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Evaluation of the maturation of individual Dengue virions with flow virometry

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ABSTRACT

High-throughput techniques are needed to analyze individual virions to understand how viral heterogeneity translates into pathogenesis since in bulk analysis the individual characteristics of virions are lost. Individual Dengue virions (DENV) undergo a maturation that involves a proteolytic cleavage of prM precursor into virion-associated M protein. Here, using a new nanoparticle-based technology, "flow virometry", we compared the maturation of individual DENV produced by BHK-21 and LoVo cells. The latter lacks the furin-protease that mediates prM cleavage. We found that prM is present on about 50% of DENV particles produced in BHK-21 cells and about 85% of DENV virions produced in LoVo, indicating an increase in the fraction of not fully matured virions. Flow virometry allows us to quantify the number of fully mature particles in DENV preparations and proves to be a useful method for studying heterogeneity of the surface proteins of various viruses.

Introduction

Viruses pursue different strategies in their infection of the host: while some of them, for example hepatitis A, rely on the exact reproduction of their antigenic spectra, others, like HIV are antigenically heterogeneous even when they are produced in homogeneous cell lines (Arakelyan et al., 2013) or the virus has been cloned (Roederer et al., 2014). While the characterization of viral preparations by most of the existing bulk techniques effectively describes the composition and properties of an average virion in the preparation, little is known about the variability between virions. Since for enveloped viruses whose surface proteins mediate viral interactions with their cellular targets, this heterogeneity may impact the biological properties of viruses. Recently, we developed a new technique, "flow virometry" that allows the analysis of the antigenic composition of individual virions (Arakelyan et al., 2013). In this study we focus on the surface protein composition of individual DENV, an important human pathogen (St John et al., 2013) that is well characterized structurally (Kuhn et al., 2002; Mukhopadhyay et al., 2005).

DENV is a positive single-stranded RNA *Flaviviridae*. Its 11Kb genome encodes a single large polyprotein, which is subsequently cleaved into three structural and seven non-structural proteins that are essential for viral replication. The structural proteins are capsid (C), prM/membrane (prM/M) and envelope (E) (Lindenbach and Rice, 2003). The E protein contains three distinct domains, the structurally central domain DI, the dimerization domain DII, which contains the fusion loop, and the immunoglobulin-like carboxy terminal domain DIII involved in the attachment to host cell receptors (Kielian and Rey, 2006).

DENV and the other flaviviruses assemble in the ER as immature viruses (Mukhopadhyay et al., 2005; Mackenzie and Westaway, 2001). The mildly acidic conditions of the trans-Golgi network promote virus maturation, in which the E protein changes its conformation allowing the cleavage of prM into pr and M by a furin-like protease (Murray et al., 1993; Randolph et al., 1990; Yu et al., 2008; Zybert et al., 2008). The pr portion of prM covers the DII domain of E, preventing premature viral fusion or inactivation in the acidic intracellular compartments through which virions traffic (Guirakhoo et al., 1992). Similarly to other viruses, an infectious DENV preparation contains immature virions (Zhang et al., 2007). However, it remains to be understood whether there are any virions that are fully matured, i.e., in which all prM molecules are cleaved, or all the virions are either fully immature with all prM molecules in uncleaved form or "mosaic", with the same virions carrying both M

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protein and prM. To answer this question, it is necessary to analyze these proteins on the surface of single virions.

Towards this goal, here, we adapted a high throughput flow virometry technique (Arakelyan et al., 2013) to characterize the degree of maturation of individual virions in a preparation of infectious DENV. Briefly, flow virometry of DENV includes five main steps: (i) labeling DENV with a fluorescent lipidic dye DiI and then with fluorescent 2H2 antibodies that reveal prM protein; (ii) capturing labeled virions with 15 nm-nanoparticles (MNPs) coupled with 3H5-1 antibodies against E protein; (iii) separating the DENV–MNPs complexes from unbound antibodies using magnetic columns; (iv) eluting DENV–MNPs complexes from the columns, and (v) analyzing the eluted complexes with a flow cytometer. We found that in DENV population produced by BHK-21 cells approximately half of virions are fully mature as no prM could be detected on their surface. In contrast, in DENV population produced in furin-deficient LoVo cells, such a mature fraction constitutes only ~15%. The high throughput flow virometry applied here to DENV can be further used for the detailed characterization of the heterogeneity of envelope proteins of various viruses and may prove important in the development of vaccine against DENV.

Results

For this flow virometry study we defined DENV as a membrane particle that carries E protein. Therefore, to reveal DENV we first labeled our preparation with a fluorescent lipidic dye DiI (adapted from Zaitseva et al., 2010) and then stained virions for the presence of prM with fluorescently labeled 2H2 anti-prM antibodies (Henchal et al., 1982). To capture DiI fluorescent particles that carry E protein we used MNPs coupled with 3H5-1 antibodies (Gentry et al., 1982) specific for the DIII domain of E protein. The DENV–MNPs complexes were separated from free antibodies on magnetic columns: due to the magnetic properties of MNPs these complexes were retained while free antibodies were not. Preparations of DENV were analyzed with a flow cytometer triggered on viral fluorescence.

Visualization of single particles

To verify that the events registered in flow analysis represent single virions rather than their aggregates, we first analyzed in a flow cytometer free DiI-labeled DENV (Fig. 1) prepared as described in *Methods*.

In general, while the number of single particles measured by flow cytometry depends linearly on the dilution of the preparation, aggregates or coincident detection do not show a linear relation with concentration (van der Pol et al., 2012). To distinguish between DENV aggregates and single virions, we diluted our preparation in serial two-fold dilutions and evaluated the relation between event frequencies and dilution factors. We excluded aggregates from our analysis by the gating strategy presented in Fig. 1A. Then we plotted the number of single events as a function of the dilution factor (Fig. 1B). There is a linear relation between the number of events and the dilution factor. Also, the mean fluorescence intensity (MFI) of events did not change over the four orders of magnitude of the dilution series, and was on average 2679.6 ± 29 arbitrary fluorescence units, with a coefficient of variation (CV) of 5.07% (Fig. 1C).

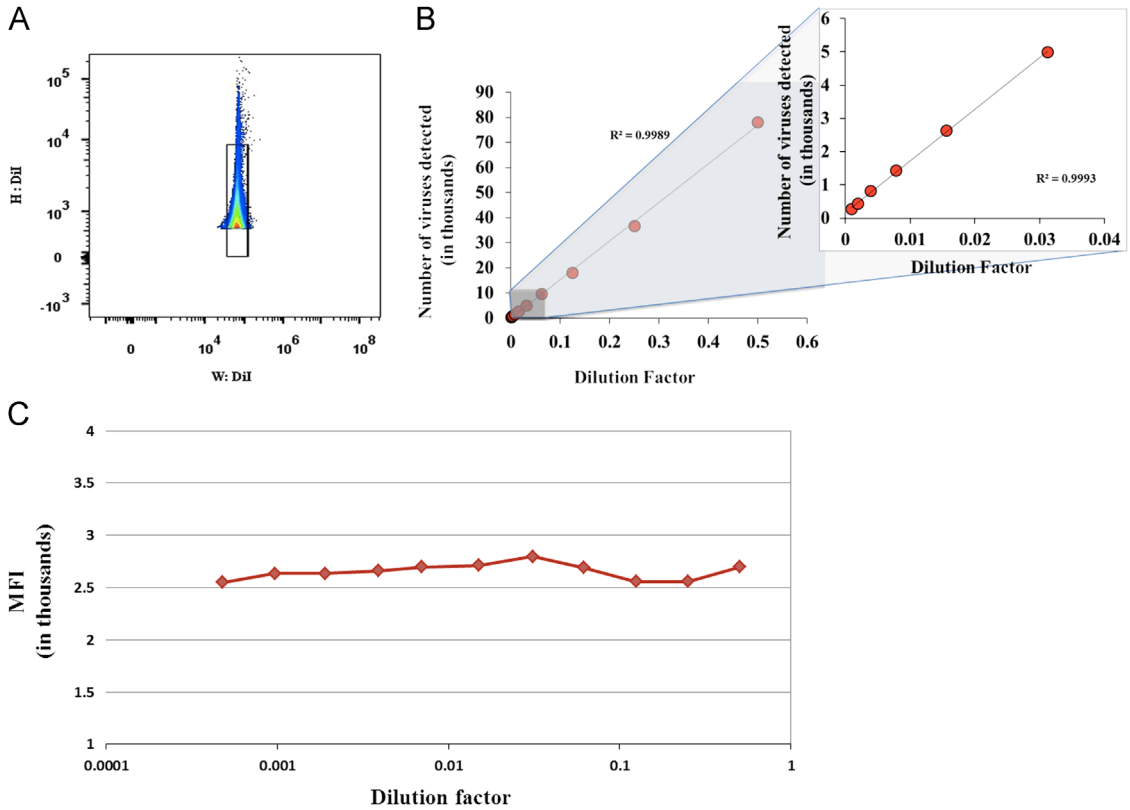

Fig. 1. Analysis of individual virions. DiI-stained DENV preparation was serially diluted two fold from 1:2 to 1:2048. The events were acquired in a fixed volume of 160 μl in duplicate using a High Throughput Sampler (HTS) at a flow rate of 0.5 μl per second on a LSRII flow cytometer, set to be triggered by DiI fluorescence. (A) Gating strategy to exclude aggregates. A singlet gate was defined by plotting fluorescence height versus fluorescence width. This gate excludes events with a high width and high height that represent aggregates. (B) Number of particles as a function of the dilution factor. DiI-labeled virions were serially diluted and the number of events for each dilution was evaluated by flow cytometry based on volumetric control. Right panel is a blow up of the left part of the *X* axis. (C) Median fluorescence intensity (MFI). DiI-labeled DENV was serially diluted with a factor two and MFI was evaluated for each dilution. *Note that the number of events is inversely linearly dependent on the number of dilutions and the MFI is constant in the large range of dilutions suggesting that the events correspond to single particles detection and not to swarm detection.*