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Antibodies against dengue virus E protein peptide bind to human plasminogen and inhibit plasmin activity

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SUMMARY

Both mice and rabbits immunized with dengue virus E protein peptide spanning amino acids 100–119 (D4E) produced antibodies that reacted not only with the D4E peptide itself but also with human plasminogen, as shown by ELISA and Western blot. Sera from dengue virus-hyperimmunized mice and dengue patients also contained antibodies against D4E and plasminogen. Furthermore, such sera all contained plasmin inhibitory activity. Using affinity-purified anti-D4E antibodies and free D4E peptide for competitive inhibition, we demonstrated that the inhibition of plasmin activity was due to anti-D4E antibodies rather than other substances in the sera. Taken together, these results suggest dengue virus E protein amino acids 100–119 are a cross-reactive immunogenic region, and antibodies against this region may interfere with human fibrinolysis.

Keywords dengue virus plasminogen plasmin antibody response

INTRODUCTION

Dengue viruses are mosquito-borne flaviviruses which have been subgrouped into four antigenically related serotypes, dengue virus types 1, 2, 3 and 4 [1]. After infection, dengue virus is found in mononuclear cells of different organs and peripheral blood of patients. Primary infection with dengue virus usually results in mild dengue fever (DF), characterized by fever, headache, muscle or joint pain, and rash. Secondary infection with serotypes different from that of the primary infection, however, might lead to fatal haemorrhagic fever and shock syndrome (DHF/DSS) [2]. The pathogenesis of DHF/DSS is still not completely understood, even though antibody-dependent enhancement (ADE) has been proposed as one of the mechanisms [3,4]. According to the ADE hypothesis, antibody generated during primary infection can not neutralize the virus during secondary infection, but such antibodies may enhance virus uptake by macrophages through Fc receptors, and even worsen the disease. Therefore, antibody against a conserved region among different serotypes of dengue virus may play an important role in the pathogenesis of DHF/DSS.

Recently, a computer-based comparison revealed that there is a 20-amino acid sequence similarity between the dengue type 4 E protein (D4E) amino acids 100–119 and plasminogen amino acids 759–779 (PL+) [5]. D4E is highly conserved in different serotypes of dengue viruses, and PL+ is also conserved among different clotting factors such as urokinase and tissue plasminogen activator. Markoff *et al*. have demonstrated that antibodies against PL+ peptide were present in three-quarters of 40 Thai patients acutely infected with dengue virus types 1, 2, 3 or 4, but not in patients infected with Japanese encephalitis virus [5]. There was also a positive correlation between detection of plasminogen cross-reactive antibodies and the appearance of haemorrhage in patients with dengue virus infection [6].

The effect of these plasminogen-cross-reactive antibodies on plasminogen or plasmin function is, however, unclear. Plasmin is a serine protease, which can digest fibrin and plays an important role in fibrinolysis [7,8]. Plasmin consists of two polypeptide chains connected by disulphide bridges. It is formed from its proenzyme, plasminogen, a single-chain molecule, by limited proteolysis at two different sites on the polypeptide chain [9]. The A chain of plasmin (amino acids 1–560 of plasminogen) contains five homologous triple-loop structures, or 'kringle' domains. These kringle domains contain lysine-binding sites which mediate its interaction with fibrin and α_2-antiplasmin [8]. The B chain of plasmin (amino acids 561–790 of plasminogen) contains the active catalytic site His-602, Asp-645 and Ser-740 [10]. Therefore, the binding site of plasminogen-cross-reactive antibodies seems to be very close to the active site of plasmin.

In an attempt to understand the effect of plasminogen-cross-reactive antibodies on plasmin function, we immunized mice and

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rabbits with D4E peptide, then studied the plasminogen cross-reactivity of specifically elicited antibodies as well as sera from dengue virus-hyperimmune mice and dengue patients. Furthermore, using an *in vitro* chromogenic substrate assay, we studied the effect of the plasminogen-cross-reactive antibodies on plasmin activity.

MATERIALS AND METHODS

Synthetic peptides

Two peptides, each corresponding to amino acids 100–119 of D4E and amino acids 759–779 of human plasminogen (PL+), were synthesized by the Peptide Synthesis Centre (National Science Council, Taipei, Taiwan). The sequences of these two peptides are GWGNGCGLFGKGGVVTCAKF and SWGLGCARPNKPGVY-VRVSRF, respectively [5]. An additional peptide (CM1) was synthesized to serve as a negative control in peptide competition assay. The sequence of CM1 is ANRYLAMKEDGRLLASKC. The purity of these peptides was confirmed by high performance liquid chromatography (HPLC) and amino acid composition analysis.

Peptide conjugation

D4E peptide was conjugated to keyhole limpet haemocyanin (KLH; Pierce, Rockford, IL) or bovine serum albumin (BSA; Sigma, St Louis, MO) [11]**.** Briefly, 20 mg of KLH or BSA and 3 mg of peptide were dissolved in 2 ml of 0·1 M phosphate buffer pH 7·0. One millilitre of 0·2% glutaraldehyde was then added drop by drop to the mixture with constant stirring. After reacting at room temperature for 24 h, the peptide conjugate was thoroughly dialysed against 0·1 M phosphate buffer.

Mice

Female BALB/c mice (6–8 weeks old) were used in this study. The mice were originally purchased from the Jackson Laboratory (Bar Harbor, ME) and bred in the Laboratory Animal Centre, National Cheng Kung University.

Virus

Dengue type 2 virus (Taiwan local isolated strain) used in this study for mice immunization and Western blot analysis was propagated in mice by intracerebral inoculation. About 8–9 days after inoculation, paralysis occurred and the mice were killed and the brain tissue was removed aseptically. Virus stock was collected from the supernatants of homogenized mouse brains after centrifugation at 16 100 ***g*** . Virus titre was determined by plaque assay on BHK-21 cells. Briefly, serial 10-fold dilutions of virus stock were added to BHK-21 monolayer, which were then incubated at 37°C, 5% CO_2 for 5 days under a 1% methylcellulose (Sigma) overlay with 2% fetal bovine serum (FBS). Plaque numbers were counted after staining with crystal violet.

Animal immunization

D4E peptide conjugated with KLH (D4E–KLH) (50 μg/mouse) was mixed with Freund's complete adjuvant (FCA) and intraperitoneally injected into BALB/c mice. Mice were boosted with D4E–KLH in PBS (50 μg/mouse) in the same route 30 days later. Sera were collected from the axially plexus of the mice at different times as indicated. In addition, two rabbits were also hyperimmunized with D4E–KLH and sera were collected 10 days after boosting. Dengue virus-hyperimmune sera were collected from mice after immunization with 100 μl of dengue virus in PBS (about 10^7 plaque-forming units (PFU)/ml) via the footpad and the base tail every week for 3 weeks.

Human sera

Sera were obtained from 16 patients with dengue fever at the convalescent stage (1–4 months after disease occurred) in Southern Taiwan during 1996. All these patients (eight females and eight males, age range 11–59 years) showed clinical symptoms such as fever, rash and bone pain. Diagnoses were confirmed by anti-dengue ELISA-IgM or haemagglutination-inhibition with or without virus isolation by the National Quarantine Service Department of Health in Taiwan. The serotypes of isolated dengue viruses were determined by type-specific MoAbs, and included type 2 (one patient), type 3 (five patients) and type 4 (one patient). Sera from normal individuals without antibody against dengue virus served as controls.

ELISA

Anti-D4E, anti-plasminogen and anti-PL+ antibody levels were determined by ELISA. Briefly, 96-well flat-bottomed ELISA plates were coated with 50 μl of D4E–BSA, plasminogen (Boehringer Mannheim, Indianapolis, IN), or PL+ peptide (0·1 mg/ml in PBS) for 2 h at 37°C. After washing with PBS, plates were blocked with a blocking buffer (1% BSA in PBS) for 1 h at 37°C. Serum samples from mice, rabbits and humans were diluted 100-fold with blocking buffer and incubated in the plates for 2 h at 37°C. Bound antibodies were detected by horseradish peroxidase (HRP)-conjugated anti-mouse, rabbit or human immunoglobulin antibodies (Sigma) and OPD (Sigma). The developed colour was read by a Vmax microplate reader (Molecular Devices, Menlo Park, CA) at 490 nm.

SDS–PAGE and Western blot analysis

D4E peptide, plasminogen (Boehringer Mannheim), and dengue virus stock were separated by 12% SDS–PAGE with Laemmli's discontinuous buffer system [12]. Electrophoresis was conducted for 1 h at 100 V and the separated proteins were detected by staining with coomassie blue solution. In Western blot, proteins were transferred from SDS–PAGE to nitrocellulose sheets [13]. D4E peptide, plasminogen and dengue virus were detected using D4E-immunized rabbit sera and alkaline phosphatase-conjugated anti-rabbit immunoglobulin antibodies (Sigma) and substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) (Sigma).

Purification of immunoglobulin by protein A and PL+ affinity column

IgG from normal and D4E-immunized rabbit sera were purified using protein A affinity column (Pharmacia Biotech, Piscataway, NJ). Unbound components were washed away with 0·85% saline. Bound IgG was then eluted with 0·1 M glycine pH 3·0, and the pH was neutralized to pH 7·0 with 1 M Tris–HCl pH 8·0. In addition, PL + peptide was conjugated to Sepharose 4B (Pharmacia Biotech) according to manufacturer's suggested procedure. Anti-PL+ antibodies were then isolated from D4E-hyperimmune rabbit sera and the serum of one dengue patient. This was done by affinity purification on, and 3 M $MgCl_2$ elution from, the PL+ affinity column. The eluted immunoglobulins were dialysed against PBS–azide. The concentration of immunoglobulin was adjusted by ultrafiltration (Amicon, Beverly, MA) and determined by BCA protein assay (Pierce).

Assay for plasmin activity

Human plasmin was prepared by activating human plasminogen with Sepharose-bound urokinase according to a method described previously [14]. The catalytic activity of plasmin was assayed using D-Val-Leu-Lys-p-nitrophenylanilide as substrate (S-2251) (Sigma) [15]. Sera were diluted 100-fold in an activating buffer (0·05 M sodium dihydrogen phosphate, 0·02 M L-lysine, 1 mM EDTA and 0·1 M sodium chloride, pH 6·8) with 25% glycerol. The plasmin (0·1 μM) was then preincubated with diluted sera (20 μl) for 1 h at 37°C. Then, 50 μl of substrate S-2251 were added and further reacted for 30 min at 37°C. The increase of absorbance at 405 nm was monitored using the Vmax microplate reader. In some experiments, plasmin was preincubated with heat-inactivated sera (56°C, 30 min) or purified immunoglobulin with or without the presence of 1 μg D4E, PL + or CM1 peptides before assay.

Statistical analysis

Statistical analysis of data was performed using Student's *t*-test, and differences were considered significant if P values were $<0{\cdot}05$.

RESULTS

Anti-plasminogen-cross-reactive antibodies in D4E-immunized animals

Anti-D4E as well as anti-plasminogen antibodies were detected in D4E-immunized BALB/c mice (Fig. 1). The kinetics of production of these two antibodies were similar. There was also a significant increase in plasminogen-cross-reactive antibodies in D4E-immunized rabbit sera (optical density (OD) $0{\cdot}83 \pm 0{\cdot}46$) compared with normal rabbit sera (OD $0{\cdot}21 \pm 0{\cdot}12$). In addition, different doses of IgG purified by protein A affinity column from both normal and D4E-immunized rabbit sera and affinity-purified rabbit anti-PL+ antibodies were tested for their plasminogen-binding ability by plasminogen-coated ELISA (Fig. 2). As expected, affinity-purified rabbit anti-PL+ antibodies showed the greatest binding ability, followed by IgG from D4E-immunized and normal rabbits (Fig. 2). These anti-PL+ antibodies not only bound to plasminogen but also D4E peptide, as shown by ELISA (data not shown). Even though there was no significant difference in binding to plasminogen between IgG from D4E-immunized and normal rabbits, the former showed much stronger binding to D4E peptide (data not shown).

Anti-D4E and anti-PL+ antibodies present in dengue patients

Direct assay of antibody levels in human sera against human plasminogen using plates coated with D4E–BSA or plasminogen were not interpretable because of high background. As an alternative, antibodies binding to synthetic peptides D4E and PL+ were assayed. A significant increase in both anti-D4E and anti-PL+ levels was observed in dengue patients compared with normal controls (Table 1). Similar anti-D4E and anti-PL+ antibodies were also detected in patient sera using anti-human IgG-specific antibodies (data not shown). Since sera used in this study were collected 1–4 months after DF occurred, these results indicate anti-D4E and anti-PL+ antibodies are present in patient sera even after the disease is cured.

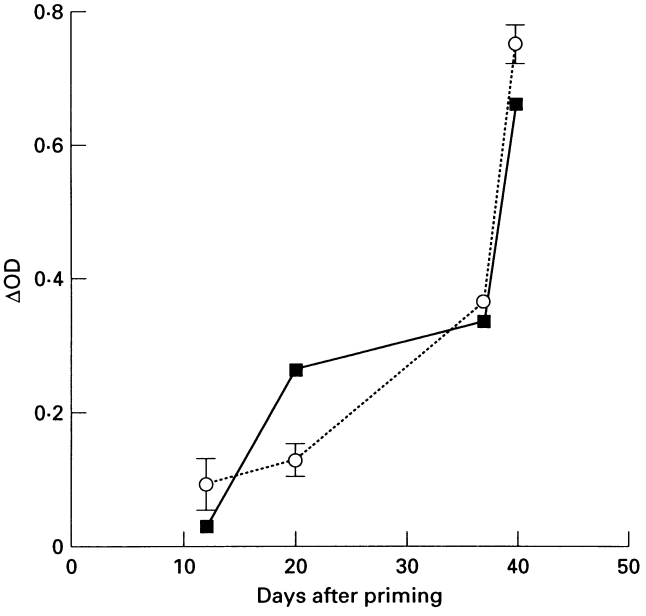

Fig. 1. Development of anti-dengue virus E protein (D4E) (■) and anti-plasminogen (○) antibodies in D4E-immunized mice. BALB/c mice were intraperitoneally injected with D4E peptide and sera were collected from the axial plexus. Sera were diluted 100-fold before determination of antibody concentration by ELISA, as described in Materials and Methods. Plates were coated with D4E–bovine serum albumin (BSA) or human plasminogen (0·1 mg/ml). Data represent the mean ± s.d. of five experiments.

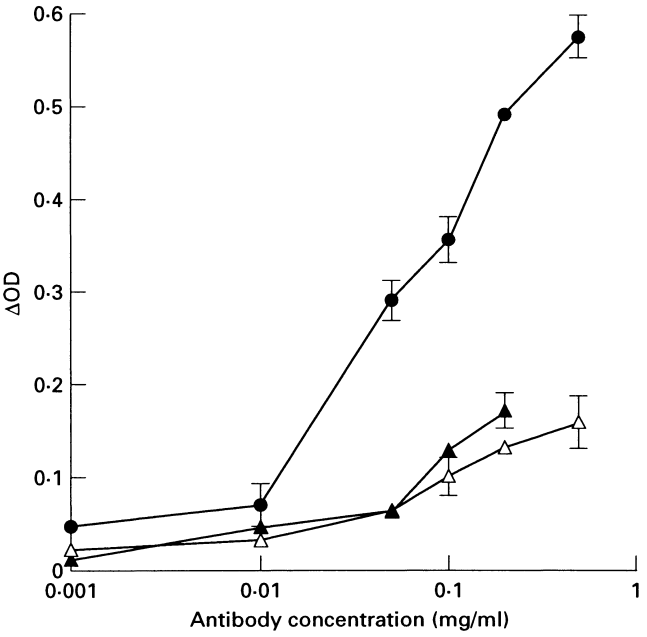

Fig. 2. Plasminogen-binding ability of affinity-purified anti-PL+ antibodies (●), IgG from dengue virus E protein (D4E)-immunized rabbits (▲) and IgG from normal rabbits (Δ). Anti-PL+ antibodies were purified from D4E-immunized rabbit sera by PL+ affinity column, IgG from D4E-immunized and normal rabbit sera were purified by Protein A column, as described in Materials and Methods. Different doses of antibodies were incubated with human plasminogen (0·1 mg/ml)-coated plates and bound antibodies were detected by ELISA, as described in Materials and Methods. Data represent the mean ± s.d. of duplicate experiments.

Table 1. Anti-dengue virus E protein (D4E) and anti-PL + antibodies in human sera†

	Antibody concentration (OD)	
Number of samples	Anti-D4E‡	Anti-PL+§
Normal (19)	0·36 ± 0·21	0·28 ± 0·17
Patients (16)	0·66 ± 0·19*	0·79 ± 0·25*

*$P < 0·001$.

†Antibody concentration was determined by ELISA as described in Materials and Methods. All sera were diluted 100-fold. Data represent the mean ± s.d. of eight separate experiments.

‡Plates were coated with D4E peptide (0·1 mg/ml in PBS).

§Plates were coated with PL + peptide (0·1 mg/ml in PBS).

Antibodies in D4E-hyperimmune sera bound to Western blots of D4E peptide, plasminogen and dengue virus

Western blot analysis showed that D4E-hyperimmune sera contained antibodies which recognized not only D4E peptide but also plasminogen and three components of dengue virus with molecular masses of about 52·5, 55 and 57 kD, respectively (Fig. 3). Since the molecular weight of E protein is about 51–60 kD [1], it is likely the proteins recognized by anti-D4E antibodies were E protein-related. Anti-D4E antibodies, however, did not react with the supernatant of uninfected brain homogenate by Western blot (data not shown).

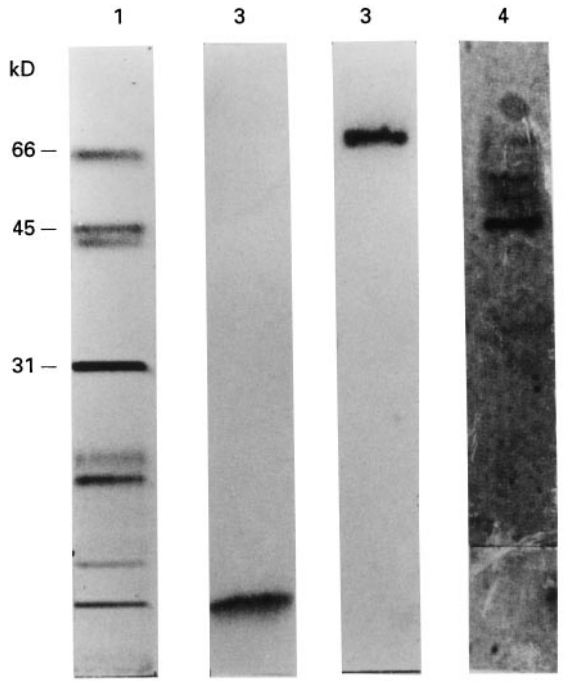

Fig. 3. Western blot analysis of dengue virus E protein (D4E)-hyperimmune serum. Samples of D4E (lane 2), plasminogen (lane 3), and dengue virus stock (lane 4) were run on 12% SDS–PAGE and transferred to nitrocellulose paper as described in Materials and Methods. Rabbit anti-D4E-hyperimmune sera were diluted 20-fold in PBS as primary antibody. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was used as secondary antibody. Lane 1: molecular mass standard.

Inhibition of plasmin activity by sera from immunized animals and dengue patients

Incubation of plasmin with sera from naive mice caused about a 10% decrease in plasmin activity compared with the activity of plasmin incubated without serum. This may be due to the plasmin inhibitors in serum. In addition, a 53% inhibition was noticed when plasmin was preincubated with murine D4E-hyperimmune sera. When sera from mice with different concentrations of antibodies against plasminogen were used, a positive correlation between the degree of plasmin function inhibition and the concentration of anti-plasminogen antibodies in murine sera was found (Fig. 4). Plasmin activity decreased with an increase in the concentration of antibodies. Significant inhibition of plasmin activity was also found in sera of dengue patients. Normal human serum reduced plasmin activity to 90·22 ± 1·56%, while dengue patients' sera reduced it to 46·92 ± 2·62% ($n = 33$, $P < 0·001$). A similar inhibitory effect was also seen in sera of D4E-immunized rabbits, but not normal rabbits. Normal rabbit sera reduced plasmin activity to 84·76 ± 6·66%, while D4E-immunized rabbit sera reduced it to 61·59 ± 7·09%.

Purified IgG from D4E-immunized rabbits inhibits plasmin activity

To confirm that the inhibition was due to the antibodies developed after D4E immunization, IgG purified by protein A affinity column from both normal and D4E-immunized rabbit sera was compared for inhibition of plasmin activity. There was about twice as much inhibition when the plasmin was preincubated with 5 μg/ml IgG from D4E-immunized sera as was found when normal sera were used (25·3% *versus* 15·8% inhibition) (Fig. 5). In addition, when affinity-purified rabbit anti-PL + antibodies were used, inhibition of plasmin activity reached as high as 40% in the presence of 5 μg/ml anti-PL + antibodies (Fig. 5). A dose-dependent inhibition of plasmin activity was noticed.

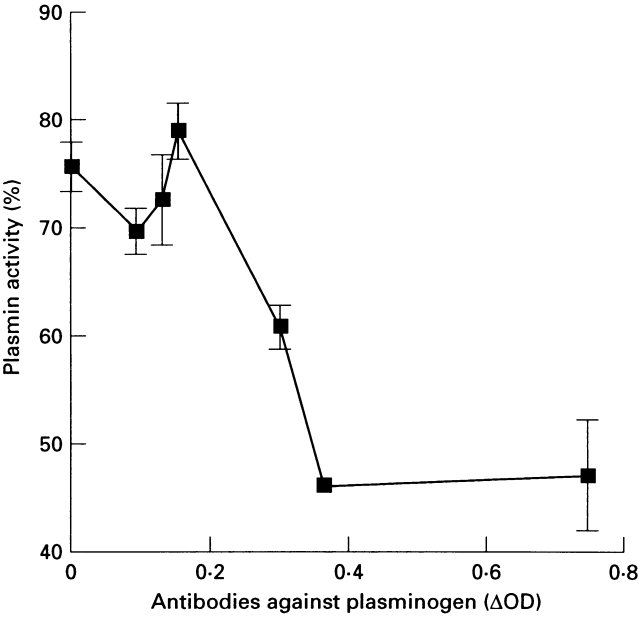

Fig. 4. Correlation between anti-plasminogen antibody concentration and plasmin activity inhibition. Twenty microlitres of 100-fold diluted sera from different mice were preincubated with 0·1 μM plasmin for 1 h at 37°C. Thirty minutes after adding substrate, absorbance was read at OD 405 nm. Data represent the mean ± s.d. of six samples.

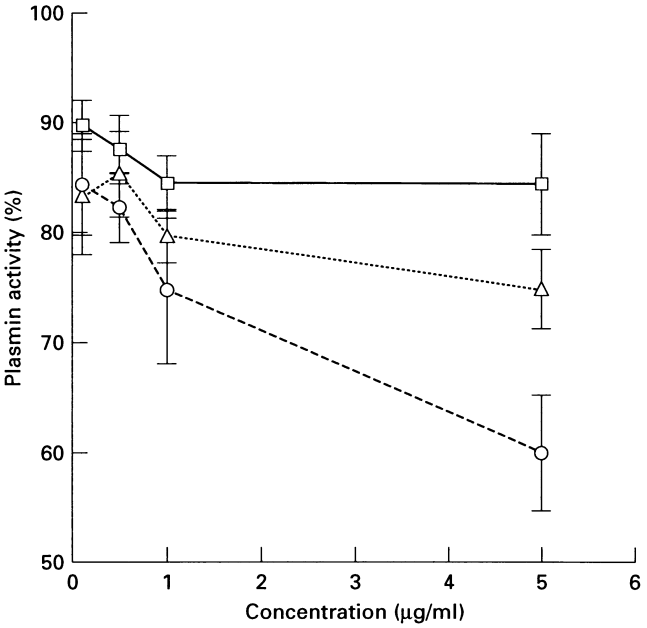

Fig. 5. Inhibition of plasmin activity by affinity-purified antibodies from normal (□) and dengue virus E protein (D4E)-immunized rabbit sera (IgG, Δ; anti-PL+, ○). Antibodies were purified from rabbit sera by protein A and PL+ affinity columns as described in Materials and Methods. Different doses (0·1, 0·5, 1 and 5 μg/ml) of rabbit immunoglobulins were preincubated with 0·1 μM of plasmin for 1 h at 37°C. Thirty minutes after adding substrate, absorbance was read at OD 405 nm. Data represent the mean ± s.d. of six samples.

Free D4E and PL+ peptide specifically compete for antibody-induced plasmin inhibition

To substantiate further that the inhibition of plasmin function was not due to other inhibitors in the sera, sera were heat-inactivated before incubation with plasmin. This procedure completely blocked the inhibition of plasmin activity by normal sera, but not dengue virus-hyperimmune sera, which still caused a 25% decrease in plasmin activity (Table 2). In addition, the effect of murine antisera on plasmin activity was competitively inhibited in the presence of free D4E or PL+ peptides (Table 2). Anti-PL+ antibodies purified from either dengue patients or D4E-immunized rabbit sera also had an inhibitory effect on plasmin activity. Moreover, rabbit and human antibody-mediated plasmin inhibitions were blocked by free D4E or PL+ peptides (Table 2), but not by negative control peptide CM1 (data not shown).

Table 2. Peptide competition for antibody-induced inhibition of plasmin function

	Plasmin activity (%)*		
	Without peptide	D4E 1 μg	PL + 1 μg
Normal sera	104·27 ± 0·86		
Rabbit sera†	81·12 ± 1·23	112·46 ± 8·86	96·81 ± 8·69
Mice sera‡ ×10§	76·00 ± 2·54	106·36 ± 4·55	104·00 ± 7·26
×100§	89·37 ± 0·64	100·90 ± 2·25	99·27 ± 3·62
Anti-PL+ from rabbit¶	76·00 ± 4·12	102·54 ± 3·76	96·00 ± 5·52
Anti-PL+ from patient¶	90·64 ± 2·96	95·27 ± 4·63	116·36 ± 8·93

*Plasmin activity was determined as described in Materials and Methods. All sera were incubated at 56°C for 30 min to inactivate protease. Data represent the mean ± s.d. of two to four separate experiments.

†Rabbits were immunized and boosted with dengue virus E protein (D4E) peptide as described in Materials and Methods.

‡Mice were immunized with dengue virus as described in Materials and Methods.

§Mice sera were diluted 10- or 100-fold with activating buffer, respectively.

¶Anti-PL+ antibodies were purified by affinity column as described in Materials and Methods. Concentrations of antibodies from rabbits and patient used in this experiment were 5 and 2 μg/ml, respectively.

DISCUSSION

In this study we demonstrate that anti-D4E antibodies not only bound to human plasminogen but also inhibited plasmin activity. Plasminogen-cross-reactive antibodies were found in D4E-immunized animals (mice and rabbits), dengue virus-hyperimmunized mice and dengue virus-infected patients. Therefore, antibodies against this plasminogen-cross-reactive site were also generated against dengue virus in mice and natural infection in humans. In addition, anti-D4E antibodies were detected in patients infected with different serotypes of dengue virus. This is consistent with a previous report, indicating that the detection of plasminogen-cross-reactive antibodies was not related to the serotype of the infecting virus [5]. However, unlike the previous report, which suggests that anti-D4E antibodies are transient and occur mostly during acute stage [5], we found that they were still detectable even 1–4 months after infection. Nevertheless, these results suggest amino acid 100–119 of dengue virus E protein is a highly conserved immunogenic region among different serotypes of dengue viruses for the antibody response.

The mechanisms by which plasminogen-cross-reactive antibodies might interfere with plasmin function are still unknown. Since the N-terminus of the cross-reactive site of PL+ lies 19 amino acids downstream from the serine-active site in plasmin, the inhibition of plasmin activity by plasminogen-cross-reactive antibodies could result from steric hindrance of binding of substrate. Similar inhibitory effects to plasmin were also found in a previous study using MoAbs generated against human plasminogen, indicating this is not a unique phenomenon [16]. An alternative effect of these plasminogen-cross-reactive antibodies on plasmin function would be that the binding of the antibodies induces conformational changes in such a way that the plasmin formed is less inhibitable by inhibitors such as α_2-antiplasmin and α_2-macroglobulin. Preliminary results suggest that this may also be true, since PL+ -binding antibodies affect plasminogen activity in the presence of α_2-antiplasmin [6]. Therefore, different effects of these plasminogen-cross-reactive antibodies on plasmin activity may occur at different levels in the fibrinolytic system.

The significance of plasminogen-cross-reactive antibodies in the pathogenesis of dengue virus infection and DHF/DSS is, however, unclear. Since D4E-immunized mice did not show any sign of abnormal haemorrhage and DF patients still have these antibodies even after recovery, it is unlikely anti-D4E antibodies alone have a significant pathogenic effect. However, it has been reported that dengue virus infection enhances the production of plasminogen activator inhibitor (PAI) from monocytes without altering plasminogen activator (PA) or procoagulant activity [17,18]. Increase in the production of PAI in dengue virus-infected

cells would inhibit plasminogen activation by PA and decrease production of plasmin. Such an effect of dengue virus on monocytes provides the potential for dengue-infected monocytes to modulate fibrinolysis at a localized site. D4E antibodies cross-reacting with plasminogen will provide an additional mechanism by which plasmin activity is inhibited in dengue patients. The inhibition of plasmin activity during dengue virus infection may enhance the clot formation at virus-infected sites and disturb the balance between the coagulation and the fibrinolysis, which may, in combination with other factors such as vasculopathy and thrombocytopenia, lead to haemorrhage, as found in patients [6].

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