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Identification of 50 kDa snake venom proteins which specifically inhibit platelet adhesion to collagen

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Of 32 snake venoms tested, the crude venoms of four (*B. atrox, B. jararaca, A. halys blomhoffii* and *C. basiliscus*) showed strong inhibitory activity in an assay of platelet adhesion to collagen. Active 50 kDa proteins were purified to homogeneity from each venom and found to be rich in cysteine on amino acid analysis. A monoclonal antibody raised against the purified *B. atrox* protein crossreacted strongly with the 50 kDa proteins from *B. jararaca* and *A. halys blomhoffii* and weakly with the protein from *C. basiliscus*, indicating that all four proteins possess a similar epitope. The proteins inhibited platelet aggregation induced by collagen but not by other agonists.

Adhesion; Collagen; Platelet; Snake venom

1. INTRODUCTION

Interference with blood coagulation is one of the main causes of the pathological manifestation in snakebites by the families of *Crotalidae* and *Viperidae* [1]. Recently, another mechanism by which snakebites may interfere with hemostasis was discovered in trigramin, a naturally-occurring low molecular weight peptide from the venom of *Trimeresurus gramineus* [2]. This peptide inhibits platelet aggregation by specifically and competitively inhibiting fibrinogen binding to the receptors associated with the GPIIb/GPIIIa complex in activated platelets. Having recently developed a method to study the adhesion of platelets to collagen in the absence of platelet aggregation [3], we screened a number of crude snake venoms to determine whether any possessed proteins that inhibited platelet adhesion to collagen, a process thought to be mediated by receptors associated with the GPIa/GPIIa complex [4–6]. We report here the isolation and identification of very similar 50 kDa proteins present in 4 different snake venoms which have the ability to specifically inhibit the adhesion of human platelets to collagen.

2. MATERIALS AND METHODS

Snake venoms were purchased from Sigma and reconstituted in 0.9% NaCl at 10 mg/ml. The following venoms (all at a final concentration of 10 μg protein/ml) were screened: *Agkistrodon acutus, A. bilineatus, A. contortrix contortrix, A. contortrix laticinctus, A. contortrix mokason, A. halys blomhoffii, A. piscivorus leuostoma, A. piscivorus piscivorus, A. rhodostoma, Austrelaps superba, Bitis arietans, Bothrops asper, B. atrox, B. jararaca, B. neuwiedi, B. nummifer, B. schlegeli, Bungarus fasciatus, Bungarus multicinctus, Crotalus adamanteus, C. atrox, C. basiliscus, C. durissus terrificus, Dendroaspis jamesonii, Naja melanoleuca, Naja naja atra, Ophiophagus hannah, Pseudechis australis, Sepedon hemachatus, Sistrurus miliarius barbouri, Trimeresurus gramineus* and *Vipera ruselli*. The adhesion assays were performed using human platelets labeled with [^{3}H]oleic acid as described previously [3]. Antagonists to TxA_2, platelet activating factor, serotonin, ADP and fibrinogen (Arg-Gly-Asp-Ser) were always present. The snake venom proteins were added to platelet suspensions 1 min before the addition of collagen and incubation was continued for 3 min at 37°C. Following incubation, the platelet suspensions were decanted into a manifold containing a 10-μm nylon disk under vacuum. The disks containing the platelets that had adhered to collagen were rinsed and then the radioactivity determined. Platelet aggregation studies are described elsewhere [7]. Purified proteins from the 4 snake venoms were reduced, pyridylethylated with 4-vinylpyridine and subjected to amino acid analysis. A monoclonal antibody (mAb) directed against the protein from *B. atrox* was prepared in mice using standard fusion and limiting dilution and cloning procedures [8]. Hybridoma screening and titering were conducted using ELISA microtitre plate assay. The mAb 2C31 was developed in ascites tumors and purified using a recombinant Protein G-agarose column. It was identified to be of the IgG1 subclass. Western blotting of the 4 active snake venoms was performed following separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12%: non-reduced) as described [9]. After transfer to Immobilon (Millipore), the proteins were incubated with mAb 2C31 followed by incubation with a secondary alkaline phosphatase-conjugated antibody. The blot was developed with 5-bromo-4-chloro-3-indolyl-phosphate/Nitroblue tetrazolium.

3. RESULTS

The crude venoms from 4 snakes, three of which were South American vipers (*B. atrox, B. jaracaca* and *A. halys blomhoffii*) and one which was a North American Rattlesnake (*C. basiliscus*), showed strong inhibitory activity in the platelet adhesion assay. Several of the

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other crude venoms caused spontaneous platelet aggregation even in the presence of the receptor antagonists which may have masked inhibitory activity in the adhesion assay.

The active proteins from the 4 inhibitory snake venoms were purified initially by chromatography on a Sephadex G-100 column (2 × 45 cm) equilibrated and eluted with 0.9% NaCl at a flow rate of 6 ml per hour. In the case of all four proteins, inhibitory activity in the platelet adhesion assay eluted early from this column, usually in 15 ml. Further purification of the snake venom proteins was accomplished by reverse-phase HPLC on a Vydac C4 column (semi-preparative, 1 × 25 cm) using a 60-min linear gradient of acetonitrile in 0.1% trifluoroacetic acid. For all four snake venom proteins, biological activity eluted at approximately 70% acetonitrile in 0.1% trifluoroacetic acid. The purification of the four proteins was followed by SDS-PAGE, as shown for *B. atrox* in Fig. 1. The purified proteins migrated as a single band with an apparent M_r of 50 kDa in both reduced and non-reduced gels, suggesting that the inhibitory protein is composed of a single polypeptide chain. However, after storage at −20°C for a week or more, larger aggregates of the venom proteins were visible, apparently reflecting disulfide interchange.

The purified proteins from the four snake venoms were subjected to amino acid analysis. The three South American venom proteins (*B. atrox, B. jararaca* and *A. halys blomhoffii*) exhibited a strikingly high degree of similarity in amino acid composition, while less similarity was found with *C. basiliscus*. In particular, the South American venom proteins contained 13 methionine residues per molecule, while the protein from *C. basiliscus* contained only 5. Comparison of the 50 kDa proteins with other proteins revealed that the snake venom proteins are unusual in the large number of cysteine residues they possess (an average of 30 residues per molecule or 6.5 mol %). Attempts to determine the amino acid sequences of the proteins purified from *B. atrox, B. jararaca* and *A. halys blomhoffii* revealed that, while they lacked carbohydrate, they all had blocked NH_2-terminal residues. Using mAb 2C31 raised against the purified *B. atrox* protein, Western blot analysis of each of the four crude snake venoms revealed a single homogeneous protein with an M_r of approximately 50 kDa (Fig. 2). In the case of *C. basiliscus*, larger amounts of the venom were required for detection by immunoblotting as compared to the other snake venoms, indicating that the inhibitory protein in this venom was less immunoreactive.

The purified proteins inhibited platelet adhesion in a competitive fashion as shown for *B. atrox* in Fig. 3. The concentration required for 50% inhibition of adhesion was approximately 10 μg/ml, or 0.2 μM assuming a molecular weight of 50 kDa. Increasing the preincubation time with snake venom protein beyond one min did not increase the extent of inhibition (results not shown). In three experiments, the purified proteins obtained after HPLC were tested for their ability to inhibit platelet aggregation in citrated platelet-rich plasma. At 5 μg/ml, the *B. atrox* totally abolished platelet aggregation induced by 1 μg/ml collagen but had no effect on the rate of platelet aggregation induced by ADP, epinephrine, platelet-activating factor or the thrombox-

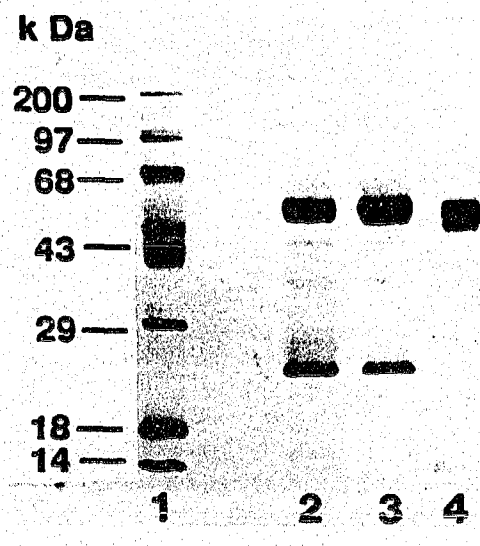

Fig. 1. Coomassie blue stain following SDS-PAGE (12%; non-reduced). Lane 1, molecular weight standards; lane 2, crude *B. atrox* venom; lane 3, *B. atrox* after Sephadex G-100 column chromatography; lane 4, *B. atrox* after HPLC.

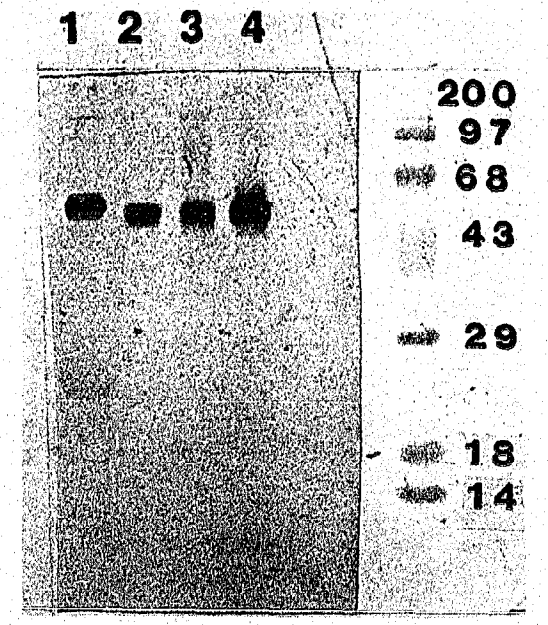

Fig. 2. Western blot analysis of crude snake venoms following SDS-PAGE (12%, non-reduced). Lane 1, *C. basiliscus* (20 μg); lane 2, *B. atrox* (200 ng); lane 3, *B. jararaca* (200 ng); lane 4, *A. halys blomhoffii* (200 ng). Molecular weight standards are shown on the right.

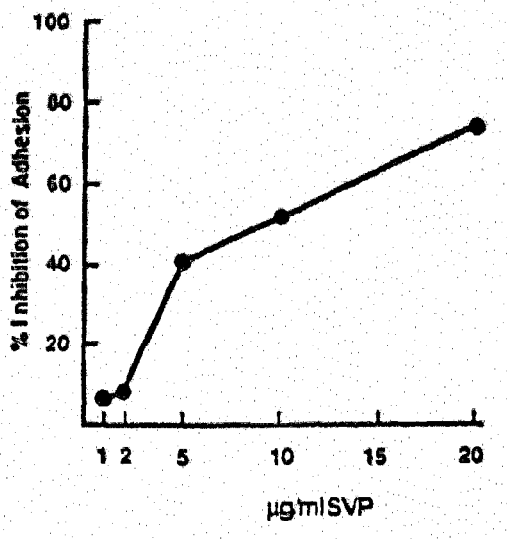

Fig. 3. Concentration-response curve for inhibition of platelet adhesion to collagen (25 μg/ml) by the purified anti-adhesive protein from *A. atrox* (SVP).

ane mimetic SQ 26655 (Fig. 4). In addition, the purified proteins inhibited both calcium mobilization and release of [^{14}C]serotonin in fura 2-loaded platelets stimulated by collagen in the presence of the feedback pathway antagonists (results not shown).

4. DISCUSSION

The adhesion of platelets to collagen is thought to play a major role in hemostasis and thrombosis. When platelets adhere to collagen they release adenine nucleotides and amines from their dense granules and, in addition, arachidonic acid is liberated from membrane phospholipids and converted to TxA_2 [10]. These agents induce platelet aggregation and the further release of dense granule constituents [11]. While it is currently accepted that Arg-Gly-Asp containing peptides [12], including snake venom proteins such as trigramin [2], inhibit platelet aggregation by competitively and specifically inhibiting platelet adhesion to fibrinogen, until now no specific inhibitors of platelet adhesion to collagen have been described. We have yet to determine how the snake venom proteins reported here specifically inhibit platelet adhesion to collagen without affecting aggregation induced by other agonists. However, we have been unable to detect proteolytic activity in the purified protein from *B. atrox* using either general protein substrates (casein, azo-casein and azo-collagen) or substrates specific for trypsin (*N*-benzoyl-Arg-*p*-nitroanilide), chymotrypsin (succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide) and collagenase (4-phenylazobenzyloxy-carbonyl-Pro-Leu-Gly-Pro-D-Arg). The possibility exists that the proteins initially possessed proteolytic ativity and that this was lost because of the use of trifluoroacetic acid and acetonitrile in the HPLC. As the 50 kDa proteins retain inhibitory activity against platelet adhesion to collagen even after HPLC it seems most likely that they act by binding to receptor sites either on platelets or on collagen.

The snake venom proteins described here differ from trigramin [2] in both molecular weight and amino acid composition. Moreover, they do not function by interacting at the fibrinogen receptor present on GPIIb/GPIIIa in activated platelets as all of our experiments were performed in the presence of Arg-Gly-Asp-Ser, an inhibitor of this receptor. Furthermore, the proteins reported here differ from another inhibitor of

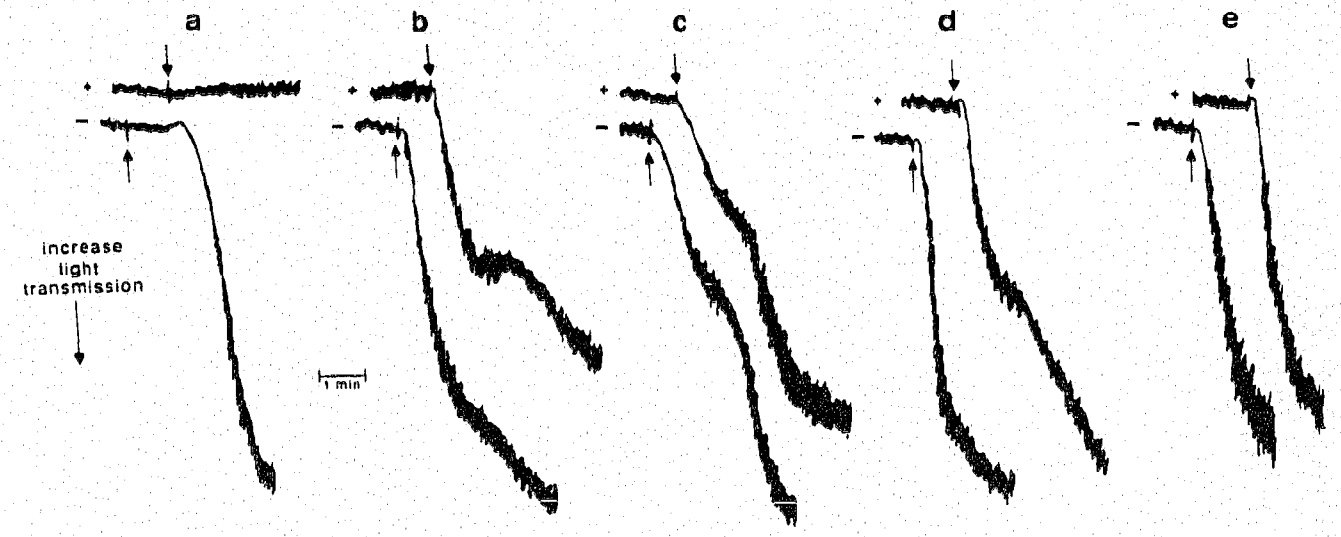

Fig. 4. Effects of the purified snake venom protein (5 μg/ml) from *B. atrox* (indicated by + sign) on aggregation in human citrated platelet-rich plasma induced by (a) collagen (1 μg/ml), (b) ADP (5 μM), (c) epinephrine (5 μM), (d) platelet-activating factor (0.25 μM) and (e) SQ 26655 (0.2 μM).

collagen-induced platelet aggregation recently described in *Bothrops jararaca* [13], as this latter protein had a molecular weight of approximately 13 kDa. Interestingly, HR1B, a 57-60 kDa hemorrhagic protein isolated from *Trimeresurus flavidoridis* was recently characterized as being a member of a new subfamily of metalloproteinases [14]. Additionally this protein contains a domain in which the Arg-Gly-Asp sequence is replaced by Glu-Ser-Glu-Cys. It is possible that the 50 kDa proteins described here contain a metalloproteinase domain at the NH_2-terminus [15] and a disintegrin-like sequence in the rest of the molecule. Attempts to elucidate the mechanism of action of our proteins are currently in progress.

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