A role for autophagolysosomes in dengue virus 3 production in HepG2 cells

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We have recently proposed that amphisomes act as a site for translation and replication of dengue virus (DENV)-2 and that DENV-2 entry and replication are linked through an ongoing association with membranes of an endosomal–autophagosomal lineage. In this report, we present the results of an investigation into the interaction between DENV-3 and the autophagy machinery. Critically, treatment with the lysosomal fusion inhibitor L-asparagine differentiated the interaction of DENV-3 from that of DENV-2. Inhibition of fusion of autophagosomes and amphisomes with lysosomes resulted in decreased DENV-3 production, implying a role for the autophagolysosome in the DENV-3 life cycle. Evidence based upon the co-localization of LC3 and cathepsin D with double stranded RNA and NS1 protein, as assessed by confocal microscopy, support a model in which DENV-3 interacts with both amphisomes and autophagolysosomes. These results demonstrate that the interactions between DENV and the host cell autophagy machinery are complex and may be determined in part by virus-encoded factors.

INTRODUCTION

The mosquito-borne dengue virus (DENV) is believed to cause 100 million new infections each year when female Aedes mosquitoes carrying the virus feed on immunologically susceptible humans (Guzman & Kouri, 2002). While the majority of these infections may either be asymptomatic or result in a relatively mild febrile disease, significant consequences can develop with the disease progressing to the life threatening dengue haemorrhagic fever or dengue shock syndrome (Halstead, 1988; Malavige et al., 2004). DENV comprises four antigenically related but distinct viruses termed DENV-1 to DENV-4, with each virus comprising many distinct genotypes (Holmes & Twiddy, 2003). Despite a large number of studies, no direct link between a specific DENV genotype and the cause of the disease has yet been published, possibly as a consequence of an imperfect understanding of the interaction between specific DENV genotypes and susceptible human cells.

It has been shown that DENV enters both insect and mammalian cells through endocytosis into clathrin-coated pits (Acosta et al., 2008; Krishnan et al., 2007) and that subsequent uncoating of the virus occurs in the endosome (Krishnan et al., 2007; van der Schaar et al., 2007) in response to the low pH environment (Heinz et al., 2004; Modis et al., 2004; Mukhopadhyay et al., 2005); however, the details of how and where DENV is translated and replicated remain poorly understood (Clyde et al., 2006). Replication in both insect and mammalian cells is accompanied by the expansion of intracellular membranes (Clyde et al., 2006; Ko et al., 1979; Miller & Krijnse-Locker, 2008; Salonen et al., 2008) but a causal link between membrane amplification and virus translation and replication remains to be elucidated.

Several viruses have been shown to induce autophagy (Espert et al., 2007; Lee & Iwasaki, 2008) which characteristically induces the formation of double- and single-membrane vesicles (Xie & Klionsky, 2007). Autophagy is an evolutionarily conserved lysosomal degradation pathway whose primary function is the catabolic salvage of cellular constituents from old organelles or aggregated proteins (Levine & Klionsky, 2004; Meijer & Codogno, 2006). Studies have shown that autophagy can be induced in response to a number of stimuli, such as nutrient depletion or starvation and bacterial and viral infection, as well as during ageing and in a number of disease conditions, including neurodegenerative diseases and cancer (Lerena et al., 2008; Levine & Klionsky, 2004; Meijer & Codogno, 2006; Mizushima et al., 2008). Induction of autophagy initially activates the formation of double-membraned autophagic vacuoles called autophagosomes (Dunn, 1990a) and it is believed that the membranes involved in autophagosome formation are sequestered from the endoplasmic reticulum (Dunn, 1990a) or trans-Golgi network (Kihara et al., 2001). During maturation, autophagosomes can fuse with endosomes to form amphisomes (Gordon & Seglen, 1988) and both autophagosomes and amphisomes fuse with lysosomes in the final maturation step to form autophagolysosomes.
(Dunn, 1990b), which represent the primary degradative vesicle (Xie & Klionsky, 2007). A schematic representation of autophagic vesicles is given in Fig. 1. Both autophagosomes and amphisomes are double-membrane vesicles, while autophagolysosomes are single-membrane vesicles.

While the process of autophagy is regulated by a large number of genes which were first identified in yeast (Xie & Klionsky, 2007), two conjugation pathways [the covalent linkage of Atg5 and Atg12 (Mizushima et al., 1998, 2002; Ohsumi, 2001) and the covalent lipidation of Atg8 by phosphatidylethanolamine (Kabeya et al., 2000; Ohsumi, 2001)] play a critical role in the formation of autophagic vesicles. Atg8 is also known as microtubule-associated protein 1 light chain 3 (LC3), and the formation and association of lipidated LC3 (the LC3-II form) with autophagic membranes (Kabeya et al., 2000) is the only validated marker of autophagosome formation (Bampton et al., 2005; Kimura et al., 2007). The co-localization of LAMP1 (a marker of endosomal and lysosomal membranes) with LC3 is frequently used to identify autophagosomal vacuoles formed prior to lysosomal fusion (autophagosomes and amphisomes), as the subsequent fusion of autophagosomes with lysosomes results in the degradation of LC3 (Eskelinen, 2005; Mizushima & Yoshimori, 2007).

Recently, DENV-2 has been shown to induce autophagy (Lee et al., 2008); modulation of autophagy with biochemical inhibitors such as 3MA, which inhibits the formation of autophagosomes (Seglen & Gordon, 1982), and activators of autophagy such as rapamycin, which induces autophagy by inhibiting the negative regulator of the autophagy pathway, the mammalian target of rapamycin (mTOR) (Noda & Ohsumi, 1998), result in modulation of DENV-2 virus yield (Lee et al., 2008; Panyasrivanit et al., 2009). Our previous study (Panyasrivanit et al., 2009) located at least part of the DENV-2 translation/replication complex autophagosomal membranes, and infection in the presence of L-asparagine (l-Asn), which inhibits fusion of lysosomes with autophagosomes (Seglen et al., 1996), resulted in an increase in virus output, indicating that amphisomes were the critical autophagic structure. This result was confirmed by co-localization experiments with mannose 6-phosphate receptor (MPR), an endosomal/amphisomal marker (Taylor et al., 2005), and we have proposed that virus entry and translation–replication can be explained in terms of a continual association with endosomal–autophagosomal membranes (Panyasrivanit et al., 2009; see also Fig. 1). However, to date, neither the study by Lee et al. (2008) nor our previous study (Panyasrivanit et al., 2009) has investigated a DENV other than DENV-2. This study sought to address this issue and determined that significant differences exist in the way that DENV-3 interacts with the autophagy apparatus compared with DENV-2. This finding will have an impact on studies attempting to find intracellular targets aimed at disrupting DENV translation and replication.

**METHODS**

**Cells, viruses and infection.** The human hepatoma cell line HepG2 (ATCC no. HB-8065) was cultivated at 37 °C as described previously (Panyasrivanit et al., 2009; Thepparit et al., 2004; Thepparit & Smith, 2004). DENV-3 (strain 16562) and DENV-2 (strain 16681) were propagated in the Aedes albopictus-derived cell line C6/36 (ATCC no. CRL-1660). Viruses were partially purified by centrifugation to remove cellular debris and stored frozen at −80 °C. Virus titre was determined by plaque assay on monolayers of the rhesus monkey kidney cell line LLC-MK2 as described previously (Sithisarn et al., 2003).

Cells were appropriately treated with 3-methyladenine (3-MA), L-Asn and rapamycin (all Sigma-Aldrich) as described previously.
(Panyasrivanit et al., 2009). Cells were infected with DENV-2 or DENV-3 in the presence or absence of appropriate inhibitor at 10 p.f.u. per cell for 2 h in Dulbecco’s modified Eagle’s medium and grown with or without biochemical treatment, as appropriate, for the times indicated.

**Indirect immunofluorescence.** Approximately 30,000 HepG2 cells were seeded onto glass coverslips and grown for 24 h under standard conditions. Coverslips were then directly infected for 2 h with DENV-3 or DENV-2 at 10 p.f.u. per cell or pretreated with autophagy modulators, as above, before infection. Cells were then grown for the times indicated in the presence or absence of an autophagy modulator as appropriate. Subsequently, cells were processed and incubated with two or three primary antibodies followed by incubation with two or three secondary antibodies, as described previously (Panyasrivanit et al., 2009).

Primary antibodies used were a rabbit polyclonal anti-MAP-LC3 antibody (sc-28266, Santa Cruz Biotechnology) or goat polyclonal anti-MAP-LC3 antibody (sc-16756, Santa Cruz Biotechnology), a rabbit polyclonal anti-LAMP1 antibody (ab24170, Abcam) a rabbit polyclonal anti-MPR antibody (ab32815, Abcam), a mouse monoclonal anti-dengue NS1 antibody (Puttikhunt et al., 2003), a mouse monoclonal anti-double-stranded (ds)RNA antibody (J2, English & Scientific Consulting), a goat polyclonal anti-ribosomal protein L28 (sc-14151, Santa Cruz Biotechnology) and a rabbit polyclonal anti-cathepsin D antibody (Ab-2) (IM 16, Calbiochem).

Secondary antibodies used were a Rhodamine Red X-conjugated goat anti-rabbit IgG (111-295-144, Jackson ImmunoResearch laboratories), Cy3-conjugated rabbit anti-goat IgG (81-1616; Invitrogen), an FITC-conjugated donkey anti-rabbit IgG (sc-2090, Santa Cruz Biotechnology), an FITC-conjugated goat anti-mouse IgG (02-18-06; KPL), an Alexa Fluor 594-conjugated chicken anti-mouse IgG (A21201, Molecular Probes) and an Alexa Fluor 647-conjugated donkey anti-rabbit IgG (A31573, Molecular Probes).

**Fluorescence confocal microscope imaging.** Fluorescent confocal microscope images were captured using the Olympus Fluoview 1000 (Olympus Corporation, Shinjuku-ku, Tokyo) equipped with Olympus Fluoview software v. 1.6 exactly as described previously (Panyasrivanit et al., 2009). Image analysis and calculation of Pearson correlation coefficients and confidence intervals (CIs) were carried out as described previously (Panyasrivanit et al., 2009).

**Western blot analysis.** Total protein of either mock-infected or DENV-2- or DENV-3-infected HepG2 cells in the presence or absence of autophagy modulators was extracted at appropriate time points. Proteins were separated by SDS-PAGE and transferred to solid membranes. Membranes were then incubated with 5% skimmed milk in Tris-buffered saline (TBS) for 2 h at room temperature. Membranes were incubated with a 1:50 dilution of a mouse monoclonal anti-dengue NS1 antibody, a 1:1000 dilution of a rabbit polyclonal anti-MAP-LC3B antibody (ab48394; Abcam) or a 1:800 dilution of a mouse monoclonal antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sc-32233, Santa Cruz Biotechnology) in 5% skimmed milk in TBS. Membranes were then incubated with a 1:4000 dilution of horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (A9044, Sigma) or a 1:3000 dilution of HRP-conjugated goat anti-rabbit IgG antibody (31460, Pierce) in 5% skimmed milk in TBS for 1 h at room temperature. Signals were detected using the ECL Plus Western blotting Analysis kit (GE Healthcare).

**Virus titration assays.** Extracellular and intracellular virus titres were analysed by standard plaque assay as described previously (Sithisarn et al., 2003; Thepparit & Smith, 2004), using either six or three (as indicated) independent replicates with duplicate assays for extracellular virus titre and three independent replicates with duplicate assays for intracellular virus titre.

**Statistical analysis.** Virus production data were analysed using the Graph Pad prism program (GraphPad Software). Statistical analysis of significance was undertaken by Paired sample test using spss (SPSS Inc.).

**RESULTS AND DISCUSSION**

While we have shown that the virus production kinetics of DENV-2 and DENV-3 in liver cells are essentially identical at an m.o.i. of 1 (Thepparit et al., 2004), previous experiments on DENV-2 and autophagy (Panyasrivanit et al., 2009) were undertaken at 10 p.f.u. per cell. To confirm that the different virus:cell ratio did not alter the kinetics of virus production, virus production profiles of DENV-2 and DENV-3 at 10 p.f.u. per cell were determined. This showed that while the absolute levels of virus produced were different, as noted previously for an m.o.i. of 1 (Thepparit et al., 2004), the time for de novo extracellular virus production did not differ between DENV-2 and DENV-3 (Fig. 2a).

To assess whether modulation of autophagy with biochemical inhibitors altered levels of extracellular DENV-3, HepG2 cells were infected with DENV-3 at 10 p.f.u. per cell in the presence or absence of 3-MA, L-Asn and rapamycin. Extracellular virus production at 24 h post-infection (p.i.) was assessed by standard plaque assay and the experiment was performed independently in parallel six times, with duplicate assays of virus titre. Infection in the presence of rapamycin, an autophagy inducer, did not alter DENV-3 output compared to the control (DENV-3 only) (Fig. 2b) but examination of earlier time points (16 and 20 h) showed significantly increased virus production in the presence of rapamycin compared with control infection (P<0.001 and P=0.016, respectively) (Fig. 2c). Infection in the presence of 3-MA showed a significant reduction in virus output compared with control infections at all time points examined (P=0.013, P=0.007 and P<0.001 for 16, 20 and 24 h p.i., respectively) (Fig. 2b, c).

Infection in the presence of L-Asn resulted in a significantly decreased extracellular virus production when assessed at 24 h (Fig. 2b; P<0.001) and 16 h (Fig. 2c; P=0.01) p.i., and a reduced virus output when assessed at 20 h p.i. (Fig. 2c). Similar, but not statistically significant, results were observed at infection ratios of 0.1 and 1 p.f.u. per cell (Fig. 2d).

The reduction in DENV-3 titre seen in the presence of L-Asn is in contrast with the increased production of DENV-2 when the infection is undertaken in the presence of L-Asn (Panyasrivanit et al., 2009). Similarly, intracellular virus yield was also reduced in the presence of L-Asn (P=0.024; Fig. 2e), suggesting that DENV-3 interacts with the autophagy machinery in a manner distinct from that of DENV-2.

We next established whether DENV-3 infection induces autophagy as shown with DENV-2 (Lee et al., 2008;
Fig. 2. Interaction of DENV-3 and autophagy. (a) DENV-2 and DENV-3 virus production at 10 p.f.u. per cell. (b, c) Extracellular DENV-3 production at 24 h (b) and at 16 and 20 h (c) p.i. at 10 p.f.u. per cell in the presence or absence of autophagy modulators. (d, e) Extracellular (d) and intracellular (e) DENV-3 production at 24 h p.i. at 0.1 and 1 p.f.u. per cell in the presence or absence of autophagy modulators. In (a)–(e), the mean virus titres (±SD) are shown. Extracellular virus data are derived from six (b) or three (a, c, d and e) independent replicates, with duplicate assays of each replicate; intracellular data are derived from three independent replicates assayed in duplicate. Statistical significance of virus output compared with the control is indicated by an asterisk (*P<0.001). (f, g) Western blot analysis of LC3 and GAPDH expression in control cells (Ctrl) either untreated or treated with 3-MA, rapamycin (+R) or L-Asn (+A). (h, i) Western blot analysis of LC3 and GAPDH expression in either mock-infected (M), control (Ctrl) or DENV-3-infected cells.
Panyasrivanit et al., 2009) by using Western blotting to detect the increased formation of the autophagy-associated form of LC3, LC3-II, in response to DENV-3 infection. To verify the action of 3-MA, l-Asn and rapamycin on autophagy in control cells, we confirmed the reduction of LC3-II in response to 3-MA (Fig. 2f), as well as the increase of LC3-II in response to both rapamycin and l-Asn (Fig. 2g). In l-Asn-treated cells, the normal degradation of LC3-II that occurs upon fusion of autophagosomes with lysosomes is prevented as fusion with lysosomes is inhibited, resulting in accumulation of LC3-II (Eskelinen, 2005; Mizushima & Yoshimori, 2007). In response to DENV-3 infection, a significant increase in LC3-II was observed for up to 3 days p.i. (Fig. 2h) and examination of earlier time points showed that increased LC3-II was detectable as early as 6 h p.i. (Fig. 2i).

To confirm the induction of autophagy by DENV-3 infection, confocal microscopy was used to detect increased co-localization of LC3 and LAMP1. In this analysis, cells

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**Fig. 3.** Induction of autophagy in response to DENV-3 infection. (a) HepG2 cells were grown on glass coverslips and either mock-infected or infected with DENV-3 either directly or in the presence of rapamycin or l-Asn. Cells were examined simultaneously for the localization of LC3 (far-red), LAMP1 (green) and separately for NS1 (red) (a) or simultaneously for LC3 (blue), LAMP1 (green) and NS1 (red) in infected cells only (b).
were additionally stained with NS1 simultaneously to ensure that co-localization of LC3 and LAMP1 was assessed in infected cells. This experiment was undertaken in parallel with cells infected with DENV-3 in the presence of rapamycin or L-Asn and mock-infected cells. Results parallel with cells infected with DENV-3 in the presence of rapamycin or L-Asn and mock-infected cells. This experiment was undertaken in order to ensure that co-localization of LC3 and LAMP1 was assessed in infected cells. A low level of co-localization was observed between LC3 and NS1 in DENV-3-infected cells, as was the co-localization between LC3 and LAMP1 seen in either DENV-3- or DENV-2-infected cells treated with mock-infected cells (Pearson correlation coefficient 0.15, 95% CI 0.08–0.24). The increase in co-localization of NS1 and LC3 in DENV-3-infected cells in the presence of L-Asn compared with control infection (Fig. 4c). The low level of co-localization between NS1 and LC3 in DENV-3-infected cells and the increase in co-localization seen when lysosomal fusion is inhibited with L-Asn suggests that, in contrast to DENV-2 where pre-lysosomal vacuoles (amphisomes or autophagosomes) are the site of translation/replication (Panyasrivanit et al., 2009), post-lysosomal fusion vacuoles (autophagolysosomes) may play a role in DENV-3 infection (Fig. 1).

We therefore investigated whether autophagolysosomes were associated with NS1 in DENV-3-infected cells using cathepsin D, a constituent of mature autophagolysosomes (Eskelinen et al., 2002). A similar level of co-localization between cathepsin D and NS1 was observed in DENV-3-infected cells as the level previously found between LC3 and NS1 (Fig. 4d; Pearson correlation coefficient 0.17, 95% CI 0.12–0.22). Importantly, co-localization between Cathepsin D and NS1 was significantly reduced in the presence of L-Asn (Fig. 4d; Pearson correlation coefficient 0.084, 95% CI 0.04–0.12; P<0.05). Therefore, both the increase in co-localization between LC3 and NS1 and the reduction of co-localization between cathepsin D and NS1 when DENV-3 infection occurs in the presence of L-Asn suggest that NS1 is divided between both pre- and post-lysosomal fusion autophagic vacuoles.

These results serve to differentiate between DENV-2 and DENV-3 infections. In DENV-2 infections, NS1 is predominantly associated with pre-lysosomal fusion vacuoles; treatment with L-Asn to inhibit lysosomal fusion and the formation of autophagolysosomes results in only a marginal and non-significant increase in co-localization between LC3 and NS1 (Panyasrivanit et al., 2009) and only a marginal increase in the levels of NS1 (Fig. 4c). In contrast, in DENV-3-infected cells, NS1 is associated with both pre- and post-lysosomal fusion vacuoles and treatment with L-Asn results in a significant increase in the levels of NS1 (Fig. 4b), a significant increase in co-localization between LC3 and NS1 (Fig. 4a) and a decrease in co-localization between cathepsin D and NS1 (Fig. 4d).

To investigate the location of the DENV-3 replication complex on autophagic pre-lysosomal fusion vacuoles, the localization of dsRNA was investigated in relation to LC3
and to LC3 and MPR. Limited co-localization was observed between dsRNA and LC3 (Pearson correlation coefficient 0.22, 95% CI 0.19–0.25) which significantly increased in the presence of L-Asn (Pearson correlation coefficient 0.48, 95% CI 0.44–0.52; \(P<0.001\)) (Fig. 5a); triple localization of LC3, dsRNA and MPR identified the pre-lysosomal fusion structures as amphisomes (Fig. 5b), which is also seen in DENV-2 (Panyasrivanit et al., 2009). As with DENV-2, ribosomal proteins were found to be co-localized with DENV-3 dsRNA (Fig. 5c), suggesting the capacity for translation exists at the replication complex on amphisomes.

To investigate the localization of the replication complex on post-lysosomal fusion vacuoles, the co-localization of cathepsin D and dsRNA was investigated at 24 h p.i. A degree of co-localization was observed between dsRNA and cathepsin D (Pearson correlation coefficient 0.28, 95% CI 0.23–0.33); this decreased significantly when infection occurred in the presence of L-Asn (Pearson correlation coefficient 0.06, 95% CI 0.01–0.11; \(P<0.001\)) (Fig. 6a), similar to the results seen for NS1. This again suggests that DENV-3 utilizes both pre- and post-lysosomal fusion vacuoles as translation/replication sites, in contrast with DENV-2, which has been proposed to use only pre-lysosomal fusion vacuoles. 

**Fig. 4.** NS1 in DENV-3-infected HepG2 cells. HepG2 cells grown on glass coverslips (a and d) or in culture (b and c) were either mock-infected or infected with DENV-3 (a, b and d) or DENV-2 (c) in the presence or absence of L-Asn. Cells were examined for co-localization of NS1 with LC3 (a) or NS1 with cathepsin D (d) or for the level of NS1 by Western blotting with GAPDH as a control (b and c). LC3 and cathepsin D are shown as red; NS1 is shown as green.
lysosomal vacuoles. To provide further evidence to support this hypothesis, we investigated the co-localization of DENV-2 dsRNA and cathepsin D (Fig. 6a) and found, as expected, that DENV-2 dsRNA shows a significantly lower level of co-localization with cathepsin D (Pearson correlation coefficient 0.19, 95% CI 0.18–0.20; P = 0.007) compared with DENV-3 dsRNA. To provide further evidence, both earlier and later time points in the DENV replication cycle were examined. At 15 h p.i., co-localization between cathepsin D and dsRNA was significantly higher in DENV-3-infected samples (Pearson correlation coefficient 0.227, 95% CI 0.19–0.27) than in DENV-2-infected samples (Pearson correlation coefficient 0.104, 95% CI 0.07–0.13; P < 0.001) (Fig. 6b). Similarly, at 36 h p.i., co-localization was higher in DENV-3-infected samples (Pearson correlation coefficient 0.229, 95% CI 0.17–0.29) than in DENV-2-infected samples (Pearson correlation coefficient 0.093, 95% CI 0.06–0.12; P = 0.003) (Fig. 6b).

Fig. 5. Location of dsRNA in DENV-infected cells. HepG2 cells grown on glass coverslips were infected with DENV-3 in the presence or absence of L-Asn and examined for either the co-localization of LC3 (red) and dsRNA (green) (a), MPR (green), LC3 (far-red, false-coloured as blue in this figure) and dsRNA (red) (b) or ribosomal protein L28 (red) and dsRNA (green) (c).
We have previously shown that DENV-2 uses prelysosomal fusion amphisomes as a site for translation and replication, and that infection in the presence of L-Asn results in an increase of intracellular and extracellular virus, suggesting that lysosomal fusion results in loss of virus viability (Panyasrivanit et al., 2009). As shown here, this is in contrast with DENV-3 which uses both amphisomes and autophagolysosomes as part of its replication strategy. Infection in the presence of L-Asn decreases both intracellular and extracellular virus yields, suggesting that interaction with the autophagolysosome is required for completion of the DENV-3 life cycle. However, while the interaction with the autophagolysosome is beneficial in terms of DENV-3 production, it is detrimental to the replication complexes, as demonstrated by the increase of NS1 in DENV-3 infections in the presence of L-Asn. Overall, our results are consistent with both DENV-2 and DENV-3 utilizing the endosomal–autophagosomal fusion pathway to gain entry into the cell and undertake translation and replication (Fig. 1). However, in detail, DENV-2 and DENV-3 employ significantly different translation/replication strategies.

Both this study and our previous study (Panyasrivanit et al., 2009) have investigated the interaction between DENV and autophagy in liver cells. While the role of liver cells in the pathogenesis of the disease has been somewhat controversial, a significant body of work exists that supports the
involvement of the liver in dengue infections (Seneviratne et al., 2006) and we have shown previously that human primary liver hepatocytes are able to be productively infected by DENV (Suksanpaisan et al., 2007). However, studies on other cell types are urgently required, particularly cells of a monocyte/macrophage lineage, as these cells are the primary mediators of dengue infection.

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