Published in final edited form as:

Anal Biochem. 2010 July 15; 402(2): 151–160. doi:10.1016/j.ab.2010.03.016.

Recognition of DAF and $\alpha_v\beta_3$ by inactivated Hantaviruses, towards the development of HTS flow cytometry assays

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Abstract

Hantaviruses cause two severe diseases in humans: hemorrhagic fever with renal syndrome (HFRS) or hantavirus cardio-pulmonary syndrome (HCPS). The lack of vaccines or specific drugs to prevent or treat HFRS and HCPS, and the requirement for conducting experiments in a biosafety level 3 laboratory (BSL-3) limit the ability to probe the mechanism of infection and disease pathogenesis. In this study we have developed a generalizable spectroscopic assay to quantify saturable fluorophore sites solubilized in envelope membranes of Sin Nombre virus (SNV) particles. We then use flow cytometry and live cell confocal fluorescence microscopy imaging to show that UV-killed SNV bind to the cognate receptors of live virions, namely, decay accelerating factor (CD55/DAF) expressed on Tanoue B cells and $\alpha_v\beta_3$ integrins expressed on Vero E6 cells. SNV binding to DAF is multivalent and of high affinity ($K_d \approx 26\text{pM}$). Self-exchange competition binding assays between fluorescently labeled SNV and unlabeled SNV are used to evaluate an infectious unit-to-particle ratio of $\sim 1:14000$. We have configured the assay for measuring the binding of fluorescently labeled SNV to Tanoue B suspension cells using a high throughput flow cytometer. In this way, we establish a proof of principle high throughput screening assay for binding inhibition. This is a first step towards the development of HTS format assays for small molecule inhibitors of viral-cell interactions, as well as dissecting the mechanism of infection in a BSL-2 environment.

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Keywords

hantaviruses; spectroscopy; High throughput flow cytometry; quantum dots; electron transmission microscopy; mass action; fluorescence labeling; lipid membranes; membrane rafts; fluorescence calibration beads; fluorescence quenching; R18; integrins; decay accelerating factor (DAF/CD55); $\alpha_v\beta_3$ integrins

Introduction

Sin Nombre virus (SNV), an enveloped virus with a genome comprised of three negative-sense RNA segments, is a New World hantavirus (Bunyaviridae: Hantavirus), and is the primary etiologic agent of the often-lethal hantavirus cardiopulmonary syndrome (HCPS) in North America [1-4]. HCPS is characterized by pulmonary edema due to capillary leak, followed by cardiogenic shock. SNV is carried by the deer mouse (*Peromyscus maniculatus*) and transmitted to humans through inhalation of virus-contaminated excreta [1-4]. The high case-fatality rate, the absence of vaccines and therapeutics, and the ability of hantaviruses (Andes) to be transmitted via the aerosol route has led to their categorization as Category A pathogens, and potential agents of biological warfare [5,6].

Hantaviruses causing HFRS or HCPS primarily infect polarized epithelial or endothelial cells through binding $\alpha_v\beta_3$ integrins [7]. In polarized epithelial cells, such as those that line the airways, the distribution of proteins is segregated into apical and basolateral localization. Accordingly the expression of $\alpha_v\beta_3$ integrins is confined to the basolateral surfaces of polarized cells [8-10], where incoming virions have no direct access. A recent study has shown that hantaviruses first bind to decay-accelerating factor (DAF/CD55), which is abundantly expressed on the apical surface of epithelial cells [11]. Nevertheless the precise mechanism by which hantaviruses infect cells is not well understood.

Recent studies have shown that SNV, killed with a calibrated dose of UV radiation, still possess the phenotypic characteristics of live virions including functional binding and the ability to induce nuclear expression of host antiviral mRNAs [12]. We have undertaken this study in order to establish the use of UV-inactivated SNV as a model system to examine the entry and trafficking events of a BSL-3 agent under BSL-2 conditions. We have used transmission electron microscopy (TEM) to show that the structural integrity of the viral capsid is conserved after UV treatment. After labeling the envelope membrane with a fluorescent lipid derivative, octadecyl rhodamine B (R18), we used absorption and fluorescence measurements to determine the degree of fluorescent labeling of the envelope membrane of SNV. We show that UV-killed SNV^{R18} specifically interacts with the cognate attachment factor (DAF) and entry receptor ($\alpha_v\beta_3$) in a manner that parallels the results obtained from the literature [7,11,13]. We further extend this study to establish a proof of principle assay for a high throughput screening (HTS) flow cytometry-based cell-binding assay to DAF-expressing Tanoue B cells. Such an assay is a first step towards HTS discovery assays for inhibitors of infection.

Materials and methods

Materials

1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(carboxyfluorescein) (fluorescein PE) and dioleoyl-L- α -phosphatidylcholine (DOPC), cholesterol, and egg sphingomyelin were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). 5 μ m diameter glass beads were obtained in dry form from Duke Scientific Corp (Palo Alto, CA). Octadecyl rhodamine B chloride (R18), and 5-octadecanoylamino fluorescein (F18) were purchased from Molecular

Probes (Eugene, OR) and used without further purification. Streptavidin-coated and biotin-coated polystyrene particles (6.7 μ m in diameter, 0.5% w/v) were purchased from Spherotech Inc. (Libertyville, IL). Streptavidin-coated quantum dots, QD585, were purchased from Invitrogen Corp (Carlsbad, CA). Phosphate-buffered saline (PBS) was purchased from Mediatech, Inc, Herndon, VA). Dimethyl sulfoxide (DMSO) and Sephadex G-50 were purchased from Sigma. TRIS (10 mM or 25 mM Tris, 150 mM NaCl, pH 7.5), HHB (30 mM HEPES, 110 mM NaCl, 10 mM KCl, 1 mM MgCl₂·6H₂O and 10 mM glucose, pH 7.4) buffer, and Hanks Balanced Saline Solution (HBSS) (0.35 g NaH₂CO₃, 0.049 g MgSO₄, 1 mM CaCl₂ or 1 mM MnCl₂) were prepared under sterile conditions and stored in 50 ml tubes at 20°C.

Cell Culture

Tanoue B cells and Vero E6 cells were maintained in either sterile filtered RPMI media, or minimum essential media (MEM). All media contained 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, 100 μ g/mL streptomycin, 10mM HEPES, pH7.4, 20 μ g/mL ciprofloxacin, 2 mM L-glutamine, and were kept at 37°C in a CO₂ water jacketed incubator of 5% CO₂ and 95% air (Forma Scientific, Marietta, OH).

Production of Sin Nombre Virus

Experiments involving live Sin Nombre virus were carried out under strict biosafety level 3 containment and practices in a registered laboratory under a protocol approved by the University of New Mexico's Institutional Biosafety Committee (Public Health Service registration number C20041018-0267) as previously described [14]. Briefly, we propagated SNV strain SN77734 [15] to titers not less than 10⁶ focus forming units (FFU)/ml in Vero E6 cells in MEM with 2.5% FBS. We inoculated the virus into subconfluent or freshly-confluent T75 flasks at a multiplicity of infection (m.o.i.) of 0.01 and harvested virions every 3-4 days until the first time-point at which the titer exceeded 10⁶ FFU/ml, as established by a focus assay [14]. We typically obtained titers in the range of 1-5 $\times$ 10⁶FFU/ml with this protocol. We have used a focus reduction neutralization test (FRNT) for hantaviruses in lieu of the plaque reduction neutralization test, since hantaviruses plaque somewhat inconsistently [16,17]. We then destroyed stocks that were prepared after that time-point by autoclaving. We stored virus stocks at -80°C in 1ml or 10 ml aliquots until use in a BSL-3 suite.

Viral Inactivation and Ultraviolet Dosimetry

SNV samples were inactivated by UV irradiation as previously described [12]. For clarity, we will briefly describe the procedure here and include some details left out in the original paper [12]. To calibrate UV irradiation in terms of killing time and intensity, the 254 nm output ($\approx$ 5 mW/cm²) of a TS-254R Spectroline UV Transilluminator (Spectronics Corp., Westbury, NY) was measured with a dosimeter stationed at points of equidistance removed from the light-source, as were the virus preparations. In a typical measurement, 1 ml virus preparations (peak titers of $\approx$ 10⁶ FFU/ml) were divided into ten 100 μ l aliquots in a 96-well plate on the floor of a laminar flow cabinet. The virus samples were then irradiated for different time intervals from the UV lamp positioned 3.8 cm above the 96-well plate. UV killing was established by focus assays [18]. As in previous measurements, it was established that UV inactivation was complete after 10 seconds of UV irradiation. Standard stock (1.5-2 $\times$ 10⁶ FFU/ml) preparations of UV-inactivated virions were usually UV-treated for 20 seconds [12].

Purification and concentration of SNV by density centrifugation

Crude UV-inactivated SNV samples were purified by adding 10 mL of Vero E6 SNV supernatant to 2 mL of a 50% solution of iodixanol in water in a 14 × 89 mm Beckman polyallomer tube, followed by centrifugation in a TH641 rotor for 5h at 4° C at 35,000-rpm (17,500 × g). This was followed by the removal of the bottom 4 ml of tube contents and transfer to 5 mL Optiseal tubes (30% Optiprep gradient), which were then spun in the near-vertical NVT90 rotor for 5-16h at 65,000 rpm (300,000 × g) at 4°C. Individual fractions were collected and a refractometer was used to identify the fraction that contained SNV by its putative density of 1.2 g/cm³ [19]. The presence of SNV was subsequently confirmed by Western blotting analyzing for viral protein components N, Gn, and Gc and used polyhistidine-track tagged recombinant SNV-N to generate the standard curve [12,15,18]. The purified (1 µg/ml N protein) SNV samples were stored in 1.0 mL aliquots at -80°C.

Electron Microscopy

Electron micrographs were taken using viruses inactivated by 20 sec UV irradiation or fixation in 0.5% glutaraldehyde for 2hours in a BSL3 laboratory. Viral inactivation was confirmed with a focus assay as described above. UV-irradiated SNV samples were crosslinked by the addition of glutaraldehyde to a final concentration of 0.5%, followed by incubation for ten minutes at room temperature. Samples were then diluted to the desired concentration in Tris EDTA buffer. Virus solutions were pipetted onto carbon coated TEM grids and incubated for five minutes at room temperature. The solution was then wicked off the grid with Whatman 1 filter paper until a thin film remained. The grids were then negatively stained by placement on droplets of 2% uranyl acetate for two minutes. Samples were imaged in a Hitachi H7500 TEM equipped with an Advanced Microscopy Sciences XR60 camera.

Fluorescent Labeling of SNV

Hantaviruses consist of a nucleocapsid surrounded by a lipid bilayer membrane derived from membrane lipids of the host cell [20]. As such, SNV is amenable to staining with fluorescent derivatives of lipophilic lipid probes such as octadecylrhodamine (R18). Virus particles were labeled according to modified literature methods [21,22]. To minimize filtrate sample volumes during gel purification, stock solutions were first concentrated to 1 µg in 250 µL of HHB buffer (30 mM Hepes, 110 mM NaCl, 10mMKCl, 1mM MgCl₂, 10 mM glucose, and 0.1% HSA pH7.4) using YM-100 Microcon centrifugal filter devices (Millipore Corp. Bedford, MA) (at 4,000 × rpm). The Microcon filters were typically pre-coated with human serum albumin (HSA) in order to reduce sample loss to non-specific binding. SNV was then transferred to a microfuge tube into which a 0.5 µL aliquot of ≈ 2 mM DMSO-solubilized R18 was added. It is important that the HHB buffer contain 0.1% HSA (97µM), as a large excess of serum albumin is necessary to limit probe aggregation and mediate the transfer of monomeric R18 from the aqueous phase to membranes [23]. The mixture was then vortexed, and incubated at 37°C in the dark under mild vortexing for 30 min. R18 labeled virions (*i.e.* SNV^{R18}) were purified of excess R18 by gel filtration using a 3 mL column of Sephadex G-50-150 gel. The volume of samples was then concentrated to 300-400 µL using YM-100 Microcon centrifugal filter devices (Millipore Corp. Bedford, MA) (at 4,000 × rpm). The concentration of purified SNV^{R18} samples was determined from absorption measurements and fluorescence dequenching assays as described below. Samples were then aliquoted and stored in 0.1% HSA HHB buffer, and used within two days of preparation and storage at 4°C. For long-term storage and use, small aliquots suitable for single use were stored at -80°C.

Standard calibration curve for quenching of R18 in supported bilayer membranes

The quantum yield of R18 solubilized in lipid bilayer membranes can be correlated to its mole fraction composition in the host membrane [23]. We have developed standard calibration curves to measure and correlate the dequenching of self-quenched R18 to its mole fraction in a single component, dioleoyl-L- α -phosphatidylcholine (DOPC) lipid bilayer membrane, and a 1:1:1 ternary mixture of DOPC/sphingomyelin/cholesterol (DSC) lipid membrane, supported on beads [23]. Such calibration curves can be used to estimate the average number of R18 probes per labeled SNV particle. Briefly, to make vesicles for calibration samples, small unilamellar vesicles (SUVs) were prepared from 1 mM solutions of DOPC and 1:1:1 D/S/C in chloroform (210 μ L total volume) in microfuge tubes. R18 was then serially titrated (0-10mol%) into the DOPC/SPM/Chol samples. The chloroform was removed by vacuum drying the samples using a CentriVap Benchtop Centrifugal Vacuum Concentrator (Labconco, Kansas City, MO) at room temperature for an hour. After addition of 400 μ L of 50 mM Tris buffer (pH 7.5), the vesicle suspension was sonicated to optical clarity using a Branson sonifier (Branson Cleaning Equipment Co., Shelton CT).

The vesicles were transferred to cuvettes in 400 μ L volumes with mini magnetic stir bars to maintain vesicles in suspension for fluorescence measurements using a Photon Technology International QuantaMaster™ Model QM-4/2005 spectrofluorometer (Lawrenceville, NJ), following our standard protocols for such measurements [24,25]. With the instrument set in kinetic mode, fluorescence intensities of the quenched vesicle suspensions were measured as a time course for 30 seconds, after which a 20 μ L aliquot of 20% Triton X-100 was added to solubilize vesicles and dequench R18. The increase in fluorescence intensity of the samples was corrected for dilution, and then correlated to the degree of self-quenching of R18 at the various mole fractions in membranes. The results were plotted as a graph representing relative quantum yield versus mole-fraction of R18. In this way the standard calibration curve was then used to determine the mole fraction of R18 embedded in the envelope membrane of SNV on the basis of the increase in the fluorescence of R18 after similar detergent solubilization [23].

Flow Cytometry Analysis

Flow cytometric measurements were performed using a standard BD FacsScan or a HyperCyt® high throughput (HT) flow cytometer [26-29]. HyperCyt® is a platform that uses a peristaltic pump in combination with an autosampler. The system enables measurement of assays up to rates in excess of one endpoint assay well per second. In the multiwell format the sampling probe of the autosampler moves from one well to the next as a peristaltic pump sequentially aspirates sample particle suspensions from each well. Between wells, the continuously running pump draws a bubble of air into the sample line to generate a tandem series of bubble-separated samples for delivery to the flow cytometer. The data were typically collected at a sampling rate of $30 \times 2\mu$ L samples per minute or a single 384 well plate every 13 minutes.

Expression of PSI domain peptides

We expressed PSI domain peptides as previously described by [7].

Specific Cell binding assays to DAF and $\alpha_v\beta_3$

Binding assays were performed by incubating SNV^{R18} with Tanoue B cells (particle to cell ratio $5 \times 10^3:1$) in 96 well tissue culture plates under mild vortexing for 15 mins. Adherent Vero E6 cells were washed twice in HHB/HSA buffer before exposure to SNV^{R18}. Specific binding to DAF expressed on Tanoue B cells was blocked with 40 μ g/ml anti-DAF antibodies as previously described [9] while Mn^{2+} or 6 μ M PSI domain peptides were used

to block specific binding to low affinity state $\alpha_v\beta_3$ [7] expressed on Vero E6 cells. Tanoue B cells were pipetted from the wells and analyzed on the flow cytometer. The adherent Vero E6 cells were washed once in HHB/HSA buffer, and incubated in 0.25% Trypsin/0.5mM EDTA at 37°C for 5mins before harvest. Cells were diluted to ~100 per μL in HHB/HSA buffer before flow cytometric analysis. Some samples were fixed in 2% PFA at 0°C for 30mins before mounting in Vector Shield containing 1 $\times$ DAPI for microscopic imaging.

Equilibrium Binding measurements to DAF

For cell-binding measurements, 5,000 Tanoue B cells, were pipetted in 10 μL volumes of HHB/HSA buffer in a 384 well plate and were then incubated with increasing titers of R18-labeled SNV^{R18} for an hour at room temperature. Control samples were pre-blocked with a ten-fold excess of unlabeled SNV. For competition binding assays [30,31], variable concentrations of unlabeled SNV were added to wells followed by the addition of a fixed amount of SNV^{R18}.

The binding data were analyzed with IDLeQuery software developed locally by Dr. Bruce Edwards (UNM Pathology Dept) [26-29]. The mean channel fluorescence (MCF) or median fluorescence intensity (MFI) of SNV^{R18}-bearing cells in each sample was converted to site density of SNV^{R18}/cell using QD585 quantum dot fluorescence calibration beads [24]. For clarity, we will briefly describe the applicability of quantum dot fluorescence calibration beads to the quantitative assessment of virus particles bound to cells [24,25]. The typical data output from a flow cytometer is normally expressed in terms of the mean fluorescence intensity of a population of cells. Standard fluorescence calibration beads are used to convert the average fluorescence value of cells to number of fluorophore equivalents using Equation 1, where: I_i are the measured intensities of the

$$\frac{I_1}{I_2} = \frac{\varepsilon_{\lambda_{ex},1}}{\varepsilon_{\lambda_{ex},2}} \cdot \frac{\phi_1}{\phi_2} \cdot \frac{\%T_1}{\%T_2} \cdot \frac{\rho_1}{\rho_2} \quad (1)$$

virus-bearing cells (I in Eqn. 1), and standard QD585 fluorescence calibration beads (2 in Eqn. 1) irradiated by the same 488nm, 15mW laser; $\varepsilon_{\lambda_{ex},i}$ are the molar extinction coefficients of R18 ($4,750 \text{ M}^{-1}\text{cm}^{-1}$) and QD585 quantum dots ($530,000 \text{ M}^{-1}\text{cm}^{-1}$) at the excitation wavelength; ϕ_i are the respective quantum yields of self-quenched R18 and QD585 dots, lot# 452664A ($\phi_2 = 0.12$); It is important to note that the emission quantum yield of quantum dots varies from batch to batch [24,25] hence the lot number of the streptavidin functionalized dots used to tag the calibration beads must be specified. The intrinsic quantum yield of R18 is 0.3 [23], which decreases in proportion to the degree of self-quenching as will be shown in the results section. ρ_i are the site densities of R18 on the cells and quantum dots on the calibration beads respectively; %T is the percent fraction of fluorescence light transmitted through the bandpass filter and is used to account for the spectral mismatch between R18 fluorophore (51%) and QD585 Quantum dots (65%) as described elsewhere [23].

Determining an unknown concentration of unlabeled SNV from competitive binding with SNV^{R18}

The reversible equilibria between a pre-established complex of receptor (R) and unlabeled SNV in competition with labeled SNV^{R18} is:

The non-covalent labeling of the virus envelope with a relatively small mole fraction of fluorescent lipid derivatives is not expected to significantly alter the binding of Gn/Gc proteins to cellular receptors (see Fig. 1D). Therefore, in the “self exchange” competition-binding assay performed with a single concentration of labeled SNV^{R18} and a range of concentrations of unlabeled SNV, the affinity constants k_{-1}/k_1 (K_{d1}) and k_{-2}/k_2 (K_{d2}) are assumed to be equal. In this way an unknown concentration of the unlabeled SNV can be determined from mass action [30,31].

Results

Some structural characteristics of viral particles

Hantaviruses are spherical or ovoid particles of sizes that span the range of 70-210 nm [32-35]. The TEM images of UV-treated virions exhibit an average diameter of 193 ± 40 nm (Figure 1A-C). These TEM images of UV-treated virions were indistinguishable from samples of glutaraldehyde-inactivated particles (not shown). The hantavirus genome is comprised of three negative-sense RNA segments that are packaged within a lipid bilayer envelope membrane that bears viral glycoproteins Gn and Gc (also known as G1 and G2) (Figure 1D). The glycoproteins are anchored in the lipid bilayer membrane by an α -helical transmembrane ribbon [36], and form spikes that project 5-10 nm above the virion surface [36]. Gn and Gc mediate viral attachment, cellular entry and fusion with intracellular organelles [4]. The genome segments designated large (L), medium (M), and small (S) (Figure 1D) are encapsidated by nucleocapsid (N) protein with one L protein associated with each segment [4]. All three gene segments are required for infectivity. However, it has been found that non-equimolar ratios of L, M, and S can be packaged in mature virions, and thus contribute to the observed differences in virion sizes [36] (Figure 1A-C). Western blot assays have shown that UV irradiation does not significantly alter their expression levels of N, Gn and Gc proteins as shown (Figure 1E).

Determination of degree of labeling of virus particles from calibration standard curves

We have recently developed an assay to quantify the amount of exogenous R18 incorporated into bead-supported bilayer membranes [23]. Here we show the application of these concepts to an assay for determining the level of R18 incorporated into the envelope membranes of virus particles. Figure 2A shows a plot of the relative quantum yield of R18 as a function of its mole-fraction in a DOPC bilayer and DSC bilayer membrane. Unlike DOPC membranes, the degree of self-quenching in DSC membranes was temperature dependent, being lower at 41°C compared to 23°C. Phase segregation of DOPC and cholesterol rich sphingomyelin domains contributes to the enhancement of self-quenching of R18 at room temperature due to partial exclusion of R18 from the liquid-ordered phase. Self-quenching in DOPC membranes was temperature independent in this range because of the sub-zero melting point of DOPC. Cholesterol and sphingomyelin (Sphingomyelin melting temperature, $T_m \approx 41^\circ\text{C}$) are known to confer smaller molecular area to membranes [37,38].

The dequenching of SNV^{R18} in Triton X-100 under similar conditions to vesicles was analyzed likewise and plotted along the y-axis as shown in Fig. 2. The dequenching of R18 incorporated into envelope membrane of SNV was shown to be temperature dependent and well correlated to the behavior of the DSC calibration curve (Figure 2). The DSC lipid composition of the calibration curve is based on the so-called canonical model of lipid raft membranes [39] because the viral envelopes of viruses that emerge from the plasma

membrane contain phospholipids and cholesterol in characteristic proportions of lipid rafts [40]. New world hantaviruses such as SNV are believed to emerge from the plasma membrane, whereas their old world counterparts bud from the lumen [41,42] of the endoplasmic reticulum which has very low cholesterol and lacks sphingomyelin [43]. This suggests that the DSC membrane is a good model of a plasma membrane-derived viral envelope membrane of SNV.

The concentration of SNV^{R18} was determined from absorbance measurements (assuming a value of 95,000 cm⁻¹M⁻¹ for the extinction coefficient of R18). The site density of probes per virus particle was subsequently calculated using Equation 3 where $d_{SNV} = 1930\text{\AA}$ (Figure 1C) and $d_{Gn/Gc} = 10\text{\AA}$ are the respective diameters of the intact SNV particles and the heterodimeric glycoproteins Gn/Gc; n is the number of glycoprotein spikes which are assumed to be up to 1500, [36]; $A = 50\text{\AA}$ is the averaged area of the lipid headgroups and is based on a recent characterization of the composition of the membrane envelope of HIV virions [40].

$$R18/SNV = \left[\frac{(\pi d_{SNV}^2 - n\pi d_{Gn/Gc}^2)}{A_{lipid}} \right] R18mole\% \quad (3)$$

The characteristic mole fraction of R18 (R18mole%) that emerged under our labeling conditions was $\approx 2.7 \pm 0.3\%$ ($N > 5$) or 1.1×10^4 probes/SNV. The typical yield of SNV^{R18} from a 1.0mL batch of $\approx 1 \mu\text{g/mL}$ N protein virus preparation was $\approx 10^8$ SNV^{R18}/μl in a final volume of $\approx 300 \mu\text{L}$.

An interesting point of note about the labeling of SNV with R18 is that the degree of labeling was consistently found to reach the same limiting value of $\approx 3\text{mol}\%$ from over 5 labeling experiments. This result is consistent with a recent study [23] in which it was shown that the transfer of R18 from the aqueous phase to DOPC and DSC membranes supported on glass beads was limited to no more than 3 % mole fraction of the total lipids in the bilayer membranes. We suggest that the transfer of exogenous lipid probes to virus particles is governed by saturable free volume or area in the envelope membranes [23,44,45].

UV killed SNV binds to cognate attachment factor, DAF, and entry receptor, $\alpha_v\beta_3$ integrin

The capacity of UV irradiated SNV^{R18} to recognize the same attachment factor and entry receptor as live virions was tested by binding assays where specificity was demonstrated by blocking access to these receptors on two cell types. Tanoue B suspension cells express DAF but not the entry receptor integrin $\alpha_v\beta_3$. This made them attractive substrates for measuring specific binding of SNV^{R18} to DAF in a flow cytometer. Anti-DAF antibodies were shown to block binding of SNV^{R18} to Tanoue B cells. The near complete blocking suggests that the interaction between SNV^{R18} and Tanoue B cells is almost exclusive to DAF (Fig. 3A). Hantaviruses bind to the plexin-semaphorin integrin (PSI) domains situated at the apex of bent β_3 subunit [7]. Mn^{2+} activation [46,47] confers an unbent high affinity state to integrins (see insert in Fig. 3B), which hinders access to the PSI domain. It is worth pointing out that Mn^{2+} had no effect on the binding of SNV^{R18} to Tanoue B cells. The lack of response to Mn^{2+} was also viewed as a confirmatory test for absence of $\alpha_v\beta_3$ integrin expression on Tanoue B cells.

Quantitative analysis of flow cytometric binding data of SNV^{R18} to DAF expressed on Tanoue B suspension cells

Raw data associated with flow cytometry measurements of specific binding of SNV^{R18} to Tanoue B cells is represented by light scattergrams and fluorescence histograms shown in Fig 4. The data shown were analyzed using FlowJo software package (flowjo.com). Fig. 4A shows paired plots of cellular granularity versus approximate cell size, as measured in terms of Side Scatter (SSC-H) versus Forward Scatter (FSC-H). Plots of SSC-H versus logarithmic channel fluorescence measurements are also shown. The data correspond to neat cells and cells incubated with 89.5pM SNV^{R18}, as indicated. The scattergram data show that the morphological features of the cells were unaffected by the virus particles, and that the cells were stained homogeneously. Figure 4B shows a pseudo 3D plot of fluorescence histograms of SNV^{R18}-bearing cells as function of increasing titers of SNV^{R18}. The fluorescence data were then analyzed as shown in Fig. 5.

Fig. 5A shows parabolic plots of mean channel fluorescence (MCF) intensity of SNV^{R18} bound to Tanoue B cells versus concentration of titers of SNV^{R18}. The site densities of R18/cell (insert) were determined from fluorescence calibration beads using Eqn. 1, assuming a quantum yield of SNV^{R18} 0.1 (based on the 3 fold self-quenching factor at room temperature (see Fig. 2)).

The log transformed data were subsequently analyzed as a sigmoidal curve (Fig. 5B) to yield an effective affinity constant (K_{d2} cf. Eqn. 2)) of 26 pM. The high binding affinity is consistent with reported multivalent interactions reported for Coxsackieviruses and DAF [48]. Competitive binding experiments were next carried out with unlabeled SNV of uncertain concentration. This is because the number of physical particles in a typical virus preparation is much higher than the number of infectious units [22,49] that are measured by a plaque or focus assay (see methods and discussion). For convenience, 1ml SNV stock was first concentrated to 250 μ l in the same way as the labeled SNV^{R18}, and then for the competition assay, increasing titers of unlabeled SNV were allowed to compete with a fixed amount of SNV^{R18}. The results are shown in Fig. 5C. The arbitrary EC50 value that is derived from this analysis was reset to $K_{d2} = 26$ pM, based on the rational assumption that labeled SNV^{R18} and unlabeled SNV bind to DAF with the same affinity constant (see Methods). This allowed the concentration of SNV titers used in the self-exchange competition assay to be defined accordingly.

Adaptation of SNV-DAF interaction to high throughput flow cytometry screening assay

Figure 6A shows typical plots of equilibrium binding and competitive dissociation data associated with SNV^{R18} binding to Tanoue B cells, measured with high throughput flow cytometry. The equilibrium binding and competitive dissociation experiments each represent a 21-well sequence of samples in a 384 well plate. Row 1 was comprised of 6 wells of blank cells for measuring autofluorescence, 5 wells for measuring SNV-blocked controls and 10 wells representing 5 concentrations of SNV^{R18} measured in duplicate. In Row 2, 5 wells were configured to measure autofluorescence, 4 wells measured controls of fixed concentration of SNV^{R18}, and 12 wells measured 6 paired samples of different concentrations of competing SNV. Row 3 is a repeat of Row 1, with the addition of 1% DMSO to all wells. 1% DMSO was added to the buffer in these assays to simulate conditions of a high throughput discovery screen, where DMSO-solubilized molecular targets are typically added to cells. The data represent time-resolved clusters of cell fluorescence events. Unlabeled SNV particles were used to block the SNV^{R18} to DAF. The median fluorescence intensity (MFI) was determined for each data cluster and plotted as a logarithmic parabolic dose response curve for binding of SNV^{R18} to cells (Fig 6B). An overlay of data taken in buffer only, and 1% DMSO buffer showed that DMSO had

negligible effects on the samples. The signal dynamic range and data variation associated with these measurements were assessed in terms of Z' factors [50];

$$Z' = 1 - \frac{3(\sigma_p - \sigma_n)}{|\mu_p - \mu_n|}$$

where, σ_p , μ_p , and σ_n , μ_n represent the standard deviations and means of the positive (p) and negative (n) controls. Positive controls are unhindered SNV^{R18} and negative controls are samples preblocked with 10× unlabeled SNV. An assay with Z' scores ranging from 0.5 to 1.0 is considered excellent, whereas a Z' score of 0 reflects “yes/no” assay. The Z' scores (shown in red over each titer point in Figure 6B) were the result of 4 readings at each point taken from the paired readings in Rows 1 and 3. Fig. 6C recapitulates the competition binding assay from Fig. 5C under HTS conditions, using data from Row 2. The concentration of titers of SNV could be determined from the calibrated EC50 value.

Discussion

We undertook this study in order to establish that UV-irradiated virus particles recognize the cognate receptors required for cellular infection *in vivo*. We view this approach as a necessary first step towards the systematic study of the interactions of BSL-3 agents under BSL-2 conditions. We fluorescently labeled UV-killed SNV with a measurable quantity of fluorescent probe. Fluorescently labeled viruses were then shown to specifically bind to cognate receptors for cellular entry and infection, namely, DAF and $\alpha_v\beta_3$ integrin. Current studies that target the elucidation of the mechanism of entry and trafficking of pathogenic hantaviruses are derived from infectivity assays, and analyzed many hours to days after infection, where the data lacks temporal resolution of the early events of the infectious process. The ability of UV-killed viruses to recognize the requisite entry receptors provides the means to establish real-time assays to image and dissect the initial cellular events that are triggered by the interaction of virus particles and cells as will be described elsewhere [51]. An important goal of this study was to establish the utility of discretely labeled virus particles as a probe for high throughput cell binding assays, with potential applications for discovery of inhibitors of binding interactions between the cognate receptors and SNV^{R18}.

Measurements and uncertainties in the spectroscopic analysis of fluorescently labeled particles

To be a useful probe for studying viral-cell interactions as well as assay development, it is important to minimize batch-to-batch variations of labeled virus particles. The titer of hantavirus particles is generally determined from focus assays in Vero E6 cells [14]. Focus or plaque assays report viral titers in terms of infectious particles and are blind to the replication-deficient particles that are otherwise capable of cellular entry, through cognate receptors [52]. Accurate measurement of physical virus particles requires the use of quantitative TEM measurements, which is beyond our present capabilities [53]. A commonly used method of correlating protein content to particles [22] presents significant uncertainties due to lack of accurate information on the distribution of N proteins in each virus particle [36] as well as the presence of soluble viral protein in the lysate. The results presented in this work concern a simple and sensitive assay for quantifying total virus particles based on the characterization of fluorescently labeled virus particles. It is worth noting that our approach does not distinguish between infectious and noninfectious particles. Nevertheless, when used in combination with the traditional plaque assay, a ratiometric determination of physical particle counts and infectious particles can emerge. Here we

explain the uncertainties in the requisite analytical steps that lead to the determination of physical particles found in a typical harvest.

Firstly, hantavirus particles span a 100-270nm size range [32-35]. The average particle size of SNV particles reported in this work was 193 ± 40 nm. This average value in size of the SNV particles was found to be stable if the cell culture conditions between inoculation and harvest were kept the same, specifically cell passage, confluency and maintenance of steady serum levels. Significant fractions of smaller and immature virions [22] were present at early passages that were characterized by $<10^6$ ffu/mL (see methods). Keeping track of the average size of particles is important for an accurate determination of the number of total lipids per average particle.

Secondly, potential errors in fluorescence dequenching assays, absorption measurements, and fluorescence calibration are addressed. Because of limiting free area [23] in the envelope membrane, the degree of labeling was shown to reach a saturation limit of $\sim 1 \times 10^4$ probes per 193nm diameter SNV particle found in our preparations. Fluorescence dequenching measurement of SNV membranes labeled with R18 (under our experimental conditions) is expected to present up to a 3 fold increase in fluorescence intensity when such particles are solubilized in 1% Triton X-100 (Fig. 2). Whenever impurities of R18 aggregates are present among the virus particles, a greater than expected increase in the fluorescence intensity of detergent solubilized membranes is observed. This is because the degree of self-quenching in free aggregates is greater than R18 solubilized in membranes. Serum albumin has been shown to diminish the presence of R18 aggregates during labeling (see ref [23] for discussion). Because hantavirus particles bear no other proteins except the Gn/Gc glycoproteins embedded in the envelope membrane, the uncertainty in their surface density does not cause significant errors in the calculation of the total lipids present in the envelope membrane (see Eqn. 3). Our estimate of Gn/Gc heterodimers is based on data derived from *La Crosse* virus, a prototypical family Bunyaviridae virion with an established 270 to 1400 glycoprotein spikes per virion [36]. The difference in the derived quantity of R18 molecules using Eqn. 3 at these limits is less than 1%. The small error is largely due to the fact that Gn/Gc proteins are membrane-anchored by a $78\text{\AA}^2$ ribbon that is not much larger than the average area of lipid headgroups ($50\text{\AA}^2$). Gn/Gc are believed to be randomly distributed [36] on the surface of the virus particle (Fig. 1D), and comprise a small fraction of the total number of the $\sim 2.3 \times 10^5$ lipids present in the outer leaflet of the envelope membrane. Therefore, the Gn/Gc proteins are not expected to significantly affect the overall organization of the envelope membrane [54]. The temperature dependent manner in which the dequenching of the viral envelope membrane and the DSC calibration curves are correlated suggests that the DSC model system is a good approximation of the envelope membrane [40].

The absorption cross-section and extinction coefficient of R18 has been shown to be modulated by the formation of self-quenching aggregates in membranes [23]. Spectral broadening and a decrease in the oscillator strength of the 564 nm absorption band of R18 [23] manifest probe aggregation. In our measurements, the absorption spectral lineshape of 3mol% R18 solubilized in SNV membranes resembles that of monomeric R18 (not shown). This result is as expected since the probability of significant probe aggregation is minimal at 3mol% [23]. Accordingly, the extinction coefficients for monomeric R18 are applicable to the fluorescence calibration analysis. For flow cytometry measurements on a FACScan, sample irradiation from a 15-mW argon ion laser is attenuated to a spot-size of $\sim 20\text{ }\mu\text{m}$ (vertical) $\times 60\text{ }\mu\text{m}$ (horizontal) at the focal point [55]. The excitation light is sufficient to uniformly excite all fluorophores on cell-borne virus particles, because of the nm pathlength across the spherical geometry of the virus particles. The putative homogeneous exposure of

fluorophores to excitation light, allows sample irradiation to be treated in the same manner as the excitation of soluble R18.

Thirdly, we have used self-exchange competition binding assays between unlabeled SNV and labeled SNV^{R18} as a means to determine the concentration of the unlabeled virus particles. Because we do not have an alternate means of directly measuring the number of physical particles, we cannot absolutely confirm the validity of the self-exchange competition assay. Nevertheless, the validity of this analysis is governed by the *a priori* assumption that $K_{d1} = K_{d2}$ in Eqn 2. Because viruses use the Gc/Gn proteins to bind to cellular receptors, the addition of 3mol% R18 into the envelope membrane is not expected to significantly perturb these proteins. Accordingly, the accuracy of the determination of the concentration of SNV is limited by the parameters used to evaluate labeled [SNV^{R18}]. For the purpose of our applications, this approach becomes a valuable tool to establish an internally consistent bookkeeping account of different batches of virus preparations. An important consequence of determining the number of physical particles in a preparation of SNV is the determination of the ratio of infectious units measured by a focus assay (see methods) to the particle count that is determined by the competition assay. The value that emerges is 1:14,000. To the best of our knowledge, we are not aware of previous data on this value for hantaviruses. Nevertheless the ratio is well within the range reported for other viruses. A recent study on Dengue (type 2 strain) and references therein [22] provides a useful discussion on the measurement and limitations of infectious units-to-particle count ratio. Overall our approach is highly sensitive and less susceptible to sample handling errors and serves the overall purpose of producing internally standardized batches of labeled SNV particles.

Development of HTS assay and Summary

The development of assays geared towards the convenient production of consistently labeled virus particles is highly desirable for high throughput discovery assays. This study has established that the cognate receptor binding properties of SNV are conserved in UV-irradiated particles. We propose to screen for small molecule inhibitors of hantavirus infection by blocking the first step in the infection process, namely the binding of hantaviruses to DAF [11]. Accordingly, labeled SNV^{R18} has been shown to be highly compatible with high throughput flow cytometry measurements using suspension cells expressing DAF (Fig. 5). Follow up studies are currently underway in which out of patent drugs are being screened for inhibitors of the binding of SNV to DAF.

Acknowledgments

This work was supported by NIH Grants K25AI060036 (TB), NSFCTS0332315 (TB&GL); U54MH074425/MH084690 (LAS); U01AI56618, U01 AI054779 (BH) 1P30CA118100, UNM School of Medicine RAC award C-2294-T (TB) and The New Mexico State Cigarette Tax to the UNM Cancer Center. Images in this paper were generated in the University of New Mexico Cancer Center Fluorescence Microscopy Facility, supported as detailed on the webpage: <http://hsc.unm.edu/crtc/microscopy/Facility.html>

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Figure 1.

Structural morphology and composition of SNV virions **A.** Low magnification wide field view of electron micrographs of Cesium Chloride-stained UV-irradiated SNV. **B.** Higher magnification of area marked with rectangle. **C.** Histogram of average size distribution of SNV virus particles shown in **A** (sample size 163 virions) mean 193 ± 40 nm. **D.** Schematic cross-section of Bunyaviridae virus. The three RNA genome segments (S, M, and L) are complexed with the nucleocapsid protein to form ribonucleocapsid structures. The nucleocapsids and RNA-dependent RNA polymerase are packaged within a lipid membrane envelope that contains viral glycoproteins Gn and Gc. **E.** Western blot representing the preservation of antigens consequent to 30-sec of UV irradiation. Purified SNV preparations were subject to irradiation (“UV”) before being treated with SDS Laemmli buffer in a boiling water bath and the proteins analyzed by SDS-PAGE. Antibodies used in the detection were anti-nucleocapsid (“N”), anti-Gc or anti-Gn.

Figure 2.

Plot of dequenching of R18 as a function of its mole% fraction in lipid vesicles (insert shows full range of experiment) in DOPC and DSC membranes at 23°C and 41°C. Horizontal tie lines are measures of dequenching of R18 solubilized in the envelope membrane of SNV at 23°C and 41°C, top and bottom, respectively. Each tie line intersects the standard calibration curves at a single point which corresponds to the mole fraction of R18 in (the envelope membrane) along the x-axis. The ovoids indicate the limiting errors in the points of intersection. Errors are based on the standard deviation in the dequenching values from over 5 labeling experiments.

Figure 3.

UV killed SNV^{R18} binds cognate receptors of live SNV. **A.** Flow cytometry measurements of SNV^{R18} binding to Tanoue B cells. Total binding is normalized to samples in the presence of 1mM Ca²⁺ and samples measured under the conditions of: 40μg/ml polyclonal H319 anti-DAF antibodies; 10× unlabeled SNV and Mn²⁺. The similarity between Ca²⁺ and Mn²⁺ conditions suggests the absence $\alpha_v\beta_3$ expression on Tanoue B cells. **B.** Models of $\alpha_v\beta_3$ in bent, low affinity (Ca²⁺) and extended conformation (Mn²⁺). Model of the extended conformation was assembled using the program *O Jones et al 1991* [56]. Figure was adapted from Xiong et al [57] and prepared with Pymol version 0.99 Delano Scientific LLC (San Francisco, CA). Micrographs show effect of cation environment on the ability of SNV^{R18} to bind to $\alpha_v\beta_3$. Ca²⁺ environment maintains $\alpha_v\beta_3$ in a bent low affinity state that leaves the PSI domain accessible to incoming virions, and enables binding of SNV to integrins. 1 mM Mn²⁺ environment activates $\alpha_v\beta_3$ into an unbent conformation that renders the PSI domain inaccessible to SNV and diminished binding. Graph shows flow cytometry measurements of binding of SNV^{R18} to Vero E6 cells. 10× SNV, 6μM PSI domain peptides, and Mn²⁺ are used to inhibit binding to Vero E6 cells.

Figure 4.

Flow cytometry measurements of SNVR¹⁸ binding to Tanoue B cells. **A.** Paired scattergram plots of cell granularity (Side Scatter, SSC-H) versus size (Forward Scatter, FSC-C) and SSC-H-versus cellular fluorescence associated with the indicated quantity of SNVR¹⁸. The ellipses shown in the scattergrams are electronic gates used to select cells (92.8% of total population of scatter events), which are considered valid for analysis. **B.** Pseudo-3D plot of log fluorescence versus concentration of SNVR18 titers.

Figure 5.

A. Equilibrium binding curve of SNVR¹⁸ to 10,000 Tanoue B cells in 10 μ l. Insert shows a plot of bound SNVR¹⁸ versus residual free SNVR¹⁸ particles. **B.** Plot of SNVR¹⁸/cell versus log free [SNVR¹⁸]. The analysis of the Sigmoidal binding data yielded a K_d of $\approx$ 26 pM. **C.** Competition binding curve of a fixed quantity of SNVR¹⁸ and various arbitrarily defined titers of unlabeled SNV. The EC₅₀ value determined from Fig. 4B is used to determine the actual concentrations of SNV titers (see text for details).

Figure 6.

A. Dot plot of SNVR¹⁸-bearing cells from an assay in a 384 well plate. **Row 1.** Data plugs bisected by the line represent duplicate titration of SNVR¹⁸ show increasing binding with increasing SNVR¹⁸ in each well. Data marked with asterisks represent samples preblocked with 10x unlabeled virions. Data points along the baseline represent autofluorescence. **Row 2.** Competition assay of SNV and SNVR¹⁸, data marked with a c represents control marker samples not mixed with SNV. Row 3 is a repeat of Row 1 in a 1% DMSO environment. **B.** Plot of median fluorescence intensity (MFI) versus added SNVR¹⁸ of Rows 1&3. Open squares represent 1% DMSO solvent, triangles neat HHB buffer. Z' values for each concentration shown in red were determined for 4 values at each point using Eqn. 4. x's represent samples preblocked with 10x unlabeled SNV. **C.** Plot of bound SNVR¹⁸/cell versus titers of unlabeled SNV nominally measured in terms of grams of N protein. The EC₅₀ value determined from Fig. 5B can then be used to convert the grams of N protein to concentration of SNV as described in Fig. 5C.