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Sphingomyelinases in the Venom of the Spider *Loxosceles intermedia* Are Responsible for both Dermonecrosis and Complement-Dependent Hemolysis

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The bite of spiders of the genus *Loxosceles* can induce a variety of biological effects, including dermonecrosis and complement (C) dependent haemolysis. The aim of this study was to characterise the toxins in the venom responsible for the different biological effects. We have previously shown that a 35 kDa protein, named F35, purified from *Loxosceles intermedia* venom, incorporates into the membranes of human erythrocytes and renders them susceptible to the alternative pathway of autologous C. Here we have further purified the F35 protein which was resolved by reversed phase chromatography into three tightly contiguous peaks termed P1, P2, and P3. P1 and P2 were shown to be homogeneous by SDS-PAGE and N-terminal aminoacid analysis, while P3 consisted of two highly homologous proteins. N-terminal sequencing of all four proteins showed a high degree of homology, which was confirmed by cross-reactivity of antisera raised against the individual purified proteins. Functional characterisation of P1 and P2 indicated the presence of sphingomyelinase activity and either protein in isolation was capable of inducing all the *in vivo* effects seen with whole spider venom, including C-dependent haemolysis and dermonecrosis. In all assays, P2 was more active than P1, while P3 was completely inactive. These data show that different biological effects of *L. intermedia* venom can be assigned to the sphingomyelinase activity of two highly homologous proteins, P1 and P2. Identification of these proteins as inducers of the principal pathological effects induced by whole venom will aid studies of the mechanism of action of the venom and the development of a effective therapy. © 1998 Academic Press

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Envenomation by spiders belonging to the genus *Loxosceles* found either in North America (*L. reclusa*), in Middle Eastern desert areas (*L. deserta*) or in South America (*L. gaucho, L. laeta, L. intermedia*) commonly results in impressive local necrotic skin lesions and more rarely causes systemic effects. Skin lesions can take months to heal and in the most severe cases death may occur. Local necrosis is preceded by oedema, thickening of the vascular endothelium, accumulation of inflammatory cells, vasodilatation, intravascular clotting, degeneration of blood vessel walls and haemorrhage, culminating in formation of a black scar. The eventual sloughing of the necrotic tissues leaves a well-demarcated draining ulcer [1-4]. Neutrophils are implicated by association in formation of the skin lesions [5]. Mild systemic effects induced by envenomation, such as fever, malaise, pruritus and exanthema are very common, while intravascular haemolysis and coagulation, sometimes accompanied by thrombocytopenia and renal failure, are restricted to approximately 16% of the victims [6-8]. In Brazil alone, more than 1000 cases of envenomation by *L. intermedia* are reported each year and, because of a lack of understanding of the mechanism of action of the venom, an effective treatment is not available. Biochemical and functional characterisation of the active components in the venom may aid the development of a suitable therapy. We have recently isolated a 35 kDa protein (termed F35) from *L. intermedia* venom which incorporates into human erythrocytes, transforming these cells into strong activators of the alternative pathway of C in autologous serum [9]. Acquisition of C activating capacity or loss of C regulation by treated erythrocytes leads to the deposition of C fragments, including C3b and factor B, C3-convertase assembly and membrane attack complex (MAC) formation with haemoly-

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sis as the final outcome [9]. These findings identified *in vitro* may explain the intravascular haemolysis observed *in vivo* in the course of natural and experimental envenomation by *Loxosceles* venom. Indeed, injection of F35 into mice induces intravascular haemolysis and also provokes a cytokine response which resembles that seen in endotoxic shock [10].

To further analyse the mechanism by which F35 produces these diverse biological activities, we have examined whether a single protein species is responsible for all the observed activities. F35 isolated by a single-step gel filtration of venom is likely to contain several different molecules of similar size. In previous reports, addressing this question, rather impure venom fractions were used [9, 11-13]. Here, F35 was submitted to further purification by reversed phase chromatography which revealed three protein peaks, P1, P2 and P3 which were biochemically and immunologically highly homologous. While P1 and P2 were homogeneous, pure proteins, P3 consisted of 2 homologous proteins separable on SDS-PAGE. Either P1 or P2 could, in isolation, mimick the biological effects of the whole venom, transforming human erythrocytes into autologous C activators *in vitro*, inducing dermonecrosis *in vivo* and exhibiting sphingomyelinase activity. P3 was inactive in all assays performed.

MATERIAL AND METHODS

Chemicals and reagents. Tween 20 (polyoxyethylene sorbitan monolaurate) was from Bio-Rad Labs. (CA, USA); BSA (bovine serum albumine), sphingomyelin and *o*-phenyldiamine dihydrochloride were obtained from Sigma Chemical Co. (MO, USA). Horseradish peroxidase-labelled secondary antibody was purchased from Zymed Lab., Inc. (CA, USA). Buffers were: dextrose-gelatine veronal buffered saline ($DGVBS^{2+}$), pH 7.4, containing 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.1% gelatine; PBS, 10 mM Na Phosphate, 150 mM NaCl pH 7.2; TBS, 20 mM Tris, 150 mM NaCl pH 7.5.

Venom. Loxosceles intermedia (Mello-Leitão) spiders were provided by "Laboratório de Imunoquímica, Instituto Butantan, SP, Brazil". The venom was obtained by electrostimulation by the method of Bucherl [14], with slight modifications. Briefly, 15-20V electrical stimuli were repeatedly applied to the spider sternum and the venom drops were collected with a micropipette, vacuum dried and stored at −20°C. Stock solutions were prepared in PBS, pH 7.2, at 1.0 mg/ml.

Animals. Male BALB/c mice weighing 18-22 g and aged 7-8 weeks, were obtained from "Biotério de Camundongos Isogênicos, Universidade de São Paulo, SP, Brasil" and adult male New Zealand white rabbits were obtained from "Biotério do Instituto Butantan". The animals were maintained and used under strict ethical conditions according to the animal welfare international recommendations.

Fractionation of L. intermedia venom. Ten milligrams of freeze-dried venom were subjected to gel filtration on a Superose 12 HR column (10/30, FPLC; Pharmacia, Uppsala, Sweden), equilibrated and eluted with TBS at room temperature. Samples were collected at a 30 ml/h flow rate, and their protein content was monitored by recording the absorbance at 280 nm. Peak fractions were then pooled and assayed for their ability to promote human erythrocyte (HuE) lysis in the presence of normal human serum (NHS) as previously described [9]. The active fractions were pooled as F35.

The pooled active fractions obtained from gel filtration chromatography were submitted to reverse phase HPLC (LKB Bromma, 2152 LC controller) using a Wide-Pore Butyl C4 column (4.6 x 250 mm) (J.T. Baker Inc., NJ, USA). The column was eluted with a 20 ml linear gradient of acetonitrile 5-70% in 0.1% aqueous trifluoroacetic acid, at a flow rate of 1 ml/min followed by a 5 ml isocratic elution with 70% acetonitrile. Protein content was detected by its absorbance at 235 nm in a L.K.B. Bromma 2151 Variable Wavelength Monitor. Proteins were freeze dried, resuspended in PBS and assayed for the enhancement of lysis of HuE by NHS. The protein content of the samples was evaluated by Lowry method [15].

Electrophoresis. SDS-PAGE was performed, as previously described [16] using 10% (w/v) minigels (Bio-Rad Labs., CA, USA) and the protein bands were revealed by the silver stain method [17].

Amino terminal sequence analysis. Purified proteins were applied to a Prospin membrane (Applied Biosystems, Warrington, UK) reduced and alkylated using standard methods. Amino terminal sequence analysis was performed using an Applied Biosystems 476A protein sequencer and 610A data analysis software. The resulting sequences were compared and alignments made using the BLAST-NCBI algorithm [18].

Isoelectrofocusing. The isoelectric point determinations of the F35 proteins obtained after reverse phase HPLC chromatography were performed using a pH gradient of 3 to 9.3 and Phast System equipment (Pharmacia, Uppsala, Sweden). Gels were stained with Coomassie Brilliant Blue.

Normal human serum and erythrocytes. Type ORh^+ human blood was obtained from healthy donors. Blood samples drawn to obtain sera were collected without anticoagulant and allowed to clot, the NHS aliquoted and stored at −80°C. Blood samples drawn to obtain HuE for subsequent use as target cells were collected in citrate, washed in $DGVBS^{2+}$, and resuspended in the same buffer at $5x10^8$ cells/ml.

Treatment of HuE with Loxosceles venom proteins. To incorporate *Loxosceles* venom components into HuE, 1 vol. of HuE at $5x10^8$ cells/ml was incubated with an equal volume of a dilution in $DGVBS^{2+}$ of whole venom or purified fractions for 30 min at 37°C. Control samples were incubated with $DGVBS^{2+}$. Purified fractions did not induce spontaneous lysis of the cells. The cells were washed five times and resuspended to the original volume in $DGVBS^{2+}$.

Haemolysis assays. 200 μl of HuE pretreated with *Loxosceles* venom purified F35 fractions or $DGVBS^{2+}$ were mixed with 100 μl of NHS and the final volume adjusted to 500 μl in $DGVBS^{2+}$. Background or total cell lysis was evaluated by incubation of HuE with $DGVBS^{2+}$ or H_2O respectively. After incubation for 1h at 37°C, the reaction was stopped by adding 2ml of 0.85% saline, unlysed cells were spun down, the absorbance of the supernatant was measured at 541 nm and expressed as the number of haemolytic sites (Z). In each experiment, HuE and NHS were always from the same donor.

Production of antisera. Groups of Balb/c mice were injected intradermally with 500 ng of HPLC-purified F35 proteins absorbed on $Al(OH_3)$. The injections were repeated four times at weekly intervals. Blood samples were collected 1 wk after the last injection, and the serum was stored at −20°C.

Dermonecrotic activity. 200 μl of *L. intermedia* venom or its purified fractions in PBS were injected intradermally in the shaved back of adult rabbits. Control sites were injected with equal volume of PBS. The size of the lesions was measured after 24 h.

Sphingomyelinase activity. Sphingomyelinase activity of F35 and the HPLC-purified fractions was assayed by measuring the enzyme catalysed hydrolysis of sphingomyelin [11]. Reaction mixtures contained 30 μg sphingomyelin and 30 μg of *L. intermedia* venom, F35, P1, P2 or P3 samples in 100 μl of 20 mM Hepes buffer (pH 7.1) with

8mM $CaCl_2$. Alternatively, reactions mixtures were incubated in the presence of 20 mM Hepes buffer (pH 7.1) containing 10 mM EDTA. Controls were prepared without venom proteins. The samples were incubated for 2h, and then extracted with 1 ml of $CHCl_3$:CH_3OH (2:1). The $CHCl_3$ layer was removed and evaporated. Each sample was taken up in $CHCl_3$ and spotted on a silica gel G plate (Merck). The plate was developed with $CHCl_3$:CH_3OH:H_2O (65:35:5) and the bands visualised by spraying with molybdate reagent [19].

Enzyme immunosorbent assay. Antigenic cross-reactivities among the HPLC-purified F35 fractions were evaluated by ELISA. Plates were coated overnight at 4°C with 100 μl of 10 μg/ml of each fraction diluted in PBS, and blocked with PBS/5%BSA for 2 h. Normal or immune sera (from mice immunised with the pure proteins) diluted in PBS/1%BSA (100 μl) were added to the plates and incubated for 1 h. After washing the plates with PBS/0.1%Tween 20, 100 μl of peroxidase goat anti-mouse IgG, IgM and IgA were added and incubated for 1h at room temperature. Plates were washed and the reaction was developed with *o*-phenylediamine dihydrochloride and H_2O_2. Absorbances were measured at 490 nm in a microplate reader (Bio-Rad 3550-UV model).

RESULTS

Purification of L. intermedia F35 Venom Proteins

In order to identify and characterise the active components in the venom of *L. intermedia*, F35, purified from *Loxosceles intermedia* venom by Superose 12 gel-filtration chromatography, was further resolved by reverse phase Butyl C4 chromatography. Three tightly contiguous peaks were obtained and labelled P1, P2, P3 according to the sequence of the elution (Fig. 1A). P1 and P2 ran as single, sharp bands on SDS-PAGE, while P3 was a doublet (Fig. 1B). By isoelectric focussing a pI of 8.3 for P1 and 8.9 for P2 was determined (data not shown). In the same experimental conditions it was not possible to determine the pI of the proteins in P3, which suggests that they have a pI outside of the working range of the system (3-9.3).

Amino Terminal Protein Sequence

The HPLC purified proteins were analysed on an automated sequencer Applied Biosystems 476A. Unambiguous sequences of P1 and P2 were followed for 39 and 29 cycles of Edman degradation, respectively. P3 yielded an unambiguous sequence for most residues but some cycles yielded two residues, suggesting that this protein peak consisted of 2 distinct but highly homologous proteins. No blank cycles were obtained for any of the three proteins (Fig. 2). A high degree of homology (89%) between P1 and P2 was observed, while the percentage of homology between P1 and P3 and P2 and P3 was between 52-86% and 58-93%, respectively, considering the first 29 aminoacids only. Database searches in protein and DNA databases revealed an 89% homology of P1 with an orphan fragment of a spider venom protein from the brown recluse spider (*Loxosceles reclusa;* PIR no A38107: [20]). The homology of P2 and P3 with this spider venom protein was 86% and 83%, respectively.

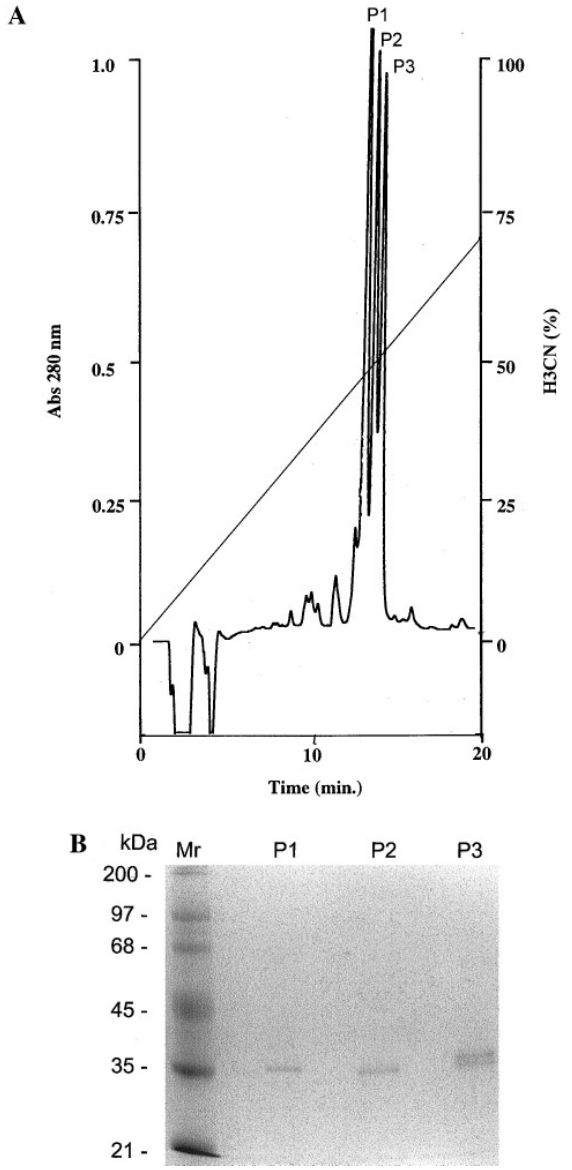

FIG. 1. Silica C4 reverse phase chromatography of the F35 *L. intermedia* venom fraction. [A] The F35 fraction obtained from gel filtration was submitted to reverse phase HPLC in a 20 ml linear gradient of acetonitrile from 5-70% in 0.1% aqueous trifluoroacetic acid at a flow rate of 1 ml/min followed by 5 ml isocratic elution with 70% acetonitrile. Protein content was detected by absorbance at 280 nm. [B] SDS-PAGE electrophoresis of the HPLC-F35 fractions (P1, P2 and P3).

A much lower, but perhaps significant homology (between 24% and 31%) was found with toxic phospholipases D (PLD) *from Corynebacterium pseudotuberculosis, Corynebacterium ulcerans* and *Arcanobacterium haemolyticum* (Genebank nos. 289914, I40839, I39484: [21, 22]). Significant homology of the purified proteins (between 62% and 79%) with the published sequences of the partially pure dermonecrotic factors from *L. gaucho, L. intermedia* and *L. laeta* was also detected (Genebank nos. 191168,

FIG. 2. Protein sequencing of P1, P2 and P3. Amino-terminal sequence of HPLC purified P1, P2 and P3 of *L. intermedia* aligned with amino acid sequences for venom toxin of *L. reclusa,* partially pure toxin of *L. intermedia, L. gaucho* and *L. laeta,* and phospholipases D from *C. pseudotuberculosis* and *A. haemolyticum* (both sequences derived from translation of cDNA sequence). Some residue insertions are required for alignment (-). Residues conserved between P1 and the other molecules are printed bold and marked (:). The sequence of P3 most homologous to P1 is used as the main sequence for P3. Residues obtained during the same cycles are printed below the P3 sequence. Sequences of *C. pseudotuberculosis* and *A. haemolyticum* start at residue 34 and 45, respectively, of the translated protein.

1911682, 1911683: [13]) (Table 1). The rather low sequence homology (68-79%) with the *L. intermedia* sequence obtained by Barbaro *et al.* is most likely due to errors in sequencing resulting from the relative impurity of that preparation [13].

Immunochemical Cross-Reactivity

Immunochemical cross-reactivities of P1, P2 and P3 were measured by ELISA. Monospecific polyclonal antisera were produce in mice by injection with purified P1, P2 or P3 proteins. These antisera were tested in ELISA using P1, P2 or P3 as antigens. As illustrated in Fig. 3, the antisera produced against P1, P2 and P3 were highly cross-reactive.

TABLE 1

Percentage Homology between P1, P2, P3 and Related Sequences Found in the Genbank Database

	P1	P2	P3
P1	100	89	52–86
P2	89	100	58–93
P3	52–86	58–93	100
L. reclusa	89	86	83
L. intermedia	79	72	68
L. gaucho	66	66	62
L. laeta	62	62	62
C. pseudo.	31	24	28
A. haemol.	34	27	31

Note. Percentage homology was calculated using the first 29 amino acids of each protein. Percentage homology of P1 and P2 with P3 was calculated using the lowest and highest matching sequences. Homology of P3 with other sequences was calculated using the sequence of P3 with the highest matching to P1.

Sphingomyelinase Activity

The venom of a spider species closely related to *L. intermedia, L. reclusa,* is known to have sphingomyelinase activity [23]. This induced us to test the purified *L. intermedia* proteins P1, P2 and P3 for sphingomyelinase activity by measuring hydrolysis of sphingomyelin. Crude venom, F35 and pure P1and P2 all possessed potent sphingomyelinase activity as shown by the breakdown of purified sphingomyelin (Fig. 4). P3 was completely inactive in this assay. The sphingomyelinase activity was inhibited by the addition of EDTA, indicating that their activities were dependent on Ca^{2+} (Fig. 4).

Induction of C-Dependent Haemolysis by Venom Proteins

To assess the ability of the *Loxosceles* spider venom fractions to induce C-dependent haemolysis, HuE were incubated with different amounts of *L. intermedia* venom, P1, P2, or P3 for 30 min at 37°C. Cells were washed and incubated for 60 min at 37°C with NHS as source of C, or buffer as control. The extent of cell lysis was determined and the Z number calculated. Fig. 5 shows that *L. intermedia* venom and the pure proteins P1 and P2 were each able to render HuE susceptible to lysis by autologous serum in a dose-dependent manner. In the same assay, P3 induced much less C-susceptibility and the low level of haemolysis after incubation with P3 was either due to a much lower enzymatic activity or possibly due to an incomplete separation from P2.

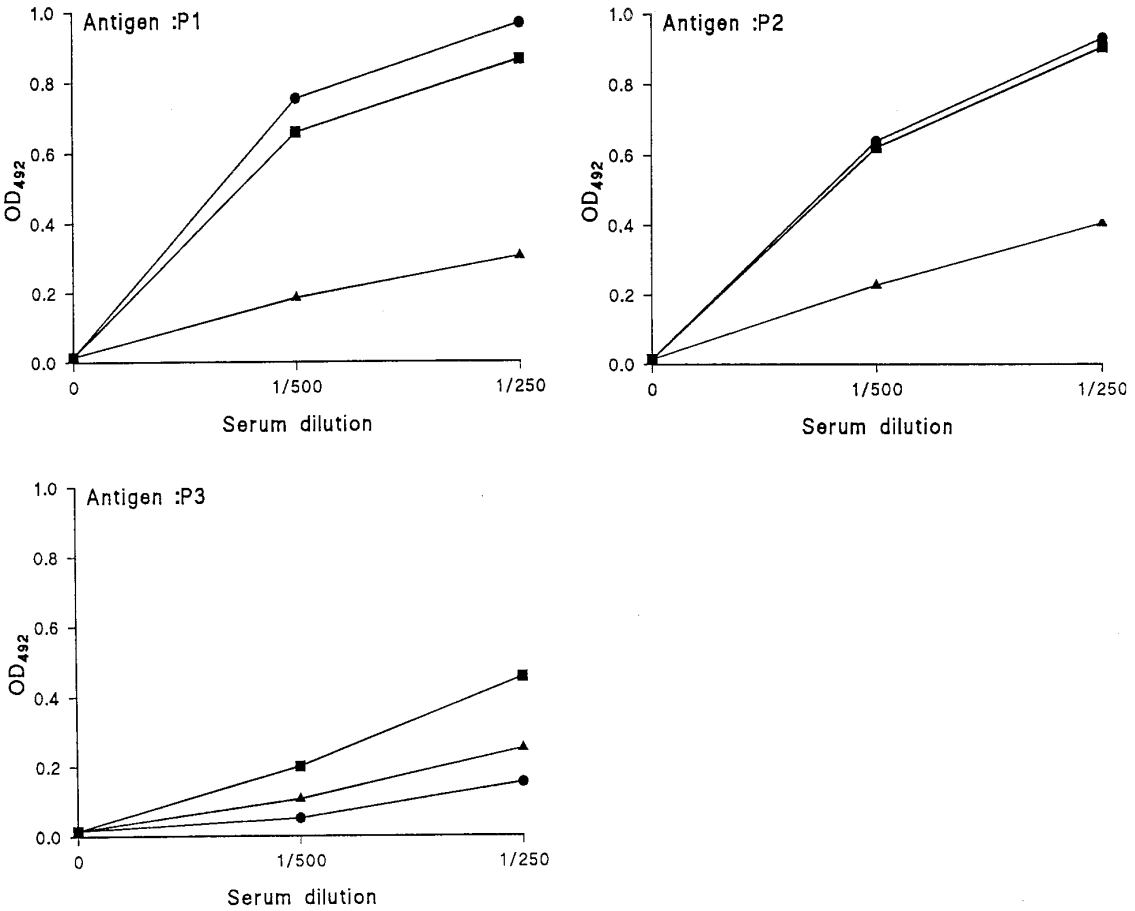

FIG. 3. Cross-reactivities among mouse sera anti-HPLC F35 proteins. ELISA plates were sensitised with P1 (Antigen P1), P2 (Antigen P2), P3 (Antigen P3) *L. intermedia* toxins, and incubated with mouse anti-P1 (●), P2 (■) or P3 (▲) sera diluted 1:250 or 1:500. The absorbance of the samples was determined at 490 nm.

Dermonecrotic Action of Venom Proteins

The ability of venom proteins to induce dermonecrotic lesions was tested by injecting rabbits with different amounts of P1, P2 or P3. The animals received buffer or *L. intermedia* venom as negative and positive controls, respectively. A typical loxoscelic lesion, as revealed by the presence of oedema, erythema and mild tenderness, developed in the skin area injected with venom within a few hours of injection. Approximately 24 h post injection, necrosis with gravitational spread and scar were observed at the inoculation site. Injection of P1 or P2 induced similar lesions but P3 was ineffective. At a given dose, P2 always induced bigger lesions than P1 and lesions also developed more quickly (Fig. 6).

DISCUSSION

Envenomation by spiders of the genus *Loxosceles* results in necrotic lesions and systemic reactions which can include haemolytic anaemia, haemoglobinemia, jaundice, haemoglobinuria, renal failure and death. In previous studies [9,10] we have demonstrated that the main toxic component of the *L.*

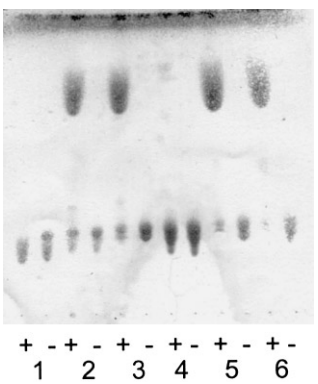

FIG. 4. Sphingomyelinase activity. Samples of sphingomyelin were incubated with the same volume of Hepes buffer (1), P1 (2), P2 (3), P3 (4), F35 (5) or *L. intermedia* venom (6) in the presence (A) or in the absence (B) of Ca^{2+}. After incubation the samples were subjected to Silica gel thin layer chromatography.

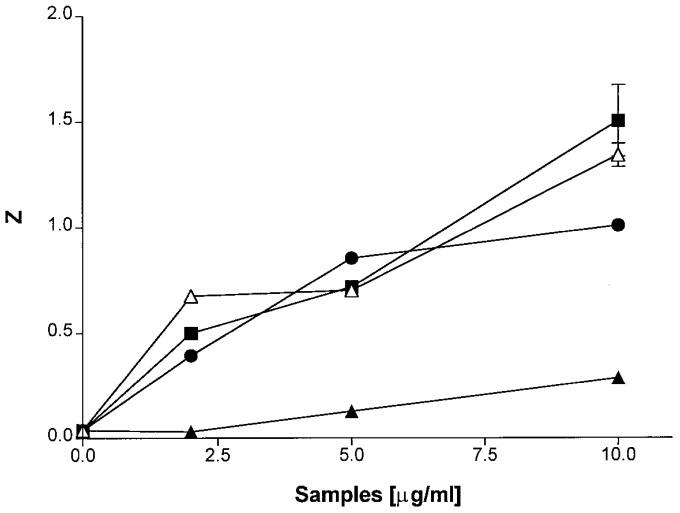

FIG. 5. Complement-dependent haemolysis induced by HPLC-F35 proteins. Human erythrocytes were treated with $DGVBS^{2+}$ or with P1 (●), P2 (■) or P3 (▲) or *L. intermedia* venom (△), washed, and incubated with NHS. The extent of haemolysis was determined spectrophotometrically at 541 nm, and expressed as the number of haemolytic sites (Z). Results are representative for 3 different experiments.

intermedia venom is a protein component of 35 kDa. This component was characterised as an inducer of C-mediated haemolysis, a dermonecrotic factor and also as a lethal factor to mice. In previous studies into the mechanism of action of envenomation by *Loxosceles* species by us and others [9,11-13], single step gel-filtration was used to purify the active components. These preparations always contained more than one protein. To verify whether all the biological activities of the whole venom could be ascribed to a single component, we further purified to homogeneity the components of the F35 protein which was obtained by Superose12 gel-filtration chromatography. By reverse phase HPLC chromatography the F35 fraction was resolved into three contiguous peaks which we termed P1, P2 and P3. SDS-PAGE analysis revealed a single, sharp band for P1 and P2 and a doublet for P3. These proteins were basic with pI values of 8.9 (P1) and 8.3 (P2) but the pI of P3 could not be determined, probably because it was above the upper limit (9.3) of the system used. The three fractions exhibited strong immunological cross-reactivity, as assessed using monospecific polyclonal antisera, suggesting that they were closely related. This was confirmed by N-terminal sequencing which yielded unambiguous single sequences for P1 and P2, confirming that these fractions only contained a single protein, with an identity close to 90% between the sequences. While most cycles of the N-terminal aminoacid analysis of P3 yielded a single sequence, some cycles yielded a double sequence confirming the suggestion from SDS-PAGE that this fraction contained two proteins. Homology with P1 and P2 was high. No blank cycles were obtained in P1, P2 or P3 sequences, indicating that none of the residues were postranslationally modified (e.g. glycosylated). N-terminal sequences of the separated proteins showed significant homologies not only among themselves but also with the published sequence of the toxin responsible for the pathological effects of *L. reclusa* venom [20] and partially pure dermonecrotic toxins from *L. gaucho, L. intermedia* and *L. laeta* venoms [13]. Some degree of homology with the phospholipase D from *C. pseudotuberculosis, C. ulcerans* and *A. haemolyticum,* which have been shown to have similar biological effects to *Loxosceles* venom, was also observed [12,13,22-24].

Phospholipases are a group of enzymes widespread in nature which promote the hydrolysis of the ester bonds in phospholipid and are classified into A1, A2, C and D by the position in the ester bond attacked [25]. Some phospholipases C and D have a specificity for the cleavage of sphingomyelin, resulting in the release of ceramides and cholines and are called sphingomyelinases. Phospholipases are frequently found as toxic components in animal venoms and bacterial toxins. The toxin venom from the spider *L. reclusa,* which is related to *L. intermedia,* has been characterised as a sphingomyelinase D [11], with four active forms, each with an estimated molecular weight of 32 kDa and pI of 8.7, 8.4, 8.2 and 7.8. Although no specific aminoacid sequence data is available for the different isoforms, a close structural relationship was suggested based on their immunological cross reactivities [11].

It has been reported that *L. reclusa* sphingomyelinase is similar in several physical and biological as-

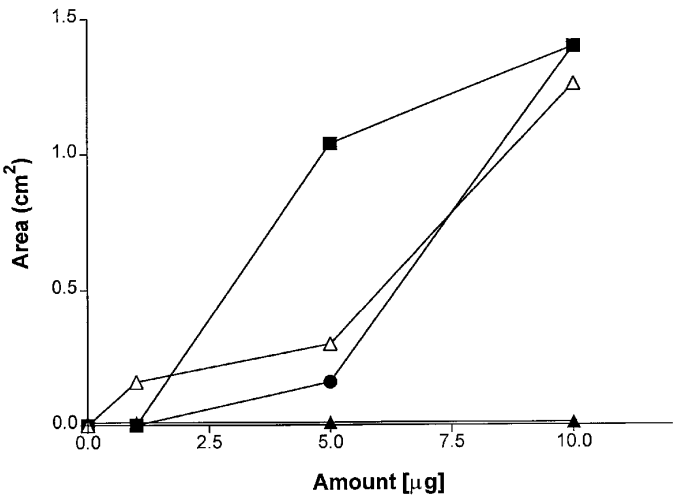

FIG. 6. Dermonecrotic activity of HPLC-F35 proteins. Adult rabbits were injected intradermally with *L. intermedia* venom (△), and various doses of P1(●), P2 (■) or P3 (▲) toxins. The animals received buffer for negative control reactions. The areas of the dermonecrotic lesions were determined 24 h after toxin injection. Results are representative for three separate experiments.

pects to the phospholipase D isolated from *C. pseudotuberculosis* (Cor-PLD) [24]. In contrast to most phospholipases, the two enzymes have a similar and unusual substrate specificity. Of the four major phospholipids in mammalian cell membranes, only sphingomyelin is hydrolysed by Cor-PLD and the same is true for *L. reclusa* 32 kDa toxin [24]. The small degree of N-terminal sequence homology between *Loxosceles* toxins and the bacterial phospholipases (Table 1) confirms the relationship between these proteins. No structural homology was found with other phospholipases.

In view of the similarities between *L. reclusa* sphingomyelin and the F35 proteins we decided to investigate the phospholipase activities of *L. intermedia* proteins. Using egg-yolk lecithin, soy bean lecithin (sources of phosphatidyl choline), glycolipid A and mVSG (membrane form of variant surface glycoprotein of *Trypanosoma brucei;* source of glycosyl phosphatidylinositol (GPI) anchors), as substrates and thin layer chromatography, agar plate methods or SDS-PAGE [26-28] we did not observe hydrolysis of these lipids by P1, P2, P3 proteins or crude spider venom, indicating the absence of general or GPI-specific phospholipase activities in these samples (data not shown). However, thin layer chromathography analysis showed that P1, P2, F35 fractions and total venom, but not P3, can hydrolyse sphingomyelin. The sphingomyelinase activity of the active samples can be inhibited by EDTA suggesting that P1 and P2 are Ca^{2+}-dependent.

In order to assess if the HPLC-purified proteins retained the original biological properties described for unfractionated venom and F35, P1, P2 and P3 were tested for dermonecrotic and haemolytic C-dependent activities. The data show that P1 and P2 toxins but not P3 possessed both these biological activities, thus showing a positive correlation between the presence of sphingomyelinase activity and the capacity to induce dermonecrosis and haemolysis for these proteins. Moreover, P2 was shown to be more active than P1 as an inducer of dermonecrosis (Figure 6). While sphingomyelinase from *L. reclusa* venom and *C. pseudotuberculosis* can induce similar biological effects [28], sphingomyelinase and phospholipase C from *Bacillus cereus* and phospholipases D from cabbage and *Streptomyces chromofuscus* were not able to induce the C-dependent haemolysis under the same conditions as P1 and P2 (our unpublished data), showing the uniqueness of the *Loxosceles* and *C. pseudotuberculosis* enzymatic activities. The mechanism of dermonecrosis is still unclear but the observation that the *Loxosceles* toxin displays sphingomyelinase activity leads us to suggest that *in vivo* ceramide, generated after sphingomyelin hydrolysis by venom toxins and shown to be a mediator of apoptosis [29], maybe the direct factor inducing the lesion.

In conclusion, we have purified 2 toxins form *L. intermedia* venom to homogeneity. The toxins showed a high degree of homology with each other and with published sequence of the sphingomyelinase venom toxin from *L. reclusa*. We show here for the first time that a variety of different biological effects (dermonecrosis, induction of haemolysis, sphingomyelinase) exhibited by *L. intermedia* venom can all be assigned to a single protein which is present in two isoforms in whole venom. Two other components that could not be separated under the conditions used, were inactive in all assays performed despite the high homology with the two biologically active toxins. The components have probably originated from gene duplication and the proteins present in P3 are probably the results of expression of modified genes. To confirm this we are currently cloning and sequencing the cDNAs encoding the proteins. The elucidation of the active components in the spider venom will aid the development of a suitable therapy. The mechanism through which a single protein can induce such a variety of different effects is the subject of further study.

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