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Short Communication

Increased Virus Uptake Alone is Insufficient to Account for Viral Burst Size Increase during Antibody-Dependent Enhancement of Dengue Viral Infection

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Abstract

Antibody-dependent enhancement (ADE) is a generally accepted hypothesis to explain increased viremia and disease severity during secondary human DENV infections. In addition to an increased number of infected cells, its mechanism has been postulated as either an increased uptake or an elevated production of viruses per infected cell during ADE infection. The latter postulate, however, has not been rigorously tested. Using Fc gamma receptor (FcγR)-positive and type I IFN-positive (THP-1 and U937) or negative (K562) human myeloid cells, and a combination of techniques including flow cytometry, plaque assay, and real time qPCR assay, we found ADE infection led to significant burst size increase in all three cell types. Furthermore, we found comparable levels of virus uptake between ADE and direct dengue infection in THP-1 cells. Collectively, our results demonstrate that burst size increase is a common feature of dengue ADE infection, and that increased viral uptake during ADE infection is insufficient to account for the burst size increase. Thus, further mechanistic study is needed to uncover the intrinsic mechanisms of ADE infection.

Keywords

Dengue; Antibody-dependent enhancement; Burst size

Abbreviations

ADE: Antibody-Dependent Enhancement; DENV: Dengue Virus; FcγR: Antibody Fc gamma receptor; FFU: Focus Forming Unit; GCP: Genome Containing Particle; IFNγ: Interferon Gamma; NS: Non Structure protein; qPCR: quantitative Polymerase Chain Reaction; RT-PCR: Reverse Transcriptase PCR

Introduction

Dengue viruses (DENV) exist as four antigenically distinct serotypes (DENV1-4), constituting the most significant mosquito-borne viral disease worldwide. Because dengue viral titers at peak viremia correlate with disease severity [1], it is critically important to define factors that contribute to the virus burden in infected individuals. The increased viremia seen in most severe dengue cases is often explained by antibody-dependent enhancement (ADE) of DENV infection, during which DENV-antibody infectious immune complexes facilitate productive infection of Fc R-bearing cells [2]. More recently, the ADE hypothesis has been supplemented by the notion that Fc R mediated DENV entry can lead to immunological suppression thereby delineating ADE into "extrinsic" i.e. increased infected cell number, and "intrinsic" components that regulate virus replication [3]. As currently proposed, intrinsic ADE hypothesis comprises several linked immune suppressive events triggered by Fc receptor engagement of DENV immune complexes: (i) reduced type I IFN production through down-regulation of viral recognition signaling pathways, and (ii) elevated secretion of inhibitory cytokine IL-10 and, (iii) the interference of normal signaling events such as Fcγ-receptor signaling and Janus Kinase-Transducer and Activator of Transcription (JAK-STAT) signaling pathways [1-3]. However, both the first two arms of the intrinsic ADE hypothesis are in discord with studies from our laboratory and those of others where no decrease in type I IFN production during ADE had been observed [4-8]. Thus, a central tenant of the intrinsic ADE hypothesis is challenged. Additionally, the contribution of IL-10 to viral production is called into question since IL-10 generally reaches the highest concentration concurrent with or following peak levels of viral infection [5,7,9,10]. Recent findings in our laboratory have demonstrated both increased IFN production and unchanged IL-10 production in DENV ADE infection [7], raising the possibility that intrinsic ADE observations may simply reflect increased uptake of virus-antibody immune complexes following Fc R engagement [11,12]. In this paper, we tested this alternative hypothesis directly.

We first determined DENV production per infected cell (burst size) by quantifying both the number of infected cells using a murine monoclonal antibody against dengue E protein and a muring IgG control antibody in combination of multicolor flow cytometry analysis as describe before [13] and the number of virions produced (infectious and total) at the time of peak infection, which is around 48 hours based on previous studies by us and others [8,11,13,14], using three cell types. These are type 1 IFN positive human macrophage-like THP-1 cells, from which most of the data in support of the intrinsic ADE notion have been derived; and two other Fc R positive control cell lines: U937 (Type 1 IFN positive) and K562 (Type 1 IFN negative), both of which have been widely used in assessing dengue ADE mechanisms [15]. Direct and ADE infections in these cells were performed essentially as described for primary monocytes [7], but modified for these cell lines using DENV2 16681 strain which has been used extensively to study dengue ADE infection. For all three cell lines (THP-1, U937, and K562), optimal MOIs (5 for THP-1 and U937 cells, and 0.5 for K562 cells) were determined prior to the experiments and used consistently throughout. Burst size was expressed as the average number of infectious virions released (determined by an immune focus staining plaque assay using Vero cells) by each infected cell (determined by flow cytometry using anti-E antibodies in combination with anti-CD14 or control antibodies) at the time of peak virus production as we previously described [13].

Results are expressed as average burst sizes achieved in THP-1, U937, and K562 cells following infection in the presence (ADE) or absence (Direct) of an optimal enhancing concentration of DENV immune human sera (these are pooled human sera from 4-6 individuals who had recovered from documented dengue virus infection, as we have described previously [11,13]) that was determined for each cell type in pilot experiments. These concentrations are 1/5,000, 1/200, and 1/1,000 for THP-1, U937 and K562 cells, respectively. Figure 1 shows that in all three cell types, ADE infection led to significantly increased burst size compared to that measured in direct infection; 8 ± 2 vs. 19 ± 4 in THP-1 (Figure 1A, P=0.005, n=8), 1 ± 0 vs. 5 ± 1 in U937 (Figure 1B, P=0.018, n=4), and 3 ± 1 vs. 8 ± 3 in K562 (Figure 1C, P=0.031, n=6). Notably, burst size measurements with monocytic THP-1 and U937 cells were similar to those that we obtained with human primary monocytes (5 ± 4 and 36 ± 24 for direct and ADE infection, respectively) [7]. Under our specific experimental conditions, the magnitude of enhancement may vary between 2 to 5 folds in individual assays. As expected, ADE infection also increased the percentage, and thus the number, of infected cells for each of the three cell types tested

(Table 1). These results suggest a general principle that dengue burst size is greater under ADE than direct infection conditions.

Having established that ADE infection increases DENV burst size, we next investigated whether increased viral uptake facilitated by Fc Rs alone is sufficient to account for the increased burst size, as we hypothesized previously [7]. Also, a kinetics experiment demonstrated that viral RNA amount increases only after 12 hours of infection in THP-1 cells, not before (Figure 2). Thus we addressed this by the measurement of internalized virus in THP-1 cells using NS1 gene specific quantitative RT-PCR 8 hrs after infection, i.e., before new DENV RNA replication is expected [11,16]. Low percentages of infected cells at this early time-point, as determined by FACS, were verified by fluorescent microscopy in two experiments (data not shown). Intracellular viral loads were then compared to the amount of virus produced by each infected cell at 48 hrs post infection, which is the time of peak virus production in our experimental system. In five experiments, we detected similar levels of viral uptake per infected cell between direct and ADE conditions (Direct: $1,639 \pm 298$
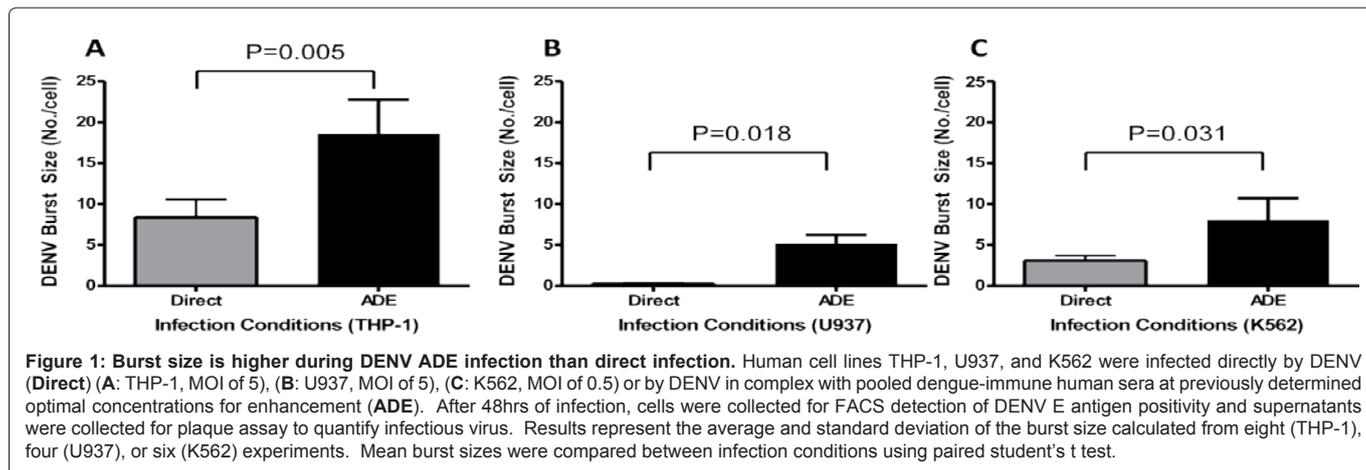

Figure 1: Burst size is higher during DENV ADE infection than direct infection. Human cell lines THP-1, U937, and K562 were infected directly by DENV (**Direct**) (**A**: THP-1, MOI of 5), (**B**: U937, MOI of 5), (**C**: K562, MOI of 0.5) or by DENV in complex with pooled dengue-immune human sera at previously determined optimal concentrations for enhancement (**ADE**). After 48hrs of infection, cells were collected for FACS detection of DENV E antigen positivity and supernatants were collected for plaque assay to quantify infectious virus. Results represent the average and standard deviation of the burst size calculated from eight (THP-1), four (U937), or six (K562) experiments. Mean burst sizes were compared between infection conditions using paired student's t test.

Table 1: The proportion of infected cells under direct and ADE conditions.

Cell Types	Percentage of infected cells (%)*	
	Direct	**ADE**
THP-1	6.1±2.7	19.3±8.4
U937	0.14±0.11	1.6±1.0
K562	10.8±5.2	25.5±14.1

*Results shown are mean and standard deviation of 4, 5 and 6 measurements for THP-1, U937 and K562 cells, respectively

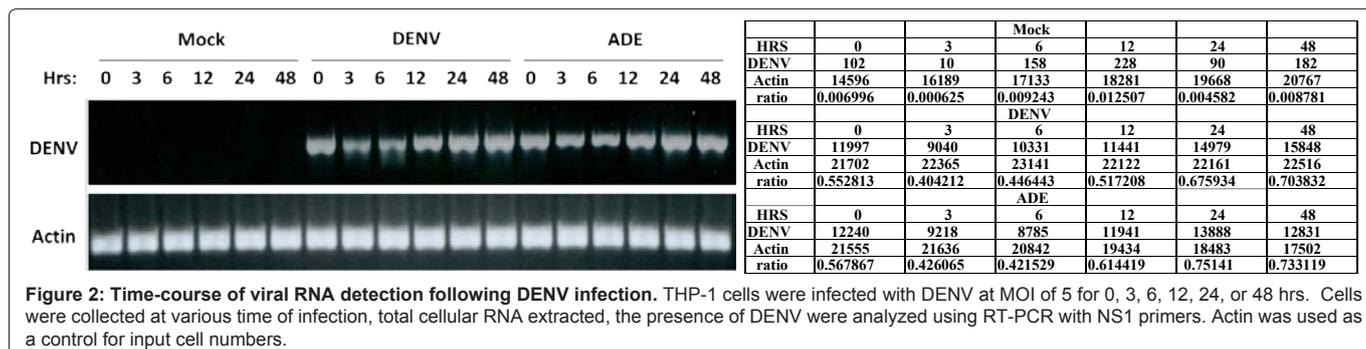|	Mock					
HRS	0	3	6	12	24	48
DENV	102	10	158	228	90	182
Actin	14596	16189	17133	18281	19668	20767
ratio	0.006996	0.000625	0.009243	0.012507	0.004582	0.008781
	DENV					
HRS	0	3	6	12	24	48
DENV	11997	9040	10331	11441	14979	15848
Actin	21702	22365	23141	22122	22161	22516
ratio	0.552813	0.404212	0.446443	0.517208	0.675934	0.703832
	ADE					
HRS	0	3	6	12	24	48
DENV	12240	9218	8785	11941	13888	12831
Actin	21555	21636	20842	19434	18483	17502
ratio	0.567867	0.426065	0.421529	0.614419	0.75141	0.733119

Figure 2: Time-course of viral RNA detection following DENV infection. THP-1 cells were infected with DENV at MOI of 5 for 0, 3, 6, 12, 24, or 48 hrs. Cells were collected at various time of infection, total cellular RNA extracted, the presence of DENV were analyzed using RT-PCR with NS1 primers. Actin was used as a control for input cell numbers.

copies/cell vs. ADE: 1,168 ± 226 copies/cell) according to methods we have described previously [11,13]. A greater than 2-fold increase in burst size during ADE, as measured by focus forming assay of infectious virus (Direct: 10 ± 3 vs. ADE: 23 ± 6, P=0.020), but not in total RNA copy number (Direct: 10,541 ± 3,272 vs. ADE: 16,953 ± 5,780) (Table 2). Our data suggest that ADE increases the infectivity only as measured in FFU, but not GCP. Since FFU is a measurement of the quantity of infectious virus, and GCP is a determination of the amount of viral genetic materials, these results raised the possibility of differing post-entry modulation between direct and ADE infection conditions, and that increased burst size during ADE infection is not a result of increased viral uptake. These new results are in agreement with our previously published work with DENV infection in primary human monocytes, in which we have consistently observed higher levels of infection and viral production [7,11,13]. Moreover, we compared in the same experiment the production of infectious DENV FFU (by plaque assay on Vero cells with supernatant collected from THP-1 cells) with total produced GCP (genome containing particle, as quantitated via qRT-PCR) and determined infectious particle to GCP ratio to be around 1:1,000 for THP-1 cells in our experimental system. Our burst size range of 1-6 for direct infection translated into a range of 1,000-6,000 GCPs per infected cell, and 5-20 (5,000-20,000) for ADE infection. This ratio is within the same range of other published data: 1:2,000 at 24 hours post infection and 1:80,000 at 48 hours post infection for primary macrophages [17] and 1:2,600 in cell lines [18]. Our findings are also in agreement with studies on HCV, another flavivirus, in which particle-to-FFU ratio were found to be 1000:1 - 2000:1 [3,4].

Although increased initial viral infectivity during ADE has been suggested [11,12], viral uptake has not been previously quantified. We addressed this issue directly with sensitive real time RT-PCR to precisely measure viral uptake, and correlated uptake with production of both total and infectious virions. We found similar viral uptake per infected cell during DENV direct and ADE infections. Given a DENV half-life of 2-3 hrs in normal culture media [19], our viral titers are unlikely to represent cumulative viral production over the life of infected cells. To our knowledge, this is one of the first reports of DENV burst size in human cell lines. We believe it provides a key determination missing from previous papers attributing higher viral titers to ADE infection.

Overall, our data support the notion of an intrinsic ADE mechanism, the exact nature of which remains unclear. Differing entry could lead to altered deployment of viral anti-IFN signaling proteins, such as nonstructural protein 4 (NS4) or NS5 [20,21], which could affect viral production per cell. Additionally, mechanisms that either do not require IFNs [22] or nitric oxide suppression [14], or that limit IFN responsiveness [23], may be activated to facilitate DENV replication in ADE infected cells. In summary, the mechanism of ADE is more complex than initially appreciated, and alternative explanations for heightened DENV replication must be further investigated.

Competing Interests

None of the co-authors has either financial competing interests, or non-financial competing interests.

Authors' Contributions

Xia Jin and Jacob J Schlesinger conceived the study. Xia Jin, Luis Martinez-Sobrido and Jacob J Schlesinger designed the experiments. Matthew Quinn and Zhihua Kou performed the experiments. Matthew Quinn and Xia Jin drafted the manuscript. All authors read and approved the final manuscript.

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Table 2: Quantitation of initial viral uptake and peak viral output.

Infection Condition (n=5)	Intracellular Copies/Cell (8hrs)	Burst Size (48hrs)	
		FFU/cell	Copies/cell
DENV	1,639±298§	10±3	10,541±3,272
ADE	1,168±226	23±6	16,953±5,780
P value	n/s	0.020	n/s

§: All numbers represent mean +/- standard deviation of results obtained from 5 independent experiments

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