A Kunjin Replicon Virus-like Particle Vaccine Provides Protection Against Ebola Virus Infection in Nonhuman Primates

Oleg V. Pyankov,[1] Sergey A. Bodnev,[1] Olga G. Pyankova,[1,2] Vladislav V. Solodkyi,[1] Stepan A. Pyankov,[1] Yin Xiang Setoh,[2] Valentina A. Volchkova,[4] Andreas Suhrbier,[2,3] Viktor V. Volchkov,[4] Alexander A. Agafonov,[1] and Alexander A. Khromykh[2]

[1]State Center for Virology and Biotechnology Vector, Koltsovo, Russian Federation; [2]Australian Infectious Diseases Research Centre, School of Chemistry and Molecular Biosciences, University of Queensland, St Lucia, and [3]QIMR Berghofer Medical Research Institute, Brisbane, Australia; and [4]Molecular Basis of Viral Pathogenicity, CIRI, INSERM, U1111-CNRS UMR5308, Université de Lyon, Université Claude Bernard Lyon 1, Ecole Normale Supérieure de Lyon, France

The current unprecedented outbreak of Ebola virus (EBOV) disease in West Africa has demonstrated the urgent need for a vaccine. Here, we describe the evaluation of an EBOV vaccine candidate based on Kunjin replicon virus-like particles (KUN VLPs) encoding EBOV glycoprotein with a D637L mutation (GP/D637L) in nonhuman primates. Four African green monkeys (*Cercopithecus aethiops*) were injected subcutaneously with a dose of 10^9 KUN VLPs per animal twice with an interval of 4 weeks, and animals were challenged 3 weeks later intramuscularly with 600 plaque-forming units of Zaire EBOV. Three animals were completely protected against EBOV challenge, while one vaccinated animal and the control animal died from infection. We suggest that KUN VLPs encoding GP/D637L represent a viable EBOV vaccine candidate.

Keywords. Ebola virus; Kunjin replicon; vaccine; nonhuman primates.

Ebola virus (EBOV) is responsible for a severe form of viral hemorrhagic fever in humans, with case-fatality rates of up to 90%. Outbreaks of Ebola disease were first documented in 1976 in the African countries Zaire (now Democratic Republic of the Congo) and Sudan [1]. Since then, a number of outbreaks have been reported in central Africa, including recent outbreaks in Uganda (31 cases and 21 deaths) in 2012 and the Democratic Republic of the Congo in 2012 (57 cases and 29 deaths [2]) and 2014 (66 cases and 49 deaths [3]). The largest outbreak of Ebola disease ever recorded started in 2014 in West Africa and has so far yielded 22 057 cases (13 675 of which are laboratory confirmed) and 8795 deaths [4]. The unprecedented scale of the current Ebola disease outbreak has demonstrated the urgent need for an effective vaccine, particularly for Ebola disease crisis responders (eg, healthcare, sanitation, and mortuary workers; laboratory technicians; and contact tracers), who are at significantly higher risk of becoming infected. Other groups at increased risk of exposure include those sharing accommodation with known or suspected EBOV-infected individuals, as well as biomedical research scientists working with EBOV. Vaccination may also provide postexposure protection for contacts; such a capability would be invaluable for Ebola disease outbreak management [5].

EBOV is an enveloped virus with single-strand, negative-sense, nonsegmented RNA molecule encoding the following genes: 3′-NP-VP35-VP40-GP-VP30-VP24-L-5′. No vaccine for EBOV is currently licensed [1]. The most advanced EBOV vaccine candidates to date are (1) a recombinant chimpanzee adenovirus 3 vaccine encoding the EBOV glycoprotein (cAd3-EBOV-GP), which is being developed by GlaxoSmithKline and the National Institute of Allergy and Infectious Diseases and is currently being fast tracked through phase 1 clinical trials; and (2) a recombinant vesicular stomatitis virus (VSV) vaccine, in which the VSV G protein has been replaced with EBOV glycoprotein (rVSVΔG-EBOV-GP), that is currently being developed by NewLink Genetics
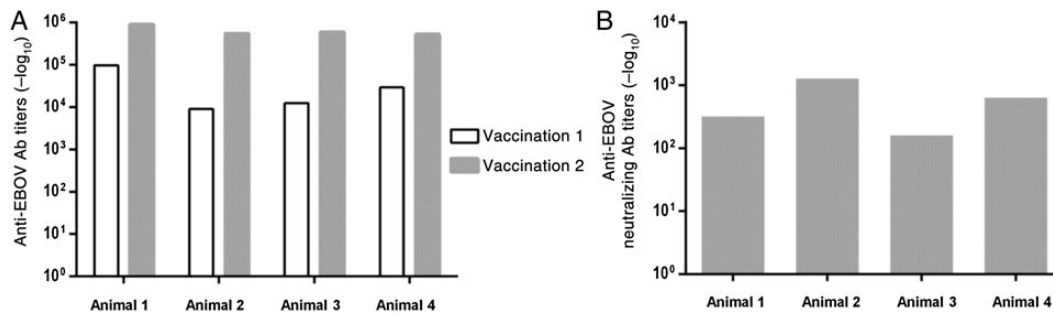
Figure 1. Induction of anti-Ebola virus (EBOV) antibodies (Abs) in immunized animals. *A*, Titers of anti-EBOV Abs determined by an enzyme-linked immunosorbent assay, using inactivated EBOV as antigen. *B*, Titers of EBOV-neutralizing Abs determined by a virus-neutralization assay.

and Public Health Agency of Canada, with phase 1 clinical trials soon to commence [6]. Both vaccines have demonstrated 100% efficacy in nonhuman primate studies. The cAd3-EBOV-GP (10^{10} plaque-forming units [PFU]) vaccine given intramuscularly protected 4 of 4 cynomolgus macaques (*Macaca fascicularis*) against challenge with 1000 PFU of Zaire EBOV 5 weeks after vaccination [7, 8]. rVSV-based vaccines against Zaire and Sudan EBOV and Marburg virus have also shown effective protection in nonhuman primate models [9]. A number of other preclinical vaccines have also been described, including inactivated virus, virus-like particles (VLPs) produced upon expression of VP40 and GP proteins, DNA vaccines expressing glycoprotein (GP) or nucleocapsid protein, and various recombinant viral and replicon vectors primarily expressing surface GP [1, 6]. Overall, vaccine studies so far indicate a critical role of antibodies in protection, with GP being the main inducer of protective antibodies in nonhuman primates [1, 6].

We have previously developed vaccine vectors based on RNA replicons of a naturally attenuated Australian strain of West Nile virus, Kunjin [10]. Kunjin replicons were constructed by deletion of viral structural genes from the Kunjin genome [10]. The lack of structural genes makes Kunjin replicon RNA incapable of productive infection, but capable of replicating and thus amplifying vaccine antigen gene(s) inserted in place of deleted structural genes. Packaging of replicon RNA into secreted VLPs significantly enhances replicon RNA transduction in vivo and thus vaccine efficacy [11]. In our previous study, a Kunjin replicon VLP expressing a D637L mutant of GP (KUN-VLP-GP/D637L) at a dose of 5×10^6 VLPs was shown to be effective in protecting 80% of guinea pigs against lethal challenge with guinea pig–adapted EBOV [12]. The D637L mutation promotes shedding of GP due to improved cleavage by TACE [13], thereby reducing cytotoxicity, which allowed manufacture of higher titers of VLPs in vitro and presumably also extended replicon-based GP expression in vivo [12].

In the current study, we extended our vaccine studies of KUN-VLP-GP/D637L to nonhuman primates, specifically African green monkeys (*Cercopithecus aethiops*) [14], a species that

is much less aggressive than macaques and therefore presents a lower risk to investigators.

VLPs were prepared as described previously [12] and concentrated by centrifugation through Amicon Ultra centrifugal filter devices, with a 100 000 molecular weight cut off (Millipore, Billerica, Massachusetts), followed by partial purification through 15% sucrose cushion. Partially purified VLPs were resuspended in Dulbecco's modified Eagle's medium (DMEM), and VLP titers were determined by immunofluorescence staining assays, using anti-EBOV GP monoclonal antibody (FE37, Abcam) and Vero cells fixed and stained at 2 days after infection with 10-fold serial dilutions of VLPs [12]. Four 4–5-kg *C. aethiops* were vaccinated subcutaneously with 10^9 VLPs per animal in 2 mL of DMEM into 4 different sites. One control animal was injected with 2 mL of DMEM. Four weeks later, animals were vaccinated for a second time with the same dose of VLPs (or DMEM).

Four weeks after the first vaccination and 3 weeks after the second vaccination, serum specimens were collected and analyzed for anti-GP antibody responses, using an enzyme-linked immunosorbent assay with inactivated EBOV as antigen and horseradish peroxidase–conjugated mouse anti-human immunoglobulin G. End point titers were determined as the highest dilution showing an OD_{450} that was statistically different (mean $\pm$ 3 SD) from that of samples from the control animal [15]. All 4 animals vaccinated with KUN-VLP-GP/D637L developed a robust anti-GP antibody response, with titers ranging from 1:9100 (animal 2) to 1:98 300 (animal 1) after the first immunization and from 1:520 900 (animal 4) to 1:893 600 (animal 1) after the second immunization (Figure 1A).

Sera were also analyzed for EBOV-neutralizing antibodies, using microneutralization assays on Vero cells. Briefly, serum specimens collected from animals 3 weeks after the second immunization was were treated at 56°C for 30 minutes, serially diluted in DMEM with 2% fetal bovine serum, and incubated with 200 PFU of EBOV for 60 minutes at 37°C. The reaction mix was then used to infect Vero cells. Forty-eight hours after infection, cells were fixed and incubated with anti-GP mouse monoclonal antibodies, followed by incubation with horseradish
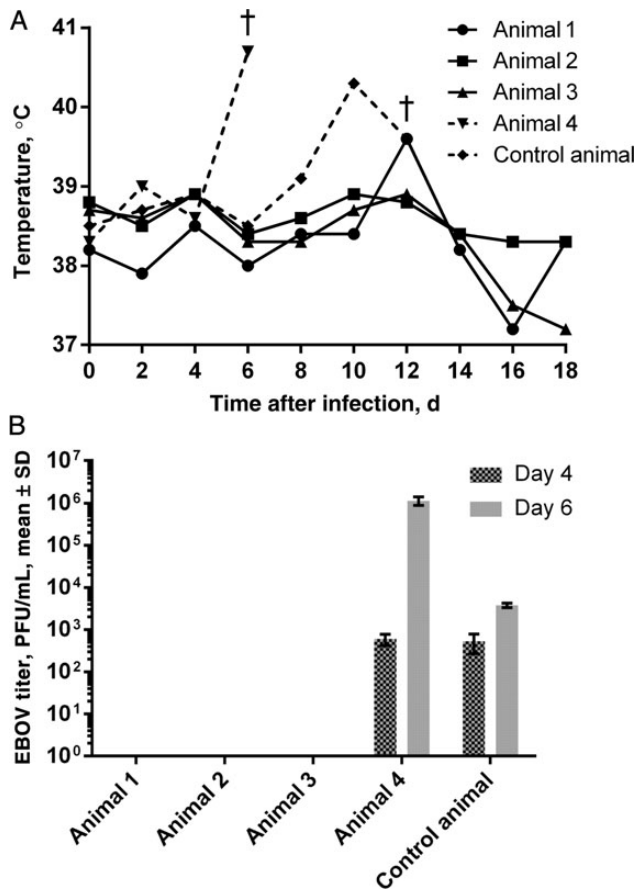
Figure 2. Monitoring of animals after challenge with Ebola virus (EBOV). *A*, Body temperature measurements of EBOV-infected animals. The dagger indicates time of death. *B*, Titers of EBOV detected in serum on days 4 and 6 after infection. Abbreviations: PFU, plaque-forming units; SD, standard deviation.

peroxidase–conjugated rabbit anti-mouse antibodies and staining with AEC substrate (Sigma). Positively stained foci were counted, and neutralization titers were determined as the dilution reducing the amount of stained cells by 50%, compared with the amount in a serum specimen from the unimmunized animal. Neutralizing titers after the second vaccination ranged from 1:160 to 1:1280 (Figure 1*B*).

Three weeks after the second vaccination, animals were challenged intramuscularly with 600 PFU of Zaire EBOV strain Mayinga passaged once in an African green monkey, amplified, and titrated on Vero cells. The temperature of infected animals was measured every second day, and serum specimens were collected on days 4 and 6 after infection to analyze EBOV titers, using plaque assays on Vero cells.

Vaccinated animals 2 and 3 showed no significant increase in temperature throughout the experiment, and vaccinated animal 1 showed a slight increase in temperature on day 12 after challenge, which returned to normal the next day (Figure 2*A*). These 3 animals also showed no detectable EBOV in serum specimens

obtained after challenge (Figure 2*B*) and were protected against EBOV challenge (Figure 2*A*). Vaccinated animal 4 unexpectedly developed an elevated temperature on day 6 after challenge and died on day 7 (Figure 2*A*), despite high antibody titers that were similar in magnitude to those seen in immunized animals 1–3 (Figure 1). The control animal showed elevated serum EBOV titers on days 4 and 6 (Figure 2*B*), had an increased temperature on day 10, and died on day 13 (Figure 2*A*), somewhat later than might be expected. Conceivably, the outbred nature of primates used in the experiment may account for observed variations in the response to immunization and to infection, with variable innate responses potentially involved [16].

This first nonhuman primate study using the KUN-VLP-GP/D637L vaccine thus illustrated that 2 immunizations with 10^9 VLPs can protect 75% of nonhuman primates against challenge with 600 PFU of the Zaire strain of EBOV. Our previous studies in guinea pigs showed a significant improvement in protection when the dose of VLPs was increased 5-fold [12]. Studies using Venezuelan equine encephalitis virus replicon VLPs in nonhuman primates also showed substantial improvement in protection when the VLP dose was increased, with 10^{10} VLPs required to induce 100% protection [17]. These latter studies might suggest that increasing the dose of the KUN-VLP-GP/D637L vaccine would also improve protection and, perhaps, also eliminate the need for a second vaccination.

In summary, the study presented herein suggests the KUN-VLP-GP/D637L vaccine represents a viable EBOV vaccine candidate, with further studies in nonhuman primates with higher doses of VLPs and larger number of animals warranted.

Notes

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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