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# Intranasal vaccination with ebola virus GP amino acids 258–601 protects mice against lethal challenge

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ABSTRACT

Ebola virus (EBOV) disease (EVD) leads to lethal hemorrhagic fever with a case fatality rate as high as 90%, thus posing a serious global public health concern. However, while several vaccines based on the EBOV glycoprotein have been confirmed to be effective in animal experiments, no licensed vaccines or effective treatments have been approved since the first outbreak was reported in 1976. In this study, we prepared the extracellular domain of the EBOV GP protein (designated as N20) by prokaryotic expression and purification via chromatography. Using CTA1-DD (designated as H45) as a mucosal adjuvant, we evaluated the immunogenicity of N20 by intranasal administration and the associated protective efficacy against mouse-adapted EBOV challenge in mice. We found that intranasal vaccination with H45-adjuvanted N20 could stimulate humoral immunity, as supported by GP-specific IgG titers; Th1 cellular immunity, based on IgG subclasses and IFN-γ/IL-4 secreting cells; and mucosal immunity, based on the presence of anti-EBOV IgA in vaginal lavages. We also confirmed that the vaccine could completely protect mice against a lethal mouse-adapted EBOV (MA-EBOV) challenge with few side effects (based on weight loss). In comparison, mice that received N20 or H45 alone succumbed to lethal MA-EBOV challenge. Therefore, mucosal vaccination with H45-adjuvanted N20 represents a potential vaccine candidate for the prevention of EBOV in an effective, safe, and convenient manner.



## 1. Introduction

Ebola virus (EBOV) is an enveloped, nonsegmented negative-sense single-stranded RNA filovirus that can cause lethal disease in humans and nonhuman primates (NHPs) with high lethality [1]. A high viral load, impaired and ineffective immune response, multiple organ failure, vascular and coagulation impairment, and progression to hemorrhagic fever are hallmarks of filovirus infection in primates [2]. A recent EBOV disease outbreak in 2013 started in Guinea and spread primarily to Liberia and Sierra Leone in West Africa. The outbreak claimed thousands of lives, which was partly due to early ignorance by the public, despite a coordinated effort by local and international organizations (e.g., World Health Organization, Centers for Disease Control and Prevention, and Chinese Center for Disease Control and Prevention) [3]. Horizontal transmission is the main mode of transmission during outbreaks, including direct contact with contaminated blood or bodily fluids, as well as indirect contact with other fomites [4]. Mucosal infection (e.g., via sexual transmission) [5] also likely occurred. Moreover, EBOV RNA may persist for long periods in the semen of EBOV disease survivors [6], which increases the risk of sexual transmission. Accordingly, mucosal immunity is of increasing concern [7,8]. Numerous studies have demonstrated that both humoral and cellular immune responses are indispensable for controlling an EBOV infection [9–11]. A number of different vaccine platforms (e.g., DNA, recombinant or subunit proteins, virus-like particles [VLPs], and recombinant viral vectors) have been established [12]. The efficacy of several potential EBOV vaccines is inspiring [12–14], particularly that of a recombinant vesicular stomatitis virus (rVSV)-based EBOV vaccine which has been successful in Phase III clinical trials and is now used during outbreaks [15–17]. However, wide application of this vaccine is difficult, especially in undeveloped areas, due to the high cost of the rVSV vaccine which is prepared by eukaryotic expression. Moreover, the vaccine

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primarily elicits humoral and cellular immunity and rarely stimulates mucosal immunity due to routine muscle vaccination. In addition, the application of viral vectors in an EBOV vaccine is associated with potential biosafety concerns [18,19]. Therefore, there is an urgent need to develop and approve an efficacious vaccine against EBOV which could simultaneously stimulate humoral, cellular, and mucosal immunity.

The EBOV genome is approximately 19 kb in length and encodes seven structural proteins, including the envelope glycoprotein (GP) [6,20]. The mature GP protein forms homo-trimmers on the surface of infected cells and virions, which is crucial for receptor binding, viral entry, and inducing host immunity [11,21]. Moreover, immunization with GP can sufficiently protect animals against lethal EBOV challenge in mice, guinea pigs, and NHPs [22–24]. Furthermore, the passive transfer of anti-GP antibodies has been shown to provide complete protection against EBOV challenge [25–28]. Therefore, GP is the primary immunogen used in the majority of vaccine candidates against EBOV infection [11]. Indeed, there are eight vaccines currently in clinical trials, all targeting the EBOV GP [12]. Several GP-based vaccine candidates currently under development include, virus-vectored vaccines [13,29], virus-like particles [30,31], and subunit vaccines [22], which confer protection against a lethal challenge in animal models (including NHPs). In this study, we selected the extracellular domain of the EBOV GP (amino acids [aa] 258–601) as a subunit vaccine to elicit humoral immunity towards EBOV.

Mucosal vaccination holds several advantages over conventional intramuscular immunization in protecting against EBOV infection, including immune protection at sites of viral entry and the convenient administration using needle-free devices [32]. However, because subunit vaccines are generally poorly immunogenic [33], a mucosal adjuvant is needed to increase the level of immunogenicity required to induce systemic and mucosal immunity. Cholera toxin (CT), an exceptionally strong mucosal adjuvant, is highly toxic [34–39]. Therefore, safer, less-toxic adjuvant are required. Agren et al. [40] used the CTA1 subunit of CT, combined with two Ig-binding domains (DD) of staphylococcal protein A to construct an nontoxic mucosal adjuvant termed CTA1-DD. CTA1-DD has been shown to bind to all immunoglobulins *in vivo* (e.g., B-cell-surface Ig) through the DD moiety [40], which correlates with the increased surface expression of CD80 and CD86 on B cells. Moreover, CTAl-DD can enhance the level of systemic IgG and mucosal IgA responses when administered intravenously or intranasally [37,41–43]. In addition, like CT, CTA1-DD can enhance specific IgG1, IgG2a, and lgG2b humoral responses, as well as both CD8+ and CD4+ T cell responses [40]. The adjuvant effect of CTAl-DD may be attributed to enhanced T-B cell reactions according to the upregulated expression of the CD80 and CD86 molecules on targeted B cells [44,45]. Importantly, in contrast to CT, CTAl-DD is completely nontoxic [46].

In this study, we evaluated the immunogenicity of the extracellular domain of GP combined with CTA1-DD when administered mucosally in mice, as well as its protective efficacy against EBOV challenge. The related humoral, cellular, and mucosal immunological characteristics were measured following intranasal vaccination with CTA1-DD-adjuvanted GP in mice, while weight loss and survival were analyzed after challenge with mouse-adapted EBOV in vaccinated mice.

## 2. Materials and methods

### 2.1. Preparation of CTA1-DD and EBOV subunit vaccine

CTA1-DD was identified according to the report by Agren et al. [40], codon-optimized, constructed into a pET30a vector, and expressed in *Escherichia coli* (*E. coli*) BL21 (DE3) in the form of inclusion bodies. The protein was dissolved in 8 M urea and extracted by DEAE exchange chromatography. After renaturation by dialysis, the concentrated protein was polished with Sephacryl™ S-200 gel filtration chromatography (designated as H45).

The artificially synthesized gene encoding 258–601 aa of Zaire EBOV GP (GenBank: AAB81004.1) with codon optimization was cloned into the pET30a vector using restriction endonuclease (*Nde*I and *Xho*I). The ligation products were transformed into *E. coli* BL21 (DE3). Positive clones were screened by Blue-White selection. Positive plasmids were identified by restriction endonuclease analysis (*Nde*I and *Xho*I), small-scale expression, sequenced, and designated as plasmid N20. Protein N20 was expressed in *E. coli* BL21 (DE3) with 1 mM IPTG as an inducer at 37 °C for 3 h. After sonic disruption (300 W, 20 s, 20 s, 20 times), the pellet was collected by centrifugation (17,300*g* for10 min at 4 °C) and resuspended in distilled water. After dissolving in urea (8 M, pH 8.0), protein N20 was crudely extracted with DEAE ion exchange chromatography (gradient elution with 100 mM, 200 mM, and 400 mM NaCl, pH 8.0). After renaturing by dialysis, protein N20 was polished with Sephacryl™ S-200 gel filtration chromatography according to the manufacturer's instructions (GE Healthcare, USA).

The H45 and N20 proteins were stored at concentrations of 1 mg/mL. The content, dose and concentration of the vaccines in each group were: H45, 100 μL, and 0.05 mg/mL (Group B); N20, 100 μL, and 0.1 mg/mL (Group B); and H45/N20, 100 μL, and 0.05 mg/mL for H45 and 0.1 mg/mL for N20 (Group D), respectively.

### 2.2. Evaluation of immunogenicity of H45 adjuvanted N20 vaccines (H45-N20) in mice

Thirty-six female Specific Pathogen Free (SPF), Balb/c mice (Vital River Laboratories, Beijing, China) aged 6–8 weeks were randomly assigned into four groups (Table 1, Groups A–D). All studies were approved by the Animal Care and Welfare Committee at the National Institute for Viral Disease Control and Prevention, China Center for Disease Control and Prevention (No. 20160908026). The mice were intranasally vaccinated according to the vaccination procedure outlined in Table 1. The mice were boosted three times at intervals of 10 days. Serum samples were collected immediately before each immunization and stored at −80 °C until further analysis. Splenocytes were isolated from euthanized mice to assess IFN-γ and IL-4 secretion by an enzyme-linked immunospot assay (ELISPOT) on day 10 after the last vaccination.

Enzyme linked immunosorbent assay (ELISA) and ELISPOT procedures were performed as described previously [47]. Briefly, anti-N20 antibody titers were determined by an endpoint dilution ELISA on micro-well plates coated with protein N20 (50 ng/well). Two 10-fold dilutions of sera were titrated in duplicate and antibodies were detected with HRP-conjugated goat anti-mouse IgG, IgG1, IgG2a, and IgA secondary antibodies (Sigma-Aldrich, USA). After the color reaction, the absorbance was read using an ELISA reader (Multiskan Go, Thermo Fisher, Finland). Titers were determined as the highest dilution at which the mean absorbance of the sample was 2.1-fold greater than the mean absorbance of the same dilution of the control serum. For the anti-N20 pilot study,

**Table 1**
Animal grouping and vaccination regimen.

| Group | Group size | Vaccination | Boost | |
|---|---|---|---|---|
| | | | Times | Interval days |
| A | 6 | PBS | 3 | 10 |
| B | 10 | H45 (5 μg) | 3 | 10 |
| C | 10 | N20 (10 μg) | 3 | 10 |
| D | 10 | H45 (5 μg)-N20 (10 μg) | 3 | 10 |

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