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ΕΡΕΥΝΗΤΙΚΗ ΕΡΓΑΣΙΑ

A preliminary report on phospholipase A2 acidic 1 precursors for the detection of antibody coated platelets

OBJECTIVE **It has been reported that several snakes of the Trimeresurus group of the pit viper family produce venoms which have effects on the platelets, such as aggregation or induction of release from of granules. *Trimerusus albolabris* is a green pit viper commonly found in Thailand. Its venom can cause platelet aggregation. This study attempted to use this function as a tool for the detection of antibody coated platelets from patients with systemic lupus erythematosus (SLE).** METHOD **Platelets from normal healthy individuals and patient with SLE were recovered and washed to remove the fibrinogen. Some of the platelets from the healthy individuals were coated with anti GP IIb/IIIa. The samples of coated and uncoated platelets and those from the patients with SLE were each mixed with a venom fraction containing phospholipase A2 acidic 1 precursors derived from *T. albolabris* and incubated at 37 °C for 30 minutes.** RESULTS **The samples were observed under light microscope for aggregation of platelets. The uncoated platelets were observed to be aggregated, but there was no aggregation in the samples of coated normal platelets and platelets from the patient with SLE.** CONCLUSIONS **This preliminary report will open up a new outlook in the area of antibody detection in SLE and it appears that *T. albolabris* venom may be of use as a tool for monitoring disease activity.**

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Προκαταρκτική αναφορά στις πρόδρομες μορφές της φωσφολιπάσης για την ανίχνευση αιμοπεταλίων συνδεδεμένων με αντισώματα

Περίληψη στο τέλος του άρθρου

Key words

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Venom from snakes demonstrates toxicity in many systems, among which are blood components such as the platelets. The venom from cobras and related species can consume complement.[1] The fraction of purified venom can alter the *in vivo* tests of platelet function and clot retraction, with prolonged lysis times and increase of PF3.[2] The possible mechanism suggested for the aggregatory activity may be membrane effects, and calcium (Ca^{2+}) may play a role in the response. The venom of pit viper is generally recognized to contain a thrombin-like enzyme. One of the best characterized types of venom that disturbs normal coagulation is Arvin, a purified fraction of venom from the Malayan pit viper, *Agkistrodon rhodostoma*. This type of venom induces defibrination with excessive bleeding[3] and with minor inhibition of platelets exposed *in vivo*.[4] Several snakes of the Trimeresurus group, such as the Okinawa pit viper *Trimeresurus okinavensis,* produce venom that contains a component which can aggregate platelets and induce the release of dense granules and alpha granule contents.[5] *T. flavoridis* (habu) venom can cause capillary

hemorrhage by damaging endothelial gaps, resulting in rapid bleeding, and it also causes thrombocytopenia and glomerulonephritis.[6,7] *T. erythrurus* and *T. popeorum* venoms also aggregate platelets *in vitro,* but that of the *T. erythrurus* is the more potent.[8] The venom of *T. mucrosquamatus,* the Chinese or Formosan habu, has multiple fractions with differing activities, some fibrinogenolytic, and others anticoagulant, with Ca^{2+} dependent activity. *In vivo* testing of *T. mucrosquamatus* venom showed that it can decrease the platelet count in rabbits to 10–20% of the total, and *in vitro* testing demonstrated aggregation with nucleotide release.[9,10]

In this study the venom fractions from the green pit viper *T. albolabris* were tested *in vitro* with samples of platelets, some of which demonstrated potent aggregating activity. Phospholipase A2 acidic 1 precursors in the venom were tested for their capability of detection of antibody coated platelet aggregation in systemic lupus erythematosus (SLE). The aggregation must be activated through the receptors GPIIb/IIa or GPIB/IX, and SLE is a disease that produces antibodies to all of these receptors, which constituted the rationale for performing this experiment.

MATERIAL AND METHOD

Preparation of washed platelets

Ten mL of blood were collected from normal healthy individuals and patients with known SLE, with EDTA as the anticoagulant. The patients with SLE were students who had been diagnosed by the physician (known cases) and the disease free control subjects were healthy teenage students in the same class. From each sample platelet rich plasma (PRP) was obtained by centrifugation at 1,500 rpm for 10 minutes. The whole PRP was mixed with 150 mL of buffer (113 mM NaCl, 4.3 mM K_2HPO_4, 24.4 mM NaH_2PO_4, and 5.5 mM glucose) and centrifuged again at 3,000 g. After centrifugation most of the supernatant was discarded, leaving 3–5 mL of pellet to which was added 100 mL of buffer (20 mM Hepes, 140 mM NaCL, 4 mM KCl, and 5.5 mM glucose) and the sample was centrifuged again at 3,000 g. At this step the concentration of platelets was adjusted to the range of 150,000–200,000 cells/µL and was ready for use in the test system. A number of platelets in a higher or lower range will affect the result.

Lyophilized *T. albolabris* venom 1 mg was dissolved in deionized water then subjected to SDS-PAGE electrophoresis, the system being composed of 12% separating gel (30% acrelamide, 1.5 M Tris pH 8.8, 10% ammonium persulfate, 10% SDS and TEMED) and 5% stacking gel (30% acrelamide, 1.0 M Tris pH 6.8,10% ammonium persulfate, 10% SDS and TEMED). Mixed equal volumes of loading buffer and venom protein were then denatured at 95 °C for 5 minutes. Each well of electrophoresis contained 5 micrograms of protein and electrophoresis was performed using a constant voltage of 140 V for 1 hour and 45 minutes. The gel was stained with Coomassie blue and then destained overnight in distilled water.

Identification of protein was made by the MALDI-TOF mass spectrophotometer. Each protein band was cut and eluted in PBS pH 7.4. The protein concentrations of the eluted proteins were determined by Bradford Protein Assay and they were tested for platelet aggregation. The selected proteins were digested into peptides then combined with matrix and left to dry. The matrix was then analyzed by the MALDI-TOF spectrophotometer. Peptide mass fingerprints were matched with those in the database of MASCOT; www.Matrixscience.com

For demonstration of platelet aggregation, the platelet rich plasma of the normal individuals and the patients with SLE were mixed with each fraction of protein and incubated at 37 °C for 30 minutes then observed under light microscopy.

In a portion of the sample from the healthy individuals, the normal platelets were coated with anti GPIIb/IIa (Dako) which was diluted to 1:10 with PBS pH 7.4 and incubated for 30 minutes at 37 °C. The platelets were washed once and diluted to achieve the concentration of 150,000–200,000 cells/µL. This was found to be the optimal dilution, and was obtained from trying different dilutions; if the platelets are too diluted this may also cause aggregation.

RESULTS

Nine bands of protein were found on SDS-PAGE electrophoresis, as seen in figure 1. Some bands were able to cause platelet aggregation to varying degrees; thus, bands number 2, 3, 4, 7 and 8 were selected for analysis by MALDI-TOF mass spectrophotometer. The results of fingerprint matching are shown in table 1. It was found that the band 7, which corresponded to the phospholipase A2, acidic 1 precursors, showed a potent aggregation of normal platelets as illustrated in figure 2. It was also found that the platelets coated with IgG from SLE and normal platelets coated with GP IIb/IIIa were not aggregated by the phospholipase A2 acidic 1 precursors, as seen in figure 2.

DISCUSSION

The incidence of SLE has not yet been documented in Thailand. Over the last five years, three students, all female, who were enrolled to study Clinical Laboratory Science in the Faculty of Allied Health Sciences at Chulalongkorn University, were diagnosed as having SLE, and they were able to complete their four-year studies. During the four-year period, their clinical symptoms fluctuated during treatment with corticosteroids. Analysis of their blood samples was made in this study even during episodes of thrombocytopenia. The potency of aggregation was

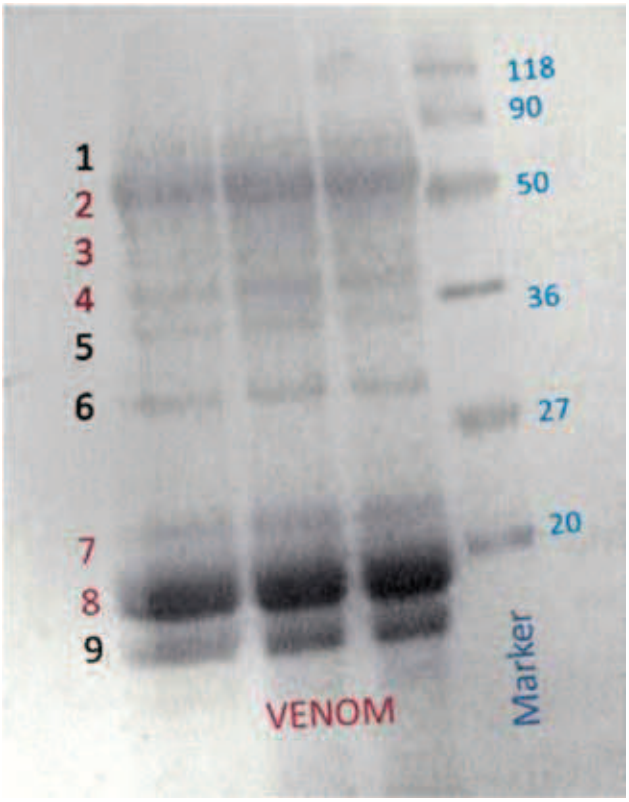

Figure 1. SDS-PAGE of *Trimeresurus albolabris* venom showing the components of venom proteins.

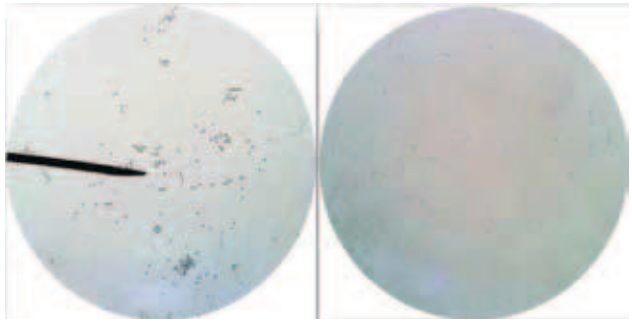

Figure 2. Aggregation patterns of phospholipase A2, acidic 1 precursor. Aggregation of platelet from normal healthy person (×1,000); aggregation pattern of SLE patient (×1,000).

observed to decrease along with the disease activity. The testing was repeated several times and the verbal accounts of the students about the disease activity were recorded at the time of appointment with their doctors who also scrutinized their blood smears and their anti DNA by latex agglutination. This experimental preliminary test may prove to be a useful tool in monitoring disease activity in LSE. The major targets of autoantibodies to platelets are platelet membrane glycoproteins, including GPIIb/IIIa and GPIb/IX.[11] A report by Lipp et al[12] postulated that anti-GPIIb-IIIa antibodies may be more closely related to the severity of thrombocytopenia. The anti GPIIb/IIa and anti-thrombopoietin receptor (anti-TPOR) antibody responses were more frequent in SLE patients with thrombocythemia than in those without thrombocythemia, but the types of clinical presentation associated with these autoantibodies are different.[13] Among patients with SLE with thrombocytopenia, 60% were shown to have antiplatelet antibodies against GPIIb/IIa, GPIb/IX or GPIV,[14] but the glycoprotein GPIIb/IIIa complex is the most abundant platelet receptor.[15] The *T. albolabris* venom Alboluxin is a potent platelet agonist acting via GPIb and GPV[16] of which anti GPIb/IX continues to be present in SLE. Both Alboluxin and phospholipase A2 acidic 1 precursor can be as a tool for the detection of antibody coated platelet in SLE. The test system is quite simple and the snake venom can be obtained from snake farms. Currently the purified protein can be produced in the research laboratory but nowadays application of the polymerase chain reaction (PCR) can overcome such purification and cloning of the protein may be an alternative. Some drawbacks may be encountered such as a very low platelet count in SLE during thrombocytopenia; it was very difficult to get the level up to 150,000–200,000 cells/μL for the experiment in some cases. As reported above these students with SLE were receiving corticosteroid treatment. In the periods when the disease was in a "silent" phase, they had no rash, normal CBC, normal urinalysis and,

Table 1. Peptides matched of eluted proteins of selected bands.

Band no	App. mass/pI	Theor. mass/pI	No of peptides matched	Sequence coverage (%)	Score	Protein
2	31,945/6.9	26,851/6.90	5	21	60	Ophanin precursor (Opharin)
3	13,139/5.5	13,447/4.47	5	58	74	Phospholipase A2
4	9,904/6.3	9,463/9.19	2	66	40	mCG147719
7	13,960/5.2	16,433/5.67	5	37	81	Phospholipase A2, acidic 1 precursor
8	9,904/5.7	7,072/5.83	2	45	40	Ring finger protein 185

most importantly, the anti DNA titer was low or negative by latex agglutination test. Although it cannot be certain that there was no pathology during the silent periods, at that time there were very few aggregations. Thus, this test using phospholipase A2 acidic 1 precursors may be of use for monitoring the disease activity in SLE.

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ΠΕΡΙΛΗΨΗ

Προκαταρκτική αναφορά στις πρόδρομες μορφές της φωσφολιπάσης για την ανίχνευση αιμοπεταλίων συνδεδεμένων με αντισώματα

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ΣΚΟΠΟΣ Έχει αναφερθεί ότι διάφορα φίδια της ομάδας *Trimeresurus* των κροταλιδών παράγουν δηλητήριο που προκαλούν συνάθροιση των αιμοπεταλίων ή απελευθέρωση των κοκκίων τους. Το *Trimerusus albolabris* είναι φίδι που απαντάται συχνά στην Ταϊλάνδη και το δηλητήριό του προκαλεί συνάθροιση των αιμοπεταλίων. Στην παρούσα έρευνα χρησιμοποιείται αυτή η λειτουργία ως εργαλείο ανίχνευσης αιμοπεταλίων συνδεδεμένων με αντισώματα στο συστηματικό ερυθηματώδη λύκο (ΣΕΛ). **ΥΛΙΚΟ-ΜΕΘΟΔΟΣ** Παρασκευάστηκαν αιμοπετάλια από φυσιολογικά άτομα, καθώς και από πάσχοντες από ΣΕΛ και έγινε πλύσιμο με σκοπό την απομάκρυνση του ινωδογόνου. Επίσης, αιμοπετάλια από φυσιολογικά άτομα συνδέθηκαν με αντι-GPIIb/IIIa. Τα φυσιολογικά αιμοπετάλια και εκείνα από ΣΕΛ συνδέθηκαν και αποσυνδέθηκαν με το δηλητήριο και το μίγμα επωάστηκε στους 37 °C για 30 min. **ΑΠΟΤΕΛΕΣΜΑΤΑ** Η συνάθροιση παρατηρήθηκε στο οπτικό μικροσκόπιο. Τα μη συνδεδεμένα αιμοπετάλια συναθροίστηκαν αλλά δεν παρατηρήθηκε συνάθροιση στα συνδεδεμένα αιμοπετάλια τόσο των φυσιολογικών ατόμων όσο και εκείνων με ΣΕΛ. **ΣΥΜΠΕΡΑΣΜΑΤΑ** Σε αυτή την πρόδρομη αναφορά δίνεται μια νέα άποψη για την αναζήτηση αντισωμάτων στο ΣΕΛ και πιθανόν να καταστεί χρήσιμη ως μέθοδος παρακολούθησης της δραστηριότητας της νόσου.

Λέξεις ευρετηρίου: Φωσφολιπάση Α2, Αιμοπετάλια

References

1. BIRDSEY V, LINDORFER J, GEWURZ H. Interaction of toxic venoms with the complement system. *Immunology* 1971, 21:299–310
2. DODDS WJ, PICKERING RJ. The effect of cobra venom factor on hemostasis in guinea pigs. *Blood* 1972, 40:400–411
3. CHAN KE. The comparison of the antitomboc action of the thrombin-like fraction of Malayan pit viper venom and heparin. *Cardiovasc Res* 1969, 3:171–178
4. PRENTICE CR, TURPIE AG, HASSANEIN AA, McNICOL GP, DOUGLAS AS. Changes in platelet behaviour during arvin therapy. *Lancet* 1969, i:645–647
5. DAVEY MG, ESNOUF MP. The isolation of a component of the venom of *Trimeresurus okinavensis* that causes the aggregation of blood platelets. *Biochem J* 1969, 111:733–743
6. OSHIO C, TSUCHIYA M, OHSAKA A. The behavior of platelets in capillary hemorrhage induced by snake venom. *Microvascular Res* 1973, 17:S49–S55
7. CATTELL V, BRADFIELD JWB. Focal mesangial proliferative glomerulonephritis in the rat caused by habu snake venom. *Am J Pathol* 1977, 87:511–524
8. MITRAKUL C. Effects of green pit viper (*Trimeresurus erythrurus* and *Trimeresurus popeorum*) venoms on blood coagulation, platelets and the fibrinolytic enzyme systems: Studies *in vivo* and *in vitro*. *Am J Clin Path* 1973, 60:654–662
9. OUYANG C, TENG CM, CHEN YC. Physicochemical properties of alpha- and beta-fibrinogenases of *Trimeresurus mucrosquamatus* venom. *Biochem Biophys Acta* 1977, 481:622–630
10. OUYANG C, TENG CM. The effect of *Trimeresurus mucrosquamatus* snake venom on platelet aggregation. *Toxicon* 1978, 16:575–582
11. KUWANA M. Autoantibodies to platelets: Roles in thrombo-

cythemia. *Inflammation and Regeneration* 2009, 29:40–46
12. LIPP E, VON FELTEN A, SAX H, MÜLLER D, BERCHTOLD P. Antibodies against platelet glycoproteins and antiphospholipid antibodies in autoimmune thrombocytopenia. *Eur J Haematol* 1998, 60:283–288
13. KUWANA M, KABURAKI J, OKAZAKI Y, MIYAZAKI H, IKEDA Y. Two types of autoantibody-mediated thrombocytopenia in patients with systemic lupus erythematosus. *Rheumatology (Oxford)* 2006, 45:851–854
14. MACCHI L, RISPAL P, CLOFENT-SANCHEZ G, PELLEGRIN JL, NURDEN P, LENG B ET AL. Anti-platelet antibodies in patients with systemic lupus erythematosus and the primary antiphospholipid antibody syndrome: Their relationship with the observed thrombocytopenia. *Br J Haematol* 1997, 98:336–341
15. CALVETE JJ. Platelet integrin GPIIb/IIIa: Structure-function correlations. An update and lessons from other integrins. *Proc Soc Exp Biol Med* 1999, 222:29–38
16. DU XIAO-YAN, MAGNENAT E, WELLS TN, CLEMETSON KJ. Alboluxin, a snake C-type lectin from *Trimeresurus albolabris* venom is a potent platelet agonist acting via GPIb and GPVI. *Thromb Haemost* 2002, 87:692–698

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