Immune correlates of protection against yellow fever determined by passive immunization and challenge in the hamster model

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Abstract

Live, attenuated yellow fever (YF) 17D vaccine is highly efficacious but causes rare, serious adverse events resulting from active replication in the host and direct viral injury to vital organs. We recently reported development of a potentially safer β-propiolactone-inactivated whole virion YF vaccine (XRX-001) which was highly immunogenic in mice, hamsters, monkeys, and humans (Vaccine 2010; 28:3827–40; New Engl J Med 2011;364:1326–33). To characterize the protective efficacy of neutralizing antibodies stimulated by the inactivated vaccine, graded doses of serum from hamsters immunized with inactivated XRX-001 or live 17D vaccine were transferred to hamsters by the intraperitoneal (IP) route 24 hours prior to virulent, viscerotropic YF virus challenge. Neutralizing antibody (PRNT50) titers were determined in the sera of treated animals 4 hours before challenge and 4 and 21 days after challenge. Neutralizing antibodies were shown to mediate protection. Animals having 50% plaque reduction neutralization test (PRNT50) titers of ≥40 four hours before challenge were completely protected from disease as evidenced by viremia, liver enzyme elevation, and protection against illness (weight change) and death. Passive titers of 10–20 were partially protective. Immunization with the XRX-001 vaccine stimulated YF neutralizing antibodies that were equally effective (based on dose response) as antibodies stimulated by live 17D vaccine. The results will be useful in defining the level of seroprotection in clinical studies of new yellow fever vaccines.

Keywords

Yellow fever; 17D; immune correlates; passive immunization; hamster; inactivated vaccine; neutralizing antibodies

Introduction

Yellow fever (YF), a mosquito-borne Flavivirus, causes hemorrhagic disease with jaundice in people inhabiting tropical areas of South America and Africa (1). The incidence of YF was dramatically reduced following development of live, attenuated vaccines in the 1930s (1, 2). The live attenuated 17D vaccine, supplied by 7 manufacturers, is currently used to...
protect travelers and is incorporated in childhood vaccination programs in many countries, with millions of doses distributed annually.

While the live 17D vaccine is highly immunogenic and effective, it can cause serious adverse events associated with replication of the vaccine virus in vital organs [yellow fever vaccine associated viscerotropic disease (YEL-AVD) and neurotropic disease (YEL-AND)]. Fortunately such events are rare (1, 3), but the severity of these serious adverse events (case fatality rate 63% for YEL-AVD) is greater than any other vaccine. The risk of serious adverse events is higher in persons with certain underlying conditions that enhance replication of 17D virus, such as elderly and immunocompromised individuals (3,4).

The reporting rate of YEL-AVD and YEL-AND in the US is 0.4 and 0.8 per 100,000 vaccinations, respectively (4). The highest rate of YEL-AVD (7.9 per 100,000) was observed during a recent immunization campaign in Peru (5). The majority of cases of YEL-AVD have been reported since 2001 when these adverse events were first described (1, 3), suggesting that earlier cases were frequently missed (6). The reporting rate of serious adverse events is higher than for other vaccines including smallpox (0.29 per 100,000) (7) or oral polio (0.11 per 100,000) (8) that have been withdrawn for safety reasons. These considerations have stimulated recent efforts to develop safer YF vaccines (9).

XRX-001 is an inactivated, whole virion YF vaccine adjuvanted with aluminum hydroxide (10). In studies of mice, hamsters and non-human primates, active immunization with XRX-001 elicited high titers of neutralizing antibodies (nAb) (10). XRX-001 was also shown to elicit neutralizing antibodies in 100% of healthy human subjects, with geometric mean antibody titers >100 (11). To confirm the critical role of nAb stimulated by the XRX-001 vaccine in protection against YF virus disease, and to identify the minimum protective nAb level required to prevent disease, passive antibody experiments were performed in a hamster model. Similar studies have previously been performed with various Flaviviruses, including YF (12–16). These studies have demonstrated that passive transfer of nAbs provides protection against disease even when administered post-virus exposure, and that neutralizing activity is essential for protection.

The hamster-adapted Jimenez strain of YF virus causes viscerotropic disease in hamsters after IP inoculation (17). This model has been used extensively in the evaluation of both antiviral drugs (18–21) and vaccines (9, 16). Outcome measures include serum levels of alanine aminotransferase (ALT), viremia and liver tissue virus, weight change, survival, and other serum chemistry parameters (22). Using this model, we have determined the efficacy and minimum protective level of nAb stimulated by immunization with the inactivated XRX-001 and live 17D vaccines. The results of these studies will help to define the level of antibody that determines seroprotection in clinical trials.

**Materials and Methods**

**Animals**

Female golden hamsters (*Mesocricetus auratus*) with an average weight of 100 g were sourced from Charles River Laboratories, Wilmington MA. After a 24-hour quarantine and 7-day acclimation period, animals were randomly assigned to cages and individually marked with ear tags. All work with animals was performed in the Biosafety Level 3 (BSL-3) area of the AAALAC-accredited Laboratory Animal Research Center at Utah State University (USU). Hamsters 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 IACUC at USU.
Viruses and virus infectivity assays

Yellow fever 17D virus was prepared by performing a single passage of a commercial vial of YF-VAX® vaccine (sanofi pasteur, Swiftwater PA) in a monolayer culture of Vero cells (see source in the section on Vaccine) and by harvesting cell culture fluid at the appearance of cytopathic effects (CPE). The virus was quantified by plaque assay in Vero cells grown in 12 well plates under methylcellulose overlay, as previously described (10). After 5 days of incubation at 37°C and 5% CO₂, plates were fixed and stained with 0.3% crystal violet-formaldehyde and plaques were counted.

Hamsters were challenged with the Jimenez strain (South American genotype I, isolated in Panama, 1974). This virus had been adapted by serial passage in hamster liver, as described by Tesh et al. (17). A seed stock was prepared from livers of hamsters removed 3 days after virus injection and homogenized in a 2 X volume of sterile phosphate buffered saline. The liver homogenate had a titer of 10⁶.⁰ 50% cell culture infectious doses (CCID₅₀)/mL. Hamsters were challenged IP with 0.2 mL of a 10⁻⁴ dilution of virus stock, which is approximately 6.25 LD₅₀.

Vaccine

XRX-001 vaccine was manufactured in conformance to current Good Manufacturing Practices (cGMP) using a well-characterized Vero Cell Bank (WHO 10–87, passage 134, obtained from the American Type Culture Collection with permission of the US Food and Drug Administration) grown in serum-free medium. For vaccine production, Vero cells grown in serum-free medium on microcarrier beads were infected, the cell culture fluid was harvested when CPE appeared, and host cell DNA was digested with nuclease. The virus was purified by multiple filtration procedures, inactivated with β-propiolactone, further purified by cellufine sulfate chromatography, and adsorbed to 0.2% aluminum hydroxide. The potency of the final product is between 8.0–9.0 log₁₀ virus equivalents (VE)/0.5 mL, VE being the inactivated antigen equivalent by ELISA of infectious virus determined by plaque assay (10,11).

Neutralization tests

Antibody levels in serum were quantified using the PRNT₅₀ as previously described (23). Briefly, samples of test sera were heat-inactivated (56° C, 30 min), serial twofold dilutions mixed with an equal volume of YF 17D virus containing 50–70 plaque forming units (PFU), incubated for 16–20 hours at 2–8° C, and inoculated onto wells of Vero monolayers grown in 12-well plates. After adsorption (1 hour, 37° C), monolayers were overlaid with 0.85% methylcellulose in Dulbecco’s minimal essential medium (DMEM) containing 10% fetal calf serum. Plates were fixed and stained with crystal violet-formaldehyde after 5 days incubation at 37° C. The endpoint was the highest dilution of serum inhibiting plaques by 50% or more compared to virus mixed with heat-inactivated fetal calf serum (negative control). XRX-001 immune serum served as a positive control.

Preparation of immune serum for use in passive immunization

Hamsters, 3–4 weeks of age were vaccinated by the intramuscular (IM) route on days 0 and 21 with XRX-001 at a dose of 8 log₁₀ VE in 0.25mL. Hamsters in the YF-VAX® vaccine control group received 0.1mL (~4.0 log₁₀ PFU) of undiluted YF-VAX® by the subcutaneous (SC) route and 10 hamsters in the placebo group received 0.2% alum in 0.9% NaCl by the IM route. Terminal blood collection was performed on day 49. Serum samples from individual hamsters within each group were pooled and the nAb titer of the pooled serum samples determined by PRNT₅₀. In one study (Experiment 2), serum from hamsters...
that received neither active nor placebo vaccinations was used as a control (‘normal hamster serum’).

Passive antibody transfer study design

For passive immunization studies, dilutions of pooled serum from the animals vaccinated with XRX-001, YF-VAX®, saline-alum placebo, or normal hamster serum were prepared as shown in Table 1. These dilutions were made based on estimates of the passive titer of nAbs in vivo, taking into consideration the dilution effects of hamster blood volume (assumed to be 7mL), resulting in a 1:15 dilution of the 0.5mL inoculum, and distribution in the intravascular space (assumed to be 50% of the IP inoculum), for an estimated total dilution in vivo of ~1:30. We were particularly interested in the protective capacity of very low passive antibody titers, because titers following active immunization <40 were previously shown to protect against challenge 10). A volume of 0.5 ml of each serum dilution was administered IP to groups of 10 hamsters 24 h prior to virus challenge. The PRNT$_{50}$ titers of the diluted serum pools used to passively immunize the hamsters are shown in Table 1, -24 h column, as well as the estimated passive titer following dilution in vivo.

Twenty-four hours after antibody treatment, animals were challenged with YF (Jimenez) virus and then followed for mortality, weight change, ALT levels, and viremia. Hamsters were challenged IP with 0.2 mL of a 10$^{-4}$ dilution of vírus stock, which is approximately 6.25 LD$_{50}$. Serum samples were collected from the retro-orbital sinus approximately 4 hours before and 4 and 21 days after challenge to determine passive antibody titer by PRNT$_{50}$. ALT was determined on day 6 after challenge using a kit (Teco Diagnostics, Anaheim, CA) according to the manufacturer’s directions and modified for use in 96-well plates. Viremia on day 4 post challenge 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 mean titer (CCID$_{50}$/mL) determined by CPE endpoint. In previous studies to develop the hamster model, the senior author determined that peak viremia occurs on day 4.

Two experiments were performed using the methods described above, except that: 1) the dose of passive antibody was adjusted in the second study in order to more accurately find the passive titer correlating with protection, and 2) normal hamster serum was used as a control in lieu of serum raised to placebo (saline + alum). In Experiment 1, antibody titers following passive immunization and challenge were determined in individual hamster sera if sufficient samples allowed; otherwise sera were pooled for PRNT$_{50}$. The basis for pooling sera obtained 4 hours before challenge was an analysis of hepatic dysfunction (ALT levels) on day 6; sera from hamsters showing protection or no protection by ALT assay were pooled for antibody determinations. In Experiment 2, all hamsters were tested individually. In both experiments, hamsters were individually identified, so that each outcome measure (survival, weight, viremia, ALT, and antibody level) could be compared.

Statistical methods

Treatment group comparisons for continuous variables (weight change, ALT, viremia) were performed by one-way ANOVA with a Bonferroni multiple comparison post-test analysis comparing the antibody treated groups to the aluminum hydroxide-saline control group. Logistic regression analysis was performed by using the continuous dependent variable of antibody titer and the independent variables of viremia and ALT level.

Results

To eliminate the possibility that non-immune hamster serum afforded non-specific protection against YF virus, a study was performed in which groups of 10 hamsters were
treated with normal hamster serum diluted 1:10 and administered IP 24 hours before virus challenge. A control group (n=10) received no treatment. There was no effect of normal serum treatment on any outcome measure: survival, weight change, viremia, or ALT (data not shown).

Hamsters immunized with two injections (Day 0, 21) of XRX-001 (8 log₁₀ VE) or with 1 injection of YF-VAX® (~4.0 log₁₀ PFU) and bled on Day 49 developed high titer nAbs for use in the passive immunization study. Pooled serum from hamsters immunized with XRX-001 vaccine had a PRNT₅₀ titer of 41,000. A 9-fold lower titer (4,800) of nAb was present in pooled serum from animals vaccinated with YF-VAX®, and no nAb was detected in animals immunized with saline + alum. These results are similar to those from a previous, independent study in which XRX-001 vaccine was found to be highly immunogenic in hamsters, with two doses eliciting 11-fold higher nAb levels than a single dose of YF-VAX® (10).

To determine the protective efficacy of YF nAb, hamsters were passively immunized with graded doses of immune serum and challenged 24 hours later with YF (Jimenez) virus. In Experiment 1, groups of 10 hamsters were passively immunized with 0.5 mL of each of the following dilutions of XRX-001 antibody: 1:10, 1:100, 1:1000 and 1:10,000 (Table 1A). Likewise, groups of 10 hamsters received 0.5 mL of serum from animals immunized with YF-VAX® at serum dilutions of 1:10, 1:100 and 1:1,000 (Table 1A). The negative control group received serum from hamsters treated with saline + alum. In Experiment 2, groups of 6–26 hamsters were passively immunized with 0.5 mL of each of the following dilutions of XRX-001 antibody: 1:10, 1:30, 1:100 and 1:300 (Table 1B). Groups of 10 hamsters received 0.5 mL of serum from animals immunized with YF-VAX® at serum dilutions of 1:10 and 1:30. Passive titers approximately 20 hours after treatment (4 hours before challenge) in hamsters receiving the highest dose of XRX-001 serum were similar in Experiments 1 and 2 (GMTs of 75 and 70, respectively). Animals receiving XRX-001 antibody diluted 1:30 or 1:100 had passive GMTs of 10–20. Few hamsters receiving YF-VAX® serum were seropositive, and titers were low. Detectable nAb persisted in the animals for at least 4 days after passive immunization with the highest dose (1:10 dilution) of the XRX-001 serum pool (Groups 1 and 9, Table 1A; Group 1, Table 1B). Passive antibody 4 days after passive transfer was observed in a minority fraction of animals treated with XRX-001 serum given at a dilution of 1:30 or 1:100. None of the remaining serum samples, including serum from animals treated with the highest dose (1:10 dilution) of YF-VAX® serum had detectable nAb titers at this time-point.

Figures 1A and B shows survival curves and weight change by treatment group in Experiment 1, and Figures 1C and D show corresponding data for Experiment 2. Hamsters treated with XRX-001 antibody at the highest doses (1:10, 1:30, and 1:100) and YF-VAX® antibody at 1:10 were protected from death. In Experiment 1, animals in the XRX-001 1:10 and 1:100 groups and the YF-VAX® 1:10 treatment group in Experiment 1 were protected against weight loss, while animals in other groups lost weight (Fig. 1B). In Experiment 2, animals in the XRX-001 1:10 and 1:30 (but not 1:100) were protected against weight loss (Fig. 2D). Weight gain was less in Experiment 2 than in Experiment 1, probably due to a more severe challenge in Experiment 2. In Experiment 1, an unexpected result, which made interpretation of the survival data difficult, was that the control group (saline + alum) had only 30% mortality, whereas typically mortality in this model is 50–70% (18). Nevertheless, some animals that received the lowest doses of antibody had the expected mortality ratio. In Experiment 2, mortality in the control group (80%) was in accordance with expectations (18).
Individual hamster data for Experiment 1 on viremia and ALT are shown in Figures 2A and 2B, respectively. Hamsters receiving the highest dose of antibody (XRX-001 1:10) were completely protected from hepatic dysfunction and viremia. Partial, but significant protection was seen in groups that received XRX-100 antibody at 1:100 and YF-VAX® antibody at 1:10. Results in Experiment 2 were generally similar. Complete protection was observed in the XRX-001 1:10 treatment group and also in the 1:30 treatment group, except for one outlier (this hamster is discussed below). Partial protection against viremia was observed in the other active treatment groups. The pattern of protection against hepatic dysfunction was similar, but statistical significance was not reached.

To define the immune correlates of protection, we determined the passive antibody levels 4 hours prior to challenge. The pre-challenge passive titer was considered to be the most useful indicator of the level of antibody needed to protect the animals from virus replication and cell damage during the acute phase of infection (days 4–6), because antibody present during the latter phase would potentially be complexed with virus and cleared, providing an inaccurate measure of passive titer. Moreover, since treatment was homologous hamster serum, clearance without virus adsorption would be minimal during the acute period of infection.

The results for Experiment 1 indicated that nAb at a titer of ≥40 provided full protection against ALT enzyme elevation and viremia (Group 1, treated with XRX-001 1:10) (Fig. 3A). Animals with passive nAb titers of 1:20 in Groups 2 (XRX-100, 1:100) and Group 5 (YF-VAX® 1:10) showed partial protection, with significantly reduced weight loss (refer to Fig. 1B), ALT and viremia levels (Fig. 2B) compared to groups receiving lower passive antibody doses and controls. Some animals with undetectable passive nAb titers (<10) before challenge also appeared to be protected (i.e. did not experience viremia or hepatic dysfunction). However, in groups showing partial protection (e.g. Group 2, XRX-001, 1:100), we could not distinguish individual animals that were protected from hepatic dysfunction from those that were not protected based on antibody levels in pooled samples (Fig. 3A). Because there was insufficient serum to test individual animals (except in Group 1), a second study (Experiment 2) was performed to more completely assess the relationship between passive titer.

In Experiment 2, the proportion of hamsters with nAb (and the passive antibody GMTs) pre-challenge in the XRX-001 1:10 and 1:30 treatment groups were 100% (GMT 70) and 83% (GMT 20), respectively. One hamster in the XRX-001 1:30 treatment group was not protected and had no detectable nAb pre-challenge (the outlier noted in Fig. 2C and D); it is possible that this animal escaped being treated. Partial protection was seen in hamsters receiving XRX-001 at higher dilutions and in the YF-VAX® 1:10 treatment groups; these groups had low or undetectable passive antibody titers (Table 1B). The results were consistent with those seen in Experiment 1.

To more fully explore the correlations between passive nAb titers and outcome measures in Experiment 2, we analyzed viremia and ALT by pre-challenge PRNT$_{50}$ value in individual hamsters (Fig. 3B,C). As already noted (Table 1B), but shown here for individual animals, passive nAb titers showed a direct relationship to dose of antibody administered. Animals with passive titers ≥40 were completely protected from viremia and hepatic dysfunction, a finding consistent with data from Experiment 1. All animals with titers ≥40 were in the XRX-001 1:10 and 1:30 highest-dose treatment groups, and all these animals survived challenge. With the exception of the single seronegative outlier in group 3 (1:30) noted above, all other animals in that treatment group were solidly protected even though passive nAb titers were 10 or 20. In the 1:100 treatment group, animals with passive titers of 10–20 showed breakthrough viremias and ALT elevations, but there was only one death. In the
lowest dose group (1:300), most hamsters had no detectable serum nAb, viremias and ALT elevations were higher, and 5/9 animals died. Overall, it could be concluded that having any detectable nAb pre-challenge (titer ≥10) afforded a degree of protection, but complete protection required a titer ≥40. In groups that received YF-VAX® immune serum, passive titers were too low to draw firm conclusions regarding immune correlates.

Regression analyses confirmed the relationships between passive antibody titer and protection determined by weight gain, viremia, and ALT measurements (Fig. 4). Complete protection was associated with a nAb titer ≥40, and partial protection with titers of 10–20.

Neutralizing antibody titers in individual animals were evaluated at the end of the experiment (Day 49, 21 days after challenge). In Experiment 1, the majority (3/9, 67%) of animals in Group 1 treated with a high dose (1:10 dilution) of XRX-001 serum had PRNT₅₀ titers below the level of detection (Table 1A), suggesting that they had been protected from active infection with the challenge virus (sterile immunity). In contrast, a higher proportion of animals treated with lower doses of XRX-001 antibody was seropositive on Day 49, suggesting that these animals had sustained active infections after challenge (Table 1A). Similar trends were observed for YF-VAX® antibody treated animals, but small sample size in some groups limited the analysis. In Experiment 2, there were similar trends, with the lowest seroconversion rate in the XRX-001 1:10 treatment group (Table 1B). However, seroconversion rates were higher than in Experiment 1, consistent with a more active challenge infection and higher lethality in the second study.

Discussion

Neutralizing antibody is the accepted mediator of immunity to Flavivirus infection and disease (24,25). The presence of pre-formed neutralizing antibodies protects by interrupting virus replication at an early stage and prevents infection of vital organs and clinical disease. Neutralizing antibodies are long-lasting following natural infection and protection against disease on re-exposure is complete, although re-exposure often is accompanied by a boost in antibody indicating limited infection. Studies employing passive transfer of polyclonal immune serum or monoclonal antibodies to Japanese encephalitis (JE) (10, 26–28), West Nile (WN) (16, 29–31), tick-borne encephalitis (TBE) (32–35) and YF (36,37) demonstrated that nAbs against the envelope protein confers protective immunity against virus challenge.

The minimum protective titer of nAb induced by YF 17D vaccine has been estimated by dose response studies in rhesus monkeys that were actively vaccinated and subsequently challenged with virulent YF virus (38). In this model, a \( \log_{10} \) neutralization index (LNI) ≥ 0.7 was associated with protection. Based on these data, an LNI of ≥ 0.7 was accepted by the US Food & Drug Administration (FDA) as indicative of seroprotection in two pivotal clinical trials of YF 17D vaccine (39,40). Similarly, based on studies in animals that were actively vaccinated, the minimum protective level of nAb in the PRNT₅₀ assay required for protection against challenge with JE virus was established to be ≥10 (26) and this cut-off was used for regulatory approval in the US of inactivated JE vaccines (41,42). It is possible that, for neurotropic infections like JE, where serum antibodies play a role in protection against neuroinvasion by a small inoculum from the bloodstream, low neutralizing antibody levels (1:10) are protective, whereas for systemic (viscerotropic) infections like yellow fever and dengue, higher levels of antibody, e.g. ≥40 may be required to protect against disease expression.

Hepburn et al. studied the nAb response of human subjects to re-vaccination with YF 17D vaccine (43). She found that subjects with nAb levels <40 had substantial increases in nAb levels following re-vaccination, whereas those with titers ≥40 had minimal increases in
antibody following re-vaccination, indicating that they were protected against infection and expansion of antigenic mass that triggered a serological response. This finding, while apparently consistent with our findings in the hamster model, is of uncertain relevance to challenge with virulent wild-type virus. Moreover, none of the animal or human studies described above took into account the possibility that cellular immunity could contribute to protection.

Passive immunization with serum generated by immunization of hamsters with both inactivated (XRX-001) and live, attenuated (YF-VAX®) vaccines protected hamsters against viscerotropic YF virus challenge in a dose-dependent fashion. A passive titer (by PRNT\textsubscript{50}) of $\geq 40$ shortly before virus challenge provided complete protection from subsequent illness (weight loss), death, viremia, and hepatic dysfunction, whereas titers of 10 and 20 provided partial protection. It should be emphasized that the passive antibody titers were measured 4 hours before YF virus challenge, whereas the outcome measures of infection—weight loss, viremia and ALT—were measured 4–6 days after challenge. As shown in Table 1, passive antibody titers had waned by the time that YF pathogenesis (weight loss, viremia and ALT) was progressing, suggesting that the minimum protective titer was over-estimated by measurements made 4 days earlier, prior to challenge. However, on day 4 after challenge, the titer of passive antibodies in the control group (Experiment 1, Group 9, Table 1A) that received high dose XRX-001 antibody without YF virus challenge was similar to the corresponding treatment group (Experiment 1, Group 1) that was challenged, indicating that virus absorption of antibody was not a factor in clearing passive antibody.

In Experiment 1, the low mortality in the placebo group was surprising when other disease parameters were taken into consideration. Mortality is generally correlated with increased serum ALT levels (18), although that was clearly not the case in this study. All but one of the placebo-treated animals developed PRNT\textsubscript{50} nAb titers of $>160$ at the end of the experiment, indicating active infection with YF virus. The single animal with PRNT\textsubscript{50}$<160$ had high ALT enzyme changes indicating the liver was infected and it sustained weight loss between 5 and 6 days after challenge, providing evidence that all of the placebo control animals had in fact been infected. Nevertheless, since weight loss, serum ALT, and viremia were included as outcome measures, the effect of passive immunization could be clearly delineated and protective doses identified (Fig. 3A). In a separate study we excluded the possibility that normal (non-immune) hamster serum provided partial protection. To confirm the results from Experiment 1, and to provide sharper definition of the immune correlates of protection for each animal, we conducted Experiment 2. In the latter study, the mortality of control animals receiving non-immune serum (80%) was in line with expectations from published studies (18–20). Viremia levels, ALT, and weight loss in the control groups were somewhat greater in Experiment 2 (Fig 1B vs. 1D; Fig 2A vs. 2C and Fig 2B vs. 2D), in support of the conclusion that the challenge in Experiment 2 was more severe, explaining the higher mortality in that study. In Experiment 2, nAb titers before challenge of $\geq 40$ were solidly protective, while titers of 10–20 provided partial protection. The conclusions regarding the immune correlates of protection were therefore consistent across both Experiments 1 and 2 despite the more severe challenge in Experiment 2.

In our study, no clear differences were observed in the quality (protective activity) conferred by similar doses of antibody raised to inactivated and live YF vaccines. The weight change, ALT and viremia levels in Experiment 1, Group 2 (XRX-001 1:100) and Group 5 (YF-VAX® 1:10) which received similar passive doses of antibody were virtually identical. In Experiment 2, the XRX-001 serum appeared to be slightly more protective than YF-VAX® antibody at the same dose. We did not determine IgG subclass distribution or other possible qualitative differences between antibodies against inactivated and live vaccines, but
apparently such differences did not modulate virus neutralization or protective capacity. A significant potential difference between active immunization with inactivated and live YF vaccines may be the generation of MHC-I dependent cellular immunity. However, the present study demonstrates that antibody alone is fully protective against challenge with YF virus.

Acknowledgments

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Figure 1.
A. Experiment 1 Survival and B time-course weight change between day 3 and 6 of hamsters passively immunized with various dilutions of serum from hamsters immunized with XRX-001 or YFVAX®. C and D. Corresponding data for Experiment 2 (**P<0.001, ***P<0.001, *P<0.05, †P=0.08, as compared with placebo controls).
Figure 2.
A. Experiment 1: viremia on day 4 post challenge for individual hamsters passively immunized with various dilutions of immune serum generated after immunization with XRX-001 or YF-VAX®.
B. Experiment 1: serum ALT 6 days post challenge for individual hamsters passively immunized with various dilutions of immune serum generated after immunization with XRX-001 or YF-VAX®.
C. Experiment 2. Viremia on day 4 post-challenge.
D. Experiment 2: serum ALT on day 6 post challenge.
Differences between groups were performed by one-way ANOVA with a Bonferroni multiple comparison post-test analysis comparing the antibody treated groups to the
aluminum hydroxide-saline control group [***P<0.001, **P<0.01, as compared with saline +alum (Experiment 1) or normal hamster serum (Experiment 2) controls].
Figure 3.
A. Experiment 1. Relationship between passive neutralizing antibody 4 hours before challenge and ALT elevations determined on day 6. Individual sera were tested in the XRX-001 1:10 treatment group, whereas serum pools representing hamsters that developed no ALT elevation or had ALT elevations were tested. The passive titers are shown.

B. Experiment 2. Correlation between neutralizing antibody 4 hours before challenge and viremia (day 4 after challenge), alanine aminotransferase (ALT) concentrations (Day 6 after challenge), and death/survival, by individual animal. Hamsters were treated with a hamster serum pool from animals previously immunized with XRX-001. Different color symbols are used to distinguish treatment (dose) groups (see Table 1B). LOD= limit of detection. ULN= upper limit of normal.

C. Experiment 2. Same display of data as in Fig 3B. Hamsters were treated with a hamster serum pool from animals previously immunized with YF-VAX®. Different color symbols are used to distinguish treatment (dose) groups (see Table 1B). LOD= limit of detection. ULN= upper limit of normal.
Figure 4.
Logistic regression, showing correlations between passive antibody titers 4 hours before challenge and A. Weight gain; B. Viremia; and C. ALT elevation. For the viremia display, logarithmic units were used for both dependent and independent variables.
Table 1A. Experiment 1: antibody levels at various times prior to and after infection with YF virus as determined by PRNT\textsubscript{50} assay.

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum Pool used for treatment</th>
<th>Serum pool Dilution</th>
<th>PRNT\textsubscript{50} geometric mean titer (GMT) at different times before and after virus challenge</th>
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<td>Treatment - 24 h</td>
<td>Estimated passive titer\textsuperscript{a} -4 hours\textsuperscript{b}</td>
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<td>1</td>
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<td>7</td>
<td>YF-VAX\textsuperscript{®}</td>
<td>1:1,000</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>Saline + Alum</td>
<td>1:10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>9</td>
<td>XRX-001 (not challenged)</td>
<td>1:10</td>
<td>4,100</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Estimated titer based on dilution of 0.5 mL inoculum in 7 mL blood volume of the hamster (1:15 dilution) and 50% distribution in the intravascular space, for a total dilution of 1:30

\textsuperscript{b} Time shown is hours (days) before/after challenge.

Table 1B. Experiment 2: antibody levels at various times prior to and after infection with YF virus as determined by PRNT\textsubscript{50} assay.

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum Pool used for treatment</th>
<th>Serum pool Dilution</th>
<th>PRNT\textsubscript{50} geometric mean titer (GMT) at different times before and after virus challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Treatment - 24 h</td>
<td>Estimated passive titer\textsuperscript{a} -4 hours\textsuperscript{b}</td>
</tr>
<tr>
<td>1</td>
<td>XRX-001</td>
<td>1:10</td>
<td>4,100</td>
</tr>
<tr>
<td>3</td>
<td>XRX-001</td>
<td>1:30</td>
<td>1,367</td>
</tr>
<tr>
<td>5</td>
<td>XRX-001</td>
<td>1:100</td>
<td>410</td>
</tr>
<tr>
<td>7</td>
<td>XRX-001</td>
<td>1:300</td>
<td>137</td>
</tr>
<tr>
<td>9</td>
<td>YF-VAX\textsuperscript{®}</td>
<td>1:10</td>
<td>480</td>
</tr>
<tr>
<td>11</td>
<td>YF-VAX\textsuperscript{®}</td>
<td>1:30</td>
<td>48</td>
</tr>
<tr>
<td>13</td>
<td>Normal hamster serum</td>
<td>1:10</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Estimated titer based on dilution of 0.5 mL inoculum in 7 mL blood volume of the hamster (1:15 dilution) and 50% distribution in the intravascular space, for a total dilution of 1:30

\textsuperscript{b} Time shown is hours (days) before/after challenge.
Serum sample volumes were insufficient to test individual animals for all groups, so sera from 2–8 animals were pooled and tested by PRNT50. See Figure 2 for serum pools and GMT results.

Geometric means were calculated for the positives (>10) only.

Not tested.

Estimated titer based on dilution of 0.5 mL inoculum in 7 mL blood volume of the hamster (1:15 dilution).

Time shown is hours (days) before/after challenge.

Geometric means were calculated for the positives (>10) only.