1,5 iodonaphthyl Azide-Inactivated V3526 Protects against Aerosol Challenge with Virulent Venezuelan Equine Encephalitis Virus.

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Abstract:

Venezuelan equine encephalitis virus (VEEV) is a New World alphavirus. VEEV is highly infectious in aerosolized form and has been identified as a bio-terrorism agent. There is no licensed vaccine for prophylaxis against VEEV. The current IND vaccine is poorly immunogenic and does not protect against an aerosol challenge with virulent VEEV. We have previously shown that VEEV inactivated by 1,5 iodonaphthyl azide (INA) protects against footpad challenge with virulent VEEV. In this study, we inactivated an attenuated strain of VEEV, V3526, with INA and evaluated its protective efficacy against aerosol challenge with wild type VEEV. We demonstrated that among three routes of immunization, intramuscular immunization with INA-inactivate V3526 (INA-iV3526) provided complete protection against aerosol challenge with virulent VEEV. Our data suggests that INA-iV3526 can be explored further for development as an effective vaccine candidate against aerosol challenge of virulent VEEV.

Key words: Venezuelan equine encephalitis, vaccine, inactivated, iodonaphthyl azide, aerosol challenge, and protection.

1. Introduction:

Venezuelan equine encephalitis virus (VEEV) is an alphavirus and a bio-threat agent that is highly infectious in aerosol form. Therefore, a VEEV vaccine should ideally be able to protect against the aerosol challenge with virulent VEEV [1]. Live-attenuated TC-83 vaccine, which is currently under investigational new drug (IND) status, and its formalin-inactivated form (C-84) provide incomplete protection against aerosol challenge in non-human primates [2]. V3526 is a live-attenuated strain of VEEV that was shown to be safe in environmental hazard studies [3, 4]. However, its development as a vaccine candidate was stopped due to causation of adverse reactions in the vaccinees during the clinical trial [5]. We have previously shown that V3526 can be completely inactivated by hydrophobic alkylating compound, 1,5 iodonaphthyl azide (INA) [6]. In this study, we evaluated the protective efficacy of INA-inactivated V3526 (INA-iV3526) against aerosol challenge with virulent Trinidad donkey strain of VEEV (VEEV-TrD). Our results demonstrated that INA-iV3526 provides a complete protection from aerosol challenge with VEEV-TrD when administered intramuscular (IM) and, therefore, is a strong candidate for further development as a VEEV vaccine.

2. Methods:

Inactivation of V3526: INA was kindly provided by Dr. Robert M Blumenthal, National Cancer Institute, Frederick, MD under a material transfer agreement with Uniformed Services University of the Health Sciences, Bethesda, MD. Sucrose gradient purified V3526 was inactivated by INA (INA-iV3526) as previously described in detail [6]. Briefly, 500 µg of viral protein was mixed with 100 µM INA and incubated at room temperature for 20 min. Inactivation was done using 100W UV lamp for 5 min with intermittent vortexing. Formalin-inactivation of V3526 (F-iV3526) was performed by treating with 0.1% v/v formalin at 37°C for 24 hours as described previously [7].

Immunization and challenge procedures: Three different routes of immunizations were tested, intranasal (IN), intramuscular (IM) and subcutaneous (SC). Animals (n= 10/group) received a primary immunization on Day 0 followed by a booster on Day 28. Two immunization doses were tested for each of the INA and formalin inactivated V3526 (*i.e.*, 1 and 3 µg for IN, and 3 and 5 µg for SC and IM). For IN immunization, 10 µl of inactivated virus suspension was inoculated in each nostril to achieve the final doses. For SC immunization, 200 µl of inactivated virus suspension was inoculated under the skin over the neck. Formalin-inactivated TC-83 (C-84) was administered as routinely given via SC inoculation. For IM immunization, 50 µl of inactivated virus suspension was inoculated in the thigh muscle of the hind leg. Mock immunization was done with equal volumes of saline. Serum was collected by retro-orbital bleed on Day 49 after the first immunization. All the animals were challenged 4 weeks after the last immunization with VEEV-TrD by aerosol route as described earlier [7]. Spray factors and inhaled dose for each of the group is given in supplementary table 1.

Ethics Statement: Animal experiments involving aerosol challenge with infectious VEEV-TrD were carried out in ABSL-3 containment facility at United States Army Medical Research Institute of Infectious Diseases (USAMRIID), Frederick, MD. Research was conducted under an IACUC approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory animals, National Research Council, 2011.

Microneutralization assay: Microneutralization test (MNT) for determining neutralizing antibody response was done as described before [8]. Endpoint titers were determined as the highest dilution that produced an OD greater than the mean OD plus 3 standard deviations of the negative controls.

Serum IgG ELISA: IgG ELISA was done as described before [9]. The negative control was 1:100 diluted normal mouse serum (Sigma Aldrich, St. Louis, MO). Endpoint titers were determined as the highest two-fold dilution that produced an OD greater than the mean OD plus 3 standard deviations of the negative controls.

Statistics: Kaplan-Meier survival analysis was used to analyze survival in immunized and control groups post-challenge with wild type VEEV. A p value < 0.05 was considered statistically significant. Two tail student's t-test was used to calculate the significance for neutralizing antibody and total IgG titer data.

3. Results and Discussion:

V3526 is a furin-cleavage mutant of V3000 (a full length clone of VEEV-TrD) with excellent immunogenic profile [3, 10]. The efforts to develop V3526 as vaccine candidate were stopped after it was found to cause adverse reaction in the vaccinees during the clinical trials [5]. Inactivation of V3526 by INA, therefore, was undertaken to impart another layer of safety over an already attenuated virulence profile. INA is a hydrophobic alkylating compound which covalently binds to the lipoproteins in the viral envelope without affecting the structural integrity of the surface epitopes [6, 11]. Several viruses including VEEV have been shown to be inactivated by INA, while retaining their immunogenic properties [6, 11-17].

Overall, protection in animals correlated with an increased total serum IgG titer and presence of neutralizing antibodies. IN immunization provided the lowest level of protection against aerosol challenge with VEEV-TrD (Figure 1A) and was concomitant with the lack of neutralizing and total virus-specific IgG response in these mice (Figure 2 A and 3A). A similar observation has earlier been reported for Eastern equine encephalitis virus (EEEV), where intranasal inoculation of INA-inactivated EEEV did not protect mice against challenge with the virulent EEEV [9]. In contrast, IN immunization with 1 and 3 µg of F-iV3526 provided 60% and 70% protection,

respectively, against virulent VEEV-TrD challenge (Figure 1A). This protection was concomitant with the presence of neutralizing and total serum IgG titers (Figure 2A and 3A).

SC immunization with γ -radiation inactivated-V3526 or F-iV3526 was previously shown to provide 90% protection against the aerosol challenge with VEEV-TrD [7, 18]. In this study, immunization with increased doses (3 and 5 μ g) of F-iV3526 and INA-iV3526 still provided only 90% protection (Figure 1B). Therefore, although protection provided by SC vaccination with F-iV3526 and INA-iV3526 was significant over non-immunized control (Kaplan-Meier $p < 0.05$), the SC route may not provide complete protection against aerosol challenge with virulent VEEV.

IM immunization with INA-iV3526 provided complete protection against aerosol challenge with VEEV-TrD. Only immunization with either 3 or 5 μ g of INA-iV3526 conferred 100% protection from challenge with virulent VEEV-TrD (Figure 1C). Saline administered mice died 4-6 days after challenge. These results corroborate an earlier vaccination study where γ -radiation inactivated-V3526 in combination with adjuvants showed protection from aerosol challenge with VEEV-TrD [18]. F-iV3526, at doses lower than the dose used for immunization in this study, had also been shown to provide partial protection against aerosol challenge with VEEV-TrD [7]. In this study, F-iV3526 at doses of 3 and 5 μ g protected 60% and 80% of the immunized mice, respectively (Figure 1C). F-iV3526 therefore, either may not provide or may require an even higher dose of immunization for complete protection against exposure to aerosolized VEEV-TrD. INA-iV3526 protected all mice in the 3 or 5 μ g immunized groups and that was significantly higher than the protection conferred by the 3 μ g F-iV3526 immunized group (Kaplan-Meier $p \leq 0.05$). Although 100% protection was observed in the INA-iV3526 group compared to 80% protection in the 5 μ g F-iV3526 group, this difference was not statistically significant.

The neutralizing serum antibody responses in the INA-iV3526 vaccinated mice were consistently lower than those in the mice vaccinated with the F-iV3526 by SC (Figure 2B) or IM (Figure 2C) route. However, neutralizing antibody titer in C-84 immunized (SC) group was higher than the INA-iV3526 immunized groups (Figure 2B). This observation was also noted in a previous study with C-84 [7, 18]. In SC groups, level of protection was same between the INA-iV3526 and F-iV3526 groups, *i.e.*, 90%. Interestingly, C-84 provided the lowest protection in spite of exhibiting higher neutralizing antibody titers (Figure 2B). In IM groups, INA-iV3526 immunization resulted in increased protection in comparison to the F-iV3526 immunization even though the neutralizing antibody titers were higher in F-iV3526 immunized group. These results are confounding as neutralizing antibodies are conventionally believed to be a good correlate of protection against alphaviruses [19]. A plausible explanations for this result may be that INA-iV3526 preserved an important viral envelope epitope that conferred protection even at low neutralizing antibody titers such that the quality of responses was improved. Alternatively, it is possible that INA-iV3526 induced an efficient T-cell response which has been suggested to protect mice from challenge with virulent VEEV [20]. Level of total serum IgG were similar

among all the SC and IM immunization groups (Figure 3B and 3C). Also, as expected, animals across all immunization groups initially lost weight after challenge with VEEV-TrD, followed by weight gain in surviving mice 6-7 days after the challenge (Supplementary Figure 1).

In conclusion, IM vaccination with INA-iV3526 presents a strong case for further evaluation of INA-inactivated V3526 as a potential VEEV vaccine candidate.

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Figures:

Figure 1:

Figure 1. Protective efficacy of INA-iV3526 against aerosol challenge with VEEV-TrD:

Mice were immunized with INA-iV3526 or F-iV3526. Four weeks after the second immunization, animals were exposed to aerosolized VEEV-TrD. Percent survival was noted for each of the groups immunized IN (A), SC (B) or IM (C). * *P-value* ≤ 0.05 in comparison to Saline group, ^ *p-value* ≤ 0.05 in comparison to F-iV3526 3 μg group, and # *p-value* ≤ 0.05 in comparison to C-84 group.

185 **Figure 2:**

186 **Figure 2. Neutralizing antibody titers:** Neutralizing antibody titers were determined micro
 187 neutralization assay. Positive titers were determined as titers that were 2 times that of the
 188 standard error of the saline controls. (A) IN immunization; (B) SC immunization; and (C) IM
 189 immunization. Values are presented as $\pm$ SEM. * P -value ≤ 0.05 in comparison to INA-iV3526
 190 groups.

191 **Figure 3:**

192
193 **Figure 3. Serum IgG titers:** Total serum IgG titers were determined by ELISA. Positive titers
194 were determined as titers that were 2 times that of the standard error of the saline controls. (A)
195 IN immunization; (B) SC immunization; and (C) IM immunization. Values are presented as $\pm$
196 SEM. * P -value ≤ 0.05 in comparison to INA-iV3526 groups unless indicated otherwise.

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