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Complete inactivation of Venezuelan equine encephalitis virus by 1,5-iodonaphthylazide

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Abstract

Hydrophobic alkylating compounds like 1,5-iodonaphthylazide (INA) partitions into biological membranes and accumulates selectively into the hydrophobic domain of the lipid bilayer. Upon irradiation with far UV light, INA binds selectively to transmembrane proteins in the viral envelope and renders them inactive. Such inactivation does not alter the ectodomains of the membrane proteins thus preserving the structural and conformational integrity of immunogens on the surface of the virus. In this study, we have used INA to inactivate Venezuelan equine encephalitis virus (VEEV). Treatment of VEEV with INA followed by irradiation with UV light resulted in complete inactivation of the virus. Immuno-fluorescence for VEEV and virus titration showed no virus replication *in-vitro*. Complete loss of infectivity was also achieved in mice infected with INA treated plus irradiated preparations of VEEV. No change in the structural integrity of VEEV particles were observed after treatment with INA plus irradiation as assessed by electron microscopy. This data suggest that such inactivation strategies can be used for developing vaccine candidates for VEEV and other enveloped viruses.
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Venezuelan equine encephalitis virus (VEEV) is a member of arbovirus group, family togaviridae in genus alphavirus and is transmitted by mosquito in nature by subcutaneous inoculation [1–3] and causes biphasic infection [4–6]. Clinical signs of disease in humans include fever, headache, malaise and myalgia [7,4]. Recent outbreaks of VEEV have resulted in its identification as an emerging pathogen [8]. It is highly infectious in aerosol [9] and has been developed as a bioweapon [10].

Current live attenuated TC-83 vaccine for VEEV is under new-investigational drug status and is given to laboratory personal at risk. Formaldehyde inactivated TC-83, known as C84, is used as a booster following immunization with live attenuated TC-83 vaccine [11,12]. These vaccines have limitations such as, adverse reaction, short-lived immunity and several non-responders [13–15,11]. Therefore, it is important to develop new safe vaccine candidates for VEEV.

INA selectively penetrates into the hydrocarbon core of biological membranes. Upon irradiation, with UV light these compounds directly bind to proteins and lipids embedded in the bilayer [16–21]. The resulting inactivation of multiple components in the membrane is efficient and selective so that ectodomain of proteins or lipids outside the bilayer are not affected [22,16]. Recently this approach was used to produce inactivated HIV and SIV virions for vaccine application [23]. INA has also been used for inactivation of Ebola and Influenza virus (Raviv et al., personal communication). In this study, we demonstrate a complete

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and efficient inactivation of VEEV upon treatment with INA followed by irradiation with UV light. Inactivation of VEEV by INA was associated specifically with irradiation. There was complete absence of VEEV induced cytopathic effect *in vitro* and clinical sign of disease *in vivo* by INA treated plus irradiated VEEV.

Materials and methods

Animals. Seven- to eight-week-old male Swiss CD-1 mice were obtained from Charles River Laboratories, Wilmington, MA. Mice were housed in micro isolator cages and were provided food and water ad libitum with a 12 h light/dark cycle. All experiments were carried out in bio-safety level 3 (BSL-3) facility and in accordance to Guide for the Care and Use of Laboratory Animals (Committee on Care And Use of Laboratory Animals of The Institute of Laboratory Animal Resources, National Research Council, NIH Publication No. 86-23, revised 1996).

Virus. Molecularly cloned, virulent strain of VEEV, V3000 [5], kindly provided by Dr Franziska B Grider, USUHS, Bethesda, MD was used in the present study.

Inactivation of VEEV with INA. Purified VEEV stocks with known virus titer were suspended in 1× DPBS at a protein concentration of 0.5 mg/ml in a clear transparent tube and from this point reduced lighting conditions were used. INA was provided by Drs. Raviv and Blumenthal, NCI, Frederick, MD under a material transfer agreement. INA was added to the virus suspension to a final concentration of 10, 50, and 100 μM in 3–4 installments and samples were incubated for 20 min in the dark at room temperature (RT). Samples were then centrifuged at 1000 rpm for 1 min to remove precipitated INA crystals. Supernatant was transferred to new tube and glutathione was added to a final concentration of 20 mM. Virus suspension was then irradiated using 100 W mercury UV lamp in the following setup: A clear glass plate was placed immediately in front of the lamp (to filter lower UV wavelengths of light) and water filled 75 cm^2 transparent tissue culture flask (used as a heat filter) was placed approximately 6–7 cm apart from the glass plate. Finally, samples were placed 6–7 cm away from the flask in such a way that samples are completely illuminated with the light passing through the flask. Irradiation was done for 90 s, vortexed and again irradiated for 90 s. There after full light conditions were used and samples were stored at −80 °C. For infection, *in vitro* and *in vivo*, virus titers were back calculated and dilutions were made accordingly.

Treatment groups and controls. To determine the effect of INA treatment procedure and irradiation alone on virulent VEEV, following groups with proper controls were created. VEEV only, VEEV plus irradiation, VEEV plus DMSO (0.5%), VEEV plus DMSO plus irradiation, VEEV plus INA (100 μM) only, VEEV plus INA (10 μM) plus irradiation, VEEV plus INA (50 μM) plus irradiation, VEEV plus INA (100 μM) plus irradiation, PBS only and DMSO only. Since INA was dissolved in DMSO and highest concentration of DMSO (0.5%) was achieved in the 100 μM INA treatment, we used 0.5% DMSO in controls.

Infectivity assay—cytopathic effects (CPE). Vero cells (ATCC, Manassas, VA) were plated in 24-well tissue culture plates and infected with VEEV at a multiplicity of infection (MOI) of 0.5. After 72 h post-infection (p.i.) plates were washed twice with 1× PBS and fixed with 10% NBF followed by staining with 0.5% crystal violet (CV) (Sigma–Aldrich, St. Louis, MO) for 10 min.

Cell proliferation (MTT) assay. Cell proliferation was determined using the Cell proliferation kit I {MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)} (Boehringer Manheim, Indianapolis, IN) according to manufacturer's protocol. Briefly, cells were plated in 96-well tissue culture plates and infected with VEEV samples at MOI of 0.5. At each time point (12, 24, 48, and 72 h) 10 μl of MTT dye was added to each well followed by addition of solublization buffer after 4 h. Plates were incubated overnight and color development was quantified using ELISA reader at 570 nm.

Immuno-fluorescence for VEEV antigen. Cells were plated in 8 chamber slides and infected with VEEV at MOI of 0.5. At 12, 24, 48 h p.i. slides were fixed in chilled acetone:methanol (1:1) solution for 10 min and stored at −20 °C. For immuno-fluorescence staining, slides were rinsed in 1× PBS and blocking was done with 1% BSA for 1 h at RT. Excess BSA was removed and slides were incubated with primary polyclonal rabbit anti-VEEV antibody (kindly provided by Dr Franziska B Grider, USUHS, Bethesda, MD) at a dilution of 1:500 in 1% BSA for 1 h/RT. Slides were then rinsed with 1× PBS and incubated with secondary FITC conjugated goat anti-rabbit IgG (Molecular probes, Eugene OR) at a dilution of 1:250 for 1 h at 37 °C. Slides were washed with 1× PBS and mounted with vectashield mounting medium containing DAPI (Vector laboratories, Inc. Burlingame, CA). Immunofluorescence was observed under fluorescent microscope. Experiments were done in replicates of four and repeated twice.

Electron microscopy (EM). Two hundred mesh gold grids (Electron Microscopy Sciences, Hatfield, PA) were glow discharged for 15 min in a vacuum evaporator and samples were placed on the grids for 10 min at RT and grids were fixed in 4% electron microscopy grade paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 10 min at RT. Grids were then rinsed with distilled water and negative staining was done with 10 μl of 1% uranyl acetate (UA) for 90 s. Observations were made under Philips CM100 electron microscope operating at 80 kV.

Virus titer. Virus titer was determined in the cell supernatant of VEEV infected Vero cell cultures at 72 h p.i. as a 50% tissue culture infective dose ($TCID_{50}$) by cytopathic effect (CPE) assay as described before [24].

Challenge procedure and survival study in vivo. Mice were anesthetized using inhalation anesthesia, isoflurane and 1000 plaque forming units (pfu) of VEEV samples in 25 μl volume of 1× DPBS was injected in the left rear footpad. Animals were observed twice a day for clinical symptoms of disease such as ruffled fur, hunched posture, lethargic, hind limb paralysis, and mortality.

Results

INA plus irradiation inhibited cell death induced by VEEV

Qualitative screening for inhibition of infection was done by visualizing the CV staining (Fig. 1A). Cell proliferation assay was performed as quantitative measurement of inhibition of VEEV infection (Fig. 1B). Significant inhibition of VEEV infectivity was observed at 72 h p.i. with all three doses of INA (10, 50, and 100 μM) combined with irradiation. Significant infectivity was observed in VEEV plus irradiation, VEEV plus DMSO treatment, VEEV plus DMSO plus irradiation, VEEV plus INA (100 μM) similar to the virulent VEEV infected cells. In replicate experiment VEEV samples treated with 10 μM dose of INA combined with irradiation showed partial infectivity where as 50 and 100 μM treatment doses of INA consistently inhibited the infection when combined with irradiation.

Infection and replication of VEEV was inhibited upon treatment with INA plus irradiation

VEEV specific immuno-fluorescence staining was done to localize the VEEV antigen in the infected Vero cell cultures. VEEV specific staining was positive in the following samples: VEEV plus irradiation; VEEV plus DMSO treatment; VEEV plus DMSO plus irradiation, and VEEV plus INA (100 μM) and was similar to virulent VEEV infected cells at all time points (12, 24, and 48 h p.i.). VEEV staining was diffused in the membrane, cytoplasm and also in the nucleus (Fig. 2). There was no detectable VEEV staining