Host reverse transcriptase is required to maintain persistent dengue virus infections in C6/36 insect cells

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ABSTRACT

It has been long known that C6/36 cells challenged with Dengue virus serotype 2 (DENV-2) show initial cytopathic effects (CPE) but overcome these within 3 split passages to resume normal growth and morphology. It is also known that establishment of persistent infections requires combined action of host reverse-transcriptase (RT) activity and RNA-mediated interference (RNAi). We hypothesized that RT inhibition in C6/36 cells would also prevent persistent infections with DENV-2 and that treatment of stable, grossly normal C6/36 cells persistently infected with DENV-2, even after long passaging, would revert to CPE and die under continuous exposure to the RT inhibitor tenofovir disoproxil fumarate (TDF). Toxicity tests using TDF with naive C6/36 cells revealed limited effects at 0.1 mM but challenge with DENV-2 in its presence resulted in death, as expected based on previous reports. However, TDF treatment of stable, grossly normal C6/36 cell cultures persistently infected with DENV-2 for up to 30 split passages also induced severe CPE and death. The results indicate a key role of host RT not only in the establishment of persistent infections but also in their long-term maintenance. Persistent infections in shrimp and insects are sometimes characterized with low to very low levels of viral infection that are not detectable by routine histological

analysis, *in situ* hybridization, immunohistochemistry or even by very sensitive PCR. Our results suggest that treatment of grossly normal insects or crustaceans with TDF may be a simple approach to induce higher replication by accommodated viruses. This could improve detection by histological or molecular methods and provide a way to invoke a disease state. It could also be used to increase viral quantities for purification purposes.

Key words: Dengue virus serotype 2 (DENV-2); reverse transcriptase (RT); persistent infection; C6/36 cells; *Aedes albopictus* C6/36 cells; endogenous viral element(s) (EVE); P-element Induced Wimpy (PIWI) protein

INTRODUCTION

Recent research results have proven that insects and crustaceans are capable of a specific, adaptive immune response to viral pathogens based on nucleic acids instead of antibodies [1, 2]. These results support a proposal from 1998 that insects and crustaceans could tolerate one or more viruses in an adaptive response (termed viral accommodation) via unknown mechanisms [3]. Viral accommodation was characterized by persistent infections with one or more pathogenic viruses without gross or histological signs of disease. These are not chronic or latent infections but instead continuous, persistent infections that may last for a host's lifetime during which they are continuously infectious.

We now know that host generated reverse transcriptase (RT) acting on invading viral RNA is a key enzyme in the viral accommodation response [4, 5]. It results in the production of highly variable fragments of viral copy DNA (vcDNA) in both linear (lvcDNA) and circular (cvcDNA) forms [6]. These can give rise to small interfering RNA (siRNA) to feed both cellular and systemic antiviral responses based on RNA interference (RNAi) pathways [1, 2]. The vcDNA produced may also give rise to endogenous viral elements (EVE) in the host genome that can produce negative sense RNA to also feed RNAi pathways. These responses usually prevent disease but not infection by the cognate virus. EVE are heritable when they occur in germ cells [1, 2]. The term EVE was introduced around 2012 to describe non-retrovirus genome fragments discovered in mammalian genomes [7], and it was widely adopted thereafter. However, to our knowledge, the existence of EVE of non-retroviral viruses in the genome of insects (called "viral inserts" at the time) was first reported for a

DNA virus in 1999 [8] and then for RNA viruses in 2004 [9] and 2007 [10, 11]. Similar reports were published for EVE in shrimp in 2006 [12-14]. Thus, these reports predated those for mammals around 2012 and afterwards.

Our previous work has shown that stable, grossly normal lines of persistently infected *Aedes albopictus* C6/36 mosquito cells can be produced in the laboratory by split-passage with the DNA virus Aedes albopictus densovirus (AalDNV) and with the RNA viruses Dengue virus serotype 2 (DENV-2) and Japanese encephalitis virus (JEV). These can be single, dual or triple infections [15, 16]. However, the underlying mechanisms leading to these persistent infections and their maintenance in C6/36 cells have not been fully revealed.

Based on the progress described for insects and crustaceans above, we hypothesized that establishment and maintenance of stable, persistent DENV-2 infections in the *Aedes albopictus* cell line would be dependent on host generated RT not only to establish, but also to maintain tolerance to DENV-2 in persistent infections. To test this hypothesis, we used the RT inhibitor tenofovir disproxil fumarate (TDF) to confirm that it would not only prevent the establishment of a stable *Aal*-DENV-2 infected cell line but would also destabilize a previously established, stable *Aal*-DENV-2 infected cell line. TDF is in a class of medication called a nucleoside reverse transcriptase inhibitor (NRTI). It functions as a defective adenosine nucleotide that interferences with RT activity. The experiments described herein were possible with DENV-2 because it causes transient cytopathic effects in naive C6/36 cells that dissipate within 2 to 3 split passages after which the cells resume normal growth and morphology for unlimited passages despite 100% persistent infection with DENV-2 [16]. Such experiments are not possible with AalDNV, because challenge and establishment of persistent infections does not cause any visible effects with C6/36 cells.

MATERIALS AND METHODS

Insect cell lines and viral inoculum

Aedes albopictus C6/36 cells (ATCC, catalogue number CRL-1660) were grown in Leibovitz's (L-15) medium containing 10% heat-inactivated fetal bovine serum (FBS), 10% tryptose phosphate broth (TPB) and 1.2% antibiotic (Penicillin G and Streptomycin). DENV-2 (NGC strain) used in this work was obtained from the US Armed Forces Research Institute

of Medical Sciences (AFRIMS), Bangkok through the courtesy of Ananda Nisalax and was stored in 20% fetal bovine serum at -80ºC at the Division of Medical Molecular Biology, Office of Research and Devleopment, Faculty of Medicine, Siriraj Hospital, Mahidol, University, Bangkok. After thawing at room temperature, the stock was used as inoculum for monolayers of naive C6/36 cells in Leibovitz's (L-15) medium containing 1% heat-inactivated fetal bovine serum (FBS), 10% tryptose phosphate broth (TPB) and 1.2% antibiotic (Penicillin G and Streptomycin). At days 5-7 after challenge, the supernatant solution was removed and used as inoculum for subsequent trials.

Overall design of the experiments

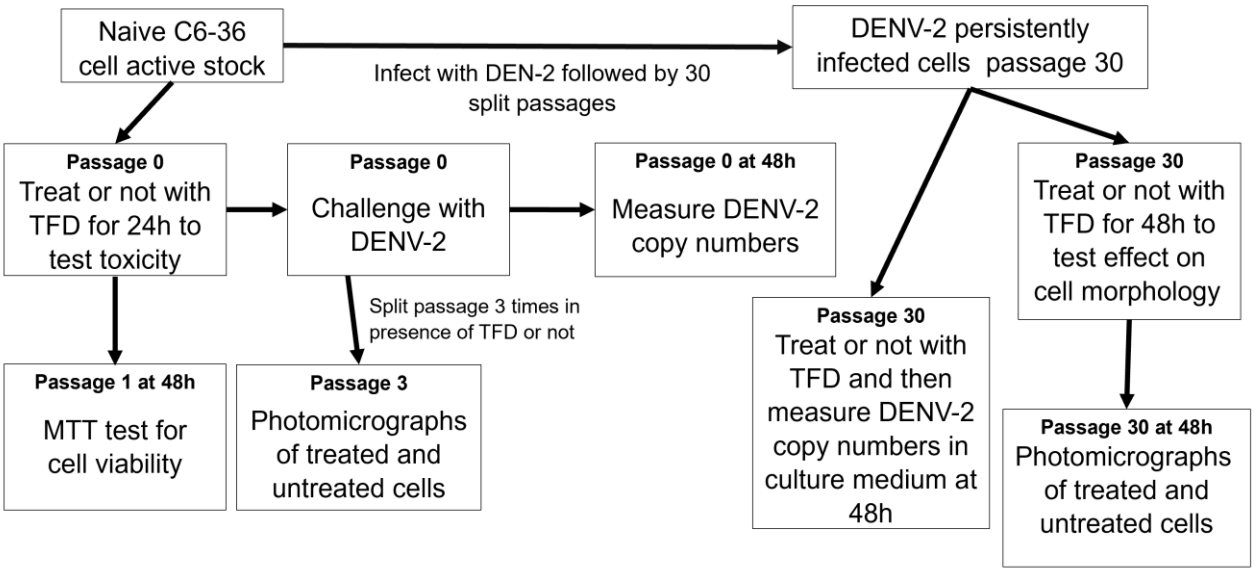

Figure 1. A flow diagram of the experiments carried out in this study.

Acute and persistent infections of C6/36 cells with Dengue-2 virus

Persistent infections of DENV-2 in C6/36 cells were achieved in 6-well plates (Costar, Corning) as previously described [16]. Briefly, culture plates were seeded with naive C6/36 cells at a density of 10^6 cells/well and incubated for 24 h at 28ºC to produce confluent monolayers. This could be called passage zero (P0). The cell monolayers were then challenged with DENV-2 at a multiplicity of infection (MOI) of 0.1. After incubation for 2 hours with gentle shaking at room temperature, the medium was removed and fresh medium containing 2% FBS was added for further incubation at 28°C for 2 days. Then, the supernatant solution was removed, and cells were suspended by knocking in L-15 containing

10% FBS at 1:3 dilution and transferred to a new culture well at ½ density for 2-days cultivation (to full confluence). This transfer constituted the 1st passage. For subsequent passages, the decantation, suspension, dilution and transfer processes were carried out sequentially at 2-day intervals to establish persistently infected cultures. As previously reported [16], minor cytopathic effects (CPE) occurred for the first three passages (the acute infection stage) but were absent thereafter (the extended persistent infection stage). Three replicates were done in 6-well plates. Mock-infected cells were run in parallel to the viral infected cells to serve as negative controls.

MTT assay for TFD toxicity to C6/36 cells

Tenofovir [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] hereafter called TDF is as a nucleoside reverse transcriptase inhibitor in purified chemical powder form that was obtained from the Thailand Government Pharmaceutical Organization. Monolayers of C6/36 cells cultured for 24 hours were treated with TDF at concentrations of 0.1, 0.25 and 0.5 mM in culture media for 24 hours. The concentration used was calculated from the manufacturers recommend dose for human use. After 24h treatment with TDF, cells layers were examined and photographed with an inverted Olympus IX71 light microscope equipped with a digital camera that yielded photomicrographs with embedded scale bars. Then, culture media were removed and cell viability was tested using the MTT reduction assay [17]. Briefly, cell monolayers treated with different concentrations of TDF were incubated with 100 µl MTT solution (0.5 mg/ml) for 2 h at 28 °C protected from light. After incubation, the formazan salts formed were dissolved in dimethyl sulfoxide for 15 min followed by measuring intensity at 490 nm using a microplate reader (TECAN, Switzerland). Cultures of untreated cells were used as the negative control with their intensity considered as 100% relative viability.

Naive cell inhibition of reverse transcriptase (RT) upon DENV-2 challenge

Monolayers of C6/36 cells at Passage 0 were treated with TDF at different concentrations as described above for 24 hours. The treated monolayers were then challenged with DENV-2 at a multiplicity of infection (MOI) of 0.1. After incubation for 2 hours with gentle shaking at room temperature, the medium was removed, cells were washed 2 times with PBS and fresh medium containing 10% FBS was added for further incubation at 28°C for 48h (2 days)

(acute infections in C6/36 cells). Then the supernatant solution was removed for DENV-2 copy number measurement, and cells were re-suspended and serially passaged as described above but with TDF in the medium at concentrations of 0.1, 0.25 and 0.5 mM. The negative control was untreated, unchallenged C6/36 cells and the positive control was untreated C6/36 cells challenged with DENV-2. There were 3 replicates for each group.

Inhibition of reverse transcriptase in cultures persistently infected with DENV-2

The persistently DENV-2 infected cultures were established by serial passage as described above. Monolayers of C6/36 cells persistently infected for 30 passages were treated with various concentrations of TDF for 48 hours. Then morphology of the whole culture was observed under the inverted light microscope (Olympus IX71) and DENV-2 copy number in the culture medium was measured in by real-time RT PCR.

One step real-time RT-PCR for DENV-2 quantification

Culture media or cells were collected for RNA extraction using an RNA extraction kit (Qiagen). DENV-2 copy number was quantified by one step real time RT-PCR as previously described (16). Briefly Primers D2S (5'- GTT CGT CTG CAA ACA CTC CA-3') and D2C (5'- GTG TTA TTT TGA TTT CCT TG-3') were used to obtain an expected amplicon of 230 bp and a standard curve of DENV-2 copy number was prepared by serial dilution of a plasmid containing the dengue virus envelope gene target. Each treatment was compared by gene copy number. One step real time RT-PCR was performed using 100 ng of total RNA and KAPA SYBR FAST One-Step qRT-PCR Kits (Kapa Biosystems) according to the directions provided by the manufacturer. Copy numbers were quantified per 100 ng total RNA template.

Statistical analyses

Statistical analyses were performed using Graphpad prism v.9 (GraphPad Software, San Diego, California USA, www.graphpad.com). The quantity of viable cells and DENV-2 copy numbers were compared using one-way analysis of variance (one-way ANOVA) or paired t-tests, as appropriate. Differences with p-value ≤ 0.05 with 95% confidence were considered statistically significant.

RESULTS AND DISCUSSION

TDF had relatively low toxicity for C6/36 cells at 0.1 mM

Reverse transcriptase activity from endogenous retrotransposons is known in naïve C6/36 cells and is upregulated by RNA virus infection [4]. After 24 hours of normal, naïve C6/36 cell treatment with TDF at the concentrations of 0.1, 0.25 and 0.5 mM, the number of viability of the cells decreased significantly (**Fig. 2**). The effect of TDF was not severe at the lowest concentration tested (0.1 mM) that caused approximately 25% loss of relative viability by the MTT assay, and even treatment with 0. 5 mM resulted in slightly less than 50% decrease in viability. This dose-related decrease in viability was associated with increasing numbers of small, dense, dying or dead cells with increasing concentrations of TDF (**Figs. 3B to 3D**). Thus, some cell death was significant for all treatments indicating that TDF itself had a negative effect on cell viability and a concentration of 0.1mM was selected as the concentration of TDF to use in tests on the effect of RT inhibition on cellular response to DENV-2. C6/36 cells do produce telomerase (a type of reverse transcriptase), and telomerase inhibition has been related to induced mortality via apoptosis in cultured cancer cells [18]. Thus, we may speculate that death (occurrence small, dense, dead cells) was caused by TDF inhibition of host telomerase leading to apoptosis in naive C6/36 cells.

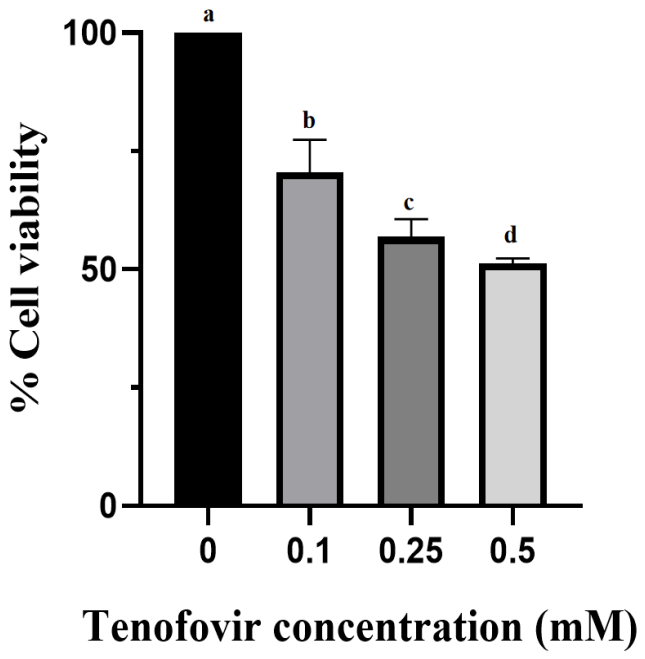

Figure 2. Effect of TDF on cell viability in C6/36 cells. Cells were incubated with different concentrations of tenofovir for 24 h followed by cell viability measurement. Baseline cell viability in the control wells not exposed to tenofovir was set at 100%. Data are expressed as percentage of the control. Bars with different letters were significantly different ($p<0.05$) from one another by one-way ANOVA.

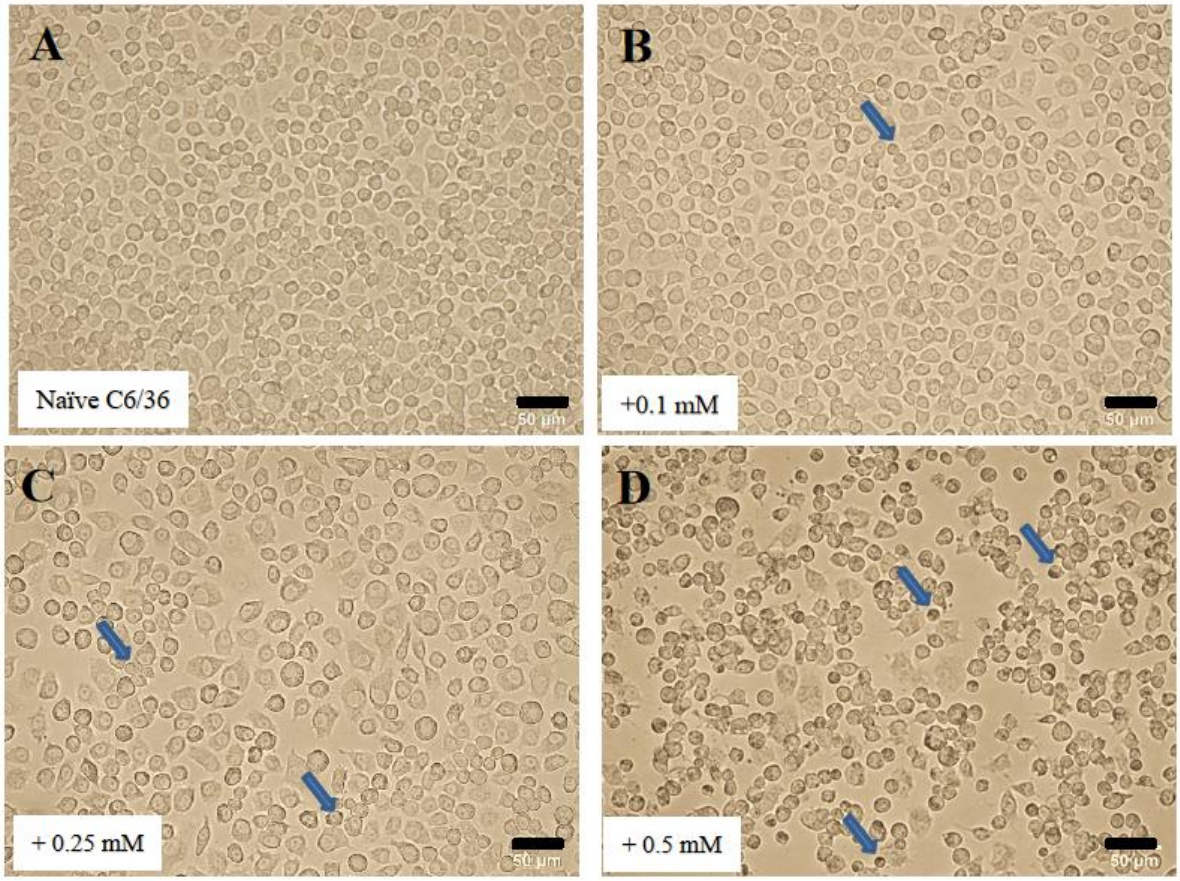

Figure 3. C6/36 cell morphology upon treatment of naive C6/36 cell cultures treated or not with TDF for 24 h. **A.** Untreated naive cells. **B.** Cells treated with 0.1 mM and showing morphology similar to untreated cells except for the presence of a few smaller and dense, dead cells (blue arrows). **C.** Cells treated with 0.25 mM and showing a mixture of normal cells like those in A and B in lower numbers but with more small dead cells (blue arrows) than in A. **D.** Cells treated with 0.5 mM and showing the largest number of small dead cells. These photomicrographs correspond to the percentage of cell viability shown in Fig. 2.

RT inhibition by TDF prevents DENV-2 persistent infections

Based on earlier work that RT inhibition by azidothymidine (AZT) prevents persistent infections with DENV-1 in C6/36 cells [4], we needed to test whether TDF (a different inhibitor) would have the same effect on DENV-2 (a different virus). When monolayers of naive C6/36 cells were treated with TDF for 24 hours followed by DENV-2 challenge, cytopathic effects (CPE) were more severe, and viable cell numbers were lower in all the TDF treatment groups than in the untreated control. By passage 3, the number cells had rapidly declined in the TDF treatment groups and the severity of CPE increased with increasing concentration of TDF (**Figs. 4B to 4D**). The untreated control cells showed minor cytopathic effects that corresponded to those previously reported and would be absent from the 4th passage onward [16]. Since all cells eventually died with DENV-2 challenge in the presence of TDF, persistently infected cultures with normal growth and morphology could not be obtained. These CPE-related phenomena were not described for the DENV-1 experiments with C6/36 cells previously reported [5].

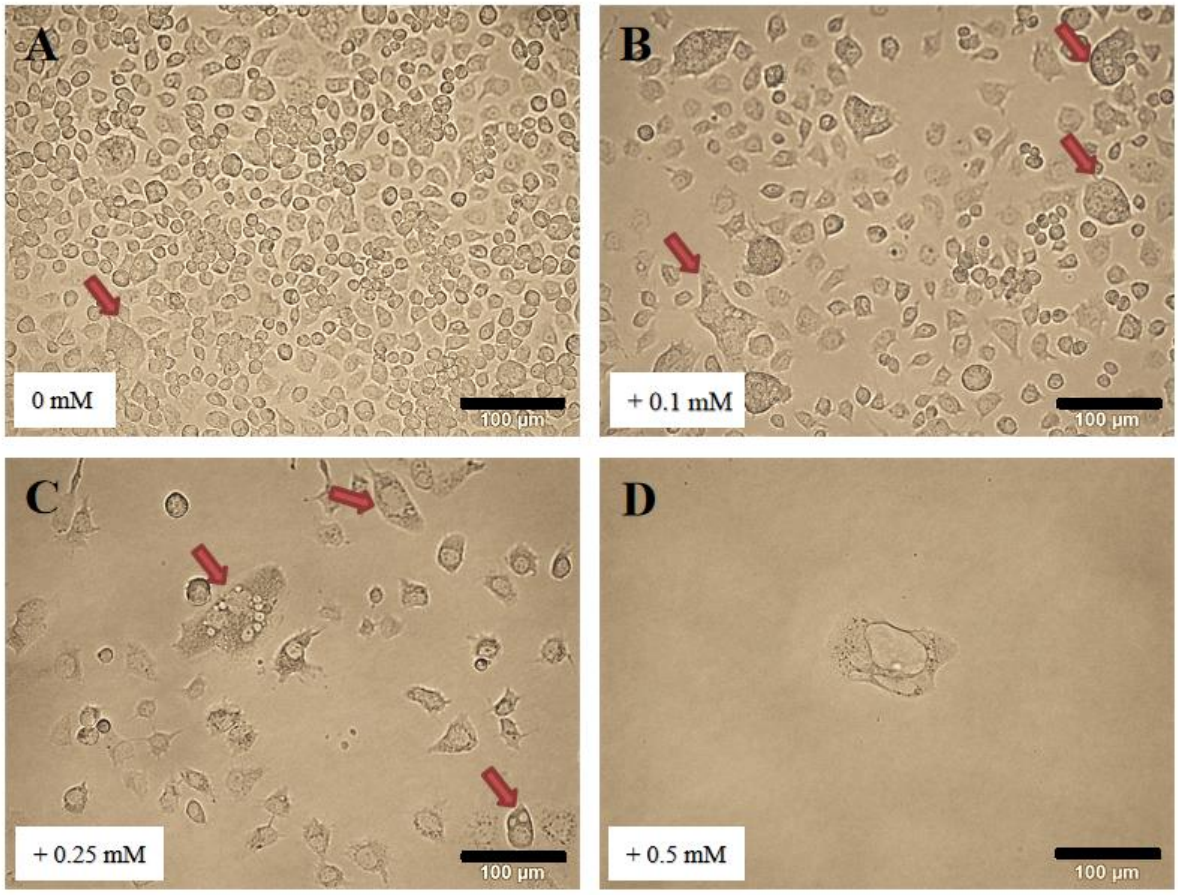

Figure 4. Morphology of naive C6/36 cells at Passage 3 in media containing TDF or not and challenged with DENV-2. **A.** DENV-2-challenged cells not exposed to TDF showing some minor CPE (red arrows) remaining at Passage 3, i.e., the cells are in the process of accommodating DENV-2 as previously reported [16]. **B.** Treatment with 0.1 mM TDF showing a decrease in cell numbers and an increase in CPE (red arrows) rather than a decrease in CPE as in (A) that would have occurred in the absence of TDF. **C.** Treatment with 0.25 mM TDF showing even fewer cells and more severe CPE. **D.** Treatment with 0.5 mM TDF showing very severe CPE with few cells remaining.

TDF increased DENV-2 production from naïve cells in a dose dependent manner

Measurement of DENV-2 copy numbers in the supernatant of C6/36 cell cultures at 48h after challenge with DENV-2 in the presence or absence of TDF revealed a significant increase in DENV-2 copy numbers dependent on the concentration of TDF (**Fig. 5**). The highest mean count was 9.60 x10^6 copies/µl with 0.5 mM TDF treatment while those in the untreated group and the 0.1 m TDF treatment were about 3.05x10^6 copies/µl at 48 hours (Passage 0) after DENV-2 challenge. The increase in DENV-2 replication was positively related to the concentration of TDF. The results suggested that reduced cellular RT activity was associated with increased viral replication, as previously reported for DENV-1 in C6/36 cells [5]. In contrast, in that report [5], the RNA virus Chikungunya did not respond to RT inhibition by increased viral replication. Thus, results may vary depending on the time of exposure before challenge and/or the concentration of RT inhibitor drug used in a specific host-viral strain interaction.

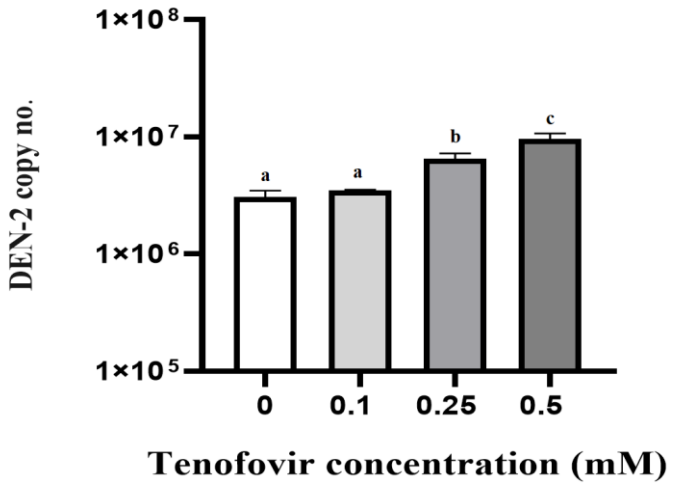

Figure 5. DENV-2 copy number in culture supernatant of naive C6/36 cells at 48 h in acute infection stage treated or not with TDF at different concentrations. Bars with different letters were significantly different ($p \leq 0.05$) from one another by one-way ANOVA.

TDF treatment destabilized persistently DENV-2-infected C6/36 cells

Our C6/36 cells persistently infected with DENV-2 by split-passaging 30 times and not treated with TDF (**Fig. 6A**) showed morphology similar to that of untreated, naive C6/36 cells as shown above in Fig. 3A. By 48 hours after exposure to TDF, all cultures exposed to TDF showed pathology with severity dependent on the TDF concentration. Those treated with 0.1 mM TDF showed CPE comparable to that which occurs when naive C6/36 cells are challenged with DENV-2 (see Fig. 4A above). It is as if addition of TDF at 0.1 mM (**Fig. 6B**) returned the persistently infected cells to a state resembling that of naive C6/36 exposed to DENV-2. At 0.25 mM TDF, the CPE severity (**Fig. 6C**) resembled that of DENV-2-challenged naive cells exposed to TDF at 0.1 mM (Fig. 4B), showing mostly cells with severe CPE together with scattered apoptotic cells. However, with the 0.5 mM treatment, the vast majority of the DENV-2 persistently infected cells became small and dense. The results suggested that reverse transcriptase was an essential element in the maintenance of persistent DENV-2 infections. Moreover, DENV-2 replication increased in the presence of TDF, except for the 0.5 mM treatment.

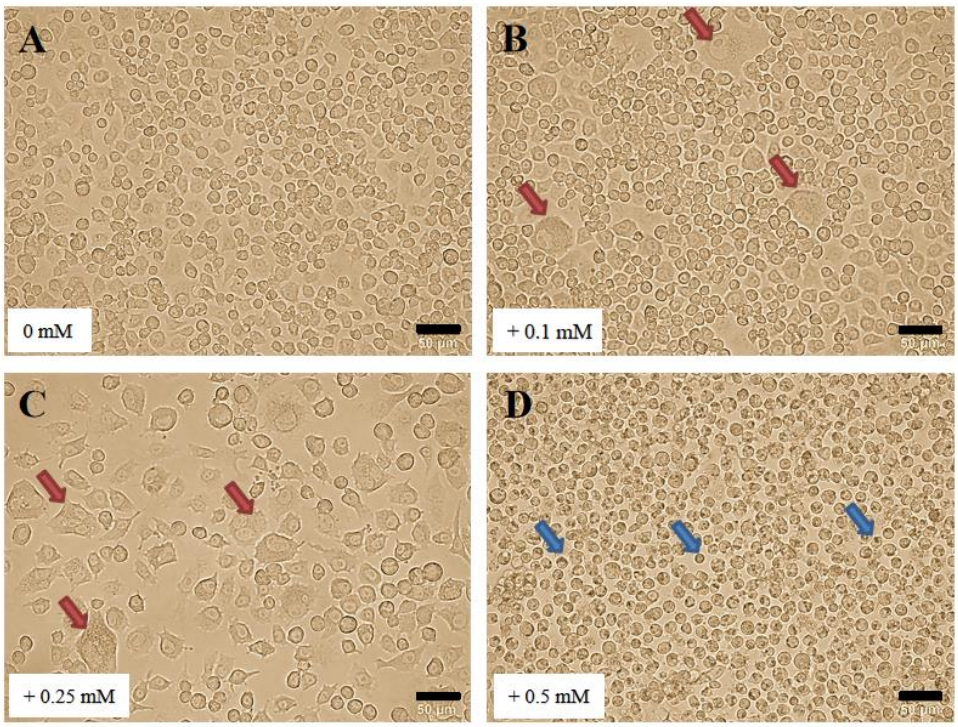

Figure 6. Cell morphology at 48 hr after C6/36 cultures persistently infected with DENV-2 for 30 passages were treated or not with tenofovir. **A.** Untreated persistently infected cells showing normal morphology comparable to that of the naïve, untreated C6/36 cells shown in Fig. 3A. **B.** Treatment with 0.1 mM TDF showing minor CPE similar to that previously reported for naïve, untreated C6/36 for the first 3 passages after challenge with DENV-2 [16] and resembling the photomicrograph of such cells shown here in Fig. 4A, i.e., as if the DENV-2 response had reverted to that of naive cells. **C.** Persistently infected cells treated with 0.25 mM tenofovir and showing more severe CPE, similar to that in Fig. 4B showing the response of newly infected cells in the presence of 0.1 mM TDF. **D.** Persistently infected cells treated with 0.5 mM TDF and showing a dominance of small dead cells, similar to those seen in Fig. 3D for naive C6/36 cells treated with 0.5 mM TDF.

The DENV-2 copy number with 0.1 mM TDF treatment was three times higher (mean 7.64 $x10^6$ copies) than that in the untreated persistent infections (3.04 $x10^6$ copy) at the same passage (**Fig 7**). The formation of syncytia and the higher DENV-2 copy numbers with 0.1 mM TDF treatment revealed that inhibiting RT activity destabilized C6/36 cells tolerant to DENV-2 with severity dependent on concentration of the RT inhibitor.

If it turns out that the requirement for RT activity is a general phenomenon upon which host insects and crustaceans depend to develop and maintain tolerance to viruses in persistent infections, it may have practical applications. For example, shrimp and insects often tolerate one or more lethal viruses that go unnoticed because they cause no signs of disease. However, those animals can transmit the viruses to naive individuals that may become infected and sometimes even die [19]. This is very important for translocation of commercial insects (e.g., bees and silkworms) and crustaceans (e.g., shrimp) where simple quarantine cannot be used to reveal viral pathogens. In addition, the tolerated viruses are sometimes present in very low numbers and may escape detection if very sensitive methods are not used. Thus, it is possible that treatment with an RT inhibitor during quarantine would result in disease expression or facilitate the detection of both known and unknown viral pathogens. This might be especially useful in the development of specific pathogen free (SPF) stocks derived from wild animals since signs of disease following TDF treatment would indicate the presence of even unknown viral pathogens.

Variable effect of TDF on DENV-2 replication in persistently infected C6/36 cells

C6/36 cells persistently infected with DENV-2 were challenged with various concentrations of TDF for 48 h before measurement of DENV-2 copy numbers in the culture supernatant. With TDF at 0.1 mM there was a significant increase ($p \leq 0.05$) in DENV-2 copies (7.64 x 10^6) in the culture supernatant when compared to the untreated, persistently infected C6/36 cells (3.0 x 10^6 copies/µl) (**Fig. 7**). However, with TDF at 0.25 mM, there was a significant decrease to 4.34 x 10^6 copies/µl compared to the 0.1 mM treatment, although it was still significantly higher than that of the untreated control ($p \leq 0.05$). In contrast, treatment with 0.5 mM resulted in a lower quantity of DENV-2 than did the untreated naive cells (4.34 x 10^5 copies/µl). These results differed from the response of naive C6/36 cells challenged with DENV-2 where copy numbers increased in proportion to the TDF concentration (**Fig. 5**). Those increasing copy numbers were associated with increasing CPE and cell lysis **(Fig 4**). In contrast, the response of the persistently infected cells was associated with less severe CPE than the naive cells but more severe mortality (**Fig. 6**). We speculate that the higher occurrence of death in the persistently infected cells resulted in the reduction of DENV-2 copy numbers at the TDF concentrations of 0.25 and 0.5 mM when compared to 0.1 mM.

TDF significantly increased DENV-2 numbers to 9.60 x10^6 copies/µl upon 0.5 mM treatment of DENV-2-challenged naive cells and to 7.64 x 10^6 with 0.1 mM treatment of persistently infected cells. Thus, low levels of TDF treatment might be a useful technique for increasing virus quantities when large amounts are needed for preparation of viral stocks or for nucleic acid extraction.

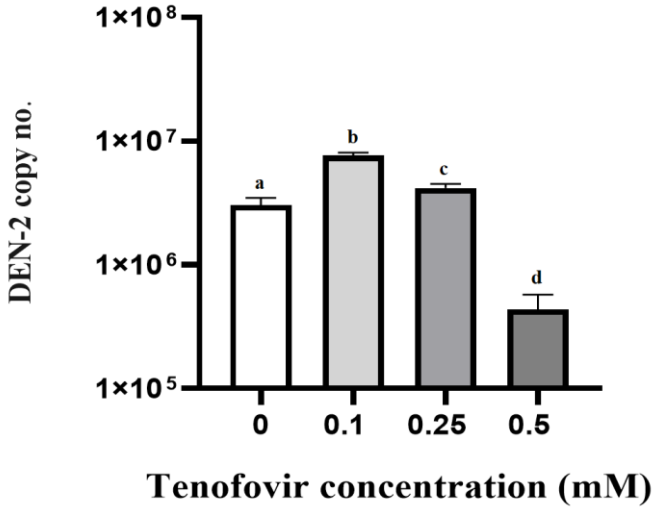

Figure 7. DENV-2 copy numbers in the culture supernatant of C6/36 cultures persistently infected with DENV-2 for 30 passages at 48h after being treated or not with TDF at different concentrations. Bars with different letters were significantly different ($p \leq 0.05$) from one another by one way ANOVA.

CONCLUSIONS

We have shown that the development and maintenance of stable cultures of C6/36 cell lines persistently infected with DENV-2 is correlated with host derived RT activity as previously reported for DENV-1 [5]. We have also shown that RT activity is required to maintain DENV-2 persistent infections. Thus, we speculate that treating insects and crustaceans with an RT inhibitor may expose known or unknown, accommodated viruses in screening programs. It may also increase viral yields for research purposes. Recent studies have shown that host RT gives rise to viral copy DNA (vcDNA) from both RNA and DNA viruses in both linear and circular forms. The vDNA, in turn, gives rise to small interfering RNA (siRNA) transcripts that lead to an RNAi response against both RNA viruses [2] and DNA viruses [20, 21]. In addition, the vcDNA also gives rise to host-cell genomic EVE, some of which produce negative-sense RNA that can also give rise to an RNAi response via PIWI proteins [22, 23]. Although C6/36 cells lack the gene Dicer-2 that is usually involved in the RNAi response, their ability to accommodate up to 3 viruses is unhindered [15]. Thus, they constitute a good model for further studies on the detailed mechanisms underlying this capability.

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REFERENCES

1. Flegel, T., *Research progress on viral accommodation 2009 to 2019.* Developmental & Comparative Immunology, 2020. **112**: p. 103771.

2. Bonning, B.C. and M.-C. Saleh, *The interplay between viruses and RNAi pathways in insects.* Annual Review of Entomology, 2021. **66**(1).
3. Flegel, T. and T. Pasharawipas, *Active viral accommodation: a new concept for crustacean response to viral pathogens.* Advances in shrimp biotechnology, 1998: p. 245-250.
4. Goic, B., et al., *RNA-mediated interference and reverse transcription control the persistence of RNA viruses in the insect model Drosophila.* Nature immunology, 2013. **14**(4): p. 396-403.
5. Goic, B., et al., *Virus-derived DNA drives mosquito vector tolerance to arboviral infection.* Nature communications, 2016. **7**(1): p. 12410.
6. Tassetto, M., M. Kunitomi, and R. Andino, *Circulating immune cells mediate a systemic RNAi-based adaptive antiviral response in Drosophila.* Cell, 2017. **169**(2): p. 314-325. e13.
7. Feschotte, C. and C. Gilbert, *Endogenous viruses: insights into viral evolution and impact on host biology.* Nature Reviews Genetics, 2012. **13**(4): p. 283-296.
8. Lin, C.-L., et al., *Persistent Hz-1 virus infection in insect cells: evidence for insertion of viral DNA into host chromosomes and viral infection in a latent status.* Journal of virology, 1999. **73**(1): p. 128-139.
9. Crochu, S., et al., *Sequences of flavivirus-related RNA viruses persist in DNA form integrated in the genome of Aedes spp. mosquitoes.* Journal of General Virology, 2004. **85**(7): p. 1971-1980.
10. Maori, E., et al., *Isolation and characterization of Israeli acute paralysis virus, a dicistrovirus affecting honeybees in Israel: evidence for diversity due to intra-and inter-species recombination.* Journal of General Virology, 2007. **88**(12): p. 3428-3438.
11. Maori, E., E. Tanne, and I. Sela, *Reciprocal sequence exchange between non-retro viruses and hosts leading to the appearance of new host phenotypes.* Virology, 2007. **362**(2): p. 342-349.
12. Tang, K.F. and D.V. Lightner, *Infectious hypodermal and hematopoietic necrosis virus (IHHNV)-related sequences in the genome of the black tiger prawn Penaeus monodon from Africa and Australia.* Virus research, 2006. **118**(1-2): p. 185-191.
13. Flegel, T.W., *Hypothesis for heritable, anti-viral immunity in crustaceans and insects.* Biology Direct, 2009. **4**: p. 1-8.
14. Saksmerprome, V., et al., *Additional random, single to multiple genome fragments of Penaeus stylirostris densovirus in the giant tiger shrimp genome have implications for viral disease diagnosis.* Virus research, 2011. **160**(1-2): p. 180-190.
15. Kanthong, N., et al., *Persistent, triple-virus co-infections in mosquito cells.* BMC microbiology, 2010. **10**(1): p. 1-8.
16. Kanthong, N., et al., *Mosquito cells accommodate balanced, persistent co-infections with a densovirus and Dengue virus.* Developmental & Comparative Immunology, 2008. **32**(9): p. 1063-1075.
17. Wu, X., et al., *Inhibitory effect of small interfering RNA on dengue virus replication in mosquito cells.* Virology journal, 2010. **7**: p. 1-8.
18. Mondello, C. and A.I. Scovassi, *Telomeres, telomerase, and apoptosis.* Biochemistry and cell biology, 2004. **82**(4): p. 498-507.
19. Flegel, T.W., *The special danger of viral pathogens in shrimp translocated for aquaculture.* Science Asia, 2006. **32**(3): p. 215-221.

20. Zhu, M., et al., *BmCPV-Derived Circular DNA vcDNA-S7 Mediated by Bombyx mori Reverse Transcriptase (RT) Regulates BmCPV Infection.* Frontiers in Immunology, 2022. **13**.
21. Taengchaiyaphum, S., et al., *Shrimp parvovirus circular DNA fragments Arise from both endogenous viral elements and the infecting virus.* Frontiers in Immunology, 2021. **12**: p. 729528.
22. Whitfield, Z.J., et al., *The diversity, structure, and function of heritable adaptive immunity sequences in the Aedes aegypti genome.* Current Biology, 2017. **27**(22): p. 3511-3519. e7.
23. Tassetto, M., et al., *Control of RNA viruses in mosquito cells through the acquisition of vDNA and endogenous viral elements.* Elife, 2019. **8**: p. e41244.