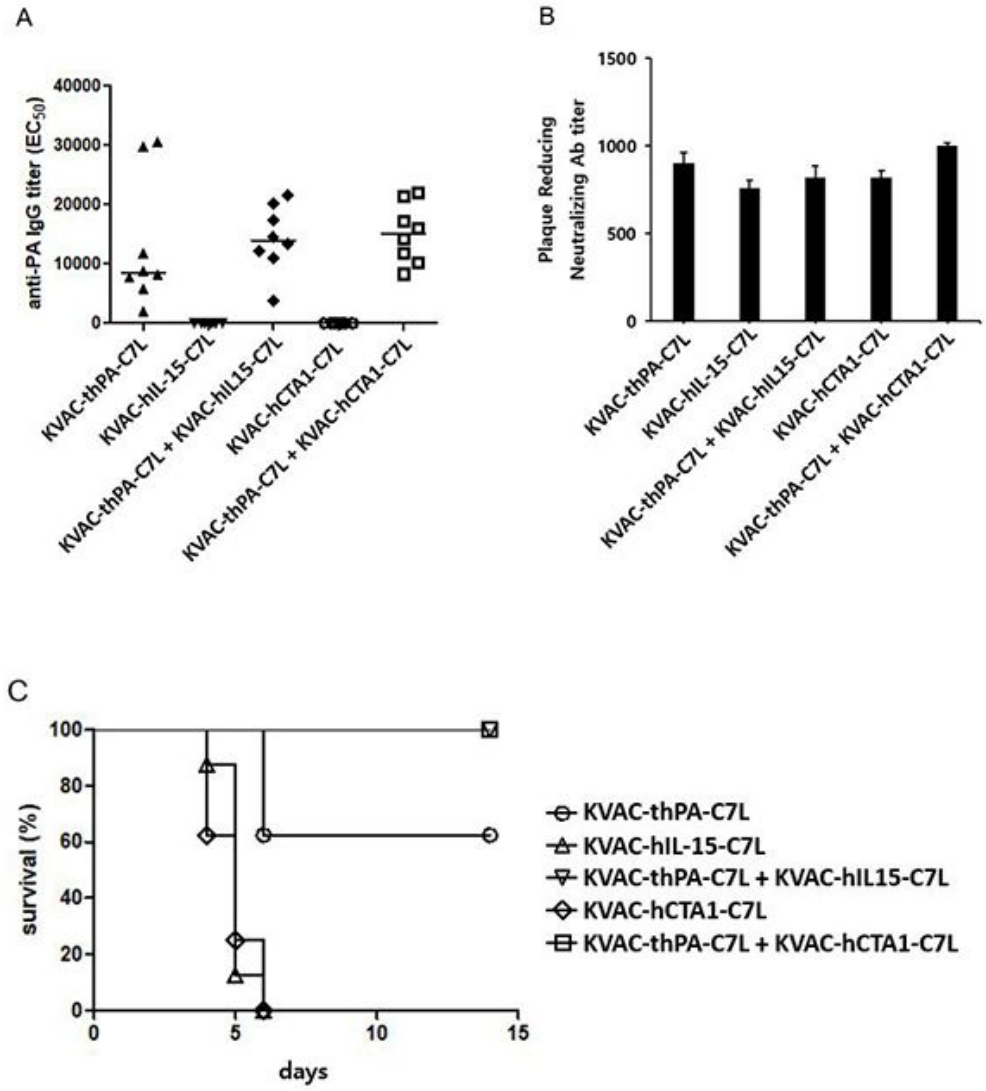

Figure 3

Immunogenicity and protective efficacy of the bivalent vaccine with or without adjuvant-expressing viruses in a mouse model (A) Anti-PA IgG titers were determined by ELISA. The Y-axis represents EC50 values. The horizontal bars indicate median of individual groups. The median values of anti-PA IgG titer are 8,505 for KVAC-thPA-C7L, 13,950 for KVAC-thPA-C7L + KVAC-hIL15-C7L, and 15,100 for KVAC-thPA-

dy titers were determined by PRNT assay. The Y-axis

represents PRNT50, the reciprocal of the dilution fold of sera reducing plaque formation in half. (C) Immunized mice were challenged with 50×LD50 of B. anthracis Sterne spores by s.c. injections. Survival rates were observed for 14 days.

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