



Inactivated yellow fever 17D vaccine: Development and nonclinical safety, immunogenicity and protective activity

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ABSTRACT

In the last 10 years new concerns have arisen about safety of the live, attenuated yellow fever (YF) 17D vaccine, in particular viscerotropic adverse events, which have a case-fatality rate of 64%. A non-replicating cell culture-based vaccine would not cause these adverse events, and potentially could be used in persons with precautions or contraindications to use of the live vaccine, including age <9 months and >60 years, egg allergy, immune suppression, and pregnancy. We developed a whole virion vaccine from the 17D strain inactivated with β -propiolactone, and adsorbed to aluminum hydroxide. The inactivated vaccine was highly immunogenic in mice, hamsters, and cynomolgus macaques. After a single dose in hamsters and macaques, neutralizing antibody titers were similar to those elicited by the live 17D vaccine (YF-VAX[®], Sanofi Pasteur). After two doses of inactivated vaccine, neutralizing antibody titers in hamsters were significantly higher than after a single dose of YF-VAX[®] [geometric mean titer (GMT) 20,480 vs. 1940, respectively ($P < 0.001$, ANOVA)]. Hamsters given a single dose or two doses of inactivated vaccine or a single dose of YF-VAX[®] were fully protected against hepatitis, viremia, weight loss and death after challenge with YF virus (Jimenez strain). A clinical trial of the inactivated vaccine (XR-001) has been initiated.

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1. Introduction

Yellow fever (YF), a mosquito-borne flavivirus disease, is endemic and epidemic in tropical areas of South America and Africa. The disease caused by YF virus is the original 'viral hemorrhagic fever', characterized by fever, nausea, vomiting, epigastric pain, hepatitis with jaundice, renal failure, hemorrhage, and shock. The case-fatality rate is 20–50% and there is no specific treatment [1]. Because most YF cases are not reported, the WHO estimated that

the true incidence may be 200,000 cases and 30,000 deaths annually [2], but this estimate needs revision as vaccine coverage is improving in Africa. Yellow fever virus is maintained in nature by wild monkeys and tree-hole breeding mosquitoes. For this reason, the disease cannot be eradicated, and human vaccination is the most effective means of prevention and control. Since development of the live, attenuated 17D vaccine in 1936 [3,4], over 550 million persons have been vaccinated [4a]. Up to 30 million doses of YF vaccine are now distributed annually (A. Homma, pers. comm., 2009). In the United States, approximately 400,000 travelers and military personnel receive the YF vaccine annually [4b].

The live, attenuated YF 17D vaccine is administered as a single subcutaneous injection, and is highly effective, causing a protective antibody response in 99% of subjects [5,6]. Antibodies appear rapidly (within 10 days) and boosters are given every 10 years, although immunity is probably life-long in most people. The vaccine causes a low-level viremia between days 2–4 after vaccination. Common adverse events (fever, myalgia, headache)

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occur in 15–20% of subjects during the first week, but are generally mild.

The safety of 17D vaccine was questioned in 2001 when a new syndrome, YF vaccine associated viscerotropic disease (YEL-AVD) was first reported [7–9]. Based on a retrospective review of adverse event reporting, a total of 59 cases of YEL-AVD (38 fatal, case-fatality rate 64%) have been identified going back to 1973 [10]; however, all but 2 cases, have occurred since 1990 (Ref. [5] and M Gershman, CDC, pers. comm., 2009). In contrast, during this same interval, only 6 fatal cases of naturally acquired YF were reported [5] despite modest levels of vaccine coverage (~31% in U.S. travelers [11]), suggesting the possibility that for many travelers, the risk of dying from the vaccine exceeds that of dying from infection with wild-type virus. The caveat, of course, is that the risk of natural infection may be high in areas with active transmission. During epidemics, the incidence of infection has ranged from 20 to 40% of the affected human population, and 1 in 7 people infected develop illness [5]. There is little question about the benefit of vaccination in such circumstances. However, in the absence of an overt outbreak, it is difficult to assess risk of travelers being exposed to wild-type virus.

YEL-AVD is a fulminant, life-threatening infection of liver and other vital organs resembling YF disease [5,7–9,12]. The pathogenesis of this adverse event involves replication of vaccine virus in and damage to visceral organs, exceeding the host's ability to clear the virus. The reported incidence of YEL-AVD approximates 0.4 per 100,000 vaccinations [13]. Elderly persons and individuals with a history of thymectomy are at considerably higher risk of serious adverse events. In persons 60–69 years the reporting rate of YEL-AVD is 1.0 per 100,000, and in persons ≥ 70 years the rate is 2.3 per 100,000 [13]. In 2007, during a mass immunization campaign in Peru the incidence of YEL-AVD was 7.9 per 100,000 [14]; no clear explanation was found for the substantially higher incidence in this occurrence.

The incidence and death following YEL-AVD are higher than those following vaccination with other live viral vaccines; oral polio vaccine causes vaccine associated paralytic poliomyelitis (VAPP) at an incidence of 0.11 per 100,000 [15] and few deaths and smallpox vaccine causes post-vaccinal encephalitis at a rate of 0.29 per 100,000 with a 20–35% case-fatality rate [16]. Both of these vaccines were discontinued in many areas due to safety concerns.

The occurrence of YEL-AVD caused by the live YF 17D vaccine, has spurred development of a safer alternative (inactivated) vaccine. An inactivated vaccine would also potentially reduce the incidence of other serious adverse events associated with the live YF 17D vaccine, including YF vaccine associated neurotropic adverse events (YEL-AND) and allergic reactions caused by egg proteins (residual from the hens' eggs used for manufacture) and gelatin (added to stabilize the vaccine). Finally an inactivated vaccine could potentially be used in elderly persons and in those with contraindications to the live 17D vaccine, including immunosuppressed individuals, persons with a history of thymectomy, infants <9 months of age, and even pregnant and nursing women.

In this paper we report the development of an inactivated YF vaccine and the results of nonclinical testing. A clinical trial has been initiated in human volunteers and will be reported separately.

2. Materials and methods

2.1. Viruses and virus infectivity assays

For use in neutralization tests and as a standard for plaque assays, a stock of YF 17D was prepared by passing an aliquot of virus from a commercial vial of YF-VAX® in a monolayer culture of Vero cells and harvesting cell culture fluid at the appearance of cytopathic effects (CPE). The virus was quantified by plaque assay on Vero cells.

For challenge of hamsters, the YF Jimenez strain (South American genotype I, isolated in Panama, 1974) had been adapted by serial passage in hamster liver, as described by Tesh et al. [17]. A seed stock was prepared from a clarified suspension of liver tissue of the infected hamsters on day 3 post-virus injection, and homogenized in a 2× volume of sterile phosphate buffered saline. The liver homogenate had a titer of $10^{6.0}$ 50% tissue culture infectious doses (TCID₅₀)/mL.

Yellow fever 14-FA (isolated from a human patient in Angola 1971, Angola genotype, passaged 7 times in suckling mouse brain and once in C6/36 and in Vero cells) was obtained from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA), University of Texas Medical Branch (UTMB), Galveston TX. The titer was determined by immunofocus assay (the preferred method at UTMB). Briefly, serial 10-fold dilutions of virus were plated in Vero cell monolayers, overlaid with agarose, incubated for up to 5 days, fixed with formalin, permeabilized with ethanol, and immunostained using a polyclonal hyperimmune anti-YF mouse ascitic fluid obtained from the WRCEVA reagent collection, followed by a peroxidase labeled goat anti-mouse IgG secondary antiserum (Sigma, St. Louis MO). Antibody binding was visualized by the addition of VIP-Vector® substrate solution (Vector Laboratories, Burlingame, CA) prior to counting the stained foci.

2.2. Vaccine

The XRX-001 vaccine produced is a whole virion vaccine inactivated with β -propiolactone (BPL) and adsorbed to aluminum hydroxide. Research lots and clinical grade vaccine were manufactured using the same methods, the latter in conformance to current Good Manufacturing Practices (cGMP). A Vero Cell Bank was manufactured for Xcellerex at WuXi AppTec (Philadelphia, PA) from WHO 10–87 Vero cells obtained from the American Type Culture Collection, with permission from the Food & Drug Administration. The cells were grown in serum-free medium and fully tested for identity, adventitious agents, and tumorigenicity. Yellow fever 17D virus was obtained from a commercial lot (YF-VAX®, Sanofi Pasteur, Swiftwater, PA) and adapted for growth by 10 serial passages at terminal dilution in Vero cell cultures, followed by a single round of plaque purification. Growth curves in duplicate monolayer cultures of Vero cells grown in 75 cm² flasks were performed at low (0.001 PFU/cell) and high (1.0 PFU/cell) multiplicities of infection (MOI) to compare the unadapted (P1) and adapted (P11) virus. RNA was extracted from P1 and P11 virus for full genomic sequencing. The P11 RNA was used with lipofectamine to transfect Vero cells grown in serum-free conditions. The progeny virus was used to produce a Master Virus Seed, and the Master Virus Seed to produce a Working Virus Seed according to cGMP. Seed viruses were tested for sterility, mycoplasma and adventitious viruses. The Working Virus Seed was used to infect Vero cells grown on Cytodex®-1 microcarrier beads (GE Healthcare) in an XDR-50® Xcellerex single-use bioreactor at an MOI of 0.01 plaque-forming units (PFU)/cell. The virus-containing supernatant was partially purified and concentrated by passage through a 90 μ m sieve tube to remove cells and beads, depth filtration, digestion with nuclease (Benzonase®), ultra-filtration and diafiltration, followed by sterile filtration. The virus was then inactivated by addition of 0.1% BPL (Ferak, Berlin, Germany) followed by 16 h incubation at room temperature. Inactivation was followed by a final cellulose sulfate chromatographic purification step, dilution to the target potency, formulation with proprietary stabilizers, sterile filtration, adsorption to 0.2% (w/v) aluminum hydroxide (Alhydrogel®, Brenntag Biosector, Denmark), and filling into glass vials. The drug substance and final filled vaccine meet Xcellerex' specifications for potency, identity, sterility, endotoxin level, residual Vero cell DNA

and protein, pH, osmolarity, appearance, stability, and other parameters.

2.3. Potency assay

A monoclonal ELISA was used to quantitate YF antigen and also serves as a test for identity. 2E10 IgG2a mouse monoclonal antibody (developed by JJ Schlesinger, University of Rochester, Rochester, NY) is directed against a neutralizing epitope in the upper surface of Domain II of the E glycoprotein at amino acids 71/72 [18]. For use in potency assays, the 2E10 hybridoma was used to prepare purified antibodies from mouse ascitic fluids at Maine Biotechnology Services (Portland ME). 2E10 antibody at a concentration of 1 µg/mL in carbonate–bicarbonate buffer, pH 9.6 was coated on the 384-well microtiter plates. Equal volumes of samples were added to quadruplicate wells and YF antigen was captured on the plates, followed by a detection antibody reagent, the 2E10 monoclonal conjugated to horseradish peroxidase (HRP) (2E10-HRP). FemtoMax Super Sensitive Chemiluminescent HRP Substrate was added to each well. The range of the method was 4.6. The limit of quantitation (LOQ) of YF antigen in 2E10 ELISA was 4.6 log₁₀ virus equivalents (VE)/mL, limit of detection (LOD) 4.1 log₁₀ VE/mL, and range of the assay up to 7.0 log₁₀ VE/mL. Intra-assay (5%) and inter-assay (10%) precision of the 2E10 ELISA were considered excellent. An infectious YF standard was used to prepare the standard curve for the ELISA; the titer of this standard has been measured in multiple independent plaque assays by plaque assay. There was a 1:1 correlation between ELISA value and the infectivity titer of the virus in PFU/mL and thus potency was expressed as virus equivalent (VE)/mL. A modification of the assay was developed to determine potency of aluminum hydroxide-bound antigen. To determine the relationship between potency in the 2E10 ELISA and virus protein concentration, sucrose gradient purified YF 17D virus was tested for protein content by MicroBCA protein assay (Thermo Scientific) using bovine serum albumin as a standard and for YF antigen by 2E10 ELISA.

2.4. Inactivation kinetics and test for residual live virus

The kinetics of inactivation by BPL was studied in several experiments using a version of the cGMP process at 1/40th scale of all steps, with matching types of containers. After addition of BPL to virus bulk to a final concentration of 0.1%, samples were taken at various time points, dialyzed to remove BPL and assayed for infectivity by Vero plaque assay and for retention of 2E10 epitope by the potency assay (described above). A 'no treatment' control was virus bulk without BPL treatment frozen and held at $\leq -60^\circ\text{C}$, thawed once at the appropriate time interval and assayed together with treated samples. BPL decay was measured by gas chromatography. To determine whether significantly more YF virus could be inactivated by this process step, additional virus was spiked into the reaction mixture 1 h after addition of BPL and a second spike was performed at 10 h. In addition to inactivation of YF virus, an evaluation of the BPL inactivation step for inactivating a high concentration of a relatively resistant non-enveloped DNA virus (porcine parvovirus) was performed. A standardized test performed according to Good Laboratory Practices (GLP) for residual live virus was applied to all manufactured lots by Microtest Laboratories Inc. (Agawam, MA). Five % of the total volume of the bulk drug substance and appropriate controls were inoculated into fluid cultures of Vero cells and observed for CPE for 7 days at which time a blind passage was performed into fresh cultures which were observed for another 7 days, blind passaged a second time and observed for 21 days. The LOD in this assay was determined to be 0.14 TCID₅₀/mL. Before testing, test samples were evaluated in a spiking experiment with live virus to show that the inactivated virus

did not interfere with detection of replication-competent YF 17D virus.

2.5. Neutralization tests

Sera from immunized and control mice, hamsters and non-human primates were tested for antibody using a 50% plaque reduction method as previously described [19]. Briefly, samples of test sera were heat-inactivated, serial 2-fold dilutions prepared, mixed with an equal volume of YF 17D virus-containing 60–80 plaque-forming units, incubated for 16–20 h at 2–8 °C, and inoculated onto duplicate wells of Vero monolayers grown in 12 well plates. After virus adsorption (1 h, 37 °C), monolayers were overlaid with methylcellulose. Cells were fixed with formaldehyde and stained with crystal violet–methanol after 4 days incubation at 37 °C. The endpoint was the highest dilution of serum inhibiting plaques by 50% or more compared to diluent–virus controls. A positive result was 50% reduction in plaques at the lowest final dilution of serum (after mixing with an equal volume of virus); for different studies in mice the lowest dilution of serum in one experiment was 1:4 or 1:8 or depending on the volume of serum available, and 1:20 in a second experiment, whereas and for hamsters and monkeys it was 1:40.

A second (immunofocus) method was used to test for neutralizing antibodies against wild-type YF viruses. Tests were performed at UTMB under BSL3 containment. For the PRNT₅₀ assays, 2-fold serial dilutions of serum were mixed 1:1 with diluted virus-containing ~50 foci and incubated at room temperature for 60 min. Virus only and media only controls were included with each dilution series. Duplicate dilution series were prepared for each sample. After incubation, virus/serum mixtures were transferred into individual wells of 24-well plates containing Vero cell monolayers, incubated at room temperature for 60 min and then overlaid with agarose. Plates were incubated for 5 days and foci were detected by immunostaining as described (Section 2.1). Means of foci counts in the virus only control wells were used to determine the 50% neutralization cutoff titers for each test.

2.6. Animals

All animals used in this study, including Sprague–Dawley rats (Charles River Laboratories, Raleigh, NC), 7 weeks old; inbred BALB/c and outbred CD-1 mice (Harlan Laboratories, Indianapolis, IN) 6–8 weeks of age; Syrian hamsters (*Mesocricetus auratus*), (Charles River Laboratories, Wilmington, MA) 3–4 weeks of age; and cynomolgus monkeys (*Macaca fascicularis*) of Chinese origin (Three Springs Scientific, Perkasie, PA), 4–7 years of age were cared for in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council) under an animal use protocol approved by the Institutional Animal Care and Use Committee at each of the participating institutions.

2.7. Toxicology study

This study was performed for Xcellerex by Bridge Global Pharmaceutical Services, Inc. (Gaithersburg, MD). The vaccine was administered on study days 1, 8, 15 and 22 to 10 male and 10 female 7-week-old Sprague–Dawley rats as two 0.25 mL intramuscular (IM) injections of XR-001 for a total dose of 8×10^8 VE. An equal number of rats received 0.9% saline. The four-dose regimen of active XR-001 constitutes 2 more doses than the number intended for the Phase 1 clinical trial. Half of the animals (5 males and 5 females) from each group were sacrificed 1 day after the last vaccination (acute phase) and the remaining were sacrificed 14 days after the last vaccination (recovery phase). Animals were assessed

twice daily for clinical signs. Body weight was measured 24 and 48 h and 7 days after vaccinations. Body temperature and Draize dermal scoring were performed prior to and after each vaccination. Immunogenicity was assessed by PRNT₅₀ at necropsy to confirm that the animals were able to mount antibody responses to the vaccine. As a measure of acute phase (proinflammatory) reactions, fibrinogen and IL-6 levels were measured in blood 2 days after the first immunization and then again at sacrifice. At sacrifice a battery of blood chemistry, hematology and coagulation tests were performed. Full gross necropsies were performed as specified by the WHO guidelines [22], bone marrow smears were taken, and organs were weighed and histopathology specimens were taken.

2.8. Mouse immunization studies

Mouse inoculations were performed at BRM Inc. (Worcester MA) and neutralizing antibody tests were performed at Xcellerex. Female inbred BALB/c and outbred CD-1 mice (Harlan Laboratories, Indianapolis, IN) mice 6–8 weeks of age, were inoculated by the subcutaneous (SC) route with live YF-VAX® in a volume of 100 μ L injected in the interscapular region. For intramuscular (IM) administration, a volume of 100 μ L of inactivated vaccine was injected into both posterior thigh muscles (50 μ L/leg). Mice were terminally bled under anesthesia by cardiac puncture and serum samples were tested for antibody by PRNT₅₀ as described above.

2.9. Hamster immune response and protection against lethal challenge

The immune response and challenge study in hamsters was performed at Utah State University and neutralizing antibody tests were performed by Xcellerex. Syrian hamsters (*Mesocricetus auratus*), 3–4 weeks of age (Charles River Laboratories, Wilmington, MA), were inoculated IM with graded doses of XRX-001, with YF-VAX® SC or with placebo (0.2% aluminum hydroxide in 0.9% NaCl). Antibodies were measured by PRNT₅₀ on day 49 (28 days after the last immunization), and animals were then challenged by the intraperitoneal route with 20 TCID₅₀ of the Jimenez strain of YF virus [16] and followed for illness, weight change, serum alanine aminotransferase (ALT) levels, and viremia. ALT and viremia were determined on day 6 after challenge. Weight change was determined between days 3 and 6 after challenge. These parameters have been determined to be sensitive indicators of protection against challenge [20]. ALT was determined using a kit (Teco Diagnostics, Anaheim, CA) according to the manufacturer's directions and modified for use in 96-well plates. Viremia was determined by testing four replicates of each of a series of serial 10-fold dilutions of serum in Vero cells grown in 96-well plates and TCID₅₀ endpoint was based on highest dilution of serum that produced CPE. Survival data were analyzed using the Wilcoxon log-rank survival analysis and the significance of the differences observed in viremia, ALT and PRNT₅₀ were determined by ANOVA, followed by Bonferroni's group comparison test.

2.10. Monkey immunogenicity study

This study was performed at BioQual Inc. (Rockville, MD) and neutralizing antibody tests were done at Xcellerex and UTMB (wild-type YF virus). *Macaca fascicularis* (cynomolgus) monkeys of Chinese origin (Three Springs Scientific, Perkasie, PA), 4–7 years of age, weighing 3–8 kg, were tested for neutralizing antibodies to dengue and Japanese encephalitis [21] at the Centers for Disease Control & Prevention, Ft. Collins, CO and those used on study were shown to be seronegative. Monkeys were allowed to acclimate for 40 days in a controlled-environment indoor facility (protected from mosquitoes) before commencing this study. Monkeys were ran-

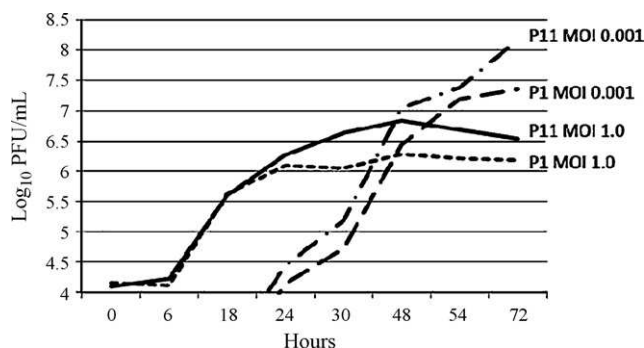


Fig. 1. Growth curves in Vero cell monolayers of YF-VAX® passed once in Vero cells (P1) and virus adapted by 10 serial passages at terminal dilution followed by a single plaque purification (P11) at high and low MOI. Values are means of duplicate cultures.

domly assigned to be inoculated with the experimental XRX-001 vaccine or YF-VAX® and bled from the femoral vein at specified intervals. Animals were also observed twice daily for clinical signs and were weighed weekly. Food consumption was also monitored.

3. Results

3.1. Adaptation of yellow fever 17D to replicate in Vero cells

The clonal Passage 11 (P11) virus showed a 2.5-fold (0.4 log₁₀) increased replication capacity in a one-step growth curve (MOI 1.0 PFU/cell) in Vero cells grown in a monolayer culture (75 cm² flask) compared to the 17D at Passage 1 (P1) (Fig. 1). At low MOI (0.001 PFU/mL), the difference in yield was 0.82 log₁₀, or 6.6-fold. In stirred tank bioreactors with Vero cells growing on microcarrier beads, the adapted virus consistently grew to titers >8.0 log₁₀ PFU/mL (data not shown).

3.2. Yellow fever virus antigenic potency correlation to viral protein concentration

There was a linear relationship between the 2E10 monoclonal ELISA potency assay and MicroBCA protein content of sucrose gradient purified YF virus (Fig. 2). A clinical lot of XRX-001 (Lot 900-03-09-006) having a potency of 8.6 log₁₀ VE/mL (8.3 log₁₀ VE/0.5 mL dose) had a protein content of 8.7 μ g/mL (4.4 μ g/0.5 mL dose).

3.3. Inactivation of yellow fever virus by BPL

In preliminary studies, the hydrolysis of BPL was determined by gas chromatography measurements. BPL decayed to unde-

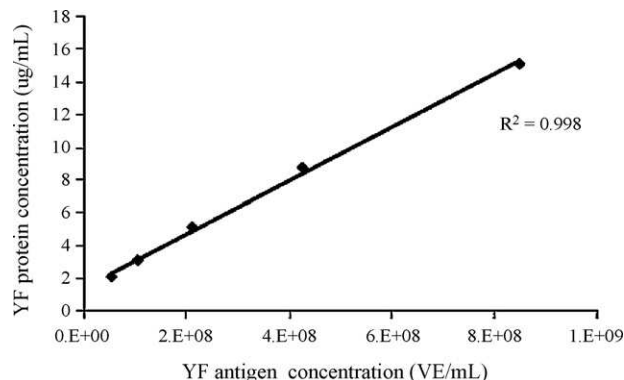


Fig. 2. Correlation between the 2E10 potency assay and content of viral protein, XRX-001 drug substance.

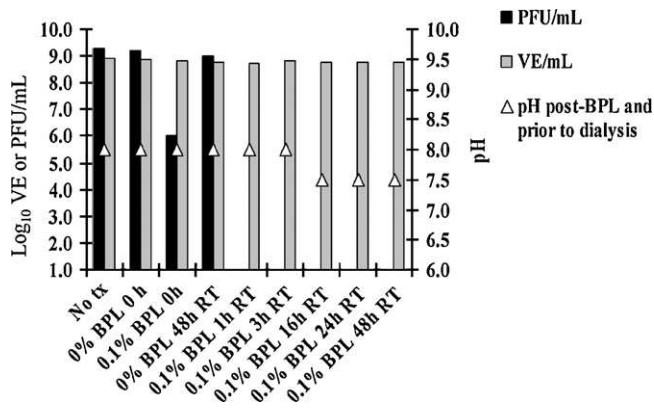


Fig. 3. Inactivation kinetics of yellow fever 17D. BPL was added to a final concentration of 0.1% to live virus bulk ($9.0 \log_{10}$ PFU/mL) in 50 mM HEPES, 145 mM NaCl, 7% sorbitol, pH 8. The inactivation solution was mixed at room temperature (RT) and sampled at various times. The no-treatment control was a virus sample that was not treated with BPL and had been frozen at $\leq -60^\circ\text{C}$ until thawed and tested at the time points shown. The black bars represent the infectivity in PFU/mL measured by plaque assay; the gray bars represent the antigenic potency by 2E10 monoclonal ELISA. The triangles represent the pH of the sample at each time point.

tectable levels within 10–12 h at room temperature (data not shown). To determine the efficacy of BPL to inactivate YF 17D virus, inactivation studies were performed at a reduced (1/40th) scale at controlled room temperature (22.5°C). The starting material was live virus bulk purified and concentrated by depth filtration, Benzonase® digestion, ultra-filtration and diafiltration, having a titer of $9.0 \log_{10}$ PFU/mL.⁴ Inactivation of virus was rapid, with no detectable infectivity ($\text{LOD } 0.4 \log_{10}$ PFU/mL) after 1 h (Fig. 3). There was, however, no impact on antigenic potency of the preparation as measured by 2E10 monoclonal ELISA. The pH was monitored throughout, because hydrolysis of BPL to β -hydroxypropionic acid can reduce pH and because pH below 5.5 could itself inactivate virus confounding the interpretation of the effect of BPL. However, due to the buffer use, pH remained >7.5 throughout the study (Fig. 3).

To determine whether sufficient BPL was present to inactivate an additional quantity of YF virus, the experiment was repeated with the addition of a spike of $8.0 \log_{10}$ PFU at either 1 or 10 h. Samples were taken at 16 h after initiating BPL inactivation to determine the amount of YF virus that was inactivated. No infectious virus was detected when an additional $8 \log_{10}$ PFU were added at 1 h to the $9 \log_{10}$ PFU/mL starting virus concentration. When the additional $8 \log_{10}$ PFU of virus was added at 10 h, only $2 \log_{10}$ PFU of the additional material were inactivated (data not shown). This observation is consistent with the hydrolysis of BPL which showed that the BPL concentration drops to less than 0.01% by 10 h, corresponding to the time when fresh virus was added.

To determine the ability of the inactivation process to kill an adventitious virus, a high titer stock of porcine parvovirus (PPV) was used to spike the purified YF virus bulk just prior to BPL inactivation. The PPV spiked material was inactivated with BPL for 16 h, and at the end of the time of inactivation, a sample was removed and assayed for the residual amount of infectious PPV on swine testicle (ST) cells. Controls for the study included PPV spiked into buffer with and without added BPL, and YF virus bulk spiked with a cell lysate (mock spike). The results of the study indicated that PPV infectivity was reduced by at least $4.45 \log_{10}$, as no PPV was detected after 16 h.

⁴ The development study described here used a plaque assay to measure infectivity. As described in Section 2, a GLP assay for residual live virus applied to vaccine lots utilized a CPE based assay and the LOD was expressed in TCID_{50} units.

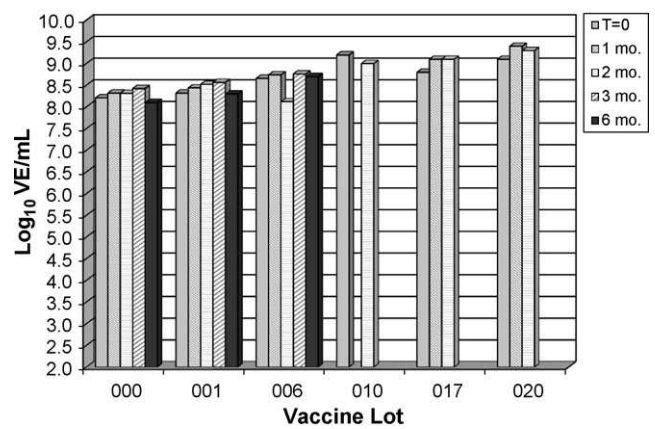


Fig. 4. Stability of XRX-001 vaccine lots at $2-8^\circ\text{C}$. Lots of vaccine are being tested in an ongoing stability study; therefore some time points were not yet reached and no data are provided in the figure.

3.4. Stability

Stability of the inactivated YF vaccine adsorbed to aluminum hydroxide was assessed under real time conditions (ongoing) as well as under accelerated conditions (25°C) for 8 weeks. All lots evaluated to date met potency specifications (time $0 \pm 0.5 \log_{10}$ VE/mL) under recommended storage conditions of $2-8^\circ\text{C}$ for up to 6 months, the longest time period tested to date (Fig. 4). Similarly there were no changes in potency for 8 weeks at 25°C (data not shown). All other specifications including the % not bound to aluminum hydroxide, pH, endotoxin, and appearance were also met under real time and accelerated conditions. Binding to aluminum hydroxide was very efficient and desorption at high pH or high salt was inefficient. In the formulated vaccine, $>98.7\%$ of the antigen in the vaccine was bound to aluminum hydroxide and binding was stable at $2-8^\circ\text{C}$ for at least 6 months and at 25°C for at least 8 weeks.

3.5. GLP toxicology study

There were no test article-related deaths in rats inoculated with the XRX-001 vaccine. All animals gained weight throughout the study; there were no elevations in body temperature; and there were no clinical abnormalities observed except for minor local injection site changes. Three XRX-001-treated animals showed swelling and/or erythema at the injection site after the 2nd or 3rd dose. XRX-001 vaccine had a minimal effect on dermal Draize observations. All edema noted was minimal (grade 1, slight swelling, indistinct border), while erythema was generally minimal (grade 1, light pink, indistinct) and was only recorded as mild (grade 2, bright pink/pale red, distinct) at one observation point in one animal. Edema and erythema were only noted in one limb of any rat and were short in duration (lasting 1–3 days). There were no test-article-related effects on hematology parameters, coagulation assays, or fibrinogen. IL-6 values were highly variable. On study day 3, IL-6 levels were increased 2-fold or more in 11/20 XRX-001 treated but only in 2/20 placebo treated animals. The mean IL-6 values were modestly higher in the XRX-001 vaccine group relative to concurrent controls and pre-treatment baseline values, but the difference was not statistically significant. The modest increase in IL-6 was consistent with immune stimulation from a vaccine test article. At the terminal necropsy, there was a statistically significant XRX-001 vaccine-related decrease in albumin/globulin (A/G) ratios due to increases in serum globulin values compared to the placebo controls. At the recovery necropsy, XRX-001-treated animals also had increased globulin values. At both necropsy time points, how-

Table 1
Experimental design and neutralizing antibody responses, first mouse study, by treatment group.

Group	Mice	Vaccine/route	Schedule		% PRNT ₅₀ positive (+/total)	Range of titers	Geom mean PRNT ₅₀ titer*
			Vaccination	Sacrifice/antibody test			
1	BALB/c	8.0 log ₁₀ VE BPL-inactivated in 0.2% aluminum hydroxide/IM	Days 0, 14	Day 28	100% (5/5)	32–128	74
2	BALB/c	8.0 log ₁₀ VE BPL-inactivated in 0.2% aluminum hydroxide/IM	Days 0, 14, 28	Day 42	100% (5/5)	2048–4096	3566
3	BALB/c	7.0 log ₁₀ VE BPL-inactivated in 0.2% aluminum hydroxide/IM	Days 0, 14	Day 28	100% (5/5)	64–≥128	97
4	BALB/c	7.0 log ₁₀ VE BPL-inactivated in 0.2% aluminum hydroxide/IM	Days 0, 14, 28	Day 42	100% (5/5)	4096	4096
5	BALB/c	8.0 log ₁₀ VE BPL-inactivated in Freund's complete/incomplete/SC	Days 0, 14, 28	Day 42	100% (5/5)	4096	4096
6	BALB/c	7.0 log ₁₀ VE BPL-inactivated in Freund's complete/incomplete/SC	Days 0, 14, 28	Day 42	60% (3/5)	16–128	64
7	BALB/c	8.0 log ₁₀ VE BPL-inactivated no adjuvant/IM	Days 0, 14, 28	Day 42	100% (5/5)	128	128
8	BALB/c	Live YF-VAX®/SC	Day 0	Day 28	20% (1/5)	<4–16	5.3
9	CD1	8.0 log ₁₀ VE BPL-inactivated in 0.2% aluminum hydroxide/IM	Days 0, 14	Day 28	100% (5/5)	1024–4096	2048
10	CD1	8.0 log ₁₀ VE BPL-inactivated in 0.2% aluminum hydroxide/IM	Days 0, 14, 28	Day 42	100% (5/5)	2012–4096	3131
11	BALB/c	0.2% aluminum hydroxide/IM	Days 0, 14, 28	Day 42	20% (1/5)	<4–16	5.3

GMT calculated using highest titer determined for each mouse, e.g. if titer was ≥2048, that value was assigned.

ever, the serum globulin increase was minimal and values were within the normal reference range for Sprague Dawley rats. The increase in serum globulin levels is likely due to administration of the vaccine. There were no other abnormalities in clinical chemistry.

Enlargement of both iliac lymph nodes was observed on terminal necropsy in 4/5 males and 4/5 females in the XRX-001-vaccinated group, and generally correlated with microscopic lymphoid hyperplasia. No test-article-related findings were noted at the recovery necropsy. XRX-001 vaccine increased absolute and relative (to brain) spleen weights in females euthanized at the terminal necropsy. This finding was associated with microscopic finding of lymphoid hyperplasia.

Test article-related microscopic findings in the injection sites of animals euthanized at the terminal necropsy included granulomatous inflammation, subacute inflammation, and edema. In the iliac lymph nodes of animals euthanized at the terminal necropsy, minimal to moderate lymphoid hyperplasia was observed in one or both lymph nodes in all XRX-001-vaccinated animals and was characterized as an increase in the number and size of germinal centers and increased cellularity (lymphocytes) in the paracortex and small clusters of foamy macrophages (or histiocytes). In the spleen, minimal lymphoid hyperplasia was observed in 4/5 males and 5/5 females in the XRX-001-vaccinated group, and this finding correlated with increased spleen weights (in females). For the recovery animals, test article-related inflammation at and adjacent

to injection sites was similar in appearance to the observations in terminal necropsy animals. However, a decrease in severity of granulomatous inflammation, and absence of edema, in injection sites at recovery necropsy, suggests ongoing resolution of test article effects.

Overall, the histopathological changes were characterized by inflammatory changes at injection sites and lymphoid hyperplasia in draining lymph nodes and spleen expected following repeated inoculation of a vaccine, and did not indicate any toxicities of concern for human vaccination with the test article.

All animals developed neutralizing antibodies against YF (titers ranged from 5120 to >81,920).

3.6. Immunogenicity of the YF vaccine in mice

In the first study, BALB/c or CD-1 mice were given, at 14-day intervals, 2 or 3 IM inoculations of XRX-001 vaccine adsorbed to 0.2% aluminum hydroxide (Table 1). A control group received undiluted YF-VAX® SC. Two dose levels of XRX-001 (7.0 and 8.0 log₁₀ VE) were tested in BALB/c mice, whereas only the high-dose level was tested in CD-1 mice. Other groups of mice were primed with inactivated vaccine and complete Freund's adjuvant (CFA) by the SC route and boosted with vaccine with incomplete Freund's adjuvant (IFA). An additional control group received inactivated vaccine with no adjuvant. Sera were tested by PRNT₅₀ 14 days after the last immunization with inactivated vaccine and 28 days after live YF

Table 2

Experimental design and neutralizing antibody responses, second study in CD-1 mice (to determine accelerated immunization schedule), by treatment group.

Group	Vaccine, actual dose in 0.1 mL	Schedule		% PRNT ₅₀ positive (+/total)	Range of titers	Geom mean PRNT ₅₀ titer ^a
		Vaccination	Sacrifice/antibody test			
1	BPL-inactivated in 0.2% aluminum hydroxide, 7.5 log ₁₀ VE	Day 0	Day 7	0% (0/5)	<20	<20
2	BPL-inactivated in 0.2% aluminum hydroxide, 6.5 log ₁₀ VE	Day 0	Day 7	0% (0/5)	<20	<20
3	BPL-inactivated in 0.2% aluminum hydroxide, 7.5 log ₁₀ VE	Days 0, 7	Day 14	70% (14/20)	<20–160	26.5
4	BPL-inactivated in 0.2% aluminum hydroxide, 6.5 log ₁₀ VE	Day 0, 7	Day 14	13.3% (2/15)	<20–20	11
5	BPL-inactivated in 0.2% aluminum hydroxide, 5.4 log ₁₀ VE	Days 0, 7	Day 14	0% (0/10)	<20	<20
6	BPL-inactivated in 0.2% aluminum hydroxide, 7.5 log ₁₀ VE	Days 0, 14	Day 21	95% (19/20)	<20–>1280	88.8
7	BPL-inactivated in 0.2% aluminum hydroxide, 6.5 log ₁₀ VE	Days 0, 14	Day 21	20% (3/15)	<20–20	11.5
8	BPL-inactivated in 0.2% aluminum hydroxide, 5.4 log ₁₀ VE	Day 0, 14	Day 21	0% (0/10)	<20	<20
9	Live YF-VAX [®] undiluted	Day 0	Day 14	20% (1/5)	<20–20	11.5
10	Live YF-VAX [®] diluted 1:10	Day 0	Day 14	0% (0/5)	<20	<20
11	Live YF-VAX [®] diluted 1:100	Day 0	Day 14	0% (0/5)	<20	<20
12	PBS–0.2% aluminum hydroxide	Days 0, 14	Day 21	0% (0/5)	<20	<20

^a For calculation of GMT, the reciprocal titer was used one dilution below or above a value for which the endpoint was not determined, e.g. 10 was used for <20 and 2560 was used for >1280.

17D immunization. Negative control animals received aluminum hydroxide adjuvant only.

Neutralizing antibody response rates and titers are shown in Table 1. All mice, regardless of strain, receiving 2 or 3 doses of 7.0 or 8.0 log₁₀ VE of the inactivated vaccine without adjuvant (Group 7), or with aluminum hydroxide adjuvant (Groups 1–4, 9, 10), developed neutralizing antibody responses. Vaccine administered by the SC route with Freund's adjuvant was less immunogenic; only 3 (60%) of 5 mice receiving 7.0 log₁₀ VE of inactivated virus responded (Group 6). Two doses of inactivated vaccine (on days 0 and 14) were less immunogenic than 3 doses given on days 0, 14 and 28. Since mice receiving 2 doses were only followed for 14 days after the second dose before being sacrificed, it could not be determined whether antibody titers would have continued to rise without the third boost or whether the latter was principally responsible for the rise in antibody titers after the day 28 booster dose. One mouse in the control group had a low titer (1:16) of antibody, which was probably non-specific.

Neutralizing antibody titers of 2048–4096 were found in 5 of 5 BALB/c mice immunized with 3 doses of XRX-001 at the 7.0 and 8.0 log₁₀ VE dose levels (Groups 2 and 4, Table 1). CD1 mice immunized with 2 doses of XRX-001 at the 8.0 log₁₀ VE/dose level achieved higher titers (Group 9; titers 1024–4096) than did sim-

ilarly immunized BALB/c mice (Group 1; titers of 32 to ≥128). YF-VAX[®] was poorly immunogenic, with only 1 of the 5 mice developing a low level of neutralizing antibodies. Low antibody titers in mice receiving undiluted YF-VAX[®] are not unexpected [19].

A second study employed an accelerated vaccination schedule to investigate kinetics of the immune response (Table 2). CD-1 mice were given either a single IM dose or two IM doses at 7- or 14-day intervals, of graded doses of XRX-001 inactivated vaccine adsorbed to 0.2% aluminum hydroxide. Positive control groups received undiluted, 1:10, or 1:100 diluted YF-VAX[®]. A negative control group received 0.2% aluminum hydroxide only. Neutralizing antibodies were determined by PRNT₅₀ at 7 or 14 days after the last immunization.

No antibodies were observed 7 days after a single dose of vaccine (Table 2). When vaccine was administered as two doses separated by 7 days and antibodies measured 7 days after the second dose, 70% of mice receiving 7.5 log₁₀ VE seroconverted (Group 3). The 50% immunizing dose given on days 0 and 7 was 7.11 log₁₀ VE (data from Groups 3–5 calculated by the method of Reed and Muench) [22].

When the vaccine was administered in two IM inoculations 14 days apart and antibodies measured 7 days after the second inoculation, 95% of the mice receiving 7.5 log₁₀ VE seroconverted (Group 6, Table 2). The 50% immunizing dose given on days 0 and 14 was

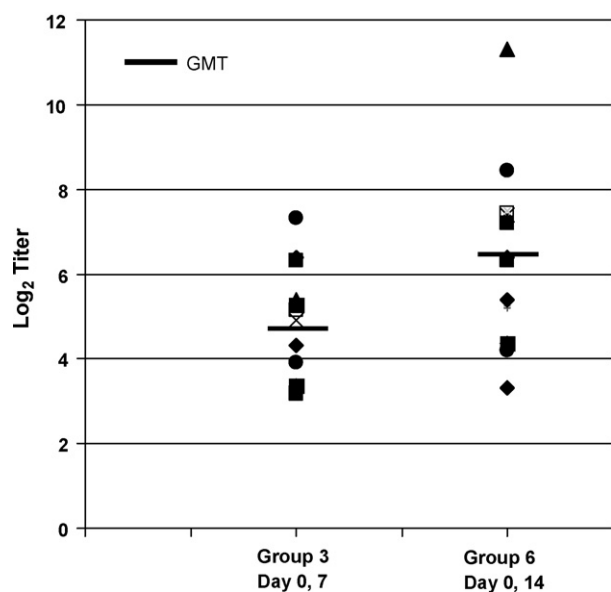


Fig. 5. Individual neutralizing antibody titers, mice in Groups 3 and 6 (XRX-001 high dose given on days 0 and 7 or 0 and 14, respectively). Sera were taken 7 days after the second dose.

6.97 log₁₀ VE (data from Groups 6–8). Thus, a longer interval (14 days) between two inoculations was more immunogenic than the accelerated schedule dosing 1 week apart. Neutralizing antibody responses in individual mice inoculated 1 or 2 weeks apart are shown in Fig. 5. As noted previously, YF-VAX[®] was poorly immunogenic in mice.

3.7. Hamster immune response and protection against lethal challenge

Three groups of 10 Golden hamsters (*Mesocricetus auratus*), 3–4 weeks of age were vaccinated IM on days 0 and 21 with three different doses of XRX-001 [high, mid and low dose, roughly 8, 7 and 6 log₁₀ VE (Table 3)]. One group of hamsters received a single injection of the high vaccine dose on day 21. Control groups received YF-VAX[®] undiluted or YF-VAX[®] diluted 1:10 and a final group received placebo (0.2% aluminum hydroxide in 0.9% NaCl). Antibodies were measured by PRNT₅₀ before challenge on day 49 with the Jimenez strain of YF virus. Animals were then observed for illness, weight change, increased serum alanine aminotransferase (ALT) levels, and viremia.

All animals given a single inoculation (day 21) or two inoculations (days 0 and 21) of the high-dose XRX-001 vaccine (7.7–7.9 log₁₀ VE) seroconverted and developed high levels of neutralizing antibodies (Table 3). Two inoculations resulted in very high neutralizing antibody responses (>10,240) in all animals and antibody titers were significantly higher than those evoked by 0.1 mL of undiluted live YF-VAX[®] (GMT 1940) ($P < 0.001$, ANOVA). A single inoculation of XRX-001 (Group 7) elicited neutralizing antibody titers (GMT 2,195) similar to those observed following live YF-VAX[®] (GMT 1940).

The neutralizing antibody response to XRX-001 showed a clear relationship to dose, with seroconversion and GMT diminishing with decreasing dose. The mid-dose (6.7–6.9 log₁₀ VE) was moderately effective, showing 80% seroconversion and reduced GMT (453) compared to high-dose XRX-001 (GMT 20,480). The low dose XRX-001 (5.7 log₁₀ VE) was poorly immunogenic. The 50% immunizing dose (ID₅₀) of 2 doses given 21 days apart was determined to be 6.23 log₁₀ VE (method of Reed and

Muench) [21]. Individual hamster antibody responses are shown in Fig. 6A.

After challenge, 7 (78%) of 9 hamsters inoculated with placebo died (Table 3; Fig. 6B). In contrast, all of the animals given one or two inoculations of the high-dose of XRX-001 or two doses of the mid-dose of XRX-001, or with YF-VAX[®] (undiluted or diluted 1:10) survived. In addition, death occurred in only 1 of 10 (10%) of the animals vaccinated with the lowest dose of XRX-001, indicating partial protection in this dose group despite low antibody responses. The differences in survival ratios between the vaccinated and placebo groups were statistically significant ($P < 0.05$, Wilcoxon log rank test). It is likely that hamsters in the low dose group had antibody titers below the lowest serum dilution (1:40) used in the PRNT₅₀.

ALT levels 6 days after challenge were elevated in 5 (62.5%) of 8 placebo treated hamsters indicating severe hepatitis (Table 3; Fig. 6C). Animals that received either 1 or 2 inoculations of high-dose XRX-001 had normal ALT levels. Two animals in the mid-dose XRX-001 treatment group developed elevated ALT levels, and these two hamsters were the same animals that did not seroconvert after vaccination. Nine (90%) of 10 hamsters in the low dose XRX-001 treatment group developed elevated ALT ($P < 0.001$ vs. the high-dose group); thus while the majority of animals in this group survived, they had hepatic dysfunction.

Viremia was observed in 7 (78%) of 9 hamsters in the placebo group (Table 3; Fig. 6D). None of the animals given 1 or 2 inoculations of high-dose XRX-001 vaccine, mid-dose XRX-001 vaccine, or YF-VAX[®] developed detectable viremia (LOD of the assay is 1.8 log₁₀ TCID₅₀/mL). Three of 10 animals in the low dose XRX-001 treatment group developed viremia, but the levels were reduced compared to the placebo group.

Weight loss after challenge was observed in 5 (55.6%) of 9 hamsters in the placebo group and in 7 (70%) of 10 animals in the low dose XRX-001 group (Table 3; Fig. 6E). Fewer animals in the other vaccinated groups showed weight loss.

3.8. Immunogenicity of the YF vaccine in non-human primates

Two groups of 3 cynomolgus macaques received a clinical lot of XRX-001 (Lot 900-03-006) adsorbed to 0.2% aluminum hydroxide. The dose of XRX-001 injected IM was 8.3 log₁₀ VE in 0.5 mL. One group received inoculations on days 0 and 21, and the second group received inoculations on days 0, 10, and 21. A control group of 2 monkeys were inoculated SC with 0.5 mL of undiluted YF-VAX[®]. Monkeys were bled for antibody determinations before vaccination on day 0, after 1 vaccination for Groups 1 and 3 (day 21), after 2 vaccinations for Group 2 (day 21), and 21 days after the final vaccination (day 42).

With one exception (Monkey #60964) YF neutralizing antibody titers were high (≥ 640) on day 21, after 1 dose of inactivated or live vaccine (Table 4). Two doses of inactivated vaccine on days 0 and 10 also elicited an antibody response similar to 1 dose of live vaccine. There did not appear to be a benefit from a 3-dose immunization schedule (Group 2) over a 2-dose schedule (Group 1). With the exception of one animal in Group 1 (#60964), which showed a rise in titer between day 21 and 42, antibody titers were either not substantially increased or actually decreased on day 42 after the second or third dose of vaccine.

The day 42 sera of all monkeys were also tested by PRNT₅₀ against a wild-type strain of YF virus representing a heterologous genotype (14-FA, Angola genotype) (Table 4). Both XRX-001 and live 17D vaccine elicited neutralizing antibody responses to homologous 17D (derived from West Africa II genotype) and to heterologous [23] 14-FA virus.

Table 3

Experimental design, neutralizing antibody responses and protective efficacy of XRX-001 and YF-VAX in Golden hamsters.

Group	Vaccine/route	Schedule (dose)		% PRNT ₅₀ positive (+/total) and GMT Day 49 (pre-challenge)	Survival ratio (%)	ALT No. elevated/tested (% elevated) Geo.mean (IU/mL)	Viremia No. Pos./tested (% Pos.) mean (log ₁₀ CCID ₅₀) ^a	No. with weight loss/tested (%) between day 3 and 6 post-challenge mean change (grams)
		Vaccination	Challenge					
1	XRX-001 Lot 900-03-09-001C (high dose), 0.2% aluminum hydroxide/IM	Days 0, 21 (7.9, 7.7 log ₁₀ VE in 0.25mL) ^b	Day 49	10/10 (100%) GMT 20,480	10/10 (100%)	0/10 (0%) GM 63.7	0/10 (0%)	3/10 (30%) Mean +3 g
3	XRX-001 Lot 900-03-09-001B (mid-dose), 0.2% aluminum hydroxide/IM	Days 0, 21 (6.9, 6.7 log ₁₀ VE in 0.25mL) ^b	Day 49	8/10 (80%) GMT 453	10/10 (100%)	2/10 (20%) GM 80.1	0/10 (0%)	2/10 (20%) Mean +2 g
5	XRX-001 Lot 900-03-09-001a (low dose), 0.2% aluminum hydroxide/IM	Days 0, 21 (5.7, 5.7 log ₁₀ VE in 0.25 mL) ^b	Day 49	3/10 (30%) GMT 37	9/10 (90%)	9/10 (90%) GM 169.0	3/10 (30%) Mean 3.0 ^a	7/10 (70%) Mean –5 g
7	XRX-001 Lot 900-03-09-001C (high dose), 0.2% aluminum hydroxide/IM	Day 21 (7.7 log ₁₀ VE in 0.25 mL)	Day 49	9/9 (100%) GMT 2195	10/10 (100%)	0/10 (0%) GM 60.8	0/10 (0%)	1/10 (10%) Mean +4 g
9A	YF-VAX [®] Lot UF432AA undiluted/SC	Day 21 (3.8 log ₁₀ PFU in 0.1 mL)	Day 49	5/5 (100%) GMT 1940	5/5 (100%)	1/5 (20%) GM 81.0	0/5 (0%)	1/5 (20%) Mean +3 g
9B	YF-VAX [®] Lot UF432AA diluted 1:10/SC	Day 21 (2.8 log ₁₀ PFU in 0.1 mL)	Day 49	4/4 (100%) ^c GMT 905	5/5 (100%)	0/5 (0%) GM 61.5	0/5 (0%)	1/5(20%) Mean 0 g
11	Saline 0.2% aluminum hydroxide/IM	Day 0, 21 (0.1 mL)	Day 49	0/7 (0%) ^d	2/9 (22%)	5/8 (62.5%) ^d GM 129.7	7/9 (78%) Mean 5.9 ^a	5/9 (55.6%) Mean –1 g

^a Mean log value calculated for animals with detectable viremia only. The LOD of viremia is 1.8 log₁₀ TCID₅₀.^b Day 0 and day 21 inocula potencies were measured separately.^c One animal not tested for antibody (serum QNS).^d Two animals not tested for antibody (serum QNS) and 1 was not tested for ALT.

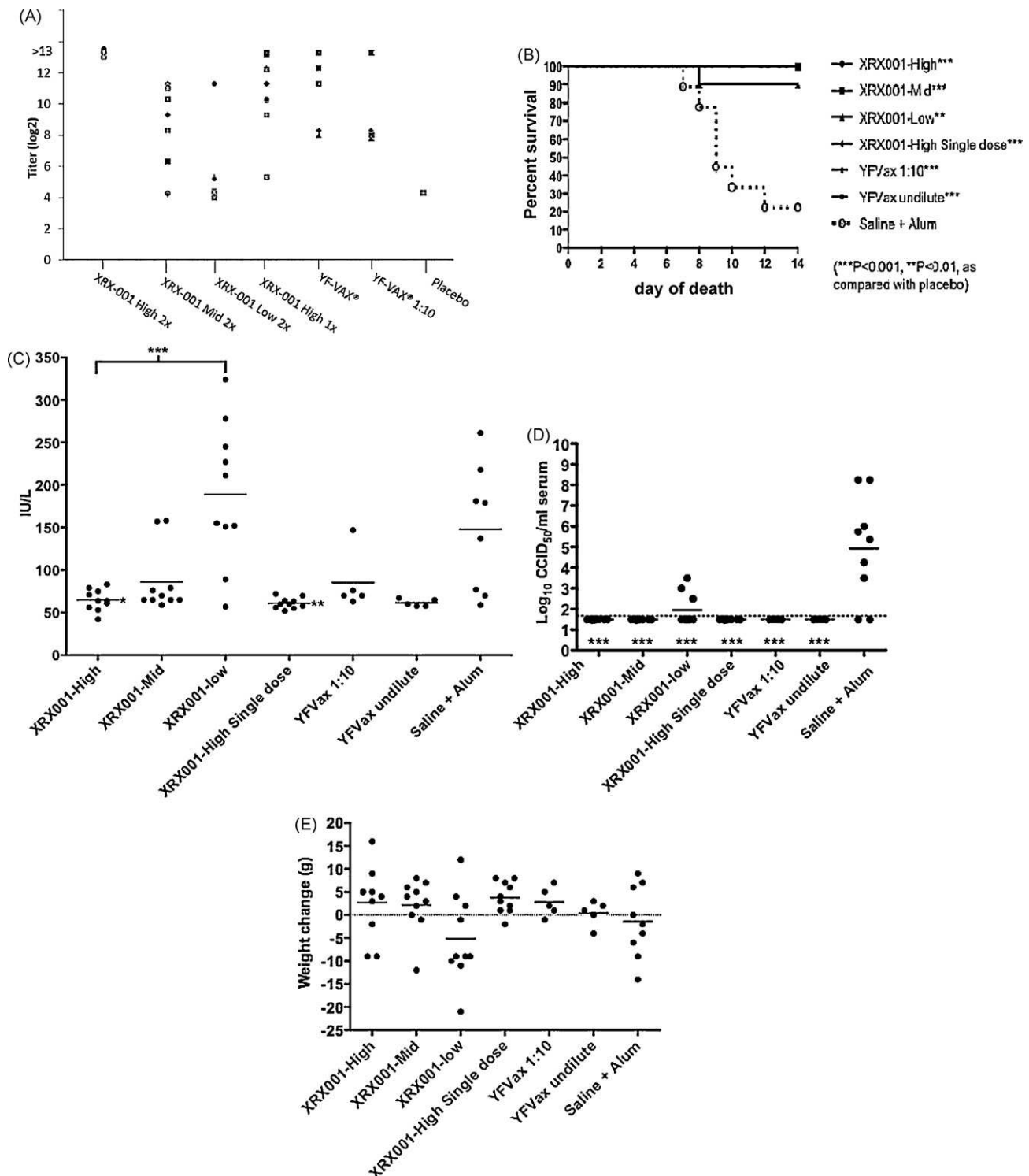


Fig. 6. (A) Individual neutralizing antibody levels, by treatment group (refer to Table 3), hamsters inoculated with XRX-001 or YF-VAX®. (B) Survival, hamsters immunized with XRX-001 or YF-VAX and challenged on day 0 with hamster-virulent YF virus (Jimenez). *P* values, Wilcoxon log-rank test. (C) Alanine aminotransferase (ALT) levels, serum of hamsters vaccinated with XRX-001 or YF-VAX® 6 days after challenge with hamster-virulent YF (Jimenez) virus. The low and high XRX-001 dose groups were statistically different (*** $P < 0.001$). Hamsters receiving a single (high) dose of XRX-001 or two (high) doses of XRX-001 were statistically different compared to the placebo group (** $P < 0.01$, * $P < 0.05$). ANOVA with Bonferroni correction. (D) Viremia titers 6 days after challenge of vaccinated hamsters with virulent YF virus. *** $P < 0.001$ compared with the placebo group. Cell culture ID₅₀ (CCID₅₀) = TCID₅₀ (terminology used in text). Dotted line is limit of detection in the assay. (E) Weight change between days 3 and 6 after challenge, by treatment group.

4. Discussion

Historical attempts to develop inactivated YF vaccines were made in 1928 using chemically inactivated spleen or liver sus-

pension from monkeys infected with wild-type virus [24], and later (in 1935) using mouse brain, chick embryo or monkey serum virus inactivated by heat, ultraviolet or formaldehyde [25]. Overall, these preparations were problematic due to residual live virus

Table 4

Neutralizing antibody response rates and titers, cynomolgus macaques vaccinated with XRX-001 or YF-VAX®.

Group	Treatment	Animal #	PRNT ₅₀ Titer to YF 17D			Immunofocus titer to 14-FA ^a
			Day 0	Day 21	Day 42	Day 21
1	XRX-001 2 doses (8.3 log ₁₀ VE in 0.5 mL) days 0, 21	60,964	<40	80	2560	60
		29,162	<40	640	1280	30
		29,448	<40	5120	320	1280
2	XRX-001 (8.3 log ₁₀ VE in 0.5 mL) 3 doses days 0, 10, 21	61,325	<40	320	640	1280
		10,443	<40	1280	80	1600
		26,367	<40	640	1280	120
3	YF-VAX® (undiluted 0.5 mL) day 0	26,372	<40	2560	1280	240
		26,390	<40	640	320	160

^a Mean of replicate tests.

or were ineffective due to inadequate antigenic potency, reflecting the rudimentary virological methods available at the time. With the development of successful live, attenuated YF vaccines (the French neurotropic and 17D vaccines) in the 1930s [3–5] there was no further interest in pursuing inactivated vaccines against this disease. The impetus for such an effort came only recently, 65 years after development of the live 17D vaccine, when new concerns arose over its safety due to reports of YEL-AVD. In addition to the Xcellerex program, BioManguinhos (Rio de Janeiro) is engaged in the development of an inactivated YF vaccine [26].

A robust manufacturing method has been developed facilitating the production of inactivated YF vaccine (XRX-001) in continuous cultures of Vero cells, a substrate used for several licensed vaccines [inactivated polio and Japanese encephalitis (IXIARO®) vaccines, and live, attenuated smallpox vaccine (ACAM2000)]. Yields of YF 17D in Vero cells were enhanced by adapting the virus by serial passage, which resulted in a 3- to 6-fold increase in virus titer in cell culture fluid, and was associated with a single amino acid change in the consensus sequence. Using a single-use, stirred tank bioreactor system (the Xcellerex XDR®) and Vero cells grown on microcarrier beads, the titer of virus in cell culture fluid exceeded 8.0 log₁₀ PFU/mL; with the concentration during downstream purification, the titer of the final drug substance was consistently >9.0 log₁₀ VE/mL before dilution to the desired potency in the final vial. Biosafety during manufacturing was ensured by preparing the vaccine from the attenuated 17D strain (a BSL2 agent) and by immunizing personnel with the approved 17D vaccine.

The antigenic potency of the XRX-001 vaccine was shown to be within the same range of approved, aluminum-adjuvanted inactivated vaccines against two other flaviviruses: Japanese encephalitis (IXIARO®, Intercell, Vienna, Austria), which is used at 6 µg of antigenic protein/dose [27]; and tick-borne encephalitis (FSME-Immun®, Baxter, Orth, Austria), which is used at 2.4 µg/dose [28]. The XRX-001 vaccine lot used in an ongoing clinical trial has a potency in the same range: 4.4 µg/dose, which represents 8.3 log₁₀ VE, equivalent to the same number of PFU in the form of inactivated antigen. Based on its comparable antigenic potency to Japanese encephalitis and tick-borne encephalitis, aluminum-adjuvanted vaccines, it is expected that the XRX-001 vaccine will be immunogenic in humans. The vaccine does not contain proteins contaminating the current 17D vaccine implicated in allergy (ovalbumin and other egg derived proteins) or gelatin used as a stabilizer. The live, 17D vaccine is contraindicated for persons with egg allergy, and the reporting rate of anaphylaxis ranges between 1.8 and 3.2 per 100,000 [12,29].

A key issue in developing a new viral vaccine is the method selected for inactivation of the virus, the demonstration that the inactivation capacity far exceeds the amount of virus in the material being processed, and the application of sensitive methods for detecting residual live virus [30]. These concerns stem from

untoward experience with inactivated polio vaccines (the “Cutter Incident” [31]) and periodic failures during production of certain other vaccines, including Venezuelan equine encephalitis and rabies. In addition, it is well-known that chemical and physical inactivation methods can degrade epitopes and reduce immunogenicity of some vaccines [32,33]. We utilized β-propiolactone (BPL), which alkylates purine residues of nucleic acids to destroy virus infectivity. BPL has a long history of use in vaccine manufacture and has a number of advantages over formalin, including rapid virus inactivation kinetics and rapid hydrolysis to β-hydroxypropionic acid, a harmless natural fat metabolite in humans, thereby eliminating the need for neutralization or removal steps [34,35]. Inactivation of 9 log₁₀ PFU of XRX-001 was rapid (less than 1 h) and it was possible to spike an additional 8 log₁₀ PFU of virus into the reaction mixture at 1 h and still achieve full inactivation. A sensitive method for detecting residual live virus at a limit of detection of 0.14 TCID₅₀/mL in vaccine lots was qualified, involving three cycles of passages in Vero cells. No live virus has been detected in multiple lots of XRX-001. Even if there was a low level of residual live virus in a vaccine lot, below the limit of detection, it is highly unlikely to cause an untoward event because it is an attenuated strain.

Antigenic potency was maintained over the 16 h inactivation process as determined by an ELISA that detects a key (2E10) neutralization epitope and has identical sensitivity for measurement of live and inactivated virus. These results indicated that there was no degradation of the antigen, at least of the 2E10 neutralizing epitope. Others have compared methods of inactivating various viruses, including BPL, formalin and ultraviolet light. In the case of rhabdoviruses, formalin and BPL were shown either to degrade both B cell epitopes and T cell epitopes, or to degrade T cell epitopes while B cell epitopes were retained [36,37]. At this point in development, we have not studied T cell responses to the inactivated YF vaccine.

The nonclinical evaluation of XRX-001 showed no safety signals in a study in rats employing a high dose given repetitively on an accelerated schedule. The main findings were mild injection site inflammation, transient IL-6 elevations, and lymphoid hyperplasia consistent with stimulation of the immune system.

The principal safety benefit of the inactivated vaccine is its inability to replicate in tissues of the host. The pathogenesis of both YEL-AVD and the majority of cases of YEL-AND involves unchecked replication of 17D virus in critical visceral organs including the liver, kidney, and heart (in YEL-AVD) or invasion of the central nervous system via the bloodstream (in YEL-AND) [5,7–9,12]. YEL-AVD may result from deficient production of antiviral responses (such as 2',5'-oligoadenylate synthetase, C1q and double-stranded RNA-activated protein kinase) or some other defects in innate immunity [38–40]. Acquired immune deficiency, resulting from HIV/AIDS [41] or thymectomy [42] can also be responsible for serious adverse events due to unchecked YF 17D virus replication. These adverse

effects are not easily modeled in animals, although recently it was shown that interferon- α/β and γ receptor deficient mice develop a viscerotropic disease not dissimilar to wild-type YF or YEL-AVD [43]. In the future it will be important to demonstrate that the inactivated vaccine is safe in this model and in models of (adaptive) immune suppression. There is no reason to expect otherwise. Many persons desiring to travel to the tropics who require YF vaccination have risk factors for severe and serious adverse events, especially age >60 years, immune suppression, and pregnancy and would benefit from the availability of a non-replicating vaccine.

Aluminum hydroxide was selected as an adjuvant for XRX-001. Until recently, aluminum adjuvants were the only ones incorporated into vaccine formulations in the US, and their long history of use reduces the regulatory risk of the new YF vaccine. The putative mechanism of action of aluminum adjuvants has been recently reported (it activates the NALP3 inflammasome pathway) [44]. It is used as an adjuvant in two similar inactivated flavivirus vaccines (IXIARO® and tick-borne encephalitis vaccines). These vaccines, as well as XRX-001 depend on production of neutralizing antibodies as the mediator of protective immunity, and thus the Th2-orientation of CD4+ cells promoted by aluminum adjuvant is a favorable attribute. When inactivated vaccine was administered to mice with Th1-oriented Freund's adjuvant, antibody responses in mice were substantially weaker (Table 1).

Outbred (CD-1) mice developed more robust immune responses to two doses of the inactivated YF vaccine than inbred BALB/c (H-2d) mice. It is not surprising to find genetic differences in antibody responses in a mouse strain (BALB/c) that is genetically MHC restricted since T cell priming by antigen presenting cells is an MHC function involved in and T cell cooperation is critical to regulated transcription of genes that determine specific B cell expansion and differentiation.

Mice are useful for assessment of immune responses to an antigen (like XRX-001), but they respond poorly to the live 17D vaccine due to host species restriction of this virus [19]. Consequently, we relied on hamsters and monkeys to assess the relative immunogenicity of XRX-001 and live 17D vaccines. The hamster model is widely used for the development of vaccines and antiviral drugs against YF [17,20]. Hamsters develop fatal viscerotropic infection characterized by hepatitis, elevated liver enzymes, and jaundice and thus models closely the disease in humans infected with wild-type YF virus strains. The virological and pathologic characteristics of this model have been described [17,20,45]. The mortality ratio of wild-type YF virus is about 50–75% in hamsters, whereas it is 20–50% in humans. Time to death in hamsters is similar to that in humans. Unlike mice, but like human and non-human primates, hamsters respond well to the live, attenuated 17D vaccine, developing high titers of neutralizing antibodies. Therefore, the hamster model is suitable for testing both the immunogenicity and protective efficacy of a new YF vaccine and for comparing responses to the benchmark live 17D vaccine. The antibody responses evoked in hamsters 4 weeks after a single dose of XRX-001 were surprisingly robust and were similar to those following the live 17D vaccine (Table 3). Hamsters immunized with one inoculation of XRX-001 were protected against lethal challenge with the Jimenez strain of YF virus. Monkeys also developed neutralizing antibody titers after a single dose of XRX-001 similar to those following YF-VAX® (Table 4). It was of considerable interest that most monkeys did not show a significant boost in antibodies after a second or third dose of vaccine, a finding that is unexplained and will require confirmation in humans. If these findings are observed in humans, a single dose of XRX-001 in humans might provide sufficient levels of immunity to allow short term travel, an advantage seen with some other non-replicating travel vaccines (hepatitis A, typhoid Vi). However, attempts to induce protective immunity with a single (high) dose of another flavivirus vaccine (IXIARO®) were not successful [27].

XRX-001 was developed from a biologically cloned virus derived from YF 17D, vaccine, which is an uncloned, genetic swarm. This approach could raise the criticism that a clonal sub-population was selected that may not produce the same spectrum of immune responses against heterogeneous wild-type YF viruses belonging to at least 7 different genotypes [23]. However, the clonal XRX-001 vaccine elicited neutralizing antibodies in mice, hamsters and monkeys that neutralized uncloned 17D virus at high titer as well as wild-type virus belonging to a different genotype (Angola), and it protected hamsters against a challenge virus belonging to yet another different genotype (Jimenez, genotype South America I). Yellow fever virus represents a single serotype (despite the differentiation of genotypic variants), and the virus E protein contains redundant neutralizing epitopes. Thus, the use of a clonal antigen in this vaccine does not appear to limit protective efficacy against heterologous strains of YF virus.

The fact that XRX-001 vaccine had equivalent or better immunogenicity than live 17D vaccine in hamsters and monkeys was unexpected, since in general it is thought that live, replicating vaccines are more effective due to expansion of the antigenic mass and induction of a cytokine milieu which provides an adjuvant effect. The extraordinary efficacy of YF 17D vaccine for humans has been the subject of recent study, including definition of gene activation signatures [39,46–48]. The virus activates myeloid and plasmacytoid DCs to produce proinflammatory cytokines (IL-12p40, IL-6 and interferon- α via toll-like receptors (TLR) 2, 7, 8, and 9 [39,49]. Other proinflammatory mediators induced in peripheral blood mononuclear cells of vaccinees include IL-1 α and CXCL10 (CXCL10) [50]. The activation of multiple TLRs is responsible for the mixed Th1 and Th2 helper T cell response seen after 17D immunization. Production of interferon- α by plasmacytoid DCs requires TLR-mediated activation of the mammalian target of rapamycin (mTOR), a regulator of cytokine expression, and activation of downstream mediators of mTOR, p70 ribosomal S6 protein kinases [51]. The innate immune receptors RIG-I and melanoma differentiation-associated gene 5 (MDA-5) are stimulated also, as well as the genes that regulate these signaling pathways (e.g. RIG-I-like RNA helicase). Transcription factors that regulate expression of interferon- α are activated (e.g. IRF7, ETS and STAT1). In addition, genes in the inflammasome pathway and the complement pathway are switched on. Importantly, gene signatures correlate with the magnitude of the B and T cell responses in individual subjects. Complement protein C1qB, eukaryotic translation factor 2 alpha kinase 4 (EIF2AK4) and solute carrier family 2, member 6 (SLC2A6) correlated with CD8+ responses [39,46]. Activation of TNF receptor superfamily, member 17 (TNFRSF17) a receptor for B cell activating factor (BAFF), was a correlate of the magnitude of the antibody response [39,46]. Overall, these gene signatures indicate a marked up-regulation of the innate immune system that persists for about 2 weeks after vaccination. This response determined, in turn, the strength and persistence of the adaptive polyfunctional immune responses to 17D vaccine.

The inactivated YF vaccine would be an obvious control for studies of gene activation and the role of innate immune activation in adaptive immune responses. Given the equivalent immunogenicity of XRX-001 and YF 17D in animal models, it is likely that the inactivated vaccine also stimulates a strong innate immune response, although the activation signals and transcription factors may be different from those elicited by live 17D vaccine.

Finally, a word about the use of the neutralization test method (PRNT₅₀) for the assessment of immunogenicity, which we believe is an appropriate assay. The original work establishing the immune correlate of protective immunity to YF was performed in non-human primates by Mason et al. [52], who used a constant serum-varying virus method that expressed results as a log₁₀ neutralization index (LNI). In recent pivotal trials of a live YF 17D

vaccine, we also used this method [6] since it had been established by precedent [52]. However, the method is not well suited for non-clinical studies in small animals, e.g. mice and hamsters, because of the large volume of serum required. Moreover, the point of the studies performed here was to compare the immune responses to the inactivated vaccine to those evoked by live 17D; the comparisons used the same method for measuring neutralizing antibody (PRNT₅₀) and are thus valid. We could have used a 70% or 90% cut off for the PRNT, but the 50% cutoff is widely accepted, and was considered appropriate for trials with chimeric vaccines using YF as a vector [53,54] and trials leading to licensure of IXIARO® [55]. Once again, the point of our analysis was comparison of titers across treatment groups (XRX-001 and YF-VAX®). For clinical trials of the new vaccine, we intend also to use the PRNT₅₀ assay, which has now been formally validated for use in the ongoing Phase 1 trial. The relationship between PRNT₅₀ and the LNI assay is definitely of interest and will be determined and reported subsequently. However, since clinical efficacy will likely be based on non-inferiority trials of XRX-001 against YF 17D, and we believe it will be acceptable to use the PRNT₅₀ assay applied to sera from subjects in both treatment groups.

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