Antibodies against thrombin in dengue patients contain both anti-thrombotic and pro-fibrinolytic activities

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Summary

Dengue virus (DENV) infection may result in severe life-threatening Dengue haemorrhagic fever (DHF). The mechanisms causing haemorrhage in those with DHF are unclear. In this study, we demonstrated that antibodies against human thrombin were increased in the sera of Dengue patients but not in that of patients infected with other viruses. To further characterise the properties of these antibodies, affinity-purified anti-thrombin antibodies (ATAs) were collected from Dengue patient sera by thrombin and protein A/L affinity columns. Most of the ATAs belonged to the IgG class and recognized DENV nonstructural protein 1 (NS1). In addition, we found that dengue patient ATAs also cross-reacted with human plasminogen (Plg). Functional studies *in vitro* indicated that Dengue patient ATAs could inhibit thrombin activity and enhance Plg activation. Taken together, these results suggest that DENV NS1-induced thrombin and Plg cross-reactive antibodies may contribute to the development of haemorrhage in patients with DHF by interfering with coagulation and fibrinolysis.

Keywords

Coagulation factors, autoantibodies, haemostasis

Introduction

Dengue viruses (DENVs) are mosquito-borne flaviviruses that have been subgrouped into four antigenically related serotypes: DENV type 1, 2, 3, and 4 (1). DENV is a positive-stranded RNA virus with an envelope. DENVs have three structural proteins, including a core protein, a membrane-associated protein, and an envelope (E) protein, and seven non-structural (NS) proteins, including NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5. DENV infection is prevalent in tropic and sub-tropic areas where mosquito vectors reside (2). It has been estimated that greater than 2.5 billion people (i.e. two-fifths of the world's population) are now at risk for DENV infection. Over 50 million DENV infections globally occur each year. More than 100 countries are under threat of DENV infection. Primary infection with DENVs generally causes Dengue fever (DF) with symptoms such as fever, headache, and bone pain. Most patients will spontaneously recover; however, in some cases, particularly during secondary infections with different serotypes of DENVs, Dengue haemorrhagic fever or Dengue shock syndrome (DHF/DSS) may develop. In DHF/DSS patients, all types of haemorrhagic manifestations occur along with typical symptoms of DF (3, 4). The case-fatality rate for DHF/DSS can reach 20% if untreated. Antibody-dependent enhancement (ADE) has been proposed as an explanation for why DHF/DSS mostly occurs in secondary infections with different serotypes of DENV (5, 6). How-

ever, the pathogenic mechanisms causing haemorrhage in DHF/DSS remain unclear.

Clinical studies have shown that almost all DHF/DSS patients have abnormal haemostasis, which is demonstrated by thrombocytopenia (platelet counts less than 100,000/μl), prolonged activated partial thromboplastin time (aPTT), prothrombin time (PT), and thrombin time (TT). In addition, the patient fibrinogen level is decreased, and fibrin degradation products are increased in Dengue patients' plasma (7-10). Thus, clinical evidence indicates that coagulation is inhibited while fibrinolysis is hyper-activated in Dengue patients, which may contribute to haemorrhage in DHF/DSS.

In our previous study, we found that there is sequence homology between the DENV E and NS1 proteins and different coagulation factors such as (pro-)thrombin, fibrinogen, and plasminogen (Plg) (11). In addition, we found that monoclonal antibodies generated from DENV-immunised mice can cross-react with human Plg and enhance its activation (12). It is known that plasmin (Plm) and thrombin share homologous trypsin/thrombin-like serine protease domains (13) and that some mAbs generated from patients with antiphospholipid syndrome (APS) can bind various serine proteases including Plg and thrombin and tip the balance toward a hypercoagulant state (13-17). Therefore, we hypothesised that some anti-Dengue antibodies may bind to thrombin and inhibit its activity to thus promote haemorrhage in

patients infected with DENV. In this study, we first detected the presence of antibodies that could cross-react with thrombin in Dengue patient sera followed by the affinity purification of anti-thrombin antibodies (ATAs) from these patients to characterise their properties.

Materials and methods

Patient sera

In this study, Dengue patient sera were collected at the acute stage of disease during a DENV type 2 infection outbreak (n=32) in Kaohsiung and a DENV type 3 infection outbreak (n=48) in Vietnam as previously described (18). Sera from the acute stage of enterovirus 71 (EV71) (n=6) or hepatitis C virus (HCV) (n=21) infection and normal human sera from healthy donors (n=14) were included as controls.
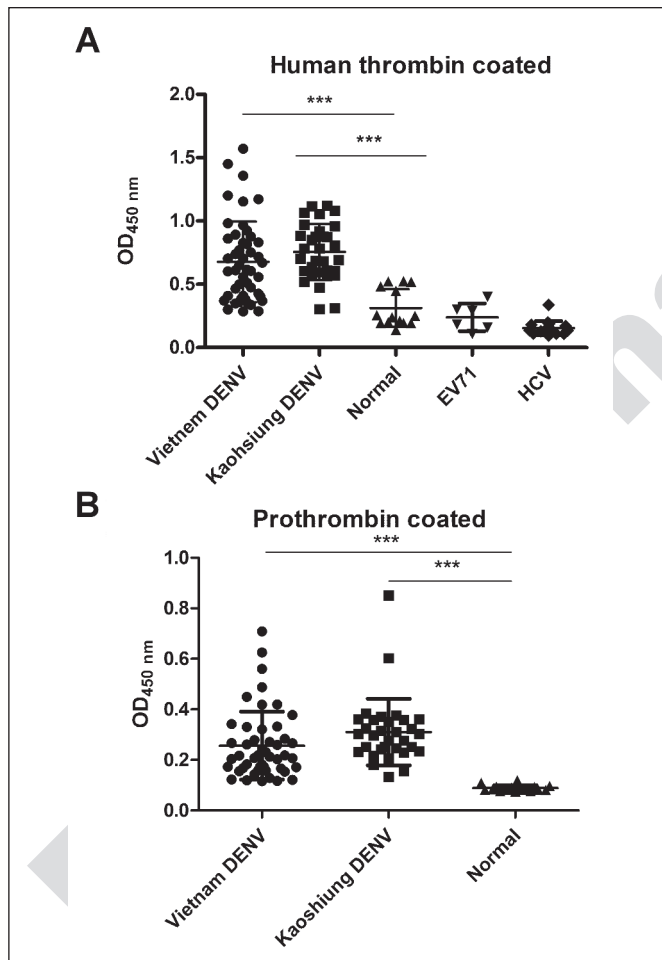
Figure 1: Antibodies in Dengue patient sera bind to thrombin, prothrombin and Plg. Sera from Vietnam (n=48) and Kaohsiung (n=32) Dengue patients or healthy donors (n=14), EV71 patients (n=6), and HCV patients (n=16) were used to detect the presence of IgG binding to human thrombin (A) or human prothrombin (B) by ELISA as described in *Materials and methods*. Each dot represents one serum sample. Error bars represent the mean ± SD. ***, p< 0.005 (Student's t test).

Virus and antibody purification

DENV type 2 (PL046 strain) was used in this study. To prepare DENV-infected cell lysate, C6/36 cells (10^6 cells) was seeded in a 6-well plate and infected with DENV at a 0.2 multiplicity of infection for 48 hours (h). The cells were lysed by RIPA buffer (10 mM Tris HCl, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, pH 7.4). The DENV complex was prepared as previously described (12). Briefly, virus supernatants were incubated with one-third of the volume of the precipitation buffer (28% PEG 8000, 8% sodium chloride) at 4°C overnight and centrifuged at 10,000 x g for 30 minutes (min). The DENV complex was resuspended in 10 mM phosphate-buffered saline (PBS; pH 8.0) and dialysis was performed in the same buffer. To obtain affinity purified ATAs, we used bovine thrombin (Sigma-Aldrich, St. Louis, MO, USA) conjugated cyanogen bromide (CNBr) Sepharose 4B (GE Healthcare, Uppsala, Sweden) followed by a second purification with protein A/L resin (GenScript Corp., Piscataway, NJ, USA). The purified antibodies were dialysed against PBS and stored at -20°C.

Cloning and expression of recombinant proteins

The recombinant NS1 and E protein domain III (DIIIE) proteins were cloned and expressed as previously described (19, 20). Briefly, primers that contained Nde I and Xho I restriction enzyme sites were designed to amplify different lengths of NS1 and full-length DIIIE DNA. The amplified DNA products were digested with restriction enzymes followed by insertion into the pET-43.1a vector (Novagen, Madison, WI, USA). Plasmid constructs were transformed into Rosetta (DE3) *Escherichia coli* for expression. Protein induction and purification were performed as previously described (20).

Enzyme-linked immunosorbent assay (ELISA)

Human thrombin, bovine thrombin, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Human Plg was obtained from Haematologic Technologies (Essex Junction, VT, USA). The desired proteins in PBS (5 μg/ml) were coated onto 96-well ELISA plates (GeneDireX, Las Vegas, NV, USA) at 4°C overnight followed by blocking with 1% BSA in PBS and washing with PBS plus 0.05% Tween 20 (PBST). Antibodies or sera were incubated in wells at 37°C for 1 h and washed with PBST. Bound antibodies were detected with horseradish peroxidase (HRP)-conjugated goat secondary antibodies directed against human IgG or IgM (Sigma-Aldrich). Colour development was performed by adding 50 μl of TMB substrate and 50 μl of 2 N sulfuric acid. The optical density (OD) values at 450 nm were measured with a VersaMax microplate reader (Molecular Devices, Crawley, West Sussex, UK). In a competitive inhibition assay, ATAs were pre-incubated with different concentrations of rNS1 or rDIIIE followed by the removal of recombinant proteins using magnetic nickel agarose beads before addition into thrombin or Plg-coated ELISA plates.

Western blotting analysis

For Western blotting, infected or non-infected C6/36 cell lysates and proteins were separated in 4–20% RunBlue gels (Expedeon, San Diego, CA, USA). The separated proteins were transferred onto a PVDF membrane and blocked with Noise Cancelling Reagents (Millipore, Billerica, MA, USA). Antibodies were incubated with membranes at 4°C overnight and washed with PBST (0.05% Tween 20 in PBS). The membranes were detected with HRP-conjugated goat anti-human IgG.

Chromogenic assay of thrombin and Plm activity

The chromogenic substrates S-2251 (for Plm activity) and S-2238 (for thrombin activity) were purchased from Chromogenix (Milano, Italy). To determine the effect of antibodies on human thrombin activity, human thrombin (0.4 NIH units/ml) was incubated with antibodies (10 μg/ml) at 37°C for 1 h. Thrombin activity was detected by adding S-2238 (0.5 mM in final concentration) and incubating for an additional 1 h. The OD value at 405 nm was measured every 10 min with a VersaMax microplate reader. To detect the effect of antibodies on urokinase-induced Plg activation,

control Ig or ATAs (10 or 30 μg/ml) were incubated with Plg (10 μg/ml) followed by the addition of urokinase (1 NIH unit/ml) and S-2251 (0.5 mM final concentration). The mixture was incubated at 37°C for 1 h, and the OD_{405} value was monitored. To test the effect of ATAs on Plg activation, control Ig (30 μg/ml) or ATAs (30 μg/ml) were incubated alone or in the presence of Plg (10 μg/ml) at 37°C for different periods of time as indicated. The mixtures were then incubated with S-2251, and the Plm activity was determined by monitoring the OD_{405} value as described above.

Thrombin time (TT)

In the TT assay, human (0.4 NIH units/assay) thrombin was incubated with antibodies in a final volume of 100 μl. The mixtures were incubated at 37°C for 1 h followed by addition into 200 μl of human PPP. The clotting time was determined by an automated blood coagulation analyser (CA-50, Sysmex Corp., Kobe, Japan).
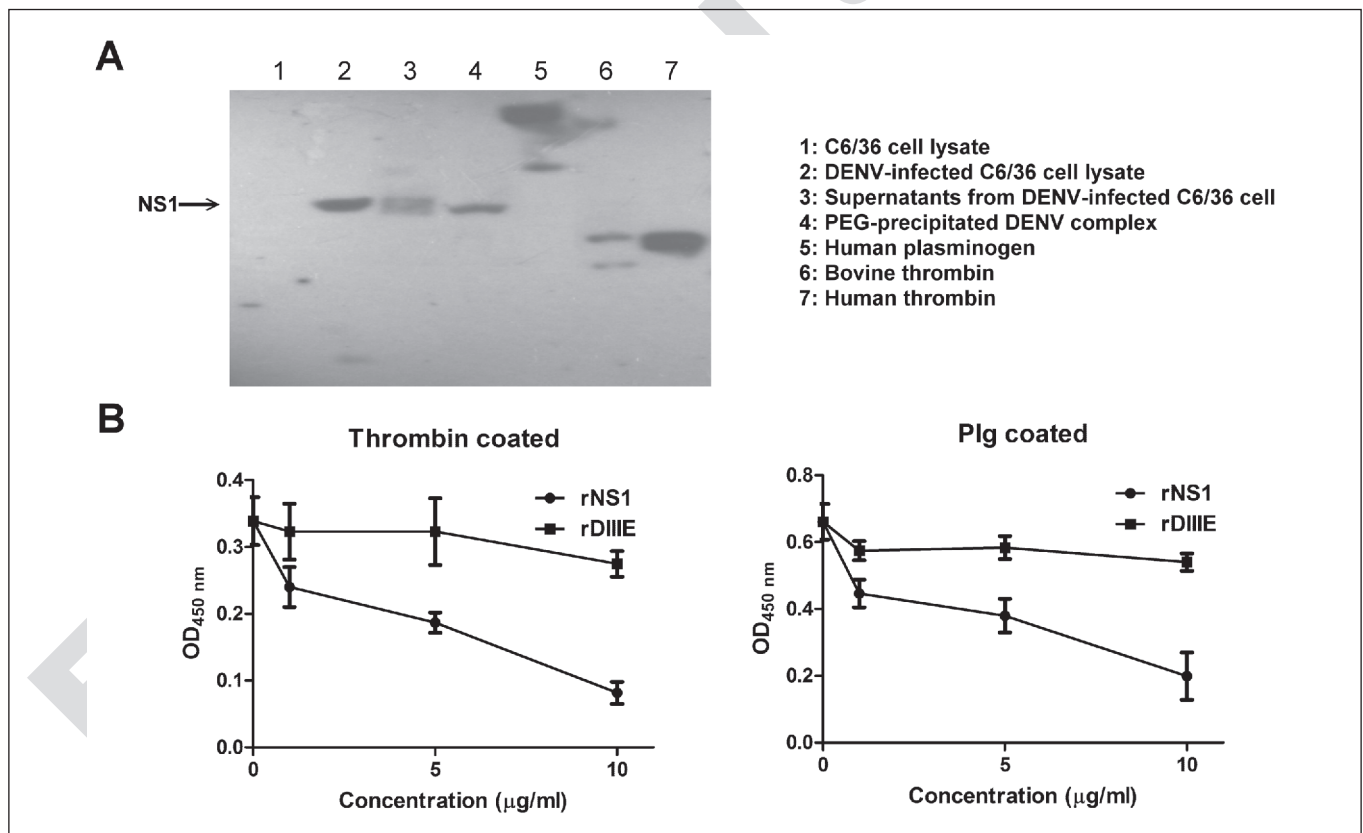
Figure 2: ATAs from Dengue patients bind to Dengue NS1 and Plg. A) Dengue patient ATA binding to DENV proteins, thrombin and Plg was determined by Western blotting. Lane 1: C6/36 cell lysate; Lane 2: DENV-infected C6/36 cell lysate; Lane 3: Supernatants from DENV-infected C6/36 cells; Lane 4: PEG-precipitated DENV complex; Lane 5: Plg; Lane 6: bovine thrombin; Lane 7: human thrombin. B) Competitive inhibition of Dengue patient ATA binding to thrombin and Plg. ATAs (10 μg/ml) were pre-incubated with different concentrations of the rNS1 or E protein domain III proteins followed by adsorbing with magnetic nickel agarose beads. The binding ability of unbound antibodies to thrombin or Plg was detected by ELISA.
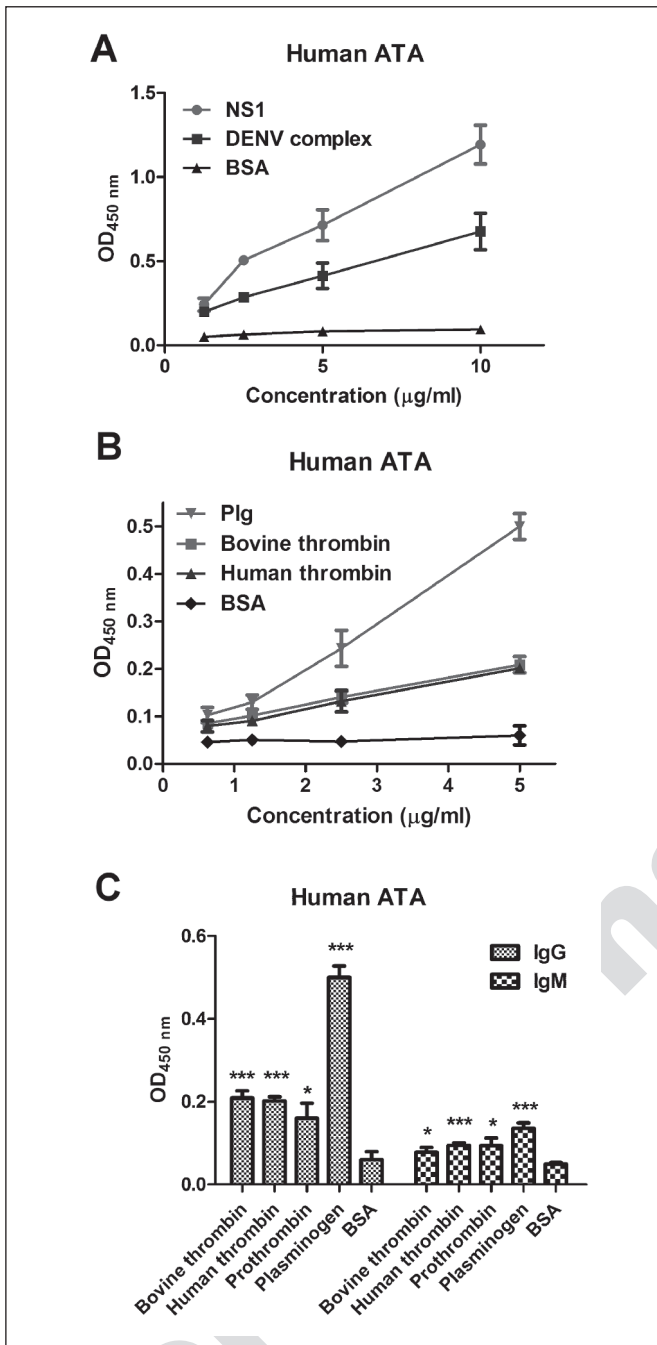
Figure 3: Comparison and characterisation of the binding ability of Dengue patient ATAs. A) Dose-response curves of Dengue patient ATA binding to the DENV complexes or NS1 by ELISA. B) Dose-response curves of Dengue patient ATA binding to human/bovine thrombin, Plg, or BSA by ELISA. C) Comparison of the binding ability of different isotypes of ATA (IgG or IgM) to different coagulation factors by ELISA. The results are presented as the mean ± SD from three independent experiments. **, p<0.01; ***, p< 0.005 (Student's t-test).

Fibrin formation and clot lysis assay

In the fibrin formation assay, human thrombin (50 µl; 2 NIH units/ml) was incubated with antibodies (50 µl; 10 µg/ml) at 37°C for 1 h followed by addition into human PPP (100 µl; 10-fold diluted with PBS). Fibrin formation was determined by monitoring the turbidity with the OD_{350} values using a VersaMax microplate reader. In the clot lysis assay, human PPP (200 µl; 10-fold diluted) was first mixed with antibodies (50 µl; 10 µg/ml) and urokinase (3 NIH units/ml; Sigma-Aldrich) followed by addition into human thrombin (50 µl; 2 NIH units/ml), and the turbidity changes were periodically monitored. To calculate the percentage of clot lysis, we found that the clot was lysed after 1 h. Thus, we calculated the lysis percentage with the following formula: $\text{clot lysis} (\%) = \dfrac{A - C}{A - B} \times 100\%$ where A represents the OD_{350} value at 1 h, B represents the OD_{350} value at 0 h, and C represents the OD_{350} value at 2, 3, or 4 h.

Statistical analysis

Data were expressed as the mean ± SD of three independent experiments. The Student's t-test or two-way ANOVA were used to analyse significance: ***: $p < 0.001$, **: $p < 0.01$, and *: $p < 0.05$. In linear regression analysis, the slope of the regression line significantly different from zero was analysed with GraphPad Prism 5 software.

Results

Anti-thrombin antibodies in Dengue patient sera

Sera from 80 Dengue patients in the acute stage, including 48 patients from Vietnam (mainly DENV 3 infection) and 32 patients from Kaohsiung (DENV 2 infection), were diluted 10^2-fold to detect the presence of antibodies against human thrombin by ELISA. Significant increases in antibody binding to human thrombin (►Figure 1A) and prothrombin (►Figure 1B) were found in Dengue patient sera compared with that in EV71- and HCV-infected patient sera or normal human sera. In addition, as we previously demonstrated, there were Plg cross-reactive antibodies in the sera from these Dengue patients (12). A positive correlation between the anti-thrombin and anti-Plg antibodies was found in the Dengue patients from Vietnam and Kaohsiung (R=0.45 and 0.34, respectively) (data not shown).

Purification and characterisation of ATAs from Dengue patient sera

To further characterise ATAs in Dengue patients, we pooled 15 ml of sera from Dengue patients as described in *Materials and methods*. Antibodies in the eluent from a thrombin column were further purified with a protein A/L column. After two rounds of purification, 585 µg of ATA was collected. Next, we characterised the binding specificity of these ATAs for different antigens by Western blotting analysis. In addition to Plg and thrombin, we

found that the ATAs also recognised NS1 in DENV-infected C6/36 cell lysates, supernatants, and PEG-precipitated DENV complexes (▶Figure 2A). Furthermore, Western blotting analysis confirmed the presence of three Dengue structural proteins (capsid, pr-M, and E protein) in the DENV complexes (data not shown). However, these structural proteins were not recognised by ATAs. To further characterise the region of ATA binding to NS1, full-length and N- and C-terminal-truncated recombinant NS1 (a.a. 90-293 and a.a. 90-323, respectively) were used. We found that ATAs could bind to all of the different rNS1 protein lengths (data not shown). The cross-reactivity of ATAs with NS1 and thrombin/Plg was further confirmed by competitive inhibition. Pre-incubation with rNS1, but not rDIIIE, could inhibit ATA binding to thrombin or Plg in a dose-dependent manner (▶Figure 2B).

Comparison and characterisation of the binding ability of ATA to different antigens

To compare the binding ability of ATAs to different antigens, an ELISA was performed. The dose-dependent binding of ATAs to rNS1 and PEG-precipitated DENV was observed (▶Figure 3A). In addition, the dose-response curves of ATA binding to bovine and human thrombin were similar (▶Figure 3B). However, the ability of the ATAs to bind Plg was unexpectedly higher than their binding to thrombin at the same concentration. Isotype analysis indicated that most of the ATAs were of the IgG isotype, and only a few were of the IgM class (▶Figure 3C).

Inhibition of thrombin activity by ATAs from Dengue patients

To determine the effect of Dengue patient ATAs on thrombin activity, we performed thrombin chromogenic, fibrin formation, and thrombin time (TT) assays. A significant inhibition in human thrombin activity by ATAs, but not by normal human Ig, was found by chromogenic assay (▶Figure 4A). In addition, fibrin formation in the presence of ATAs was also delayed compared with that in the presence of control Ig (▶Figure 4B). Furthermore, ATAs, but not control human Ig, could significantly prolong the TT at a concentration as low as 10 μg/ml (▶Figure 4C).

Dengue patient ATAs enhance fibrinolysis

Because ATAs from Dengue patients also cross-reacted with Plg, we tested whether they could enhance Plg activation as we previously reported (12). We found that ATAs, but not control Ig, could enhance urokinase-induced Plg activation in a dose-dependent manner (▶Figure 5A). Consistently, the lysis of fibrin clots was faster in the presence of the ATAs, but not in the presence of control Ig, in the clot lysis assay (▶Figure 5B). Moreover, S-2251 hydrolysis was also significantly increased when ATAs were incubated with Plg alone for more than 20 h (▶Figure 5C). Although a much longer time was required for the ATAs to activate Plg in the absence of urokinase, these results indicated that, as we
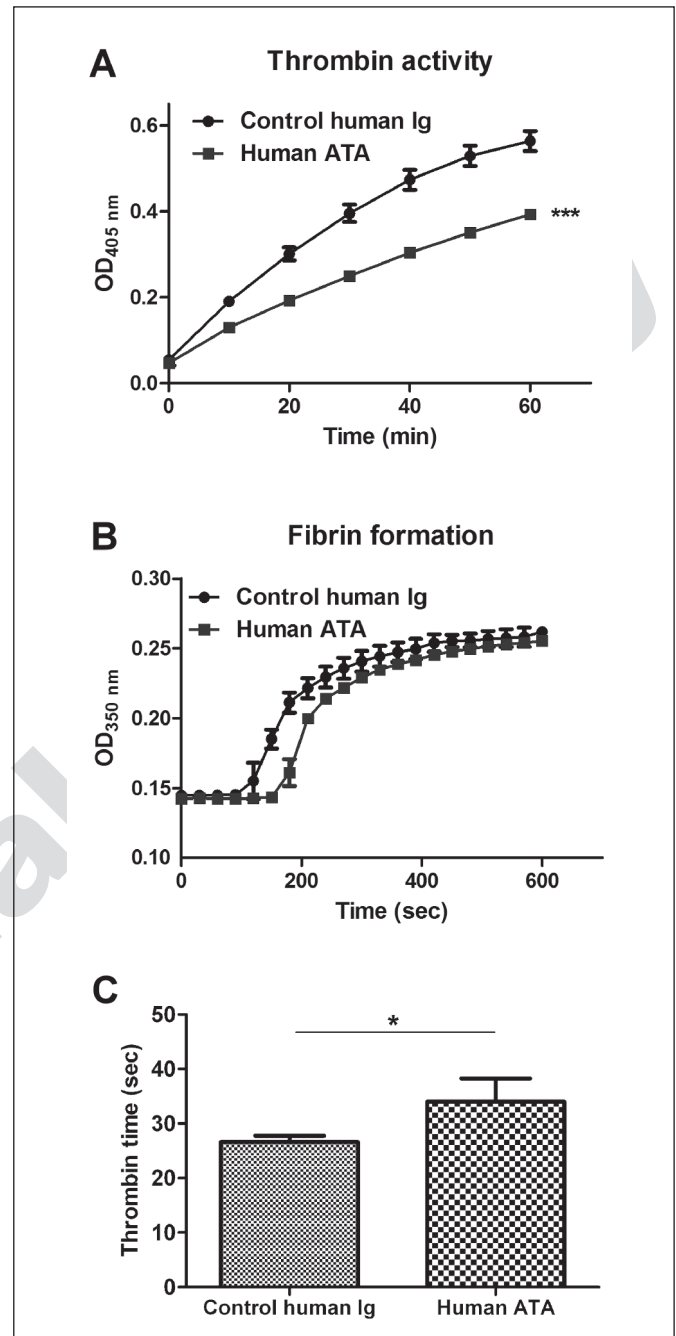
Figure 4: Dengue patient ATAs inhibit thrombin activity. A) Human thrombin was pre-incubated with control Ig or the ATA as indicated for 1 h. Thrombin activity was determined by S-2238 as described in *Materials and methods*. Significance was analysed by two-way ANOVA. *, $p < 0.05$; ***, $p < 0.001$. B) Human thrombin was pre-incubated with control Ig or the ATA as indicated for 1 h before addition into human PPP. The fibrin formation was monitored by the change in turbidity at OD350 as described in *Materials and methods*. C) Dengue patient ATAs (10 μg/ml in final concentration) were pre-incubated with human thrombin for 1 h followed by addition into human PPP to detect thrombin time as described in *Materials and methods*. The significance was analysed by Student's t-test. *, $p < 0.5$; **, $p < 0.01$.

previously found, some of these Plg-cross-reacting anti-DENV antibodies could directly enhance Plg activation.
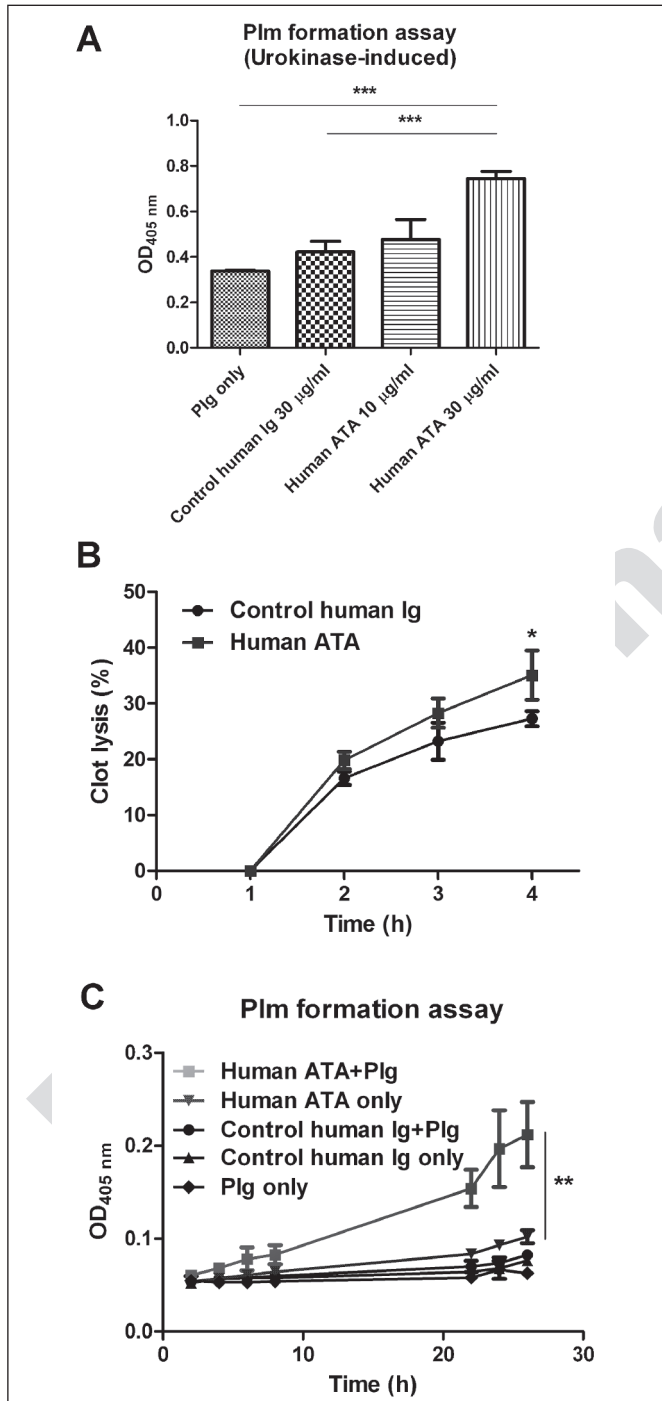
Discussion

To test our hypothesis that Dengue patients have antibodies against thrombin that might interfere with coagulation, we searched for the presence of anti-thrombin antibodies in Dengue

Figure 5: Dengue patient ATAs enhance fibrinolysis. A) Plg was incubated with control human IgG or ATA before addition with urokinase and S-2251 complex. After 1 h of incubation, Plm formation was measured by monitoring the OD value at 405 nm. *, p<0.5; **, p<0.01 (Student's t-test). B) Human PPP was pre-incubated with urokinase, control Ig or ATAs before human thrombin addition. The fibrin clot was formed 10 min after thrombin addition, and it started to lyse after 1 h. The percentage of clot lysis was calculated as described in *Materials and methods*. The significance at each time point was analysed by Student's t-test. *, p<0.5 All results are presented as the mean ± SD from three independent experiments. C) S-2251 was incubated with Plg or the ATA alone, or Plg mixed with the ATA or control Ig. Plm formation was measured as described above at the indicated time after incubation. Significance was analysed by two-way ANOVA. **, p<0.01.

patient sera and studied their effects on coagulation and fibrinolysis activities. The results showed that anti-thrombin antibodies were significantly increased in Dengue patients from Kaohsiung (mainly DENV type 2 infection in adults) and Vietnam (mainly DENV type 3 infection in infants) compared with normal controls or patients infected with other viruses. Moreover, a positive correlation between anti-thrombin and anti-Plg antibodies was found in these patients. To further characterise the properties of these anti-thrombin antibodies, we used thrombin and protein A/L affinity columns to purify ATAs from the sera of Dengue patients. We found that most of these ATAs belonged to the IgG class and recognised not only thrombin but also the Dengue NS1 protein and human Plg.

It is known that the DENV E and NS1 proteins have amino acid sequence homology with many of these coagulation factors (11). Therefore, it is possible that ATAs are induced by molecular mimicry between DENV antigens and these coagulation factors during DENV infection. Because ATA pre-incubation with rNS1, but not rDIIIE, reduced the binding of ATAs to thrombin and Plg, NS1 might be the major antigen to induce ATAs in Dengue patients. During DENV infection, NS1 can be secreted into plasma and form a complex with prothrombin to inhibit its activation (20). Therefore, NS1 may directly interfere with haemostasis or indirectly through its antibodies.

The influence of Dengue patient ATAs on thrombin formation and its activity was also studied. Our results indicated that ATAs could inhibit human thrombin activity, which was demonstrated by TT, thrombin chromogenic and fibrin formation assays *in vitro*. Because ATAs could also bind to prothrombin, we were interested in whether ATAs could also interfere with prothrombin activation. However, based on the results from aPTT and PT assays, we found ATAs had no effects on prothrombin activation (data not shown). Therefore, ATAs could interfere with thrombin activity, but not prothrombin activation.

Previously, Plg cross-reactive antibodies have been found in Dengue patient sera and were correlated with the occurrence of a haemorrhage (21, 22). In addition, there is sequence homology between type 4 DENV E protein amino acids 100-119 (D4E; GWGNGCGLFGKGVVTCAKF) and Plg amino acids 759-779 (PL+; SWGLGCARPNKPGVYVRVSRF). Affinity-purified antibodies directed against the synthetic peptide PL+ from D4E pep-

tide immune sera could inhibit Plm activity (23). However, there are other regions of the DENV that can induce Plg cross-reactive autoantibodies that can enhance Plg activation rather than inhibit Plm activity (12, 23). Therefore, DENV-induced antibodies that recognize different Plg epitopes might have different effects on Plg activation. Because Dengue patient ATA could also recognise Plg, we were interested in determining the effects of ATAs on Plg activation. Our results indicate that ATA could enhance Plg activation as demonstrated by chromogenic and clot lysis assays. However, because the amount of Dengue patient ATA was not enough to perform passive transfer studies *in vivo*, we could not determine the effect of Dengue patient ATAs on coagulation and fibrinolysis *in vivo*.

It is known that thrombin and Plm are major players in the activation of coagulation and fibrinolysis, respectively. Thrombin activated by either intrinsic or extrinsic pathways converts fibrinogen to fibrin and leads to clot formation. In contrast, Plm is activated from Plg by serine proteases such as tissue Plg activator or urokinase. Plm can digest fibrin into fibrin degradation products and lead to fibrin network disintegration. In this study, we found that ATAs not only can inhibit thrombin activity but also enhance urokinase-induced Plg activation. The mechanisms for ATA-induced thrombin inhibition and Plg activation remain unclear. However, ATAs incubated with Plg alone could directly convert Plg into Plm, indicating that some of these antibodies may contain serine protease activity, as we previously found for Plg cross-reactive DENV monoclonal antibodies in mice (12).

What is known about this topic?

- Dengue virus (DENV) infections may result in severe life-threatening Dengue haemorrhagic fever (DHF). However, the mechanisms to cause haemorrhage during DENV infection remain elusive.
- There are sequence homologies between DENV envelop protein (E), nonstructural protein 1 (NS1) and different coagulation factors.
- Antibodies cross-react with plasminogen (Plg) have been found in Dengue patients. In addition, monoclonal antibodies against Plg from DENV-immunised mice could interfere with fibrinolysis. However, the effects of these DENV-induced coagulation factors cross-reactive antibodies in dengue patients on coagulation and fibrinolysis are unclear.

What does this paper add?

- We report that anti-thrombin antibodies (ATA) which can cross-react with dengue NS1 and Plg are significantly increased in dengue patients' sera as compared to those in normal sera or other virus-infected patients' sera.
- Affinity-purified ATA collected from DENV patients' sera by thrombin and protein A/L affinity columns could inhibit thrombin activity and enhance Plg activation *in vitro*, suggesting DENV NS1-induced thrombin and Plg cross-reactive antibodies may contribute to the development of haemorrhage in DHF.

Clinically, the development of acquired anti-thrombin antibodies is not uncommon (24). Most symptomatic cases have been associated with haemorrhage, although thrombosis has been reported in APS patients (17, 25-27). Autoantibodies that can recognise both thrombin and Plg have also been found in APS patients. However, completely opposite effects for these autoantibodies were found in APS (13). In APS, anti-thrombin antibodies can interfere with the inactivation of thrombin by antithrombin and impair the fibrinolytic activity of Plm, thus converting normal haemostasis into thrombosis (17, 28). In contrast, our results indicate that the anti-thrombin antibodies induced by DENV can simultaneously inhibit thrombin activity and enhance Plg activation. It is possible that, due to specificity differences, anti-thrombin antibodies in APS and Dengue patients may play different roles in disease pathogenesis (29).

Antibodies directed against DENV NS1, which can cross-react with endothelial cells and platelets, leading to endothelial damage, and platelet dysfunction have been previously reported (11, 30-32). The titres of these endothelial cells and platelets cross-reactive anti-NS1 antibodies were higher in acute phase (3-7 days after fever onset) of DHF/DSS patients than those in DF patients. Furthermore, sera collected in the convalescent phase (1-3 weeks after acute phase) showed a decrease in the titres of these autoantibodies when compared to those collected in the acute phase, even though the total anti-NS1 levels continually remain high (33, 34). In this study, we further extend the cross-reactivity of anti-DENV NS1 antibodies to different coagulation factors. The molecular mimicry between DENV NS1 and different host proteins may represent a strategy employed by DENV to evade immune attack. Although investigation of sequential serum samples is required to further support the importance of DENV NS1-induced coagulation factor cross-reactive autoantibodies in the development of DHF/DSS, the pathogenic roles of autoantibodies induced by NS1 should not be neglected. Moreover, NS1 epitopes mimicking host proteins should be avoided in the design of Dengue vaccine to avoid potentially dangerous immune responses to the vaccine (35, 36).

Conflicts of interest
None declared.

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