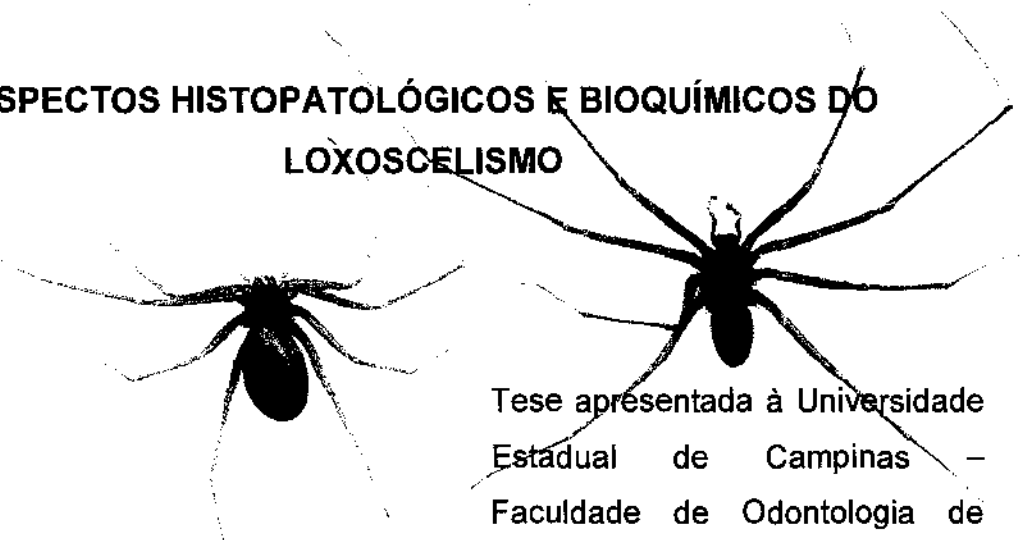
SILVIO SANCHES VEIGA

ASPECTOS HISTOPATOLÓGICOS E BIOQUÍMICOS DO LOXOSCELISMO



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As memórias de meus pais, Amélia da Veiga Sanches e Eugênio Sanches Garcia, vítimas do câncer.

O espírito é imortal.

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SUMÁRIO

Resumo

As aranhas marrons (gênero *Loxosceles*) são de grande importância médica, com os acidentes (Loxoscelismo) ocorrendo por todo o mundo. As atividades biológicas do veneno da aranha marrom usualmente incluem lesões dermonecróticas no local da picada, acompanhadas de hemólise, hemorragia e insuficiência renal. Neste trabalho, nós descrevemos a histologia da glândula produtora de veneno da L. intermedia (espécie que prevalece em Curitiba) através de métodos citoquímicos e imunohistoquímicos, microscopia eletrônica de transmissão e de varredura. A organização da glândula se assemelha à arquitetura geral das glândulas produtoras de veneno das aranhas. Nós observamos que as glândulas da L. intermedia possuem duas camadas de musculatura estriada, uma camada externa e uma interna em contato direto com uma estrutura semelhante à membrana basal, separando a região muscular das células epiteliais. As células musculares são multinucleadas, com núcleos localizados na periferia celular e com seus citoplasmas ricos em retículo endoplasmático liso e miofibrilas. Utilizando microscopia eletrônica de varredura, nós mostarmos que as células musculares da camada externa têm morfologia ramificada. As células epiteliais têm retículo endoplasmático rugoso acentuado, coleções mitocondriais, complexo de Golgi evidente, interdigitações de membranas e vesículas secretoras contendo o veneno, sendo este uma mistura complexa de proteínas.

Em outra investigação, através de eletroforese de poliacrilamida gradiente linear 3-20% na presença de dodecil sulfato de sódio e coloração monocromática pela prata, fomos capazes de mostrar que o veneno de L. intermedia é uma mistura complexa de proteínas que variam entre 5 a 850 kDa. O tratamento do veneno com tripsina ativa duas moléculas gelatinolíticas com 85 kDa e 95 kDa de massa molecular. Experimentos utilizando inibidores de proteases confirmaram que somente inibidores de serino-proteases foram eficientes no bloqueio da atividade proteolítica destas enzimas. Essas enzimas apresentam pH ótimo entre 7,0 e 8,0. A análise da especificidade funcional dos substratos dessas enzimas

mostrou que elas degradam eficientemente gelatina (colágeno desnaturado) mas não têm atividade proteolítica sobre hemoglobina, imunoglobulina G, albumina, fibrinogênio e laminina, sugerindo especificidade de atividade proteolítica.

Estudando o veneno de L. intermedia, nós fomos capazes de detectar atividade proteolítica sobre a fibronectina e o fibrinogênio (duas importantes proteínas plasmáticas), mas ausência de atividade proteolítica sobre a laminina e os colágenos tipo I e IV. Analisando inibidores de proteases, mostramos que os quelantes de metais divalentes como o EDTA e a 1,10-fenantrolina bloquearam completamente as atividades proteolíticas do veneno, enquanto inibidores de serino-proteases, tiol-proteases e proteases ácidas não tiveram nenhuma atividade efetiva, indicando envolvimento de metaloproteases. Através de zimogramas detectamos uma molécula com atividade gelatinolítica com massa molecular de 35 kDa. A sensibilidade ao acetato de 4-aminofenil mercúrio e à 1,10-fenantrolina comprovaram tratar-se de uma metaloprotease. Zimogramas copolimerizados com fibronectina ou fibrinogênio detectaram atividade proteolítica de uma outra enzima com massa molecular de 28 kDa, também sensíveis ao tratamento organomercurial e com a fenantrolina comprovando tratar-se de outra metaloprotease.

Com a finalidade de descartarmos a crítica racional contra a presença de enzimas proteolíticas no veneno obtido por eletroestimulação, como sendo decorrente da contaminação com egesto abdominal, os venenos de *L. intermedia* e *L. laeta* foram diretamente coletados das glândulas produtoras de veneno por microdissecção e homogeneização suave. Eletroforese corada pelo método da prata mostrou não existir diferenças significativas no perfil protéico entre os venenos glandulares e eletroestimulado. Experimento de zimograma com o veneno glandular detectou atividade gelatinolítica na região de 32-35 kDa, idêntico ao veneno obtido por eletrochoque. A atividade inibitória da fenantrolina sobre esta atividade proteolítca adicionalmente à caracteriza como uma metaloprotease autócrina do veneno. Experimentos de clivagem proteolítica com o veneno glandular mostraram atividades fibronectinolítica e fibrinogenolítica, bloqueadas por quelantes de metais divalentes, de modo idêntico ao veneno obtido por

eletrochoque. Adicionalmente, quando o fibrinogênio foi incubado com extrato abdominal de *L. intermedia*, a fibrinogenólise originou fragmentos peptídicos de baixa massa molecular completamente diferentes da fibrinogenólise causada pelo veneno. Experimentos de zimograma utilizando veneno glandular de *L. laeta* também detectaram atividade gelatinolítica na região de 32-35 kDa, atividade esta inibida pela fenantrolina, confirmando a presença de metaloproteases no veneno de ambas as espécies.

Através de metodologias específicas para análises de resíduos de oligossacarídeos como lectin-blotting, cromatografia de afinidade lectínica e tratamento com glicosidases, fomos capazes de identificar no veneno de *L. intermedia* várias glicoproteínas N-glicosiladas, com resíduos de alta-manoses e glicoconjugados complexos fucosilados. Trabalhando com venenos deglicosilados enzimatica ou quimicamente, pudemos detectar que as atividades trombocitopênicas, fibronectinolítica e fibrinogenolíticas do veneno não dependem dos resíduos de açúcares das glicoproteínas do veneno. No entanto, a atividade gelatinolítica da metaloprotease Loxolisina B, uma glicoproteína do tipo alta-manose, após deglicosilação química ou enzimática, diminui a níveis residuais de 28% quando comparada com a molécula glicosilada, sugerindo a importância destas modificações pós-traducionais na atividade gelatinolítica. A massa molecular diminui em aproximadamente 2 kDa após deglicosilação. Análises do veneno deglicosilado quimica ou enzimaticamente, mostram atividade dermonecrótica diminuída quando comparadas com o veneno glicosilado.

Observações por microscopia de luz mostram que o veneno de *L. intermedia*, obtido por eletrochoque, reproduz os principais sinais clínicos do loxoscelismo em laboratório, exibe atividade degradatória de membranas basais do tumor de EHS. A degradação de membranas basais do tumor de EHS pôde ser confirmada através das colorações citoquímicas pelo PAS e Azul de Alcian, bem como diminuída imunomarcação para a laminina. Análises por microscopia eletrônica confirmaram os resultados descritos acima, mostrando atividade degradatória e disruptiva do veneno sobre membranas basais de EHS. Utilizando moléculas purificadas de membranas basais, através de cinéticas de proteólise e

eletroforeses de poliacrilamida ou agarose, nós mostramos que o veneno não tem atividade degradatória sobre a laminina ou colágeno do tipo IV, mas é capaz de clivar a entactina e o núcleo protéico do heparam sulfato proteoglicano. Quando o tumor de EHS foi incubado com o veneno, nós detectamos a liberação de laminina no sobrenadante, corroborando a ocorrência de atividade disruptiva da membrana basal. A atividade entactinolítica do veneno foi bloqueada pela fenantrolina (inibidor de metaloproteases), mas não por outros inibidores proteolíticos, corroborando que metaloproteases do veneno estão envolvidas na atividade degradatória e disruptiva de membranas basais. A degradação de membranas basais pode explicar as atividades deletérias do veneno sobre a integridade glomerular e os vasos sangüíneos, resultando em problemas renais e hemorragia.

O efeito do veneno de *L. intermedia* sobre células endoteliais foi investigado *in vivo* e *in vitro*. Análises morfológicas e ultraestruturais por microscopia de luz e eletrônica de transmissão, mostraram que o veneno atua *in vivo* sobre células endoteliais de vasos sangüíneos da pele de coelhos, causando instabilidade destes vasos, vacuolização citoplasmática endotelial e bolhas. Do mesmo modo, o tratamento de células endoteliais em cultura de aorta de coelhos com veneno, mostrou perda de adesão das células, aumento da liberação de compostos celulares para o meio de cultura e diminuição da matriz extracelular endotelial. Experimentos por eletroforeses de poliacrilamida e agarose mostraram que o veneno é ativo sobre proteoglicanos da superfície celular e matriciais das células endoteliais, liberando estas moléculas para o meio de cultura. Adicionalmente, quando moléculas purificadas como heparam sulfato proteoglicano e o complexo protéico laminina-entactina foram incubados com o veneno, pudemos detectar atividade proteolítica sobre estes substratos. Estes resultados sugerem que o veneno de *L. intermedia* tem atividade citotóxica sobre células endoteliais tanto *in vivo* como em cultura, removendo importantes constituintes destas células ou da matriz extracelular endotelial os quais são importantes na organização e atividades biológicas destas células.

Coelhos intradermicamente expostos ao veneno mostraram hemorragia no local da injeção iniciando-se aproximadamente 1 hora após a inoculação e

atingindo atividade máxima entre 2 e 3 dias. Biópsias analisadas por microscopia de luz e microscopia eletrônica de transmissão mostraram vacúolos subendoteliais e degeneração das membranas citoplasmáticas de células endoteliais de vasos sangüíneos, exudação de plasma para o tecido conectivo, formação de fibrina e trombos dentro dos vasos sangüíneos. A incubação de fibrinogênio purificado com veneno de *L. intermedia* mostra atividade proteolítica parcial sobre as cadeias Aα e Bβ do fibrinogênio *in natura*, mas significante atividade proteolítica sobre as cadeias Aα, Bβ e γ quando estas estavam separadas ou o fibrinogênio estava desnaturado. Estudos de cinética proteolítica, mostraram que a cadeia Aα é mais susceptível à proteólise. A fibrinogenólise causada pelo veneno pode ser bloqueada pelo EDTA e a fenantrolina, mas não por outros inibidores de proteases. Plasma humano incubado com o veneno tem seus parâmetros de coagulação como tempo de protrombina, tempo de tromboplastina parcial ativada e tempo de trombina aumentados. Através de cromatografias de gel filtração, isolamos no veneno uma toxina de 30 kDa com atividade fibrinogenolítica. Por estes dados podemos propor que as desordens hemorrágicas em nível local ou sistêmicas que acontecem durante o loxoscelismo são conseqüências de uma atividade direta sobre o fibrinogênio além de citotoxidade sobre células endoteliais dos vasos sangüíneos.

Em outra investigação, coelhos albinos foram submetidos a um protocolo experimental de envenenamento agudo, utilizando veneno de *L. intermedia*, com ênfase na determinação das características histopatológicas das lesões dermonecróticas. Coelhos receberam injeções intradérmicas do veneno e foram monitorados 4 horas, 12 horas, 1, 2 e 5 dias após o envenenamento. Amostras para análise histológica foram coletadas de 3 coelhos por tempo de experimentação e processadas para análise histopatológica. Os principais achados macroscópicos nas primeiras 4 horas foram inchaço e hemorragia local e em nível microscópico edema da derme, hemorragia, degeneração das paredes dos vasos sangüíneos, exudação de plasma, trombose, acúmulo de neutrófilos dentro e fora dos vasos sangüíneos com maciça diapedese, coleção difusa de células inflamatórias (leucócitos PMN) na derme, além de edema da musculatura

subcutânea. Dentro das próximas horas até 5 dias pós-envenenamento, as alterações histopatológicas progrediram para um massivo infiltrado neutrofílico dentro da derme e mesmo na musculatura subcutânea, destruição dos vasos sangüíneos, trombose, hemorragia, mionecrose e necrose coagulativa por volta do quinto dia. Sinais de reparo tecidual da lesão dermonecrótica foram aparentes no segundo dia pós-envenenamento, com proliferação de fibroblastos e formação de tecido de granulação.

Por análise microbiológica, fomos capazes de isolar e identificar diferentes bactérias, entre as quais o *Clostridium perfringens* (envolvido em condições dermonecrotizantes) a partir do veneno e quelíceras de aranhas marrons coletadas diretamente da natureza. Trabalhando com veneno de *L. intermedia* associado ou não ao *C. perfringens*, utilizando coelhos como modelo experimental para a dermonecrose, pudemos observar que esta conjugação resultou em aumento do quadro dermonecrótico, quando comparado com a dermonecrose induzida pelo veneno sozinho, sugerindo um provável papel desta bactéria nos quadros dermonecróticos graves mostrados por alguns pacientes.

Abstract

The brown spider, genus *Loxosceles*, is becoming of great medical importance, with envenomation (Loxoscelism) occurring throughout the world. The biological activities of the brown spider venom usually include dermonecrotic lesions at the bite site accompanied by hemolytic and haemorrhagic effects and also by renal failure. We described the histology of the venom gland of *L. intermedia* using immunohistochemical and staining methods, by transmission electron microscopy and by scanning electron microscopy. The organization of the venom gland of *L. intermedia* follows the general architecture of spiders venom glands. We observed that the venom glands present two layers of striated muscle fibers, an external layer and an internal layer in touch with an basement membrane structure and a fibrillar collagen matrix separating the muscular region from epithelial cells. Muscle cells are multinucleated, with nuclei peripherally placed and their cytoplasm rich in sarcoplasmic reticulum, myofibrills and continuous Z lines. By using scanning electron microscopy we can detect muscular cells from external layer as branching cells. Epithelial cells have their cytosol extremely rich in rough endoplasmic reticulum, mitochondria collection, Golgi apparatus, interdigitating membranes and secretory vesicles that ultimately accumulate the venom, a complex protein mixture.

In the present investigation, when we submitted *L. intermedia* venom to linear gradient 3-20% SDS-PAGE stained by a monochromatic silver method we detected a heterogeneous protein profile in molecular weight, ranging from 850 kDa to 5 kDa. In an attempt to detect zymogen molecules of proteolytic enzymes, venom aliquots were treated with several exogenous proteases. Among them, trypsin activated two gelatinolytic molecules of 85 kDa and 95 kDa in the venom. In experiments of hydrolysis inactivation using different protease inhibitors for four major class of proteases, we detected that only serine-type protease inhibitors were able to inactivate the 85 kDa and 95 kDa enzymes in the venom. An examination of the 85 kDa and 95 kDa gelatinolytic activities as a function of pH showed that these proteases had no apparent activities at pH below 5.0 and higher

than 9.0 and displayed little activity at pH 6.0, with the optimal pH for their activities ranging from 7.0 to 8.0. Evaluation of the functional specificities of the 85 kDa and 95 kDa venom proteases showed that these proteases efficiently degrade gelatin (denatured collagen) but have no proteolytic activity on hemoglobin, immunoglobulin, albumin, fibrinogen or laminin, suggesting specificity of their proteolytic actions. We describe here two serine-proteases activities in *L. intermedia* venom probably involved in the harmful effects of the venom.

By studying Loxosceles intermedia (Brown spider) venom we were able to detect a proteolytic action on fibronectin and fibrinogen but an inability to degrade full length laminin, type I and type IV collagens. By studying enzyme inhibitors we observed that divalent metal chelators as EDTA and 1,10-phenanthroline completely blocked this cleaving action whereas serine-protease inhibitors, thiol-protease inhibitor and acid-protease inhibitor showed little or no effect on the proteolytic activity of the venom indicating involvement of a metalloproteinase. Zymogram analysis of venom detected a 35 kDa molecule with gelatinolytic activity. The metalloproteinase nature was further supported by its sensitivity to 4-aminophenyl mercuric acetate (APMA) treatment which decreased its molecular weight to 32 kDa, inhibition of its gelatinolytic effect by 1,10-phenanthroline and its elution from gelatin-sepharose affinity beads. In addition, zymogram experiments using fibronectin and fibrinogen as substrates detected a fibronectinolytic and fibrinogenolytic band at 28 kDa which changed its electrophoretic mobility to 20 kDa band after organomercurial treatment. The inhibitory effect of 1,10 phenanthroline and APMA sensitivity on this proteolytic effect confirmed the presence of a second metalloproteinase in the venom. The data presented herein describe two invertebrate metalloproteinases in L. intermedia venom with different specificities one gelatinolytic and another, fibronectinolytic and fibrinogenolytic, probably involved in the harmful effects of the venom.

In the present investigation, in order to dispute the rational criticism against the presence of proteolytic enzymes in the electrostimulated venom obtained from spiders of the genus *Loxosceles,* as a consequence of contamination with abdominal secretions, venoms of *L. intermedia* and *L. laeta* were directly collected

from venom glands by microdissection and gentle homogenization. Gel electrophoresis stained by silver method carried out to compare *L. intermedia* electrostimulated venom and venom gland extract demonstrated no significant differences in protein profile. Zymogram analysis of *L. intermedia* venom gland extract detected a gelatinolytic activity in the 32-35 kDa region. The inhibitory effect of 1,10-phenanthroline on this proteolytic activity further supported its metalloprotease nature. In proteolytic digestion experiments *L. intermedia* venom gland extract was also able to cleave purified fibronectin and fibrinogen. The inhibitory effect of 1,10-phenanthroline on these degrading activities confirmed the presence of metalloproteases in the venom. In addition, when purified fibrinogen was incubated with *L. intermedia* abdominal extract, the fibrinogenolysis was completely different, generating low mass fragments that ran away from the gel, a proteolytic event not blocked by 1,10-phenanthroline. Zymogram experiments using *L. laeta* venom gland extracts further detected a gelatinolytic band at 32-35 kDa, also inhibited by 1,10-phenanthroline, confirming the presence of metalloproteases in both species.

Using lectin-immunelabeling, lectin-affinity chromatography, glycosidases and protease-K treatments we were able to identify several proteins in venom as N-glycosylated, with high-mannose oligosaccharides structures, medial Golgi complex-type glycoconjugates but the absence of trans Golgi complex sugars or glycosaminoglycans residues. Working with enzymatic or chemically deglycosylated venom we detected that platelet aggregation (thrombocytopenic activity) as well as fibronectinolytic and fibrinogenolytic (hemorrhagic effects) were sugar-independent when compared to glycosylated venom. Nevertheless zymograph analysis in gelatin co-polimerized gels showed that the loxolysin-B a gelatinolytic metalloproteinase of venom with 32-35 kDa a high-mannose glycoprotein eluted from a Con-A-sepharose column, after enzymatic N-deglycosylation or sodium metaperiodate oxidation strongly had decreased its molecular weight in aproximately 2 kDa and gelatinolytic effect to a 28% residual activity when compared with glycosylated molecule, pointing for a post-translational glycosylation dependent gelatinolytic effect. Analysis of chemically or enzymatic N-

deglycosylated venom for its dermonecrotic effect detected only a residual activity when compared with glycosylated control. Thus, the present report suggest oligosaccharide moistures playing a role in destructive effects of brown spider venom, open a possibility for a cabohydrate-based therapy.

Light microscopy observations showed that the *L. intermedia* venom obtained through electric shock, which reproduces two major signals of Loxoscelism in laboratory, exhibits activity toward EHS-basement membrane structures. Basement degradation was seen by a reduced PAS and Alcian Blue staining as well as by a reduced immunostaining for laminin when compared to control experiments. Electron microscopy studies confirmed the above results, showing the action of the venom on EHS-basement membranes and demonstrating that these tissue structures are susceptible to the venom. Using purified molecules of the basement membrane, we determined through SDS-PAGE and agarose gel that the venom is not active toward laminin or type IV collagen, but is capable of cleaving entactin and endothelial heparan sulfate proteoglycan. In addition, when EHS tissue was incubated with venom we detected a release of laminin into the supernatant, corroborating the occurrence of some basement membrane disruption. Venom degrading effect upon entactin was blocked by 1,10-phenanthroline, but not by other protease inhibitors as PMSF, NEM or pepstatin-A. By using light microscopy associated with PAS staining we were able to identify that 1,10-phenanthroline also inhibits EHS-basement membrane disruption evoked by venom, corroborating that a metalloprotease of venom is involved in these effects. Degradation of these extracellular matrix molecules and the observed susceptibility of the basement membrane could lead to loss of vessel and glomerular integrity, resulting in hemorrhage and renal problems after envenomation.

The effect of brown spider (*Loxosceles intermedia*) venom on endothelial cells was investigated *in vivo* and *in vitro*. Morphological and ultrastructural observations by light microscopy and transmission electron microscopy showed that the venom acts *in vivo* upon vessel endothelial cells of rabbits that were intradermally injected, evoking vessel instability, cytoplasmic endothelial cell

vacuolization and blebs. Likewise, treatment of rabbit endothelial cells in culture with the venom led to loss of adhesion of the cells to the substrate. Endothelial cells in culture were metabolically radiolabelled with sodium [^{35}S]-sulfate and the sulfated compounds (proteoglycans and sulfated proteins) from medium, cell surface and extracellular matrix (ECM) were analysed. Agarose gel electrophoresis and SDS-PAGE showed that the venom is active on the ECM and on cell surface proteoglycans, shedding these molecules into the culture medium. In addition, when purified heparan sulfate proteoglycan (HSPG) and purified laminin–entactin complex were incubated with the venom we observed a partial degradation of the protein core of HSPG as well as the hydrolysis of entactin. The above results suggest that the *L. intermedia* venom has a deleterious effect on the endothelium of vessels both *in vivo* and in culture, removing important constituents such as HSPG and entactin that are involved in the adhesion of endothelial cells and of subendothelial extracellular matrix organization.

Rabbits intradermally exposed to the venom showed a local hemorrhage starting 1 hour after inoculation and reaching maximum activity between 2 and 3 days. Biopsies examined by light and transmission electron microscopy showed subendothelial blebs, vacuoles and endothelial cell membrane degeneration in blood vessels, plasma exudation into connective tissue, fibrin and thrombus formation within blood vessels. *L. intermedia* venom, incubated with fibrinogen partially degrades Aα and Bβ chains, of intact fibrinogen and significantly cleaves all Aα, Bβ and γ chains when they were separated or when fibrinogen was denatured by boiling. Proteolytic kinetic studies showed that the Aα chain is more susceptible to venom hydrolysis than the Bβ chain. The fibrinogenolysis was blocked by EDTA and 1.10-phenanthroline, but not by other protease inhibitors. Human plasma incubated with the venom, had their coagulation parameters as prothrombin time, activated partial thromboplastin time and thrombin time increased. Through molecular sieve chromatography, we have isolated a venom toxin of 30 kDa with fibrinogenolytic activity. We may propose that the local and systemic hemorrhagic disorders evoked in loxoscelism are consequences of direct

venom fibrinogenolysis together with cytotoxicity to subendothelial structures and endothelial cells in blood vessels.

In the present investigation we submitted albino rabbits to an acute experimental envenomation protocol using *Loxosceles intermedia* (brown spider) venom, with emphasis on the determination of the features of lesion pathogenesis induced by this spider, which is the cause of several accidents through out the world. Rabbits received intradermal injections of the venom and were monitored over the first 4 hours, and then 12 hours and 1, 2 and 5 days after envenomation. Histologic specimens from 3 rabbits per time point were collected from euthanized animals and processed for histological examination by light microscopy. Major macroscopic findings observed during the first 4 hours were swelling and a haemorrhagic spot at the injection sites and microscopic finding at the dermis level were oedema, haemorrhage, degeneration of blood vessel walls, plasma exudation, thrombosis, neutrophil accumulation in and around blood vessels with an intensive diapedesis, a diffuse collection of inflammatory cells (polymorphonuclear leukocytes) in the dermis, and subcutaneous muscular oedema. Over the following hours and up to 5 days after envenomation the changes progressed to massive neutrophil infiltration (no other leukocytes) into the dermis and even into subcutaneous muscle tissue, destruction of blood vessels, thrombosis, haemorrhage, myonecrosis, and coagulative necrosis on the 5th day. Lesion repair was apparent at 2 days, with proliferation of fibroblasts and the formation of a granulation tissue.

Isolation and identification of many different bacteria, among them *Clostridium perfringens*, of great medical importance due to its involvement in dermonecrotizing and systemic conditions, was carried out from the venomous apparatus (fangs and venom) of spiders obtained directly from nature, through microbiological cultures in aerobic and anaerobic conditions. Working with *Loxosceles intermedia* venom (alone) and with the venom conjugated with *Clostridium perfringens* using rabbits as experimental models for dermonecrosis, allowed for the observation that venom and anaerobic bacteria conjugated resulted in a striking increase of the dermonecrotic picture when compared to venom alone,

suggesting a role for *Clostridium perfringens* in the severe dermonecrotic picture of these patients and opening the possibility for the association of antibiotic therapy in treating loxoscelism.

Revisão da Literatura

Dentre os quadros clínicos produzidos por acidentes que envolvem animais peçonhentos, aqueles causados pelos aracnídeos são de grande importância médica. Em algumas localidades, tanto a freqüência de acidentes quanto suas conseqüências podem caracterizá-los como fator de interesse a nível de saúde pública (MARTINEZ-VARGAS,1987; RIBEIRO et al.,1993). Nos últimos anos, o Estado do Paraná e em especial a Região Metropolitana de Curitiba, vêm sofrendo um aumento significativo no número de acidentes produzidos por aracnídeos, popularmente conhecidos como "aranhas-marrons". Estas aranhas pertencem ao gênero Loxosceles e para este, são atualmente conhecidas aproximadamente 70 espécies. As aranhas do gênero *Loxosceles* têm vasta distribuição pelo mundo tropical, subtropical e temperado, principalmente no Continente Americano, a começar pelos países da América do Norte, América Central e das Antilhas e em proporções não poucas vezes alarmantes, em determinadas regiões do Peru, Chile, Argentina, Uruguai e Brasil.

Das aranhas do gênero *Loxosceles* que ocorrem no continente americano, aproximadamente 18 espécies ocorrem na América do Norte, América Central e Antilhas e 30 na América do Sul. No Brasil ocorrem 7 espécies heterogeneamente distribuídas que são *Loxosceles gaucho*, encontrada em São Paulo e Rio Grande do Sul; *Loxosceles similis*, encontrada na Paraíba, São Paulo e Minas Gerais; *Loxosceles adelaide*, encontrada no Rio de Janeiro; *Loxosceles hirsuta*, encontrada no Rio Grande do Sul, Santa Catarina e Paraná; *Loxosceles intermedia*, encontrada do Rio Grande do Sul ao Rio de Janeiro; *Loxosceles amazônica*, encontrada no Amazonas e Ceará e *Loxosceles laeta*, encontrada nas regiões sul e sudeste.

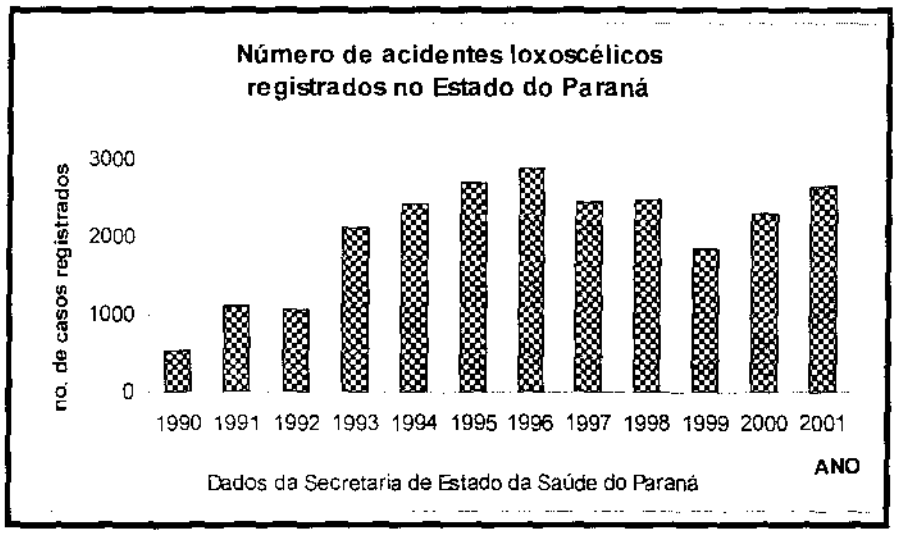
No Paraná através dos levantamentos epidemiológicos realizados pelos órgãos responsáveis da Secretaria de Saúde, verificou-se que no ano de 1992, do total de aranhas-marrons coletadas na cidade de Curitiba e Região Metropolitana, 92% foram identificadas como *L. intermedia* e 8% como *L. laeta* (RIBEIRO et al., 1993; MANFREDINI et al., 1993). Com relação à sua distribuição geográfica, o

aracnídeo apresentou notória preferência pela zona urbana do município de Curitiba (80%). Nas regiões mencionadas, a densidade populacional dessa espécie vem aumentando significativamente a partir de 1989.

Essa proliferação reflete-se também no aumento do número de acidentes que ocorrem com esta espécie. De 100 casos de acidentes notificados em 1986, passou-se para mais de 1000 em 1992 (MANFREDINI et al., 1993; BARBARO et al., 1995) e 2400 em 1996 (COUTINHO, 1996).

No Brasil, o loxoscelismo não é muito freqüente, com exceção do estado do Paraná, particularmente Curitiba e região metropolitana. No período de 1988-1989 foram notificados 595 casos, que corresponderam a 62,2% das notificações de acidentes por *Loxosceles* spp. e entre 1989 e 1990 o total de casos chegou a 923, perfazendo 77,4% dos acidentes no País, com incidência maior nos períodos mais quentes do ano. Em 1999, foram registrados 1.454 casos no Paraná (dados fornecidos pelo Centro de Saúde Ambiental - SESA - PR). Já em 2001, foram registrados e confirmados 2664 casos.

Analisando-se as notificações oficiais dos acidentes com aracnídeos nessa região, percebe-se que cerca de 52% destes são causados pela espécie *L. intermedia*. Do total de acidentados, a maioria encontra-se na faixa etária de 20 a 39 anos, indicando que a camada populacional mais sujeita aos episódios de acidente é aquela economicamente mais ativa. As estatísticas indicam também que as lesões ocorreram principalmente no tronco (18%) e nos membros(35%), partes do corpo geralmente cobertas e, portanto, potencialmente mais susceptíveis ao contato e compressão do animal sobre a pele, uma vez que a maioria dos acidentes ocorre quando o indivíduo encontra-se dormindo, ou, ao vestir-se (RIBEIRO et al., 1993; MANFREDINI et al.,1993). Embora fatores múltiplos possam estar relacionados, é provável que o aumento de acidentes envolvendo a "aranha-marrom" em Curitiba e região Metropolitana nos últimos anos deva-se a desequilíbrios ambientais e ecológicos, tais como desmatamentos e extinção de predadores naturais, o que poderia impedir a manutenção da densidade populacional da espécie em níveis estáveis (BARBARO et al.,1995).

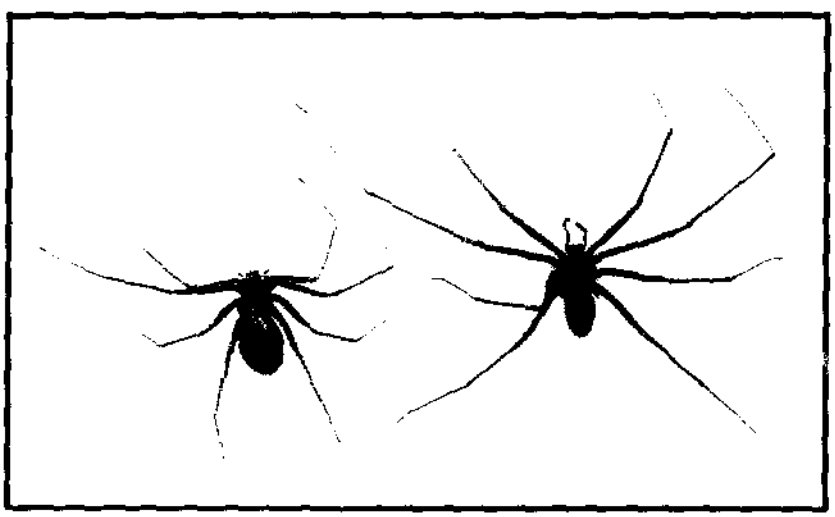


BIOLOGIA DO GÊNERO LOXOSCELES

Estas aranhas apresentam comprimento corporal variando de 8 a 15 mm, com patas medindo de 8 a 30 mm, com pelos curtos e escassos. Sua coloração varia do marrom claro ao marrom escuro. Os machos apresentam corpo mais delgado e patas mais longas que as fêmeas. Os olhos dispostos em 3 pares, com localização em semi-círculo, são característica de segurança para identificação do gênero. São animais sedentários, que constróem teias irregulares, semelhantes à algodão esfiapado. Sua alimentação é constituída de pequenos insetos. Instalam-se e reproduzem-se com facilidade, em ambientes diversificados. Após o acasalamento, a ooteca é tecida pela fêmea e a postura pode gerar de 60 a 200 ovos. A "aranha-marrom" é uma animal não agressivo e possui hábitos noturnos (HITE et al., 1960; BUCHERL, 1960-62; GAJARDO-TOBAR, 1966; LUCAS et al., 1983/84; RODRIGUES et al., 1986; JORGE et al., 1991; FUTRELL, 1992). É destacada a sua preferência por locais secos e escuros, e ela costuma abrigar-se sob cascas de árvores, nas fendas de muros, em cavernas, pilhas de tijolos e junto a entulhos acumulados. A aquisição de hábitos intra- domiciliares por este animal guarda relação direta com a maior incidência de acidentes dentro das residências humanas (LUCAS et al., 1983/84; LUCAS, 1988; JORGE et al., 1991; FUTRELL,1992; COUTINHO, 1996).

A "aranha-marrom" suporta temperaturas que variam de 8 à 43^{0}C; sendo maior o registro de acidentes, durante os meses mais quentes do ano (74%) (SCHENONE and LETONJA, 1975; SCHENONE et al., 1989). No outono e inverno, o número de acidentes com este aracnídeo é substancialmente reduzido (SCHENONE and LETONJA, 1975). Ambos os sexos são venenosos e podem sobreviver até 276 dias sem alimento (LOWRIE, 1980; GERTSCH and ENNIK, 1983; FUTRELL, 1992).

Loxosceles intermedia



Fêmea **Macho**

CARACTERÍSTICAS DO VENENO LOXOSCÉLICO

O veneno da "aranha-marrom" é um líquido com características protéicas, produzido por glândulas apócrinas situadas no cefalotórax do animal, que comunicam-se com o exterior através do aparelho inoculador, constituído por um par de quelíceras (GAJARDO-TOBAR, 1966; MARTINEZ-VARGAS, 1987).

A quantidade de veneno seco,varia entre espécies. Por exemplo, animais da espécie *L.rufipes*, secretam na média, 700 μg por extração (LUCAS,1988). Uma adulto da espécie *L. reclusa*, libera no volume total de

veneno(aproximadamente 4 μl), de 65 a 100 μg de proteína (FORRESTER et al., 1978).

Vários são os estudos realizados com o objetivo de esclarecer os mecanismos de ação do veneno das aranhas deste gênero no estabelecimento dos quadros clínicos produzidos pela picada do animal.

BARBARO et al. (1995) estudaram o veneno de *L. gaucho* e entre as enzimas presentes, encontraram a hialuronidase e a esfingomielinase D, atribuindo a estas a responsabilidade principal pelo quadro clínico de dermonecrose e o óbito dos animais em experimentação. Os estudos realizados com o veneno da *L. laeta* mostraram resultados contraditórios, em relação ao efeito do mesmo sobre glóbulos vermelhos. KENIERIN et al. (1975) não constataram o efeito lítico do veneno sobre hemácias humanas e de outros animais, ao contrário dos resultados obtidos por outros estudos que mostraram o aparecimento de anemia hemolítica, correlacionada com a esfingomielinase D. Esta enzima agiria degradando a esfingomielina (componente da membrana do eritrócito),e esta reação seria dependente de Ca^{++} e pH ótimo de 7,1 (FORRESTER et al., 1978; KURPIEWSKI et al., 1981; REKOW et al., 1983; REES et al., 1988).

Além desses efeitos, a provável ação do veneno sobre a atividade ATPásica da membrana celular e consequentemente, sobre o mecanismo de troca de íons foi discutida por SUAREZ et al. (1971). Esta constatação, parece ter sido corroborada pelos trabalhos realizados pelo Ministério da Saúde do Brasil em 1992, que sugerem que este efeito possa contribuir para a atividade hemolítica do veneno, pelo aumento do fluxo de líquido para o interior da célula (BRASIL,1992).

Em estudos que comparam a capacidade hemolítica do veneno da *L. rufipes* sobre hemácias de diferentes espécies animais (KELEN et al., 1960/62), mostrou-se que o veneno da *L. rufipes* hemolisou com intensidade eritrócitos humanos e de bovinos. Tal situação, porém, não se repetiu em eritrócitos de outras espécies como cão, cavalo, coelho, cobaia, rato e camundongo.

Essas diferenças, evidentemente, podem estar relacionadas ao tipo de metodologia empregada, à técnica de extração do veneno, à sua estocagem e, ao pH e composição de eletrólitos das soluções experimentais utilizadas, além de outros fatores correlatos (BRAVO et al., 1993; SATAKE, 1994).

Alguns estudos foram realizados com ênfase na imunologia do veneno da Loxosceles. Entre estes estão os realizados por BRAVO et al. (1993), que afirmam ser o efeito hemolítico do veneno independente da ação de anticorpos. Ainda foram feitos experimentos para avaliar o efeito de frações protéicas do veneno, supostamente tóxicas, influenciando na fisiologia dos neutrófilos e na ação do complemento. Nestes estudos o veneno da espécie *L. reclusa* demostrou capacidade de inibir a quimiotaxia dos neutrófilos. No entanto, nenhuma alteração foi observada quanto à capacidade fagocitária destas células. Este estudo ainda encontrou alterações na eficiência dos mecanismos de coagulação, por induzir a diminuição do complemento sérico, o prolongamento do tempo da atividade parcial da tromboplastina e redução em 44% dos fatores de coagulação XII, XI, IX e VIII. O tempo de protrombina do plasma foi também prolongado de 1,5 a 2,0 segundos (BABCOCK, 1981; BABCOCK et al., 1986). Mais recentemente, nosso grupo de trabalho pode mostrar que o veneno tem princípios que atuam diretamente sobre células endoteliais de vasos sangüíneos (VEIGA *et al.*, 2001) além de degradar membranas basais e o fibrinogênio (VEIGA *et al.*, 2000 e ZANETTI *et al.*, 2002). Tais ações deletérias do veneno parecem ser decorrentes de metaloproteases presentes no mesmo (FEITOSA *et al.*, 1998; VEIGA *et al.*, 1999 e DA SILVEIRA *et al.*, 2002).

Contudo, tais estudos são muito preliminares para explicar em definitivo, o processo de estabelecimento e evolução dos quadros clínicos produzidos pelo veneno de *L. intermedia* .

Os venenos aracnídicos em geral consistem de misturas de proteínas com ações enzimáticas ou tóxicas. BARBARO et al.(1994a) e BARBARO et al. (1994b) compararam a reatividade cruzada dos venenos das diferentes espécies de Loxosceles mostrando, por análises em SDS-PAGE, que os venenos das aranhas *L. gaucho*, *L. intermedia* e *L. laeta* possuem pelo menos 9 bandas

protéicas, estando as principais localizadas na faixa de 32 a 35 kDa. O isolamento e caracterização destas frações protéicas é objetivo perseguido por vários pesquisadores, num esforço para detectar as bases moleculares da ação do veneno produzido por estes animais.

No entanto, outros animais peçonhentos de importância médica, possuem venenos que são também muito estudados, na tentativa de se esclarecer tanto a composição bioquímica, quanto a atividade biológica dos mesmos. Assim, alguns estudos desenvolvidos com venenos de cobras, demonstraram a presença abundante de enzimas proteolíticas em sua composição, mostrando que estas podem influir na toxicidade desses venenos. Isto é válido principalmente para a família Crotalidae e Viperidae (KINI,1995).

Os componentes da matriz extracelular são alvo de atividade para algumas das proteases presentes em venenos de cobras, que por esta ação produzem vários efeitos nocivos (principalmente os ligados à hemostasia), destacando-se a degradação proteolítica dirigida sobre moléculas da matriz extracelular solúveis no plasma, como a fibronectina e o fibrinogênio (WILLIANS et al.,1983). Existem estudos que afirmam ser este tipo de degradação o responsável pelos distúrbios hematológicos apresentados em vários tipos de acidentes com ofídios (ARAGON-ORTIZ and GUBENSEK, 1987; HITE et al., 1992; BODE et al., 1993; GUTIERREZ et al.,1995), principalmente quando envolvem enzimas do tipo metaloproteases. Dentre a família de metaloproteases, dois tipos de moléculas merecem destaque, pela importância e prevalência dos estudos: as metaloproteases de matriz extracelular e as reprolisinas. Estas moléculas são endopeptidases que caracterizam-se por clivar praticamente todas as moléculas da matriz extracelular. Podem atuar sobre a regulação e organização das moléculas da matriz extracelular, influenciando assim, todos os processos nos quais estejam envolvidas, entre eles, o desenvolvimento de processos fisiológicos e patológicos nos tecidos, a participação na reestruturação tecidual em doenças invasivas como o câncer ou degenerativas como periodontites, artrites e osteoporose (BIRKEDAL-HANSEN et al.,1993). Elas são secretadas na matriz sob forma "latente" e são ativadas através de quebra proteolítica nos domínios pró-peptídicos, mais

especificamente sobre pontes de cisteína e, para isso, requerem a participação de íons de metais bivalentes.

Estudos realizados até os dias atuais mostram que o veneno de *Loxosceles intermedia* é rico em proteínas com atividades enzimáticas e pode ser comparado a alguns venenos de cobras, onde os estudos demonstraram a presença abundante de enzimas proteolíticas em sua composição (MARTINEZ-VARGAS, 1987). Entre elas foi descrito uma esfingomielinase de 32 kDa envolvida aparentemente na agregação plaquetária (KURPIEWSKI et al., 1981) e outras moléculas de 33, 34 e 35 kDa associadas com atividades nocivas do veneno (BARBARO et al., 1992; GEREN et al., 1976; TAMBOURGI et al., 1995; FEITOSA et al., 1998; VEIGA et al., 1999 e DA SILVEIRA *et al.*, 2002). O veneno contém ainda outros fatores hemorrágicos, uma metaloprotease de 20-28 kDa (loxolisina A) com efeitos sobre a fibronectina e o fibrinogênio e uma metaloprotease gelatinolítica de 32-35 kDa (loxolisina B) provavelmente associada com a atividade dermonecrótica do veneno (FEITOSA et al., 1998).

QUADRO CLÍNICO CAUSADO PELA PICADA DE *Loxosceles intermedia*

O quadro clínico produzido pelo envenenamento é genericamente denominado como Loxoscelismo e pode variar em função de fatores ligados ao estado geral do indivíduo envolvido no acidente como seu estado nutricional, suceptibilidade ao veneno e idade, como também a quantidade de veneno inoculado, o local da picada e o tempo decorrido até a tomada de medidas terapêuticas (GAJARDO-TOBAR, 1966; SCHENONE and LETONJA,1975; BARBARO et al.,1994b).

O envenenamento causado pela picada da Loxosceles pode estabelecer, em geral, dois tipos de quadros clínicos: quadro cutâneo ou dermonecrótico e quadro cutâneo-visceral ou sistêmico.

O quadro cutâneo caracteriza-se por uma lesão dermonecrótica saliente, que evolui a partir do local da picada e representa em média 80% das formas de

Loxoscelismo (SCHENONE and LETONJA,1975; MARTINEZ-VARGAS,1987; SCHENONE et al.,1989).

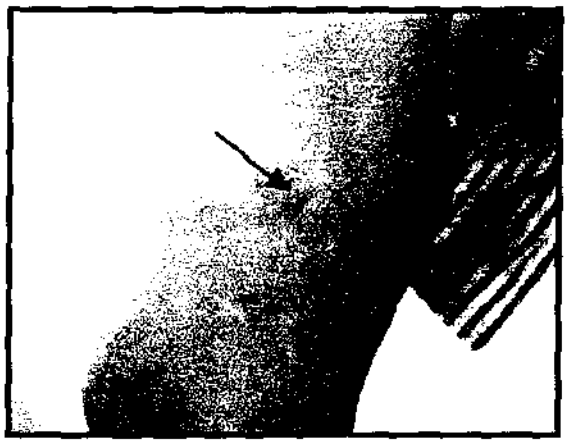
CAVENESS em 1872, descreveu as primeiras características de uma lesão cutânea atribuída ao veneno da "aranha-marrom". Contudo somente em 1947 foram publicados outros relatos que correlacionavam lesões necróticas à ação do veneno desta aranha (MACCIAVELO, 1947). O reconhecimento definitivo da ação patogênica do veneno só foi feito em 1957, a partir de estudos sobre lesões necróticas produzidas pela espécie *L. reclusa* (ATKINS et al., 1957).

Durante muito tempo, as lesões produzidas pelo veneno de *Loxosceles sp.* foram atribuídas às aranhas do gênero Lycosa (BRAZIL and VELARD, 1926). No entanto, avaliações clínicas mais detalhadas e estudos experimentais mais controlados, levaram a constatação de que algumas características dos acidentes loxoscélicos, como, por exemplo, a lesão dermonecrótica, mostravam ser específicas do gênero e que os sinais clínicos apresentavam evolução diferente daqueles atribuídos a outros aracnídeos.

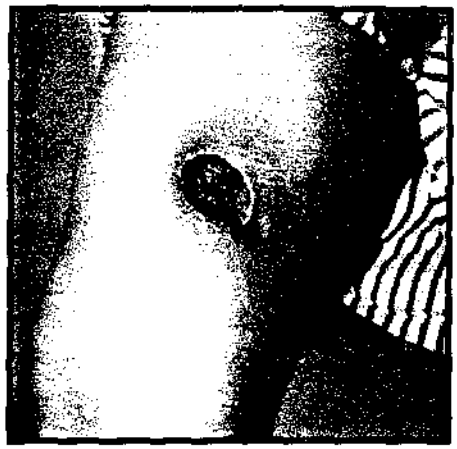
Uma importante característica do quadro de Loxoscelismo, é a dor, que aparece no quadro cutâneo e é relatada como "queimação" ou ardência, evidenciando-se horas após o acidente, e se acentuando com o passar do tempo. Esta dor, é geralmente acompanhada de prurido, edema e eritema. Em seguida, pode surgir uma lesão de 1 a 30 cm de diâmetro, circundada por um halo vermelho e zonas pálidas, denominada de "placa marmórea".

Conforme relato de vários autores, a lesão do loxoscelismo, pode evoluir para a formação de úlcera necrótica ou "mancha gangrenosa", que por ser de difícil cicatrização, necessita por vezes, que o tecido necrosado sofra excisão e cirurgia reparadora subsequente. Em algumas situações, apesar da aplicação de tais procedimentos, a incapacitação permanente do membro afetado pode ocorrer, devido a grande evolução da lesão dermonecrótica (GAJARDO-TOBAR, 1966; SCHENONE and LETONJA, 1975; BARBARO et al.,1994a; BARBARO et al., 1994b).

Quadro Dermonecrótico



Aspecto Inicial da Lesão



Aspecto Avançado

Pacientes portadores de loxoscelismo por *L. reclusa*, mostraram em biópsias, a presença de células inflamatórias, vasodilatação, degeneração da parede dos vasos sanguíneos, hemorragia na derme e tecido subcutâneo. Além disso pode ser observado o acúmulo de leucócitos polimorfonucleares, necrose e formação de abcessos, que podem ocorrer 3 a 5 dias após o acidente (SMITH et al., 1970 ; FUTREL, 1992). Estudos da histopatologia experimental em coelhos, realizados por nosso grupo, confirmaram que, para a *L. intermedia* (aranha marrom que prevalece em nossa região), os processos hemorrágico e inflamatório são conservados e aparentemente idêntico aos causados pela *L. reclusa* (OSPEDAL *et al.*, 2002).

Deve-se salientar, além disso, que dados disponíveis na literatura em relação à ação dermonecrótica do veneno de aranhas deste gênero (REES et al., 1988), bem como análise dos acidentes ocorridos especificamente com a espécie *L. intermedia* na cidade de Curitiba, alertam sobre o fato de que o problema resultante da picada pode durar vários anos (há relatos de até oito anos), sendo que, em muitos casos, há recorrência freqüente, de difícil controle médico. A agressão causada ao acidentado, embora raramente mortal, pode produzir, assim, ferimentos por longos períodos de tempo, com ulcerações cutâneas

persistentes. Além disso a necrose que poderá formar-se é determinante para o advento de outros problemas, como por exemplo, a contaminação secundária da ferida por agentes bacterianos e fúngicos patogênicos, como recentemente mostrado por nosso grupo (MONTEIRO *et al.*, 2002).

Como a biologia e a bioquímica do veneno dessa aranha (*L. intermedia*) são quase que totalmente desconhecidas, o diagnóstico do acidente tem sido difícil e o tratamento realiza-se, via de regra, sem bases científicas sólidas. Não se sabe, por exemplo, qual (ou quais) das frações do veneno total é (são) a (s) causadora (s) dos problemas decorrentes da picada. Verifica-se assim, que os cuidados médicos são realizados em bases ainda bastante empíricas, com todos os problemas que daí podem advir ao paciente.

O quadro conhecido como cutâneo-visceral é menos freqüente, representando em média 14% dos casos e com evolução ocorrendo geralmente nas primeiras 24 horas pós-acidente (SCHENONE and LETONJA,1975; MARTINEZ-VARGAS,1987; SCHENONE et al.,1989; COUTINHO,1996). Este inclui o aparecimento de astenia, febre, emese, cefaléia, alterações sensoriais, insônia, podendo chegar eventualmente ao coma. As manifestações sistêmicas incluem alterações no quadro hematológico, podendo levar à coagulação intravascular disseminada (CID), agregação plaquetária e anemia hemolítica (FORRESTER et al., 1978; BASCUR et al., 1982; RESS et al., 1988).

Estudos histopatológicos analisaram efeitos do veneno da "aranha-marrom", e nestes foram observados a oclusão de vênulas e artérias, tanto em lesões humanas como em coelhos. Além disso, nos pacientes que foram a óbito em conseqüência de loxoscelismo sistêmico, as autópsias realizadas, mostraram alterações degenerativas nos parênquimas de vários orgãos. Edema, congestão, hemorragia e erosões da mucosa digestiva, estavam presentes e associadas a estas lesões, além de lesões tubulares renais do tipo nefrose hemoglobinúrica (PIZZI, et al., 1957; SCHENONE and LETONJA, 1975; SCHENONE et al., 1989). PIZZI et al. (1957) verificaram que em órgãos como o pulmão, fígado e rins ocorriam efeitos sobre o sistema de vasos sanguíneos (vênulas e artérias). MARTINEZ-VARGAS, 1987 relata que em 62% dos casos de loxoscelismo

cutâneo-visceral, o rim acha-se comprometido, atingindo o estado de insuficiência renal aguda (IRA). A taxa de mortalidade mais elevada foi encontrada naqueles pacientes nos quais procedimentos dialíticos não foram realizados e estes, apresentaram com frequência padrões histológicos característicos de necrose tubular aguda. Recentemente foi demonstrado por COUTINHO (1996), que a inoculação de doses sub-letais do veneno em ratos exercia evidentes influências nas condições fisiológicas e estruturais normais do rim, que poderiam induzir ao quadro de IRA. Nos animais inoculados, verificou-se a presença de hemólise intravascular, diminuição do hematócrito e elevação dos níveis plasmáticos de bilirrubina.

Esse procedimento também afetou a integridade estrutural do epitélio tubular, onde foram encontradas lesões degenerativas, com a presença de necrose, células hipertrofiadas e intensa vacuolização citoplasmática. As alças de Henle, não apresentaram alterações significativas naquele estudo. Com relação aos glomérulos foi observada hipertrofia das células da membrana parietal, além de descamação celular. Este quadro histológico é conhecido também para casos de envenenamento humano com animais peçonhentos de outras espécies (LIMA,1966).

Estes dados levam a crer na possível atividade hemolítica do veneno. BASCUR et al. (1982), estudando o efeito do veneno de *L. laeta* sobre a coagulação, demonstraram a queda nos valores do fibrinogênio e aumento dos produtos de degradação da fibrina, efeitos estes encontrados na manifestação da coagulação intravascular disseminada (CID). COUTINHO (1996) afirma que o efeito hemolítico contribui decididamente na injúria renal, sugerindo que a presença de aglomerados de hemoglobina livre, precipitados ao longo do néfron, possam retardar o fluxo do fluido tubular e produzir uma resposta patológica, representada pela redução do ritmo de filtração glomerular, desencadeando a IRA

Sabe-se que as membranas basais desempenham diversas funções vitais, tanto em tecidos embrionários, como em adultos, em condições normais e patológicas. No caso específico da membrana basal glomerular, está estabelecido que a mesma age como uma barreira seletiva, regulando a filtração,

tanto em carga, quanto em tamanho de moléculas do plasma que passam para o espaço urinário; e que os sítios aniônicos regularmente distribuídos na mesma são representados por proteoglicanos e glicosaminoglicanos, principalmente heparan sulfato e ácido hialurônico. Estudos mostram que a remoção enzimática do heparan sulfato e não do ácido hialurônico, pode produzir alterações na permeabilidade da membrana basal glomerular (KANVAR and FAQUHAR.,1979). Assim, a ausência de proteoglicanos ou um bloqueio de suas funções, influencia o processo de filtração renal, levando em geral a situação de proteinúria. Dados recentes do nosso grupo, mostram que o veneno degrada heparam sulfato proteoglicano "in vitro", podendo assim justificar molecularmente alguma ação renal (VEIGA *et al.*, 2000).

No estudo realizado por COUTINHO (1996), verificou-se também intensa proteinúria nos animais envenenados, sugerindo-se que esta decorra de alterações na membrana basal glomerular, mais especificamente sobre a atividade seletiva exercida por esta em função da presença de sítios aniônicos existentes na mesma. Dados experimentais, obtidos em estudos-piloto, em nosso laboratório, com o veneno de *L. intermedia,* indicaram a existência de proteínas catiônicas, quando o perfil eletroforético do veneno foi ensaiado utilizando-se técnicas de focalização isoelétrica (IEF e SDS-PAGE). Assim, o efeito tóxico neste caso, seria traduzido por uma desorganização eletrostática da membrana glomerular e o processo de filtração seria prejudicado, levando à excreção de proteínas plamáticas que, no plasma, exibem carga negativa (DE BARROS SILVA et al., 1992).

Trabalhos claramente conclusivos demonstram que o proteoglicano envolvido nos processos filtrantes da membrana basal glomerular é um heparam sulfato. KANVAR and FAQUHAR (1979), em trabalho clássico, comprovaram essa situação.

Além do proteoglicano heparam sulfato, há outros componentes da membrana basal glomerular que devem ser observados nas situações, onde a mesma sofre processos agressivos. Trata-se das glicoproteínas laminina, colágeno tipo IV, entactina e a fibronectina. São macromoléculas que

desempenham uma ativa função, tanto fisiológica quanto estrutural. Na literatura consultada não há estudos conclusivos a respeito da relação entre os venenos e tais glicoproteínas. Considerando-se que, em cerca de 9% dos casos de loxoscelismo, ocorre IRA, é lícito supor que uma das estruturas que certamente sofrerá evidente agressão deverá ser a membrana basal glomerular.

Como se percebe pelo exposto, estudos mais abrangentes são indispensáveis para se entender em maior profundidade e abrangência a biologia do veneno desse aracnídeo, a lesão dermonecrótica, bem como o quadro cutâneo-visceral.

POTENCIAL BIOTECNOLÓGICO

A utilização de venenos de animais peçonhentos ou toxinas purificadas destes venenos tem originado aplicabilidades biotecnológicas na indústria farmacêutica, além de ferramentas aplicadas nas diversas áreas da ciência. No caso dos venenos loxoscélicos, buscaremos identificar e aplicar biotecnologicamente algumas toxinas. Os peptídeos de baixa massa molecular podem ser modelos biológicos de inibidores ou efetores de canais iônicos. Algumas toxinas dermonecróticas poderão servir como ferramentas diretas ou modelos para desenho de drogas que atuem nos processo inflamatórios. Toxinas com atividades proteolíticas ou hialuronidásicas, poderão ter aplicabilidades em patologias obstrutivas. Dentro deste projeto, além dos objetivos já enunciados poderemos também, através de nossos resultados, chegarmos a algumas aplicabilidades biotecnológicas do veneno ou toxinas oriundas deste.

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Resultados (Artigos)

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Structural and ultrastructural description of the venom gland of *Loxosceles intermedia* (brown spider)

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Abstract

The brown spider, genus *Loxosceles*, is becoming of great medical importance, with envenomation (Loxoscelism) occurring throughout the world. The biological activities of the brown spider venom usually include dermonecrotic lesions at the bite site accompanied by hemolytic and haemorrhagic effects and also by renal failure. The objective of the present study was to describe the histology of the venom gland of *L. intermedia* using glands from adult spiders which were investigated by light microscopy, using immunohistochemical and staining methods, by transmission electron microscopy, and by scanning electron microscopy. The organization of the venom gland of *Loxosceles intermedia* follows the general architecture of spiders' venom glands. Using light microscopy and transmission electron microscopy we observed that the venom glands of *L. intermedia* present two layers of striated muscle fibers, an external layer and an internal layer in touch with an extracellular matrix which is a basement membrane structure and a fibrillar collagen matrix separating the muscular region from epithelial cells of the venom gland.

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Muscle cells are multinucleated, with nuclei peripherally placed and their cytoplasm rich in sarcoplasmic reticulum, myofibrills and continuous Z lines. By using scanning electron microscopy we can detect muscular cells from external layer as branching cells. Epithelial cells have their cytosol extremely rich in rough endoplasmic reticulum, mitochondria collection, Golgi apparatus, interdigitating membranes and secretory vesicles that ultimately accumulate the venom, a complex protein mixture.

1. Introduction

Many studies on venomous animals and their venoms have been published in the literature due to a direct connection of these venoms with many biological areas and to the pathological problems they may cause (Futrell, 1992; Bjarnason and Fox, 1995; Kamiguti et al., 1996).

The objective of the present study was to describe the histology and ultrastructural cytology of the venom gland of *L. intermedia* spider. Very little is known about the biology of this spider and the venom secreted by it. With respect to this, the present study is intended as a contribution to a more in-depth investigation of these venomous animals.

Spiders of the genus *Loxosceles* provoke clinical signs and symptoms named Loxoscelism in victims of their bites. The skin lesions are characterized by dermonecrosis, erythema, edema and haemorrhage, with the possible occurrence of local necrotic ulcers of difficult cicatrization (Forrester et al., 1978; Wasserman and Anderson, 1984; Futrell, 1992). The systemic effects of the venom include the appearance of fever and malaise and may lead to hemolysis, disseminated intravascular coagulation, platelet aggregation and renal failure (Kurpiewski et al., 1981; Bascur et al., 1982; Rees et al., 1988; Futrell, 1992).

The high incidence of bites in humans is due to the fact that brown spiders have acquired domiciliary habits. Although the spiders are not aggressive they bite when compressed by people as they put on or take off their clothes (Lucas, 1988; Futrell, 1992).

Spiders of the genus *Loxosceles* range 8–15 mm in body length and their legs measure 8–30 mm. They are sedentary and have night habits. They build irregular webs that look like cotton thread. They eat little insects and reproduce easily even in unfavorable environments (Hite et al., 1960; Futrell, 1992). They can withstand temperatures ranging from 8 to 43°C, with the occurrence of bites being higher in the hotter months of the year (74%). Both males and females are venomous and they can survive up to 276 days without water or food (Lowrie, 1980; Gertsch and Lennik, 1983; Futrell, 1992).

Martinez-Vargas (1987) reported that the venom is produced by apocrine glands situated in the cephalothorax which communicate with the outside through two ducts that lead into the inoculator apparatus formed by a pair of chelicerae. The

venom is essentially of a protein nature, with enzymatic or toxic features and can be compared to the venom of snakes, which has been shown to contain several different enzymes in its composition (Futrell, 1992).

The venom of the brown spider is formed by many enzymes, among them a sphingomyelinase of 32 kDa, apparently involved in platelet aggregation (Kurpiewski et al., 1981) and other molecules of 33, 34 and 35 kDa related to harmful activities of the venom (Geren et al., 1976; Barbaro et al., 1992; Tambourgi et al., 1995; Feitosa et al., 1998; Veiga et al., 1999). The venom also contains other active factors, a metalloproteinase of 20–28 kDa (loxolysin A) with fibronectinolytic and fibrinogenolytic effects and a gelatinolytic metalloproteinase of 32–35 kDa (loxolysin B) probably associated with the dermonecrosis and haemorrhagic effects (Feitosa et al., 1998). In a study of the oligosaccharide profile of glycoprotein constituents of *L. intermedia* venom, it was verified that the venom has several proteins post-translationally modified by asparagine-linked sugars and at least one glycoprotein with serine/threonine-linked sugars, with N-linked sugars playing a role in the gelatinolytic effect of loxolysin B and also playing an important role in the dermonecrotic effect of the venom (Veiga et al., 1999).

In the present report, we describe the venom gland of *L. intermedia* submitted to morphological, ultrastructural and immunological investigations. This technical information could be important for a better understanding of Loxoscelism, providing some basis for the knowledge of this type of envenomation.

2. Materials and methods

2.1. Animals

Adult *L. intermedia* were collected in the city of Curitiba and in its surroundings (Paraná, Brazil). Spiders were anesthetized with chloroform (Merck, Rio de Janeiro) and the venom gland was removed for histological and ultrastructural examination.

2.2. Histological methods for light microscopy

Venom glands were fixed in Carnoy's solution (Beçak and Paulete, 1976) for 2 h, dehydrated in an ethanol (Merck) series, cleared in xylene (Merck), embedded in paraffin (Merck) and cut into 4 μm thick sections. The sections were stained with hematoxylin and eosin (Merck), periodic acid-Schiff (PAS, Merck, McManus, 1948), alcian blue (Merck, Beçak and Paulete, 1976) and picrosirus (Merck, Junqueira et al., 1979).

2.3. Immunohistochemical analysis

Paraffin sections mounted on glass slides were deparaffinized in xylene overnight

and rehydrated in a graded ethanol series and water. The sections were then washed with PBS, incubated in 3% H_2O_2 (Merck) at room temperature for 15 min to inhibit the activity of endogenous peroxidase, washed with PBS, and nonspecific protein-binding sites were blocked with 1% bovine serum albumin (Sigma, St. Louis) in PBS at room temperature for 30 min under humidified conditions. After washing in PBS, sections were incubated for 2 h at 37°C with a primary polyclonal anti-laminin antibodies diluted 1:500 (a gift from Dr. R.R. Brentani, Ludwig Institute for Cancer Research, São Paulo, Brazil) or primary polyclonal anti-entactin antibodies (Rb_2 α-ET), diluted 1:500 (produced in our laboratory using purified EHS-entactin, as described Harlow and Lane, 1988), or primary polyclonal antibodies against crude venom diluted 1:250 (a gift from Dr. C.C. Olórtegui, from Fundação Ezequiel Dias, Belo Horizonte, Brazil). Excess antibody was removed with PBS and then the materials were incubated with goat anti rabbit IgG peroxidase conjugate (Sigma) diluted 1:100. Following further washing in PBS, diaminobenzidine (Sigma) was used to visualize the immunoreactivity. Sections were washed in PBS and water, dehydrated in ethanol, cleared in xylene, and mounted in Entellan (Merck). Negative control reactions were performed by incubating the sections with pre-immune serums diluted in PBS under the same experimental conditions as described for hyperimmune serums.

2.4. Transmission electron microscopy

For electron microscopy the venom glands were fixed in modified Karnovsky's fixative (Karnovsky, 1965; Gremski and Cutler, 1986) for 2 h. After fixation, the tissues were washed in 0.1 M cacodylic acid buffer (EMS, Fort Washington), pH 7.3, postfixed in 1% OsO_4 (Polysciences, Warrington) in 0.1 M cacodylic acid buffer, pH 7.3, for 1 h, dehydrated with ethanol and propylene oxide (Merck), and embedded in Epon 812 (EMS). Thin sections and ultrathin sections were then obtained with a diamond knife on an LKB ultramicrotome. Thin sections were stained with toluidine blue (Merck) and examined by light microscopy. Ultrathin sections were contrasted with uranyl acetate (Merck) and lead citrate (Merck) and examined in a JEOL-JEM 1200 EX II transmission electron microscope at an accelerating voltage of 80 kV.

2.5. Scanning electron microscopy

Venom glands were fixed in modified Karnovsky's fixative (Karnovsky, 1965) for 2 h. After fixation, the tissues were washed in 0.1 M cacodylic acid buffer, pH 7.3, and postfixed in 1% OsO_4 in 0.1 M cacodylic acid buffer, pH 7.3, for 1 h. They were then dehydrated in ethanol, critical-point dried, sputter-coated with gold and examined with a MEV XL-30 Philips scanning electron microscope.

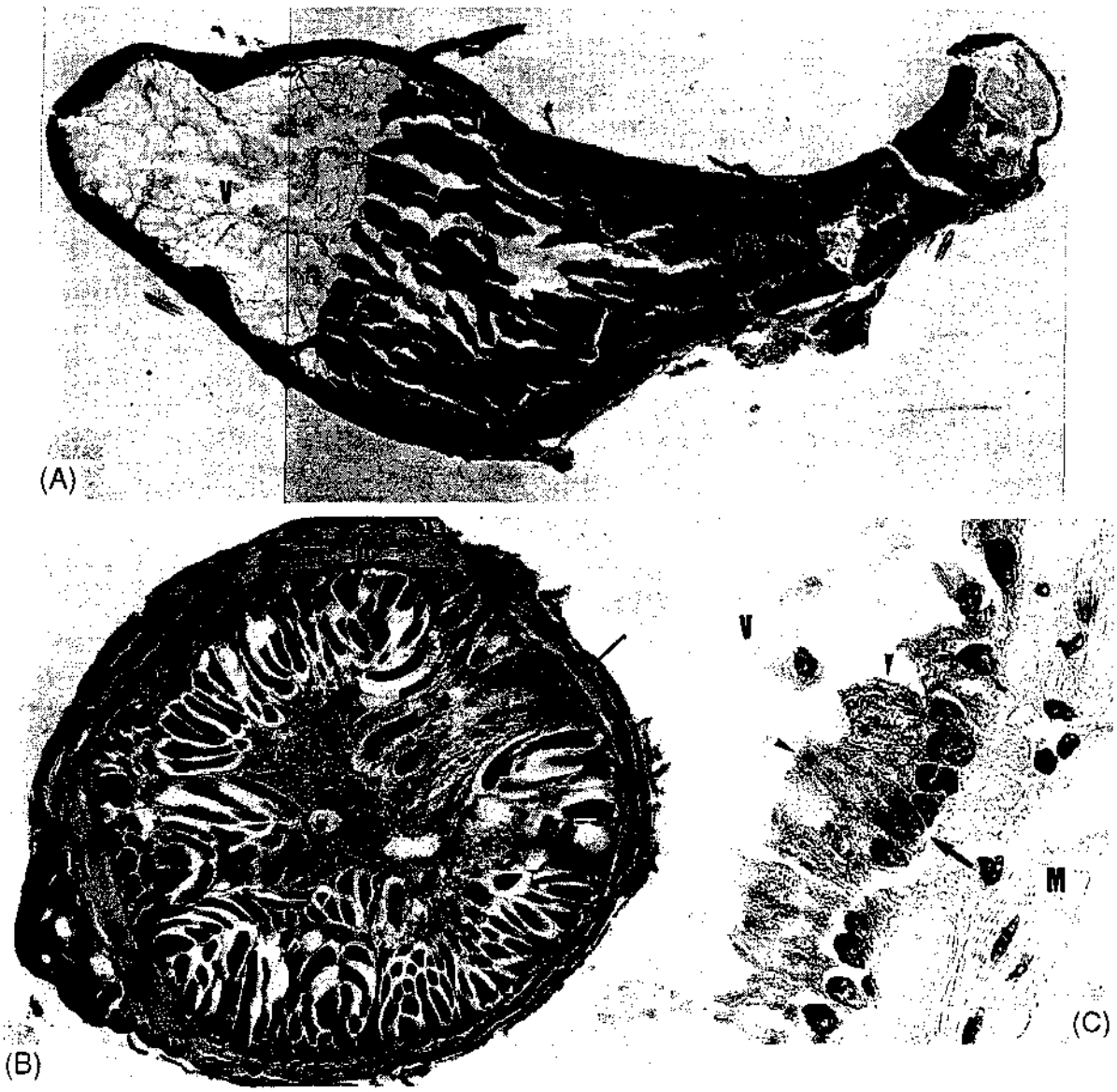


Fig. 1. Hematoxylin–eosin staining and venom immunolabeling of *L. intermedia* gland sections. The venom glands are similar in architecture to those of other spiders. The secretory epithelium (arrows) and two layers of muscular tissue (M) can be seem. The venom (v) is accumulated inside of gland (A) longitudinal section (magnification 100×) and cross-section (B) (magnification 200×) of glands stained with H.E. (C) depicts an insert of a longitudinal section stained with hematoxylin at a higher magnification (400×), where we can observe some characteristics of epithelium as simple, mononucleated, with basal nuclei (arrow) and an hyperactive cytoplasm (arrow heads). Muscular tissue (M) and the lumen of gland (V) can also be observed. (D) and (E) (magnification 200×) show longitudinally sectioned gland, immunolabelled with antibodies against crude venom (arrow heads). M represents the position of muscular tissue (D), and the same reaction using pre-immune serum (E).

3. Results

3.1. Histological analysis of the venom glands of L. intermedia by light microscopy

Examination of *L. intermedia* venom gland sections stained with hematoxylin and eosin showed that they have two layers of striated muscle fibers, one external

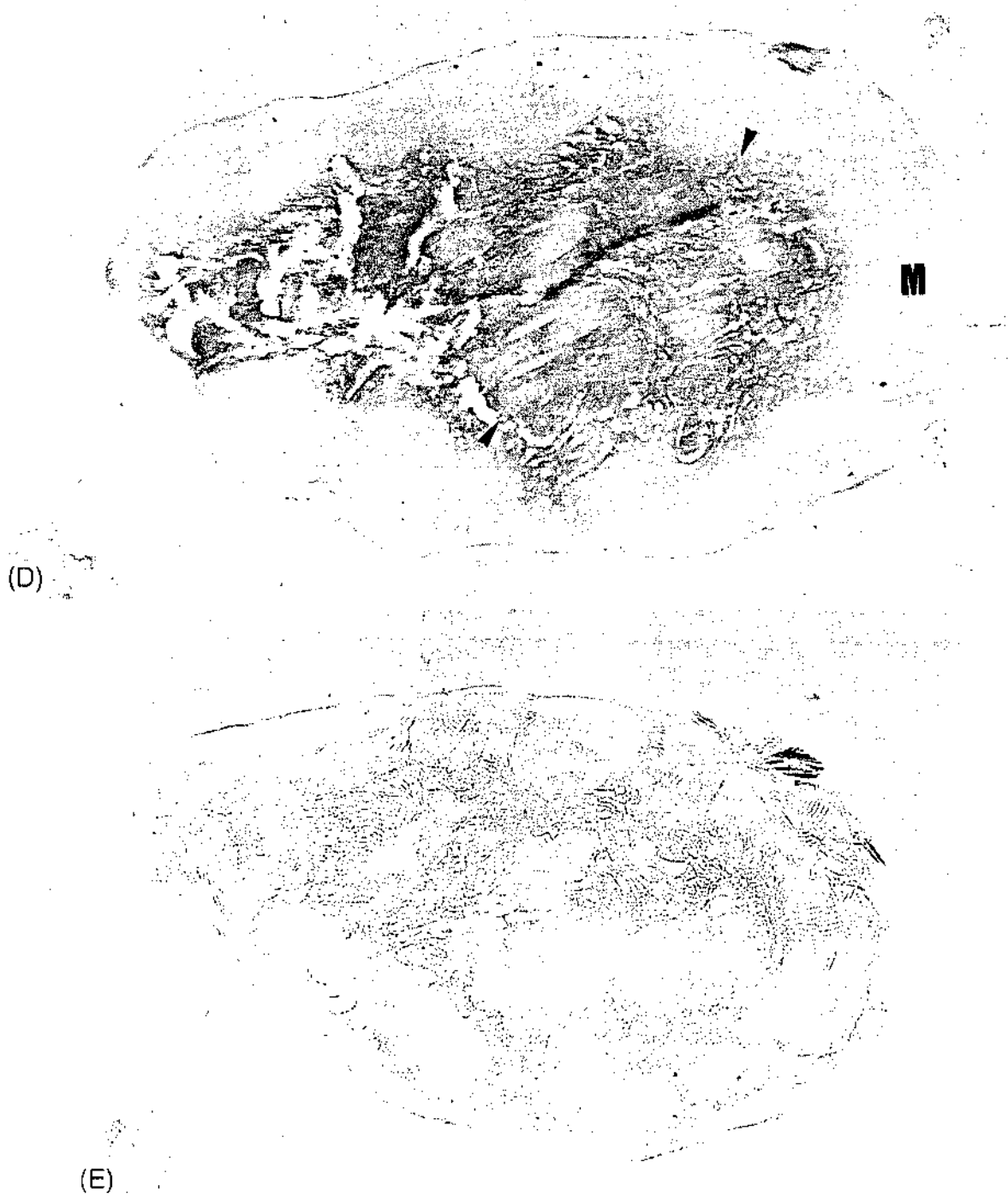
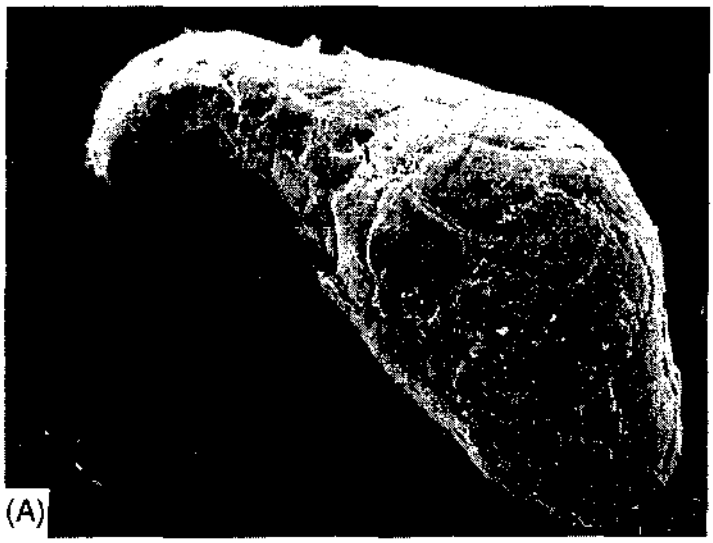


Fig. 1 (*continued*)

layer and one internal layer in touch with an underlying structure that separates the muscular region from the epithelial region of the venom gland (Fig. 1A and B), resting internally on the secretory epithelium, that is a simple glandular epithelium, mononucleated cells, with its nuclei aligned peripherally placed close to the underlying tissue (that separates epithelial from muscular cells). Through a longitudinally sectioned venom gland stained with hematoxylin its possible to



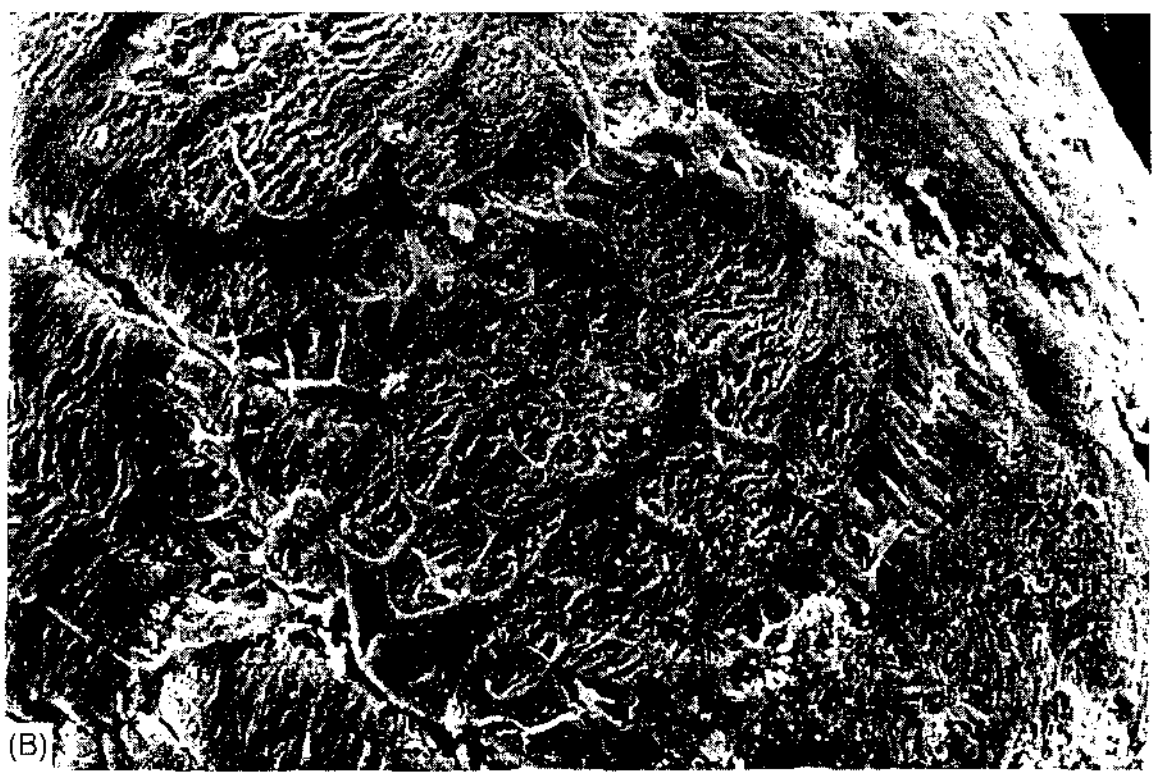


Fig. 2. Morphological and ultrastructural description of muscle cells from *L. intermedia* venom gland. The venom glands are bulbous in shape. (A) depicts scanning electron micrograph of a venom gland at a magnification of 70 ×. The gland is covered with muscle bundles that completely encapsulate it. The asterisk region is shown at a higher magnification (325×) where we observe these muscular cells as branching cells (B). (C) shows a longitudinally sectioned gland stained with hematoxylin and eosin at a magnification of 630 ×. M represents the muscular tissue, arrow heads point nuclei peripherally placed, arrow points the epithelium and V represents the lumen of the gland. (D) and (E) represent transmission electron micrographs of a muscular cell longitudinally sectioned, where a peripherally placed nucleus (closed arrow) can be seen. Closed arrow heads represent continuous Z lines and open arrow heads represent striated myofilaments at a magnification of 4600× (D). (E) shows a muscular tissue section at a higher magnification (30.000×). The closed arrow shows longitudinally placed myofibrils and the closed arrow head depicts cross-sectioned myofilaments at the same section, at right angles to one another, supporting two muscular layers at the same tissue, one placed perpendicular to another. The open arrow represents smooth endoplasmic reticulum (sarcoplasmic reticulum).

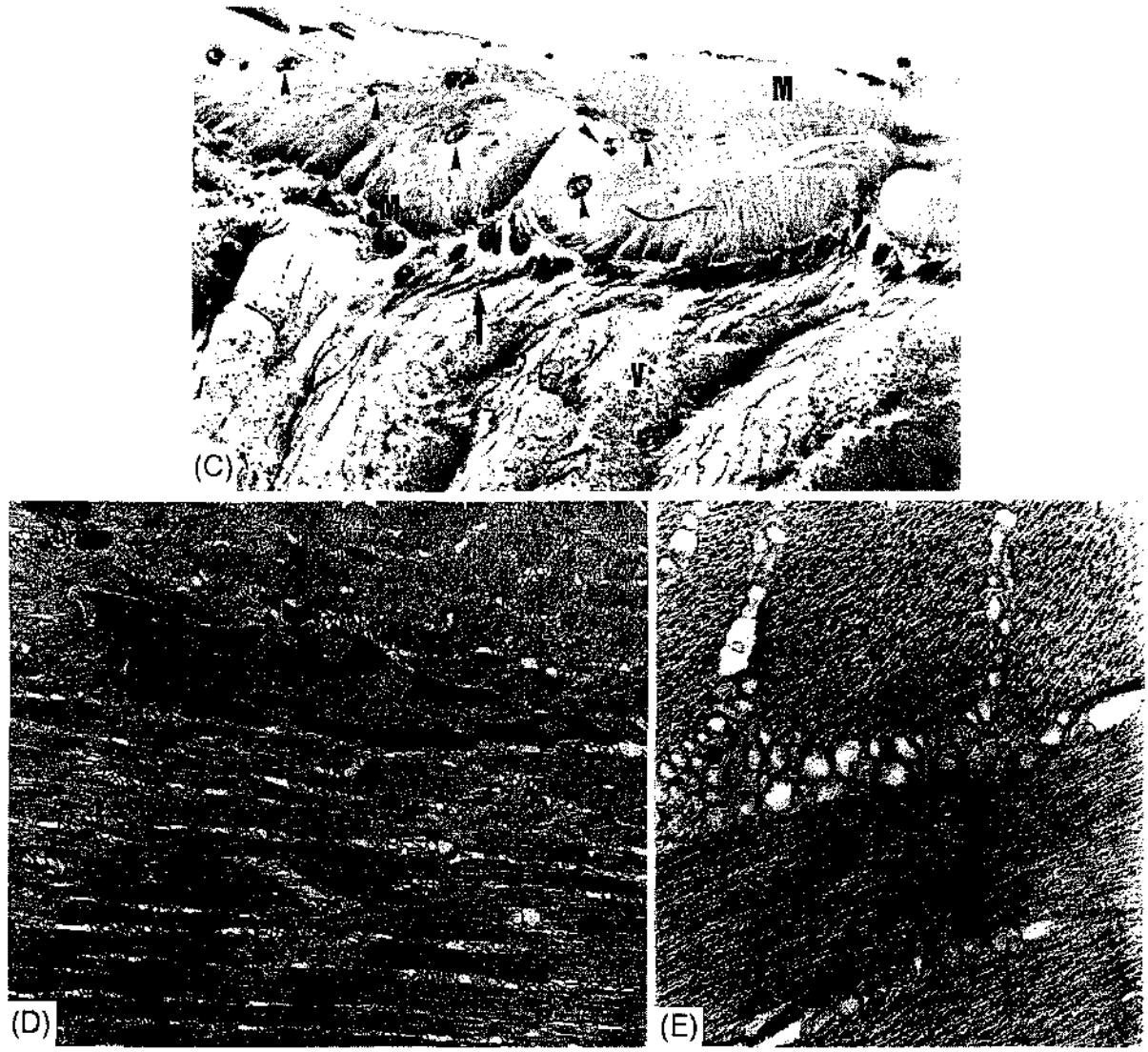


Fig. 2 (*continued*)

Fig. 3. Ultrastructural description of epithelial cells from *L. intermedia* venom gland. (A) depicts a cross-sectioned gland venom of *L. intermedia* observed through transmission electron microscopy at a magnification of 6400 ×. M represents muscular tissue, arrows show the underlying tissue that separates the muscle area from the secretory epithelial cells (ec), where the nuclei (N) can be seen and deposit of venom at the lumen of the epithelial cells (V). (B) Epithelial cells present a lengthened nucleus (N) located close to their basal portion. M represents muscle tissue, arrows depict the underlying structure that separates epithelial cells from muscle cells and i represent interdigitating epithelial cell membranes (magnification 14.000×). (C) Epithelial cytoplasm is rich in rough endoplasmic reticulum (er), mitochondria collections (mt) and venom granules (g) (magnification 10.000×). (D) Golgi apparatus (Ga) and secretion vesicles (Se) are easily recognized (magnification 36.000×). Electron micrographs of the *L. intermedia* venom gland suggest that the secretion mechanism is of the holocrine type. (E) depicts some epithelial cells (ec) adhered onto underlying tissue (arrows) and other epithelial cells undergo a degeneration, where we can observe detaching cells from underlying tissue (arrow heads). m represents muscular tissue and V depict venom deposits (magnification 2600×). Cell debris (Cd) are shown eliminated with the venom secretion (F) (magnification 6800×).

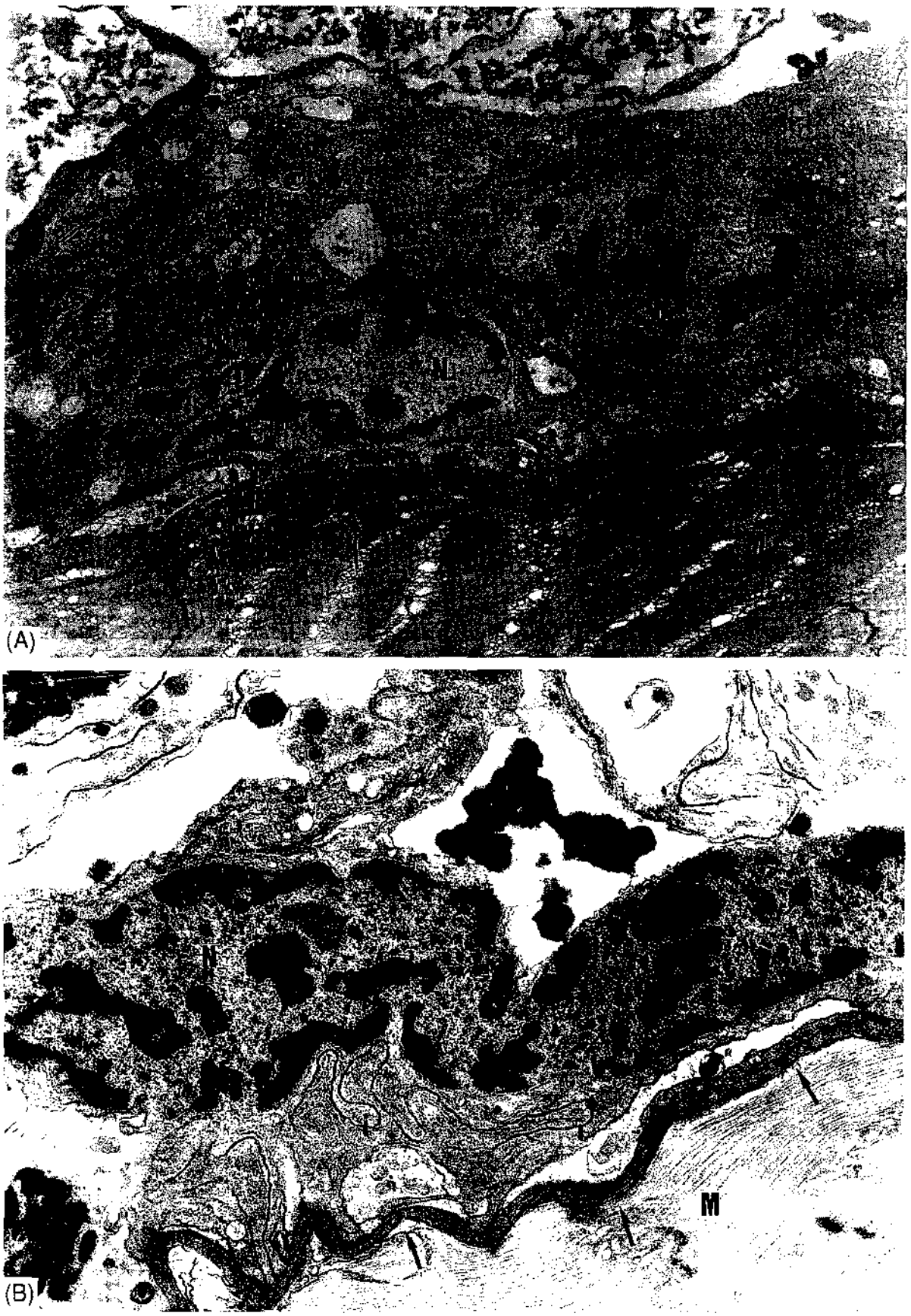


Fig. 3 (*Caption opposite*)

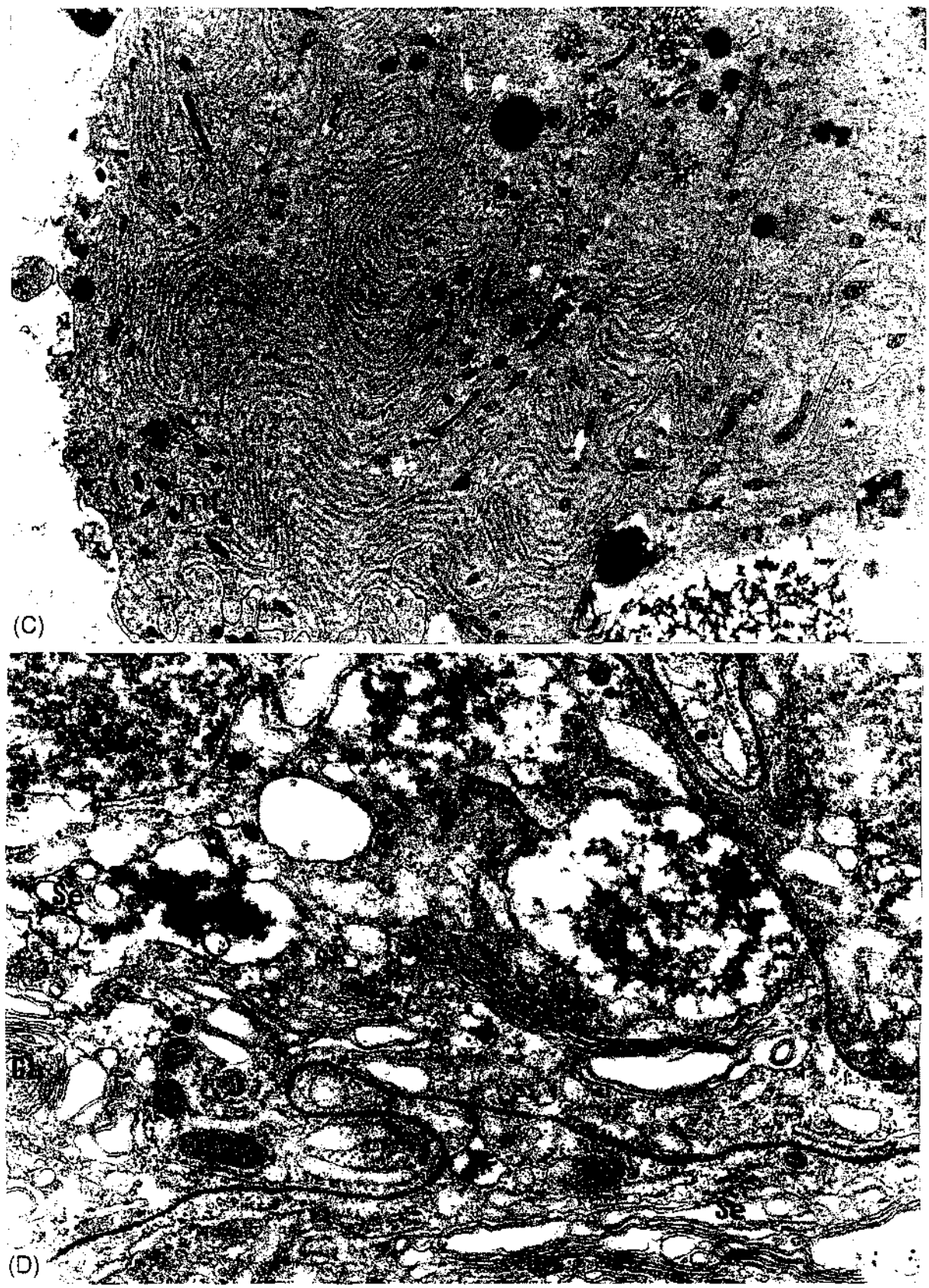


Fig. 3 (*continued*)

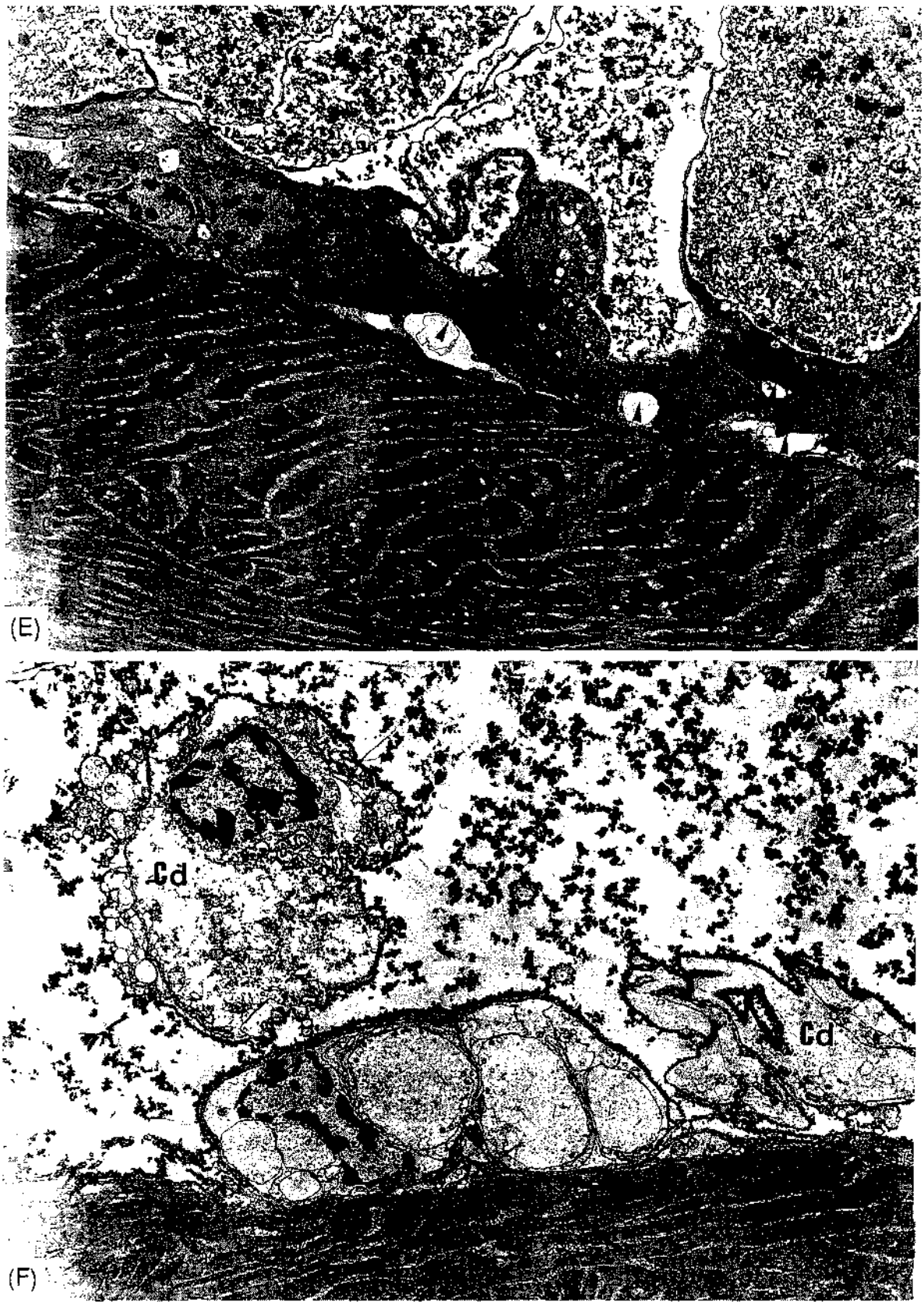


Fig. 3 (*continued*)

observe that epithelial cell cytoplasm are arranged side by side containing projections toward the lumen of gland what suggest hyperactivity (Fig. 1C). The lumen of the gland is rich on secretory vesicles containing venom comproved through an immunolabelling using a polyclonal antibody against crude venom (Fig. 1D and E).

3.2. Morphologycal and ultrastructural studies of muscle cells from L. intermedia venom glands by light, scanning and transmission electron microscopies

The results obtained by scanning electron microscopy show that *L. intermedia* venom gland is bulbous in shape (Fig. 2A). Blocks of muscle bundles completely encapsulating the gland can also be observed, we can conclude that external muscular venom gland cells are branching cells in morphology (Fig. 2B). By using gland sections stained by hematoxylin and eosin we can see these muscular cells as transversely striated, multinucleated with the nuclei peripherally aligned (Fig. 2C). Through transmission electron microscopy micrographs we can see these muscular cells with longitudinally aligned nuclei. The myofilaments form defined sarcomeres, delimited by continuous Z lines. Muscle cells depict in its cytoplasm bundles with a large number of co-aligned myofilaments (Fig. 2D), these muscle filaments seem to be related to contractility and represent the major cytoplasmic content of muscle cells. We can observe perpendicular arrangement of filaments at the same sectioned material, corroborating the two layers of striated muscle cells described in Fig. 1 and a large number of smooth reticulum (sarcoplasmatic reticulum) (Fig. 2E).

3.3. Morphologycal and ultrastructural studies of epithelial cells from L. intermedia venom glands by transmission electron microscopy

The secretory epithelium of *L. intermedia* venom glands is formed by a simple glandular epithelium (Fig. 3A). The cells present a lengthened nucleus placed close to their basal portion (Fig. 3B), their cytoplasm is rich in rough endoplasmic reticulum, contain collections of mitochondria (Fig. 3C), Golgi apparatus and many interdigitations are observed among the cells, increasing the surface of the secretory cells (Fig. 3D). In the apical region of the cells near the gland lumen there are many secretory granules of different sizes and electron densities (Fig. 3D). The products of secretion are kept in pockets limited by cytoplasmic membranes (Fig. 3E). Transmissiom electron micrograph of epithelium venom gland shows several epithelial cells adhered to underlying tissue that separate these cells from muscle and other epithelials cells undergo a degeneration (Fig. 3E and F) and being eliminated among products of venom secretion (Fig. 3F), suggesting a holocrine mechanism controlling venom secretion.

3.4. Molecular analysis of the underlying structure between epithelial and muscular cells

We analyzed the structure that separates the muscle tissue from the secretory epithelial tissue in the gland.

Fig. 4A shows gland sections where this underlying structure is visualized by periodic acid-Schiff (PAS) staining, a technique widely employed to demonstrate the presence of glycoproteins in histological sections. It is possible to observe the positivity of this structure throughout its extension, showing a marked presence of oligosaccharide residues in this region of the gland. Fig. 4B depicts a gland section stained with alcian blue. The underlying structure was found to be positive to alcian blue, showing the presence of glycosaminoglycan sulfated residues in this region, colocalized with the PAS positive structure. By using a monospecific anti-laminin polyclonal antibody (Fig. 4D) and a monospecific anti-entactin polyclonal antibody (Fig. 4F) we can see positivity for laminin and entactin in this underlying region, also colocalized together with the structures rich in glycoproteins and glycosaminoglycans (positive for PAS and alcian blue techniques). Fig. 4C and E represent negative controls for immunolabelling, where we used pre-immune serums. Transmission electron microscopy permitted us to observe fibrils in this region suggesting it as of collagen (Fig. 5A and B), what as confirmed by picrosirius staining. After polarization of light microscopy, we could detect the presence of these molecules in this underlying structure (Fig. 5C).

4. Discussion

The venom glands of *Loxosceles intermedia*, a spider belonging to the suborder Labidognatha (Araneomorphae), family Loxoscelidae, are paired structures located in the cephalothorax that communicate with the outside through two ducts that lead into the inoculator apparatus, formed by a pair of chelicerae. These characteristics are also found in *Latrodectus mactans* (Smith and Russell, 1967), *Loxosceles reclusa* (Foil et al., 1979) and *Ctnedus medius* (Brazil and Vellard, 1925), araeneomorphae spiders. The venom glands of *Plesiophirctus collinus*, a mygalomorphae spider, are located dorsally in the basal article of the chelicerae, between adductor and abductor muscles, and in *Heteropoda venatoria* and *Lycosa indagastrix*, araeneomorphae spiders, the venom glands are located in the cephalothorax, held up by the adductor and abductor muscles (Ridling and Phanuel, 1986).

The position of the stings in the spiders allows us to divide them into two suborders: Araneomorphae whose stings inoculate venom perpendicular to the longitudinal axis of the body, and Megalomorphae, whose stings are parallel to each other and to the a longitudinal axis of the body. All the species on the American continent that can cause human envenomation requiring specialized medical treatment belong to the Araneomorphae suborder (Brazil and Vellard, 1925; Soerensen, 1996).

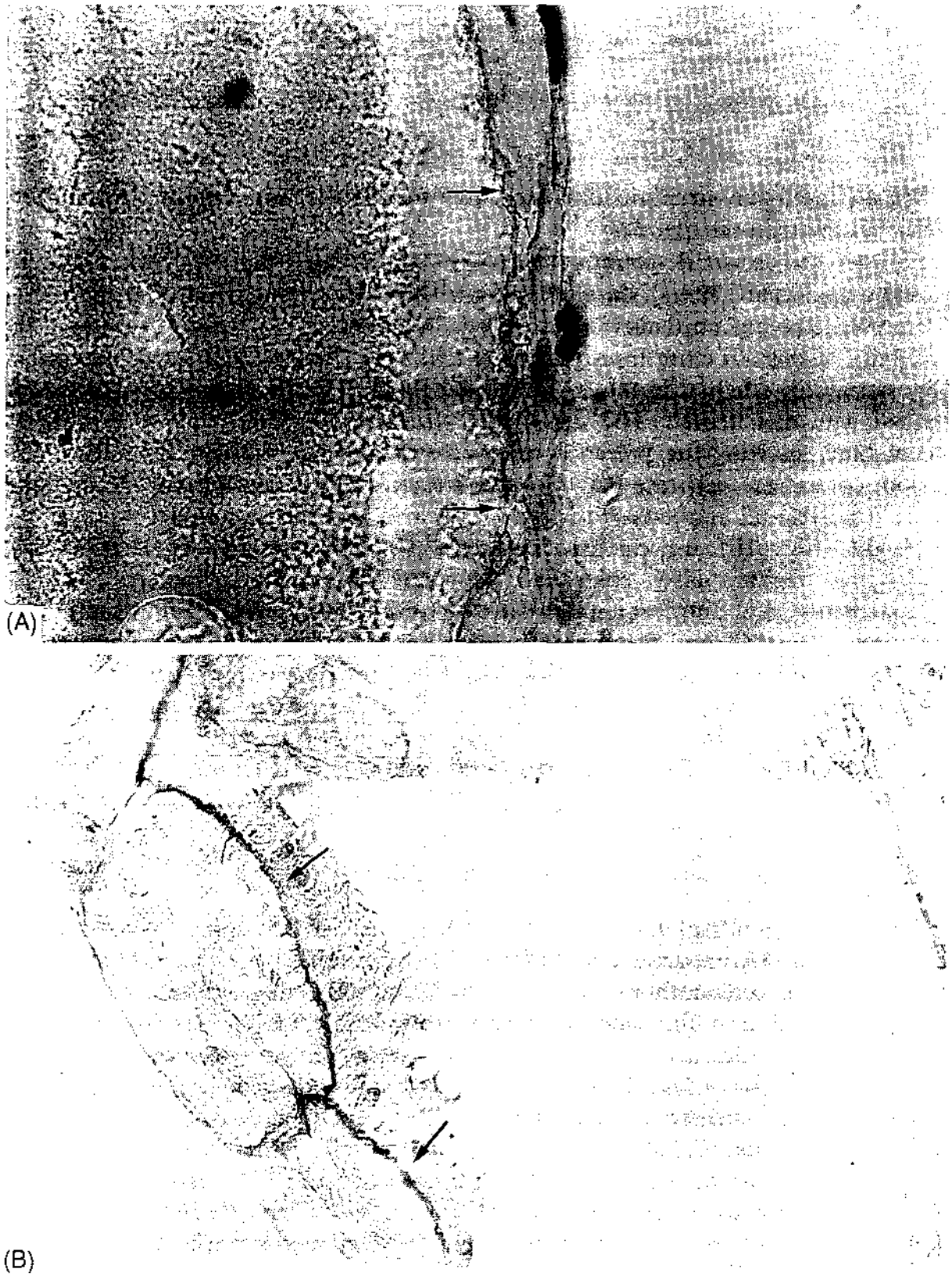


Fig. 4. Molecular analysis of the underlying tissue between muscle and epithelium of *L. intermedia* venom gland. (A) *L.intermedia* gland sections were stained with PAS. We can see a structure rich in glycoproteins (arrows) (magnification 400×). (B) Alcian blue staining at pH 1.0 of *L. intermedia* gland sections shows that the material reacted with the dye (magnification 600×). The positive reaction (arrows) demonstrates the presence of sulfated glycosaminoglycans in this area, which colocalize with other glycoproteins. Immunohistochemical analysis of *L. intermedia* gland sections using anti-laminin antiserum (D) (magnification 600×) and anti-entactin antiserum (F) (magnification 600×). The results show a positive pattern in the underlying region, indicating that this is a basement membrane structure (arrows). (C) and (E) (at the same magnifications) are negative controls for immunolabelling reactions using pre-immune serums. Arrow heads point the underlying structure position.

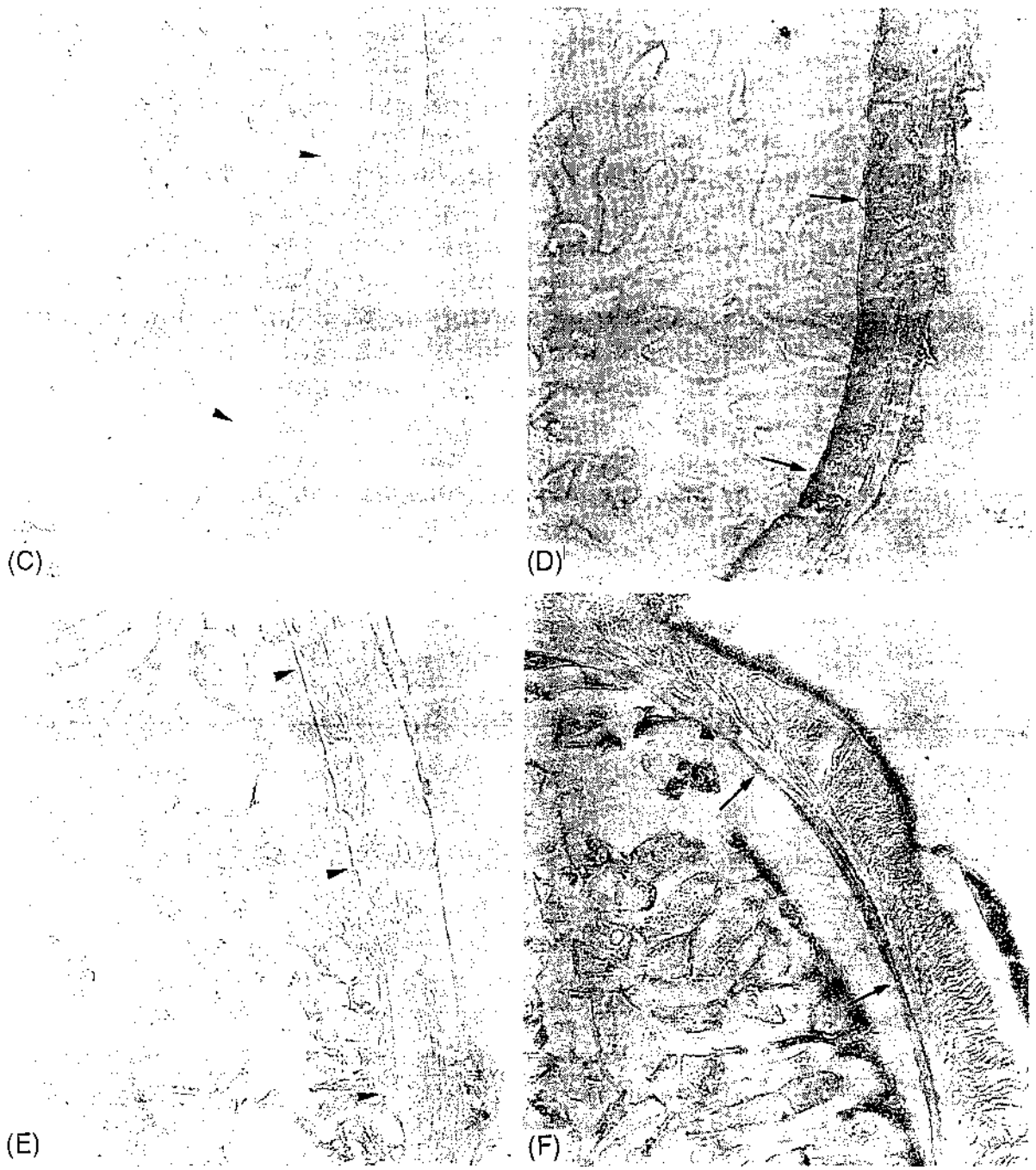


Fig. 4 (*continued*)

Differences in the shape and position of the gland in different species of spiders have been described by Bertkau (1891). In the genus *Atypus* the glands are composite, in *Filistata* they are of the multilobular type and in *Scytodes* they are bilobular. The venom glands of *L. intermedia* are bulbous in shape (Fig. 2A), a characteristic also observed in *L. reclusa* (Foil et al., 1979). Venom glands of cylindrical shape are found in *H. venatoria* and *L. indagastrix* (Ridling and Phanuel, 1986), as well as in *L. mactans* (Smith and Russell, 1967). In *Ctenedus medius*, a megalomorphae spider, the glands have the aspect of a sack (Brazil and

Vellard, 1925), as also observed in the yellow scorpion, *Buthus famulus* (Kanwar et al., 1981). In *P. collinus* the glands are carrot-like in shape (Ridling and Phanuel, 1986).

Anatomically *L. intermedia* venom glands present two layers of striated muscle fibers, an external layer and an internal layer in touch with an underlying structure that separates muscle cells from secretory epithelium of the gland (Figs. 1C, 2C and E). The same is found in *L. reclusa* (Foil et al., 1979) and in *C. medius* (Brazil and Vellard, 1925). According to Ridling and Phanuel, 1986), the venom glands of *P. collinus*, *H. venatoria* and *L. indagastrix* have two main layers, the external muscle layer and internal secretory layer. *L. mactans* venom glands are covered by a layer of striated muscle fibers (Smith and Russell, 1967). In *B. famulus*, the venom glands are incompletely encapsulated by a muscle layer (Kanwar et al., 1981).

In *L. intermedia* the muscular tissue that covers the venom glands is formed by muscle bundles with a large number of co-aligned contractile filaments; the microfilaments appear to consist of actin and myosin (Fig. 2E) and seem to be

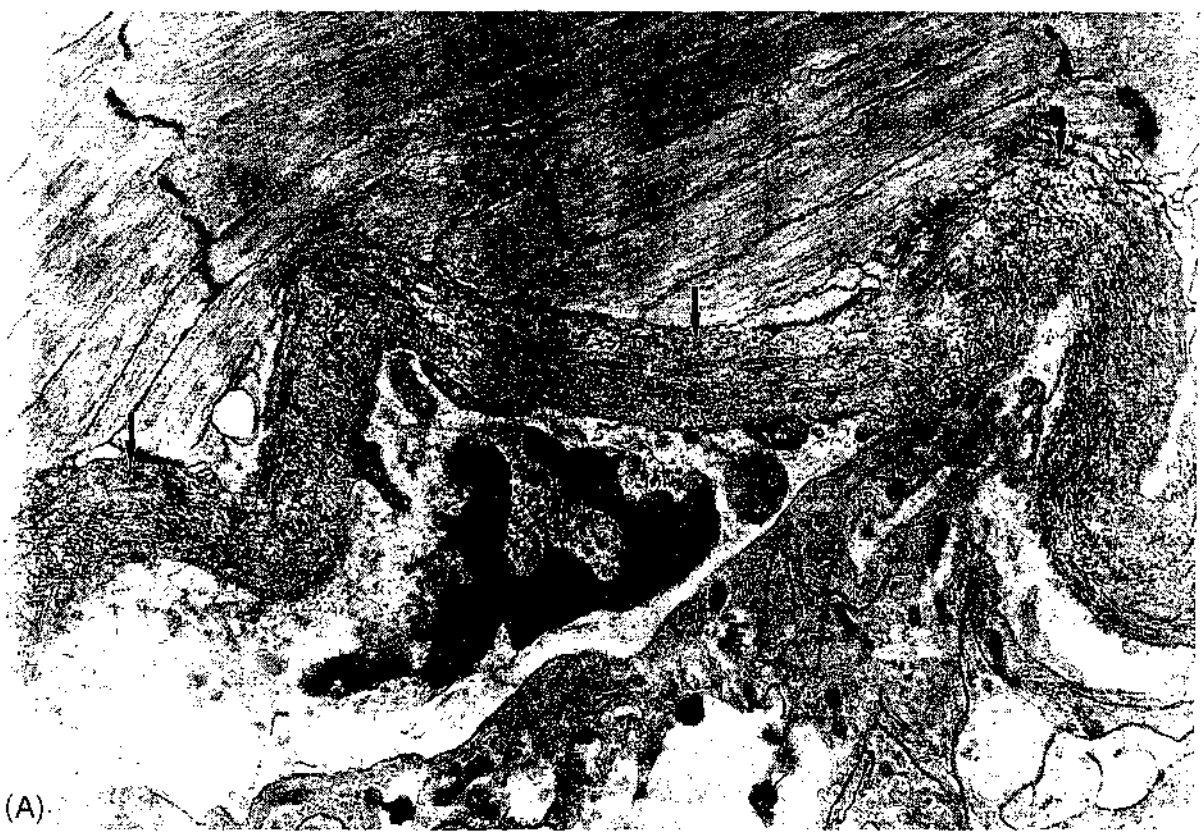


Fig. 5. Transmission electron microscopy and picrosirius staining of the underlying tissue between muscle and epithelium of *L. intermedia* venom gland. (A) Through transmission electron micrographs of a gland section (magnification 20.000×) we can observe the underlying tissue as rich in fibrillar structures (arrows). An insert of these fibrillar structures (arrow heads) can be seen at higher magnification (36.000×) (B). We can conclude that this fibrillar structure is an extracellular matrix rich in fibrils, containing collagen (positive for picrosirius staining, arrows) (magnification 600×), that colocalized with the basement membrane structure (C).

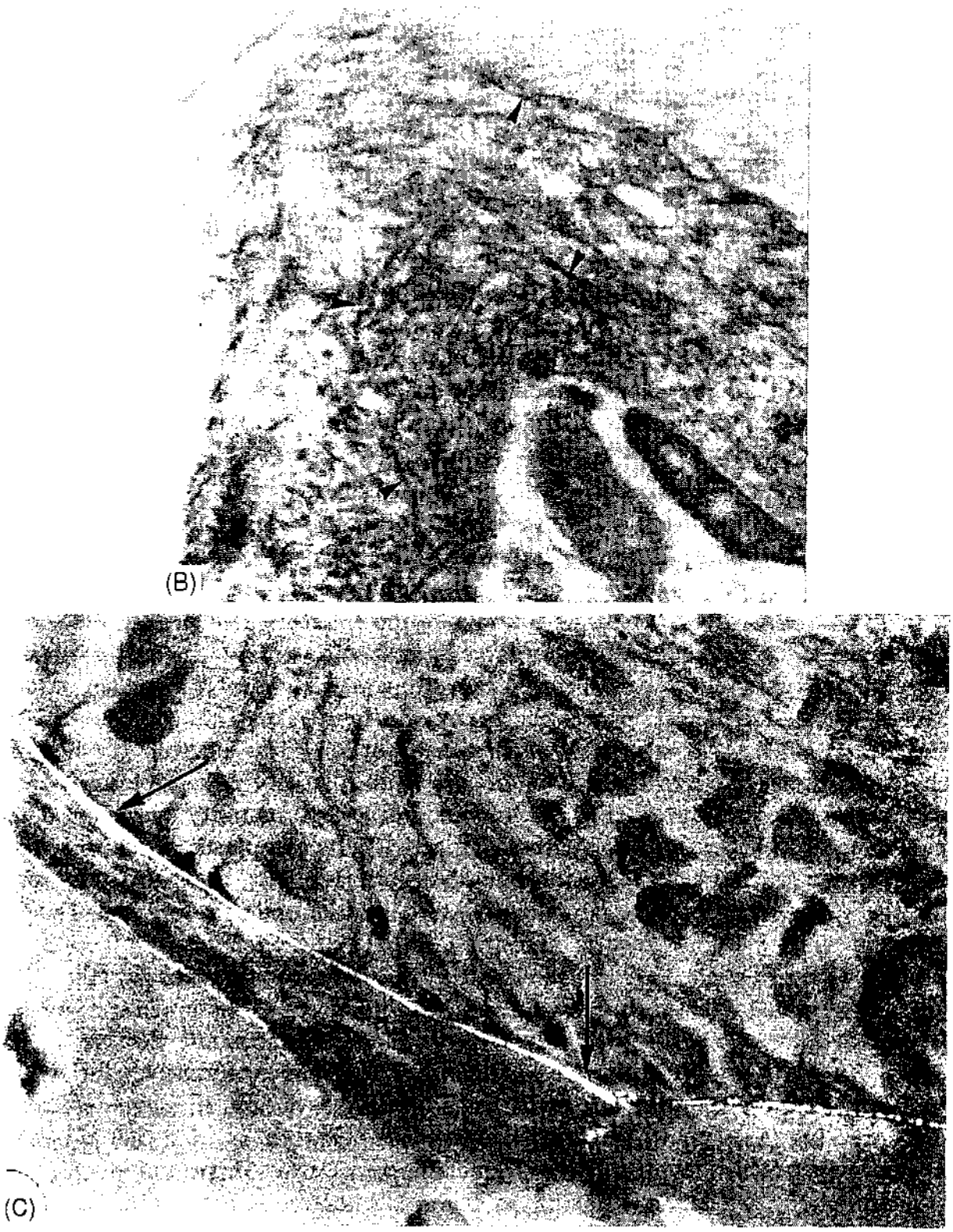


Fig. 5 (*continued*)

related to contractility, occupying most of the cytoplasm of the muscle cells. The internal muscle fibers are presumably used to force the exit of the venom through the reduction of the contraction gland. The same description was reported for *L.*

reclusa (Foil et al., 1979), *L. indagastrix* and *P. collinus* (Ridling and Phanuel, 1986). In *L. mactans*, Smith and Russell (1967) reported that the venom glands are covered by a layer of intrinsic muscle fibers which, when analyzed in cross sections, present muscle fibers running in a transverse slightly oblique direction, as well as a striated musculature with contracting units of myofibrils.

The secretory epithelium of *L. intermedia* is of the simple glandular type (Fig. 1C). The same type of epithelium is found in *L. reclusa* (Foil et al., 1979), in *H. venatoria* (Ridling and Phanuel, 1986) and in *C. medius* (Brazil and Vellard, 1925). In *L. mactans* many cell layers are found, varying in position and shape (Smith and Russell, 1967). *P. collinus* and *L.* indagastrix have many basal processes that reach into the lumen of the gland, thus increasing the surface area to accommodate many secretory cells (Ridling and Phanuel, 1986). In the yellow scorpion *B. famulus* the glandular epithelium has two types of cells, small basal cells and large columnar cells, the main cells that secrete the venom (Kanwar et al., 1981).

According to Foil et al. (1979), the epithelial cells that produce the venom in the *L. reclusa* gland have a basal nucleus, a cytoplasm rich in rough endoplasmic reticulum, mitochondria and Golgi bodies. The apical portion of these cells is filled by a big vacuole, limited by a thin cytoplasmic membrane.

In *L. intermedia* the cytoplasmic portions of the epithelial cells present a plasma membrane with invaginations, with a large amount of interdigitating cell processes increasing the secretory cell surface, suggesting a high level of cellular stress caused by the dynamics of venom excretion (Fig. 3D). The cells present a lengthened nucleus located near their basal portion. The nucleus contains a characteristic more electron-dense heterochromatin, that is visible in close contact with the nuclear membrane (Fig. 3A and B), and a large quantity of euchromatin indicating a nucleus in intense synthesizing activity. Its cytoplasm is rich in rough endoplasmic reticulum, as well as in polyribosomes (Fig. 3C), organelles necessary for the synthesis of proteins abundantly stored in the apical cytoplasm. This is coherent with previous reports showing that *L. intermedia* venom is rich in proteins (Feitosa et al., 1998; Veiga et al., 1999). Many secretory vesicles (Fig. 3D) are also observed in close contact and near the prominent Golgi apparatus, which is present in the form of a squashed stack with a characteristic dilation in its edges. Golgi portions are easily recognized, and are responsible for post-translational modifications such as cleavage and glycosylation, in agreement with our previous data showing that *L. intermedia* venom is rich in N-linked glycoproteins (Veiga et al., 1999). Mitochondria of oval shape are distributed throughout the cellular axis, mainly in the apical central portion (Fig. 3C). The products of secretion are stored in pockets limited by membranes (Fig. 3E).

In *L. reclusa* (Foil et al., 1979) the apical portion and lateral surface of mature cells are limited by irregularly distributed microvilli. Keegan and Lockwood (1971) described the presence of similar apical microvilli in the secretory epithelium of the venom glands of the scorpion *Centruroides marx*. These authors proposed that the microvilli can increase the cell surface as another pathway for

the release of vacuolar secretory processes or to move the contents in the direction of the duct. These structures were not found in *L. intermedia*.

According to some authors, in spiders of the genus *Loxosceles* the venom is produced by apocrine glands (Martinez-Vargas, 1987). However, the results obtained here by transmission electron microscopy, which show cellular debris (Cd) and cellular structures being eliminated with the venom secretion, suggest that the venom glands of *L. intermedia* are of the holocrine type (Fig. 3E and F). The holocrine secretion mechanism was described for *Lactrodectus mactans* (Ancona, 1931), *Ctenus medius* (Bordas, 1905), *P. collinus*, *H. venatoria* and *L. indagastrix* (Ridling and Phanuel, 1986), whereas Barth (1962) described a more complex and non-degenerative sequence of secretion events in glands of certain species of *Lactrodectus*. Our data strongly suggest that venom secretion mechanism of *L. intermedia* is of the holocrine type.

In the present study, by using light microscopy and transmission electron microscopy examination, we showed herein the presence of an underlying structure between the muscle layers and the secretory epithelium. A very thick extracellular matrix from which extensions project into the gland interior was described in *C. medius* (Brazil and Vellard, 1925). According to Ridling and Phanuel (1986), in *L. indagastrix* and *P. collinus* the muscle surface is surrounded by the basement membrane, which forms a continuous layer inside the musculature. These species also present some processes that project into the central lumen and form a network of fibrils, whereas these processes are absent in *H. venatoria*. In *L. mactans* this extracellular matrix has collagen fibrils (Smith and Russell, 1967) which were not found in *L. reclusa* (Foil et al., 1979).

In the venom gland of *L. intermedia*, the underlying structure is an extracellular matrix. The extracellular matrix is a complex network of secreted molecules, specially glycoproteins and proteoglycans that fills the extracellular spaces among the cells (Hay, 1991; Kreis and Vale, 1993). This underlying structure is found to be very rich in glycoproteins in sections stained with PAS (Fig. 4A). In alcian blue-stained material at pH 1.0 the reaction was positive, showing the presence of sulfated proteoglycans (Fig. 4B). Immunohistochemistry using anti-laminin or anti-entactin antibodies showed the presence of laminin and entactin as part of this extracellular matrix structure (Fig. 4D and F). These data strongly suggest that this extracellular matrix is a basement membrane that is an special kind of extracellular matrix, characterized by its ubiquitous thin sheet of molecules produced and secreted adjacent or surround a large variety of cells as epithelial, muscle, nervous and fat cells (Beck et al., 1990; Yurchenco and Schittny, 1990). In the case of *L. intermedia* venom gland, based on histological and ultrastructural data (Figs. 4D, F and 5A) we may postulate that both epithelial and muscular cells produce their basement membranes and part of the underlying tissue between these cells is a mixture of these basement membranes. On the other hand, by using transmission electron microscope at high magnification (Fig. 5A and B) and picrosirius staining method (Fig. 5C), we were able to detect collagen fibrils in this underlying tissue. Since basement membranes do not have fibrillar collagens, but network-forming collagen (as type IV collagen) instead we may postulate that

colocalized with the basement membrane. there is also a connective extracellular matrix, rich in fibrillar collagen, produced by other cells different from epithelial or muscular cells and of origin unknown at the present moment.

Acknowledgements

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Identification of high molecular weight serine-proteases in *Loxosceles intermedia* (brown spider) venom

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Abstract

High molecular weight serine-proteases have been identified in *Loxosceles intermedia* (brown spider) venom. The mechanism by which *Loxosceles* spp venoms cause dermonecrotic injury (a hallmark of loxoscelism) is currently under investigation, but it seems to be molecularly complex and in some instance proteases might be expected to play a role in this skin lesion. In the present investigation, when we submitted *L. intermedia* venom to linear gradient 3–20% SDS-PAGE stained by a monochromatic silver method we detected a heterogeneous protein profile in molecular weight, ranging from 850- to 5-kDa. In an attempt to detect zymogen molecules of proteolytic enzymes, venom aliquots were treated with several exogenous proteases. Among them, trypsin activated two gelatinolytic molecules of 85- and 95-kDa in the venom. In experiments of hydrolysis inactivation using different protease inhibitors for four major class of proteases, we detected that only serine-type protease inhibitors were able to inactivate the 85- and 95-kDa enzymes in the venom. An examination of the 85- and 95-kDa gelatinolytic activities as a function of pH showed that these proteases had no apparent activities at pH below 5.0 and higher than 9.0 and displayed little activity at pH 6.0, with the optimal pH for their activities ranging from 7.0 to 8.0. Evaluation of the functional specificities of the 85- and 95-kDa venom proteases

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showed that these proteases efficiently degrade gelatin (denatured collagen) but have no proteolytic activity on hemoglobin, immunoglobulin, albumin, fibrinogen or laminin, suggesting specificity of their proteolytic actions. We describe here two serine-proteases activities in *L. intermedia* venom probably involved in the harmful effects of the venom.

1. Introduction

Envenomation by the bite of brown spiders (*Loxosceles* genus) produces severe cutaneous injury (Forrester et al., 1978; Schenone et al., 1978; Babcock et al., 1981; Rees et al., 1984). This characteristic skin lesion (hallmark of accidents) begins with an acute local inflammatory reaction characterized by edema, followed by inflammatory cell accumulation, hemorrhage into the dermis at the bite site and an impressive necrotic lesion of cutaneous tissue with gravitational spreading and formation of a black eschar (Atkins et al., 1958; Wasserman and Anderson, 1983).

The mechanism by which the venom causes dermonecrotic injury is currently under investigation, but since the venom is composed of a mixture of several proteins, this mechanism seems to be complex and dependent on many different proteins acting synergistically.

Inflammatory cells accumulated at the envenomation site (especially polymorphonuclear leukocytes) might be expected to play a role in the lesion caused by the venom (Smith and Micks, 1970; Majeski et al., 1977), with neutrophils and vessel endothelial cells being a central target for molecular constituents present in the venom (Patel et al., 1994). In addition, adult plasma components are also required for the deleterious effects of the venom since in vitro experiments using a purified putative dermonecrotic toxin from the venom diluted in synthetic buffer or neonate plasma did not induce platelet activation, an event that may be responsible for vessel thrombosis, tissue ischemia and then skin degeneration (Rees et al., 1988). The serum amyloid P component (a pentraxin protein) which is deficient in neonate but not in adult plasma, seems to be the target for platelet activation and indirect ischemic effects and is likely to have a role in the necrosis provoked by the venom (Gates and Rees, 1990). The activation of the autologous complement alternative pathway (C system of plasma) also seems to participate in the noxious effects of the venom (Tambourgi et al., 1995). The depletion of the complement constituents by different molecules resulted in reduction of clinical signals in skin injected with venom (Smith and Micks, 1970). On the other hand, hemorrhagic and necrotic harmful effects caused by *L.* spp similar to those observed after snake bites (Fox and Bjarnason, 1995; Bjarnason and Fox, 1995) can be directly associated with hydrolytic enzyme activities (proteases) present in the venom, that could trigger the severe necrotic lesions by acting on different protein substrates. The first evidence of protease activities in *L.* spp venoms was described by Eskafi and Norment (1976) studying *L. rechusa* venom. Jong et al. (1979), studying the same venom, reported the

presence of a proteolytic enzyme (approximately 30-kDa) that appears to have a broad substrate specificity. Recently, working with *L. intermedia* venom and using zymograph experiments, Feitosa et al. (1998) and Veiga et al. (1999) demonstrated that the venom contains two metalloproteases with distinct substrate specificity, one of 32–35-kDa with gelatinolytic activity and the other of 20–28-kDa with fibronectinolytic and fibrinogenolytic activities, suggesting its involvement in the deleterius effects of the venom. The 32–35-kDa metalloprotease is a glycoprotein type high-mannose, with *N*-glycosylation responsible for 2-kDa of the molecular weight of this molecule and in such way controlling the hydrolytic activity of this enzyme (Veiga et al., 1999). Barbaro et al. (1996) reported some levels of proteolytic (caseinolytic) activity in the venoms of *L. gaucho*, *L. laeta* and *L. intermedia*.

In this present report we further studied a new proteolytic activity in *L. intermedia* venom. We present here the identification of high molecular weight serine-proteases of 85- and 95-kDa with substrate specificity, which could be involved in the deleterious effects of the venom as a function of their proteolytic activities.

2. Materials and methods

2.1. Reagents

Laminin was purified from Engelbreth–Holm–Swarm (EHS) tumors, produced in 2-month-old C57-BL10 female mice as described (Paulsson et al., 1987). Gelatin was purchased from Gibco Diagnostics (Madson, USA). Casein, bovine serum albumin (BSA), hemoglobin and fibrinogen were obtained from Sigma (St. Louis, USA). Rabbit immunoglobulin type IgG was purified from fresh rabbit serum (obtained from the Federal University of Parana Animal House under appropriate conditions) using protein-A-Sepharose (Pharmacia, Uppsala, Sweden) as described by Harlow and Lane (1988). The proteases used for tentative in vitro activation of venom (see below), such as trypsin, chymotrypsin, pepsin, elastase, V8-protease, collagenase, protease type XIV and proteinase K, were purchased from Sigma. The protease inhibitors used, i.e. ethylenediaminetetraacetic (EDTA), 1,10-phenanthroline, aprotinin, phenylmethylsulfonyl fluoride (PMSF), benzamidine, leupeptin, soybean trypsin inhibitor (STI), *N*-ethylmaleimide (NEM), iodoacetamide and pepstatin-A were all obtained from Sigma.

2.2. Spider venom extraction

L. intermedia venom was obtained from LIPAPE (Laboratório Interdisciplinar de Pesquisa em Animais Peçonhentos, Setor de Ciências Biológicas, Universidade Federal do Paraná). The venom was extracted from spiders captured from nature and kept for a week without any kind of food. The venom was extracted from the fangs of spiders by electrostimulation (15 V) applied to the cephalothorax and

collected with a micropipette, dried under vacuum and frozen at −85 C until use. The pure venom is a transparent liquid, whereas venom contaminated with stomach egestion contents becomes cloudy and was always discarded. A pool of venom collected from approximately 500 spiders was used in each experiment (Barbaro et al., 1992; Feitosa et al., 1998).

2.3. Electrophoresis

For the analysis of crude venom proteins, samples were run on SDS containing polyacrylamide gel (SDS-PAGE) using a 3–20% polyacrylamide linear gradient under non-reducing conditions as previously described by Laemmli (1970). Gel was stained with silver nitrate as described (Wray et al., 1981). The molecular mass markers used were myosin (205-kDa), β-galactosidase (116-kDa), phosphorylase B (98-kDa), bovine serum albumin (67-kDa), ovalbumin (44-kDa) and carbonic anhydrase (29-kDa) purchased from Sigma. For an extremely high molecular weight protein standard we used laminin (approximately 850-kDa) purified as described in the reagents section. For an extremely low molecular weight protein standard we used cytochrome C (12-kDa) obtained from Sigma.

2.4. Zymograms using different protein substrates

Ten percent SDS-PAGE was prepared as described above and copolymerized with different substrates such as gelatin, casein, hemoglobin, BSA, rabbit immunoglobulin type IgG and fibrinogen at final concentrations of 3 mg ml. For gel copolymerized with laminin the procedure was the same, except for the presence of 3 M urea, since laminin is insoluble in water at concentrations higher than 1 mg ml. Samples of 40 μg of venom pre-incubated or not with hexogenous proteases (see below) were electrophoresed at 25 mA at 4 C. After electrophoresis, gels were washed twice for 30 min in 2.5% Triton X-100 (Sigma) to remove SDS.

Table 1
Venom proteolytic activation by treatment with exogenous proteases[a]. (+) gelatinolytic activation; (−) non gelatinolytic activation

Protease	Cleavage site	Results
Trypsin	after Lys and Arg	+
Elastase	after uncharged and non aromatic aminoacids	−
Pepsin	broad specificity	−
Collagenase	after X in ProXGlyPro	−
Proteinase K	broad specificity	−
Proteinase type XIV	broad specificity	−
Chymotrypsin	after Phe, Thr, Tyr	−
Protease V8	after Asp or Glu	−

[a] For venom activation 1 μg of one different protease listed above was incubated with 40 μg of crude venom, at 37 C for 1 h.

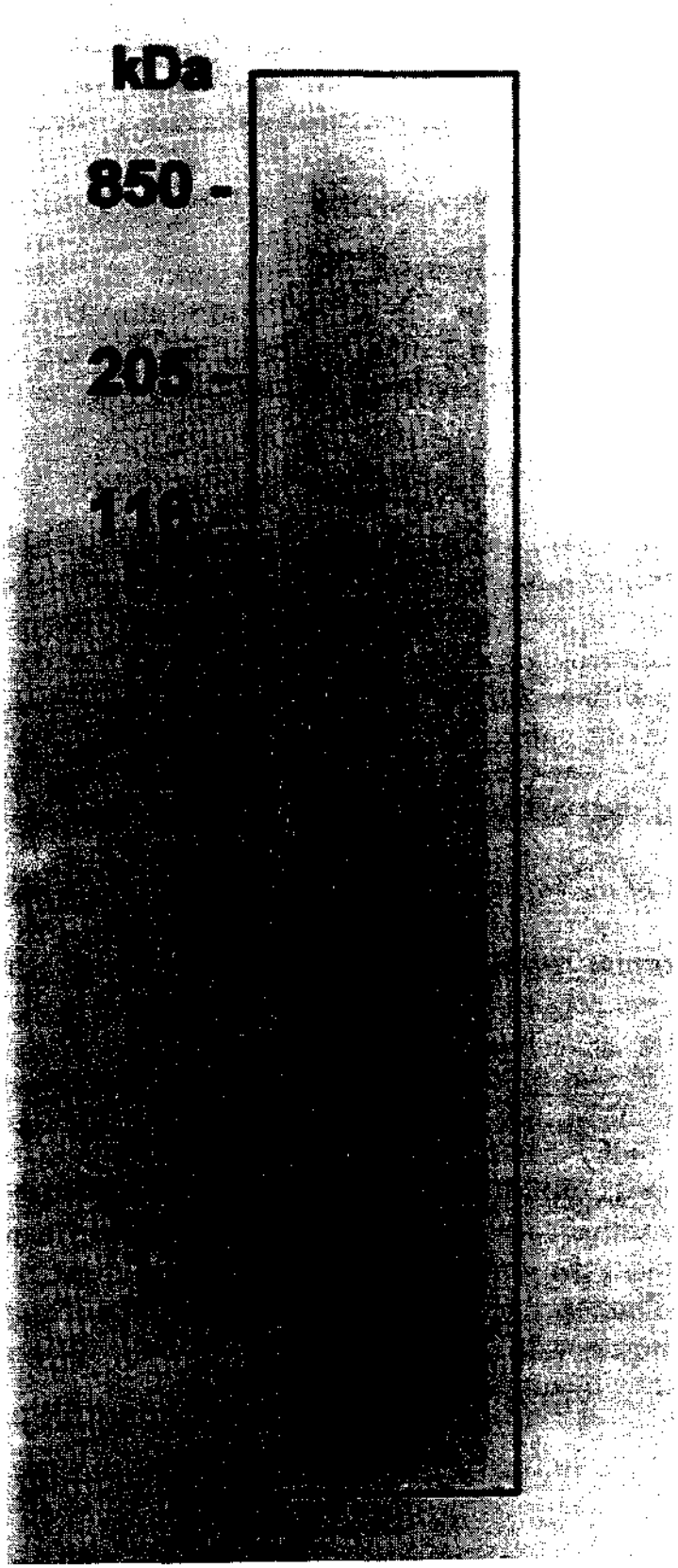


Fig. 1. SDS-PAGE profile of L. intermedia venom proteins. *L. intermedia* crude venom proteins were separated by a continuous 3–20% (w/v) linear gradient polyacrylamide gel under nonreducing conditions and stained by the monochromatic silver method. Molecular mass markers are shown on the left.

incubated overnight at 37°C in 50 mM Tris–HCl, pH 7.3, 200 mM NaCl, 5 mM $CaCl_2$ and 5 mM $MgCl_2$ and then stained with Coomassie Brilliant Blue R and destained with 50% methanol in water. Clear zones of substrate lysis against a blue background stain indicated the presence of degrading enzymes.

2.5. Assays for proteolytic activation of venom in vitro using exogenous proteases

Aliquots of *L. intermedia* venom (40 μg) were incubated with different hexogenous proteases (1 μg) for 1 h at 37°C (see Table 1). The materials were then immediately dissolved in Laemmli buffer in the absence of reducing agents and electrophoresed for zymogram using gelatin as substrate, as shown above.

2.6. Zymograms under different pH conditions

To study optimal pH conditions for the 85- and 95-kDa proteases, we developed zymograms using gelatin as protein substrate under the same experimental conditions as described above, except that different buffer systems were used for incubation overnight at 37°C. For pH 3.0, 4.0 and 5.0, zymographs were developed in 50 mM acetate buffers. For pH 6.0, 7.0 and 8.0, zymographs were developed in 50 mM phosphate buffers. For pH 9.0, 10.0 and 11.0, zymographs were developed in 50 mM Tris–HCl buffers. Lysed gelatin zones from the zymogrphs had their optical densities checked and graded.

2.7. Determination of the biochemical nature of 85- and 95-kDa venom proteases

In order to determine the biochemical nature of 85- and 95-kDa proteases of *L. intermedia* venom, we performed zymograms containing 40 μg of trypsin-activated venom and gelatin as substrate, using 50 mM Tris–HCl buffer at pH 7.3 containing excess of one type of proteinase inhibitor (Harlow and Lane, 1988) such as EDTA (10 mM), 1,10-phenanthroline (5 mM), PMSF (10 mM), leupeptin (0.02 mM), aprotinin (0.002 mM), benzamidine (10 mM), soybean-trypsin inhibitor (0.0005 mM), NEM (5 mM), iodoacetamide (10 mM) and pepstatin-A (0.007 mM). As positive control, a zymogram was developed in the presence of activated venom under the same conditions as described above, but in the absence of any protease inhibitor. The relative efficacies of different inhibitors upon 85- and 95-kDa gelatinolytic activities were measured by using lysed gelatin zones that were densitometrically quantified.

3. Results

3.1. Heterogeous molecular weight profile of L. intermedia crude venom proteins

Figure 1 depicts the profile of *L. intermedia* crude venom proteins on a linear gradient 3–20% SDS-PAGE under nonreducing conditions, stained by the highly

sensitive monochromatic silver method (Wray et al., 1981). It can be seen that the venom is enriched in proteins with low molecular weights in the range of 40–5-kDa. Nevertheless, the presence of high molecular weight proteins (850–60-kDa) was also observed to a lesser extent.

3.2. Proteolytic activation of L. intermedia venom by treatment with an exogenous protease

As previously reported in different cases, venom proteases can be produced as zymogen molecules, and in such structural chains are encoded pro-peptide sequences, which are later proteolytically processed to yield activated forms (Bjarnason and Fox, 1995; Fox and Bjarnason, 1995). Table 1 summarizes

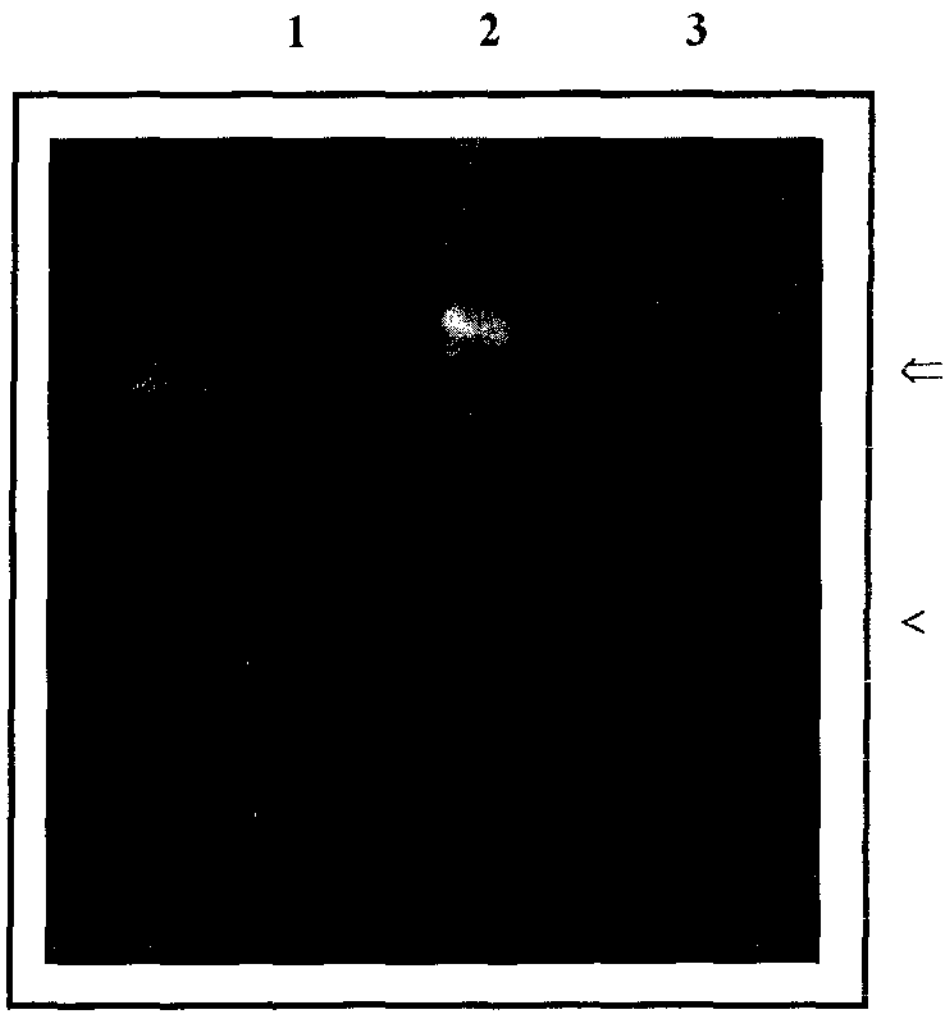


Fig. 2. Effect of trypsin treatment on *L. intermedia* venom proteolytic activity. Samples containing 40 μg of venom were incubated with trypsin (1 μg) for 1 h at 37 C and then submitted to a gelatin copolymerized 10% SDS-PAGE zymogram. The figure illustrates the zymograph of the venom before trypsin treatment (lane 1), the zymograph of the venom after trypsin treatment (lane 2) and the zymograph of trypsin without venom, under the same experimental conditions as the negative control (lane 3). The arrow indicates the 85- and 95-kDa proteases and the arrowhead indicates loxolysin B previously described (see Introduction).

experiments where *L. intermedia* crude venom was incubated with different exogenous proteases and then processed for zymograph experiments with copolymerized gelatin. The results indicate that among several tested exogenous proteases, only trypsin was able to produce proteolytic activation of the venom. Figure 2 depicts a zymograph of *L. intermedia* crude venom before any kind of exogenous activation (lane 1). The arrowhead shows Loxolysin B (35-kDa), a metalloprotease previously characterized in venom (Feitosa et al., 1998; Veiga et al., 1999). Lane 2 shows a zymograph of venom previously treated with trypsin, and lane 3 shows a zymograph of trypsin under the same experimental conditions but without venom (control). The arrow points 85- and 95-kDa gelatinolytic activated proteases. It can be seen that after these exogenous treatments, only trypsin displayed some activity toward *L. intermedia* crude venom, activating two gelatinolytic signals at the 85- and 95-kDa positions (arrow).

3.3. Biochemical nature of the 85- and 95-kDa gelatinolytic proteases of L. intermedia venom

In order to obtain more information about the 85- and 95-kDa proteases of *L.*

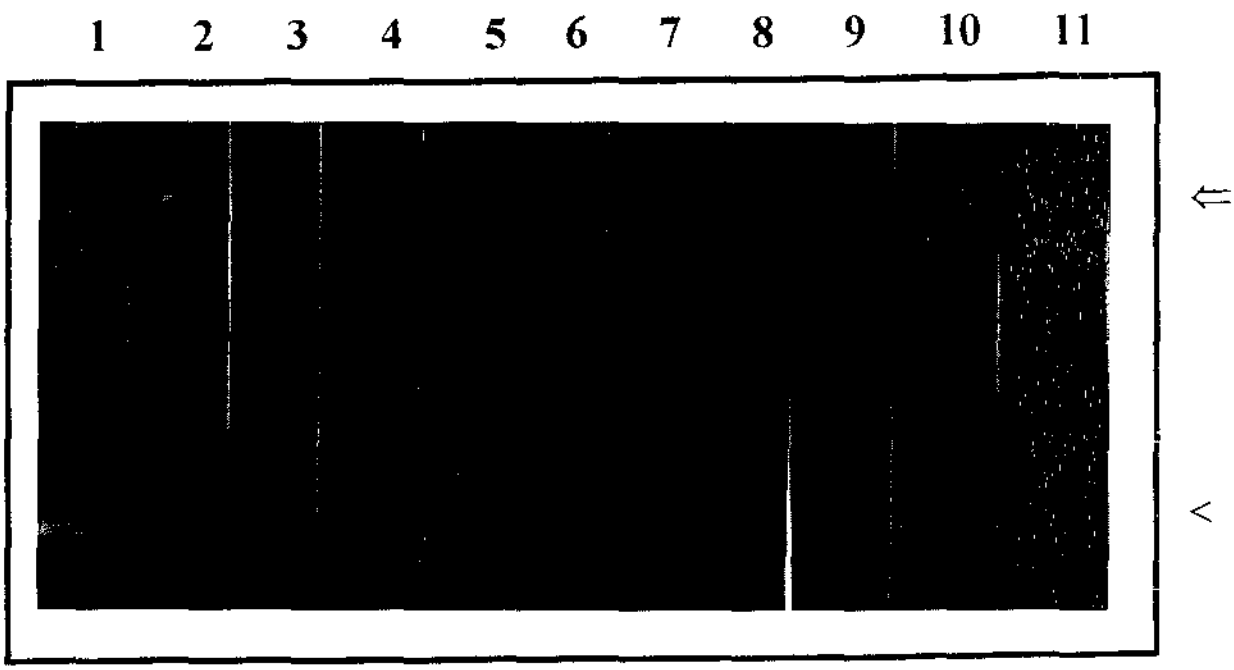


Fig. 3. Enzyme inhibitors effects on L.intermedia venom 85- and 95-kDa proteolytic activities. Aliquots of venom (40 μg) were treated with 1 μg of trypsin for 1 h at 37 C and then applied to a 10% SDS-PAGE gelatin copolymerized zymogram. Zimographs were developed overnight at 37 C in the absence of enzyme inhibitors (lane 1, control) or in the presence of different enzyme inhibitors such as EDTA (lane 2), 1,10-phenanthroline (lane 3), PMSF (lane 4), aprotinin (lane 5), benzamidine (lane 6), soybean-trypsin inhibitor (lane 7), leupeptin (lane 8), NEM (lane 9), iodocetamide (lane 10) and pepstatin-A (lane 11). The arrowhead shows a 35-kDa protease (loxolysin B, a metalloproteinase previously described by Feitosa et al. (1998) and Veiga et al. (1999)) and the arrow indicates the position of the 85- and 95-kDa proteases.

intermedia venom, we performed experiments of inactivation of hydrolysis, using different inhibitors of four major class of proteolytic enzymes, such as EDTA and 1,10-phenantroline (divalent chelators), sulphydryl (thiol-protease) inhibitors such as NEM and iodoacetamide, aspartic (acid-protease) inhibitor such as pepstatin-A and serine-type protease inhibitors such as PMSF, leupeptin, benzamidine, aprotinin and soybean-trypsin inhibitor.The relative efficacies of inhibitors effects were measured as can be seen in Fig. 3 and Table 2, just serine-type protease inhibitors evoked inactivation of the 85- and 95-kDa gelatinolytic activities in the venom.

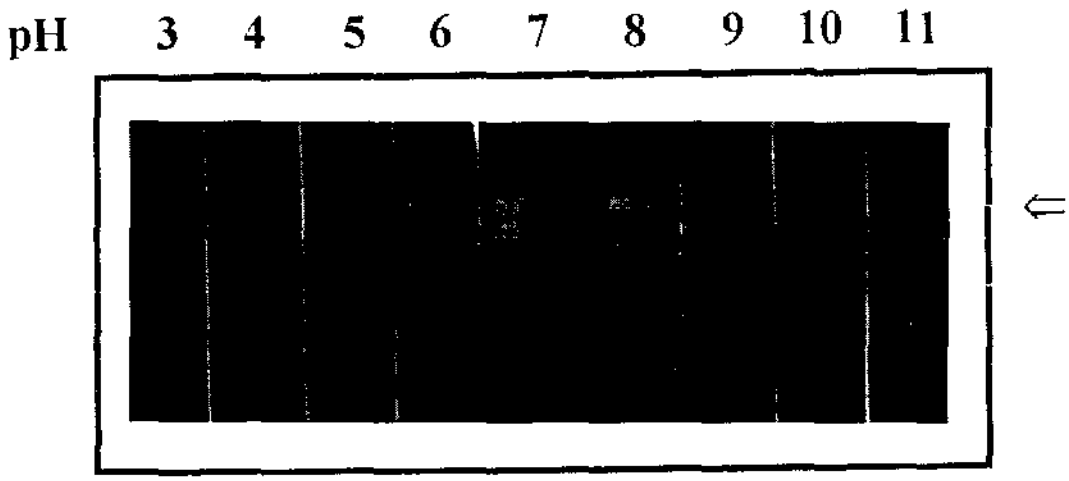
3.4. Optimal activity of L. intermedia venom 85- and 95-kDa proteases as a function of pH

The activities of *L. intermedia* venom trypsin-activated 85- and 95-kDa proteases were examined as a function of pH. Aliquots of *L. intermedia* venom were treated by exogenous trypsin as described in Materials and methods and then assayed for gelatinolytic effects in the regions of 85- and 95-kDa through zymograph experiments carried out under conditions of different buffer pH values. Zymograms containing polymerized gelatin were developed in 50 mM acetate buffers, pH 3.0, 4.0 and 5.0; in 50 mM phosphate buffers, pH 6.0, 7.0 and 8.0; and in 50 mM Tris–HCl buffers, pH 9.0, 10.0 and 11.0. Lysed gelatin zones from zymographs were measured and graded. As illustrated in Fig. 4A and B, both proteases appear to possess a pH range of optimal activities under weak acidic, neutral or weak basic experimental conditions.

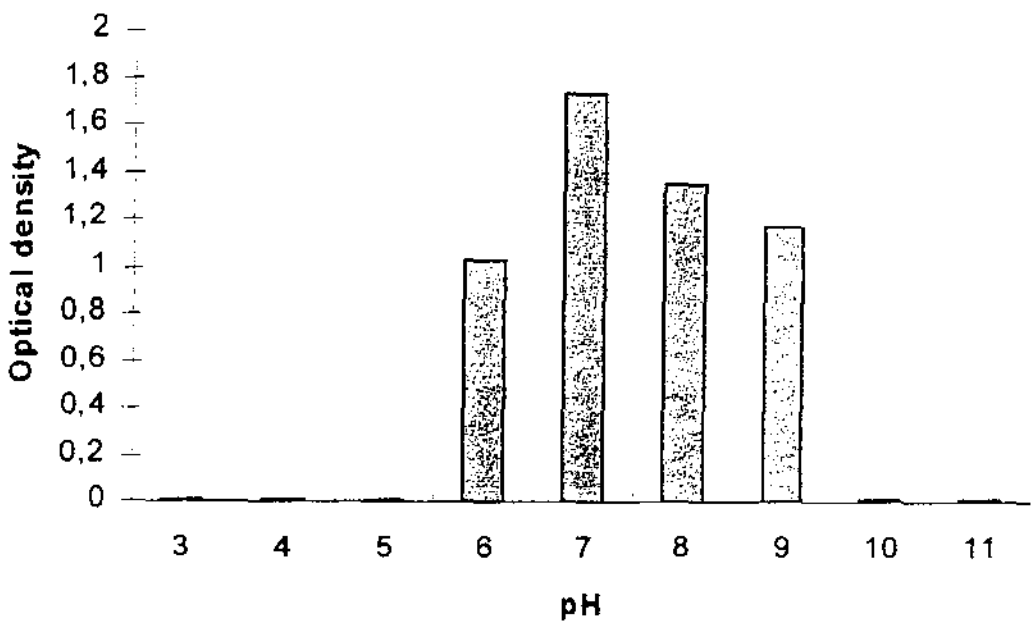
Table 2
Protease inhibitors effects on *L. intermedia* 85- and 95-kDa venom proteases[a]

Inhibitors	Percentage of activity of 85- and 95-kDa[a]
Control	100.0
EDTA	104.9
1,10-Phenantroline	100.7
PMSF	0
Leupeptin	0
Aprotinin	0
Benzamidine	0
STI	0
NEM	75.1
Iodoacetamide	99.1
Pepstatin-A	98.0

[a] Enzyme activities were measured from lysed zones of zymograms of Fig. 3 that were densitometrically quantified by a scanning laser densitometer.



A



B

Fig. 4. *L. intermedia* venom 85- and 95-kDa protease activities as a function of pH. (A) Venom aliquots (40 μg) were activated by trypsin treatment (1μg) for 1 h at 37 C and then subjected to 10% SDS-PAGE gelatin copolymerized zymograms. Zymographs were developed at 37 C overnight in the presence of different buffers and pHs as described in Materials and methods. pH values are listed at the top of the figure. The arrow shows the position of the 85- and 95-kDa proteases. (B) Lysed zones from zymographs of experiments shown in (A) were densitometrically quantified by a scanning laser densitometer and graphically plotted. Gelatinolytic activities are reported as optical densities (ordinate) in function of pH (abscissa).

3.5. Proteolytic actions of L. intermedia 85- and 95-kDa venom serine-proteases on different substrates

In order to evaluate some functional specificities of the 85- and 95-kDa venom proteases, we performed zymograph experiments with various copolymerized protein substrates. Aliquots of trypsin-activated venom were submited to copolymerized zymograms containing different proteins (3 mg/ml) such as gelatin, casein, serum albumin, hemoglobin, immunoglobulin (IgG), laminin and fibrinogen, under optimal experimental conditions. As shown in Fig. 5, the 85- and 95-kDa proteases efficiently degraded gelatin, acted on casein to a lesser extent and had no hydrolytic activity on hemoglobin, immunoglobulin (type IgG), bovine serum albumin (BSA), laminin or fibrinogen, suggesting some specificity in their proteolytic actions.

4. Discussion

Although it has been well documented that brown spider bites are associated with necrotic skin lesions and tissue degrading activities at the bite site (Atkins et al., 1958; Wasserman and Anderson, 1983; Futrell, 1992), the real molecular mechanisms responsible for this hallmark signal of loxoscelism are still little understood, and more information about them should be obtained (see

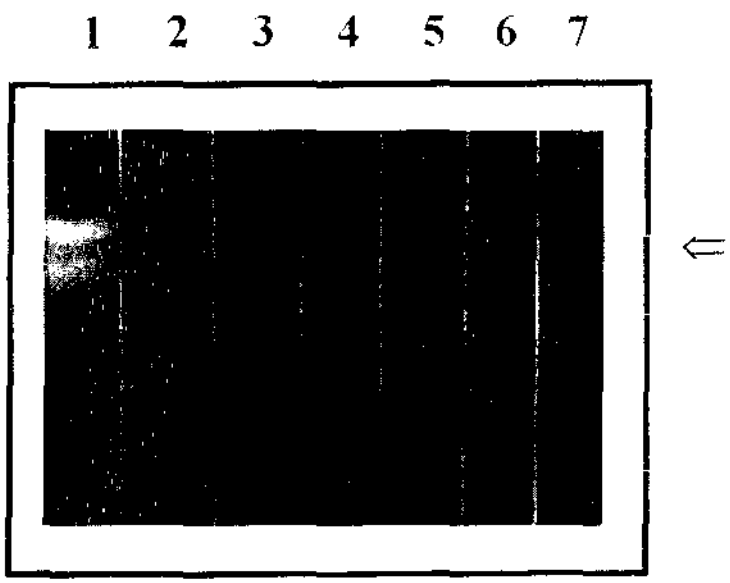


Fig. 5. Action of *L. intermedia* venom 85- and 95-kDa proteases on different protein substrates. Venom samples (40 μg) were activated for 85- and 95-kDa proteases by trypsin treatment as described above and then submitted to 10% SDS-PAGE zymograms containing different copolymerized protein substrates. Gelatin (lane 1), casein (lane 2), hemoglobin (lane 3), BSA (lane 4), immunoglobulin IgG (lane 5), laminin (lane 6) and fibrinogen (lane 7). Zimographs were developed overnight at 37°C. The arrow indicates the electrophoretic positions of the 85- and 95-kDa proteases.

Introduction). Studying *L. intermedia* venom through a wide gradient (3–20%) SDS-PAGE and the high sensitive silver stain protein method, we show here that the venom is a heterogeneous mixture of proteins, ranging in size from 850 to 5-kDa (Fig. 1). In addition, this result also indicates that the high molecular weight proteins from 850- to 60-kDa are poorly expressed proteins and the low molecular weight proteins are extensively expressed. These data contradict previous studies that reported only some low molecular proteins in other *L.* spp spider venoms (Norment et al., 1979; Barbaro et al., 1992), a discrepancy that can be explained by the less developed protein detection methods previously employed.

The necrotic skin effect evoked by *L. intermedia* venom significantly appears just a few hours after the bite, a fact suggesting some kind of activation of venom principles for their late degrading effects. In the present study we were able to activate the proteolytic effect of *L. intermedia* venom by an in vitro enzyme treatment (Table 1 and Fig. 2). Among several proteolytic enzymes assayed for venom activation, only trypsin was able to produce such effect, evoking two gelatinolytic molecules of 85- and 95-kDa in the treated venom. There are no previous descriptions of such behavior in the venom of other spiders, but we may speculate that trypsin treatment could specifically degrade the pro-peptide domains of the zymogen molecules, liberating these proteinase structures, as reported for several snake venom proteinases (Bjarnason and Fox, 1995). In vitro resistance to proteolytic enzymes but sensitivity to trypsin could be explained by specific amino acid sequences in the region between the pro-peptide and proteinase domains in zymogen, since trypsin hydrolyzes the peptide bond just after lysine or arginine (see Table 1).

When studying the 85- and 95-kDa enzyme activities in the presence of several inhibitors (Fig. 3 and Table 2), we observed that divalent metal chelators such as EDTA or 1,10-phenanthroline did not inhibit the gelatinolytic activities of these enzymes, indicating the absence of divalent metal ions at the catalytic sites of these proteases. *N*-ethylmaleimide (NEM) and iodoacetamide also had no inhibitory effects on the 85- and 95-kDa proteases, suggesting that sulphydryl groups were not necessary for these enzyme activities. Pepstatin-A, an acid (aspartic)-protease inhibitor, was also ineffective in blocking these enzyme activities, again demonstrating that an acid-amino acid is not a functional molecule at the active site of these *L. intermedia* venom proteases. On the other hand, when serine-type inhibitors such as PMSF, aprotinin, benzamidine, soybean-trypsin inhibitor and leupeptin were used, the protease activities of the 85- and 95-kDa molecules were inhibited to 0%, indicating that serine is a functional amino acid at the active site of these enzymes and is critical for their activity, demonstrating their biochemical nature as serine-type proteases.

When evaluating the 85- and 95-kDa protease activities as a function of pH, we observed that these brown spider venom proteases have no detectable activities in the acidic 3.0 to 5.0 pH range and in the basic 10.0 to 11.0 pH range. At pH 6.0 they begin to display hydrolytic activities and apparently have a pH range of optimal activity from pH 7.0 to softly basic pH ranges such as 8.0, decreasing the gelatinolytic activities with increasing of pH conditions. These results demonstrate

that these 85- and 95-kDa serine-proteases are active under normal human physiological conditions, in which the pH is 7.2–7.3.

With respect to substrate specificity, it is interesting to observe that the 85- and 95-kDa proteases have no broad activities, efficiently hydrolyzing gelatin (denatured collagen) and hydrolyzing poorly casein, but displaying no effects on laminin, BSA, hemoglobin, immunoglobulin IgG or fibrinogen, a fact precluding substrate specificity in vivo. The real significance of this narrow effect is still obscure. Proteases with specific substrates were previously described in *L. intermedia* venom, which contains a 32–35-kDa metalloprotease that degrades gelatin but not BSA, casein, fibronectin or fibrinogen and a 20–28-kDa protease that degrades fibrinogen and fibronectin, but not gelatin, BSA or casein (Feitosa et al., 1998). The specificity of action of proteases should be related to spider self-protection since, for example, the venom gland of *L. intermedia* is extremely rich in laminin (basement membrane) separating muscle tissue involved in venom secretion from epithelial cells involved in venom production (dos Santos et al., 1999). *L. intermedia* venom, although having other proteolytic activities, seems to have no laminolytic effect (Feitosa et al., 1998). This narrow proteolytic effect of the 85- and 95-kDa proteases speaks against the possibility of these enzymes representing contamination of venom with egested stomach contents, since in this case we would expect the activated enzymes to have broad substrate specificities.

Literature described several examples of synergism among different proteases. The degradation of extracellular matrix during normal or pathologic conditions seems to be controlled events where molecules as native collagen suffers an initial effect of collagenases from fibroblasts or PMN leukocytes, partially denaturating this molecule, that then is sequentially degraded by gelatinases A and B, or other matrix metalloproteases, that does not act on native collagens (see Birkedal-Hansen et al., 1993; Kleiner and Steler-Stevenson, 1993). For venom proteases also there are literature examples (Baramova et al., 1989; Fox and Bjarnason, 1995), that described some reprolysins (the major noxious proteases of snake venoms) that hydrolysis gelatins from different types of collagens, but have no action on full-lenght collagens.

As discussed in the Discussion, we may postulate that serine-proteases described herein complement the degradation effect triggered by other proteases of venom (Feitosa et al., 1998; Veiga et al., 1999) or even so other proteases released by damaged tissue cells, since during dermonecrosis, injured cells also appears to contribute to skin destruction (Futrell, 1992). Some proteases may act evoking a partial denaturation of native collagen and gelatinase-like proteases (Feitosa et al., 1998) or and serine-like proteases described herein could complement the degrading effect.

After the identification of 85- and 95-kDa serine-type proteases, an important question is: what is (are) the real activator(s) of the venom in the blood, or tissues of bitten animals that stimulate the activation of the 85- and 95-kDa proteases? This is an open question, but we may speculate that, as described for other zymogen proteases, kallikrein and thrombin or other proteases present in different tissues may participate in this event (Kleiner and Steler-Stevenson, 1993; Mignatti

and Rifkin, 1993). The information presented here about a new protease (serine-type) in *L. intermedia* venom acting synergistically with other principles in the venom may explain the noxious effects of the venom and strengthen the previously data that point proteolytic enzymes in *L.* spp venoms.

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DETECTION AND CHARACTERIZATION OF METALLOPROTEINASES WITH GELATINOLYTIC, FIBRONECTINOLYTIC AND FIBRINOGENOLYTIC ACTIVITIES IN BROWN SPIDER (*LOXOSCELES INTERMEDIA*) VENOM

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L. Feitosa, W. Gremski, S. S. Veiga, M. C. Q. B. Elias, E. Graner, O. C. Mangili and R. R. Brentani. Detection and characterization of metalloproteinases with gelatinolytic, fibronectinolytic and fibrinogenolytic activities in Brown spider (*Loxosceles intermedia*) venom. *Toxicon* **36**, 1039–1051, 1998.—By studying *Loxosceles intermedia* (Brown spider) venom we were able to detect a proteolytic action on fibronectin and fibrinogen but an inability to degrade full length laminin, type I and type IV collagens. By studying enzyme inhibitors we observed that divalent metal chelators as EDTA and 1,10-phenanthroline completely blocked this cleaving action whereas serine-protease inhibitors, thiol-protease inhibitor and acid-protease inhibitor showed little or no effect on the proteolytic activity of the venom indicating involvement of a metalloproteinase. Zymogram analysis of venom detected a 35 kDa molecule with gelatinolytic activity. The metalloproteinase nature was further supported by its sensitivity to 4-aminophenyl mercuric acetate (APMA) treatment which decreased its molecular weight to 32 kDa, inhibition of its gelatinolytic effect by 1,10-phenanthroline and its elution from gelatin-sepharose affinity beads. In addition, zymogram experiments using fibronectin and fibrinogen as substrates detected a fibronectinolytic and fibrinogenolytic band at 28 kDa which changed its electrophoretic mobility to 20 kDa band after organomercurial treatment. The inhibitory effect of 1,10 phenanthroline and APMA sensitivity on this proteolytic effect confirmed the presence of a second metalloproteinase in the venom. The data presented herein describe two invertebrate metalloproteinases in *L. intermedia* venom

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Abbreviations: APMA, *p*-aminophenylmercuric acetate; EDTA, ethylenediaminetetraacetic acid; Me_2SO, dimethyl sulfoxide; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

with different specificities one gelatinolytic and another, fibronectinolytic and fibrinogenolytic, probably involved in the harmful effects of the venom.

INTRODUCTION

Loxoscelism is a term utilized to represent accidents with spiders of the genus *Loxosceles*, family *Loxoscelidae* (Barbaro *et al.*, 1994), that are disseminated and occur in all regions of America, Africa and Europe. The venom of this spider is remarkable for its dermonecrotic action at the bite site and for disseminated intravascular coagulation, platelet aggregation and hemolytic action as systemic effects (Forrester *et al.*, 1978; Bascur *et al.*, 1982; Rees *et al.*, 1988). Platelet aggregation and intravascular disseminated coagulation are dependent on extracellular matrix molecules such as plasma fibronectin and fibrinogen (Williams *et al.*, 1983; Ruoslahti, 1988) and hemostatic troubles triggered by the venom could be ascribed to the presence of proteolytic enzymes for these substrates, such as matrix metalloproteinases and reprolysins (Kleiner and Steler-Stevenson, 1993; Bjarnason and Fox, 1995).

Matrix metalloproteinases represent a group of endopeptidases that cleave practically all of extracellular matrix molecules. Their effects are involved in regulation of assembly, remodeling and size of extracellular structures in developmental and pathological conditions as normal tissue differentiation, repairing and organization, or invasive and destructive diseases as periodontal degeneration, rheumatoid arthritis and tumor invasion (Matrisian, 1990; Woessner, 1991; Mignatti and Rifkin, 1993).

The matrix metalloproteinase family is composed of collagenases, stromelysins, gelatinases and putative metalloproteinases. Such classification is according to homologies and differences regarding to substrate specificities or showing structural similarity to prototype five-domain modular structure characteristic of these molecules (Birkedal-Hansen *et al.*, 1993; Kleiner and Steler-Stevenson, 1993). The prototype domain structure of matrix metalloproteinases is composed by a hydrophobic signal sequence, an amino-terminal propeptide that should be removed by enzyme activation, a divalent metal binding domain that is the catalytic sequence involved directly in proteolytic activity of these enzymes, a flexible proline-rich region and a carboxy-terminal hemopexin/vitronectin-like domain that seems to be involved in substrate specificity. Gelatinases have an additional domain responsible for gelatin binding activity (Huhtala *et al.*, 1990, 1991).

Reprolysins are proteolytic metalloproteinases that cleave several protein substrates. They are involved in destructive effects of snake venoms. Although the name reprolysin strictly is applied only to 14 snake venom proteases whose primary sequences are known, in light of recent developments and based in evidences as molecular masses, substrate specificities, partial amino-acid sequences and biological activities, the term reprolysin is applied to all related metalloproteinases from snake venoms (Bjarnason and Fox, 1994, 1995). At the present moment about 102 reprolysins have been purified from several species of snakes. Its molecular masses range from 15 to 100 kDa and these variety appear to present molecules with multimodular proteins containing up to four modules, termed proteinase domain, disintegrin like domain, high Cys domain and lectin-like domain (Bjarnason and Fox, 1994, 1995). The small reprolysin proteases ranging from 20 to 30 kDa appear to be composed only of proteinase domain, those with 30 to 50 kDa appear to have an additional disintegrin domain, those with molecular

masses from 50 to 80 kDa have a third Cys rich domain and those with 80 to 100 kDa have a four protein module lectin-like domain (Bjarnason and Fox, 1994, 1995). Most of the described reprolysins have been found to provoke hemorrhage (Bjarnason and Fox, 1995) and some of the reprolysins have the ability to cleave extracellular matrix molecules such as fibronectin, entactin, laminin and type IV collagen, do not cleave type I native collagen but cleave denaturated gelatin (Baramova *et al.*, 1989; Bjarnason and Fox, 1995).

The present study was undertaken in an effort to detect the molecular basis of hemostatic and vascular lesions triggered by *L. intermedia* (Brown spider) venom. Based on proteolytic degradation studies with purified extracellular matrix molecules, by protease inhibitors assays, zymograph analysis with different substrates and affinity chromatography, we report herein the identification of two gelatin binding metalloproteinases one of these with fibronectinolytic and fibrinogenolytic activities probably involved in hemostatic troubles disseminated by the venom.

MATERIALS AND METHODS

Reagents

Laminin was purified from Engelbreth–Holm–Swarm (EHS) tumors, produced in 2-month-old C57-BL10 female mice as described (Paulsson *et al.*, 1987). Fibronectin was purified from fresh human plasma (obtained from Hospital A. C. Camargo, São Paulo, Brazil) by affinity chromatography on gelatin-Sepharose (Pharmacia, LKB Biotechology, Uppsala, Sweden) as described (Akiyama and Yamada, 1985). Type I collagen purified from rat tail tendon according to the method of Guis *et al.* (1973) was a kind gift of Dr. S. R. P. Line from UNICAMP, Piracicaba, Brazil. Human fibrinogen and pepsin-extracted human placental type IV collagen were purchased from Sigma (St. Louis, USA). Gelatin was purchased from Gibco Diagnostics (Madison, USA).

Loxosceles intermedia venom extraction

The *L. intermedia* venom was obtained from LIPAPE, Setor de Ciências Biológicas, Universidade Federal do Paraná. The venom was extracted from spiders (grown in appropriate conditions) that were submitted to an electric shock (15 V) in the cephalothorax, which was collected with a micropipette, dried and frozen at −20 C until use. A pool of venom collected from approximately 500 spiders was used in each experiment.

Degradation of extracellular matrix molecules

EHS laminin, rat tail tendon type I collagen, human plasma fibronectin and human fibrinogen, were incubated with venom at a ratio of substrate:venom (100:1) at 37 C. Aliquots of reaction mixtures were collected after 30 min, 1, 2, 4, 8 and 16 h and the proteolytic effect of venom was stopped by placing the samples at −20°C. As controls we collected samples of purified molecules (time 0) and for experimental stability samples were collected after 16 h in the absence of venom. Samples were subjected to 5 or 7.5% SDS-PAGE under reducing or non-reducing conditions as indicated in respective legends and the proteolytic effect of venom assessed by Coomassie Blue R staining. For pepsin-extracted human placental type IV collagen, the degrading effect of venom was checked under identical experimental conditions as above except that the temperature of incubation was 25 C. Purified type IV collagen denatures at temperatures above 30°C with a midpoint transition near 37 C (Mackay *et al.*, 1990). Using placental type IV collagen in identical conditions as above but at 37 C we were able to detect an aggregation with disappearance of type IV collagen major bands in SDS-PAGE, even without the presence of venom suggesting a temperature susceptible denaturation which made the experiment invalid. For type I collagen the degradation studies performed at 25 and 37 C showed no differences.

Effect of proteinase inhibitors on the fibronectinolytic and fibrinogenolytic activities of venom

Human plasma fibronectin and human fibrinogen were incubated with *L. intermedia* venom at a ratio of (100:1) substrate:venom at 37 C for 16 h in the presence of a divalent metal chelator, such as EDTA (2 mM) or 1,10-phenanthroline (3 mM), a serine-protease inhibitors, such as aprotinin (2 μg/ml) or PMSF (5 mM), a thiol-protease inhibitor, such as leupeptin (2 μg/ml), or an acid-protease inhibitor, such as pepstatin-A (1 μg/ml). As negative controls the respective substrates were incubated at 37 C for 16 h in the absence of venom and for positive controls they were incubated under the same conditions, but in the presence of venom and in

the absence of protease inhibitors. Proteolytic degradations were stopped by placing the samples at −20 C and subjecting them to 5% SDS-PAGE under reducing conditions for fibronectin or non-reducing conditions for fibrinogen. Both gels were stained by Coomassie Blue R.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were carried out in 5, 7.5 or 12% polyacrylamide gels as described by Laemmli (1970) under reducing or non-reducing conditions and stained with Coomassie Blue R. The molecular mass markers used were: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (98 kDa), bovine serum albumin (67 kDa), ovalbumin (44 kDa) and carbonic anhydrase (29 kDa) purchased from Sigma.

SDS-PAGE-zymograms

12% SDS-PAGE gels were prepared as described in Section 2.5 and polymerized at final concentrations of gelatin (3 mg ml), fibronectin (2 mg ml) or fibrinogen (2 mg ml). Samples of 40 μg of venom were dissolved in Laemmli buffer in the absence of reducing agents or for metalloproteinase activation studies samples of 40 μg of venom were pre-incubated at 37 C for 3 h in 2 mM of APMA (Sigma) freshly prepared in 0.05 N NaOH, processed as in Section 2.5 and electrophoresed at 25 mA at room temperature. After electrophoresis, gels were washed for 30 min in 2.5% Triton X-100 (Sigma) to remove SDS and incubated overnight at 37 C in 50 mM Tris HCl pH 7.3, 200 mM NaCl, 5 mM $CaCl_2$ or in the same buffer and conditions, containing 3 mM of 1,10-phenanthroline and then stained with Coomassie Blue R. Clear zones of substrate lysis against a blue background stain indicated the presence of degrading enzymes.

Gelatin-sepharose enzyme purification

Loxosceles intermedia venom (3 mg) was applied to gelatin-Sepharose affinity beads as described by Mackay *et al.* (1990) and incubated overnight with end-over-end-rotation at 4 C. Gelatin binding molecules were eluted with 7.5% dimethyl sulfoxide and the eluate dialyzed against 5 mM Tris-HCl, pH 7.3, containing 0.5 mM $CaCl_2$ and concentrated (10×) in a vacuum concentrator. Enzyme activity was verified by zymography in a 12% SDS-PAGE in a gel copolymerized with gelatin (3 mg/ml) as described above or silver stained for protein bands as described by Wray *et al.*, 1981.

RESULTS

Degradation studies of extracellular matrix molecules by Loxosceles intermedia venom

Extracellular matrix components are targets for proteolysis by several proteases present in snake venoms, which produce their noxious effects (markedly haemorrhagic accidents) by proteolytic degradation of soluble plasma matrix molecules as fibrinogen and fibronectin (Williams *et al.*, 1983). Since vascular problems are a major consequence in loxoscelism, we examined the presence of proteinases in the venom of the *L. intermedia* spider studying the degrading effect of this venom on purified extracellular matrix mol-

Fig. 1 (*Opposite.*)

Fig. 1. Proteolytic effect of *L. intermedia* venom in extracellular matrix molecules. EHS laminin (A), type I collagen (B), type IV collagen (C) and fibronectin (D) were incubated with venom at a ratio of (100:1) substrates:venom at 37°C (except for type IV collagen that was evaluated at 25°C). For laminin, type I collagen and fibronectin, degradation was assessed following incubation for 0 h (lane 1), 30 min (lane 2), 1 h (lane 3), 2 h (lane 4), 4 h (lane 5), 8 h (lane 6) and 16 h (lane 7). Lane 8 shows controls for purified molecules incubated for 16 h at 37°C in the absence of venom. Type IV collagen degradation was assessed following incubation for 0 h (lane 1), 1 h (lane 2), 2 h (lane 3), 4 h (lane 4), 8 h (lane 5) and 16 h (lane 6). Lane 7 shows controls for experimental stability type IV collagen incubated for 16 h in the absence of venom. Degradation products were determined by Coomassie Blue R stain following 5% SDS-PAGE [(A), (B) and (D)] or 7.5% SDS-PAGE [(C)] under reduced conditions. (A) The open arrow shows β1 and γ1 chains of EHS laminin that comigrate and the closed arrow depicts the α1 chain. The 150 kDa band represents entactin copurified with laminin. (B) The arrow head represents the α2 type I collagen chain, the open arrow shows the α1 chain and the closed arrow shows the dimer β chains. (C) We can see the major components, 100, 130, 160 and 170 kDa, of pepsin-extracted human placental type IV collagen. (D) The arrow shows comigratory fibronectin A and B chains. Molecular mass markers are shown on the left.

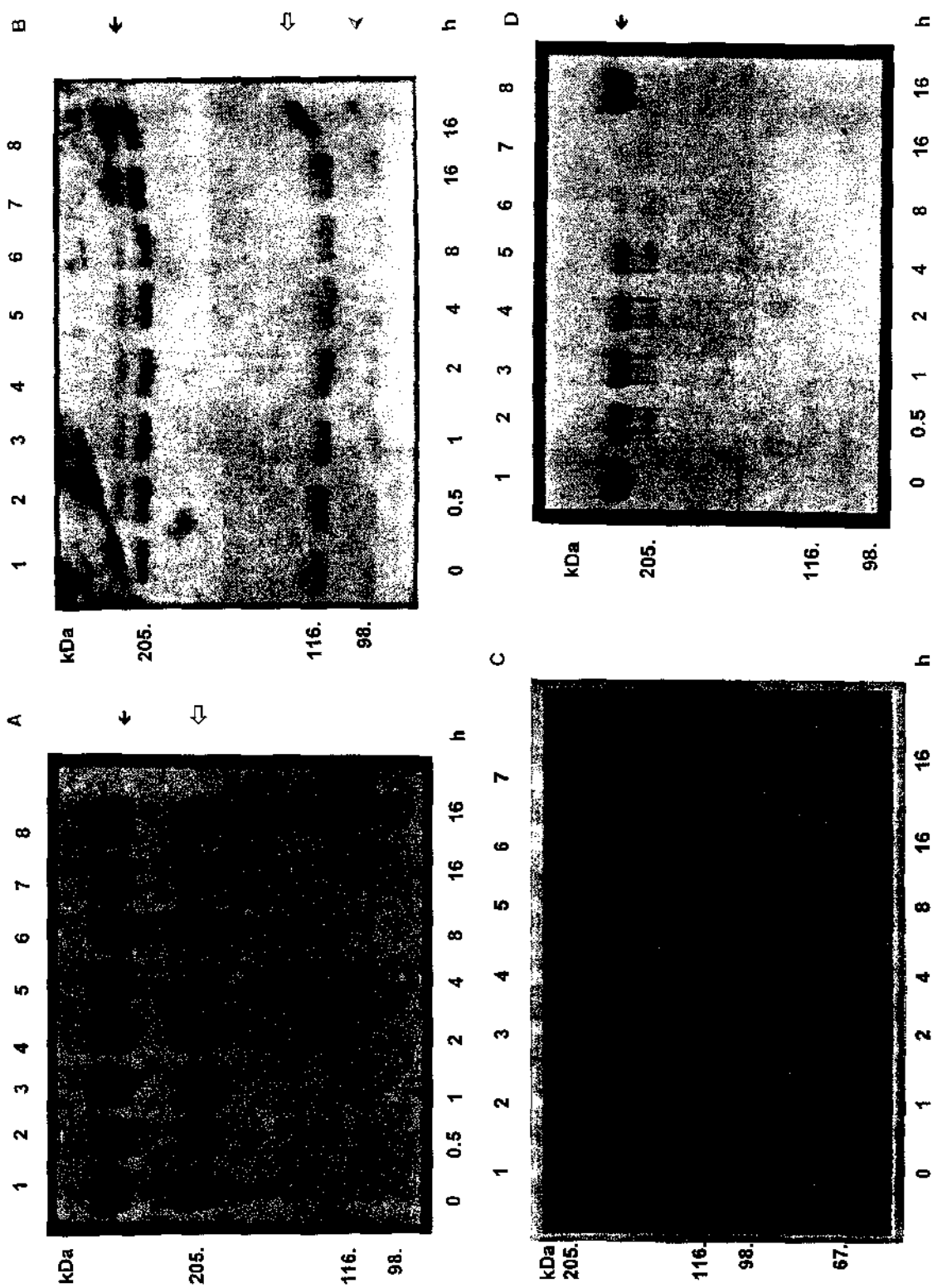

Fig. 1 (*Caption opposite.*)

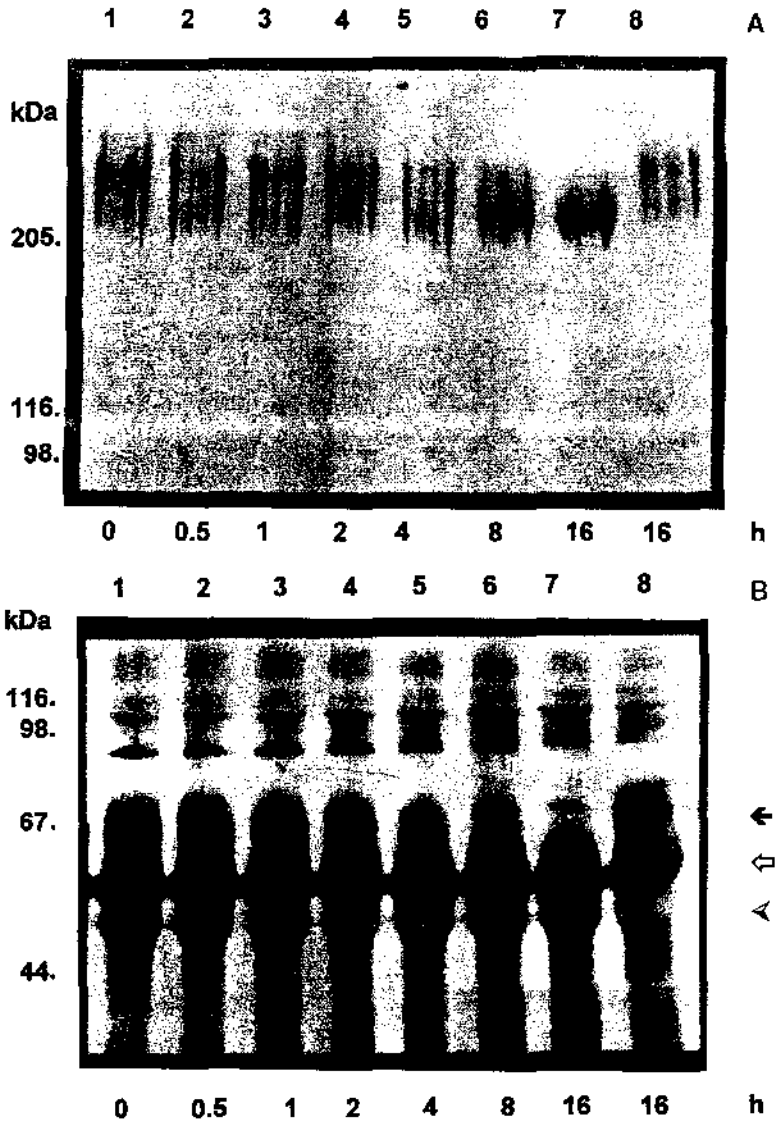

Fig. 2. Proteolytic effect of *L. intermedia* venom on human fibrinogen. Human fibrinogen was incubated with venom at a ratio of (100:1) substrate:venom at 37°C. Proteolytic hydrolysis was assessed following incubation for 0 h (lane 1), 30 min. (lane 2), 1 h (lane 3), 2 h (lane 4), 4 h (lane 5), 8 h (lane 6), 16 h (lane 7), lane 8: fibrinogen incubated for 16 h at 37°C in the absence of venom. The degree of cleaving was determined by Coomassie Blue R stain following 5% SDS-PAGE under non-reducing conditions (A) or silver stain following 7.5% SDS-PAGE under reduced conditions (B). Arrow head shows fibrinogen γ chain, open arrow B(β) chain and closed arrow A(α) chain. Molecular weights are indicated on the left.

ecules by proteolytic digestion kinetic experiments from 0–16 h (laminin, type I collagen, type IV collagen, fibronectin and fibrinogen). As shown in Fig. 1 neither laminin (A), nor type I (B) or type IV (C) collagens suffered any kind of proteolytic degradation by venom action during 16 h; on the other hand, fibronectin (D) was completely cleaved.

Partial fibrinogenolytic activity of L. intermedia venom

Since we detected a proteolytic activity of *L. intermedia* venom on fibronectin, a major cell-adhesive protein involved in platelet adhesion and aggregation, inflammatory processes and wound healing (Ruoslahti, 1988; Veiga *et al.*, 1996, 1997), we decided to

evaluate its action on fibrinogen, a soluble plasma matrix molecule involved in blood coagulation by fibrin net formation and platelet aggregation. As depicted in Fig. 2, proteolytic kinetic studies for 0–16 h, as in Section 3.1, showed a partial digestion of fibrinogen between 8–16 h, with proteolytic activity only on the A(α) chain and without effect on B(β) or γ chains.

Inhibitory effect of divalent metal chelators on proteolytic activity of L. intermedia venom

In order to obtain information about the nature of the proteolytic effects produced by *L. intermedia* venom, we studied its degrading action on fibronectin and fibrinogen in the presence of inhibitors of four major groups of proteolytic enzymes, such as

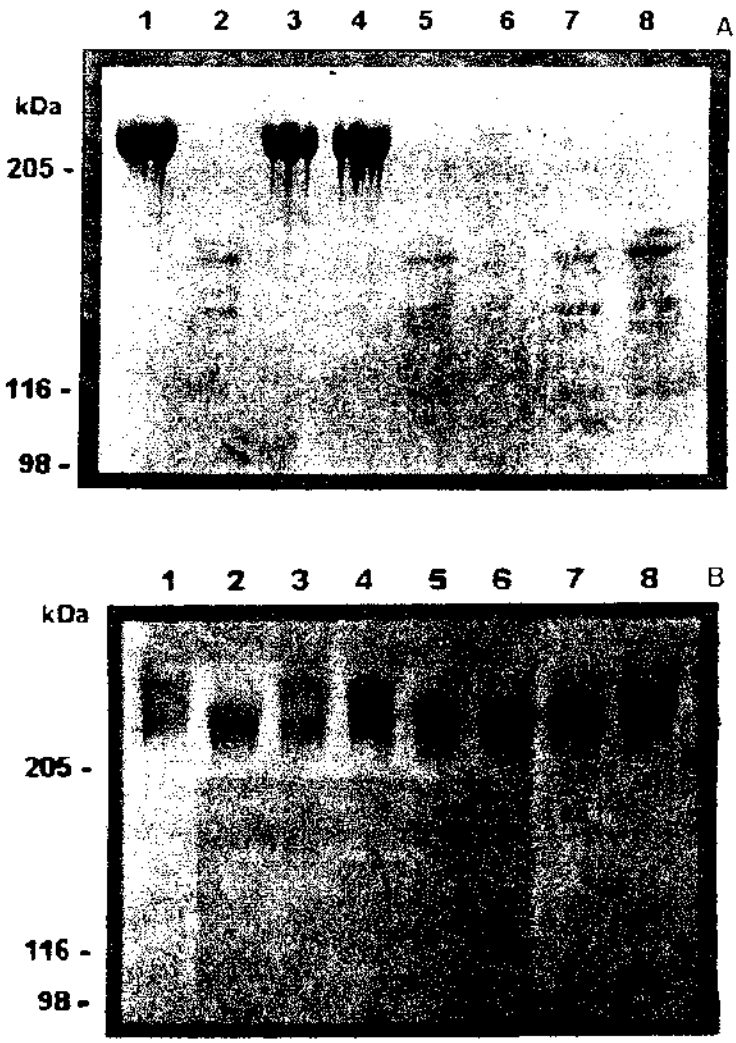

Fig. 3. Protease inhibitors action in proteolytic hydrolysis effect of *L. intermedia* venom. Human plasma fibronectin (A) or human fibrinogen (B) were incubated with venom at a ratio of (100:1) substrates:venom at 37°C for 16 h in presence of different protease inhibitors: EDTA (lane 3), 1,10-phenanthroline (lane 4), aprotinin (lane 5), PMSF (lane 6), leupeptin (lane 7) and pepstatin-A (lane 8). Lane 1 shows negative controls: fibronectin and fibrinogen incubated for 16 h in absence of venom, lane 2 represents degrading positive controls: incubation for 16 h in the presence of venom. Degrading products were submitted to 5% SDS-PAGE under reducing conditions for fibronectin (A) or non-reducing conditions for fibrinogen (B) and stained by Coomassie Blue R. Molecular mass markers are on the left.

EDTA and 1,10-phenanthroline (metalloproteinase inhibitors), aprotinin and PMSF (serine protease inhibitors), leupeptin (thiol-protease inhibitor) and pepstatin-A (acid protease inhibitor). As depicted in Fig. 3(A) and (B) proteolytic actions of *L. intermedia* venom on fibronectin and fibrinogen, respectively, were blocked only by 1,10-phenanthroline and EDTA, suggesting the involvement of divalent metals in these degrading mechanisms and pointing to the presence of metalloproteinase activity in the venom.

Presence of a 32–35 kDa gelatinolytic molecule in L. intermedia venom

Given the probable involvement of metalloproteinase(s) in the degrading effects of *L. intermedia* venom, our next goal was to characterize its molecular mass. Based on the fact that neither types I or IV collagens nor laminin suffered any kind of proteolysis

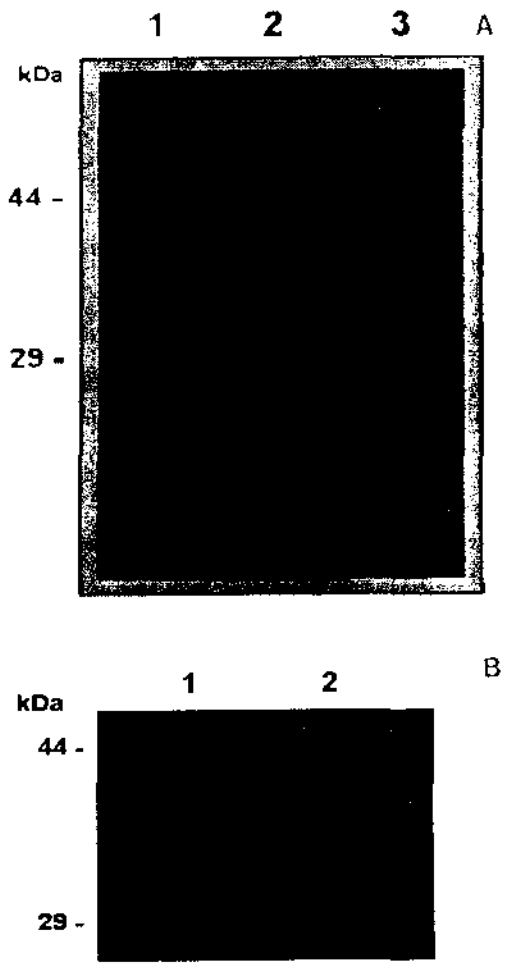

Fig. 4. 32–35 kDa gelatinolytic activities in *L. intermedia* venom. (A) Samples of 40 μg of venom were untreated (lanes 1 and 3) or pretreated for 3 h at 37°C with 2 mM APMA (lane 2) and subjected to a 12% SDS-PAGE impregnated with gelatin. Zymograms were developed overnight at 37°C in the absence (lanes 1 and 2) or presence of 3 mM 1,10-phenanthroline (lane 3). B: *L. intermedia* venom was affinity chromatographed in gelatin-Sepharose beads and bound material eluted with 7.5% Me_2SO containing buffer as described in Section 2. Eluted material was analyzed in a zymogram by 12% SDS-PAGE containing gelatin as substrate (lane 1) or silver stained for protein content (lane 2). Molecular weight markers are shown on the left.

when submitted to the venom, we performed a zymography experiment using gelatin as a substrate in the presence or absence of 1,10-phenanthroline. As shown in Fig. 4(A), there is in the venom a gelatinolytic activity in the region of 35 kDa (lane 1), that was completely blocked by divalent metal chelator (lane 3) corroborating the above results. Some metalloproteinases are secreted as latent pro-enzymes that undergo proteolytic cleavage (propeptide domain) during activation and it is true for matrix metalloproteinases (Birkedal-Hansen *et al.*, 1993; Kleiner and Steler-Stevenson, 1993) and for reprolysins (Hite *et al.*, 1992; Paine *et al.*, 1992; Bjarnason and Fox, 1995). To examine the presence of a propeptide in the 35 kDa gelatinolytic protein of *L. intermedia* venom, we treated the venom with APMA (an organomercurial drug that converts latent forms of proproteinases to active forms by autolytic cleavage) (Grant *et al.*, 1987; Nagase *et al.*, 1990). As depicted in Fig. 4(A) (lane 2) we can see that after APMA treatment the 35 kDa gelatinolytic form resulted in a 32 kDa form, strongly pointing to the presence of a propeptide domain in this enzyme.

Some metalloproteinases described in vertebrates compared to other proteinases, have additional domains similar to the gelatin-binding domains of fibronectin that confers its gelatin binding activity (Huhtala *et al.*, 1990; Collier *et al.*, 1991). In order to confirm the molecule of 32–35 kDa, present in *L. intermedia* venom, as a gelatinolytic protease, we submitted the venom to gelatin-affinity chromatography. As shown in Fig. 4(B) zymography (using gelatin as substrate) of affinity purified material from the venom, depicts a 32–35 kDa protein with gelatinolytic activity [a pattern similar to APMA-treated venom as depicted in Fig. 4(A)] and by a mass criterion using silver staining in the same material eluted from gelatin-chromatography, we can see the protein band at 32–35 kDa confirming this venom spider metalloproteinase as a gelatin binding and gelatinolytic molecule.

Fibronectinolytic and fibrinogenolytic activities of metalloproteinases in L. intermedia venom

We next examined the ability of this enzyme to cleave fibronectin and fibrinogen (the two substrates sensitive to degradation by the venom) performing zymography experiments using plasma fibronectin [Fig. 5(A)] and fibrinogen [Fig. 5(B)] as substrates under the same conditions as used in gelatin zymography, either in the absence (lane 1) or presence (lane 3) of 1,10-phenanthroline, or with venom previously treated by APMA (lane 2). We can observe that using these substrates there was no detection of gelatinolytic enzyme of 32–35 kDa, as depicted in Fig. 4, but instead a 28 kDa molecule, with fibronectinolytic and partial fibrinogenolytic activities, that had its proteolytic hydrolysis effects completely blocked by 1,10-phenanthroline and suffered a decrease in molecular weight to 20 kDa after APMA treatment. In addition, identically detected as for the 32–35 kDa form, this 20–28 kDa metalloproteinase is also eluted from a gelatin-sepharose column confirming its gelatin binding ability [Fig. 5(C)]. These results not only detected other metalloproteinase in the venom but also showed substrate specificity since these latter forms (20–28 kDa) although binding gelatin, only cleave fibronectin and fibrinogen, whereas 32–35 kDa gelatinolytic molecule has no effect on these substrates. Zymography experiments using casein as the substrate under the same conditions as described above showed no degrading signals for 32–35 and 20–28 kDa enzymes corroborating the above specificities found (data not shown).

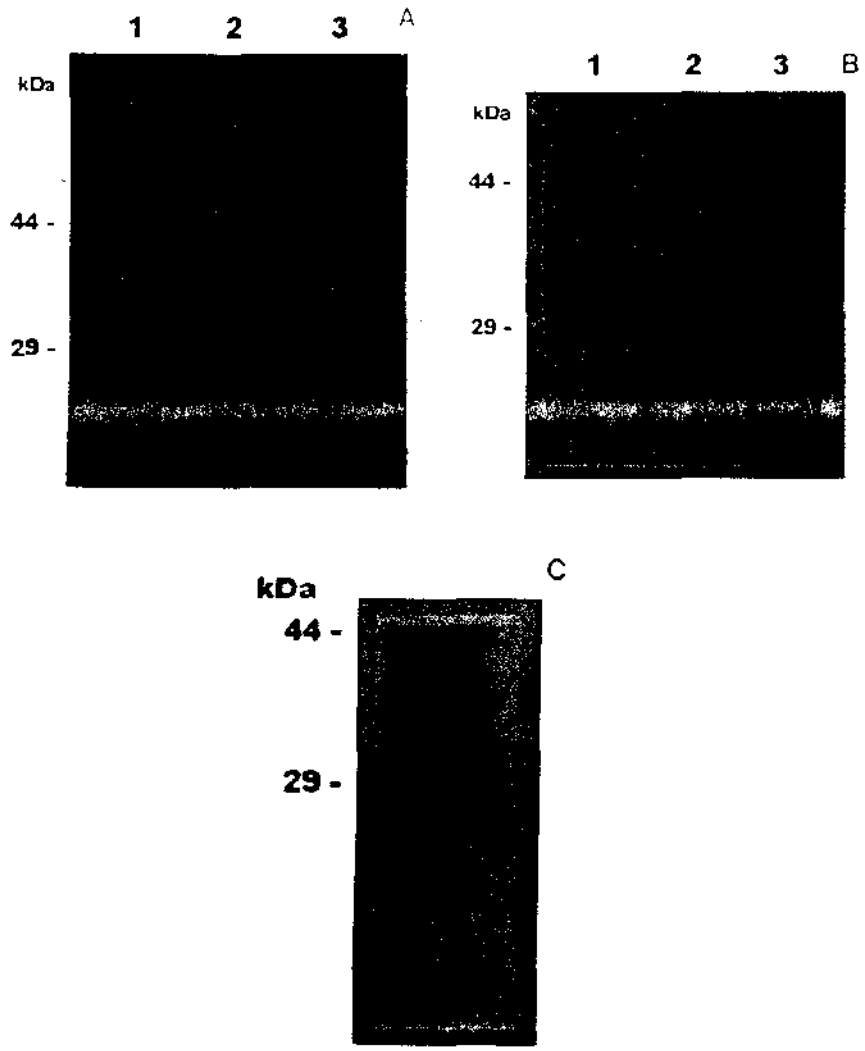

Fig. 5. Fibronectinolytic and fibrinogenolytic activities of a 20–28 kDa molecule in *L. intermedia* venom. Samples of 40 μg of venom were untreated (lanes 1 and 3) or pretreated for 3 h at 37°C with 2 mM APMA (lane 2) and subjected to 12% SDS-PAGE containing as substrates purified human fibronectin (A) or human fibrinogen (B). Zymograms were developed overnight at 37°C in the absence (lanes 1 and 2) or presence of 3 mM 1,10-phenanthroline (lane 3). The venom was affinity chromatographed in gelatin-Sepharose beads and eluted by 7.5% Me_2SO containing buffer as described in Section 2, subsequently analyzed by 12% SDS-PAGE and silver stained for protein content (C). Molecular mass markers are on the left.

DISCUSSION

Metalloproteinases have been described as proteolytic enzymes involved in haemorrhagic effects of several snake venoms (Takeya *et al.*, 1990; Sanchez *et al.*, 1991; Hite *et al.*, 1992; Bjarnason and Fox, 1995). In this report working with *L. intermedia* venom we were able to detect a selective proteolytic activity to degrade extracellular matrix molecules. The venom showed no ability to degrade full length EHS laminin, type I and type IV collagens under conditions that preserved the tertiary structure of these molecules, but partially cleaved native fibrinogen A(α) chains, with no activity on the B(β) and γ chains and completely degraded both A and B fibronectin chains (Figs 1 and 2). In addition, under the conditions used in this study in the ratio of venom:substrates

(1:100), only EDTA and 1,10-phenanthroline (metal chelators) completely blocked the cleaving action of venom on fibronectin and fibrinogen, compared to other enzyme inhibitors, such as serine-protease inhibitors, thiol-protease inhibitors and acid-protease inhibitors, that had no effect on the degrading action of the venom (Fig. 3) indicating the participation of metalloproteinase(s). This preliminary conclusion is based on the fact that the metalloproteinase family shares a diversity and selectivity in substrates and that all enzymes of this family possess a metal atom binding sequence in the catalytic domain (Emonard and Grimaud, 1990; Matrisian, 1990; Birkedal-Hansen *et al.*, 1993; Rawlings and Barrett, 1995).

The confirmation of a metalloproteinase in the venom is shown by its ability to degrade gelatin in a zymogram (35 kDa as apparent electrophoretic mobility), by its sensitivity to the zinc chelator 1,10-phenanthroline in the same zymogram, its specific activation and conversion by APMA (an organomercurial drug) to a 32 kDa form [Fig. 4(A)] and by its ability to bind to gelatin-sepharose chromatography [Fig. 4(B)]. Together with the selectivity and sensitivity features of metalloproteinases as discussed above, another important characteristic of this family of enzymes is the fact that they are produced as zymogen molecules and are secreted as latent pro-enzymes that should undergo proteolytic cleavage of an amino-terminal domain during activation (Kleiner and Steler-Stevenson, 1993; Fox and Bjarnason, 1995). We can conclude that the 35 kDa gelatinolytic molecule is a latent pro-enzyme form and the 32 kDa molecule represents an activated form obtained after APMA treatment that induces an autolytic cleavage of the propeptide domain. What are the natural substrates and targets for 32 35 kDa gelatinolytic enzyme? This is an open question. Vertebrate gelatinases appear to degrade connective tissue components (Birkedal-Hansen *et al.*, 1993) and related to this we can suppose the involvement of this enzyme in the dermonecrotic action of venom, a characteristic signal 12–24 h after *L. intermedia* accidents, but this is unclear at the present moment.

Interestingly, in addition we were able to detect in the venom, a second form of metalloproteinase, with fibronectinolytic and fibrinogenolytic features in zymogram experiments, which had an apparent molecular weight of 28 kDa and had its proteolytic abilities blocked by the metal chelator 1,10-phenanthroline and was converted by APMA treatment to a 20 kDa form (Fig. 5). The 20–28 kDa form was also eluted from a gelatin-sepharose column [Fig. 5(C)], suggesting that this enzyme has gelatin-binding domains, but with a lower ability to degrade gelatin compared to fibrinogen and fibronectin. Although capable of binding gelatin, this metalloproteinase cleaves preferentially fibronectin or fibrinogen. Different peptide substrates can discriminate the proteolytic action of different metalloproteinases (Netzel-Arnett *et al.*, 1991a) and a single amino acid substitution in substrates can alter their degrading susceptibility (Fields *et al.*, 1987; Netzel-Arnett *et al.*, 1991b; Birkedal-Hansen *et al.*, 1993). Fibrinogen is a dimeric glycoprotein of about 340 kDa formed by three different chains covalently associated by disulfide bridges, named an A(α) subunit of 64 kDa, a B(β) subunit of 56 kDa and a γ subunit of 47 kDa (Williams *et al.*, 1983). The lower degree of proteolysis in a zymogram copolymerized with fibrinogen [Fig. 5(B)] (there was no improvement even with longer digestion time) compared to with fibronectin [Fig. 5(A)] and gelatin [Fig. 4(A)] can be explained by partial degradation of fibrinogen as detected in Fig. 2 where we can see the venom action only in the A(α) chain, but without effect in the B(β) or γ chains compared to proteolytic hydrolysis of both A and B fibronectin chains [Fig. 1(D)]. Venoms of various snakes contain proteolytic enzymes that cleave fibrinogen partially triggering disseminated intravascular coagulation by fibrin formation

(Williams *et al.*, 1983). Disseminated intravascular coagulation is a disorder associated with *L. intermedia* accidents and from this we can speculate the involvement of *L. intermedia* fibrinogenolytic metalloproteinase in this syndrome.

For metalloproteinases to function as enzymes that cleave substrates '*in vivo*', they must be converted to the catalytically active form. In the case of metalloproteinases of *L. intermedia* venom, indeed a small amount of active forms of gelatinolytic and fibronectinolytic or fibrinogenolytic are present in some batches of venom and can be detected through zymography experiments (data not shown), but the behavior of accidents with *Loxosceles* spiders that usually provoke the harmful effects only 8 to 16 h after the bite point to enzyme activation in the blood, which really can be made by the plasminogen activator–plasmin cascade system as described for some metalloproteinases including interstitial collagenase, stromelysins and gelatinases (Kleiner and Steler-Stevenson, 1993; Mignatti and Rifkin, 1993). However, we cannot discard the fact that, in the *Loxosceles* system described herein, a proteolytic enzyme also activates metalloproteinases in the venom, since we could detect a 220 kDa proteolytic molecule in the venom not sensitive to metal chelators or APMA activation (data not shown) that could participate in this event.

We postulate here, in light of the first detection and characterization of metalloproteinases in *Loxosceles* venom, the trivial name Loxolysin A to 20–28 kDa and Loxolysin B to 32–35 kDa proteases, which combines a reference to the spider genus (Loxo) and the proteolytic effect (lysin).

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Identification of proteases in the extract of venom glands from brown spiders

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Abstract

In the present investigation, in order to dispute the rational criticism against the presence of proteolytic enzymes in the electrostimulated venom obtained from spiders of the genus *Loxosceles*, as a consequence of contamination with abdominal secretions, venoms of *L. intermedia* and *L. laeta* were directly collected from venom glands by microdissection and gentle homogenization. Gel electrophoresis stained by silver method carried out to compare *L. intermedia* electrostimulated venom and venom gland extract demonstrated no significant differences in protein profile. Zymogram analysis of *L. intermedia* venom gland extract detected a gelatinolytic activity in the 32–35 kDa region. The inhibitory effect of 1,10-phenanthroline on this proteolytic activity further supported its metalloprotease nature. In proteolytic digestion experiments *L. intermedia* venom gland extract was also able to cleave purified fibronectin and fibrinogen. The inhibitory effect of 1,10-phenanthroline on these degrading activities confirmed the presence of metalloproteases in the venom. In addition, when purified fibrinogen was incubated with *L. intermedia* abdominal extract, the fibrinogenolysis was completely different, generating low mass fragments that ran away from the gel, a proteolytic event not blocked by 1,10-phenanthroline. Zymogram experiments using *L. laeta* venom gland extracts further detected a gelatinolytic band at 32–35 kDa, also inhibited by 1,10-phenanthroline, confirming the presence of metalloproteases in both species.

Keywords: Proteases; *Loxosceles*; Zymogram

1. Introduction

Brown spider venoms have been reported to cause two major clinical signs in accidental bites. The skin lesions appear between 4 and 12 h after envenomation and are characterized by oedema and local haemorrhage at the bite site and may progress to an impressive necrotic lesion of cutaneous tissue with gravitational spreading and formation of a black eschar in the next 24–48 h after the accident (Futrell, 1992; Sezerino et al., 1998; Sams et al., 2001). The systemic effects generally occur 24–48 h after envenomation. They are hematologic disorders such as hemolytic anemia, thrombocytopenia and disseminated intravascular coagulation (probably evoked by the direct fibrinogenolytic activity of the venom) (Futrell, 1992; Williams et al., 1995; Sezerino et al., 1998), as well as other disturbances such as renal failure, fever, weakness, nausea, and vomiting (Futrell, 1992; Sezerino et al., 1998; Lung and Mallory, 2000). The molecular and cellular aspects of loxoscelism have not been well established. Polymorphonuclear leukocytes present at the bite site and around it have been reported to play a role in the skin lesions (Smith and Micks, 1970; Patel et al., 1994). The activation of the autologous complement alternative pathway also seems to participate in the noxious effects of the venom such as erythrocyte lysis and dermonecrosis (Futrell, 1992; Tambourgi et al., 1995, 2000). The serum amyloid P component also seems to participate in the thrombocytopenic activity of the venom and in its indirect ischemic effects (Gates and Rees, 1990).

The mechanisms by which brown spiders cause local and systemic lesions can also be attributed to proteolytic toxins

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present in the venom which, by degrading fibrinogen, fibronectin, entactin, and heparan sulphate proteoglycan and by evoking disruption of basement membrane structures (Feitosa et al., 1998; Veiga et al., 1999, 2000a, 2001a,b), can be associated with local haemorrhage, harmful necrotic effects as well as gravitational spreading of the cutaneous lesions and systemic pathogenesis involving disseminated intravascular coagulation and renal disorders.

All results describing proteolytic activities in the *L. reclusa* venom (Eskafi and Norment, 1976; Jong et al., 1979) or more recently in studies on *L. intermedia* venom (Feitosa et al., 1998; Veiga et al., 1999, 2000a,b, 2001a,b) used venoms obtained by electric shock. These descriptions are open to criticism concerning the fact that spider stomach egesta and the hydrolytic enzymes they contain may be mixed with the venom during extraction. We present here evidence confirming that the venoms obtained directly from the venom glands of brown spiders have proteolytic activities.

2. Materials and methods

2.1. Reagents

Fibronectin was purified from human plasma obtained at the University Hospital, Federal University of Parana, by affinity chromatography on gelatin–Sepharose (Pharmacia, Uppsala, Sweden) as described by Engvall and Ruoslahti (1977). Purified human fibrinogen was purchased from Sigma (St Louis, USA) and gelatin was purchased from Gibco (Madison, USA).

2.2. Venom extraction

L. intermedia venom was extracted from spiders submitted to an electric shock (15 V) applied to the cephalothorax. The venom was collected with a micropipette, dried and stored at −85 °C until use. A pool of venom collected from approximately 500 spiders was used in the experiments (Feitosa et al., 1998; Veiga et al., 2000a). For *L. intermedia* and *L. laeta* venom gland collection, adult animals were anesthetized in a chloroform chamber and their abdominal compartments were cut out and discarded using a stereo-dissecting microscope (Olympus, Japan), the dorsum of the cephalothorax of spiders was removed and a pair of glands were collected into ice-cold PBS. The glands were washed in PBS in order to remove some possible contaminants and venoms were harvested in PBS by gentle compressing of the glands. Glands were removed from the solution by centrifugation at 13,000 rpm and the venom gland extract was stored at −85 °C until use. Approximately 200 *L. intermedia* and 100 *L. laeta* spiders were used in these experiments. The protein contents of electrostimulated venom or venom gland extracts were determined by the Coomassie blue method (Bio-Rad, Hercules, USA) as described by Bradford (1976).

2.3. Scanning electron microscopy analysis

The venom apparatus of *L. intermedia* obtained as described before was fixed in modified Karnovsky's fixative for 1 h, washed in 0.1 M cacodylic acid buffer, pH 7.3, and postfixed in 1% OsO_4 in the same cacodylic acid buffer for 1 h. The material was dehydrated in ethanol, critical-point dried, sputter-coated with gold, and examined with a MEV XL-30 Philips scanning electron microscope (dos Santos et al., 2000).

2.4. Gel electrophoresis

To compare the protein profiles of *L. intermedia* electrostimulated venom and venom gland extract, SDS gel electrophoresis was performed as described by Laemmli (1970). Samples were submitted to a 3–20% (w/v) polyacrylamide gel linear gradient electrophoresis under non-reducing conditions. The molecular mass markers used were the 10 kDa protein ladder purchased from Gibco. The gel was stained by the polychromatic silver method as described by Sammons et al. (1981).

2.5. SDS-PAGE zymogram experiments

To study the proteolytic activities in the venoms, zymogram experiments were performed by 7.5% SDS-PAGE containing 3 mg/ml of copolymerized porcine skin gelatin. Samples of 40 μg of *L. intermedia* or *L. laeta* venom gland extracts diluted in Laemmli buffer under non-reducing conditions were electrophoresed (20 mA) at 4 °C. After the run, gels were washed twice in 2.5% Triton X-100 for 30 min to remove SDS and then incubated at 37 °C overnight in 50 mM Tris–HCl buffer, pH 7.3, containing 200 mM NaCl and 1 mM $CaCl_2$ and $MgCl_2$ in the absence or presence of 5 mM 1,10-phenanthroline (see legends for details). Gels were stained with Coomassie blue dye and clear zones of substrate lysis against a blue background stain indicated the presence of gelatin-degrading enzymes (Feitosa et al., 1998).

2.6. Fibronectin- and fibrinogen-degrading studies

Purified human fibronectin or purified human fibrinogen (samples of 100 μg diluted in PBS) were incubated with samples of 50 μg of electrostimulated venom, venom gland extract or spider abdominal extract (see figure legends for details) at 37 °C overnight. Control and digested materials were analyzed by a linear 3–20% (w/v) SDS-PAGE under reducing conditions for fibronectin or non-reducing conditions for fibrinogen and stained with Coomassie blue dye.

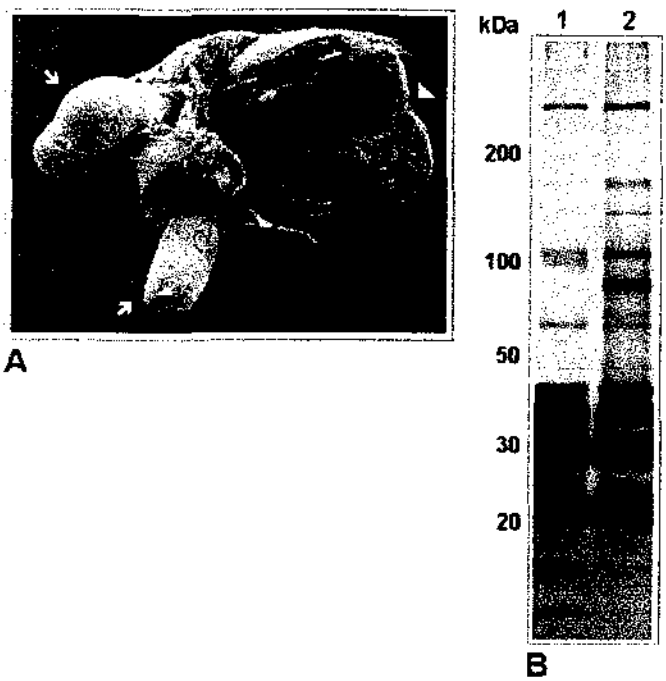

Fig. 1. Scanning electron microscopic view of the *L. intermedia* venom apparatus. Comparative protein profile of the electrostimulated venom and venom gland extract. (A) Paired venom glands (arrows) attached to chelicerae (arrow head) forming the venom apparatus are shown. The glands were collected from this apparatus, exhaustively washed and then homogenized to produce the venom gland extract. (B) Protein profile of electrostimulated venom (1) or venom gland extract (2) from *L. intermedia*, separated by polyacrylamide gel electrophoresis (3–20% w/v, linear gradient) under non-reducing conditions and stained with a polychromatic silver method. Molecular mass marker positions are shown on the left of the figure.

3. Results

3.1. Low magnification morphological view of the venom gland from L. intermedia; comparative protein profile of electrostimulated venom and venom gland extract

In order to validate the model suggested for the study of proteases directly present in the venom of *L. intermedia*, we show here by scanning electron microscopy a view of the venom gland. Fig. 1A depicts the venom apparatus consisting of two glands that are bulbous in shape and two fangs. For venom gland extraction the glands were separated without any kind of other tissue contamination and then processed as described in Section 2. Fig. 1B shows a comparative protein profile of *L. intermedia* venom obtained by electrostimulation of the cephalothorax or harvested directly from venom glands. Protein contents were determined by the Coomassie blue method (Bradford, 1976), normalized and electrophoresed by linear gradient 3–20% SDS-PAGE under non-reducing conditions, with staining by the polychromatic silver method. It can be seen that both venoms are enriched in proteins of low molecular mass in the 20–45 kDa range. The presence of high molecular mass proteins was also observed to a lesser extent.

3.2. Identification of metalloproteases in the venom gland extract of L. intermedia

Given the probable involvement of proteases in the noxious activities of *L. intermedia* venom and the criticism of stomach egesta contamination when obtaining the venom by electrostimulation, we performed a zymograph experiment using gelatin as substrate. As shown in Fig. 2, a proteolytic activity is present in the 32–35 kDa region in the venom gland sample (lane 1). In order to obtain more information on the biochemical nature of this gelatinolytic protease described, we repeated a zymogram but in the presence of 1,10-phenanthroline (since previous reports have been described metalloproteases in the venom, see Section 1 for details). As illustrated in lane 2, this treatment completely blocked the gelatinolytic activity of the venom, supporting the view that this molecule is a metalloprotease.

3.3. Fibrinogenolytic and fibronectinolytic activities in L. intermedia venom gland extract

Since we had previously described the presence of proteolytic activities on plasma extracellular matrix molecules such as fibrinogen and fibronectin in the electrostimulated venom, we carried out an experiment to check the possible

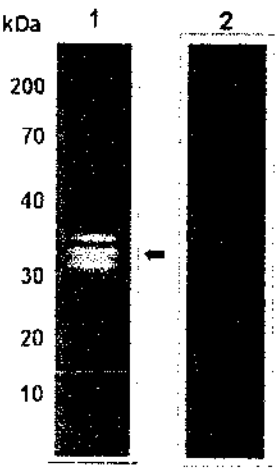

Fig. 2. Presence of gelatinolytic activity in *L. intermedia* venom gland extract. Samples of 40 μg of venom gland extract were subjected to 7.5% SDS-PAGE impregnated with gelatin. Zymograms were developed at 37 °C overnight in the absence (lane 1) or presence of 5 mM 1,10-phenanthroline (lane 2). Gels were stained with Coomassie blue dye. Molecular mass markers are shown on the left. The arrow points at gelatinolytic activity in the 32–35 kDa region.

action of the venom gland extract on these molecules. Purified human fibronectin and fibrinogen were incubated with electrostimulated venom or venom gland extract under the same experimental conditions (as described in Section 2). As can be observed in Figs. 3 and 4A for fibronectin and fibrinogen, respectively, both venoms hydrolyzed these molecules in a similar manner, with phenanthroline blocking both the fibrinogenolytic and fibronectinolytic activities of venom gland extract and electrostimulated venom, strengthening the idea of metalloproteases directly present in *L. intermedia* venom.

3.4. Comparison of the fibrinogenolytic activity of electrostimulated venom, venom gland extract and abdominal spider extract

To complete the molecular analysis indicating the presence of proteases in the venom of *L. intermedia*, we performed a degradation study using purified fibrinogen (a good substrate for proteolysis) incubated with normalized samples of electrostimulated venom, venom gland extract and with spider abdominal extract (with its digestive contents). Fig. 4B shows an increased hydrolytic activity towards fibrinogen evoked by the abdominal extract compared to venom hydrolysis under the same experimental conditions, suggesting different protease profile in these samples. The absence of an inhibitory effect of phenanthroline on abdominal extract proteolysis compared to its effect on venom proteolysis supports this hypothesis.

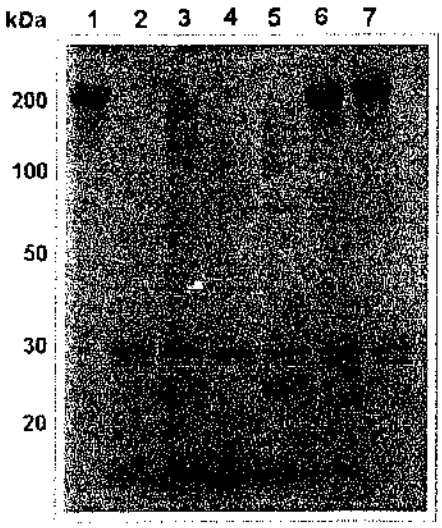

Fig. 3. Presence of fibronectinolytic activity in *L. intermedia* venom gland extract. Samples of purified human fibronectin were incubated at 37 °C overnight with electrostimulated venom (positive control for digestion) or venom gland extract. Materials were submitted to a 3–20% (w/v) SDS-PAGE linear gradient under reducing conditions and the gel was stained with Coomassie blue dye. Lane 1: fibronectin incubated under identical experimental conditions but in the absence of venom (control for substrate experimental stability); lane 2: electrophoretic mobility of the major proteins of the electrostimulated venom; lane 3: fibronectin incubated with electrostimulated venom; lane 4: electrophoretic mobility of the major proteins of the venom gland extract; lane 5: fibronectin treated with venom gland extract; lanes 6 and 7, respectively, fibronectin incubated with electrostimulated venom and with venom gland extract but in presence of 5 mM 1,10-phenanthroline. Molecular mass markers are shown on the left.

3.5. The presence of proteases in the venoms is a phenomenon similar for L. intermedia and L. laeta venom gland extracts

As all experiments described so far were performed using *L. intermedia* venom, which may suggest some species characteristic, we decided to investigate whether this phenomenon is also presented in the venom of other *Loxosceles* species such as *L. laeta*. For this purpose, venoms were obtained directly from the venom gland and samples normalized for protein contents were analyzed by a zymogram copolymerized with gelatin. As shown in Fig. 5, we can see that identically to what was observed for *L. intermedia* venom, *L. laeta* venom also possesses proteases in the 32–35 kDa region (metalloprotease).

4. Discussion

Working with samples extracted directly from venom glands of *L. intermedia* and *L. laeta*, we have described the presence of proteases in these venoms. We carried out experiments using venom gland extracts in order to counter

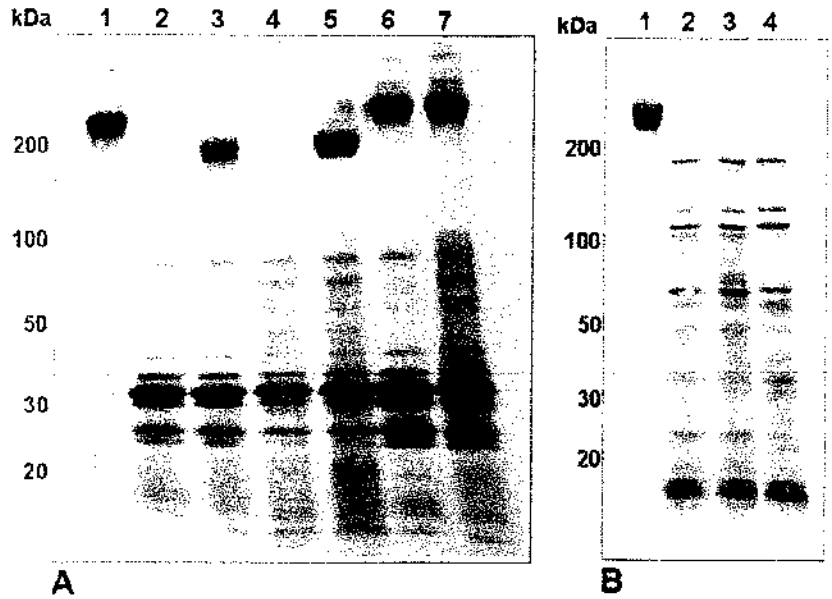

Fig. 4. Presence of fibrinogenolytic activity in *L. intermedia* venom gland extract. (A) Samples of purified human fibrinogen were incubated at 37 °C for 16 h with electrostimulated venom (positive control) or venom gland extract. The extent of fibrinogen cleavage was determined by Coomassie blue staining after 3–20% (w/v) linear gradient SDS-PAGE under non-reducing conditions. Lane 1 represents fibrinogen alone (negative control), lane 2 electrostimulated venom alone, lane 3 fibrinogen cleaved by electrostimulated venom, lane 4 venom gland extract alone, and lane 5 fibrinogen degraded by the venom gland extract. Lanes 6 and 7, respectively, depict fibrinogen incubated with electrostimulated venom or venom gland extract in the presence of 5 mM 1,10-phenanthroline. Molecular mass standards are shown on the left of the figure. (B) Fibrinogenolysis experiments were repeated by incubating fibrinogen with abdominal spider extracts instead of venom. Lane 1 shows fibrinogen alone (control for experimental stability), lane 2 shows electrophoretic mobility of major proteins from the abdominal extract, lane 3 represents fibrinogen incubated with the abdominal extract, and lane 4 fibrinogen incubated with abdominal extract but in the presence of 5 mM 1,10-phenanthroline. Molecular mass markers are shown on the left.

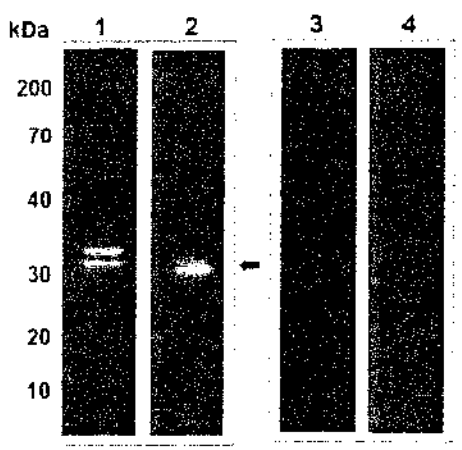

Fig. 5. Comparative gelatinolytic activities of *L. intermedia* and *L. laeta* venom gland extracts. Samples containing 40 μg of *L. intermedia* (lanes 1 and 3) or *L. laeta* (lanes 2 and 4) venom gland extracts were applied to a 7.5% SDS-PAGE gelatin copolymerized zymogram. Zymographs were developed at 37 °C overnight in the absence of metalloprotease inhibitors (lanes 1 and 2), or in the presence of 5 mM 1,10-phenanthroline (lanes 3 and 4). The arrow indicates the positions of gelatinolytic activities in both venoms at 32–35 kDa.

the very rational criticism concerning the fact that electrostimulated venom may be contaminated with stomach egesta during the process of obtaining venom. This criticism was raised against preliminary papers describing proteolytic activities of *L. recluse* spider venom on *Heliothis virescens* and *Musca domestica* larvae (Eskafi and Norment, 1976) and against the results of Jong et al. (1979) who observed hydrolytic activities of the same venom on L-aminoacyl-β-naphthylamide derivatives, or against our previous reports in which, working with electrostimulated venom of *L. intermedia*, we were able to describe proteolytic activities on gelatin, fibronectin and fibrinogen (Feitosa et al., 1998; Veiga et al., 1999, 2001a), disruption of EHS-basement membrane structures with proteolysis of entactin (Veiga et al., 2000a, 2001a), or endothelial cell cytotoxicity with hydrolysis of the heparan sulphate proteoglycan protein core (Veiga et al., 2001b).

Our working model was based on collection of the venom apparatus containing a couple of venom glands and fangs by using a low magnification light microscope (see Fig. 1A), a procedure in which the abdominal spider compartment (containing the stomach and digestive hydrolytic enzymes) was separated. The glands are then removed from the rest of the venom apparatus, washed exhaustively with PBS to clear some possible contaminant and then gently compressed to obtain the venom. Protein profile studies between

electrostimulated venom and venom gland extract from *L. intermedia* (Fig. 1B) showed that the venoms are a mixture of proteins with a very similar electrophoretic profile especially in the region of low molecular mass proteins (20–45 kDa), as previously demonstrated (Veiga et al., 2000a). The expression of some individual high molecular mass proteins by the venom gland extract, not observed in electrostimulated venom, was probably due to cell lysis during removal of the gland. Nevertheless all of them were devoided of proteolytic activities as shown in Fig. 2. The absence of some different proteins in the electrostimulated venom points against oral egesta contamination during venom harvesting.

The presence of proteolytic activities in the venom of *L. intermedia* (venom gland extract) was first demonstrated by its ability to degrade gelatin in zymogram experiments, where gelatinolytic enzymes with apparent electrophoretic mobilities in the 32–35 kDa region are visible (see Fig. 2). The inhibitory sensitivity to the zinc chelator 1,10-phenanthroline supports this hypothesis and indicates that these are metalloprotease molecules (Fig. 2, lane 2) as previously demonstrated by electrostimulated venom (Feitosa et al., 1998).

In addition, we were able to detect in the venom gland extract the same fibronectinolytic and fibrinogenolytic activities (Figs. 3 and 4) as found in electrostimulated venom and previously described (Feitosa et al., 1998; Veiga et al., 1999, 2001a). The very similar cleaving products after fibronectin exposure to electrostimulated venom and to the venom gland extract (Fig. 3), as well as similar fibrinogen fragments produced after venom treatments (Fig. 4A), strengthen the idea of the presence of direct proteases in *L. intermedia* venom. The inhibitory effect of phenanthroline on these proteolytic activities further indicates the participation of metalloproteases in the venom.

The major criticism related to the process for obtaining electrostimulated venom and to protease description or identification in this venom is the possibility of some contamination with stomach oral egesta and all hydrolytic enzymes contents present in this secretion. The results illustrated in Fig. 4A clearly show that both electrostimulated venom and venom gland extract act on fibrinogen in a similar way, partially cleaving fibrinogen under the conditions assayed. On the other hand, the spider abdominal extract digests in a different manner compared to the cleaving activities of the venoms (Fig. 4B). This difference in clevage patterns for fibrinogen treated with venom or treated with the abdominal extract indicates the absence of venom contamination with oral egesta under the harvesting conditions used. The inhibitory action of 1,10-phenanthroline on the fibrinogenolytic activities of the venoms, but its inability to block abdominal extract proteolysis (Fig. 4B) further support this conclusion.

Finally, we investigated whether these hydrolytic activities of *L. intermedia* venom gland were also presented in *L. laeta*, another brown spider species. As depicted in Fig. 5, we can see that *L. laeta* venom gland extract displays proteolytic activity on gelatin, an effect identically blocked by 1,10-phenanthroline. These results indicate that metalloproteases are present in both *L. intermedia* and *L. laeta* venoms, suggesting a conservation feature of possible biological significance.

The major clinical characteristic at the site of a brown spider bite is a necrotic lesion (Futrell, 1992; Sams et al., 2001), and the real mechanism by which the venom causes dermonecrotic lesions is currently under investigation. A 30–35 kDa protein devoid of proteolytic activity isolated from venoms of brown spiders, biochemically characterized as a sphingomyelinase D, can produce dermonecrotic lesions, platelet aggregation and red blood cell hemolysis in animals (Kurpiewski et al., 1981, Tambourgi et al., 1995). Recently, Monteiro et al. (2002) isolated the bacteria *Clostridium perfringens* in the venom and fangs of *L. intermedia*. Using experimental models for dermonecrosis the authors observed that venom and *C. perfringens* conjugated resulted in a striking increase of dermonecrotic pictures when compared to venom alone, suggesting a role for *C. perfringens* in enhancement of the dermonecrotic lesion. On the other hand, systemic disturbances that may include disseminated intravascular coagulation, renal failure, as well as weakness, vomiting and malaise have also been reported after envenomation (Futrell, 1992; Sezerino et al., 1998; Lung and Mallory, 2000). These activities point to venom spreading to the body of bite victims, an event that may be dependent on venom proteases, Gomez et al. (2001) described a direct correlation between diffusion of the venom from the envenomation site, with inflammation reaction. Interestingly, Veiga et al. (2000a) have shown that the venom acts on basement membranes causing disruption of these structures, as well as cytotoxicity on blood vessel wall endothelial cells in vivo and in vitro (Veiga et al., 2001b). Such venom degrading activities on subendothelial basement membranes may be related to loss of vessel integrity and spreading of the venom toxins to underlying tissues.

Considering the findings described before, it is possible to postulate that some pathological activities triggered by the venom such as haemorrhage into the dermis at the bite site, disseminated intravascular coagulation and the defective wound healing detected in some bite victims, could have the participation of venom proteases, since fibronectin and fibrinogen are multifunctional proteins involved in the mechanisms of hemostasis and tissue repair (Ruoslahti, 1988; Ruggeri, 1992). Finally the presence of proteases in the venom may play an indirect role in the activation of proteases released by patients which primarily appear to act on dermonecrosis, as is the case of neutrophil proteases (Smith and Micks, 1970; Futrell, 1992; Patel et al., 1994) or proteases of the complement system, which also have been shown to participate in dermonecrosis and hemolysis (Futrell, 1992; Tambourgi et al., 1995, 2000).

We cannot rule out the involvement of digestive proteases in the accidents caused by brown spiders as postulated by

Atkinson and Wright (1992) and Futrell (1992), but based on the above mentioned results, it is possible to assume that metalloproteases are self components of brown spider venoms.

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TOXICON

Oligosaccharide residues of *Loxosceles intermedia* (brown spider) venom proteins: dependence on glycosylation for dermonecrotic activity

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Abstract

Loxosceles spp. (brown spider) envenomation has been reported to provoke dermonecrosis and haemorrhage at the bite site (a hallmark of accidents) and, to a lesser extent, thrombocytopenia, hemolysis and disseminated intravascular coagulation in some cases. Using lectin-immunolabeling, lectin-affinity chromatography, glycosidase and proteinase K treatments we were able to identify several venom N-glycosylated proteins with high-mannose oligosaccharide structures, complex-type glycoconjugates such as fucosylated glycans, but no galactose or sialic acid residues as complex sugars or glycosaminoglycan residues. Working with enzymatically or chemically deglycosylated venom we found that platelet aggregation (thrombocytopenic activity) as well as the fibronectinolytic and fibrinogenolytic (haemorrhagic) effects of the venom were sugar-independent when compared to glycosylated venom. Nevertheless, zymograph analysis in co-polymerized gelatin gels showed that enzymatic N-deglycosylation of loxolysin-B, a high-mannose 32–35 kDa glycoprotein of the venom with gelatinolytic metalloproteinase activity, caused a reduction of approximately 2 kDa in its molecular weight and a reduction of the gelatinolytic effect to a residual activity of 28% when compared to the glycosylated

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molecule, indicating a post-translational glycosylation-dependent gelatinolytic effect. Analysis of the dermonecrotic effect of the chemically or enzymatically N-deglycosylated venom detected only residual activity when compared with the glycosylated control. Thus, the present report suggests that oligosaccharide moieties play a role in the destructive effects of brown spider venom and opens the possibility for a carbohydrate-based therapy.

1. Introduction

The brown spider, genus *Loxosceles*, is becoming of great medical importance with accidental envenomation (Loxoscelism) disseminated in all American countries (Dillaha et al., 1964; Futrell, 1992). Preliminary studies have shown that the venom contain several enzymes which are related to its deleterious effects. A sphingomyelinase of 32 kDa, apparently involved in platelet aggregation, has been described (Kurpiewski et al., 1981). In addition, the venom contains other haemorrhagic factors, a metalloproteinase of 20–28 kDa (loxolysin A) with fibronectinolytic and fibrinogenolytic effects (Feitosa et al., 1998), probably involved in the haemorrhage observed locally at the envenomation site and, in some instances, in systemic haemorrhage and disseminated intravascular coagulation; a gelatinolytic metalloproteinase of 32–35 kDa (loxolysin B) probably associated with the dermonecrotic activity of the venom (Feitosa et al., 1998), and other molecules of 33, 34 and 35 kDa also associated with noxious activities of the venom (Geren et al., 1976; Barbaro et al., 1992; Tambourgi et al., 1995).

Little is known about the glycosylation-enzyme activities of venoms. Several snake metalloproteinases contain potential N-linked glycosylation sequences. That is true for H2-proteinase, a non-haemorrhagic protein from the venom of *Trimmeresurus flavoviridis* (Takeya et al., 1989); for high molecular mass haemorrhagic protein HR1B from the venom of *Trimmeresurus flavoviridis* (Takeya et al., 1990); for LHFII, a protease isolated from the venom of *Lachesis muta muta* (Sanchez et al., 1991); for Jararhagin, a high molecular weight haemorrhagic protease from *Bothrops jararaca* venom (Paine et al., 1992); for low molecular weight haemorrhagic protease HR2a from the venom of *Trimmeresurus flavoviridis* (Miyata et al., 1989); for atrolysin A (Ht-a), a haemorrhagic protease isolated from *Crotalus atrox* venom (Hite et al., 1994), and for rhodostomin, a protease isolated from the venom of *Calloscelasma rhodostoma* (Au et al., 1991). Among the several metalloproteinases that potentially have N-linked glycosylation sequences, at the present time only HR1B has been shown to contain two sugar chains at positions Asn^{73} and Asn^{181} (Takeya et al., 1990) and atrolysin A (Ht-a) sugar residues such as fucose, glucosamine, galactose, mannose and sialic acid at positions Asn^{517} and Asn^{533} (Hite et al., 1994; Fox and Bjarnason, 1995). Despite being potentially N-linked glycosylated, the functions and structures of the glycoconjugates of these venom metalloproteinases are currently unknown. With

respect to other proteases in snake venoms, we may mention the Factor-X-activating protein from Russell's viper venom (RVV-X), a glycoprotein with six N-linked oligosaccharides such as high-mannose and complex bi-, tri- and tetra-antennary structures necessary both for the maintenance of polypeptide structure and for the ability to activate Factor-X (Gowda et al., 1994; Gowda et al., 1996). Batroxobin, a thrombin-like serine protease from *Bothrops moojeni*, is heterogeneously glycosylated with biantennary partially incomplete complex-type glycans and hybrid-type structures of unknown functions (Lochnit and Geyer, 1995) and the coagulant thrombin-like enzyme, bilineobin from the venom of *Agkistrodon bilineatus* that contains six N-linked glycosylation consensus sites and linked fucose, N-acetylglucosamine, galactose, mannose and N-acetylneuraminic acid oligosaccharides, with the sugar moieties in some way regulating the activity of bilineobin, since the deglycosylated enzyme generates more rapidly fibrinopeptide A than native bilineobin (Nikai et al., 1995). With respect to spider venoms and despite some similarities to snake venoms in their activities, nothing is known about the sugar chemistry or glycobiology of proteins present in the venoms.

We have examined the oligosaccharide profile and associated functions of *L. intermedia* (brown spider) venom proteins. Using lectin-blotting assays, lectin-affinity chromatography, protease and glycosidase treatments we were able to detect asparagine-linked high-mannose, complex-type structures as N-linked fucosylated molecules, one serine/threonine-linked N-acetyl-galactosilated protein, but the absence of linked glycosaminoglycan and galactose or sialic acid residues as complex structures. The platelet aggregation ability, fibronectinolytic and fibrinogenolytic activities of venom (involved in the haemorrhagic effect) were independent of sugar residues, but the gelatinolytic and remarkable dermonecrotic actions triggered by the venom were sugar-dependent.

2. Materials and methods

2.1. Reagents

Human fibronectin was purified from fresh plasma (obtained from Hospital A.C. Camargo, São Paulo, Brazil) by gelatin-affinity chromatography (Sigma, St. Louis, USA) as described (Engvall and Ruoslahti, 1977). Human fibrinogen was purchased from Sigma and gelatin was purchased from Gibco (Madison, USA). Peptide-N^4-(N-acetyl-β-glycosaminyl) asparagine amidase F (N-glycosidase F) from *Flavobacterium meningosepticum*, O-glycopeptide endo-D-galactosyl-N-acetyl-α-galactosamino hydrolase (O-glycosidase) from *Diplococcus pnemoniae*, sialidase from *Arthrobacter ureafaciens*, a glycan differentiation kit and other digoxigenin-labeled lectins were purchased from Boehringer-Mannhein Biochemica (Mannhein, Germany). Biotin-labeled *Pisum sativum* lectin was purchased from Sigma. Concanavalin-A-sepharose was purchased from Pharmacia LKB-Biotechnology

(Uppsala, Sweden). Nitrocellulose membranes were from Bio-Rad (Hercules, CA, USA).

2.2. Venom preparation

L. intermedia venom (a pool from approximately 500 spiders) was obtained from spiders grown under appropriate conditions and submitted to an electric shock of 15 V in the cephalothorax as described by Feitosa et al. (1998). In the platelet aggregation assay, zymogram experiments and dermonecrosis studies, the venom concentration used of 40 μg represents the average venom concentration injected in a spider bite.

2.3. Analytical gel electrophoresis

Analytical discontinuous electrophoresis was performed with 5% (w/v), 12% (w/v) or continuous gradient 3–20% (w/v) polyacrylamide gels under non-reducing or reducing conditions (depicted in legends) as described (Laemmli, 1970). For protein detection, gels were stained with Coomassie Blue. For Western-blotting, proteins were transferred overnight to nitrocellulose filters as described (Towbin et al., 1979). Molecular mass markers used were myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase-b, 98 kDa; serum albumin, 67 kDa; ovalbumin, 44 kDa; carbonic anydrase, 29 kDa purchased from Sigma.

2.4. Lectin-blotting reactions

Venom proteins (5 μg) were either directly dot-blotted or electroblotted onto nitrocellulose membranes as described above. Detection of glycoproteins was performed using the glycan differentiation kit or other digoxigenin-labeled lectins (Boehringer Mannhein) according to manufacturer's instructions. Reactions were developed as previously described (Veiga et al., 1995).

2.5. Deglycosylation of venom proteins

L. intermedia venom proteins (1 mg/ml in 100 mM sodium phosphate buffer, pH 7.3, 0.1% (w/v) SDS and 1% (v/v) Triton X-100) were enzymatic digested by treatment with peptide-N^4-(N-acetyl-β-glycosaminyl) asparagine amidase F (N-glycosidase) (10 units) at 37°C for 48 h. Non-deglycosylated venom used as control in experiments performed to study the biological activity of N-linked oligosaccharides was treated identically as above, but in absence of N-glycosidase. Treatment for removal of O-linked sugars was performed by sequential incubation of *L. intermedia* venom (1 mg/ml in 100 mM sodium phosphate buffer, pH 7.3, 0.1% (w/v) SDS and 1% (v/v) Triton X-100) with neuraminidase (20 mUnits) at 37°C for 2 h. and O-glycosidase (25 mUnits) at 37°C overnight. Alternatively, for

sugar moiety oxidation, Con-A-sepharose eluted material (1 mg/ml in PBS) was incubated with 5 mM sodium metaperiodate at 37°C in the dark for 15 min.

2.6. Glycosaminoglycan chain evaluation

Venom core proteins (3 mg) were digested using excess proteinase K (50 μg; Sigma) at 58°C overnight. The products obtained were analyzed by agarose gel electrophoresis in 50 mM 1,3-diaminopropane acetate buffer, pH 9.0 (Aldrich, Milwaukee, USA). After the electrophoretic run, compounds were precipitated in the gel using 0.1% cetavlon for 2 h at room temperature. After drying, the gel was stained with toluidine blue (Dietrich and Dietrich, 1976). The glycosaminoglycan chain standards used were chondroitin sulfate (CS) from shark cartilage, dermatan sulfate (DS) from pig skin and heparan sulfate (HS) from bovine pancreas.

2.7. Lectin affinity chromatography

For Con-A-lectin affinity chromatography, *L. intermedia* venom proteins (5 mg) diluted in starting buffer (20 mM Tris–HCl, pH 7.3, 500 mM NaCl, 1 mM $MnCl_2$ and 1 mM $CaCl_2$) were applied to a Con-A-sepharose column (Pharmacia) and incubated overnight at 4°C. After incubation, the column was washed with starting buffer and eluted with 200 mM α-D-methyl-mannoside (Sigma) in starting buffer.

2.8. Platelet aggregation assay

Human platelet-rich plasma was obtained by differential centrifugation from fresh human blood drawn into acid-citrate-dextrose. Platelet aggregation in the presence of venom before or after enzymatic N-deglycosylation (40 μg) was recorded at 37°C at a stirring rate of 1000 rpm using a Becton-Dickinson aggregometer as described (Plow et al., 1985).

2.9. Fibronectin- and fibrinogen-degrading studies

Human plasma fibronectin or fibrinogen (0.5 mg/ml in 50 mM Tris–HCl buffer, pH 7.3, plus 5 mM $CaCl_2$ and 5 mM $MgCl_2$) were incubated with enzymatic N-deglycosylated venom (obtained as described above), or glycosylated venom (control) processed as described above, at a substrate:venom ratio of 100:1, at 37°C overnight as previously described (Feitosa et al., 1998). Digested materials and controls were analyzed by 5% SDS-PAGE under non-reducing conditions for fibrinogen and under reducing conditions for fibronectin and stained with Coomassie Blue dye.

2.10. Zymogram experiments

Zymography was performed by 12% SDS-PAGE, containing polymerized 3 mg/ml of porcine skin gelatin. Samples (Con-A-sepharose eluted materials from *L. intermedia* venom; 40 μg) submitted to enzymatic deglycosylation and controls, as described above, were diluted in Laemmli buffer under non-reducing conditions and electrophoresed (20 mA) at 4°C. After the run, gels were washed twice in 2.5% (v/v) Triton X-100 for 20 min each and then incubated overnight at 37°C in 50 mM Tris–HCl buffer, pH 7.3, containing 200 mM NaCl, 5 mM $CaCl_2$ and 5 mM $MgCl_2$ and stained with Coomassie Blue. Clear zones of substrate lysis against a blue background stain indicated the presence of degrading enzyme (Feitosa et al., 1998).

2.11. Dermonecrosis studies

For the evaluation of dermonecrotic effects, enzymatically deglycosylated venom or chemically oxidated sugar moieties of venom proteins (samples of 40 μg diluted in PBS), obtained as described above, were injected intradermally into a shaved area of rabbit skin. As control, glycosylated venom processed identically as for deglycosylation, but in the absence of deglycosylating agents, was used at the same concentration (40 μg diluted in PBS). The dermonecrotic lesion was checked 3 h, and 1, 5 and 10 d after injection.

3. Results

3.1. Analysis of lectin glycosidase-based oligosaccharide residues of L. intermedia Venom proteins

There are no previous reports studying the structure or the biological role of oligosaccharide residues in *Loxosceles* spp. venom proteins. Initially, lectin binding and glycosidase treatments were performed in order to evaluate the existence and determine the type and importance of glycosylation in *L. intermedia* venom proteins. Crude venom proteins were directly dot-blotted (Fig. 1A) or submitted to a linear gradient SDS-PAGE, electroblotted onto nitrocellulose membranes before (Fig. 1B) or after sequential treatments with N-glycosidase, or neuraminidase and O-glycosidase (Fig. 1C), and further reacted with lectins that recognize specific sugars. Directly spotted venom was positive for *Galanthus nivalis* agglutinin (GNA) and *Canavalia ensiformis* (Con-A), suggesting the presence of high-mannose glycosylated proteins. The venom was also positive for *Pisum sativum* agglutinin (PSA) and Wheat Germ agglutinin (WGA), revealing proteins containing N-acetylglucosamine residues, and to *Aleuria aurantia* agglutinin (AAA), indicating proteins with fucosylated glycans. Reactions were negative for *Datura stramonium* agglutinin (DSA) and *Phaseolus vulgaris* agglutinin (PHA-L), demonstrating the absence of terminal β1-4 galactosyl residues or polylactosamine

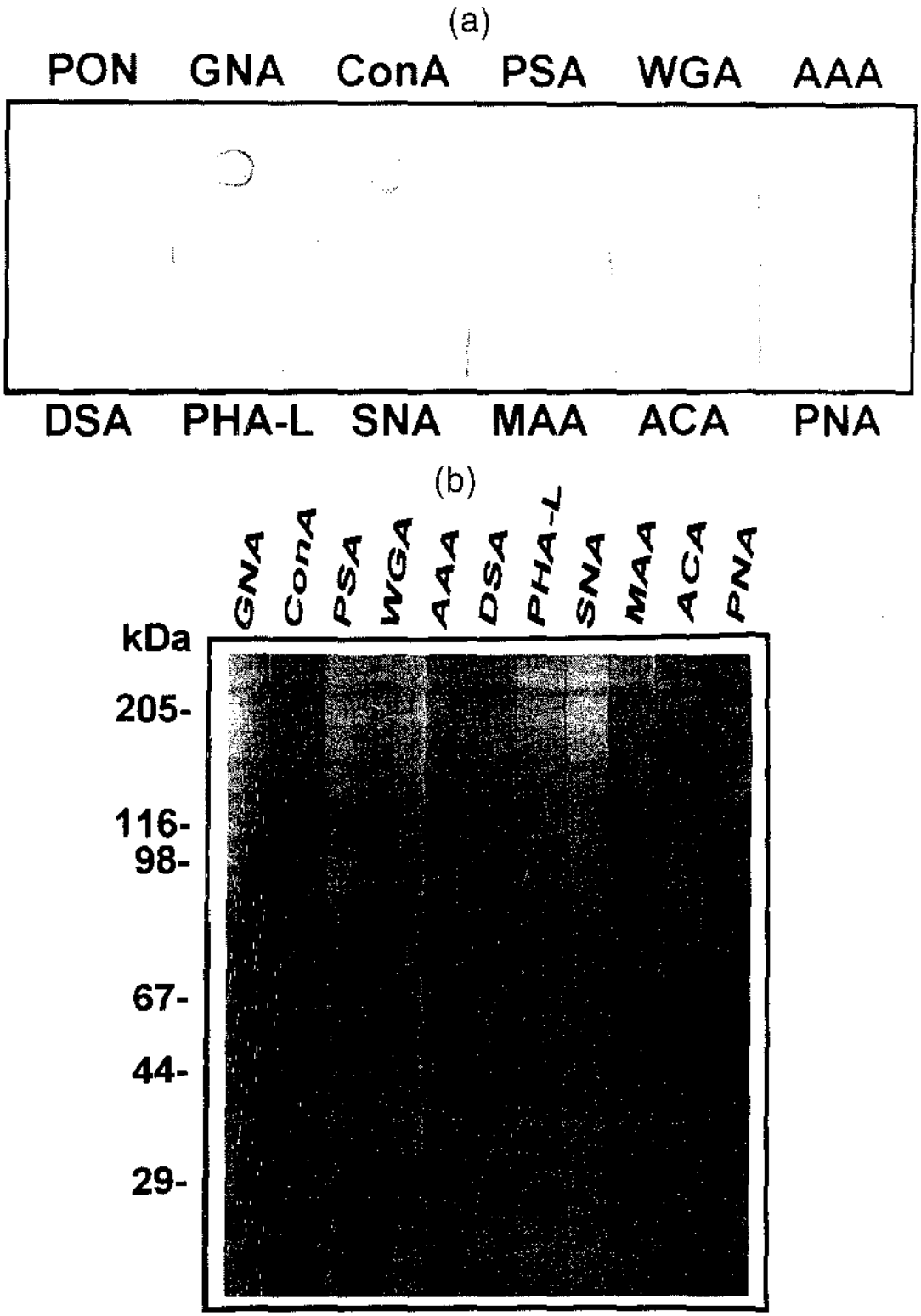

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Fig. 1. Lectin-affinity blot analysis of *L. intermedia* venom proteins. Venom (5 μg per spot or lane) was directly spotted (A) or electroblotted after a linear gradient 3–20% (w/v) SDS-PAGE under non-reducing conditions onto nitrocellulose membranes before (B) or after (C) glycosidase treatment and blotted with digoxigenin-labeled or biotin-labeled lectins. Lanes represent reactions with GNA, Con-A, PSA, WGA, AAA, DSA, PHA, SNA, MAA, ACA and PNA. Molecular mass markers are shown on the left. (D) *L. intermedia* crude venom (3 mg) was digested with proteinase-K. The materials obtained were submitted to agarose gel electrophoresis in 50 mM 1,3 diaminopropane acetate buffer, pH 9.0. Putative GAGs were precipitated in the gel with 0.1% cetavlon and stained with toluidine blue. Lane 1: glycosaminoglycan standards CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; lane 2: digested venom material.

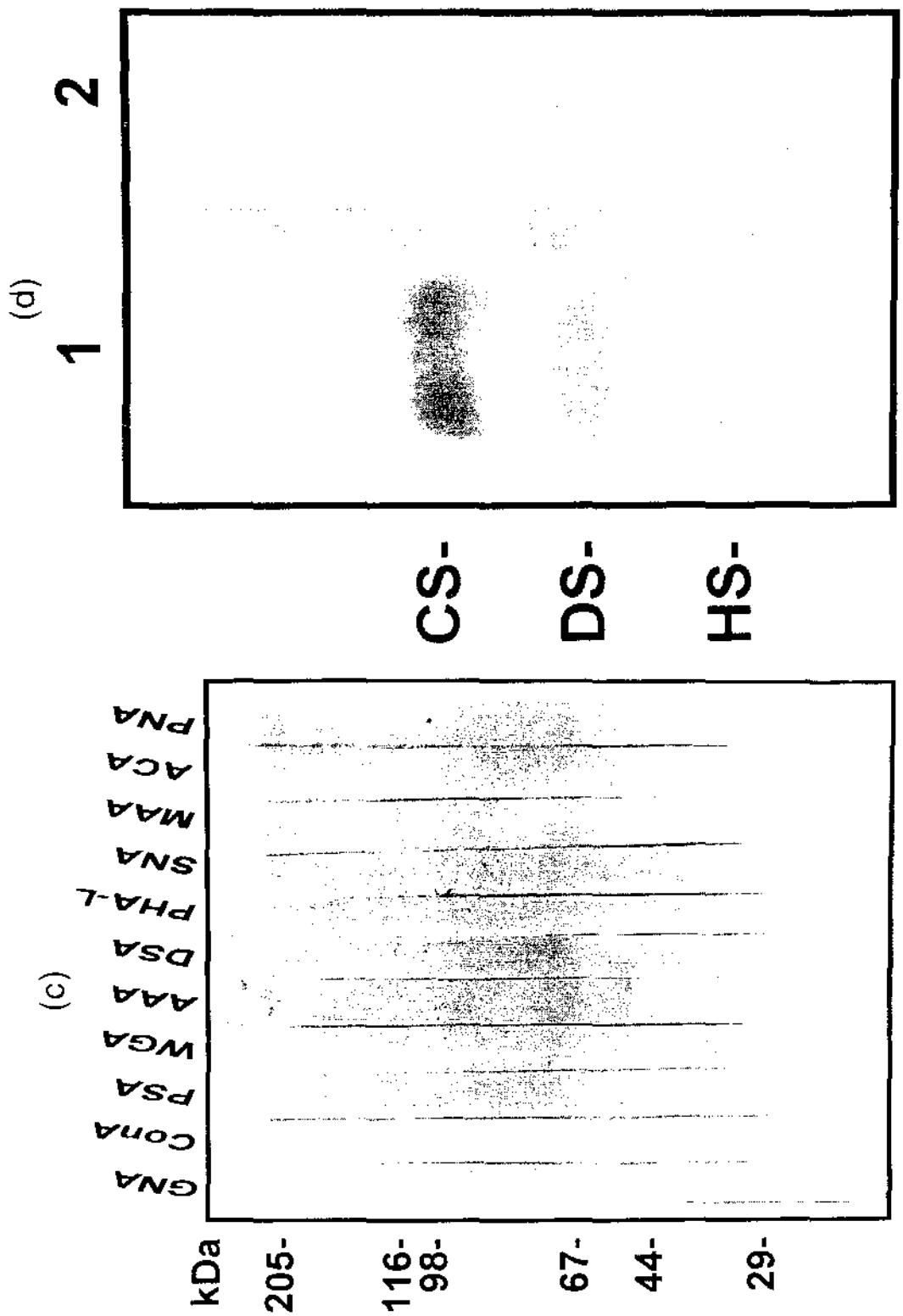

Fig. 1 (*continued*)

glycans and were also negative for *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA), showing the absence of N-acetylneuraminic acid (sialic acid) residues in venom proteins. Furthermore, venom proteins were also positive for *Amaranthus caudatus* agglutinin (ACA) and Peanut agglutinin (PNA), demonstrating the presence of glycosylated proteins with galactose β1-3-N-acetylgalactosamine residues, typically Ser/Thr-linked oligosaccharides. This carbohydrate composition analysis was further confirmed by lectin affinity Western-blotting (Fig. 1B, lanes 1 and 2) in which we were able to detect bands of 98, 69, 67, 60, 35, 28, 20 kDa that were recognized by GNA and Con-A before N-glycosidase treatment but not after digestion (Fig. 1C, lanes 1 and 2), demonstrating the presence of N-linked high-mannose structures in these venom proteins. We could also observe that there were proteins of 98, 28 and 20 kDa positive for PSA and WGA before N-glycosidase digestion (Fig. 1B, lanes 3 and 4) but negative after enzyme treatment (Fig. 1C, lanes 3 and 4), corroborating a preliminary lectin-spot assay and showing N-linked N-acetylglucosamine residues. Fig. 1B and C, lanes 5, show that the venom reacted with *Aleuria aurantia* agglutinin (AAA) before, but not after treatment with N-glycosidase, respectively. Two proteins in the region of 28 and 20 kDa were positive for N-linked fucose residues. Fig. 1B and C in lanes 6, 7, 8 and 9 respectively represent venom proteins before (1B) and after (1C) N-glycosidase digestion, reacted with *Datura stramonium* agglutinin (DSA), *Phaseolus vulgaris* agglutinin (PHA-L), *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA). These results demonstrate the absence of N-linked Gal(β1–4)GlcNac, polylactosamine sugar residues, NeuNac(2–3)Gal or NeuNac(2–6)Gal oligosaccharides. Fig. 1B and C (lanes 10 and 11) show that the venom reacted with *Amaranthus caudatus* agglutinin (ACA) and Peanut agglutinin (PNA) before (1B) but not after (1C) treatment with neuraminidase and O-glycosidase. The figure shows the presence of a protein of 67 kDa bearing O-linked Galβ(1–3)GalNac residues (see Table 1 for a summary of lectin-based carbohydrate structures).

In addition, to improve the oligosaccharide profile of *L. intermedia* venom proteins we searched for the presence of proteoglycan molecules (proteoglycans represent the best characterized sulfated molecules containing serine-linked glycosaminoglycan sugar chains in the protein core) (Kjellén and Lindahl, 1991; Hardingham and Fosang, 1992). The crude venom (3 mg of protein) was subjected to digestion with proteinase K (a serine protease of broad specificity), a procedure that cleaves GAG chains from the protein core. Digested material was applied to agarose gel as previously described (Dietrich and Dietrich, 1976) using glycosaminoglycan standards as reference. As depicted in Fig. 1D, the agarose gel electrophoretic pattern of venom showed no signal of GAG chains, demonstrating the absence of these sugars linked to proteins in the spider venom.

Table 1
Lectin based oligosaccharide structures of *Loxosceles intermedia* venom proteins

Lectin Agglutim from	Speficitity	Reactivity	*L. intermedia* venom proteins detected
Galanthus nivalis (GNA)	Manα(1–3)Man > Manα(1–6)Man > Manα(1–2)Man >	positive	20 kDa, 28 kDa, 35 kDa, 60 kDa, 67 kDa, 98 kDa
Canavalia ensiformis (ConA)	αMan > Glc > αGlcNAc	positive	20 kDa, 28 kDa, 32 kDa, 35 kDa, 69 kDa, 98 kDa
Pisum sativum (PSA)	βGlcNAc	positive	20 kDa, 28 kDa, 98 kDa
Triticum vulgaris (WGA)	$(GlcNAc\beta 1\text{–}4)_3 > (GlcNAc\beta 1\text{–}4)_2$	positive	20 kDa, 28 kDa, 98 kDa
Aleuria aurantia (AAA)	L-Fucα(1–6)GlcNAc	positive	20 kDa, 28 kDa
Datura stramonium (DSA)	Galβ(1–4)GlcNAc GlcNAc-Ser/Thr	negative	not detected
Phaseolus vulgaris (PHA-L)	β(1–6) lactosamin	negative	not detected
Sambucus nigra (SNA)	NeuNAcα(2–6)Gal GalNAc	negative	not detected
Maackia amurensis (MAA)	NeuNAcα(2–3)Gal	negative	not detected
Amaranthus caudatus (ACA)	NeuNAc-Galβ(1–3)GalNAc-Ser/Thr Galβ(1–3)GalNAc-Ser Thr	positive	67 kDa
Peanut agglutinin (PNA)	Galβ(1–3)GalNAc	positive	67 kDa

3.2. Analysis of the platelet aggregation activity of N-glycosidase-treated venom

As stated in the Introduction, a systemic effect produced by *Loxosceles* spp. bite is thrombocytopenia triggered by low molecular weight platelet aggregating venom proteins (Kurpiewski et al., 1981). In order to verify the involvement of sugar residues present in venom glycoproteins in platelet aggregation, using platelet-rich plasma we performed an in vitro platelet aggregation assay in the presence of *L. intermedia* venom before and after N-glycosidase treatment. In order to confirm the effectiveness of N-glycosidase treatment, venom proteins were blotted using lectins GNA and AAA before and after enzyme treatment (see Fig. 2A). As shown in Fig. 2B, there was no significant difference in platelet aggregation triggered by glycosylated or deglycosylated venom proteins, strongly suggesting that N-linked sugar moieties are not involved in the thrombocytopenic activity of the venom.

3.3. Analysis of glycoconjugates of L. intermedia venom proteins in the fibronectinolytic and fibrinogenolytic activities

As mentioned in the Introduction, we have previously identified in *L. intermedia* venom a 20–28 kDa metalloproteinase with fibronectinolytic and fibrinogenolytic

activities that we termed Loxolysin A (Feitosa et al., 1998). As a function of its proteolytic abilities, Loxolysin A probably is implicated in the haemorrhagic property of the venom. Since we were able to detect glycosylation as N-linked high-mannose, N-acetylglucosamine and fucose residues in this region of the protein profile in *L. intermedia* venom (see Fig. 1B and C) we decided to further characterize the importance of these N-linked sugar residues as catalytic essentials

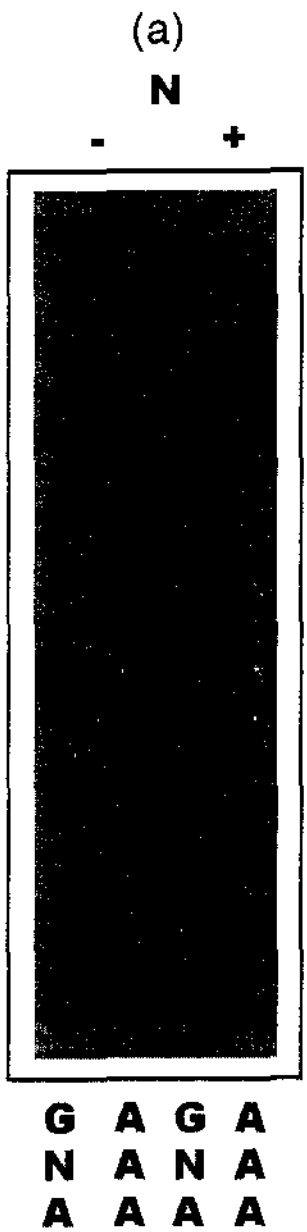

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Fig. 2. Platelet aggregation induced by *L. intermedia* venom. (A) To determine the effectiveness of N-glycosidase treatment, venom proteins before (−) or after N-glycosidase treatment (+) were blotted using GNA and AAA lectins. (B) Platelet-rich plasma was incubated with glycosylated venom in A, or N-glycosidase-treated venom in B (at the same concentration of 40 μg) or with the buffer used in the deglycosylation procedure (negative control) in C. The abscissa represents the time in seconds and the ordinate the percentage of platelet aggregation.

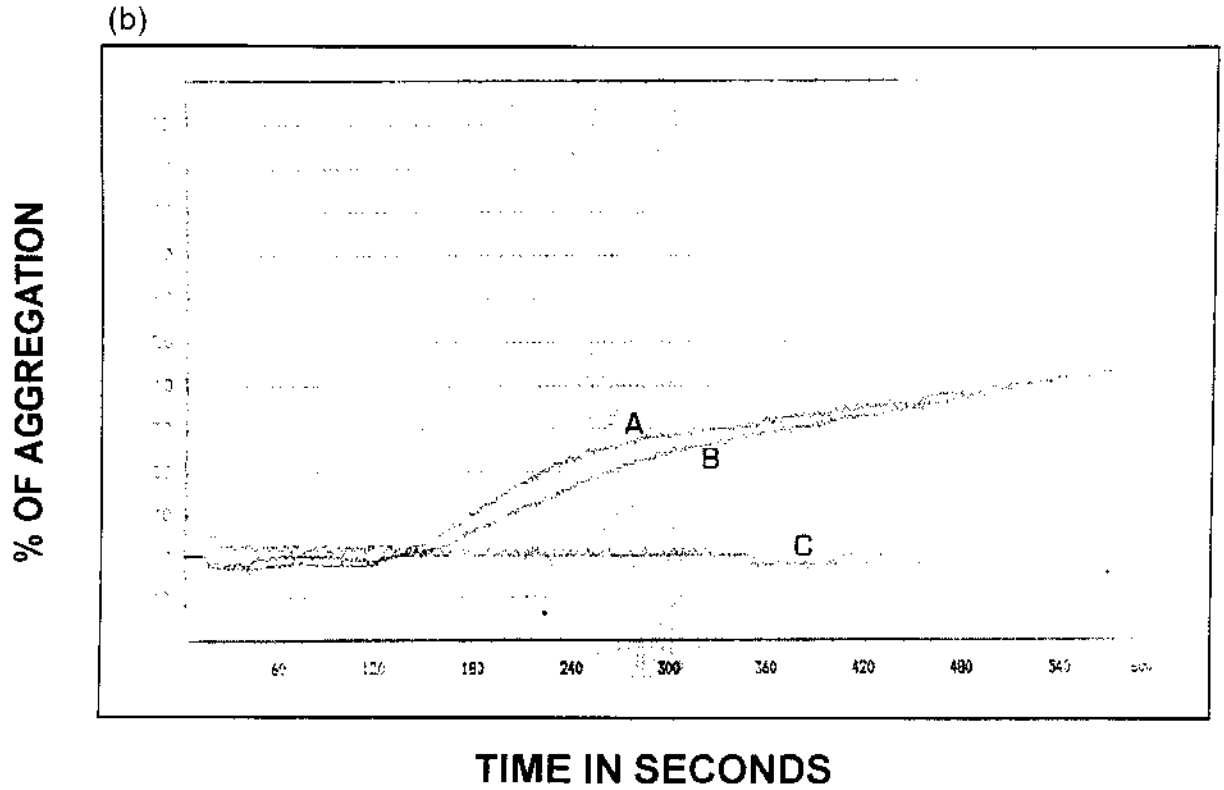

Fig. 2 (*continued*)

in these hydrolytic properties. Fig. 3A and B revealed that although some heterogeneity were obtained in fragments of fibrinogen and fibronectin degraded by glycosylated and deglycosylated venoms, neither fibrinogenolytic nor fibronectinolytic effects of venom were blocked after deglycosylation procedures, demonstrating that oligosaccharides are not involved in this degrading activity.

3.4. Glycoconjugates play a role in the gelatinolytic effect of L. intermedia venom

We have previously detected in *L. intermedia* venom a gelatinolytic metalloproteinase of 32–35 kDa named by us Loxolysin B (Feitosa et al., 1998) by a procedure identical to that described above. As depicted in Fig. 1B and C, we were able to detect N-linked high-mannose structures present in this region of *L. intermedia* venom proteins. Confirmation of the 32–35 kDa protein (Loxolysin B) as a high-mannose glycoprotein was provided by its elution using 200 mM α-D-methyl-mannoside solution from a Con-A-Sepharose affinity chromatography column and by a subsequent zymogram using gelatin as substrate (Fig. 4A). After confirming that Loxolysin B is a glycoprotein, we determined if its N-linked sugar moieties were important for its gelatinolytic activity. Fig. 4B illustrates the results of the zymograph using gelatin as a substrate of Con-A-Sepharose-eluted proteins from *L. intermedia* venom, before and after N-glycosidase treatment. We can see

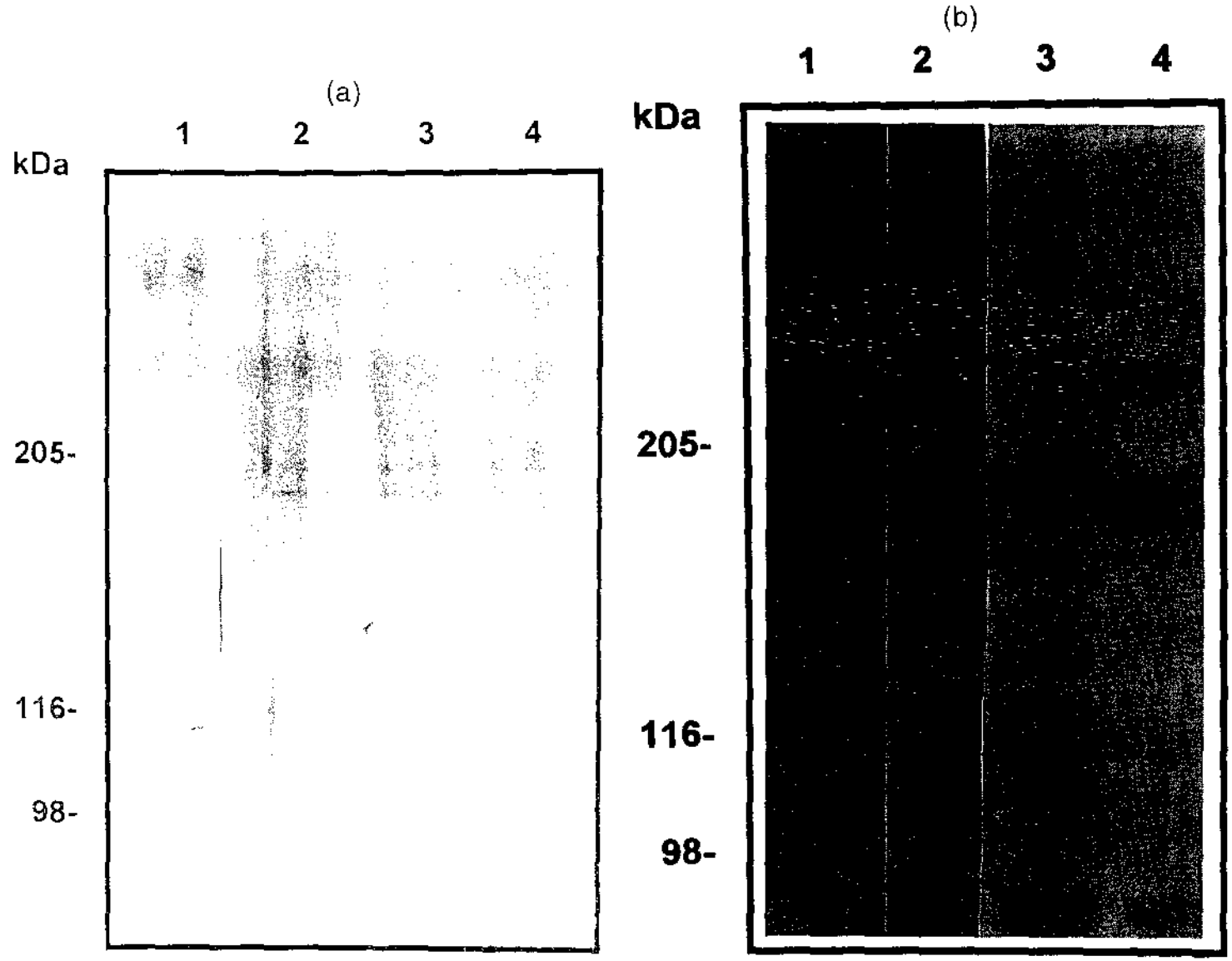

Fig. 3. SDS-PAGE profile of digested fibrinogen and fibronectin induced by *L. intermedia* venom. Purified human fibrinogen (A) or human fibronectin (B) was incubated overnight at 37 C with glycosylated venom (lanes 2), enzymatic N-deglycosylated venom (lanes 3) or metaperiodate-treated venom (lanes 4) at a 100:1 substrate:venom ratio. Lanes 1 represent substrates incubated under the same conditions of the experiment in the absence of venom (control for experimental stability). The materials used in the experiments were submitted to 5% (w/v) SDS-PAGE under non-reducing conditions (fibrinogen-3A) or reducing conditions (fibronectin-3B). Molecular weight markers are shown on the left.

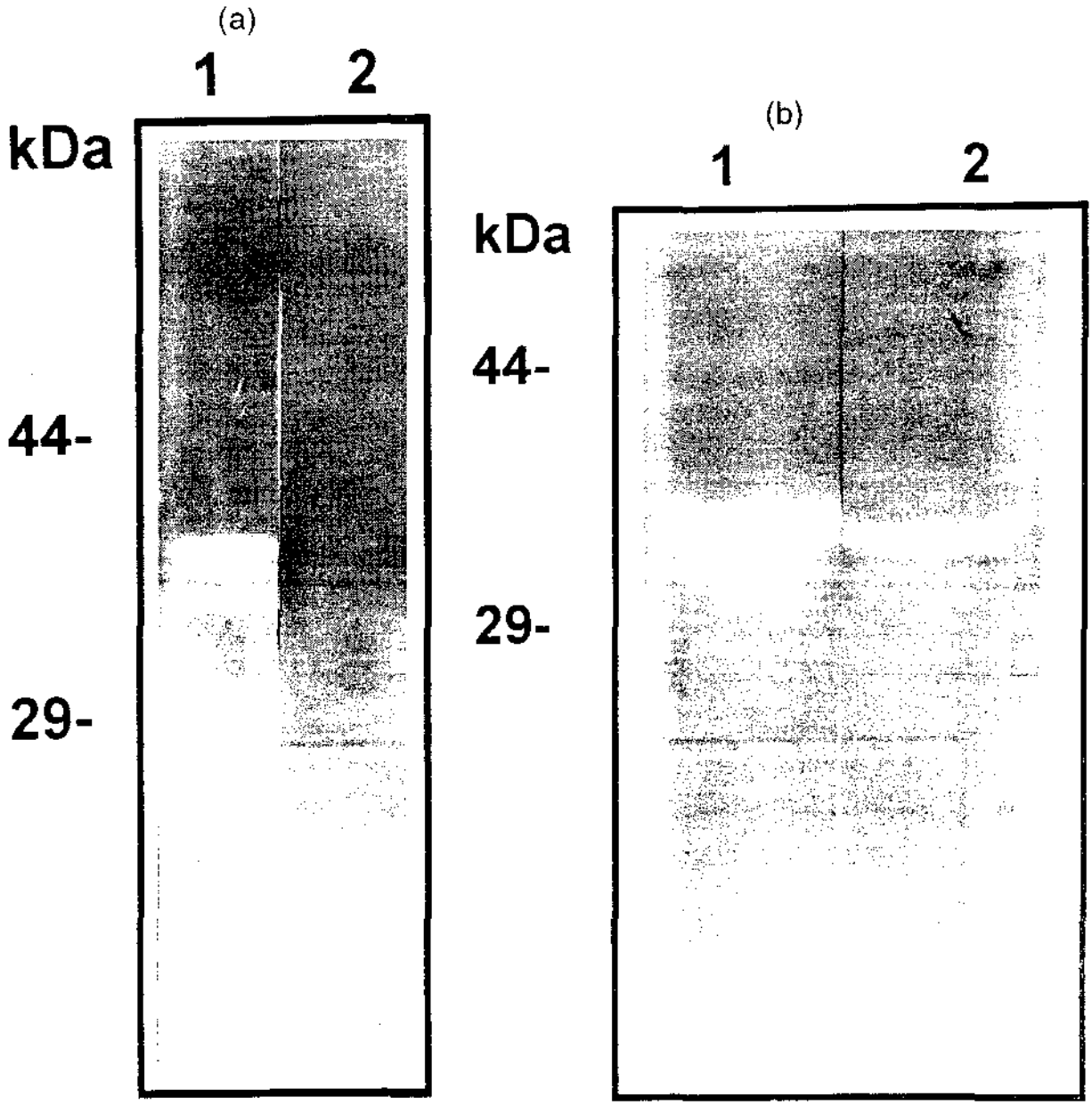

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Fig. 4. Sugar residue analysis of the gelatinolyite effect of *L. intermedia* venom. (A) *L. intermedia* crude venom was chromatographed on a Con-A Sepharose column. The D-methylmannoside-eluted material (lane 1) and flow-through proteins (lane 2) were submitted to a gelatin polymerized zymogram in 12% (w v) SDS-PAGE. Molecular mass markers are shown on the left. (B) Con-A Sepharose-eluted materials from venom were digested with N-glycosidase and submitted to a gelatin polymerized zymogram by 12% SDS-PAGE. Lane 1 represents glycosylated proteins before enzyme treatment and lane 2 after digestion. (C) Lysed gelatin zones from the zymographs in experiments shown in (B) had their optical density checked and graded (area). Gelatinolytic activity is reported as percentage of glycosylated control (100%) and shows residual gelatinolytic activity (28%) of the deglycosylated sample. Molecular mass markers are shown on the left.

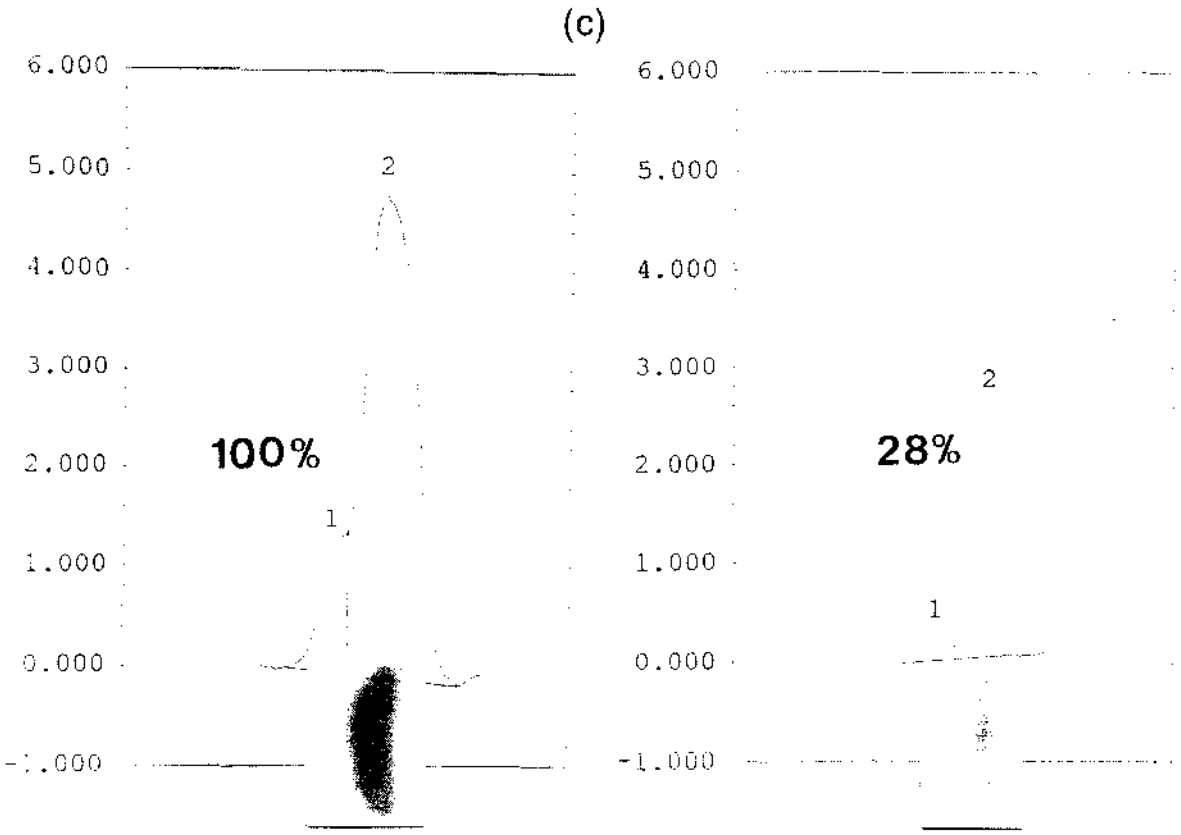

Fig. 4 (*continued*)

that after N-linked sugar removal there was a shift in SDS-PAGE mobility of approximately 2 kDa (Fig. 4B), and a strong decrease in gelatinolytic effect (28% residual activity compared with the glycosylated control) (Fig. 4C), suggesting some oligosaccharide involvement in this proteolytic activity. These data further suggest that in some way oligosaccharides do participate in the catalytic activity of *L. intermedia* metalloproteinase of 32–35 kDa in degrading gelatin.

3.5. Evidence for the role of glycosylation in the dermonecrotic lesions produced by the venom

As discussed above, a hallmark of *L. intermedia* envenoming is a dermonecrotic lesion at the bite site. Since the gelatinolytic ability of Loxolysin B is closely related to that of vertebrate gelatinases, metalloproteinases involved in normal or pathological conditions (Birkedal-Hansen et al., 1993) or reprolysins that are snake venom metalloproteinases involved in the necrotic effects produced by envenomation (Bjarnason and Fox, 1995), we postulated that Loxolysin B to some extent participates in the dermonecrotic effect of *L. intermedia* venom (Feitosa et

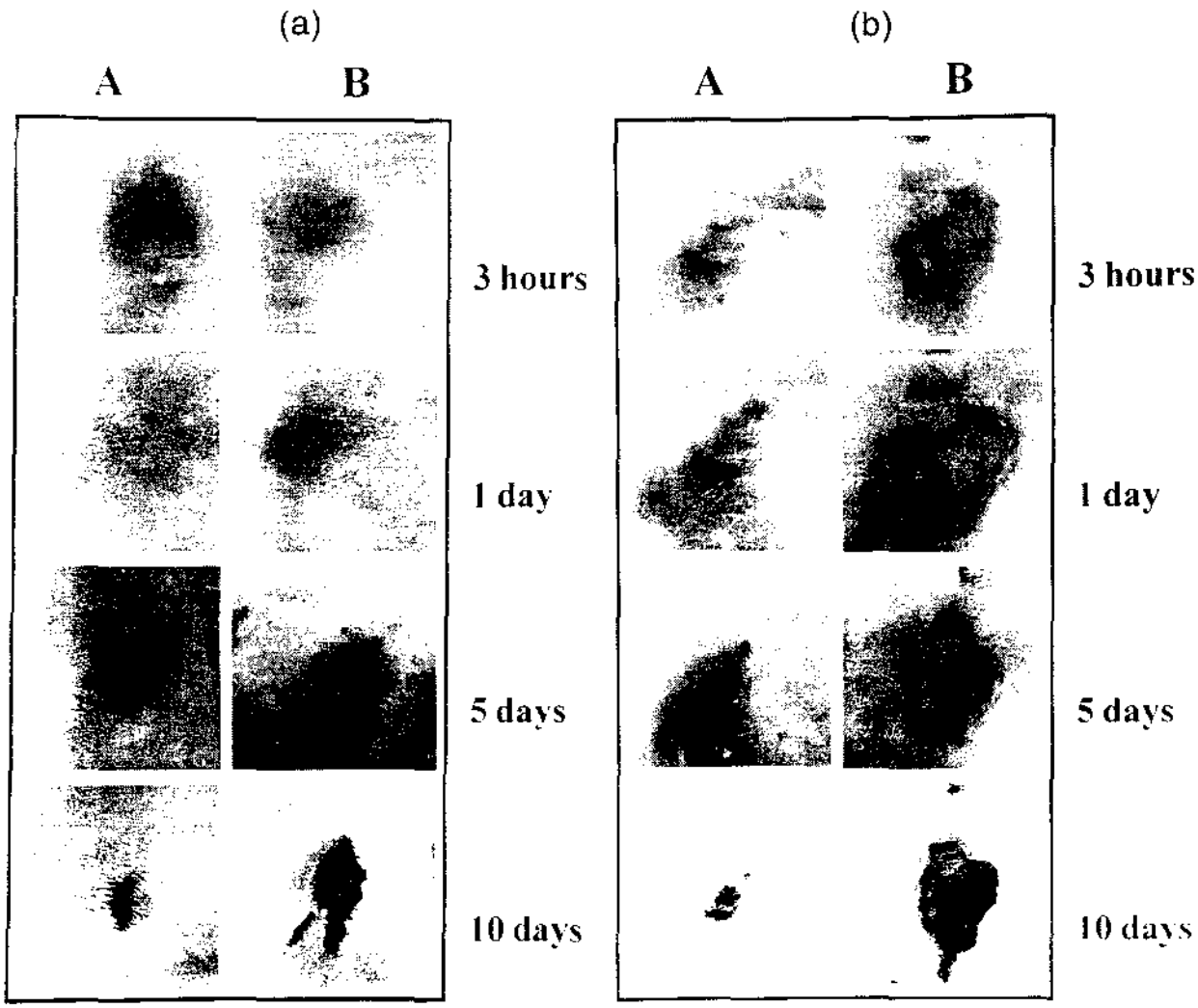

Fig. 5. Dermonecrotic lesion induced in rabbits by *L. intermedia* venom. Rabbits were intradermally injected with 40 μg crude venom (panel A) or 40 μg enzymatic N-deglycosylated venom (panel B). Reactions were observed for 3 h, 1, 5 and 10 d post injection (A). The same experiment was repeated with 40 μg of glycosylated venom (lanes A) or venom chemically treated with 5 mM sodium metaperiodate venom at 37 C in the dark for 15 min (lanes B). Lesions were evaluated 3 h, 1, 5 and 10 d after the injections (B). It is interesting to observe that, although dermonecrotic lesions (scar plaques) strongly decreased after the deglycosylation procedures, local edema, haemolysis and haemorrhage seem to be sugar-independent, in agreement with the previous results shown above.

al., 1998). Once the involvement of sugars in the in vitro gelatinolytic effect of Loxolysin B was detected, in order to determine if glycoconjugates are involved in the in vivo dermonecrotic effect, *L. intermedia* venom was deglycosylated using N-glycosidase (Fig. 5A) or had its sugar residues oxidized by sodium metaperiodate (Fig. 5B) and was intradermically injected into rabbits using glycosylated venom as a control. As shown, both enzymatic deglycosylated and chemically treated samples strongly decreased the dermonecrotic effect of venom, demonstrating sugar involvement in this noxious skin lesion.

4. Discussion

Protein glycosylation is the most abundant and diverse post-translational modification (Kornfeld and Kornfeld, 1985; Hart, 1992). The functions of protein-linked glycoconjugates in some instances largely remain enigmatic. In some cases, since protein glycosylation has a pronounced influence on protein conformation, sugar moieties are very important in molecular folding and appear to participate in intermolecular interactions, playing a central role in a range of biological activities. (Elbein, 1991; Hart, 1992; Veiga et al., 1996; Veiga et al., 1997). To date, little is known about glycosylation of the enzymes or other proteins in animal venoms.

Using lectin-blotting reactions, lectin affinity chromatography and glycosidase treatments we determined the oligosaccharide profile of *L. intermedia* venom proteins (Fig. 1A–D). It was possible to show that venom is particularly rich in proteins with N-linked high-mannose residues (positive for GNA and Con-A lectins), has three proteins in the region of 98, 28 and 20 kDa with N-linked N-acetylglucosamine (positive for PSA, WGA) and two proteins of 28 and 20 kDa with N-linked fucose moieties (positive for AAA lectin). In addition, our studies indicated that the venom has no complex Asn-linked sugars such as β1–4 galactosyl residues, polylactosamine branches or terminal sialic acid residues, but contains a protein of 67 kDa with Ser/Thr-linked Galβ(1–3)GalNac residues (positive for ACA and PNA lectins). We also did not detect the presence of glycosaminoglycan residues such as heparan-sulfate, dermatan-sulfate or chondroitin-sulfate oligosaccharides in proteinase-K-digested venom.

Since there are no previous reports on the glycobiology of spider venoms, we studied the possible involvement of oligosaccharides in the major loxoscelism effects such as thrombocytopenia, local and systemic haemorrhage and dermonecrotic lesions. Platelet aggregation and red blood cell lysis provoked by *Loxosceles* spp. appears to be related to a 32 kDa protein characterized as a sphingomyelinase D (Kurpiewski et al., 1981). As depicted in Fig. 2, using *L. intermedia* venom before and after enzymatic N-deglycosylation we detected no differences in platelet aggregation, demonstrating that carbohydrates are not involved in this biological activity of the venom.

Haemorrhagic consequences after *Loxosceles* spp. accidents appears to be molecularly more complex. Haemorrhage can be triggered directly by the thrombocytopenic effect as discussed above, or may be the consequence of deleterious effects of venom enzymes on the major plasma extracellular matrix molecules such as fibronectin and fibrinogen which are related to the blood clotting cascade (Feitosa et al., 1998), as demonstrated for haemorrhagic metalloproteinases from snake venoms (Baranova et al., 1989). As shown in Fig. 3A and B, our results demonstrate that even after enzyme N-deglycosylation or chemical sugar oxidation, the fibronectinolytic and fibrinogenolytic activities of venom essentially were the same as for glycosylated venom. It seems likely that the haemorrhagic properties of the venom are sugar independent since neither the

thrombocytopenic nor the fibronectinolytic or fibrinogenolytic effects of venom were changed after chemical or enzymatic deglycosylation.

In addition, we previously reported a gelatinolytic molecule of 32–35 kDa (Loxolysin B) in *L. intermedia* venom as shown in Fig. 4A (Feitosa et al., 1998). Loxolysin B is a high-mannose protein since it can be eluted using α-D-methyl-mannoside solution from a Con-A-Sepharose column (Fig. 4A). Enzymatic N-deglycosylation, in contrast to other venom properties, strongly reduced the gelatinolytic activity to a residual effect (Fig. 4B and C). An evaluation of the residual gelatinolytic effect compared to initial gelatinolytic activity (100% versus 28%) and changes in molecular weight (35 versus 33 kDa) indicate that this post-translational glycosylation is responsible for approximately 2 kDa in the size of Loxolysin B. These data, taken together, suggest that the gelatinolytic effect of Loxolysin B is sugar-dependent. These findings demonstrate that the activity of this spider venom metalloproteinase is modulated by carbohydrate moieties. Since many studies have shown that in some cases the volume (hydrodynamic volume) occupied by sugar chains is enormous (Perkins et al., 1988; Hart, 1992) we may postulate that Loxolysin B glycosylation is essential for protein conformation and folding, as previously described for other glyconconjugates (Elbein, 1991; Hart, 1992). Since venom is secreted in a water environment, processing Loxolysin B with oligosaccharide residues (as a function of hydrophilic balance) increases exposure of the enzyme binding site to its respective ligands or adds stability through secondary interactions between enzyme and ligands, as previously demonstrated in other models (Takeuchi and Kobata, 1991; Howard et al., 1991; Danielsen, 1992; Veiga et al., 1995). Although the function of Loxolysin B is not yet known, its gelatinolytic effect strongly indicates that this molecule is a possible target that could form part of the dermonecrotic components present in *Loxosceles* spp. venom. A protein of 34 kDa involved in the necrotic effect after envenomation was identified in *L. reclusa* venom (Geren et al., 1976; Norment et al., 1979). Working with *L. gaucho*, Barbaro et al. (1992) found that proteins of 33 and 35 kDa were responsible for its dermonecrotic activity, and studying *L. intermedia* venom, Tambourgi et al. (1995) identified a 35 kDa protein involved in its hemolytic and dermonecrotic effects. As depicted in Fig. 5A, the dermonecrotic ability of *L. intermedia* venom N-deglycosylated by N-glycosidase treatment and injected into rabbits was strongly decreased to a residual effect compared to controls. This finding was further confirmed by chemical sugar oxidation as shown in Fig. 5B. The fact that enzymatic deglycosylation or metaperiodate treatment of venom did not alter its edematogenic or haemorrhagic effects at the site of injections (as shown in Fig. 5A and B) nor did it reduce its platelet aggregation (thrombocytic activity) or its fibronectinolytic and fibrinogenolytic effects (Figs. 2 and 3), rules out protein degradation as a possible side effect of deglycosylation procedures. An attractive idea is then that post-translational glycosylation of *L. intermedia* venom proteins (especially in the gelatinolytic Loxolysin B) could play a central role in the necrotic effect produced by the venom. This glycobiological activity of the venom points to the possibility of a carbohydrate-based

dermonecrotic therapy using a carbohydrate-based drug as suggested for other pathological situations (Hodgson, 1991; Winkelhake, 1991; Hart, 1992).

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Effect of brown spider venom on basement membrane structures

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Summary

Loxoscelism or necrotic arachnidism are terms used to describe lesions and reactions induced by bites (envenomation) from spiders of the genus *Loxosceles*. Envenomation has been reported to provoke dermonecrosis and haemorrhage at the bite site and haemolysis, disseminated intravascular coagulation and renal failure. The purpose of this work was to study the effect of the venom of the brown spider *Loxosceles intermedia* on basement membrane structures and on its major constituent molecules. Light microscopy observations showed that *L. intermedia* venom obtained through electric shock, which reproduces two major signals of Loxoscelism in the laboratory, exhibits activity toward basement membrane structures in mouse Engelbreth-Holm-Swarm (EHS) sarcoma. Basement degradation was seen by a reduced periodic acid-Schiff (PAS) and alcian blue staining as well as by a reduced immunostaining for laminin when compared to control experiments. Electron microscopy studies confirmed the above results, showing the action of the venom on EHS-basement membranes and demonstrating that these tissue structures are susceptible to the venom. Using purified components of the basement membrane, we determined through SDS-PAGE and agarose gel that the venom is not active toward laminin or type IV collagen, but is capable of cleaving entactin and endothelial heparan sulphate proteoglycan. In addition, when EHS tissue was incubated with venom we detected a release of laminin into the supernatant, corroborating the occurrence of some basement membrane disruption. The venom-degrading effect on entactin was blocked by 1,10-phenanthroline, but not by other protease inhibitors such as PMSF, NEM or pepstatin-A. By using light microscopy associated with PAS staining we were able to identify that 1,10-phenanthroline also inhibits EHS-basement membrane disruption evoked by venom, corroborating that a metalloprotease of venom is involved in these effects. Degradation of these extracellular matrix molecules and the observed susceptibility of the basement membrane could lead to loss of vessel and glomerular integrity, resulting in haemorrhage and renal problems after envenomation.

Introduction

The bites of brown spiders (Loxoscelism) are remarkable for causing dermonecrosis, the most striking manifestation evoked by envenomation (Rees *et al.* 1984), with bleeding at the bite site and in some cases with systemic effects such as platelet aggregation causing thrombocytopenia (Bascur *et al.* 1982), haemolysis (Forrester *et al.* 1978), disseminated intravascular coagulation, and renal failure (Denny *et al.* 1964).

Basement membranes are ubiquitous thin sheets of macromolecules produced and secreted adjacent to several cells types such as epithelial, endothelial, muscle, fat and nerve cells (Timpl *et al.* 1987, Rohrbach & Timpl 1993). The major molecular characteristic of basement membranes is the presence of a considerable variety of biochemically complex components structurally arranged as a network. All basement membranes have a particular set of proteins such as type IV collagen, laminin, entactin and heparan sulphate proteoglycan (Yurchenco & Schittny 1990). In mammals, basement membranes play several essential roles. They are substrates for cell surface receptors which transduce important information to the cell promoting for example cell differentiation and neurite outgrowth. They are also involved in angiogenesis, platelet adhesion, and blood–urine filtration in the kidney glomerulus among several other functions (Farquhar 1991, Rohrbach & Timpl 1993).

Snake venom haemorrhagic proteases (Fox & Bjarnason 1995) produce proteolytic degradation of basement membrane constituents isolated from Engelbreth-Holm-Swarm (EHS) tumours (Baramova *et al.* 1989) as well as degradation of purified glomerular basement membrane (Ohsaka *et al.* 1973).

The first evidence of a protease activity in *Loxosceles* venoms was described by Eskafi and Norment (1976) for *Loxosceles reclusa* venom. Their results showed that

the venom possesses proteolytic activities toward *Heliothis virescens* and *Musca domestica* larvae. Jong *et al.* (1979), studying the same venom and working with L-aminoacyl-β-naphthylamide derivatives, observed that the venom hydrolysed L-leucyl-β-naphthylamide as the best substrate, whereas aliphatic and basic aminoacyl derivatives such as valine, alanine, arginine and lysine were hydrolysed less efficiently. Aromatic amino acids such as tyrosine or other aminoacyl derivatives such as proline, serine, hydroxyproline, glycine and cystine-di-β-derivatives showed no detectable substrate activity.

Loxosceles intermedia (brown spider) venom has been reported to have a degrading activity on fibronectin and fibrinogen, two soluble extracellular matrix molecules, as well as on gelatin (Feitosa *et al.* 1998, Veiga *et al.* 1999), but there are no previous reports of a basement membrane-degrading effect of spider venom proteases. Based on their pathogenetic properties, such as dermonecrotic action, thrombocytopenic activity, tissue haemorrhage and renal failure, events that could be attributable to the presence of proteolytic enzymes that degrade extracellular matrix molecules we conjecturate that brown spider (dermonecrotic spider) venoms have a potential degrading effect on this extracellular structure. In the present investigation, we studied the action of *L. intermedia* (brown spider) venom on the EHS-sarcoma tumour, a transplantable mouse tumour model which produces a highly characteristic and thick basement membrane structure as its capsule. In light and electron microscopy studies we detected a morphological disruptive activity of venom toward EHS-basement membrane. Using purified components of basement membranes we determined the hydrolytic effect of the venom on entactin and heparan sulphate proteoglycan, which indicates that the basement membrane is susceptible to the venom. Using protease inhibitors we found that only 1,10-phenanthroline abolished the entactinolytic and basement membrane disruptive effects of venom, indicating that a metalloprotease is involved in these effects.

Materials and methods

Reagents

Laminin, laminin–entactin complex and entactin were purified from EHS tumour as described by Timpl *et al.* (1979) and Paulsson *et al.* (1987). Human placental type IV collagen was purchased from Sigma (St. Louis, USA). Polyclonal anti-laminin antibodies (Rb_1aLN) and polyclonal anti-entactin antibodies (Rb_2aET) were produced using purified EHS-laminin or EHS-entactin submitted to preparative SDS-PAGE as described by Harlow and Lane (1988).

Cell line, culture conditions and purification of heparan sulphate proteoglycan and free chains

Established endothelial cell lines (from rabbit aorta) (Buonassisi & Venter 1976) were grown in Ham's F-12 tissue culture medium (Sigma) supplemented with 10% fetal calf serum (Cultilab, Campinas, Brazil) and penicillin/streptomycin (1000 U and 10 mg per litre, respectively) (Sigma). Cells were radio-labelled with sodium [^{35}S]-sulphate (IPEN, São Paulo, Brazil) (150 μCi/ml) for 24 h in an incubator with 2.5% CO_2 under humidified conditions. For heparan sulphate proteoglycan purification, radio-labelled endothelial cells were solubilized with lysis buffer (50 mM Tris–HCl, pH 7.3, 1% Triton X-100, 50 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM PMSF and 2 μg/ml aprotinin) for 15 min at 4 C. The extract was clarified by centrifugation for 10 min at 13,000*g* and frozen until use. The radio-labelled heparan sulphate proteoglycan was purified by a combination of gelatin affinity chromatography, gel filtration and ion-exchange chromatography as previously described (Nader *et al.* 1987). Radio-labelled heparan sulphate polysaccharide chains were obtained by digestion of heparan sulphate proteoglycan with 50 μg Superase (Chas Pfizer Co., NY) for 4 h at 60 C in the presence of 0.8 M NaCl, pH 8.0. At the end of the incubation, the mixture was heated for 7 min at 100 C to inactivate the proteolytic enzyme and the radio-labelled glycosaminoglycan was precipitated with two volumes of methanol at −20 C in the presence of 100 μg carrier heparan sulphate (Nader *et al.* 1989).

Spider venom extraction

The venom was extracted from spiders captured from nature and kept for a week without any kind of food. The venom was extracted from the fangs of spiders by electrostimulation (15 V) applied to the cephalothorax and collected with a micropipette, dried under vacuum and frozen at −85 C until use. The pure venom is a transparent liquid, whereas venom contaminated with stomach egestion contents becomes cloudy and was always discarded. A pool of venom collected from approximately 500 spiders was used in each experiment (Barbaro *et al.* 1992, Feitosa *et al.* 1998).

Proteolytic assays with purified constituents of the basement membrane

EHS-laminin, EHS-laminin–entactin complex (0.5 mg diluted in 50 mM Tris–HCl buffer, pH 7.3, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$) were incubated with 100 μg *L. intermedia* venom at 37 C. Aliquots of the reaction mixtures (25 μg) were collected at time 0 and after 16 h incubation. The proteolytic effect of the venom was stopped by freezing the samples at −20 C. As controls, we collected samples of venom alone, or samples of purified molecules after 16 h at the same experimental conditions but in the absence of venom, in order to check experimental stability. Samples were subjected to linear gradient 3–15% SDS-PAGE under reducing conditions and the proteolytic effect of the venom was assessed by Coomassie blue R staining. To study the effects of protease inhibitors, laminin–entactin dimer was incubated with venom overnight at the same experimental conditions

as above, in the presence of 5 mM 1,10-phenanthroline, or 10 mM PMSF, or 5 mM NEM or 5 μg/ml pepstatin-A and processed as described above. Purified EHS-entactin (aliquots of 25 μg) was incubated with increasing concentrations of crude venom (10, 20, 40 and 100 μg) at 37 C for 16 h. Samples were processed by linear gradient SDS-PAGE as described above and transferred onto a nitrocellulose membrane that was immunoblotted with a polyclonal antibody to EHS-entactin. For human placental type IV collagen, the degrading effect of venom was checked under identical experimental conditions except that the temperature of incubation was 25 C. For heparan sulphate proteoglycan, the hydrolytic effect of venom was assessed using 10,000 cpm radio-labelled heparan sulphate proteoglycan that was incubated with 10 μg venom at 37 C overnight. As a control of experimental stability, radio-labelled heparan sulphate proteoglycan was incubated under the same conditions but in the absence of venom. Samples were subjected to linear gradient 3–12% SDS-PAGE under non-reducing conditions, and the gel was dried and exposed to an X-ray film at room temperature for 10 days. To determine the action of venom on the heparan sulphate polysaccharide chains of endothelial cells, radio-labelled compounds were incubated with 10 μg venom at 37 C overnight. As a control, heparan sulphate polysaccharide chains were incubated under the same conditions in the absence of venom. The products obtained were analysed by an agarose gel electrophoresis and the gel was dried and exposed to X-ray film for 10 days.

Gel electrophoresis

SDS gel electrophoresis was performed as described by Laemmli (1970). Samples under reducing or non-reducing conditions (see legends to figures), were analysed on 4.0% or linear gradients of 3–12% or 3–15% polyacrylamide gels. For the immune reactions, proteins after electrophoresis were transferred overnight to nitrocellulose filters (Towbin *et al.* 1979). The molecular markers used were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (98 kDa), albumin (67 kDa), ovalbumin (44 kDa) and carbonic anhydrase (29 kDa) purchased from Sigma. Proteins transferred to nitrocellulose membranes were analysed by Western blotting. Filters were blocked with phosphate-buffered saline (PBS) containing 5% low-fat milk (Nestlé, São Paulo, Brazil) and incubated sequentially at room temperature for 2 h with rabbit polyclonal antiserum against laminin (Rb_1aLN) or a rabbit polyclonal antiserum against entactin (Rb_2aET) diluted 1 : 1000 and 1 : 500, respectively, in blocking solution and with alkaline phosphatase conjugate antibodies against rabbit IgG (Sigma) diluted 1 : 4000 in blocking solution for 1 h and developed in nitroblue tetrazolium (NBT) and bromochloroindolyl phosphate (BCIP) solution (Veiga *et al.* 1995).

Glycosaminoglycan analysis was performed using an agarose gel electrophoresis in 0.05 M 1,3-diaminopropane acetate buffer, pH 9.0 (Aldrich, Milwaukee, USA). After the electrophoretic run, compounds were precipitated in the gel using 0.1% Cetavlon for 2 h at room temperature (Dietrich & Dietrich 1976). After drying, the gel was stained with toluidine blue and exposed to an X-ray film (X-Omat, Kodak, USA) for 10 days at room temperature. The glycosaminoglycan standards used were heparan sulphate from bovine pancreas, dermatan sulphate from pig skin and chondroitin sulphate from shark cartilage (Seikagaku, Tokyo, Japan).

Histological methods for light microscopy

EHS tissues were fixed in modified Carnoy's fixative (Beçak & Paulete 1976) for 3 h. After fixation, the tissues were processed for histology, embedded in paraffin wax and cut into 4-μm thick sections. The sections were incubated with PBS or *L. intermedia* venom/PBS (100 μg/ml) overnight at 37 C under humidified conditions. After incubation, the sections were stained with periodic acid-Schiff (PAS) (McManus 1948) and alcian blue (Beçak & Paulete 1976). To study the effect of 1,10-phenanthroline on the hydrolytic activity of venom toward EHS-basement membranes, the materials were processed identically as above and incubated with venom in the presence of 5 mM 1,10-phenanthroline and then stained with PAS method.

Immunohistochemical analysis

EHS tissue sections mounted on glass slides were deparaffinized and incubated with *L. intermedia* venom (100 μg/ml) or with PBS (control) overnight at 37 C under humidified conditions. The EHS-sections were then washed with PBS, processed for immunohistology, incubated with primary polyclonal laminin antibody (Rb_1aLN) diluted 1 : 500 overnight at 4 C and with goat anti-rabbit IgG peroxidase conjugate (Sigma) diluted 1 : 100. Following further washing in PBS, 3,3'-diaminobenzidine was used to visualize the immunoreactivity (Gremski & Cutler 1991).

Transmission electron microscopy

EHS tissues were fixed with modified Karnovsky's fixative (Karnovsky 1965) for 2 h, washed in 0.1 M cacodylic acid buffer (pH 7.3), postfixed in 1% OsO_4 in 0.1 M cacodylic acid buffer, pH 7.3, for 1 h, dehydrated with ethanol and propylene oxide and embedded in Epon 812. Ultrathin sections were incubated with PBS or *L. intermedia* (100 μg/ml) venom/PBS overnight at 37 C under humidified conditions, contrasted with uranyl acetate and lead citrate and examined with a JEOL-JEM 1200 EX II transmission electron microscope at an accelerating voltage of 80 kV.

Scanning electron microscopy

EHS tissues were fixed in modified Karnovsky's fixative (Karnovsky 1965) for 1 h, washed in 0.1 M cacodylic acid buffer, pH 7.3, incubated with PBS or *L. intermedia* (100 μg/ml) venom/PBS overnight and postfixed in 1% OsO_4 in 0.1 M cacodylic acid buffer (pH 7.3) for 1 h. They were then

dehydrated in ethanol, critical-point dried, sputter-coated with gold and examined with a MEV XL-30 Philips scanning electron microscope.

Platelet aggregation assay

Human platelet-rich plasma was obtained by differential centrifugation from fresh human blood drawn into acid-citrate-dextrose. Platelet aggregation in the presence of venom (see legends for concentrations) were recorded at 37 °C at a stirring rate of 1000 rpm using a Becton-Dickinson aggregometer as described (Plow *et al.* 1985).

Dermonecrosis studies

For the evaluation of the dermonecrotic effect, pooled venom (sample of 40 µg diluted in PBS), obtained as described above, was injected intradermally into a shaved area of rabbit skin. The dermonecrotic lesion was checked 1 h, 6 h, 12 h, 1 day, 2 days and 5 days after injection as previously described (Veiga *et al.* 1999).

Results

EHS-basement membrane pattern and quality control of toxicity of venom

Figure 1 shows the EHS tissue structure viewed by light microscopy (A), transmission electron microscopy (B) and scanning electron microscopy (C). Figures 1(D) and (E) represent quality control of the ability of electric shock-stimulated venom to produce the major signals of Loxoscelism. Figure 1(D) shows a dermonecrotic lesion, the hallmark signal evoked by *Loxosceles* spp. envenoming. The lesion progresses from an acute local inflammatory reaction with oedema, erythema and haemorrhage into the dermis to a black eschar slough produced by necrosis and gravitational spreading. Figure 1(E) depicts the platelet aggregation activity induced by venom *in vitro*, a concentration-dependent effect. When denatured by boiling, the venom did not induce platelet aggregation, thus supporting a conformation-dependent activity.

Proteolytic effect of L. intermedia *venom on EHS-basement membrane tissue sections*

We first investigated a putative degrading effect evoked by *L. intermedia* crude venom on EHS-basement membranes. Figure 2 shows that EHS-basement membranes treated with 100 µg/ml venom in PBS overnight and stained with PAS, alcian blue, or immunohistochemically probed for laminin showed a decreased staining when compared to control samples incubated with PBS under the same experimental conditions.

Figures 1A–C. EHS-basement membrane pattern and quality control of toxicity of venom. (A) Light micrograph of EHS tumour cells stained with haematoxylin–eosin (magnification, 600×). (B, C) Transmission electron microscopy electron micrographs (magnification, 2000×) and scanning electron microscopy electron micrographs (magnification, 579×) respectively of EHS tissue. Arrows point at the tumour capsule which is a thick extracellular matrix of the basement membrane.

Effect of venom on EHS-basement membranes analysed by electron microscopy

Since light microscopy has some limitations, we undertook an additional experiment to check the possible action of *L. intermedia* on basement membranes. As shown in Figure 3, EHS-tumour tissue sections were incubated overnight at 37 °C with 100 µg/ml venom diluted in PBS,

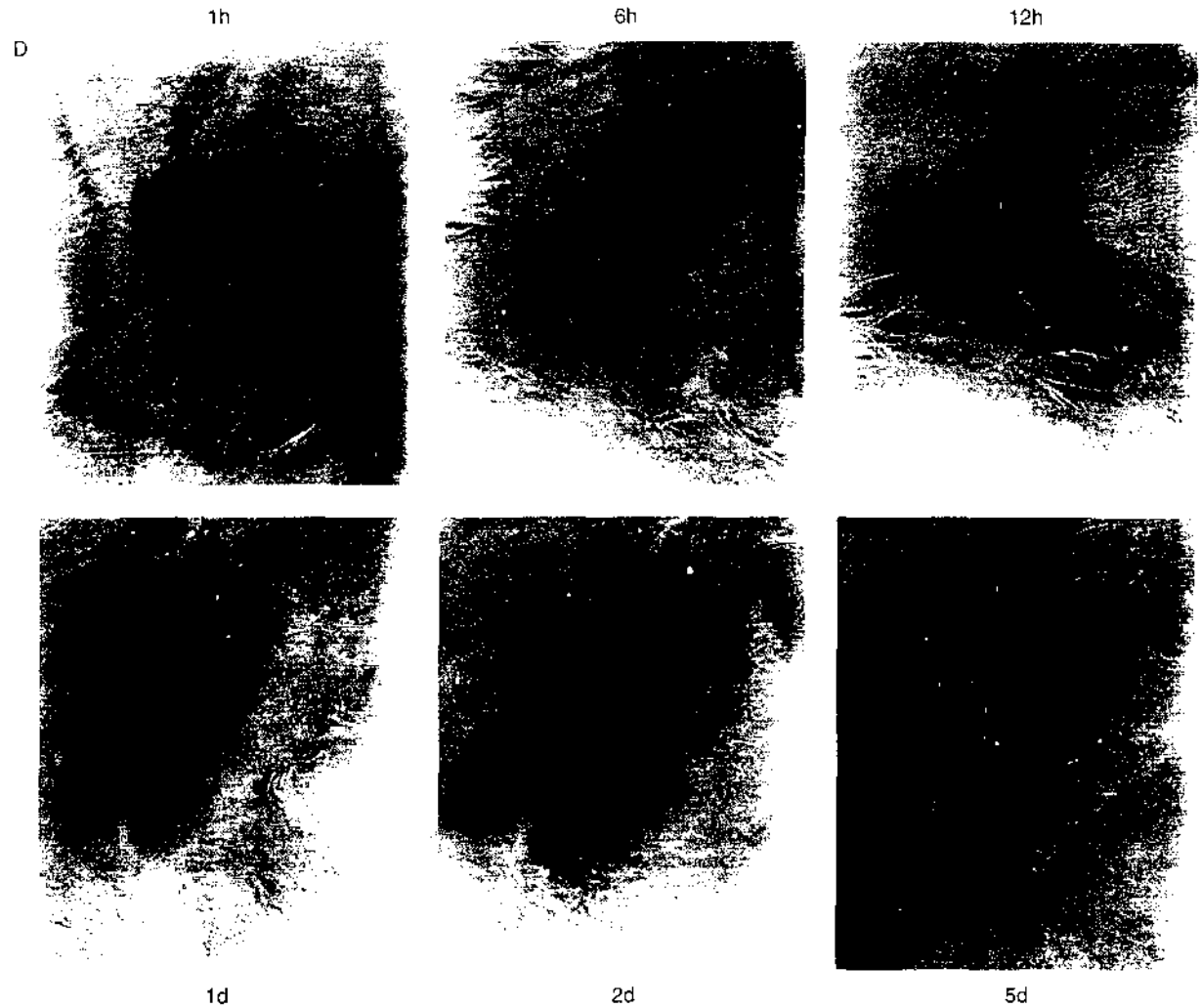

Figure 1D. Rabbits were intradermally injected with 40 μg *L. intermedia* crude venom. Lesions were evaluated 1 h, 6 h, 12 h, 1 day, 2 days and 5 days after injection.

or similarly incubated with PBS (control) and then processed for transmission electron microscopy or scanning electron microscopy. High resolution electron microscopy images confirmed that the venom has a hydrolytic activity on EHS-basement membranes, as previously observed by light microscopy. Control experiments showed a regular basement membrane surrounding the tumour. The venom-treated material showed an irregular basement membrane border (arrow) with several points of disruption. Scanning electron microscopy showed the basement membrane as a capsule in the control experiments. On the other hand, this basement membrane capsule appears disrupted in the venom-treated material.

Effect of L. intermedia *venom on purified laminin, laminin–entactin dimer, entactin and type IV collagen*

In order to confirm the venom's degrading activity towards basement membrane, we incubated purified laminin, laminin–entactin complex and purified entactin with venom at 37 C overnight. Type IV collagen was evaluated at 25 C since purified type IV collagen is denatured at temperatures above 30 C (Mackay *et al.* 1990). As shown in Figure 4(A), neither the laminin α_1 chain nor the laminin β_1 or γ_1 chains suffered any kind of proteolytic degradation by the venom. The venom also had no degrading effect on human placental type IV collagen (D), but cleaved entactin when complexed with laminin (B) or as a purified molecule (C).

Effect of venom on heparan sulphate proteoglycan and heparan sulphate polysaccharide chains

To complete the molecular analysis of the action of *L. intermedia* venom on basement membrane constituents, we checked the degrading activity of the venom on endothelial heparan sulphate proteoglycan, a major component of basement membranes. Endothelial heparan sulphate proteoglycan was incubated with venom at 37 C overnight. PBS was used as negative control. As can be observed in Figure 5(A), the venom hydrolysed the proteoglycan in the protein core, but displayed no activity toward the heparan sulphate glycosaminoglycan chain (B).

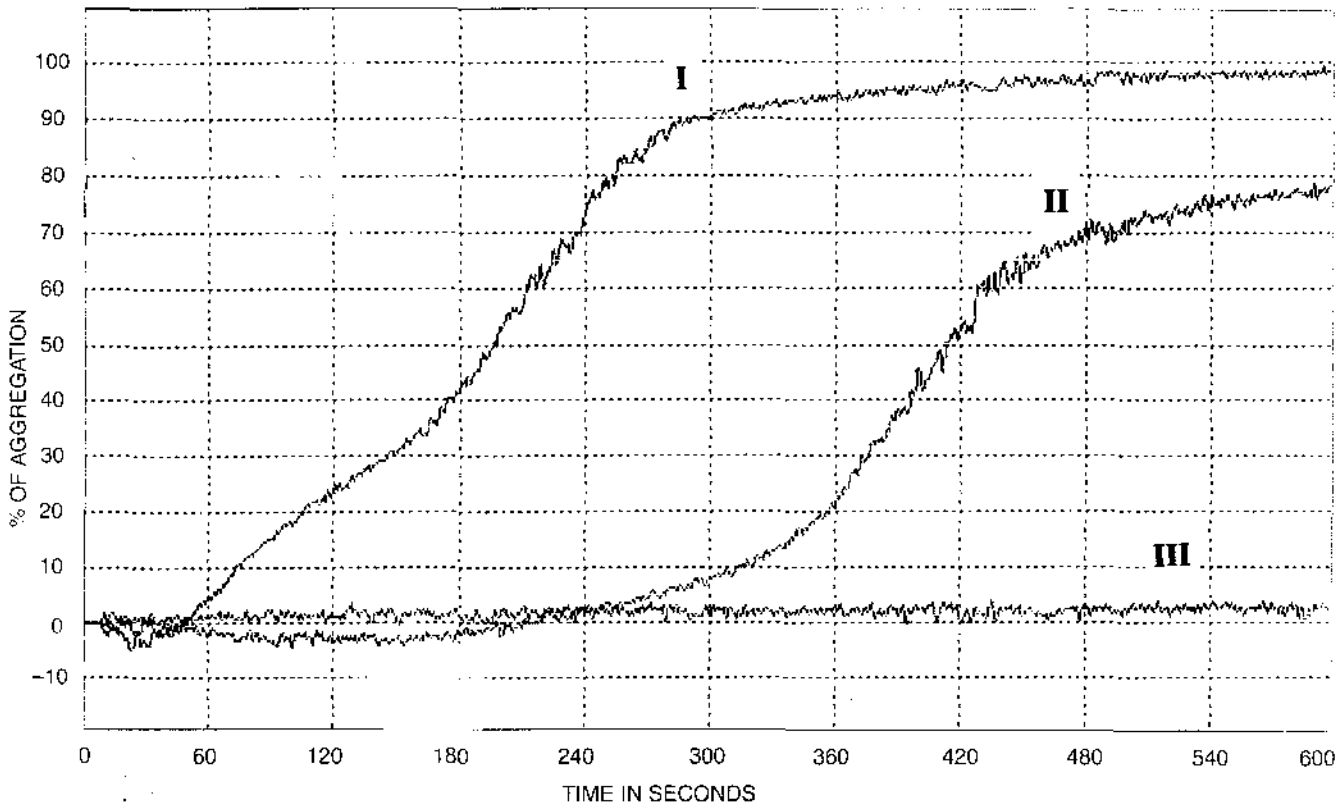

Figure 1E. Platelet-rich plasma was incubated with venom at the concentration of 100 μg (I), 25 μg (II) or 100 μg of venom previously denatured by boiling (5 min at 100 C) (III). The abscissa represents the time in seconds and the ordinate represents per cent platelet aggregation.

Disruptive effect of L. intermedia *venom on basement membrane with laminin releasing*

Based on the fact that basement membranes are an intricate network formed by specific interactions among its constituents (Yurchenco & Schittny 1990) and on the fact that the venom degrades entactin and heparan sulphate proteoglycan, but seems to have no action on laminin or type IV collagen, we investigated a possible disintegrating activity of the venom on basement membrane structures since after cleavage of entactin and heparan sulphate proteoglycan, connected molecules in the basement membrane network may suffer some kind of separation and disruption. EHS tissue (approximately 1 mg) was incubated with 100 μg venom and the supernatant probed for laminin by Western blotting using a polyclonal antibody against laminin. As can be seen in Figure 6, venom treatment of basement membranes released laminin, but, according to the previous experiment, had no apparent hydrolytic activity on this matrix molecule.

Inhibitory effect of 1,10-phenanthroline on the proteolytic activities of venom

In order to obtain information about the nature of the proteolytic effects produced by *L. intermedia* venom on EHS-basement membrane, we studied its degrading action on entactin in the presence of inhibitors of four major groups of proteolytic enzymes, such as 1,10-phenanthroline (metalloproteinase inhibitor), PMSF (serine-protease inhibitor), NEM (thiol-protease inhibitor) and pepstatin-A (aspartic-protease inhibitor). As depicted in Figure 7(A), the entactinolytic effect of venom was blocked only by phenanthroline. Our next goal was to study the activity of 1,10-phenanthroline on the destructive effect of venom toward EHS-basement membrane. As depicted in Figure 7(B), EHS-tissue sections treated with 100 μg venom in the presence of 5 mM phenanthroline and stained with PAS showed a staining profile identical to negative controls (incubated with PBS), compared to positive controls (incubated only with venom) that shows a degraded EHS-basement membrane profile.

Discussion

In the present study, we checked the possibility that brown spider venom could act directly on basement membrane structures. We carried out experiments using EHS-basement membrane, the most useful and convenient source for basement membrane studies and a model in the field (Timpl *et al.* 1987). Our working hypothesis was based on the effects triggered by brown spider venom *in vivo*, and on previous reports showing that several snake venoms can hydrolyse basement membranes. Ohsaka *et al.* (1973) described the activities of haemorrhagins isolated from *Trimeresurus flavoridis* venom on basement membrane, postulating that their noxious effects

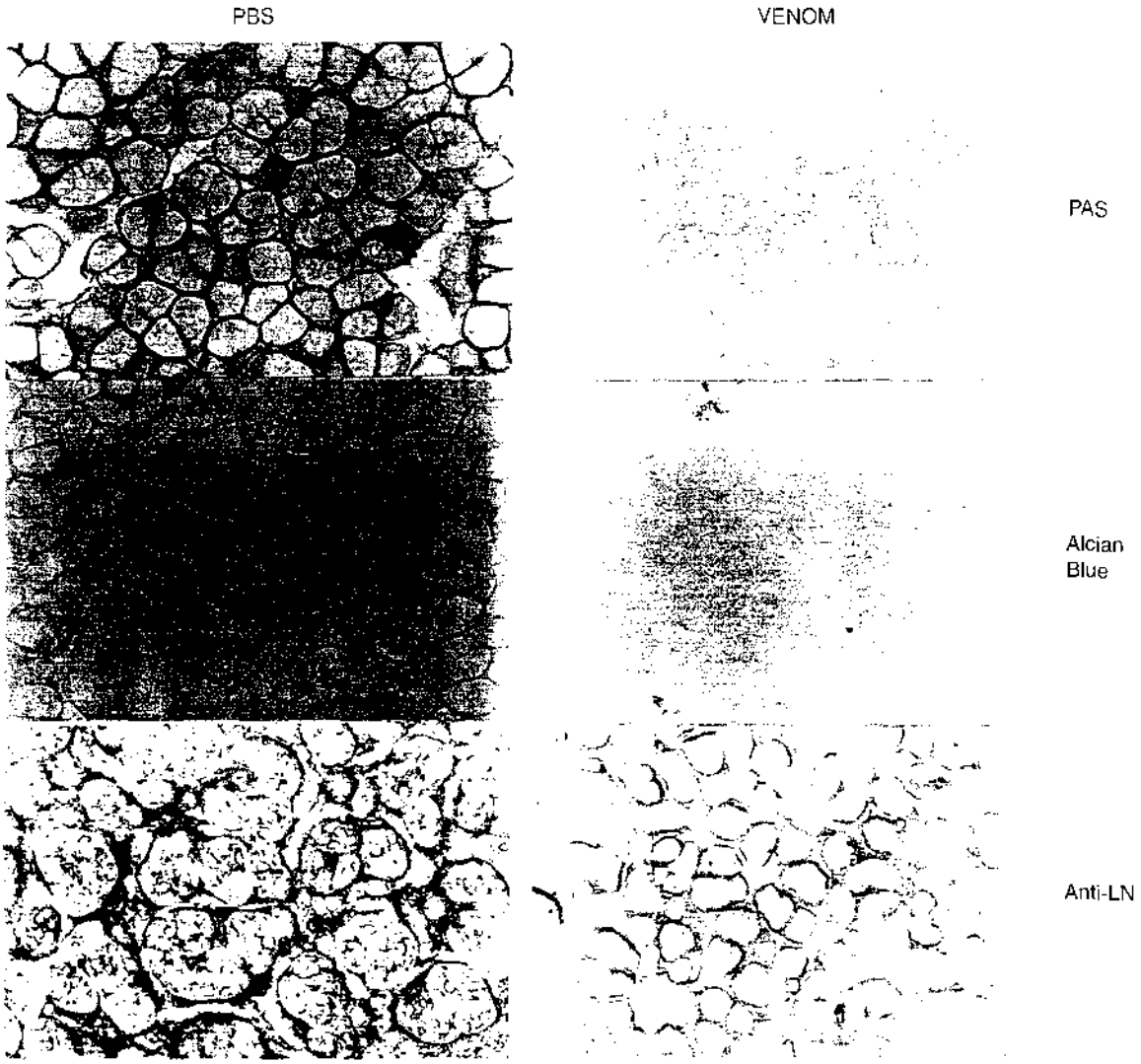

Figure 2. L. intermedia venom activity on EHS-basement membrane. EHS-tissue sections were incubated with *L. intermedia* venom overnight at 37 C (VENOM) or with PBS as control (PBS) and then stained for glycoprotein (PAS) and proteoglycan content (alcian blue) or probed for laminin by immunohistochemistry (anti-LN). Magnification of light micrographs, 200×.

could cause haemorrhage by weakening capillaries after degrading the subendothelial extracellular matrix. Civello *et al.* (1983) have reported that haemorrhagic proteinase IV from timber rattlesnake (*Crotalus horridus horridus*) venom catalysed the complete hydrolysis of basement membranes, suggesting that this cleavage effect is involved in the mechanism of the haemorrhagic action of the proteinase. Baramova *et al.* (1989) studied four haemorrhagic metalloproteinases, Ht-a, Ht-c, Ht-d and Ht-e, from western diamond black rattlesnake (*Crotalus atrox*) venom, determining their cleaving ability on type IV collagen, laminin and entactin, major constituents of basement membranes. Their data suggest that the degradations brought about by these metalloproteinases could lead to loss of capillary integrity resulting in haemorrhage.

Our initial data are based on light microscopy studies of EHS-basement membranes incubated with *L. intermedia* venom and stained for glycoprotein (PAS), proteoglycan (alcian blue) or laminin (immunohistochemistry using a polyclonal anti-laminin antibody). The results, illustrated in Figure 2, clearly indicate that the venom acts on basement membranes, with significant reduction in staining patterns compared to the staining obtained for untreated preparations (controls). Figure 2(C) (immunohistochemistry for laminin) further supports this conclusion, showing an appreciable disruption of basement membranes compared to control.

Electron microscopy studies of venom-treated EHS-basement membranes (Figure 3) further indicated venom activity toward these structures. Examination by transmission electron microscopy and scanning electron microscopy showed disrupted basement membrane regions, which were absent from control experiments.

We also tested if purified molecular basement membrane constituents represent substrates for the venom. As depicted in Figure 4, *L. intermedia* venom apparently has no activity either on laminin, or on type IV collagen, the major macromolecules of these structures. In contrast, the venom has the ability to hydrolyse entactin, a dumbbell-shaped molecule that links laminin, type IV collagen and heparan

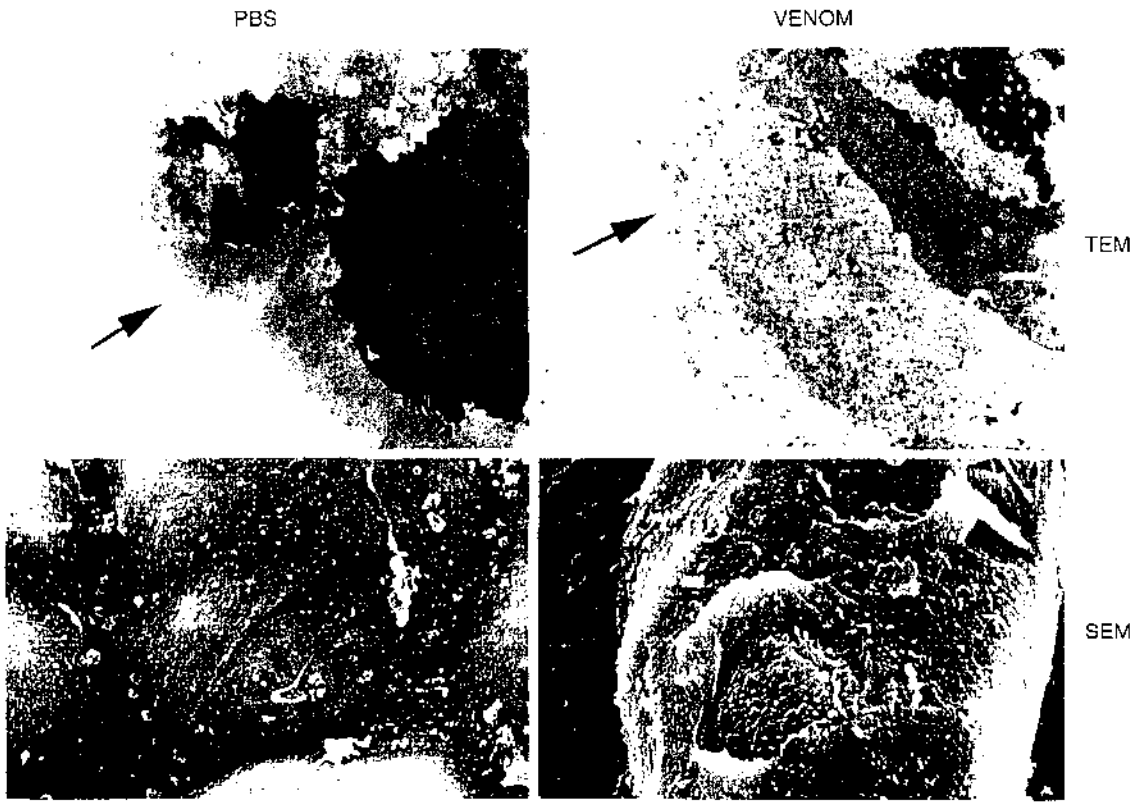

Figure 3. Action of *L. intermedia* venom on EHS-basement membrane analysed by electron microscopy imaging systems. Thin sections of EHS-tissue placed on a gold grid were incubated overnight at 37 C with *L. intermedia* venom (VENOM) or PBS as negative control (PBS), stained with osmium tetroxide, and analysed by transmission electron microscopy (TEM). Micrograph magnification, 4000×. Alternatively, EHS-tissue placed on a glass slide was incubated with venom (VENOM) or PBS as negative control (PBS), coated with gold and then examined by scanning electron microscopy (SEM). Micrograph magnification, 3865×. Arrows point to the basement membrane.

sulphate proteoglycan acting as a bridge in basement membrane organization (Yurchenco & Schittny 1990, Reinhardt *et al.* 1993). The hydrolytic action of venom on entactin (150 kDa) resulted in fragments of approximately 100 kDa and 50 kDa, when the laminin–entactin complex was checked at a 5 : 1 substrate : crude venom ratio (Figures 4(B) and 7(A)), indicating a degrading ability toward this molecule. When purified entactin was assayed against crude venom similar fragments were obtained but these fragments were subsequently degraded into a variety of lower fragments that run out the gel with increasing concentrations of venom (Figure 4(C)). Similar results of laminin–entactin dimer and purified entactin digestions have been reported for snake venom principles since, isolated entactin is degraded by atrolysin E (a reprolysin purified from *Crotalus atrox*) at positions 75, 296, 336, 402, 478, 625, 702 and 920, but the entactin–laminin dimer is cleaved in a more restricted way at positions 322, 336, 351, 840 and 953 (Baramova *et al.* 1989, Fox & Bjarnason 1995). This difference in cleavage patterns for the laminin–entactin complex and for purified entactin treated with venom can be explained by the fact that laminin, by interacting with entactin, can hide regions of entactin more susceptible to the degrading action of the venom. On the other hand, since entactin is generally considered to be denatured after the procedures used during purification (Paulsson *et al.* 1987, Fox *et al.* 1991) we can also suppose that this difference of cleavage pattern is evoked by venom, ascribed to a conformational alteration of entactin that facilitates the degrading activity of the venom by exposing normally hidden sites. *L. intermedia* venom also degrades the protein core of heparan sulphate proteoglycan from endothelial cells (Figure 5(A)), but has an apparent lack of activity on heparan sulphate polysaccharide chains (Figure 5(B)). Since proteoglycans contribute to the semipermeability properties of basement membranes and heparan sulphate proteoglycans in the kidney glomerulus seem to be important in preventing the passage of macromolecules from blood to urine (Farquhar 1991), it is possible that the renal injury with proteinuria evoked by brown spider envenomation can be attributed to this hydrolytic effect on basement membrane heparan sulphate proteoglycan.

The basement membranes mainly consist of connected molecules that can bind directly to each other (Yurchenco & Ruben 1987, Yurchenco & Schittny 1990) and, as demonstrated above, *L. intermedia* venom was free of laminin-olytic and type IV collagenolytic activities, but had the ability to hydrolyse entactin and heparan sulphate proteoglycan (Figures 4, 5 and 7). Based on the experiment described in Figure 6 (releasing of laminin from EHS-basement membranes) and structural and ultrastructural techniques

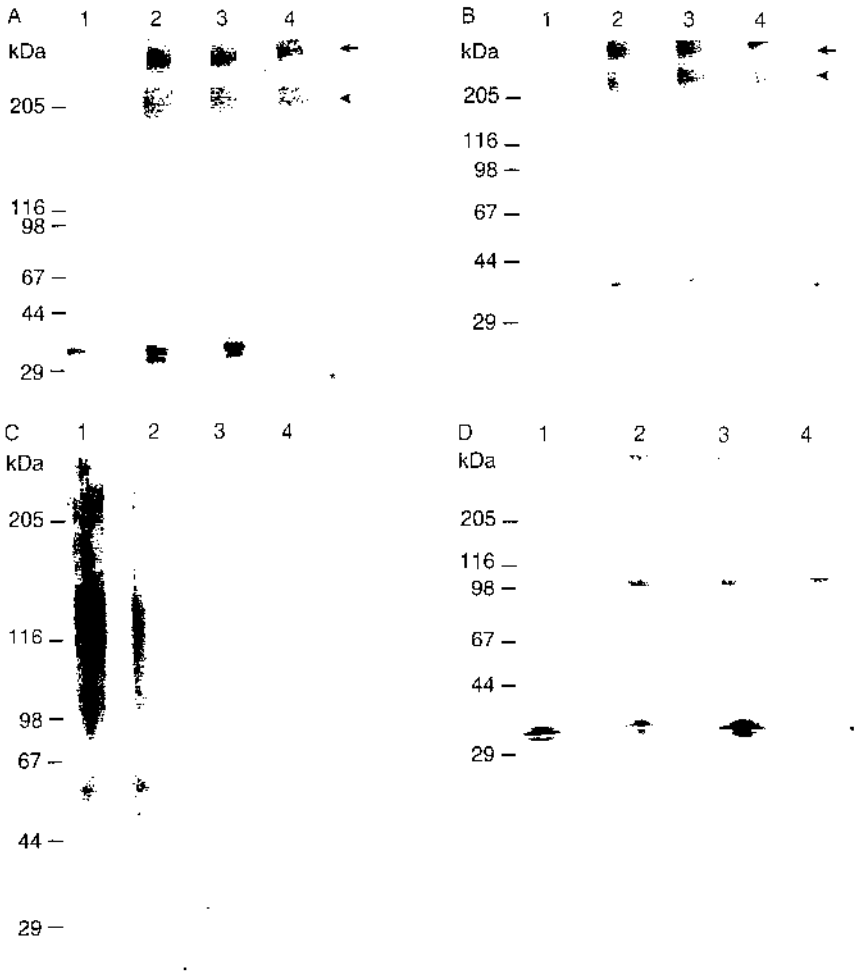

Figure 4. Effect of *L. intermedia* venom on purified laminin, laminin–entactin complex, purified entactin and type IV collagen. Purified laminin (A), laminin–entactin complex (B), 0.5 mg diluted in 1 ml 50 mM Tris–HCl buffer, pH 7.3, containing 1mM $CaCl_2$ and 1 mM $MgCl_2$, or type IV collagen (D), 0.5 mg diluted in 1 ml 0.5 M acetic acid and then neutralized to pH 7.3, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ were incubated with 100 µg venom at 37 C for laminin and laminin–entactin complex or at 25 C for type IV collagen. Aliquots of the incubation mixtures (25 µg of proteins) were removed at different time intervals and submitted to electrophoresis (linear gradient 3–15% SDS-PAGE) under reducing conditions. Lane 1: venom alone; lane 2: mixtures at time 0; lane 3: mixtures at time 16 h; lane 4: substrates alone at time 16 h; laminin (A), laminin–entactin complex (B) and type IV collagen (D). Gels were stained with Coomassie blue. Molecular weight standards are shown on the left. Black arrows point at the α_1 laminin chain, black arrowheads show β_1 and γ_1 laminin chains that comigrate, the grey arrow represents entactin, and the grey arrowhead shows an entactin fragment after venom hydrolysis. The major 100, 130, 160 and 170 kDa components of pepsin-extracted human placental type IV collagen can be seen in D. The asterisks represent the major venom protein electrophoretic migrations. In C, purified entactin samples (25 µg) were incubated with increasing concentrations of venom. Lane 1: entactin plus 10 µg of venom; lane 2: entactin plus 20 µg of venom; lane 3: entactin plus 40 µg of venom, and lane 4: entactin plus 100 µg of venom. Venom-degrading activity was visualized after 3–15% gradient SDS-PAGE through immunoblotting using a polyclonal antibody against entactin.

described in Figures 2 and 3, that show the disruptive effect of the venom toward EHS-basement membrane, we may propose that the degrading effects of spider venom on entactin and heparan sulphate proteoglycan render the structures of the basement membrane network more susceptible to damage, even releasing laminin at envenomation sites. Previous results obtained by using recombinant human entactin and mouse entactin indicate the N-terminal globular domain G_1 as the region of protease sensitivity in this molecule (Mayer *et al.* 1995). Interestingly this N-terminal globular domain G_1 in mouse entactin has a size of 50 kDa, and when we analyse the proteolytic pattern of the venom upon entactin (in the dimer laminin–entactin, see Figure 7(A)), a similar entactin fragment of approximately 50 kDa can be detected. Nevertheless more effort and investigation could be conducted to determine the sites of entactin cleavage by the venom.

We may speculate that laminin release from EHS-basement membranes occurs as a function of the degrading activity of venom principles on proteinase-sensitive regions of the domains of entactin, involved in laminin binding, thus disrupting the interaction with laminin and effectively dissociating the laminin network from the collagen IV network in basement membranes. Aumailley *et al.* (1989) detected that even through laminin short and long arms are found associated with type IV collagen, once entactin is extracted from laminin, significant laminin interaction

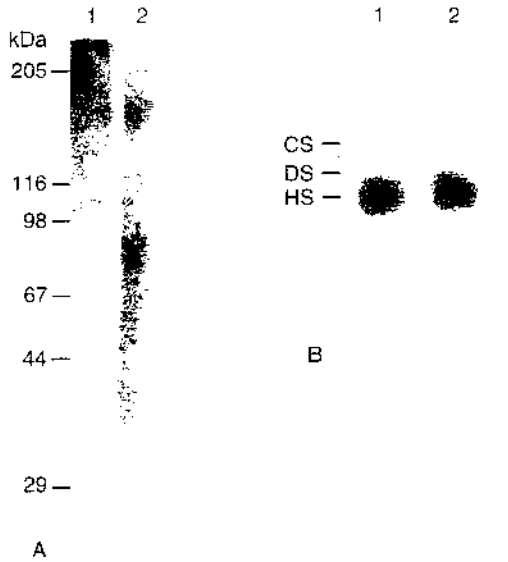

Figure 5. Action of *L. intermedia* venom on endothelial heparan sulphate proteoglycan and heparan sulphate polysaccharide chains. (A) Endothelial cells were labelled overnight with sodium [^{35}S]sulphate, 150 μCi/ml medium. The radio-labelled purified heparan sulphate proteoglycan (10,000 cpm) was incubated with venom (10 μg) or with PBS (control) at 37 °C overnight. Samples were analysed by 3–12% SDS-PAGE and the gel was dried and exposed to X-ray film. Lane 1 shows the autoradiograph of heparan sulphate proteoglycan (control) and lane 2 shows heparan sulphate proteoglycan incubated with venom. Venom components degrade the heparan sulphate proteoglycan, releasing proteoglycan fragments. (B) The same experimental procedure was repeated using heparan sulphate polysaccharide chains as substrate for the venom. The products obtained were analysed by an agarose gel electrophoresis in 0.05 M 1,3-diaminopropane acetate buffer, pH 9.0. After the run the compounds were precipitated with 0.1% cetavlon for 2 h, and the gel was then dried and exposed to X-ray film. Lane 1 shows the heparan sulphate polysaccharide chains incubated with PBS (control) and lane 2 depicts the heparan sulphate polysaccharide chains obtained after venom treatment. On the left are shown CS – chondroitin sulphate, DS – dermatan sulphate and HS – heparan sulphate glycosaminoglycan standards. Venom components had no action on the heparan sulphate polysaccharide chain.

Figure 6. Laminin-releasing activity of *L. intermedia* venom on native EHS-basement membranes. EHS-tissue (1 mg) was incubated with venom (100 μg) overnight at 37 °C. The supernatant (50 μl) was submitted to 4% SDS-PAGE under non-reducing conditions and transferred to a nitrocellulose membrane that was immunoblotted with a rabbit anti-laminin polyclonal antiserum. Lane 1 depicts purified EHS laminin (5 μg) used as control and lane 2 shows material obtained from the supernatant after treatment of the EHS-basement membrane with venom. Molecular weight standards are shown on the left.

with collagen IV cannot be detected which can be interpreted by the fact that entactin binding is the major (highest affinity) interaction between laminin and type IV collagen. Similarly, the hydrolytic effect of venom principles on the protein core of heparan sulphate proteoglycan can also be synergistically effective in dissociating the laminin network from the basement membrane. Interaction of heparan sulphate proteoglycan with other basement membranes constituents may permit coordinated regulation of basement membrane structure and function (Yurchenco & Schittny 1990). Venom degrading activity toward the protein core of heparan sulphate proteoglycan could alter basement membrane sieving, stability, porosity resulting in architectural modifications of the matrix.

Based on the observation that *L. intermedia* venom hydrolyses entactin and heparan sulphate proteoglycan and releases laminin from basement membranes, and on the fact that these molecules are important in supporting the structural–functional integrity of these tissue structures, we may speculate that the basement membrane-disrupting effect of the venom is a plausible mechanism for its haemorrhage and renal failure consequences that together with its other anticoagulant properties (Futrell 1992, Feitosa *et al.* 1998), provides a plausible mechanism for brown spider venom toxicity. The disruption of basement membrane triggered by proteases of the venom could be related to spreading of other noxious toxins, for example dermonecrotic protein, since it evokes blood vessel wall instability and increasing permeability.

The inhibitory effects of 1,10-phenanthroline on entactinolytic activity, and the disruption effect toward EHS-basement membranes of venom, indicate the participation of metalloproteinase(s) in these activities and points to the possibility of a metalloproteinase-inhibitor based therapy in Loxoscelism.

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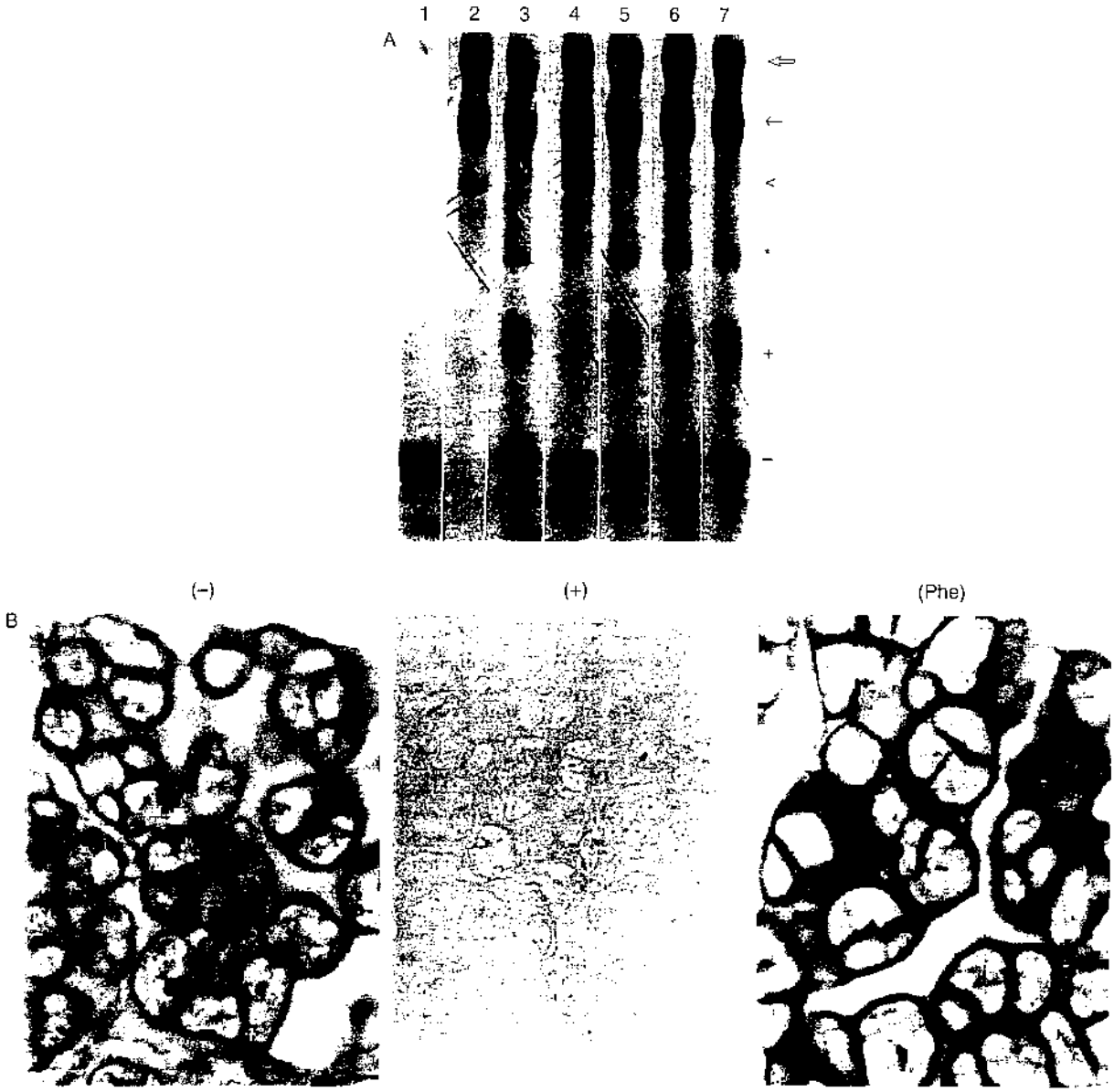

Figure 7. Inhibitory action of 1,10-phenanthroline on the proteolytic properties of *L. intermedia* venom. (A) Purified laminin–entactin complex was incubated overnight (at the same experimental conditions as described for Figure 4) with venom in presence of different protease inhibitors: phenanthroline (lane 4), PMSF (lane 5), NEM (lane 6) and pepstatin-A (lane 7). Lane 1 shows only venom, lane 2 depicts only laminin–entactin and lane 3 laminin–entactin in presence of venom and absence of inhibitors. Open arrow shows α_1 laminin chain, closed arrow β_1 and γ_1 laminin chains, arrow head intact entactin, asterisk 100 kDa entactin fragment, plus sign 50 kDa entactin fragment and minus sign major venom proteins position. (B) EHS-tissue sections were incubated with *L. intermedia* venom overnight at 37 C (+), with PBS as negative control (−) and with venom in presence of 1,10-phenanthroline (Phe) and then stained for glycoproteins (PAS). Magnification of micrographs 400×.

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Proteolytic effect of *Loxosceles intermedia* (brown spider) venom proteins on EHS-basement membrane structures

Efeito proteolítico do veneno de *Loxosceles intermedia* (aranha marrom) sobre estruturas da membrana basal de EHS

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Loxoscelism is mainly characterized by two typical clinical signals, *i.e.*, a dermonecrotic lesion and systemic effects. The dermonecrotic lesions appear at the bite site, with erythema, edema, and a local dermal haemorrhage, that can evolve to the formation of necrotic sore of difficult cicatrization with degenerative implications for the affected tissue (FORRESTER, BARRET & CAMPBELL, 1978; FUTRELL, 1992). Systemic effects are characterized by fever, malaise and bleeding that can evolve to hemolysis, thrombocytopenia, disseminated intravascular coagulation, and renal failure (KURPIEWSKI *et al.*, 1981; BASCUR, YEVENES & BORGIA, 1982; REES *et al.*, 1988; TAMBOURGI *et al.*, 1995).

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Dermonecrotic lesions, thrombocytopenic activity, disseminated intravascular coagulation and renal failure are events that could be ascribed to the presence of proteolytic enzymes that degrade extracellular matrix molecules like fibronectin and fibrinogen (FEITOSA *et al.* 1998; VEIGA *et al.* 1998, *in press*) and especially the basement membrane of subendothelial capillary cells with a consequent reduced stability of the vessel wall, changing the normal surroundings for platelet adhesion and aggregation or altering the integrity of the glomerular basement membrane involved in the filtering of molecules from blood to urine (FARQUHAR *et al.* 1991; ROHRBACH & TIMPL, 1993), as demonstrated in haemorrhage produced by snake bites (BARAMOVA *et al.* 1986; HITE *et al.* 1992). The existence of proteolytic enzymes such as metalloproteases, hyaluronidases and sphingomyelinase has been demonstrated to be present in brown spiders venom (FORRESTER, BARRET & CAMPBELL, 1978; KURPIEWSKI *et al.*, 1981; FEITOSA *et al.*, 1998; REKOW, CIVELLO & GREEN, 1983). Renal failure with proteinuria provoked by brown spider bites represents a potential action of currently unknown venom principles on renal tissue and on the renal extracellular matrix (with special emphasis on the glomerular basement membrane that acts on renal physiology like a selective barrier, establishing a filtering action between blood and urine formation).

ECM, which is structurally separated into basement membrane, connective matrix and plasma matrix, is remarkable for its complex structure consisting of secreted proteins and glycoconjugates that create a molecular network when tridimensionally assembled (YURCHENKO & SCHITTNY, 1990). ECM interactions with receptor molecules on the cell surface support several biological processes such as cell adhesion, locomotion and differentiation, playing an important physiological role in the homeostatic functions of tissues (ALBELDA & CLAYTON, 1990; MEREDITH, FAZELI & SCHWARTZ, 1993; VEIGA *et al.*, 1997). Basement membranes (a specialized kind of ECM) acts like a biomolecular filter separating many specialized tissues such as muscle, epithelial, endothelial, and nervous tissue from the respective connective tissues (MARTIN & TIMPL, 1987). Although they are widely disseminated in dif-

ferent tissues of organism, their molecular features are highly conserved. Basement membranes are composed of four main molecules, *i. e.*, laminin, type IV collagen, entactin and heparan sulfate proteoglycan (YURCHENCO & SCHITTNY, 1990; TIMPL, 1996). Basement membranes promote cell differentiation and neurite outgrowth, are involved in angiogenesis, platelet adhesion, blood-urine filtration in the kidney glomerulus where urine is formed, and also perform several other functions (FARQUHAR, 1991; ROHRBACH & TIMPL, 1993; TIMPL *et al.*, 1987). The action of molecular constituents of brown spider venom on basement membranes could explain the effects observed in Loxoscelism, with emphasis on haemorrhagic processes (subendothelial blood vessel basement membrane) and renal failure (glomerular basement membrane). In the present study, we checked the possibility that *L. intermedia* venom could act directly on basement membrane structures. Engelbreth-Holm-Swarm (EHS) tumor was used, a sarcoma producing large amounts of basement membrane that has been used for the last years as the most useful model to study this specialized ECM because of the easy extraction of basement membranes and their constituents and also because of the conserved characteristics of the latter, which render them similar to normal adult mammalian basement membrane structures (TIMPL *et al.*, 1987; TIMPL *et al.*, 1979). The EHS tissues were fixed in modified Karnovsky's fixative (KARNOVSKY, 1965) for 1 h. After fixation, the tissues were washed in 0.1 M cacodylic acid buffer, pH 7.3, and incubated with PBS or *Loxosceles intermedia* venom/PBS (100 µg/ml) overnight with shaking at 37°C and postfixed in 1 % OsO_4 in 0.1 M cacodylic acid buffer, pH 7.3, for 1 h. They were then dehydrated in ethanol, critical-point dried, sputter-coated with gold and examined with a MEV XL-30 Philips scanning electron microscope. EHS tissue incubated with PBS served as negative controls. Figure 1A shows an EHS tissue that was incubated with PBS (arrow points the basement membrane that is a capsule of tumor), and Figure 1 B shows an EHS tissue that was incubated with brown spider venom. A clearly visible disruption of the basement membrane (arrow) can be seen in EHS sections treated with venom, indicating that some of the constituents of the base-

ment membrane are degraded by the enzymes found in venom.

For transmission electron microscopy the tissues were fixed in modified Karnovsky's fixative (KARNOVSKY, 1965) for 2 h. After fixation, the tissues were washed in 0.1 M cacodylic acid buffer, pH 7.3, postfixed in 1 % OsO_4 in 0.1 M cacodylic acid buffer, pH 7.3, for 1 h, dehydrated with ethanol and propylene oxide, and embedded in Epon. Thin sections and ultrathin sections were then cut with a diamond knife on an LKB ultramicrotome. Ultrathin sections were incubated with PBS (Figure 2A) or *Loxosceles intermedia* venom/PBS (100 µg/ml) (Figure 2B) overnight at 37° C under humidified conditions. After incubation, these ultrathin sections were contrasted with uranyl acetate and lead citrate and examined with a JEOL-JEM 1200 EX II transmission electron microscope at an accelerating voltage of 80 KV. We can see extensive destruction of the basement membrane treated by venom, compared with negative control (arrows).

Paraffin-embedded EHS-tissue sections mounted on glass slides were deparaffinized in xylene overnight and rehydrated in a graded ethanol series and water (BEÇAK & PAULETE, 1976). The EHS-tissue sections were incubated with PBS (control) (figure 3A) or with *Loxosceles intermedia* venom (100 µg/ml) (Figure 3B) overnight at 37° C under humidified conditions. The EHS-sections were then washed with PBS and incubated in 3 % H_2O_2 at room temperature for 15 min to inhibit the activity of endogenous peroxidase, washed with PBS and nonspecific protein-binding sites were blocked with 1 % bovine serum albumin in PBS at room temperature for 30 min under humidified conditions. After washing in PBS, EHS-sections were incubated with a primary polyclonal anti-laminin antibody (Rb_1aLN) diluted 1:500 overnight at 4° C. Excess antibody was removed with PBS and incubated with goat anti-rabbit IgG peroxidase conjugate (Sigma) diluted 1:100. Following further washing in PBS, diaminobenzidine was used to visualize the immunoreactivity. Sections were washed in PBS and water, dehydrated in ethanol, cleared in xylene, and mounted in Entellan. We can see a reduction in the staining profile in EHS section treated with venom, compared to control, and a basement membrane disruptive effect (arrows).

Finally, in the figure 4, the major non-collagenous basement membrane molecules represented by the laminin-entactin dimer complex (0.5 mg diluted in 50 mM Tris-HCl buffer, pH 7.3, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$) purified from EHS tumors produced in 2-month-old C57-BL10 female mice as described (23) were incubated with 100 µg of *Loxosceles intermedia* venom for a period of 16 hours at 37°C (lane 1), or were incubated with PBS under the same experimental conditions as negative control (lane 2); Lane 3 shows the electrophoretic positions of the major proteins of venom. These samples were analysed by linear gradient 3-15 % SDS-PAGE under reducing conditions and stained with Coomassie Blue R for visualization (LAEMMLI, 1970). Figure shows that the α1 laminin chain (open arrow) and the β1/γ1 laminin chains that comigrate (closed arrow) were not cleaved by the venom. However, the protein pattern of the entactin chain (arrowhead), a molecule of 150 kDa, was reduced after venom treatment, and fragments of approximately 100 kDa (minus signal) and 50kDa (plus signal) can be seen. Entactin interacts with the other three major molecules of the basement membrane (laminin, type IV collagen and heparan sulfate proteoglycan), and has the important function of stabilizing this ECM structure.

Based on these results and on the cleavage of entactin by the brown spider venom constituents, we may propose that the basement membrane-disrupting effect of the venom is a possible and plausible mechanism for haemorrhage and renal failure, which, together with other anticoagulant properties of the venom (FUTRELL, 1992; KURPIEWSKI *et al.*, 1981; FEITOSA *et al.*, 1998) provides a plausible mechanism for the toxicity of brown spider venoms.

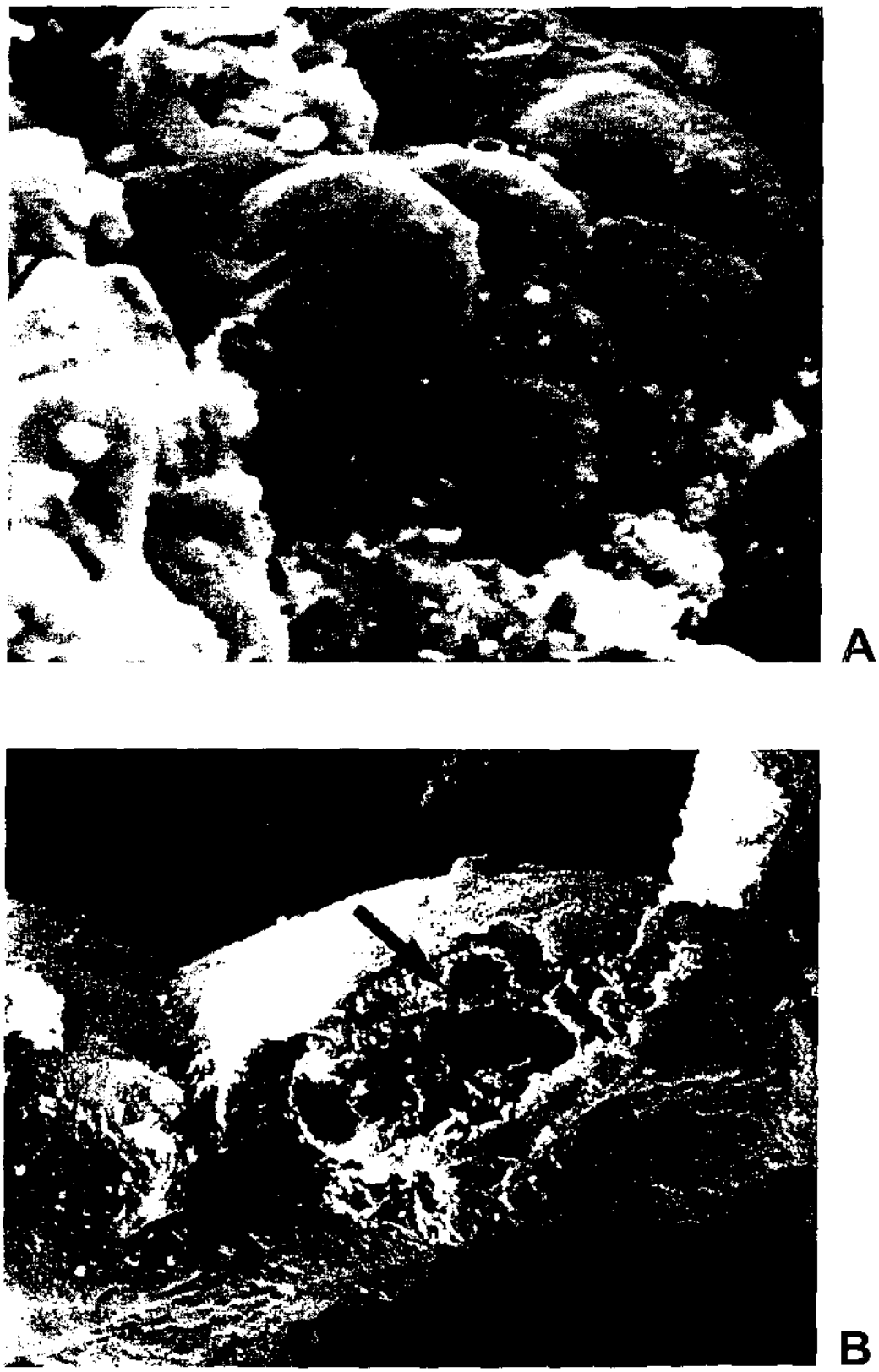

Fig. 1. Effects of *Loxosceles intermedia* venom on EHS visualized by scanning electron microscopy. A, EHS basement membrane (arrow) incubated with PBS (negative control) overnight at 37°C; B, EHS basement membrane treated with *L. intermedia* venom under the same experimental conditions as described above. The arrow points at an area where the basement membrane was disrupted by the action of the venom.

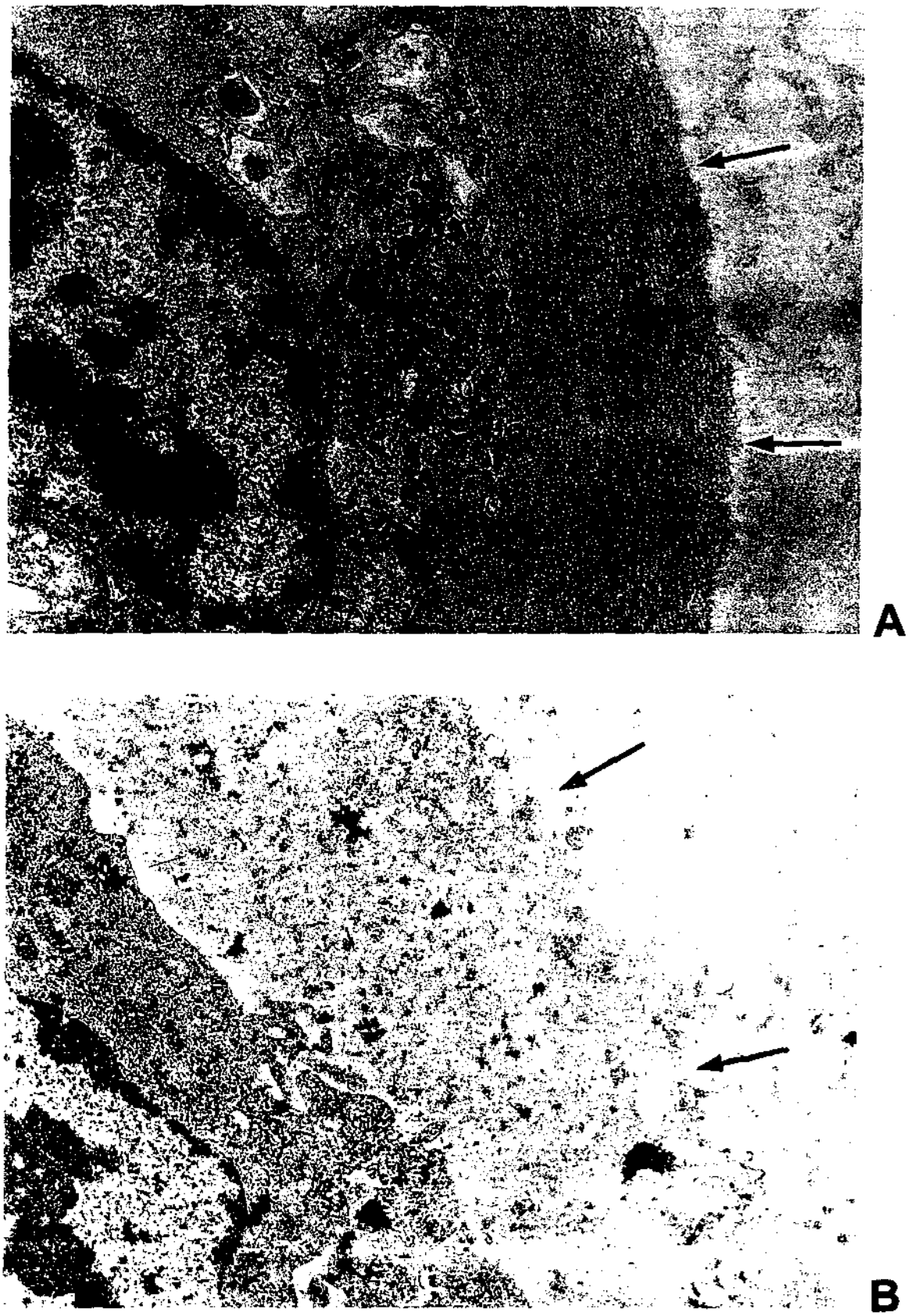

Fig. 2. Effects of *Loxosceles intermedia* venom on EHS visualized by transmission electron microscopy. A, EHS basement membrane treated overnight with PBS (negative control) at 37° C. The arrow indicates the intact basement membrane; B, EHS basement membrane that was incubated with *L. intermedia* venom under the same experimental condition. The arrow points at the basement membrane that was clearly fragmented by the venom.

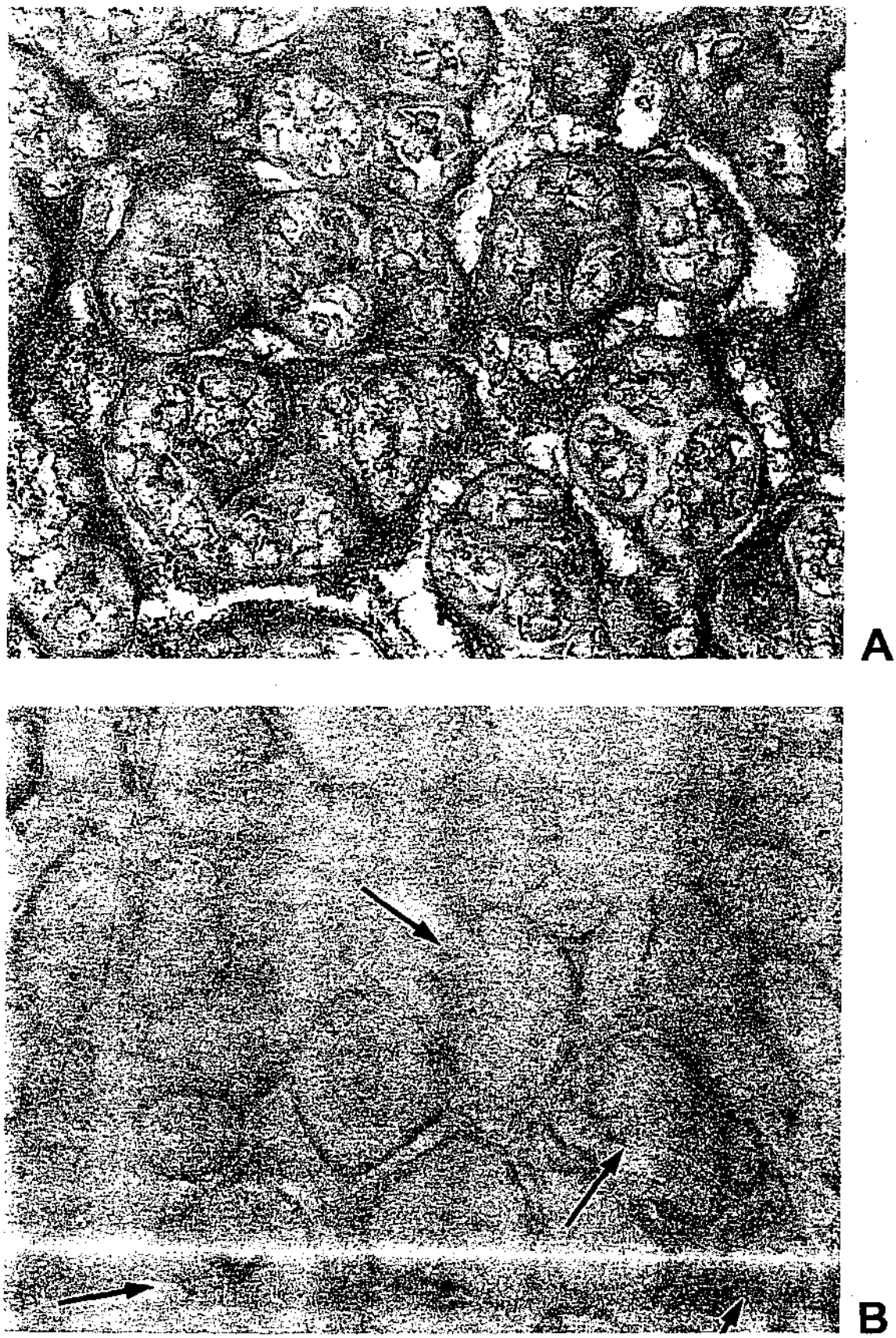

Fig. 3. Effects of *Loxosceles intermedia* venom on EHS visualized by light microscopy. A, EHS basement membrane incubated with PBS at 37° C as control and visualized by an immunohystochemistry using a rabbit polyclonal antibody against laminin; B, EHS basement membrane treated overnight with *L. intermedia* venom under the same experimental conditions. The arrows point at the basement membrane that was fragmented by the venom.

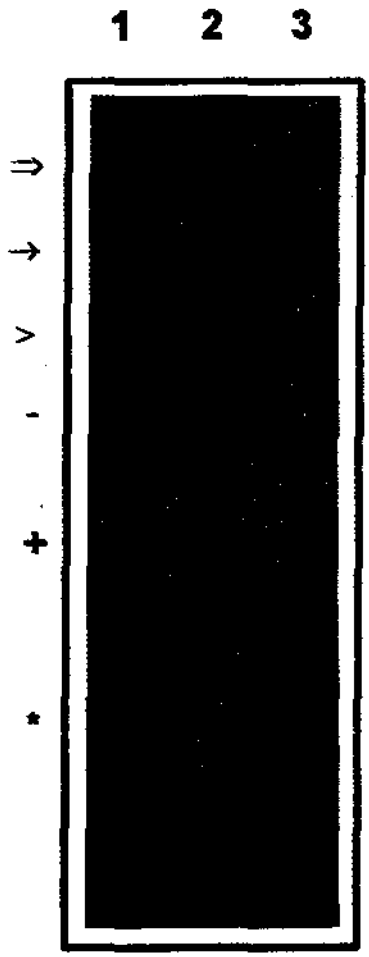

Fig. 4. Action of *Loxosceles intermedia* on purified laminin-entactin dimer complex. Laminin-entactin complex purified from EHS tumor was incubated with *Loxosceles intermedia* venom or PBS for 16 hours at 37° C. Lane 1 shows the complex treated with venom; Lane 2 shows the complex treated with PBS (negative control); and Lane 3 shows only venom. The open arrow points at the $\alpha 1$ laminin chain; the closed arrow points at the $\beta 1/y1$ laminin chains that comigrate; the arrowhead indicates entactin; the minus signal indicates the entactin fragment (100 kDa) that was produced by the action of the venom; plus signal indicates entactin fragments of approximately 50kDa (an asterisk indicates the venom profile).

SUMMARY

The envenomation induced by bites of spiders of the genus *Loxosceles* (brown spider) is known as Loxoscelism and is remarkable for a dermonecrotic lesion and systemic effects. These events are probably attributable to the presence of proteolytic enzymes in brown spider venom that degrade extracellular matrix (ECM) constituents. The objective of the present study was to determine whether brown spider venom can act on the basement membrane, a specialized kind of ECM. Using

Engelbreth-Holm-Swarm (EHS) tissue sections treated with brown spider venom and analysed by scanning and transmission electron microscopy and light microscopy, we detected a clearly visible destruction of the basement membrane structure. Using purified laminin-entactin complex, two major constituents of basement membranes, treated with venom and analysed by SDS-PAGE, we detected a partial degradation of the entactin molecule. The degradation of this molecule and the basement membrane disruption activity appear to be a plausible mechanism for some of the systemic effects triggered by the venom, as renal failure and haemorrhage.

Key words: *Loxoceles*, loxoscelism, venom proteins.

RESUMO

O envenenamento induzido por acidentes com aranhas do gênero *Loxosceles* (aranha marrom) é conhecido como Loxoscelismo e é característico por uma lesão dermonecrótica e efeitos sistêmicos. Estes eventos são atribuídos provavelmente à presença de enzimas proteolíticas no veneno desta aranha que degrada constituintes da matriz extracelular (MEC). O objetivo do presente estudo foi determinar o quanto o veneno da aranha marrom pode agir na membrana basal, um tipo especializado de MEC. Usando cortes de tecido de Engelbreth-Holm-Swarm (EHS) tratados com o veneno da aranha marrom e analisados por microscopia eletrônica de transmissão, varredura e microscopia de luz, detectamos uma destruição visível da estrutura da membrana basal. Usando complexo laminina-entactina purificado (dois dos principais constituintes das membranas basais) tratados com veneno e analisados por SDS-PAGE, detectamos uma degradação parcial da molécula de entactina. A degradação desta molécula e a atividade de rompimento da membrana basal aparenta ser um mecanismo plausível para alguns dos efeitos sistêmicos ativados pelo veneno, como a deficiência renal e hemorragia.

PALAVRAS CHAVE: *Loxoceles*, loxoscelismo, veneno.

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THROMBOSIS RESEARCH

REGULAR ARTICLE

In Vivo and In Vitro Cytotoxicity of Brown Spider Venom for Blood Vessel Endothelial Cells

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Abstract

The effect of brown spider (*Loxosceles intermedia*) venom on endothelial cells was investigated in vivo and in vitro. Morphological and ultrastructural observations by light microscopy and transmission electron microscopy showed that the venom acts in vivo upon vessel endothelial cells of rabbits that were intradermally injected, evoking vessel instability, cytoplasmic endothelial cell vacuolization, and blebs. Likewise, treatment of rabbit endothelial cells in culture with the venom led to loss of adhesion of the cells to the substrate. Endothelial cells in culture were metabolically radiolabeled with sodium [^{35}S]-sulfate and the sulfated compounds (proteoglycans and sulfated proteins) from medium, cell surface, and extracellular matrix (ECM) were analyzed. Agarose gel electrophoresis and SDS-PAGE showed that the venom is active on the ECM and on cell surface proteoglycans, shedding these molecules into the culture medium. In addition, when purified heparan sulfate proteoglycan (HSPG) and purified laminin–entactin (LN/ET) complex were incubated with the venom we observed a partial degradation of the protein core of HSPG as well as the hydrolysis of entactin. The above results suggest that the *L. intermedia* venom has a deleterious effect on the endothelium of vessels both in vivo and in culture, removing important constituents such as HSPG and entactin that are involved in the adhesion of endothelial cells and of subendothelial ECM organization.

Key Words: Brown spider; Venom; Blood vessel; Endothelial cells

The mechanisms of hemostasis are dependent on several factors that act synergistically to control circulating blood fluidity. Both muscular and endothelial cells on blood vessel walls are required for the normal arrest of hemorrhage [1]. The components of the subendothelial basement membrane as well as other adhesive extracellular matrix (ECM) proteins are also involved in hemostasis, since they are required for adhesion of circulating platelets or vessel stability [1]. The circulating platelets, through their receptors for subendothelial adhesive proteins, adhere to exposed ECM at sites of vascular injury triggering the initiation of thrombus formation [2]. In small blood vessels, subendothelial matrix and platelets can arrest bleeding, controlling local hemorrhage. Systemic bleeding requires in addition blood coagulation factors, which lead to the formation and removal of fibrin clots at sites of vascular injury [3,4]. Disruption of

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any component of this complex system may be responsible for thrombotic or bleeding disorders. Snake venoms, especially those of the Viperidae or Crotalidae families, are responsible for hemostasis disorders. These venoms contain several principles such as enzymes or nonenzyme proteins that are associated with local and/or systemic hemorrhage. Thrombin-like enzymes and metalloproteinases are widely distributed in snake venoms and have been associated with these bleeding alterations [5–7].

Loxosceles spp. (brown spiders) are of great medical importance throughout the world. They are responsible for loxoscelism, a term used to describe the clinical signals evoked by their bite [8]. Brown spider envenomations may result in two major groups of signals, i.e., local signals at the bite site characterized by erythema, swelling, hemorrhage into the dermis, necrotic lesion with gravitational spreading, and in some cases delayed wound healing, besides systemic signals, which are less common than the cutaneous ones, but generally are the cause of deaths. They are fever, malaise, weakness, vomiting, hemolytic anemia, thrombocytopenia, disseminated intravascular coagulation, hemorrhage, and renal failure [8–10].

Hemostatic problems induced by *Loxosceles* spp. venoms such as platelet aggregation disorders, disseminated intravascular coagulation, and hemorrhage into the dermis could be ascribed to the presence of toxins that alter the integrity of vessel walls, especially at the level of endothelial cells and subendothelial matrix [11–13].

We now report the effect of *Loxosceles intermedia* venom on endothelial vessel walls and endothelial cells in culture. Of particular significance were the findings that the venom alters the integrity of vessel walls by partially degrading the protein core of the heparan sulfate proteoglycan (HSPG) as well as entactin, two major glycoproteins of the basement membranes.

1. Materials and Methods

1.1. Reagents

Laminin–entactin (LN/ET) dimer was purified from Engelbreth–Holm–Swarm (EHS) tumor as described by Timpl et al. [14]. For HSPG purification, radiolabeled endothelial cells were solubilized with lysis buffer (50 mM Tris–HCl, pH 7.3, 1% Triton X-100, 50 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM PMSF, and 2 μg/ml aprotinin) for 15 min at 4°C. The extract was clarified by centrifugation for 10 min at $13{,}000 \times g$ and frozen until use. The radiolabeled HSPG was purified by a combination of gelatin affinity chromatography, gel filtration, and ion-exchange chromatography as previously described [15].

1.2. Cell Culture

Established endothelial cells [16] were maintained in F12 medium supplemented with 10% fetal calf serum (FCS) at 37°C, in the presence of 2.5% CO_2. Confluent endothelial cells were metabolically labeled for 18 h with [^{35}S]-sulfate (150 μCi/ml). The cells were washed twice with F12 medium and then exposed to the venom (40 μg/ml) in F12 for 3 h. The cells were washed twice with PBS and the sulfated compounds (proteoglycans and sulfated glycoproteins) from medium, cell surface, and ECM were analyzed. The cell surface was removed with 10 mM Tris–HCl, pH 8.4, containing 0.5% Triton X-100 (5 min, 4°C). The dishes were then washed with 25 mM Tris–HCl, pH 8.4, in 3.5 M urea. This fraction contains the ECM proteins [17]. In some instances, confluent endothelial cells were exposed to the venom for different periods of time. The cells in suspension were washed twice with F12 medium by centrifugation ($1000 \times g$, 10 min) and again plated. The cells that remained adhered to the dish were washed twice with F12 medium, removed from the dishes with pancreatin (Sigma, St. Louis, MO) and again plated onto petri dishes and maintained for 72 h in F12 medium supplemented with 10% FCS at 37°C, in the presence of 2.5% CO_2.

1.3. Spider Venom Extraction

The venom was extracted from the stings of spiders by electrostimulation (15 V) applied to the cephalothorax and collected with a micropipette, dried under vacuum, and frozen at

−85°C until use. A pool of venom collected from approximately 5000 spiders was used in the experiments [11,18].

1.4. In Vivo Studies on Rabbits

A sample of 40 μg of proteins diluted in PBS was injected intradermally into a shaved area of rabbit skin. After 4 h of venom exposure, the animals were anesthetized with sodium pentobarbital, sacrificed, and processed for histology as shown below.

1.5. Transmission Electron Microscopy

Tissues were fixed with modified Karnovsky's fixative [19] for 2 h, washed in 0.1 M cacodylic acid buffer, pH 7.3, postfixed in 1% OsO_4 in 0.1 M cacodylic acid buffer, pH 7.3, for 1 h, dehydrated with ethanol and propylene oxide, and embedded in Epon 812. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined with a JEOL-JEM 1200 EX II transmission electron microscope at an accelerating voltage of 80 kV.

1.6. Histological Methods for Light Microscopy

Tissues were fixed in modified Carnoy's fixative [20] for 3 h. After fixation, tissues were processed for histology, embedded in paraffin, and cut into 4-μm thick sections. The sections were stained with hematoxylin–eosin and analyzed by light microscopy.

1.7. Gel Electrophoresis

SDS-PAGE were performed as described by Laemmli [21]. Samples were applied to linear gradients of 3–12% or 3–15% (w/v) polyacrylamide gels under reducing or nonreducing conditions (see legends). After electrophoresis, the gels were dried and exposed to X-ray films. The molecular mass markers used were myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase b, 98 kDa; albumin, 67 kDa; ovalbumin, 44 kDa, and carbonic anhydrase, 29 kDa (Sigma). For glycosaminoglycan (GAG) studies agarose gels were performed as described by Dietrich and Dietrich [22]. After drying, the gels were stained with toluidine blue and exposed to X-ray films (X-Omat, Kodak) for 10 days at room temperature. The GAG standards used were heparan sulfate from bovine pancreas [23], dermatan sulfate from pig skin, and chondroitin sulfate from shark cartilage (Seikagaku, Kogyo, Tokyo, Japan).

1.8. Degradation of Purified Endothelial HSPG and Purified LN/ET Complex by L. intermedia Venom

For HSPG, the hydrolytic effect of the venom was assessed using 10,000 cpm of radiolabeled [$^{35}SO_4$]-HSPG incubated with 10 μg of venom at 37°C in a final volume of 50 μl. Aliquots were collected after 30 min and at 1, 2, 4, 8, and 18 h and the proteolytic effect of venom was stopped by freezing the samples at −20°C. As control, [$^{35}SO_4$]-HSPG was incubated under the same conditions but in the absence of venom. Samples were subjected to linear gradient 3–12% SDS-PAGE under nonreducing conditions, and the gel was stained with Coomassie blue, dried, and exposed to X-ray film at room temperature for 10 days.

For LN/ET digestion studies, the purified EHS-LN/ET (500 μg) was incubated with *L. intermedia* venom (100 μg) at 37°C. Aliquots were collected at 0 and 30 min, and at 1, 2, 4, 8, 12, and 24 h of incubation and the proteolysis was stopped by freezing the samples (−20°C). As control we collected a sample of purified LN/ET after 24 h but in the absence of venom. Samples were subjected to 3–15% linear gradient SDS-PAGE under reducing conditions and the proteolytic effect of the venom was assessed by Coomassie blue staining.

1.9. Quantification of Radioactive GAGs

Radiolabeled GAGs from cell surface, culture medium, and ECM from control cells or venom-treated cells obtained as described above were analyzed by agarose gel electrophoresis. The radioactivity [$^{35}SO_4$] incorporated into GAGs was determined by scraping the gel and counting in a scintillation counter using 0.5% PPO in toluene. All experiments were performed in triplicate.

2. Results

2.1. Cytotoxicity of L. intermedia Venom on Blood Vessel Endothelial Cells in Biopsies From Rabbit Skin

Based on results of experimental envenomations of rabbits using *Loxosceles laeta* venom [24] and *Loxosceles reclusa* venom [8] showing that the pathological signals of loxoscelism, edema, and hemorrhage into the dermis occur between 2 and 4 h after envenomation, we studied the behavior of blood vessel walls and endothelial cells from the skin of rabbits injected with *L. intermedia* venom, 4 h after envenomation. Fig. 1 shows the histopathological changes induced by the venom observed by light and transmission electron microscopies. Fig. 1B, C, E, and F depict the changes induced by the venom in the blood vessels, with the occurrence of subendothelial blebs, vacuoles, and degeneration of blood vessel walls. Fig. 1A and D show normal vessel endothelial cells from rabbit skin used as control (injected with PBS).

2.2. Loss of Adhesion of Rabbit Endothelial Cells in Culture Exposed to L. intermedia Venom

Endothelial cells were maintained in the presence of the venom (Fig. 2). After 3-h exposure (Fig. 2B), it is possible to note the loss of adhesion of some cells. After 6- (Fig. 2C) and 18-h (Fig. 2D) exposures, 50% and 95% of the cells are round and most of the cells are in suspension, respectively. Fig. 2A represents control cells in the absence of venom. The cells in suspension, after venom treatment for 6 and 18 h, as well as the cells that remained in the dishes after venom treatment for 18 h were again plated (Fig. 3). Examination of the cells that were in suspension for 6 and 18 h (Fig. 3A and B) or still in the dishes after 18 h (Fig. 3D) of venom treatment, 72 h after plating showed that they were adhered to the petri dishes and growing normally like the control cells (Fig. 3C). Trypan blue uptake studies showed that both the cells in suspension and adhered to the plates after 18-h treatment were 95% to 97% viable (data not shown).

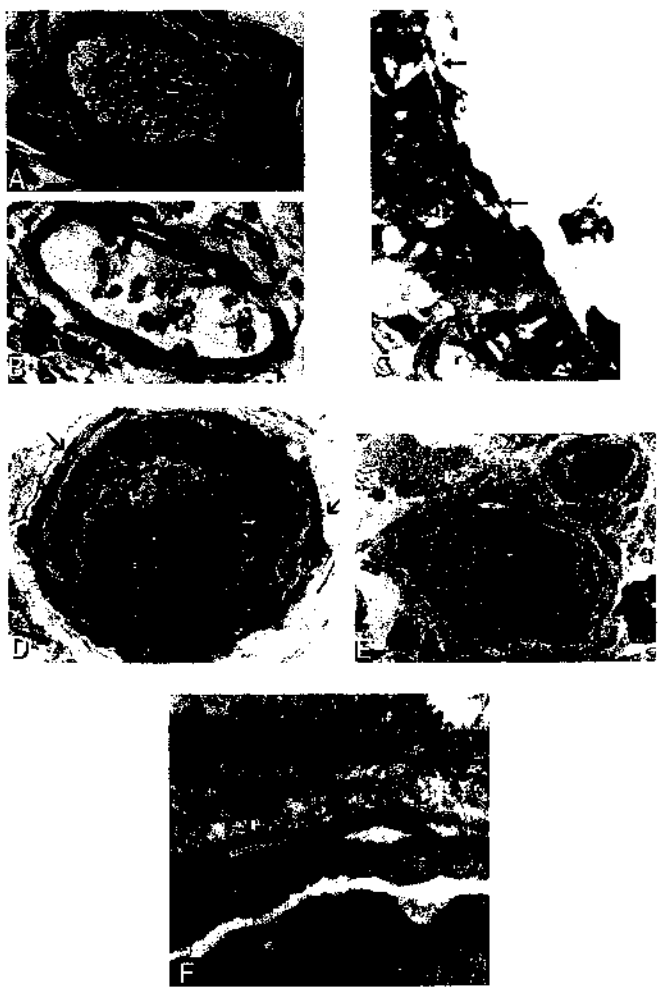

Fig. 1. Histological studies of blood vessel walls from skin of rabbits injured with *L. intermedia* venom. (A) Light micrograph (× 400) stained with hematoxylin–eosin of a normal blood vessel from rabbit skin (arrows point at endothelial cells). (B) Light micrograph (× 400) of a blood vessel from the skin of a rabbit injured with *L. intermedia* venom showing a subendothelial bleb (arrow). (C) Light micrograph at high magnification (× 1000) of a blood vessel from the skin of a rabbit injured with venom showing several subendothelial blebs and vacuoles (arrows). (D) Transmission electron micrograph of a normal blood vessel from rabbit skin (× 6000); L, lumen of the blood vessel; N, nuclei; C, cytoplasm of endothelial cells; arrows point to the subendothelial basement membrane. (E and F) Electron micrographs of a blood vessel from the skin of a rabbit injured with venom. Note the degeneration of the blood vessel wall and some subendothelial blebs (arrows); L, lumen of the blood vessel; N, nuclei of endothelial cells; asterisk shows leukocyte interacting with an endothelial cell (E, × 4000 and F, × 20,000 magnification).

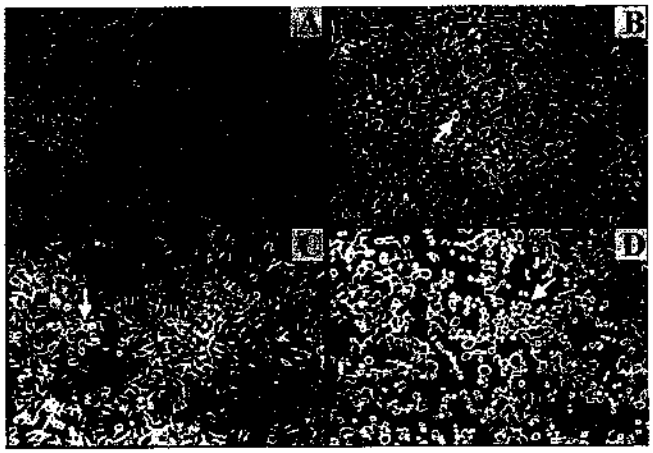

Fig. 2. Loss of adhesion of rabbit aorta endothelial cells after venom exposure. Endothelial cells were exposed to the venom for 3 (B), 6 (C), and 18 h (D). The arrows indicate rounded cells in suspension. (A) Controls cells maintained for 18 h in the absence of the venom.

2.3. *Effect of L. intermedia Venom on Sulfated Polymers From Different Cellular Components of Endothelial Cells in Culture*

To ascertain the cytotoxicity of *L. intermedia* venom on endothelial cells, experiments were performed with endothelial cells, which were radiolabeled with [^{35}S]-sulfate for 24 h and exposed to 40 μg/ml of the venom for 4 h at 37°C. ECM, cell surface, and culture medium were separated from the venom-treated cells as well as from the control cells. The sulfated polymers were extracted from these compartments and subjected to agarose gel electrophoresis (Fig. 4). A decrease of the sulfated polymers of the ECM and cell surface was observed in the venom-treated cells. This decrease was accompanied by an increase of the polymers in the medium from the venom-treated cells. To characterize the nature of the sulfated polymers, the polymers were incubated with a nonspecific proteolytic enzyme (maxatase), and again subjected to agarose gel electrophoresis (Fig. 5A). Only one radioactive component was observed with the migration of heparan sulfate. A decrease of heparan sulfate from the ECM and cell surface and an increase of the compound in the culture medium from the venom-treated cells were observed. Fig. 5B shows the quantitative analysis of this experiment. We may conclude that

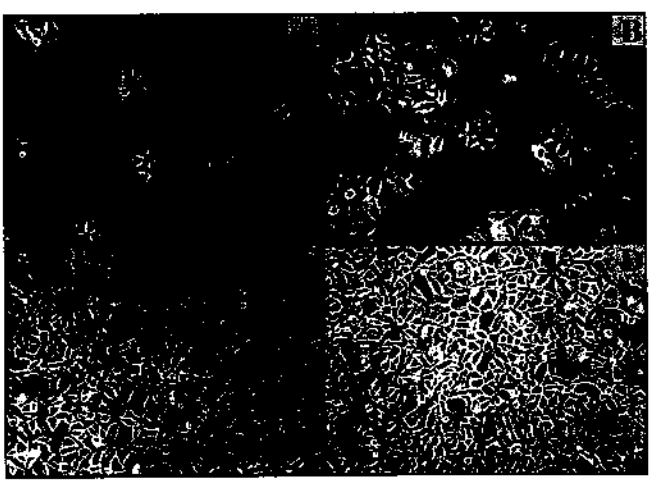

Fig. 3. Recovery of endothelial cells after venom exposure. The cells in suspension (Fig. 2) 6 (A) and 18 h (B) after venom treatment were washed twice with F12 medium by centrifugation (1000 × *g*, 10 min) and again plated. The cells that remained adhered to the plate after 18 h of venom treatment (C) as well as control cells (D) were washed twice with F12 medium, removed from the dishes with a proteolytic enzyme and again plated onto petri dishes and maintained for 72 h in F12 medium supplemented with 10% FCS at 37°C, in the presence of 2.5% CO_2.

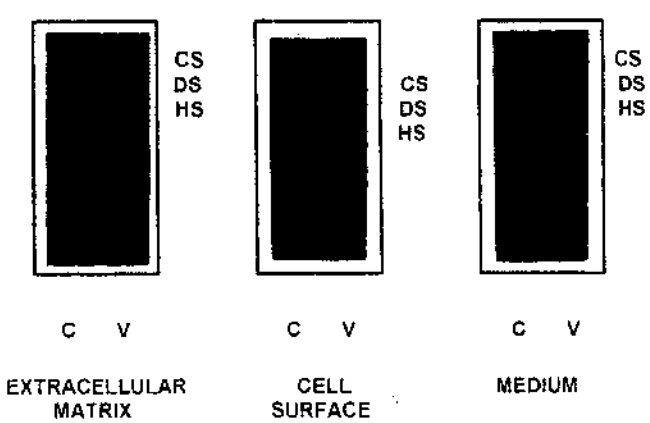

Fig. 4. Effect of *L. intermedia* venom on sulfated polymers from different cellular compartments of endothelial cells. The endothelial cells were labeled with [^{35}S]-sulfate and then exposed for 4 h to the venom or not exposed. Aliquots normalized for volumes of ECM, the cell surface, and culture medium were submitted to agarose gel electrophoresis, dried, and exposed to X-ray film. C, control fractions; V, venom-treated fractions; HS, heparan sulfate; DS, dermatan sulfate; and CS, chondroitin sulfate standard mobilities. For further details see Materials and Methods.

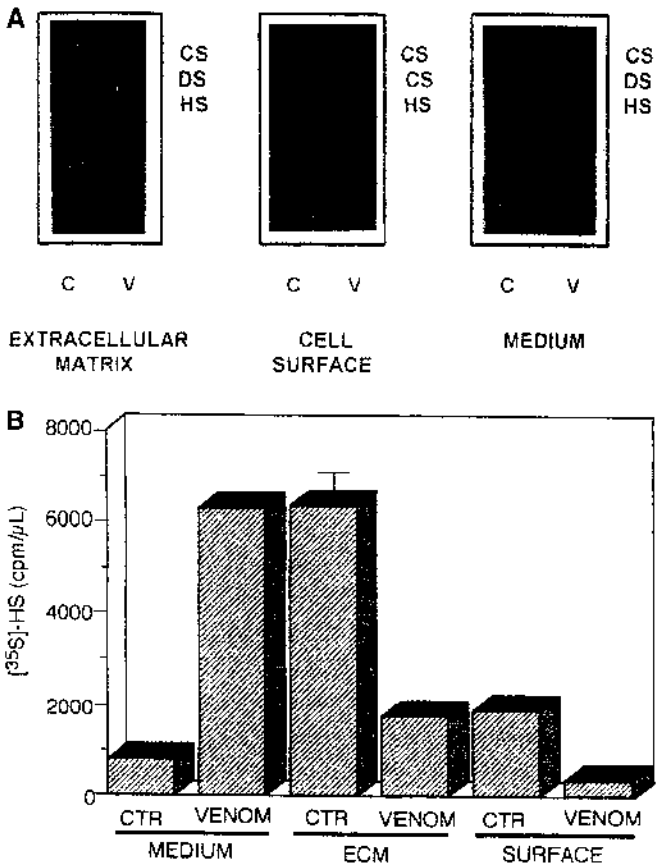

Fig. 5. Activity of *L. intermedia* venom toward proteoglycans from different cellular compartments of endothelial cells. (A) Endothelial cells were radiolabeled and treated with venom as described in Materials and Methods. Aliquots of the same volume of ECM, cell surface, or medium were digested with maxatase and the free GAGs originated were submitted to agarose gel electrophoresis, and the gels were then dried and exposed to X-ray films. C, control fractions; V, venom-treated fractions; HS, heparan sulfate; DS, dermatan sulfate; and CS, chondroitin sulfate standard positions. (B) The radioactivity incorporated into heparan sulfate was quantified as described in Materials and Methods. The results represent the mean of three individual experiments. CTR (control), samples in the absence of venom; VENOM, samples incubated with venom; MEDIUM, culture medium; ECM, extracellular matrix; SURFACE, cell surface.

most of the [^{35}S]-sulfated polymers were HSPG, which releases the free GAG chain after treatment with maxatase. We can also suggest that the venom possesses a protease that acts in a limited manner on the protein core of the HSPG releasing this compound, still as an HSPG, from the ECM and cell surface.

2.4. *Degradation of Purified HSPG From Endothelial Cells by L. intermedia Venom*

To gain more information on the nature of the action of the *L. intermedia* venom upon HSPG, [^{35}S]-HSPG from endothelial cells, purified as described in Materials and Methods, was incubated with the venom as indicated in Fig. 6. A fragment of approximately 100 kDa was formed after venom treatment, but no other fragments were formed as function of the time, suggesting that HSPG contains only one linkage susceptible to proteolysis caused by the venom.

2.5. *Degradation of Entactin in the LN/ET Complex by L. intermedia Venom*

Here, we assayed purified LN/ET complex (two major basement membrane constituent molecules) from EHS (a murine sarcoma cell, see

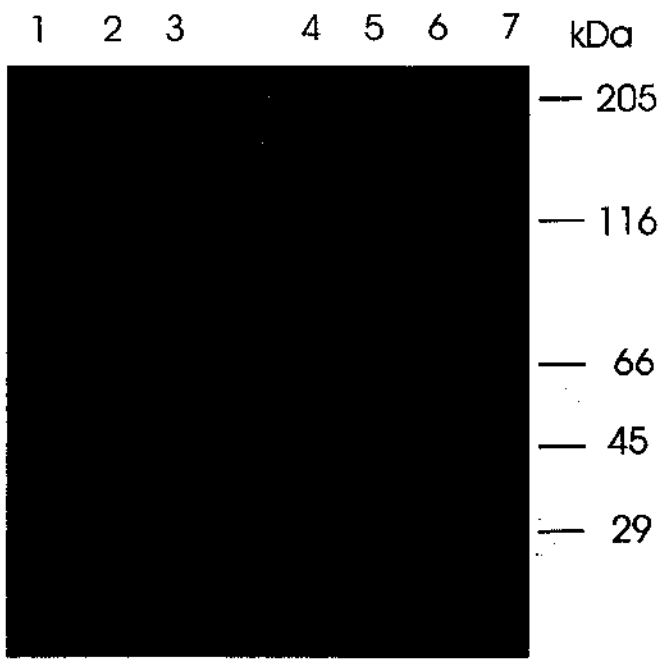

Fig. 6. Hydrolytic effect of *L. intermedia* venom on purified endothelial HSPG. HSPG purified from endothelial cells was incubated with *L. intermedia* venom for different periods of time at 37°C. Aliquots were submitted to SDS-PAGE (3–12%) under nonreducing conditions. After the run, the gel was stained with Coomassie blue, dried, and exposed to X-ray film. Lane 1, purified HSPG without venom treatment; lanes 2 to 7, respectively, HSPG exposed to the venom after 30 min and at 1, 2, 4, 8, and 18 h. Molecular mass markers are shown on the right.

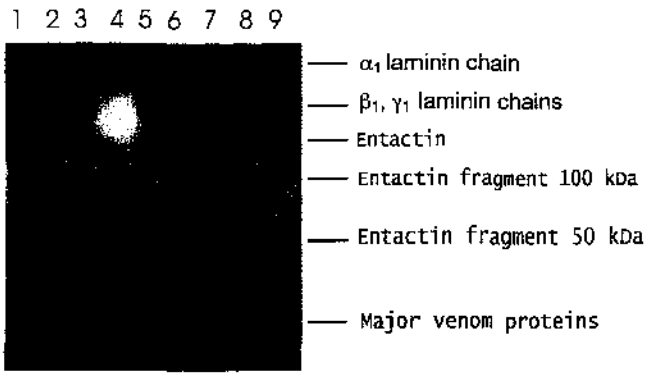

Fig. 7. Hydrolytic effect of *L. intermedia* venom on purified LN/ET dimer. LN/ET dimer purified from EHS tumor was incubated with the venom for different periods of time at 37°C as indicated. Aliquots were submitted to SDS-PAGE (3–15%) under reducing conditions. After the run, the gel was stained with Coomassie blue. Lane 1, purified LN/ET without venom treatment but incubated at 37°C for 24 h (experimental stability control); lanes 2 to 9, purified LN/ET exposed to the venom for 0 and 30 min and for 1, 2, 4, 8, 12, and 24 h.

Materials and Methods). The incubates were subjected to 3–15% SDS-PAGE (Fig. 7). Two fragments of 100 and 50 kDa were formed from entactin after 1 h on incubation or more.

3. Discussion

The hemorrhagic effects triggered by *Loxosceles* spp. venoms are currently under investigation. The venoms of different species are able to promote platelet aggregation and thrombocytopenia as evidenced for *L. reclusa* [25], *L. intermedia* [12], and *L. laeta* [26]. The platelet aggregation induced by *L. reclusa* is evoked by a low molecular mass protein characterized as sphingomyelinase D [25]. In addition, adult plasma components also appear to be required for platelet activation and aggregation [27]. Calcium or other divalent metals are required for platelet aggregation since EDTA inhibited this event [25]. Platelet activation is also an ADP release-dependent reaction [8]. On the other hand, several other active principles different from sphingomyelinase D have been identified in *Loxosceles* spp. venoms. Several enzymes such as hyaluronidase, alkaline phosphatase, phosphohydrolase, and lipase were identified in studies on *L. reclusa* venom [8]. Working with *L. intermedia* venom, we demonstrated that the venom contains metalloproteases with gelatinolytic, fibrinogenolytic, fibronectinolytic activities [11,12], and also contains two other serine proteases of 85 and 95 kDa [28].

Hemorrhagic consequences after *Loxosceles* spp. envenomation appear to be complex. Local bleeding can be evoked directly by the platelet aggregating effect, or may be a consequence of proteolytic attack of the enzymes against blood coagulation factors such as fibrinogen [11,12] or may be caused by deleterious activities of the venom on subendothelial molecules such as fibronectin [11,12], or basement membrane subendothelial structures, affecting platelet adhesion, and vessel stability [13].

Herein, by using 40 μg of proteins from venom in experimental procedures (this value represents the average concentration of venom proteins injected during accidents), we report additional evidence that brown spider venoms have deleterious activities on endothelial cells, with pathological changes important for loxoscelism, especially for endothelium-dependent vascular activities and local hemorrhage. Histological studies of skin sections processed for light or transmission electron microscopies showed subendothelial vacuoles, bleb formation, and vessel disruption 4 h after inoculation in vivo, demonstrating cytotoxic effects. Since a major characteristic of loxoscelism is an accumulation of inflammatory cells and specially polymorphonuclear leukocytes around the blood vessels infiltrated in the area of envenomation [8,24], we cannot exclude that the histopathological changes evoked in loxoscelism such as vessel instability, edema, hemorrhage, and even endothelial cell cytotoxicity such as subendothelial vacuolization and bleb formation are partially due to this massive leukocyte infiltration in the tissues around the vessels through leukodiapedesis or even leukorrhexis that eventually evokes lysis of the plasma membrane of endothelial cells. By using an endothelial cell line free of leukocytes, exposed to the venom, we were able to note the loss of adhesion of cells to the culture bottle, as well as a change in cell morphology to a round shape, confirming the direct cytotoxicity against endothelial cells. In experiments using radiolabeled venom-treated endothelial cells, we detected an

increase of HSPG into the medium and a decrease of these molecules on the cell surface and in the ECM of endothelial cells. These data were confirmed using purified HSPGs from endothelial cells incubated with the venom. It was observed that the venom is able to cleave the HSPG, originating HSPG fragments. The venom also degrade entactin from the LN/ET complex. Laminin was resistant to the hydrolytic effect of the venom, as previously shown [11,13]. The basement membranes have a particular set of proteins such as Type IV collagen, laminin, entactin, and HSPG that can bind directly to each other, originating a structure arranged as a network [29]. The degrading effects of the venom on entactin and HSPG can render the structures of the basement membrane network more susceptible to damage, together with direct venom activity upon endothelial cells, cause a disturbance of the endothelial cell adhesion to this vessel structure. It is interesting to note that experimental envenomation in rabbits originate hemorrhage into the dermis between 1 and 4 h (data not shown), that agree with the time of degrading activities of the venom upon basement membrane constituents. The above results suggest that the morphological alterations such as subendothelial vacuoles, bleb formation, and the cytotoxicity of endothelial cells evoked by the brown spider envenomation could be in part a consequence of the deleterious effects of the venom on the endothelial cell surface and subendothelial ECM, which play important roles in the organization and adherence of these cells, with the consequent occurrence of vessel instability, edema, and local hemorrhage into the dermis, venom toxins spreading to underlying tissues and delayed wound healing.

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Morphological and biochemical evidence of blood vessel damage and fibrinogenolysis triggered by brown spider venom

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The venom of the brown spider is remarkable because it causes dermonecrotic injury, hemorrhagic problems, hemolysis, platelet aggregation and renal failure. The mechanism by which the venom causes hemorrhagic disorders is poorly understood. Rabbits intradermally exposed to the venom showed a local hemorrhage starting 1 h after inoculation and reaching maximum activity between 2 and 3 days. Biopsies examined by light and transmission electron microscopy showed subendothelial blebs, vacuoles and endothelial cell membrane degeneration in blood vessels, plasma exudation into connective tissue, and fibrin and thrombus formation within blood vessels. *Loxosceles intermedia* venom incubated with fibrinogen partially degrades Aα and Bβ chains of intact fibrinogen, and significantly cleaves all Aα, Bβ and γ chains when they were separated or when fibrinogen is denatured by boiling. Proteolytic kinetic studies showed that the Aα chain is more susceptible to venom hydrolysis than the Bβ chain. The fibrinogenolysis is blocked by ethylenediamine tetraacetic acid and 1,10-phenanthroline, but not by other protease inhibitors. Human plasma incubated with the venom had coagulation parameters such as prothrombin time, activated partial thromboplastin time and thrombin time increased. Through molecular sieve chromatography, we isolated a venom toxin of 30 kDa with fibrinogenolytic activity. We propose that the local and systemic hemorrhagic disorders evoked in loxoscelism are consequences of direct venom fibrinogenolysis together with cytotoxicity to subendothelial structures and endothelial cells in blood vessels. *Blood Coagul Fibrinolysis* 13:1–14 © 2002 Lippincott Williams & Wilkins.

Keywords: brown spider, venom, hemorrhage, fibrinogenolysis

Introduction

Brown spiders (*Loxosceles* genus) are spiders of medical importance throughout the world [1,2]. *Loxosceles* venoms are a mixture of heterogeneous proteins ranging from low to high molecular mass molecules [3–5], with several of them having toxic or enzymatic activities related to the typical signs of envenomation [1,6– 9]. The hallmark of envenomation by brown spiders is a typical acute and local dermonecrotic lesion that appears some hours after envenomation. The inflammatory necrotic reaction progresses from a local erythema and edema to an impressive dermonecrosis with gravitational spreading [1,10]. In addition, during the course of skin lesion, *Loxosceles* venom also triggers systemic effects such as renal failure, intravascular hemolysis and hemorrhage, which are the most severe effects of loxoscelism associated with death in lethal accidents [1,11]. Clinical signals such as malaise, weakness, chills, fever, nausea, vomiting and convulsions, which suggest some effect at the nervous system level, have also been reported [1,11].

The hemorrhagic consequences of loxoscelism

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appear to be molecularly complex and the mechanisms by which the brown spiders cause these disorders are currently under investigation. Hemorrhage begins at the bite site inside the dermis, where histological studies have reported pronounced infiltration of polymorphonuclear leukocytes, coagulative tissue necrosis and vasculitis [1,12,13]. Preliminary studies using brown recluse spider (*Loxosceles reclusa*) venom have shown that the venom contains a sphingomyelinase D of 32 kDa involved in platelet aggregation [14]. This platelet aggregation activity has also been observed in other *Loxosceles* spider species such as *Loxosceles laeta* and *Loxosceles intermedia* venoms [8,15]. Thus, the local hemorrhage triggered by brown spider venoms can be directly attributed to thrombocytopenia, or may be the consequence of venom activity on the subendothelial extracellular matrix, as previously reported for snake venom activity on components of the subendothelial basement membrane [16]. Fibronectin and entactin are two important proteins involved in the structural and biological properties of subendothelial basement membranes [17,18], and the degrading effects of the venom on these molecules may be directly responsible for its deleterious activity on vessels during envenomation. In support of this last possibility, in a study on *L. intermedia* venom we detected a fibronectinolytic activity in the venom caused by a metalloprotease [6,7] and an entactinolytic effect also evoked by a metalloprotease [8]. Brown spider venoms also evoke disseminated intravascular coagulation causing occlusion of vessel walls in laboratory experimental animals and in human patients [1,19]. Nevertheless, the real mechanism by which *Loxosceles* venoms cause hemorrhage is poorly understood, and more effort and studies are needed to elucidate it. In the present report, we describe some evidence of blood vessel damage and fibrinogenolysis caused by exposure to the *Loxosceles* venoms at morphological and molecular levels in order to contribute to the explanation of its mechanism of activation of the hemostatic disorders.

Materials and methods

Reagents

Purified human fibrinogen was purchased from Sigma (St Louis, Missouri, USA). Polyclonal anti-B β fibrinogen chain antibodies were produced in a rabbit using purified fibrinogen submitted to preparative 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions as described by Harlow and Lane [20].

Spider venom extraction

The venom was extracted from spiders captured from nature and kept for 1 week without any kind of food. The venom was extracted from the fangs of spiders by electrostimulation (15 V) applied to the cephalothorax, and collected with a micropipette, dried under vacuum and frozen at −85°C until the time for use. Pools of venom collected from 200–500 spiders in different batches were used during all the experiments, completing a total of approximately 5000 spiders [8]. Protein content was determined by the Coomassie Blue method [21].

In vivo studies on rabbits

For the evaluation of the hemorrhagic activity of *L. intermedia* venom, pooled venom (40 μg protein samples diluted in phosphate-buffered saline) obtained as already described was injected intradermally into a shaved area of rabbit skin. The development of the lesion was checked after 30 min and after 1, 2, 4, 8, 12 and 24 h, and then daily for 25 days after exposure. For histopathological analysis, some animals were anesthetized with sodium pentobarbital and sacrificed 4 h after exposure to the venom, and tissue samples were processed as described in the following.

Histological methods for light and transmission electron microscopies

Venom-injured rabbit skins were fixed in modified ALFAC fixative for 3 h, processed for histology, embedded in paraffin and cut into 4-μm thick sections. The sections were stained with hematoxylin–eosin for light microscopy. Alternatively, tissues were fixed with modified Karnovsky's fixative for 2 h for transmission electron microscopy [22]. Ultrathin sections were contrasted with uranyl acetate and lead citrate, and examined with a JEOL-JEM 1200 EX II transmission electron microscope at an accelerating voltage of 80 kV.

Gel electrophoresis

SDS-gel electrophoresis were performed as described by [23]. Samples under reducing or non-reducing conditions were applied to polyacrylamide gels (see figure captions for details). For protein detection, gels were stained with Coomassie Blue dye. For immunoblotting, after electrophoresis, proteins were transferred onto nitrocellulose filters overnight as described by Towbin *et al.* [24]. The molecular mass markers used were from a protein

ladder of 10 kDa (Gibco BRL, Bethesda, Maryland, USA).

Determination of the fibrinogenolytic activity of L. intermedia *venom*

Purified human fibrinogen (100 μ g samples) in intact form or denatured by boiling was incubated with increasing concentrations of the venom (0.006–100 μg) at 37°C for 16 h. Samples were applied to 12.5% polyacrylamide gels under reducing conditions (see figure captions for details). Alternatively, 100 μg samples of human fibrinogen were incubated with 12.5 μg venom under the same conditions as already described, but processed for polyacrylamide gel electrophoresis (linear gradient, 3–15%) under non-reducing conditions.

Characterization of the specificity of the proteolytic effects of L. intermedia venom on fibrinogen chains

Purified human fibrinogen was submitted to preparative 12.5% (w/v) SDS-PAGE under reducing conditions. Separated Aα, Bβ and γ chains (samples of approximately 30 μg) were excised from the polyacrylamide gels and individually incubated with 100 μg/ml venom at 37°C for 16 h. The samples were submitted to 12.5% SDS-PAGE under reducing conditions as already described. Alternatively, 100 μg purified fibrinogen was incubated with 12.5 μg venom for 16 h at 37°C. As a control, fibrinogen and venom were incubated alone. The samples were submitted to 12.5% SDS-PAGE under reducing conditions. The electrophoresed material was stained with Coomassie blue or transferred overnight to nitrocellulose filters as already described and then immunoblotted using primary antibodies to the Bβ fibrinogen chain obtained as described in 'Reagents', and developed as described in detail by Veiga *et al.* [25] (see figure captions for details).

Time course of the fibrinogenolytic effect of L. intermedia *venom*

Purified human fibrinogen (100 μg samples) was incubated with the venom (12.5 μg samples) at 37°C. Aliquots of the reaction mixtures were collected after 0, 15, and 30 min and 1, 2, 4, 8, 12 and 24 h. The proteolytic effect of the venom was stopped by freezing the samples at –20°C. As control of experimental stability, fibrinogen was collected after 24 h in the absence of venom. Samples were subjected to 12.5% SDS-PAGE under reducing conditions as already described.

Effect of protease inhibitors on the fibrinogenolytic activity of brown spider venoms

Purified human fibrinogen (100 μg samples) was incubated with *L. intermedia* venom (12.5 μg samples) at 37°C for 16 h in the presence of divalent metal chelators such as ethylenediamine tetraacetic acid (EDTA) (5 mmol/l) and 1,10-phenanthroline (5 mmol/l), serine-protease inhibitors such as aprotinin (2 μg/ml) and benzamidine (10 mmol/l), thiol-protease inhibitors such as *N* -ethyl-maleimide (5 mmol/l) and iodoacetamide (10 mmol/l), and an acid-protease inhibitor such as pepstatin-A (10 μg/ml). As negative control fibrinogen was incubated at 37°C for 16 h in the absence of the venom, and as positive control fibrinogen was incubated under the same conditions in the presence of venom but in the absence of protease inhibitors. Samples were submitted to 12.5% SDS-PAGE under reducing conditions as already described. Alternatively, purified human fibrinogen (100 μg samples) was incubated with *L. intermedia*, *L. laeta* and *Loxosceles gaucho* venoms (12.5 μg samples) at 37°C for 16 h in the presence or absence of 1,10 phenanthroline (5 mmol/l). As controls, the respective venoms were incubated in the absence of fibrinogen, or fibrinogen was incubated in the absence of venoms. The materials were submitted to 12.5% SDS-PAGE under reducing conditions as already described.

Determination of hemostatic parameters in vitro

Blood coagulation parameters *in vitro* were determined using a pool of human citrated plasma from five normal donors. Samples of pooled plasma were incubated at 37°C overnight with 100 or 200 μg/ml *L. intermedia* venom. A sample of pooled plasma was processed at the same experimental conditions as already described but in the absence of venom (control for experimental stability). Assays of prothrombin time, activated partial thromboplastin time and thrombin time were performed using standardized techniques and reagents (Organon Teknika, Durham, North Carolina, USA), as described by Beutler *et al.* [26].

Chromatographic purification of fibrinogenolytic toxin from L. intermedia *venom*

The *L. intermedia* venom (10 mg) was dissolved in 50 mmol/l Tris–HCl buffer (pH 7.3), containing 150 mmol/l NaCl and then filtered through a 0.22 μm membrane (Millipore, São Paulo, Brazil) to remove some insoluble precipitated material. A column (2.5 x 150 cm) of Sephadex G-100 (Pharmacia, Uppsala, Sweden) was equilibrated with the

aforementioned buffer at 20°C, the sample was applied to the column and chromatographed using a Econo System machine (Bio Rad, Richmond, Virginia, USA), and fractions of 5 ml were eluted. The peak II eluted from G-100 column (which has the fibrinogenolytic activity) was concentrated and dialyzed against Tris–HCl buffer at 4°C, then applied to a column (2.5 × 150 cm) of Sephadex G-50 Superfine (Pharmacia) and chromatographed under the same conditions as described for the G-100 column. Purified fibrinogenolytic toxin was eluted in the fractions of peak I.

Results

Macroscopic description of experimental hemorrhage evoked by L. intermedia *venom in rabbits*

To evaluate the hemorrhagic activity of brown spider venom, aliquots of *L. intermedia* venom (40 μg proteins diluted in 100 μ l phosphate-buffered saline since 40 μg represents the average amount of venom injected in a bite) were injected intradermally into a shaved area of the skin of six New Zealand white female rabbits weighing approximately 2–4 kg. Figure 1 shows the time-dependent evolution of hemorrhage. All tested animals exhibited similar reactions of experimental loxoscelism such as edema, erythema, hemorrhage and dermonecrosis with gravitational spreading.

Microscopic studies of the nature of the hemorrhagic activity of L. intermedia *venom*

Since the skin of rabbits began to present hemorrhage between 1 and 4 h post-venom injection, in order to characterize the nature of this pathological activity we processed the hemorrhagic region of the skin for histological analysis after 4 h of venom exposure, when local hemorrhage was evident but without dermonecrosis. Figure 2 illustrates the results obtained by light and transmission electron microscopy. Structural and ultrastructural views of the venom-treated dermis are shown, with emphasis on the regions surrounding the capillaries. In Fig. 2a, the disruptive effect of the venom on endothelial cell membranes from a blood vessel wall can be seen, where edema can also be detected (disorganization of collagen fibrils). In Fig. 2b,d, one can observe the cytotoxicity of the venom on endothelial cells as demonstrated by subendothelial blebs and vacuoles or cytoplasmic membrane degeneration. In Fig. 2c, the edema is ultrastructurally confirmed by disorganization of collagen fibrils. In Fig. 2e, an extravascular fibrin network into connective tissue can be detected. A similar fibrin network can be detected within the blood vessel in Fig. 2f. From Fig. 2g,h, one can observe the thrombotic activity of the venom (thrombus inside blood vessels).

Description of the fibrinogenolytic activity of L. intermedia *venom*

Based on the fact that exposure to the venom can effectively trigger fibrin and thrombus formation within blood vessels, we postulated that the venom may have a direct action on fibrinogen, an important molecule involved in this event. Using purified human fibrinogen incubated with increasing venom concentrations (see 'Materials and methods' for details), we were able to detect a differential proteolytic activity on the Aα, Bβ and γ chains. As illustrated in Figure 3a,b, the venom degrades Aα and Bβ chains from intact fibrinogen. Interestingly, the venom significantly digested all Aα, Bβ and γ fibrinogen chains when fibrinogen was previously denatured by boiling (Fig. 3c,d), indicating some conformation-dependent resistance to proteolytic activity. Figure 3e illustrates intact fibrinogen aliquots or fibrinogen denatured by boiling, treated with the venom and resolved by linear gradient 3–15% SDS-PAGE under non-reducing conditions. Partial fibrinogenolytic activity on intact fibrinogen and a complete cleaving effect on the denatured molecule can be seen, supporting the idea of a conformation-dependent susceptibility of native fibrinogen.

Fibrinogenolytic specificity of L. intermedia *venom*

To elucidate the degrading activity of the venom on fibrinogen, we separately incubated the Aα, Bβ and γ chains of fibrinogen (see 'Materials and methods' for details) with the venom. As shown in Figure 4a, the venom did hydrolyze all fibrinogen chains to a variety of lower molecular mass fragments that ran across the gel. We also immunoblotted purified fibrinogen before and after venom treatment using a polyclonal antibody against the Bβ chain. As shown in Figure 4b, the resulting digestion fragment of the Bβ chain remained after SDS-PAGE under reducing conditions, colocalized with the γ chain, and therefore was responsible for the thickening of the γ-chain band in the gel.

Time course of the fibrinogenolytic effect of L. intermedia *venom*

Similar studies were performed using intact purified fibrinogen as substrate for *L. intermedia* venom at a ratio of 100 μg fibrinogen to 12.5 μg venom, since this was a venom concentration that efficiently

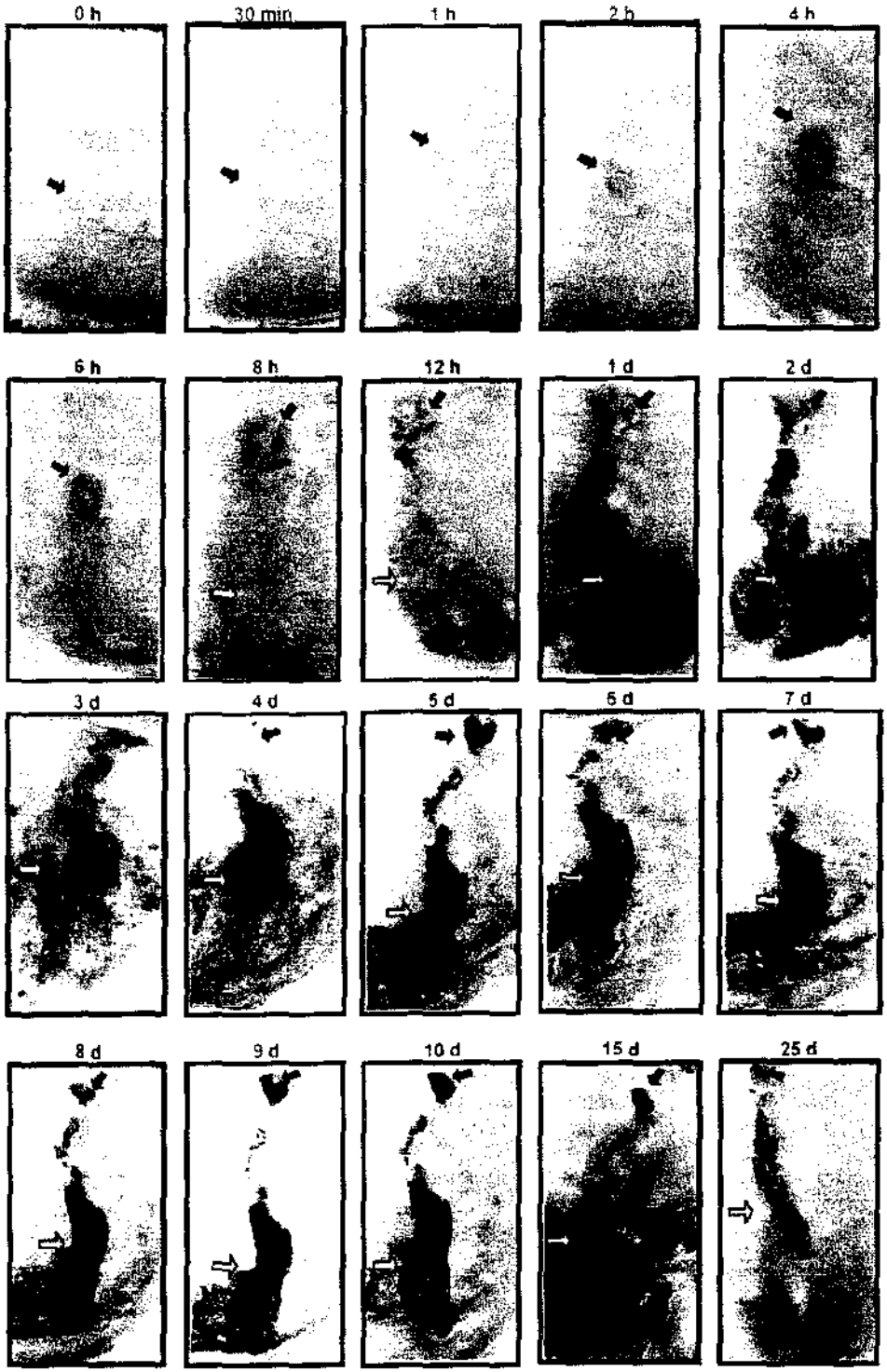


Figure 1. Time course of experimental hemorrhage and dermonecrosis into the skin of rabbits caused by *Loxosceles intermedia* venom. Rabbits were intradermally injected with 40 μg crude venom and the time course of skin lesions were evaluated macroscopically. The primary biological actions of the venom are edema and a hemorrhagic spot underlying the local of injection, starting between 1 and 2 h post-venom exposure. The hemorrhagic effect increases to a maximum activity between 2 and 3 days after venom injection. Between 8 and 12 h, the hemorrhage widens through a gravitational spreading distant from the site of injection. This is followed by a black color stain (the first sign of dermonecrosis, and represents the surface of ischemic tissue that becomes black) that colocalizes with the hemorrhage between 12 h and 1 day. Over the following days, the black stain becomes hardens and originates an eschar between 1 and 2 days. There is also an erythema surrounding the black eschar between 1 and 4 days that disappears at 5 days of experiments. The black eschar remains over the following weeks or may drop out, exhibiting the lesioned tissue like a non-healing ulcer of deep dermo and subcutaneous tissue. The black arrows point to the venom injection site and white arrows show gravitational spreading of the lesion.

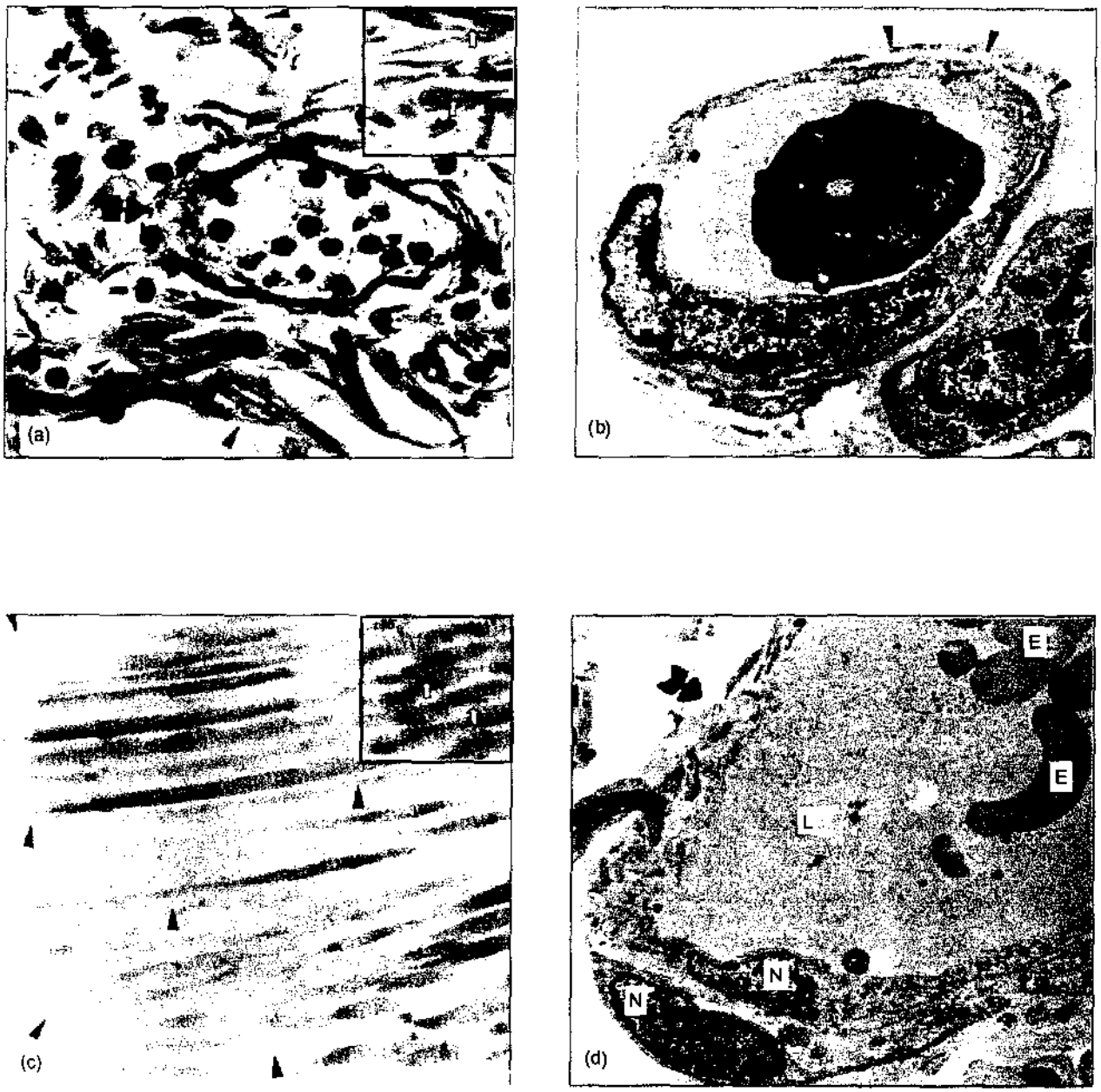


Figure 2. Histopathologic changes of blood vessels and underlying tissues of skin of rabbits triggered by *Loxosceles intermedia* venom at time 4 h. The figure shows the disruptive effect of the venom in rabbit skin. (a) Light micrograph of a blood vessel and underlying tissues of the skin of a rabbit treated by the venom. Showing the disruptive effect of the venom toward the blood vessel wall (arrow) and the collagen fibrils disorganization effect (arrowheads) (400×). The inset shows normal organization of collagen fibrils from connective tissue into the dermis (open arrows) (400×). (b) Electron micrograph of a capillary of the skin from a rabbit treated by the venom, showing subendothelial blebs (arrowheads) evoked by the venom (6000×). (c) Electron micrograph of the connective tissue of the skin from a rabbit treated by the venom, showing the edema activity (large amounts of a fluid in the extracellular compartment), evidenced by disorganization and the moving away (arrowheads) effect of the venom on collagen fibrils (15 000×). The inset depicts normally organized collagen fibrils from dermal connective tissue (open arrows) (15 000×). (d) Electron micrograph of a blood vessel of the skin from a rabbit treated by the venom, showing the disruptive activity of the venom on the endothelial cell membrane and subendothelial basement membrane (arrow). N, Endothelial cell nuclei; E, erythrocytes within the vessel; L, lumen of the vessel (4000×). (e) Light micrograph of blood vessels and connective tissue of the skin from a rabbit treated by the venom, showing the activity of the venom on blood vessels, increasing their permeability and resulting in the passage of plasma into the connective tissue, evidenced by fibrin network formation (arrowheads). Arrows point to blood vessels, (400×). (f) Light micrograph of a blood vessel of the skin from a rabbit treated by the venom, showing a fibrin network within the vessel (arrowheads) (100×). (g) Light micrograph of a blood vessel of the skin from a rabbit treated by the venom, showing a thrombus within the vessel (arrowheads) (400×). (h) Electron micrograph of a capillary of the skin from a rabbit treated by the venom, showing a thrombus within the lumen of the capillary (arrows). Arrowheads point to platelet granules (800×).

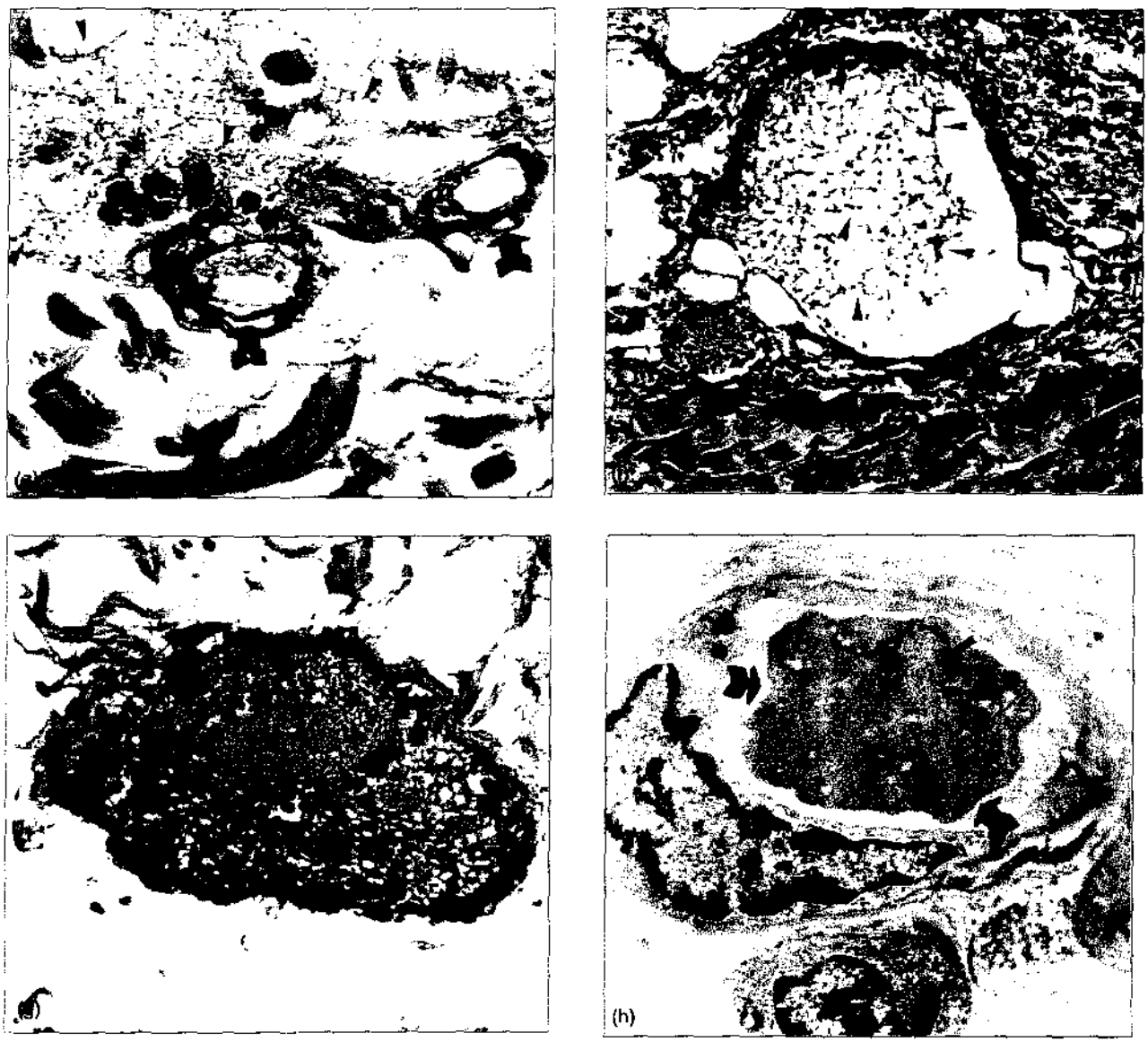


Figure 2. (*continued*)

degraded both Aα and Bβ fibrinogen chains (see Fig. 3), and aliquots of the reaction mixtures were collected at different times of incubation. Figure 5 shows that the Aα chain begins to be hydrolyzed between 30 min and 1 h after venom exposure, with progressive proteolysis with the time, whereas the Bβ chain begins to be cleaved after 4 h of venom treatment.

Inhibitory effect of divalent chelators on the fibrinogenolytic activity of the venom

To obtain more information about the nature of the fibrinogenolytic effect of *L. intermedia* venom, we studied its degrading action on purified fibrinogen in the presence of inhibitors of four major groups of proteolytic enzymes (see 'Materials and methods' for details). As can be seen in Figure 6a, the fibrinogenolytic effect of the venom was blocked only by EDTA and 1,10-phenanthroline, suggesting that the presence of a divalent metal ion is critical for enzyme activity. Our next goal was to evaluate the activity of 1,10-phenanthroline on the fibrinogenolytic effects of two other different brown spider venoms (*L. laeta* and *L. gaucho*). As shown in Figure 6b, the fibrinogenolytic activity of all tested venoms was decreased compared with that of the control (absence of inhibitors).

L. intermedia *venom activity on the blood clotting system*

To further characterize the fibrinogenolytic activity of the venom with the blood clottability, samples of

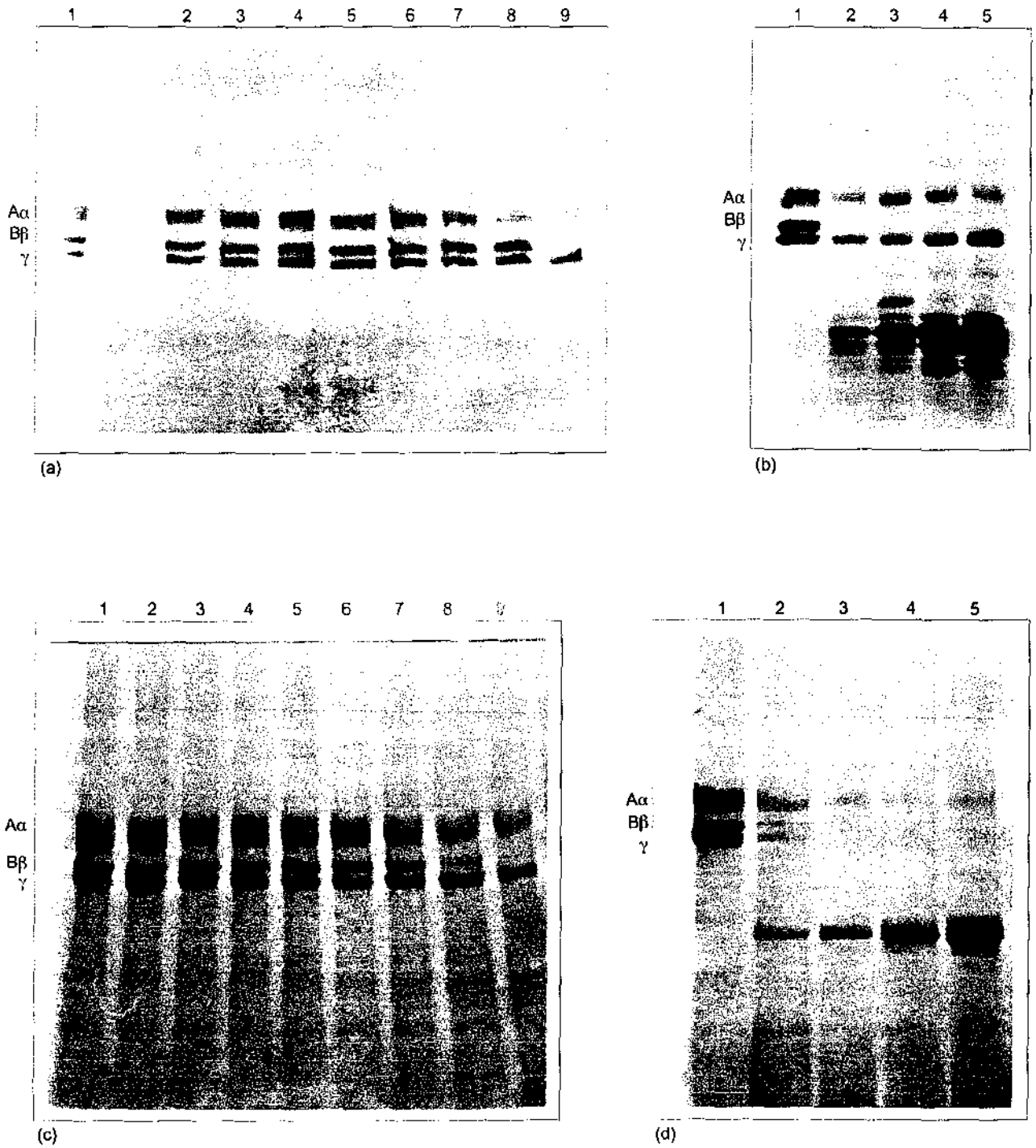


Figure 3. Proteolytic effect of *Loxosceles intermedia* venom on purified human fibrinogen. Experiments in which purified human fibrinogen as the intact molecule (a) and purified human fibrinogen denatured by boiling (c) were incubated with increasing concentrations of venom, for 16 h at 37°C. Lane 1, Fibrinogen alone as the control for experimental stability of substrate; lanes 2–9, fibrinogen incubated with 0.006, 0.012, 0.025, 0.05, 0.1, 0.2, 0.4 and 0.8 μg venom. The fibrinogenolytic activity of the venom was determined after 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and Coomassie blue staining. Using purified intact fibrinogen (b) and fibrinogen denatured by boiling as already described (d), excepted that *L. intermedia* venom concentrations were increased. Lane 1, Fibrinogen alone as control; lanes 2–5, fibrinogen exposed to 12.5 g, 25.0 g, 50.0 g and 100.0 μg venom. The materials were analyzed identically as already described. The positions of fibrinogen Aα, Bβ, and γ chains are shown. (e) Human fibrinogen (100.0 μg) was incubated with the venom (12.5 μg) under the same experimental conditions as already described, except that materials were processed in a linear gradient 3–15% SDS-PAGE under non-reducing conditions. Lane 1, Venom alone; lane 2, fibrinogen alone (control for experimental stability); lane 3, native fibrinogen incubated with the venom; lane 4, fibrinogen denatured by boiling, exposed to the venom.

Figure 3. (*continued*)

human citrated plasma (see 'Materials and methods' for details) were incubated with different concentrations of venom and some blood coagulation parameters determined. Table 1 summarizes the hemostatic parameters determined and the results.

Identification and purification of a fibrinogenolytic toxin in the L. intermedia *venom*

The fibrinogenolytic enzyme presented in the *L. intermedia* venom was purified from crude venom by sequencial gel filtration chromatography, first by using a Sephadex G-100 column followed by another Sephadex G-50 column (see 'Materials and methods' for details) as depicted in Figure 7. The pooled fractions from peak II of G-100 chromatography that concentrates the fibrinogenolytic activity were subjected to a G-50 column, which yielded a purified protein at 30 kDa with fibrinogenolytic activity in peak I.

Discussion

Although the clinical signals of loxoscelism have been well documented over the past few years, data about the hemorrhagic disorders evoked by brown

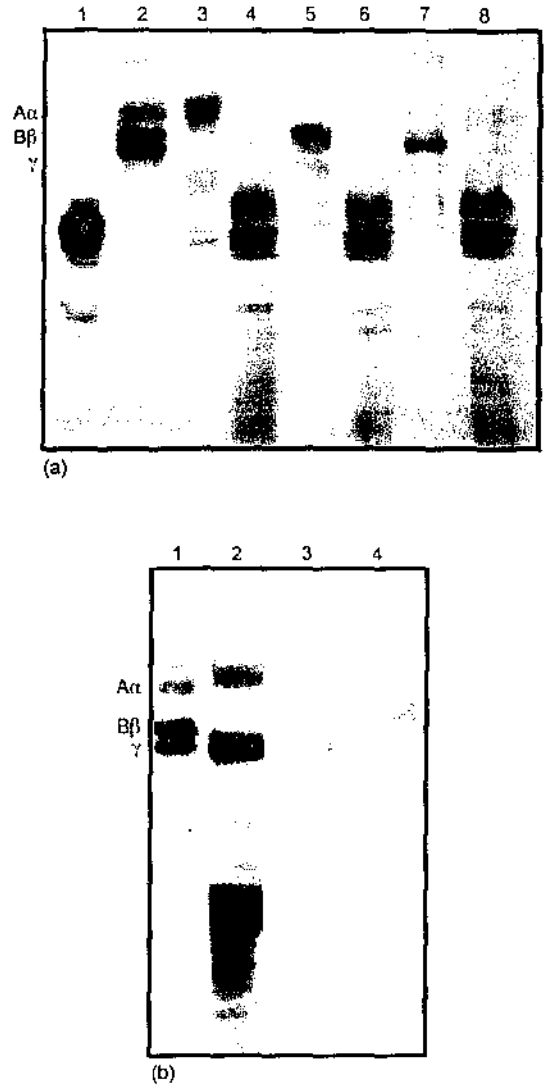


Figure 4. Characterization of the specificity of the proteolytic effects of *Loxosceles intermedia* venom on fibrinogen chains. (a) Separated fibrinogen chains were excised from a preparative polyacrylamide gel under reducing conditions and then were treated by the venom, overnight at 37°C (see 'Materials and methods' for details). The materials were electrophoresed in 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and the gel was stained by Coomassie blue dye. Lane 1, Electrophoretic positions of the major venom proteins; lane 2, fibrinogen alone as control for experimental stability of substrate; lanes 3 and 4, respectively, separated Aα chain pre- and post-venom treatment; lanes 5 and 6, respectively, separated Bβ chain pre- and post-venom treatment; lanes 7 and 8, γ chain pre- and post-venom treatment. The positions of fibrinogen Aα, Bβ, and γ chains are depicted in the figure. (b) Aliquots of 100 μg intact fibrinogen were incubated with 12.5 μg venom overnight at 37°C. The obtained materials were separated by 12.5% SDS-PAGE under reducing conditions and stained by Coomassie blue dye (lanes 1 and 2) or transferred onto a nitrocelulose membrane that was immunoblotted by using a polyclonal antibody against Bβ chain (lanes 3 and 4). Lanes 1 and 4, Fibrinogen alone as control before venom treatment; lanes 2 and 3, venom-treated fibrinogen. The positions of fibrinogen Aα, Bβ, and γ chains are depicted in the figure.

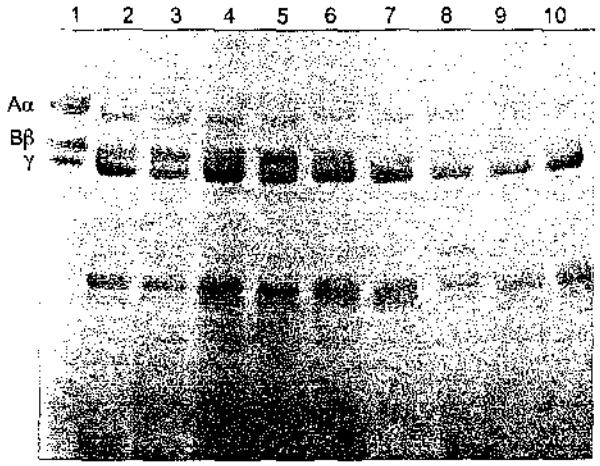
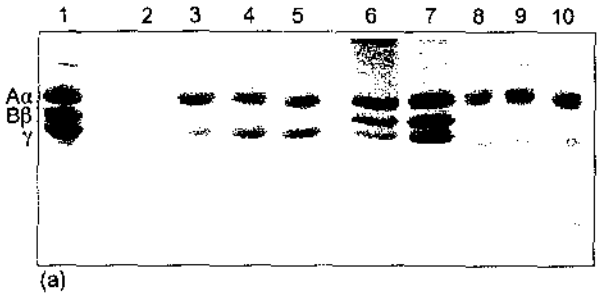


Figure 5. Time-dependent activity of *Loxosceles intermedia* venom on intact fibrinogen. Samples of intact human fibrinogen were incubated with the venom at a ratio of 100 μg fibrinogen to 12.5 μg venom at 37°C. Venom degradation activity on fibrinogen was acessed following incubation for 0 min (lane 2), 15 min (lane 3), 30 min (lane 4), 1 h (lane 5), 2 h (lane 6), 4 h (lane 7), 8 h (lane 8), 12 h (lane 9) and 24 h (lane 10). Lane 1 shows control for experimental stability, fibrinogen incubated for 24 h at 37°C in the absence of venom. Degradation of fibrinogen was determined by Coomassie blue dye following 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. The positions of fibrinogen Aα, Bβ, and γ chains are depicted in the figure.



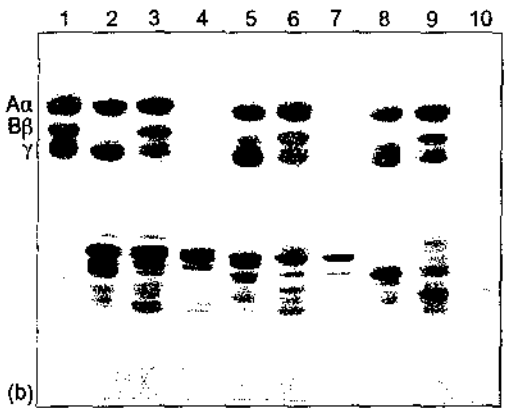


Figure 6. Protease inhibitors action on fibrinogenolytic activity of *Loxosceles intermedia* venom. (a) Intact purified human fibrinogen (aliquots of 100 μg) were incubated with venom (12.5 μg) overnight at 37°C in the presence of different protease inhibitors. The reaction products were submitted to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and stained by Coomassie blue dye. Lane 1, Control for experimental stability (fibrinogen in absence of venom); lane 2, electrophoretic positions of the venom major proteins; lane 3, degrading positive control (fibrinogen incubated with venom in absence of protease inhibitors); lanes 4–10, respectively, fibrinogen incubated with venom in the presence of 10 mmol/l benzamidine, 2 μg/ml aprotinin, 5 mmol/l ethylenediamine tetraacetic acid, 5 mmol/l 1,10-phenanthroline, 5 mmol/l *N*-ethyl-maleimide, 10 mmol/l iodoacetamide, and 10 μg/ml pepstatin-A. The positions of fibrinogen chains are depicted. (b) Once we had detected the inhibitory effect of 1,10-phenanthroline on venom activity, we repeated the same experiment as described but comparing *L. intermedia* venom with other brown spider venoms (*Loxosceles laeta* and *Loxosceles gaucho*). Lane 1, Fibrinogen alone without venom exposure; lanes 2–4, respectively, fibrinogen treated by *L. intermedia* venom in the absence of inhibitor and in the presence of 5 mmol/l 1,10-phenanthroline; lane 4, electrophoretic position of major venom proteins; lanes 5–7, respectively, fibrinogen treated by *L. laeta* venom in the absence of inhibitor and in the presence of 5 mmol/l 1,10-phenanthroline; lane 7, electrophoretic mobility of major venom proteins; lanes 8–10, respectively, fibrinogen treated by *L. gaucho* in the absence of inhibitor and in the presence of 1,10-phenanthroline; lane 10, electrophoretic profile of major venom proteins. The positions of fibrinogen chains are indicated.

spiders have been limited to earlier reports describing the effects of the venom on platelet aggregation and disseminated intravascular coagulation [1,14]. The mode of action of how venom causes hemorrhagic disorders is obscure.

In the present investigation, we studied some morphological and biochemical characteristics of the hemorrhagic disorders triggered by *L. intermedia* (brown spider) venom. Initially, we carried out experiments using (six) New Zealand rabbits (an animal model that reproduces the signals of loxoscelism found in humans) for dermal envenomation using 40 μg venom. Macroscopic examination of the rabbit skin (Fig. 1) showed hemorrhage into the dermis between 1 and 2 h after venom injection. The local hemorrhagic effect (hemorrhagic spot) increased with time, reaching maximum activity between 2 and 3 days after venom injection. Between 8 and 12 h, there was a gravitational spreading of the hemorrhage that colocalized with a black stain appearing between 12 h and 1 day. Between 1 and 2 days, this black stain hardened and formed an eschar. The hallmark signal of loxoscelism (i.e. the dermonecrosis reaction) continued until at least 25 days post-venom injection.

On the basis of microscopic examination of

Table 1. *Loxosceles intermedia* venom effects on blood coagulation parameters *in vitro*

Venom concentration (μg/ml)	PT (s)	APTT (s)	TT (s)
0	13.0 ± 1.0	33.0 ± 1.0	18.0 ± 2.0
100	35.0 ± 3.0	113.0 ± 3.0	25.0 ± 2.0
200	46.0 ± 6.0	174.0 ± 3.0	34.0 ± 3.0

Data are presented as mean ± standard deviation for three individual experiments. PT, Prothrombin time; APTT, activated partial thromboplastin time; TT, thrombin time.

histochemically processed skin sections (Fig. 2) obtained 4 h post-venom treatment (when hemorrhage but not dermonecrosis was present), we may propose some mechanisms by which the venom induces hemorrhage. The venom had an effect on the membrane of endothelial cells from the blood vessel wall, as well as a disruptive activity on the subendothelial basement membrane, a structure of extreme importance for vessel stability. This finding agrees with our preliminary results showing the action of the venom on the Engelbert–Holm–Swarm mouse sarcoma basement membrane and on molecules purified from this structure such as entactin and heparan sulphate proteoglycan [8]. Literature reports have described similar results for several snake venom proteases such as hemorrhagins isolated from *Trimerisurus flavoridis* venom [27], proteinase IV from *Crotalus horridus* [28] and the reprolysins from *Crotalus atrox* [16].

Considering these results as a whole, we may propose that the venom had a direct damaging action on the structure of blood vessel endothelial cells, and induced degeneration by causing vascular permeability and hemorrhage *per rhexis* into the underlying area. Based on Figure 2, we may also conclude that, 4 h after injection, the venom triggered fibrin deposition and thrombus formation within blood vessels, possibly leading to ischemic lesions that would increase blood vessel damage, causing vascular permeability and hemorrhage *per rhexis*.

The proposed fibrinogenolytic activity of the venom is supported by the data in Figures 3 – Fig. 4 Fig. 5. The venom partially cleaved the Aα and Bβ chains of native fibrinogen. This fibrinogenolytic activity was more marked when fibrinogen was denatured by boiling, supporting some conformational resistance of substrate-related venom enzymes. Using intact fibrinogen as substrate, we may consider the fibrinogenolytic activity of this venom to involve α - and β -chain fibrinogenases.

The Aα chain appeared to be more susceptible to venom proteolysis than the Bβ chain, since it suffered degradation when incubated with a lower venom concentration and in a more rapid manner than the Bβ chain, but there was no specificity for the Aα or Bβ chain, since both chains were degraded with time. The fibrinogenolytic activity of *L. intermedia* venom was direct and did not depend on other blood components since the results described represent the treatment of purified human fibrinogen with venom alone. We cannot rule out the possibility that the thrombogenic activity evidenced in this experimental model of loxoscelism was a consequence of the partial fibrinogenolytic effect of the venom. But, since direct plasma incubation with the venom, even when using 500 μg/ml (which is five times higher than the highest venom concentration used in this experimental animal protocol or usually injected during an accident) did not induce plasma coagulation as done by thrombin, for example (data not shown), we may propose that the thrombogenic activity triggered by the venom was the consequence of one of the following mechanisms. First, endothelial cytotoxicity directly evoked by the venom (Fig. 2), exposing the vessel wall subendothelial structures such as basement membrane and connective extracellular matrix molecules, which attract blood cells, as evidenced for other toxin venom and models [29,30] or, second, platelet aggregation evoked by the venom, with the release of vasoactive molecules, thrombin deposition and, consequently, thrombogenesis [31], as also demonstrated for several other venom examples [29,32].

The fibrinogenolytic effect of brown spider venoms appears to be dependent on a metalloprotease activity, since fibrinogen–venom proteolysis was blocked only by EDTA and 1,10-phenanthroline, two divalent metal chelators (Fig. 6). Based on the results indicating that the proteolytic effects of three different brown spider venoms (*L. intermedia, L. gaucho* and *L. laeta*) are closely similar (Fig. 6B), we may suggest that this proteolysis is a conservative event for different *Loxosceles* species, indicating some biological function of this effect related to the life cycle and the envenomation of brown spiders. Many metalloproteases that cause local or systemic hemorrhage in experimental animals have been isolated from snake venoms (for details, see [16,32,33]), but our preliminary results [6–8] and the present data are the first to indicate the presence of this kind of proteolytic toxins in brown spider venoms. On the basis of the aforementioned results and those described in Table 1, we may suggest that the metalloprotease-dependent fibrinogenolytic activity

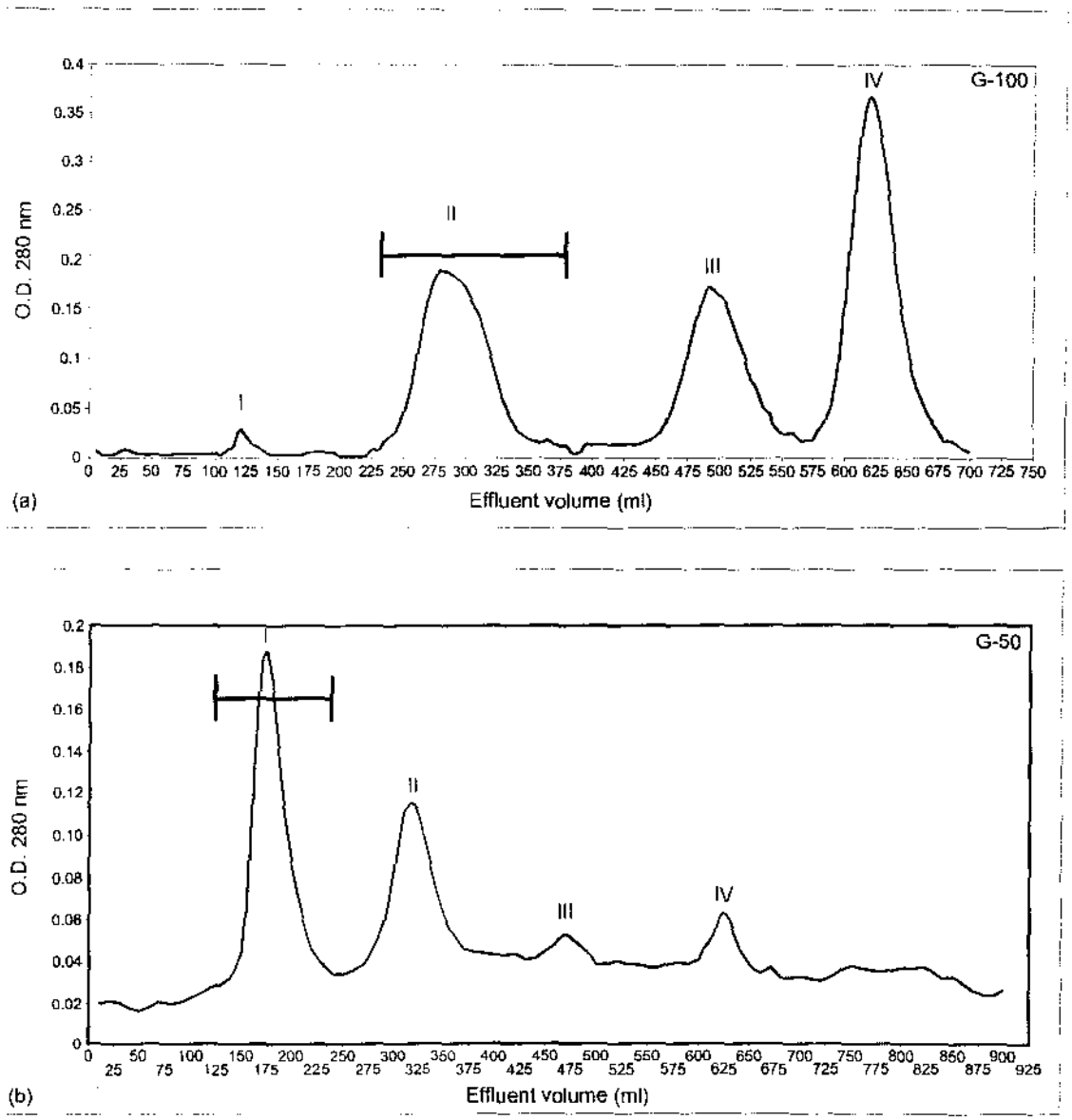


Figure 7. Chromatograms and sodium dodecyl sulfate-polyacrylamide gel electrophoresis showing the purification of fibrinogenolytic enzyme from crude venom of *Loxosceles intermedia*. (a) Chromatogram of elution of 10 mg venom using a Sephadex G-100 column. The column effluent was monitored for the presence of protein by absorbance at 280 nm and for fibrinogenolytic activity. Horizontal bar indicates pools that concentrate fibrinogenolytic activity, used for further purification in Sephadex G-50 Superfine. (b) Chromatogram showing the elution of the pooled fractions from peak II of the Sephadex G-100, separated on a Sephadex G-50 column. The eluted peak I concentrates purified fibrinogenolytic toxin. (c) Polyacrylamide electrophoresis under non-reducing conditions stained with Coomassie Blue. Lanes 1–4, respectively, fibrinogen alone (control for experimental stability of substrate), fibrinogen incubated with crude venom, fibrinogen incubated with pooled fractions of peak II of the G-100 column, and fibrinogen incubated with pooled fractions of the peak I of the G-50 column. Arrow, Intact molecule of fibrinogen; arrowhead, cleaved fibrinogen and asterisc depicts purified fibrinogenolytic toxin at 30 kDa. Molecular mass protein standard positions are shown on the left.

of the venom is able to delay or inhibit plasmatic coagulation, increasing hemorrhagic disturbances during loxoscelism.

With regard to the potency of the metalloprotease-hemorrhagic toxin from brown spiders (Fig. 7), we have not yet established the minimum hemorrhage dose. However, if we compare snake accidents in which 100–150 mg total proteins are injected at the bite site with brown spider envenomations, in which only a few micrograms of total proteins are injected at the bite site, we may assume this molecule as very efficient and potent. Thus, based on the presented data, we may assume that the venom-induced fibrinogenolytic action (depen-

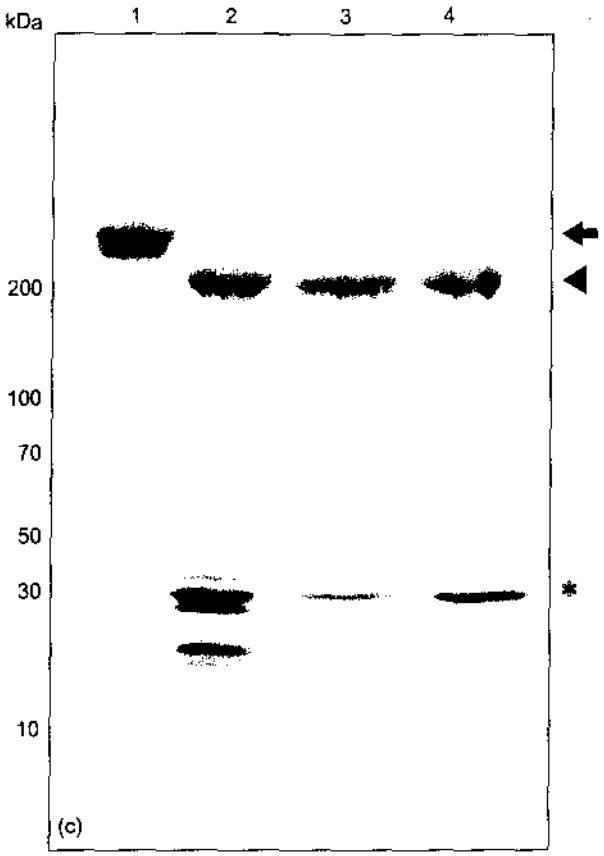


Figure 7. (*continued*)

dent of a 30 kDa metalloprotease), plus the venom effects on platelet aggregation, vessel wall endothelial cell cytotoxicity and basement membrane disruption, are determinant events related to the hemorrhagic disorders caused during loxoscelism.

Acknowledgments

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Extracellular matrix molecules as targets for brown spider venom toxins

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Abstract

Loxoscelism, the term used to describe lesions and clinical manifestations induced by brown spider's venom (*Loxosceles* genus), has attracted much attention over the last years. Brown spider bites have been reported to cause a local and acute inflammatory reaction that may evolve to dermonecrosis (a hallmark of envenomation) and hemorrhage at the bite site, besides systemic manifestations such as thrombocytopenia, disseminated intravascular coagulation, hemolysis, and renal failure. The molecular mechanisms by which *Loxosceles* venoms induce injury are currently under investigation. In this review, we focused on the latest reports describing the biological and physiopathological aspects of loxoscelism, with reference mainly to the proteases recently described as metalloproteases and serine proteases, as well as on the proteolytic effects triggered by *L. intermedia* venom upon extracellular matrix constituents such as fibronectin, fibrinogen, entactin and heparan sulfate proteoglycan, besides the disruptive activity of the venom on Engelbreth-Holm-Swarm basement membranes. Degradation of these extracellular matrix molecules and the observed disruption of basement membranes could be related to deleterious activities of the venom such as loss of vessel and glomerular integrity and spreading of the venom toxins to underlying tissues.

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Introduction

Brown spiders (*Loxosceles* genus) have been reported to cause several clinical manifestations. Envenomation provokes two major kinds of signals, i.e., local lesions at the bite site characterized by edema followed by vasodilatation, blood vessel degeneration, local hemorrhage and a significant cutaneous tissue injury with gravitational spreading, that can exacerbate to necrotic skin ulcers and degeneration (1-4), and systemic effects that begin as a malaise and can become generalized, with hemolysis, thrombocytopenia, disseminated intravascular coagulation and renal failure. These clinical signs and toxicological effects appear to be phenomena similar for several *Loxosceles* species including the more studied *L. reclusa*, *L. laeta*, *L. intermedia* and *L. gaucho* species (4-8).

Brown spider venoms are highly complex and contain many different proteins (9-11). The exact mechanisms by which the

venoms cause their deleterious effects are currently under investigation, with putative explanations involving an indirect event, as is the case for endothelial cell-dependent neutrophil activation caused by the venoms and seemingly related to the dermonecrotic lesion (4,12-14). The presence of a sphingomyelinase D-like enzyme (32-35 kDa) probably associated with necrotic, hemolytic and thrombocytopenic activities triggered by the venoms has also been identified in different *Loxosceles* species (3,4,6,10,15,16). Other enzymes such as a hyaluronidase have been postulated to be a spreading factor during the lesions (4,17), and protease activities also appear to have some participation in the noxious effects of the venoms (11,18-21). Adult plasma components appear to be required for the deleterious effects of the venoms, since a purified putative dermonecrotic toxin diluted in neonate plasma or synthetic buffer did not induce platelet activation, an event that may be responsible for thrombosis, tissue ischemia and dermonecrosis (8). Indirectly, venoms also seem to cause injury by binding to cell membranes and activating the complement system of plasma (8,22,23) (see Table 1).

The key observations that some physiological events closely dependent on the basement membrane and on connective or plasma extracellular matrix constituents are altered during loxoscelism, as is the case for platelet subendothelial adhesion and aggregation, hemostatic troubles such as disseminated intravascular coagulation, hemorrhage into the dermis, renal failure and even cutaneous tissue injury, point to the presence of molecules potentially deleterious to these structures in the venoms.

This review focuses on the specific degrading effects triggered by *Loxosceles* venoms in extracellular matrix molecules and the biological consequences of these hydrolytic activities related to the clinical signs induced by loxoscelism.

Presence of gelatinolytic enzymes in *L. intermedia* venom

Several extracellular matrix molecules have been described as targets for degradation evoked by proteases present in snake venoms, which cause hemorrhage, necrosis and edema (24-28). Soluble plasma fibrinogen is the major substrate in this family, but it is not the only one since snake venom actions on laminin, entactin, fibronectin and

Table 1. Brown spider venom properties.

Venom effects	Molecules involved	References
Dermonecrotic lesion	32 kDa++, 35 kDa+, +++	3,4,8,10,16,22
Intravascular hemolysis	32 kDa++, 35 kDa+	4,6,16,22
Platelet aggregation and thrombocytopenia	32 kDa++, 35 kDa+	4,8,15
Gravitational spreading	33 kDa++, 63 kDa++	4,17
Fibrinogenolytic activity	20-28 kDa+	19
Fibronectinolytic activity	20-28 kDa+	19
Gelatinolytic activity	32-35 kDa+, 85 kDa+, 95 kDa+	11,19,20
Entactinolytic activity	Unknown+	21,57
Basement membrane-degrading effect	Unknown+	21,57
Complement system activation	32 kDa++, 35 kDa+	4,16,22,23
Heparan sulfate proteoglycan hydrolysis	Unknown+	21

Dermonecrotic lesion, intravascular hemolysis, platelet aggregation and complement system activation are events dependent on the similar brown spider toxins of 32-35 kDa devoid of proteolytic activities.
The 32-35-kDa molecule with gelatinolytic activity is a metalloprotease.
Results obtained by using *Loxosceles intermedia* venom (+), *L. reclusa* venom (++), and *L. gaucho* venom (+++).

gelatin have been described (24,29). With respect to spider venoms and despite some similarities to snake venoms in their activities, little is known about the presence of venom proteases that degrade extracellular matrix molecules. Studying *L. intermedia* venom (the prevalent brown spider in southern Brazil) we were able to detect a 32-35-kDa metalloprotease with gelatinolytic activity. The 35-kDa protease form seems to be a latent pro-enzyme molecule that undergoes cleavage, originating the 32-kDa form (19). The 32-35-kDa proteases are high-mannose glycoproteins (20). We also detected zymogen molecules of proteolytic enzymes in the venom since trypsin activated two gelatinolytic serine proteases of 85 and 95 kDa in the venom. Other proteins such as casein, albumin, hemoglobin and laminin did not suffer any kind of cleavage. The specificity of action of these proteases should be related to spider self-protection. For example, the venom gland of *L. intermedia* is extremely rich in laminin, which separates muscle tissue (involved in venom secretion) from epithelial cells (involved in venom synthesis) (30), and the venom seems to have no lamininolytic activity (19,21). The natural substrate of the 32-35-kDa protease is unknown (since gelatin is denatured collagen and the venom does not display any activity on full length collagen), but, based on gelatinolytic activity, we may assume that this protease has properties like vertebrate gelatinases that appear to cleave connective components (31) and we may propose that this brown spider enzyme is functionally related to the deleterious effects of the venom. Native collagen can suffer an initial effect of collagenase from polymorphonuclear neutrophil leukocytes (which, as described above, are concentrated at the bite site and around it and seem to play a role in dermonecrosis), partially denaturating this molecule, which then can be sequentially degraded by these gelatinase-like venom proteases (see Table 1).

Effect of *Loxosceles* venom proteases on plasma extracellular matrix molecules

Brown spider envenomation, although causing classical skin degeneration, is also responsible for disorders of hemostasis such as hemorrhage into the dermis and disseminated intravascular coagulation. The hemorrhage mechanism has not been fully established at the molecular level. Plasma extracellular matrix molecules are multifunctional proteins involved in blood coagulation on the basis of their ability to bind heparin, as is the case for fibronectin and vitronectin (32-34), to interact with plasminogen, plasminogen activator inhibitors and the thrombin-antithrombin III complex, as is the case for vitronectin (33,35,36), and to promote platelet adhesion and aggregation, as is the case for fibrinogen, fibronectin, von Willebrand factor and thrombospondin (25,28,37-39). They also mediate fibrin formation as fibrinogen (38,39). *L. intermedia* venom degrades both the A and B chains of fibronectin (Figure 1) into a variety of smaller fragments (19,20), and partially digests fibrinogen (Figure 1) (19,20), but, interestingly, has no proteolytic activity on soluble vitronectin (see Figure 1). This fibrinogenolytic effect is closely similar to that of other brown spider venoms (*L. gaucho* and *L. laeta*) (Zanetti VC, unpublished results). The fibronectinolytic and fibrinogenolytic activities of *L. intermedia* venom are produced by a metalloprotease of 20-28 kDa (19). Based on the biological activities of brown spider venoms, we may postulate a relationship of this fibronectinolytic effect of the venom with hemorrhage and difficulties in wound healing. The hemorrhage into the dermis at the bite site, vasodilatation and injury to blood vessel walls could be associated with defective fibronectin surrounding the endothelial cells of capillaries. The hemorrhage could also be related to imperfect platelet adhesion and aggregation, since fibronectin partici-

pates in platelet attachment and aggregation and the proteolytic effect of the venom may disturb this event. Finally, the defective wound healing observed in some cases of envenomation may also be ascribed to the fibronectinolytic activity of the venom, since fibronectin-integrin interactions participate in an essential manner in this phenomenon (32). An identical hypothesis could be raised for the fibrinogenolytic ability of brown spider venom, since it has been well defined that several snake venom proteases with hemorrhagic activities readily degrade fibrinogen-fibrin into nonclotting fragments (26,27,40). Disseminated intravascular coagulation is a clinical feature detected in a few cases of brown spider envenomation. Venoms from various snakes that partially degrade fibrinogen provoke disseminated intravascular coagulation by fibrin formation (25,28). As previously postulated by Bascur et al. (7) and corroborated by Feitosa et al. (19) and Veiga et al. (20), on the basis of partial fibrinogenolytic activity triggered by brown spider venoms, we may speculate that this ability is involved in disseminated intravascular coagulation, a complication that, together with other hematologic disturbances, has been responsible for most of the deaths due to loxoscelism.

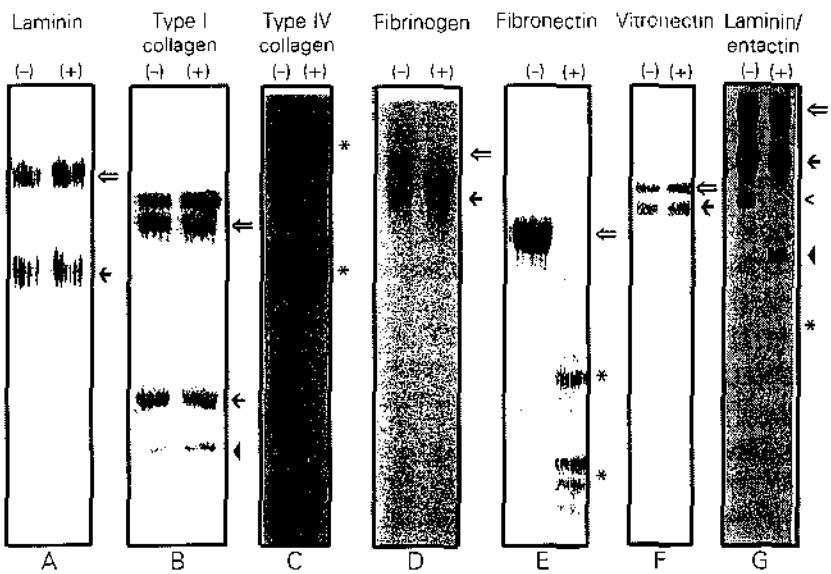

Figure 1. Proteolytic effect of *Loxosceles intermedia* venom on purified extracellular matrix molecules*. EHS-laminin (A), rat tail tendon type I collagen (B), human placental type IV collagen (C), human fibrinogen (D), human fibronectin (E), human vitronectin (F) and EHS-laminin/entactin dimer (G) were incubated with venom (+) or in the absence of venom, as controls for experimental stability (-) under the conditions described in References 19, 20, 21 and 57. The products obtained were analyzed by SDS-PAGE under reducing conditions (except for fibrinogen that was analyzed under nonreducing conditions). In *panel A* the open arrow indicates the laminin α_1 chain and the filled arrow indicates the β_1 and γ_1 laminin chains that co-migrate. In *panel B* the open arrow indicates the type I collagen ß dimers, the filled arrow depicts α_1 type I collagen chain and the filled arrowhead indicates the α_2 type I collagen chain. In *panel C* the asterisks represent the major components of trypsin-extracted human placental type IV collagens of 100, 160 and 170 kDa. In *panel D* the open arrow points to an intact fibrinogen molecule and the filled arrow indicates the fibrinogen fragment. In *panel E* the open arrow shows the co-migratory fibronectin A and B chains, and asterisks indicate fibronectin fragments. In *panel F* the open arrow points to the 75-kDa vitronectin molecule and the filled arrow points to the 65-kDa normally processed vitronectin fragment. In *panel G* the open arrow indicates the laminin α_1 chain, the filled arrow indicates the β_1 and γ_1 laminin chains that co-migrate, the open arrowhead indicates intact entactin, the filled arrowhead indicates the 100-kDa entactin fragment, and the asterisk shows the 50-kDa entactin fragment. *Based on results from References 19, 20, 21 and 57.

Fibrinogen is a large glycoprotein (340 kDa) formed by dimeric linking of three distinct chains, Aα, Bß and γ, linked by disulfide bridges. As described above, fibrinogen Aα chains are degraded by the venoms. It is interesting to observe that the α chains of fibrinogen have two RGD sequences, a peptide well defined as a major binding site for several integrins (41). The cleaving of fibrinogen RGD sequences by the venoms could reduce the interaction of integrin α_{IIb} β_3 on the platelet surface with fibrinogen, resulting in the loss of platelet adhesion and functional aggregation and contributing to the hemorrhagic effect of the venom. We may suggest this proteolysis as a conservative event for *Loxosceles* species, indicating some biological function related to the life cycle and envenomation of brown spiders (see Table 1).

Action of brown spider venom on basement membrane constituents

The basement membranes are ubiquitous specialized forms of extracellular matrices produced by several cell types such as endothelial, epithelial, fat, muscle and nervous cells. Molecularly they are characterized by the presence of a considerable variety of biochemically complex components arranged

as a network and formed by a particular set of proteins such as laminin, entactin, type IV collagen and heparan sulfate proteoglycan. Despite their tissue specificity (different isoforms), these components are present on practically all tissue basement membrane structures. In mammals, basement membranes play several essential roles in angiogenesis, cell differentiation, platelet adhesion, neuritogenesis, and blood-urine filtration through the kidney glomeruli, among several other functions (42-46). The effects of venom toxins on basement membranes have been well established for some snake venom hemorrhagic proteases that produce proteolytic degradation of basement membrane constituents isolated from Engelbreth-Holm-Swarm (EHS) tumor (29,40), as well as degradation of purified glomerular basement membrane (47). The pathogenic properties of *Loxosceles* venoms, such as dermonecrotic action, thrombocytopenic activity, local hemorrhage and renal disorders, are events attributable to the presence of proteolytic enzymes that degrade basement membrane molecules. By using the EHS tumor, a transplantable model which produces a characteristic and thick basement membrane as its capsule (48), we detected a disruptive activity of *L. intermedia* venom toward basement membrane structures. The venom apparently has no activity on laminin or type IV collagen (see Figure 1), the major macromolecules of these structures, but has the ability to hydrolyze entactin (19,21), a dumbbell-shaped molecule that links laminin, type IV collagen and heparan sulfate proteoglycan in the basement membrane organization (42,43). The hydrolytic activity of *L. intermedia* venom on entactin resulted in fragments of approximately 100 and 50 kDa when entactin was complexed to laminin and lower fragments that run out the gel when purified entactin was assayed (21). Similar results concerning this differential entactin susceptibility to the venom have been reported for other venoms such as snake venom toxins (29,40). This difference in cleavage profile for purified entactin and for the laminin-entactin complex treated with *L. intermedia* venom can be explained by the fact that laminin interacting with entactin can hide domains of entactin more susceptible to the degrading action of the venom, as previously described for the entactinolytic effect evoked by atrolysins, metalloproteases from *Crotalus atrox* snake venom (40).

Another essential family of molecules involved in basement membrane assembly and biological activities are the proteoglycans, complex molecules formed by a protein core to which one or more glycosaminoglycan chains are linked (49,50). They have been involved in several biological effects such as extracellular matrix organization (42,43), they are extracellular matrix cell surface receptors (51-53), play a role in cell-cell recognition (54), control cell growth and proliferation (50,55), participate in blood vessel stability and hemostasis (49), and have several other functions (49-51). *L. intermedia* venom degrades the protein core of purified heparan sulfate proteoglycan, but has no activity on heparan sulfate polysaccharide chains (21). On the other hand, the venom degrades chondroitin sulfate polysaccharide chains (Nader HB and Dietrich CB, personal communication). An established endothelial cell line from rabbit aorta (56) submitted to the presence of *L. intermedia* venom increases the liberation of heparan sulfate proteoglycan and sulfated proteins from its extracellular matrix and cell surface into the culture medium (Nader HB and Dietrich CB, personal communication). Based on the fact that *L. intermedia* venom degrades entactin, heparan sulfate protein core, and chondroitin sulfate polysaccharide chains, and releases laminin from basement membranes and that these molecules are extremely important for the structural and functional properties of basement membranes, we may speculate that these effects of the venom are plausible mechanisms for several deleterious conse-

quences of envenomation (see Table 1).

Concluding remarks and future expectations

The molecular mechanisms by which the brown spider venoms cause dermonecrotic injury, local and systemic hemorrhage, thrombocytopenia, hemolysis, disseminated intravascular coagulation and renal failure are currently under investigation, but since the venom is composed of a mixture of several proteins, these mechanisms seem to be molecularly complex and may be dependent on many different toxins.

In the present review, based on previous results obtained by our group (11,19-21,57), we introduce the idea of venom toxins acting as proteases upon molecular constituents of plasma extracellular matrix such as fibronectin and fibrinogen and basement membrane constituents such as entactin and heparan sulfate proteoglycan. Based on these degrading activities on plasma constituents and the disruptive effect on basement membrane structures triggered by venoms, we may speculate that these activities are plausible mechanisms for hemorrhage, delayed wound healing and renal failure and are also related to the spreading of other noxious toxins (for example, dermonecrotic protein), since disruption of the subendothelial basement membrane evokes blood vessel wall instability and increased permeability.

It is clear that more work is needed to elucidate the structure and function of these venom metalloproteases and to fully understand how they interfere with clinical signs of loxoscelism. However, it is interesting that all detected proteolytic activities and especially the disruptive effect of *L. intermedia* venom on EHS-basement membranes can be inhibited by 1,10-phenanthroline, a metalloprotease inhibitor.

On the other hand, based on biodiversity, venom toxins are excellent tools for investigating molecular mechanisms in the cell biology field, as well as for pharmaceutical applications of newly discovered medicines. In this area, venom toxins could be used as starting materials to design new drugs or directly for therapeutic use as is the case for ancrod, a defibrinogenating enzyme from *Callocelasma rhodostoma* venom, and batroxobin, a defibrinogenating enzyme from *Bothrops atrox* venom, toxins that have been used as defibrinogenating agents for a number of clinical conditions such as deep vein thrombosis, myocardial infarction, pulmonary embolus, acute ischemic stroke, angina pectoris, central retinal vein occlusion and renal transplant rejection (27,58-60). With respect to the pharmaceutical applications of brown spider venom toxins, at least one possibility can emerge from our results. The fibrinogenolytic-fibrinolytic properties of venoms directly indicate that this toxin could be useful for clinical applications in cardiac and thrombotic diseases based on reduced blood fluidity. Other possibilities can directly or indirectly arise from brown spider venom toxins (for example, as a tool in cell biology procedures to study the inflammatory response). A molecular understanding of loxoscelism may greatly enhance our therapeutic approach to this envenoming problem, as well as generate potential toxins as tools for scientific protocols and medicinal applications.

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Isolation and identification of *Clostridium perfringens* in the venom and fangs of *Loxosceles intermedia* (brown spider): enhancement of the dermonecrotic lesion in loxoscelism

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Abstract

Loxoscelism or the envenoming by the brown spiders (*Loxosceles* genus spiders), may produce extensive dermonecrosis and hemorrhage at the bite site and, eventually, systemic reactions that may be lethal. Isolation and identification of many different bacteria, among them *Clostridium perfringens*, of great medical importance due to its involvement in dermonecrotizing and systemic conditions, was carried out from the venomous apparatus (fangs and venom) of spiders obtained directly from nature, through microbiological cultures in aerobic and anaerobic conditions. Working with *Loxosceles intermedia* venom (alone) and with the venom conjugated with *Clostridium perfringens* using rabbits as experimental models for dermonecrosis, allowed for the observation that venom and anaerobic bacteria conjugated resulted in a striking increase of the dermonecrotic picture when compared to venom alone, suggesting a role for *Clostridium perfringens* in the severe dermonecrotic picture of these patients and opening the possibility for the association of antibiotic therapy in treating loxoscelism. © 2002 Published by Elsevier Science Ltd.

Keywords: *Loxosceles intermedia*; *Clostridium perfringens*; Dermonecrosis

1. Introduction

The accidents with spiders from the genus *Loxosceles* represent a public health problem in Brazil since they were first recognized (Rosenfeld et al., 1957; Cardoso et al., 1990). The accidents caused by *Loxosceles* spp spiders are characterized by a dermonecrotic lesion at the site of the spider's bite. This characteristic skin lesion begins with an acute inflammatory reaction characterized by edema, followed by inflammatory cell accumulation, hemorrhage into the dermis and an impressive lesion of cutaneous tissues, with formation of a black scar (Forrester et al., 1978; Rees et al., 1984; Futrell, 1992).

The pure venom is a transparent liquid enriched in proteins with low molecular mass in the range of 5–40 kDa (Veiga et al., 2000). A number of purified toxins of *Loxosceles* spiders have been identified as sphingomyelinase D, hyaluronidase and metalloproteases (Futrell, 1992; Wright et al., 1973; Feitosa et al., 1998).

The bacterial *Clostridium* are widely distributed in nature (soil, water, dust, plants and human and animal feces) and as spores they may remain viable for long periods of time. For this reason the environment is undoubtedly highly contaminated with spores of these bacteria (Murray and Citron, 1991; Murray et al., 2000).

The onset of anaerobic infection is favored by events like presence of tissue necrosis, local suppuration, ischemia among others, all of them favoring the spread of local oxi-reduction potential.The disease has a great virulence potential for this bacterium is highly histotoxic, and may cause extensive dermonecrotic skin lesions, muscle necrosis, shock, renal failure and death (Middlebrook and Dorland, 1984). At the microscopic level, Gram positive rods can be

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seen in huge amounts in the tissues. Immediate treatment is essential for severe infections and require surgical excision of the wound and high doses of the antibiotic penicillin (Hatheway, 1990; Murray and Citron, 1991; Murray et al., 2000).

In this study the anaerobic bacterium *Clostridium perfringens* was isolated from the venom and the fangs of the brown spider *Loxosceles intermedia*. Albine Neo-Zealand adult rabbits were used for experimental dermonecrosis and the results revealed that the extension and severity of dermonecrotic picture was importantly augmented when the spider venom was conjugated with the bacterium *Clostridium perfringens*. Once the reasons why some loxoscelism victims present a small dermonecrotic picture while others present very important lesions are still unknown, the results obtained in this experiment suggest these bacteria might be involved in the most severe dermonecrotic pictures of loxoscelism indicating the possibility of antibiotics as an associated therapy in these cases.

2. Materials and methods

2.1. Reagents

The following materials were employed: 10 μl bacteriologic loop (Kohle loop), anaerobic jar (Oxoid, Hampshire, England), gas mixture (nitrogen: 80–85%; 5–10% of carbonic gas end 5–10% hydrogen), vacuum bombing, Palladium catalyzer, anaerobic indicator strips, culture media for recovery and identification of presumptive anaerobic bacteria: brain heart infusion supplemented agar (Merck, Darmstadt, Germany); phenylethyl alcohol agar; bacteroides bile esculine agar (Sigma, St. Louis, USA); thioglycolate supplemented with vitamin K and hemin (Difco Laboratories, Detroit, USA); egg yolk agar, culture media for recovery of facultative anaerobic bacteria: blood agar; Mac Conkey (Sanofi Diagnostics Pasteur, Marnes, La-Coquette, France), light microscopic, oil immersion and stereomicroscope.

2.2. Electrostimulated spider venom extraction

Adult *L. intermedia* were collected in the city of Curitiba and in its surroundings (Parana, Brazil). The venom was extracted from the fangs of spiders by electrostimulation (15 V) applied to the cephalothorax and collected with a micropipette, dried under vacuum, and frozen at −85°C until use (Barbaro et al., 1994; Feitosa et al., 1998).

2.3. Spider fangs, glands venom and electrostimulated venom extraction for microbiological assays

Adult *L. intermedia*, collected as described above were anesthetized with chloroform (Merck, Rio de Janeiro) and under aseptic conditions the fangs, venom glands and electrostimulated venom were removed for microbiology studies. Electrostimulated venom and fangs were collected from 160 spiders and for the extraction of the venom glands were used in this experiment 50 spiders.

2.4. Microbiological laboratory identification

The methods employed for isolation and identification of aerobic, facultative, anaerobic and microaerophilic bacteria were based on studies performed by Allen and Baron, 1991; Koneman et al., 1992. The culture media for research of anaerobic organisms were supplemented with hemine (5 μg/ml) and menadione (10 μg/ml for hard media and 0.1 μg/ml for liquid media). Before inoculation, test tubes containing thioglycolate medium were boiled with half loose cap for 10 min in a water bath and then rapidly cooled in an ice bath, and tightly closing the cap. This procedure ensures the partial elimination of oxygen from the environment. The test tubes containing 2.5 ml of thioglycolate supplemented medium were inoculated with either electrostimulated venom samples, fangs or venom glands from *L. intermedia* and incubated in anaerobic jars at 37°C. The second step of the experiment, the study of dermonecrotic lesions, the material collected under aseptic conditions with syringe, from rabbit skin inoculation sites was transferred to a transport and culture medium. Anaerobic, aerobic and facultative bacteria were identified according to the Microbiology Manual from the American Association of Microbiology (Allen and Baron, 1991).

2.5. In vivo studies: dermonecrosis lesions in rabbits

For reproduction and comparison of dermonecrotic lesions electrostimulated *L. intermedia* venom (alone) and

Table 1
Isolation of aerobic, facultative and anaerobic bacteria from some body compartments of *Loxosceles intermedia* (as the data indicate, the bacterium *Clostridium perfringens* was isolated from 25% of the fangs and from 6,25% of the venom studied while no bacterium was present in the *L. intermedia* glands obtained under aseptic conditions)

Loxosceles intermedia	Samples	*Clostridium sp*	*Clostridium perfringens*	Other bacteria
Electro-stimulated venom	160 (100%)	10 (6,25%)	10 (6,25%)	26 (16,25%)
Fangs	160 (100%)	20 (12,50%)	40 (25%)	59 (36,85%)
Glands	50 (100%)	0 (0%)	0 (0%)	0 (0%)
Total material analyzed	370 (100%)	30 (8,10%)	50 (13,51%)	85 (22,97%)

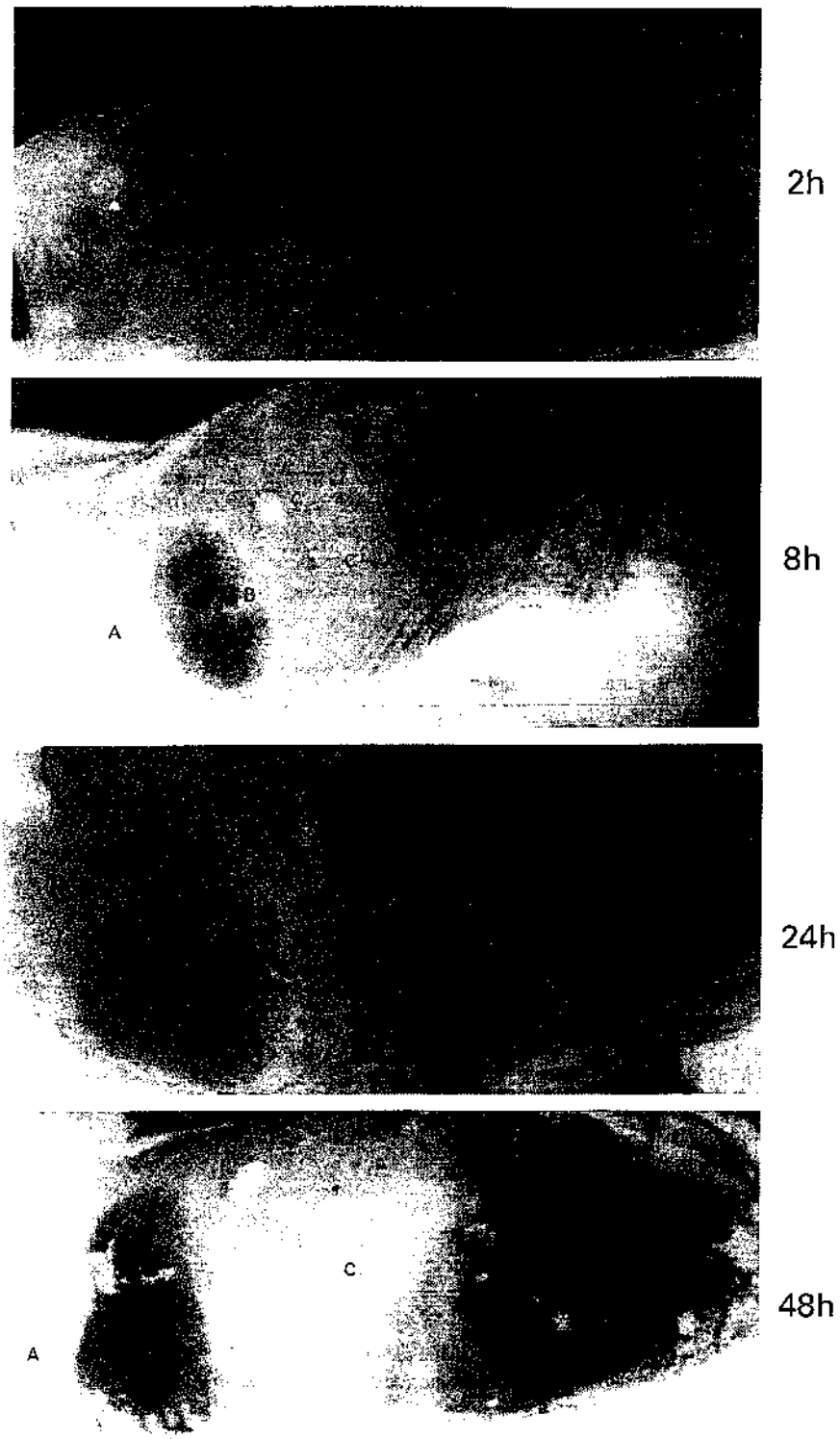


Fig. 1. Dermonecrotic lesion induced in rabbits by PBS, *L. intermedia* electrostimulated venom alone and conjugated with *C. perfringens*. Rabbits were intradermally injected and the dermonecrotic lesions were observed at intervals of 2, 8, 24 and 48 h after injection. (A) 100 μl PBS—phosphate buffer saline; (B) 20 μg *L. intermedia* Venom; (C) 3×10^3 *C. perfringens* cells/100 μl PBS; (D) 20 μg *L. intermedia* enom + 3×10^3 C. *perfringens* cells/100 μl PBS.

venom conjugated with the anaerobic bacterium *Clostridium perfringens* were used. Venom was used in the concentration of 20 μg diluted in PBS (for this concentration represents the lower limits of venom amount usually inoculated in a normal accident with the spider). The approximated amount of bacteria inoculum was 3×10^3 *C. perfringens* cells. Negative control dermonecrotic lesion rabbits had 100 μl of PBS solution inoculated in a previously depilated skin area. Dermonecrotic lesions were evaluated at intervals of 2, 8, 24 and 48 h after injection. Experimental dermonecrosis was also assayed exactly as described above but in these assays the rabbits received the antibiotic Penicillin G or *L. intermedia* venom conjugated with the bacteria *C. perfringens* inactivated through autoclave. All other conditions were exactly the same as described above. After 48 h of venom and bacterial exposure, the rabbits were anesthetized with sodium pentobarbital, sacrificed, and processed for histology as shown below.

2.6. Histological methods for light microscopy

Skin tissues of dermonecrotic rabbits were fixed and processed for histology, embedded in paraffin wax and cut into 4-μm thick sections. The sections were stained with hematoxylin and eosin (Merck) (Beçak and Paulete, 1976) and analyzed under light microscopy; some tissue samples were deparaffined and submitted to Gram stain to study the presence of bacteria.

3. Results

3.1. Microbiological analysis of the electrostimulated venom, fangs and venom glands of L. intermedia

There are no previous data to describe either the presence of bacteria in venom or in neither the fangs of *L. intermedia* nor the role they may play in the pathologic dermonecrotic picture of loxoscelism. The electrostimulated venom fangs and venom glands were cultured in microbiological aerobic and anaerobic media. *Staphylococcus* coagulase-negative, *Escherichia coli*, *Acinetobacter* sp, *Serracia marcensces*, *Propionibacterium acne*, *Bacillus* sp, *Citrobacter freundii* and *Streptococcus* sp., were isolated and identified, in addition to the anaerobic bacterium *Clostridium perfringens* (Table 1), which is potentially pathogenic and causes severe dermonecrotic lesions and which was the target of our investigation in this experiment.

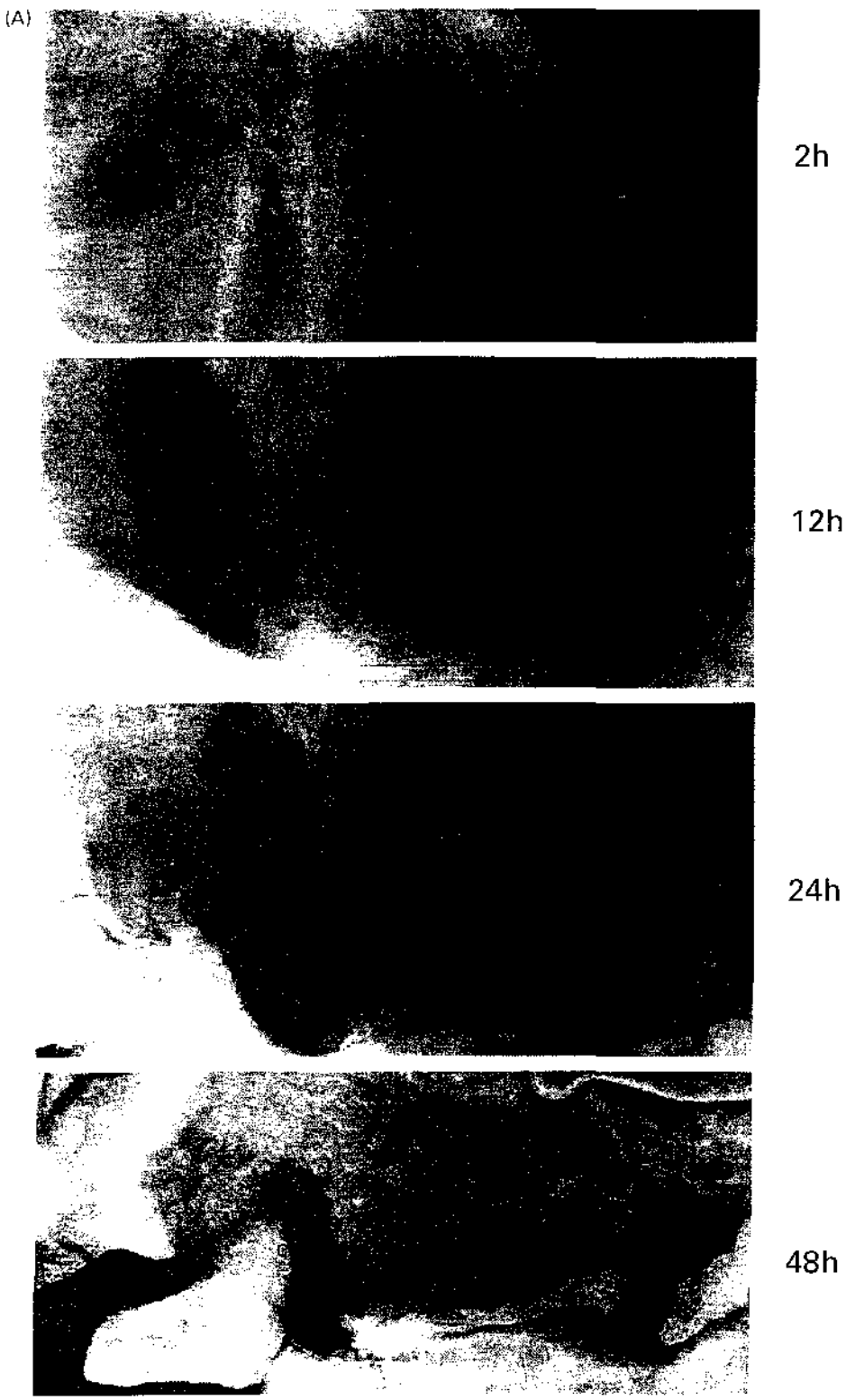
3.2. Evidence for the potentialization of dermonecrotic lesions induced by L. intermedia electrostimulated venom conjugated with C. perfringens

With the purpose of studying the influence of *C. perfringens* with the loxoscelic venom, experimental dermonecrosis was assayed in rabbits for comparison of the dermonecrotic picture induced by the venom alone with that induced when the venom was conjugated with *C. perfringens* (see Section 2). As depicted in Fig. 1, the dermonecrotic lesion induced by *L. intermedia* venom associated with the bacterium *C. perfringens* becomes highly enhanced as compared to the lesion provoked by the venom alone, suggesting a role for these bacteria as an aggravating factor of the experimental dermonecrotic picture.

3.3. Influence of antibiotic therapy and the inactivation of bacteria on autoclave on the dermonecrotic lesions induced by L. intermedia venom

With the purpose of determining the role of *C. perfringens* as an element that favors the exacerbation of the dermonecrotic process caused by *Loxosceles intermedia* venom, experimental dermonecrosis was assayed in rabbits as described above. Briefly, 24 h before and during the experimental inoculation the animals received antibiotic therapy (G Penicillin, the drug of election for anaerobic organisms (Murray and Citron, 1991; Murray et al., 2000)) (Fig. 2A). Alternatively, animals received only PBS, *L. intermedia* electrostimulated venom conjugated with autoclave inactivated bacteria *C. perfringens* and the venom alone (Fig. 2B). As illustrated in the figures, in the experiments with antibiotic therapy and bacteria inactivation there is no enhancement of dermonecrotic lesion, strongly suggesting that *C. perfringens* does play a role in the severity of loxoscelic dermonecrotic picture.

Fig. 2. Influence of antibiotic therapy and the inactivation of bacteria on autoclave on the dermonecrotic lesions. (A) Dermonecrotic lesion induced in rabbits by *L. intermedia* electrostimulated venom alone and conjugated with *C. perfringens* (rabbits were previously and during the experiments, treated with antibiotics). The dermonecrotic lesions were observed at intervals of 2, 12, 24 and 48 h after injection. (A) 100 μl PBS—phosphate buffer saline; (B) 20 μg *L. intermedia* venom alone; (C) 20 μg *L. intermedia* venom + 3×10^3 *C. perfringens* cells/100 μl PBS (conjugate venom + bacteria). There is no enhancement of dermonecrotic lesion evoked by venom plus bacteria compared to B point (venom alone). (B) Dermonecrotic lesion induced in rabbits by *L. intermedia* electrostimulated venom alone and venom conjugated with *C. perfringens* (inactivation of *C. perfringens* by autoclave). The dermonecrotic lesions were observed at intervals of 2, 12, 24 and 48 h after injection. (A) 100 μl PBS—phosphate buffer saline; (B) 20 μg *L. intermedia* venom alone; (C) 20 μg *L. intermedia* venom + 3×10^3 *C. perfringens* cells/100 μl PBS (conjugate venom + bacteria). There is no enhancement of dermonecrotic lesion evoked by venom plus bacteria compared to B point (venom alone).


(A)
2h
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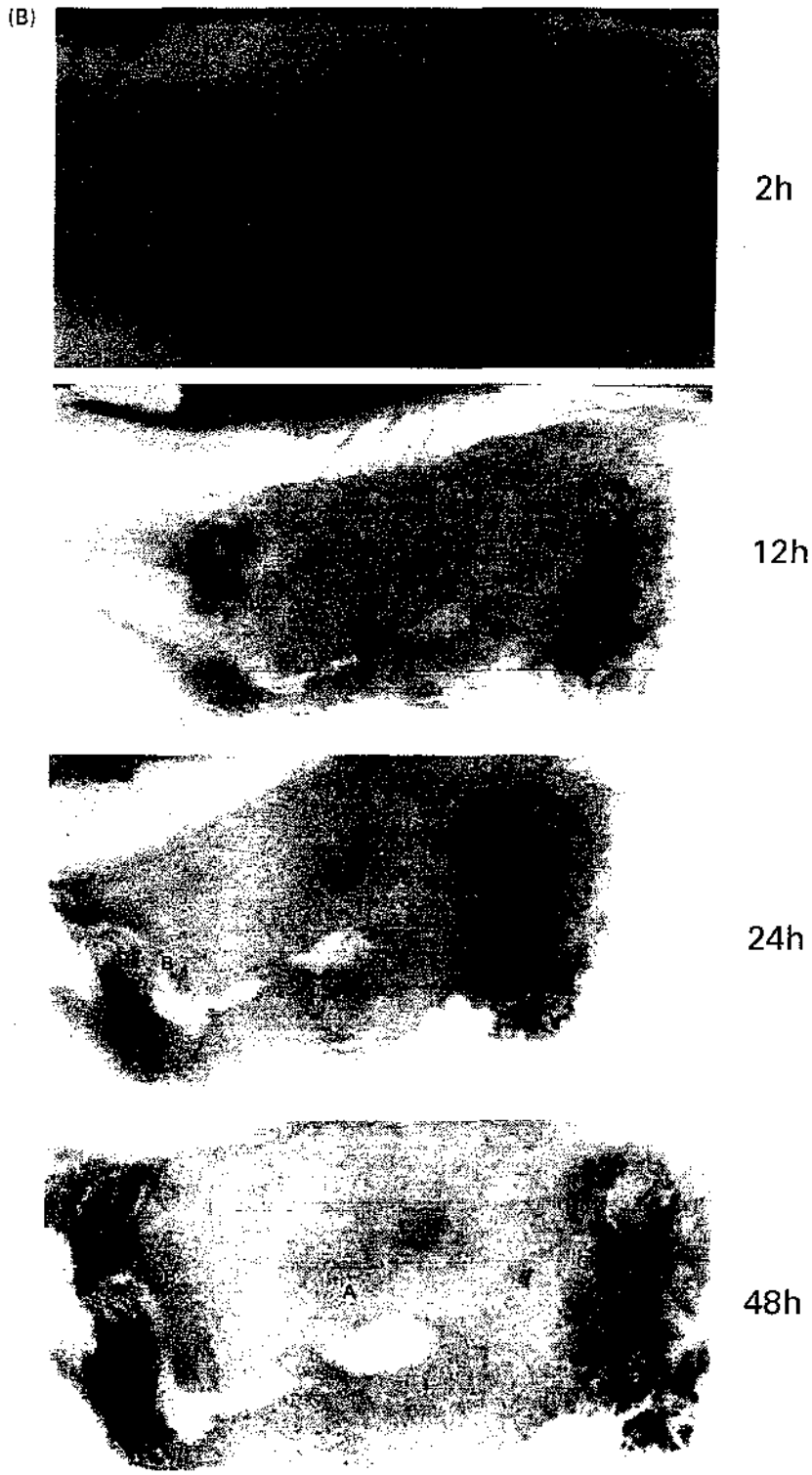

Fig. 2. (*continued*)

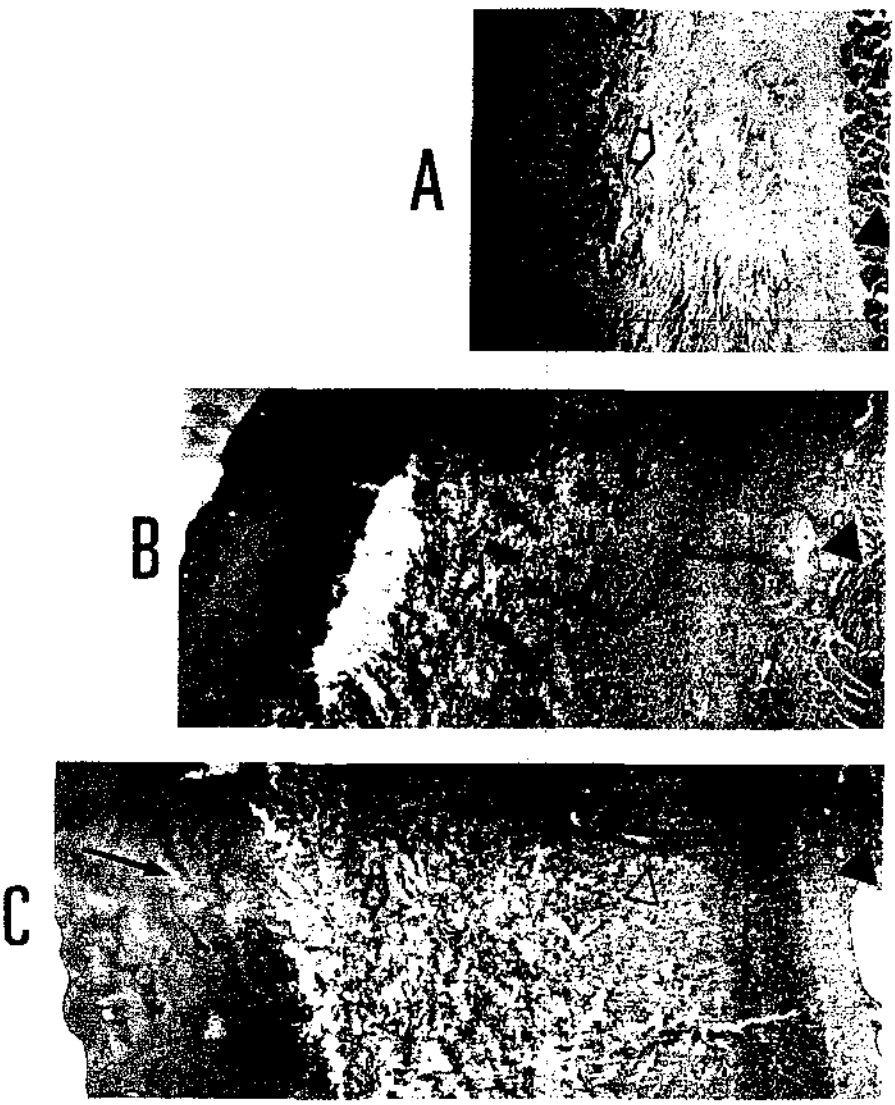

Fig. 3. Comparative histological profiles of rabbit normal skin and dermonecrotic lesions induced in rabbits treated with *L. intermedia* electrostimulated venom alone or *L. intermedia* venom conjugated with *C. perfringens*. (A) Rabbit normal skin; (B) Histopathological profile of lesion induced by *L. intermedia* venom alone; (C) Histopathological profile of lesion induced by *L. intermedia* venom conjugated with the bacteria *C. perfringens* (magnifications of 100×). Comparatively, at the same amplification, we can observe an increase of edema and inflammatory reaction of skin treated by venom plus *C. perfringens*, than venom alone. Closed arrows show epidermis, open arrows point connective tissues, closed arrowheads depict muscle tissue. Open arrowheads point the collections of inflamatory cells basically represented by polymorphonuclear leukocytes.

3.4. Histopathological studies of dermonecrotic lesions induced in rabbits treated with L. intermedia *venom alone and* L. intermedia *venom conjugated with* C. perfringens

With the purpose of proving the correctness of data described previously, which suggest that the presence of the bacteria *C. perfringens* in the dermonecrotic lesion induced by the *L. intermedia* venom favors the enhancement of the lesion, the skin of rabbits inoculated either with the venom of *L. intermedia* or with the venom conjugated with the bacteria *C. perfringens*, was processed for histology and analyzed under light microscopy. Fig. 3A shows the histologic profile of rabbit normal skin. In Fig. 3B the histological profile of the lesion induced by *L. intermedia* venom alone, where one can see the presence of a scar in the skin surface, from the lining epithelium until the skeletal muscle tissue, edema along the whole skin structure, a collection of leukocytes between the connective tissue and fat and skeletal muscle tissues. In Fig. 3C, the histopathological profile of the dermonecrotic lesion induced by *L. intermedia* venom conjugated with *C. perfringens*, revealing the same histological profile as the previous picture, except that it is more

Fig. 4. Gram stain in histological section of dermonecrotic lesion induced in rabbit by *L. intermedia* electrostimulated venom conjugated with *C. perfringens* after 48 h. (A) Histological section of dermonecrotic lesion induced in rabbit by *L. intermedia* venom + *C. perfringens* after 48 h. Stained by Gram method (amplification of 1000×). Arrows point rods of *C. perfringens* distribuited in the tissues. (B) As control an histological section of rabbit skin injected with *C. perfringens* alone after 48 h was stained by Gram method. There was no bacteria growth.

exacerbated. The scar in the skin surface is increased, the edema on the tissue skin is also increased and the leukocyte collection is also very increased in relation to the inflammatory picture induced by *L. intermedia* venom alone. These histopathological data strongly corroborate the microscopic data described previously and suggest that the bacteria *C. perfringens* enhances the dermonecrotic effects of *L. intermedia* venom conjugated with the bacteria *C. perfringens*.

3.5. Reisolation of C. perfringens *from the experimental dermonecrotic lesion induced by* L. intermedia *venom conjugated with the bacteria* C. perfringens

Finally, in order to demonstrate the role of *C. perfringens* as an enhancing element of *L. intermedia* venom dermonecrotic activities, tissue punitions were carried out from the dermonecrotic lesions of tissues inoculated with the venom alone, the bacteria alone and the venom conjugate with bacteria. As shown in Table 2, only the material from the dermonecrotic lesion induced by *L. intermedia* venom conjugated with *C. perfringens* produced a positive result in anaerobic culture, compared to the other samples that provided negative results. Gram stain histology results of dermonecrotic tissues induced by *L. intermedia* venom conjugated with *C. perfringens* in this site after 48 h were positive, as can be seen in Fig. 4. The results obtained strongly suggest the role played by these organisms as potentializers of the dermonecrotic picture and that a previous dermonecrosis induced by the venom is needed for the development of these microrganisms.

Table 2
Reisolations of *Clostridium perfringens* from experimental dermonecrosis induced in rabbits (during experimental procedures, each rabbit received three points of inoculation (A), (B) and (C), from which biopsies were collected and analyzed for anaerobic culture protocols (the experiments were repeated five times)

Samples (culture)	Anaerobic culture
(A) Biopsy of dermonecrotic lesion evoked by *L. intermedia* venom alone after 24 h	Negative
(B) Biopsy of rabbit skin inoculated with *C. perfringens* after 24 h	Negative
(C) Biopsy of dermonecrotic lesion caused by *L. intermedia* venom plus *C. perfringens* after 24 h	Positive

4. Discussion

In this study the authors evaluated the microbiological conditions in some brown spider's body compartments and its venom. Among the bacteria isolated, one of major clinical importance was the anaerobic organism *C. perfringens*. In addition, it was possible to reproduce the dermonecrotic lesion induced in rabbits with the *L. intermedia* venom and with the conjugate *C. perfringens* plus venom with a significant severing of the lesion.

Importantly, in the dermonecrotic and systemic lesions of loxoscelism there is a similarity between data reported in the literature for the brown spider and *C. perfringens* accidents. After the brown spider bite, there appears a erithematous macula in the site of venom inoculation, and also a haemorragic blister may appear. Necrosis becomes evident only by the end of the first week or during the second week after the accident evidentiating an ulcer of variable sizes. If there is no secondary infection, healing of the wound occurs as resolution. In the presence of necrosis, healing seldom occurs before 1 month and in the most severe cases there is a systemic picture named visceral-cutaneous loxoscelism. During the first few days or hours after the accident there is massive haemolysis with subsequent haemoglubinuria that may evolve to renal failure and death (Futrell and Morgan, 1977; Bascur et al., 1982).

After skin introduction by penetrating wounds, animal bites and other routes (Goldstein et al., 1984), the anaerobic bacteria *C. perfringens*, under appropriate conditions (as tissue necrosis) colonize the tissues and proliferates very intensively causing dermonecrotic pictures such as gas gangrene and serious systemic impairment such as renal

failure and death, due to the potent exotoxins that provide these bacteria with extreme pathogenicity. Gas gangrene or myonecrosis is an infection with one of the worst prognosis and after the colonization with *C. perfringens* there is progressive destruction of skeletal muscles and the formation of blisters and gas with accumulation of liquid (edema). Incubation period varies from a few hours to 4–6 weeks (usually 24–48 h), after the first moment of wound onset.The lesion becomes progressively larger, sometimes very rapidly. Blisters and vesicles, containing a dark red liquid, which later becomes brown and dermonecrotic, form on the skin, in the corresponding affected area (Middlebrook and Dorland, 1984).

Besides identification and isolation of anaerobic bacteria found in the venom and fangs of *L. intermedia* spiders, this study intended the reproduction of the dermonecrotic lesions by this potentially pathogenic bacterium. The organism was isolated and identified in spider fangs and electrostimulated venom (Table 1), which although sterile, when cultivated directly from the glands becomes contaminated in the fangs, where this organism is found with significant prevalence (as confirmed in our experiment) due to the contact of the brown spider with the environment, once *C. perfringens* spores are widely distributed in soil, water, plants and human and animal feces (Murray et al., 2000). This is very important because it is probably related with the gravity of loxoscelism pictures. The results obtained suggest that the spider venom gets contaminated during its passage through the fangs (inoculating apparatus) and when the bacterium *C. perfringens* is present the organism will be inoculated together with *L. intermedia* venom, and this may be the causing agent of the severe dermonecrosis pictures not only because of toxic components of *L. intermedia* venom itself (Feitosa et al., 1998; Veiga et al., 2000) but also due to the highly potentially pathogenic toxins produced by *C. perfringens* (Murray and Citron, 1991; Murray et al., 2000) and as the venom rapidly causes a necrotic lesion on the tissue, this results in an environment that favors the growing of anaerobic *C. perfringens* organisms.

When *L. intermedia* venom was inoculated with the bacteria *C. perfringens* there was an evident potentialization of all parameters mentioned above. This study indicates that *C. perfringens* is an important factor in the exacerbation of dermonecrotic lesions caused by *L. intermedia* also suggesting that it may contribute to the eventual systemic simultaneous impairment, which is typical when these organisms are present in the wounds. This finding provides the basis for better prophylactic and therapeutic approaches of *L. intermedia* lesions.

Patients bitten by the brown spider receive a therapeutic protocol that involves the use of anti-histaminic, corticosteroids and anti-loxoscelic sera. Plastic surgery is recommended when ulcerations are extensive or affect areas like face (Barbaro et al., 1992; Finegold, 1993). While in gas gangrene specific therapy is carried out with G-penicillin as the first choice antibiotic for anaerobic organisms like *C. perfringens*, serology and surgical intervention is recommended for the compromised dermonecrotic areas (Hatheway, 1990; Murray and Citron, 1991; Murray et al., 2000).

The data obtained with this experiment emphasize the possibility of co-infection of the lesions caused by *Loxosceles intermedia* with *Clostridium perfringens* especially in those cases with the presence of severity parameters already mentioned. Thereby, an efficient therapy for treating loxoscelism must take into account that besides the venom components with toxic activities there might be a new date adding to the therapeutic and preventive studies of the accidents with the brown spider, *L. intermedia*.

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Histopathological findings in rabbits after experimental acute exposure to the *Loxosceles intermedia* (brown spider) venom

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Abstract

Loxoscelism, the term used to describe envenomation with brown spiders, is characterized by a dermonecrotic lesion at the bite site. In the present investigation we submitted albino rabbits to an acute experimental envenomation protocol using *Loxosceles intermedia* (brown spider) venom, with emphasis on the determination of the features of lesion pathogenesis induced by this spider, which is the cause of several accidents through out the world. Rabbits received intradermal injections of the venom and were monitored over the first 4 hours, and then 12 hours and 1, 2 and 5 days after envenomation. Histologic specimens from 3 rabbits per time point were collected from euthanized animals and processed for histological examination by light microscopy. Major macroscopic findings observed during the first 4 hours were swelling and a haemorrhagic spot at the injection sites and microscopic finding at the dermis level were oedema, haemorrhage, degeneration of blood vessel walls, plasma exudation, thrombosis, neutrophil accumulation in and around blood vessels with an intensive diapedesis, a diffuse collection of inflammatory cells (polymorphonuclear leukocytes) in the dermis, and subcutaneous muscular oedema. Over the following hours and up to 5 days after envenomation the changes progressed to massive neutrophil infiltration (no other leukocytes) into the dermis and even into subcutaneous muscle tissue, destruction of blood vessels, thrombosis, haemorrhage, myonecrosis, and coagulative necrosis on the 5th day. Lesion repair was apparent at 2 days, with proliferation of fibroblasts and the formation of a granulation tissue.

Introduction

Brown spider venoms have long been recognized for their potent noxious activities. Spiders of the genus *Loxosceles* cause severe necrotic arachnidism at the bite site in humans throughout the world (Futrell, 1992; Meier and White, 1995) and also several systemic effects such as acute renal failure, disseminated intravascular coagulation and intravascular haemolysis, that, although less common than local signs of envenomation, are responsible for the death of the victims (Futrell, 1992; Kurpiewski *et al.* 1981; Lung and Mallory, 2000; Rees *et al.* 1983).

Dermonecrotic reaction, the most striking manifestation evoked during envenomation, is currently under investigation, with some venom toxins now well understood as is the case for the low molecular mass protein of 33-35 kDa characterized as sphingomyelinase D (Futrell, 1992; Kurpiewski *et al.* 1981; Tambourgi *et al.* 1998; Tambourgi *et al.* 2000) involved in dermonecrosis, platelet aggregation and haemolysis, and hyaluronidases of 33 kDa and 63 kDa, probably involved in the gravitational spreading of dermonecrosis (another hallmark of accidents) (Futrell, 1992; Veiga *et al.* 2001a; Wright *et al.* 1973). Some proteases may be involved in haemorrhage and intravascular coagulation through their fibrinogenolytic activity, in delayed wound healing (another feature of envenomation), defective platelet adhesion and haemorrhage through their fibronectinolytic activity, and in proteolysis of entactin and heparan sulphate proteoglycan protein core. They may also cause basement membrane disruption that may be related to renal failure and alteration of the integrity of blood vessel

walls with plasma exudation and haemorrhage, as well as venom spreading to the body with systemic effects (Feitosa *et al.*1998; Veiga *et al.* 2000; Veiga *et al.* 2001a; Veiga *et al.* 2001b; Zanetti *et al.* 2002).

A great problem regarding the understanding of Loxoscelism is the fact that different authors have used venoms harvested from distinct *Loxosceles* spider species in their experimental investigations. State of the art informations about brown spiders have been reported in studies using venoms from *L. deserta, L. gaucho, L. intermedia, L. laeta, L. reclusa and L. rufescens* (Barbaro *et al.* 1992; Futrell, 1992; Gomez *et al.* 1999; Pizzi *et al.* 1957; Young and Pincus, 2001). Some results described for a species of *Loxosceles* are not reported for others, basically because researchers generally use in their experiments the venom collected from the most common species in their countries and the difficulties to obtain enough venom to reproduce experimentally the pathogenesis of Loxoscelism at laboratory conditions. The histopathological findings concerning dermonecrotic lesions are good examples of this problem. Literature data describing such observations have been reported for experimental or accidental envenomations caused by *L. reclusa* and *L. laeta* (Futrell, 1992; Pizzi *et al.* 1957; Smith and Micks, 1970), but no additional information is available about exposure to the venoms of *L. deserta, L. gaucho, L. hirsuta, L. intermedia*, and others.

These questions, together with the high incidence of necrotic arachnidism in Southern Brazil (an average of more than 2,000 accidents per year only in the city of Curitiba and Metropolitan Region), prompted us to perform a detailed study concerning the pathological changes induced by exposure to the venom of *L. intermedia*, the brown spider that prevails in Southern Brazil. The present data

describe characteristics of the histopathological findings of experimental acute dermonecrotic lesions induced in rabbits by *L. intermedia* venom. Our results revealed a massive acute inflammatory reaction with coagulative necrosis of affected areas. By using this kind of analysis, we hope that some insight into Loxoscelism could be gained, opening the possibility for a differential diagnosis in case of doubtful etiology.

Materials and methods

Spider venom extraction

The venom was extracted from *L. intermedia* spiders captured in nature and kept for 5 days without any kind of food. An electrostimulation of 15 V was applied to the cephalothorax of the spiders and the venom was collected directly from the stings with a micropipette, dried under vacuum and frozen at – 85°C. A pool of venom collected from 1.000 spiders was used in the experiments (Feitosa, *et al.* 1998; Veiga *et al.* 2000).

Dermonecrosis studies in vivo on rabbits

For evaluation of dermonecrosis, pooled venom (samples of 40 μg of proteins from crude venom diluted in a final volume of 100 μl PBS, since 40 μg of proteins represent the average venom concentration injected during natural accidents), was injected intradermally into shaved areas of the skin of rabbits. Three adult New Zealand animals weighing 3 and 4 kg from the Central Animal

House of the Federal University of Parana were used for each time interval. The dermonecrotic lesions were checked 4 hours, 12 hours, 1 day and 2 and 5 days after the injections. A group of animals that received only 100 μl of PBS was used as a negative control (Lopes- Ferreira, 2001). After venom exposure, the animals were anesthetized with sodium thiopental (Abbott, São Paulo, Brazil), sacrificed and processed for histology as shown below.

Histological methods for light microscopy

Tissues were processed for histology as described (Drury and Wallington, 1980). After fixation, tissues were embedded in paraffin and cut into 4 μm thick sections. The sections were stained with hematoxylin (Merck, Darmstadt, Germany) and eosin (Sigma, St Louis, USA) and analyzed by light microscopy (Olympus BX-40, Japan).

Immunohistochemical analysis

Materials were formalin-fixed. Paraffin sections mounted on glass slides were deparaffinized in xylene overnight and rehydrated in graded ethanol series and water. The sections were then washed with PBS, incubated in 3% H_2O_2 (Merck) at room temperature for 15 min to inhibit the activity of endogenous peroxidase and washed with PBS, and nonspecific protein binding sites were blocked with 1% bovine serum albumin (Sigma) in PBS at room temperature for 30 min under humidified conditions. After washing in PBS, sections were incubated for 2 hous at 37°C with a primary polyclonal antibody against fibrinogen diluted 1:500

(produced in our laboratory using purified fibrinogen and rabbits, as described (Halow and Lane, 1988). Excess antibody was removed with PBS and the material was incubated with peroxidase-conjugated goat anti-rabbit IgG (Sigma) diluted 1:100. Following further washing in PBS, diaminobenzidine (Sigma) was used to visualize the immunoreactivity. Sections were washed in PBS and water, dehydrated in ethanol, cleared in xylene, and mounted in Entellan (Merck). Negative control reactions were performed by incubating the sections with pre-immune sera diluted in PBS under the same experimental conditons as described for hyperimmune serum.

Results

Normal macroscopic and histological characteristics of the skin of rabbits

The objective of the present report was the description of the development of the lesions caused by *L. intermedia* venom during acute cutaneous Loxoscelism experimentally induced in rabbits. We considered as acute cutaneous Loxoscelism the lesions that appeared on the skin from 0 hours to 5 days after envenomation. All tested animals developed very similar reactions.

As a control, Figure 1 depicts the normal aspect of rabbit skin. Figure 1A shows the macroscopic appearence of a shaved area of skin. In Figure 1B, we can observe histological details of the epidermis and the underlying dermis. The stratified squamous epithelium, *stratum corneum* with keratinized cells and collagen fibers are visible. Figure 1C depicts collagen bundles of dense connective tissue and details of hair-associated structures. Figure 1D illustrates

details of normal dermis or *corium* located beneath the epidermis, showing a layer of dense connective tissue rich in collagen fibers arranged as randomly interwoven bundles of varying sizes. Normal blood vessels responsible for the blood supply to the dermis are also shown (1D). Figure 1E depicts the normal aspect of skeletal muscle tissue located beneath the dermis.

Histopathological changes in rabbit skin induced by L. intermedia venom 4 hours after treatment

The initial sign of injury appeared between 1 and 4 hours after venom injection. As shown macroscopically (Figure 2A), swelling and haemorrhage (characterized as a spot) developed at the injection site. Histologically, within 4 hours after venom exposure the epidermis was preserved, showing the stratified squamous epithelium and *stratum corneum* with keratinized cells (2B). Nevertheless, oedema occurred under the dermis, as shown by marked disorganization of loose connective tissue (2B). At this stage hair follicles were preserved (2C). A biopsy collected at this time showed an intensive collection of inflammatory cells in and around the blood vessels of the dermis, characterizing massive diapedesis with infiltration of connective tissue and even deeper tissues. The inflammatory cells are basically represented by neutrophils (2D, 2E, 2F). The dense connective tissue inside the dermis presents disorganization of collagen fibers with multifocal infiltration of inflammatory cells (2H) and a proteinaceous network formation among collagen bundles (2I). Immunohistochemical reaction using an anti-fibrinogen antiserum confirmed that his proteinaceous substance is fibrin (2J), indicating an increase in vascular permeability with exudation and

of skelectal muscle by leukocytes and an outflow of fluid among muscle fibers (muscular oedema) (Figure 3H, 3I).

Histological features of dermonecrotic lesions induced in rabbits 24 hours after L. intermedia venom exposure

Twenty-four hours after experimental envenomation, macroscopically, the site of envenomation was surrounded by a white halo of ischemic tissue. The area of gravitational spreading showed haemorrhage, erythema and swelling and changed from a violaceous to a black colour (Figure 4A). Histologically, the lesion showed preserved epidermis (epithelium and keratinized cells), but with the *stratum corneum* assuming a dull dark colour. Disorganization of the collagen fibers from loose connective tissue near the epidermis was especially marked with some leukocyte infiltrates indicating oedema and an evolving inflammatory reaction (Figure 4B). The hair follicles with necrotic degeneration, as well as a collection of inflammatory cells were maximized compared to the 12 hour treated group. A fibrinoid exudate and erythrocytes surrounding hair follicle debris were also evident (Figure 4C). At 24 hours after envenomation the vascular changes were enormous; thrombosis with necrotic tissue, a massive accumulation of leukocytes inside blood vessels with peripheral orientation along the vascular endothelium (leukocytic margination), transmigration across the blood vessel wall (diapedesis), migration to surrounding interstitial connective tissue (the leukocytes were mainly neutrophils at different stages of maturation, with no other kind of leukocytes being detected) (Figures 4D, 4E, 4F). Intense interstitial oedema, fibrin network deposition among interwoven collagen bundles from dense connective tissue and a marked

accumulation of inflammatory polymorphonuclear leukocytes in the dermis were clearly visible (Figure 4G). At this time, the pathogenesis induced by envenomation in skeletal muscle was evidenced by myonecrosis of some myofibrils (myolysis) and leukocyte infiltration (Figure 4H).

Histological findings 2 days after experimental L. intermedia envenomation

Macroscopically, the evolution of the dermonecrotic reaction at the injection site 48 hours post-envenomation showed a halo of ischemic tissue varying in colour from white to red and hardened. At this time, in the area of gravitational spreading the center of the lesion acquired a hardened texture, the central area also became black, with the surrounding region showing diffuse erythema (Figure 5A). At the microscopical level, the striking feature at this time was the destruction of epidermis integrity, with a massive haemorrhage and necrosis of surrounding collagen near the epidermis (Figure 5B). The hair follicles appeared like necrotic debris of tissue, also surrounded by a massive haemorrhage (Figure 5C). Morphological changes inside the dermis revealed a radial leukocyte infiltration, diffuse points of necrosis and a marked haemorrhage into the connective tissue. The collagen fibers of the affected areas were disturbed and expanded by oedema. A fibrillar exudation was also noted (Figure 5D, 5E). A marked accumulation of inflammatory cells (PMN leukocytes) was observed in the interface of the dermis with skeletal muscle (we named this event "neutrophils palisade") (Figure 5F, 5G). The muscle tissue revealed areas of myolysis and necrosis, with a radial infiltrate of leukocytes. At this time, nevertheless, an important modification was noted in some sections of the interface of muscle and connective tissue, i.e., the presence

of granulation tissue showing abundant formation of new blood vessels (angiogenesis) and fibroblast migration and proliferation, indicating that a repair process was in progress (Figure 5H, 5I).

The histologic picture 5 days post experimental envenomation by L. intermedia

At this time macroscopically the necrotizing lesion assumed a dark colour and developed an indurated and extensive eschar, where at least two regions could be differentiated. At the injection site (like a *punctum*) there was a hard halo and at a distance there was a more prominent lesion area disseminated by gravitational spreading (Figure 6A). Microscopically there was an impressive necrotic lesion of the epidermis and surrounding regions (Figure 6B), as well as connective tissue and hair follicles inside the dermis (Figure 6C). At this stage muscle tissue under the dermis showed myolysis and calcification points (Figure 6F, 6G). At the interface of muscle tissue and the neutrophils palisade (Figure 6E), a repair process could be detected by the presence of a well-formed bed of granulation tissue with fibroblast migration and proliferation, and deposition and organization of new fibrous tissue (Figure 6D).

Discussion

Loxoscelism is the term used to describe reactions and lesions caused by bites from spiders of the genus *Loxosceles*. These spiders are distributed all around the world, although some species prevail in different countries for reasons that have not been fully elucidated. *Loxosceles deserta* is the primary species in

Africa; *L. rufescens* in the Mediterranean and Australia; *L. laeta* is located primarily in Central America and South America; *L. reclusa* is found in the Midwest States of the USA, and *L. intermedia* prevails in South Region of Brazil where this species is responsible for most cases of envenomation (Futrell, 1992; Veiga *et al.* 2001a; Young and Pincus, 2001).

The bites of brown spiders are remarkable for causing dermonecrosis, the hallmark of envenomation, that sometimes is also named gangrenous arachnidism or necrotic arachnidism (Futrell, 1992; Meier and White, 1995) and may evolve to a skin ulceration. Loxoscelism is an unresolved clinical problem; the treatments for local lesions are based on steroids, dapsone, anti-venom serum therapy and surgical excision, but they only reduce the problem when used in a time-dependent manner and when administered during the first hours post-envenomation (Futrell, 1992; Rees *et al.* 1981).

In the past, most studies on brown spider envenomation were conducted using *L. reclusa* venom or clinical data from natural accidents with this spider. Little was known about other species of brown spiders.

The literature suggests that clinical and laboratory findings regarding natural accidents resemble those obtained in animal models especially when rabbits are used, showing a significant overlap of similarities (Elston *et al.* 2000; Futrell, 1992; Veiga *et al.* 2000).

Considering the differential geographic distribution of spiders of the genus Loxosceles, some particular characteristics of each species, the endemic accidents caused by *L. intermedia* in South of Brazil and the lack of literature data focussing on the histopathological changes evoked by envenomation with this species, and in

order to provide some information for the differential diagnosis and some support for treatments, we described here the histological findings obtained after experimental acute envenomation of rabbits using *L. intermedia* venom. For acute envenomation we considered tissue reactions that occured during the first 5 days post-venom exposure. The time of collection of specimens for analysis was based on previous reports from our group (Veiga *et al.* 1999; Veiga *et al.* 2000) and the appearance of major macroscopic changes in the skin that could be of interest to physicians were described.

Histological analysis performed 4 hours after venom exposure showed macroscopically a haemorrhagic spot at the injection site; microscopically, the cells from the epidermis and hair follicle in the dermis were preserved, although early events observed within the dermis were interstitial oedema, plasma exudation indicated by fibrin deposition in connective tissue, a massive accumulation of leukocytes (PMN) inside blood vessels (no other kind of infiltrated leukocytes were detected in our model) and an evident diapedesis with inflammatory infiltrate of interstitial structures and even of muscle tissue. At this time we also detected capillary thrombosis and blood vessel wall degeneration, besides oedema of skeletal muscle under the skin. No gravitational spreading was observed in 4-hour treated rabbits.

Based on the present data, which show an early (in the first 4 hours) and massive collection of PMN leukocytes inside blood vessels and distributed in neighbouring tissues, and since these cells play an essential role in the dermonecrotic effects of brown spider venoms (Patel *et al.* 1994; Smith and Micks 1970), we may understand why most treatments induce no significant decrease in

dermonecrosis when applied later, and the importance of a differential diagnosis and of an early visit to a specialist for an effective therapy.

Over the following hours (12 h-treated group) erythema and swelling at the injection site and gravitational spreading of haemorrhage were macroscopically evident. Histological analysis showed preserved epidermis, oedema of interstitial tissue with plasma exudation, hair follicle necrosis, neutrophil infiltrate, haemorrhage, necrotic thrombosis with vessel oclusion, blood vessel degeneration, muscular oedema, and myolysis. Macroscopic analysis the 24 h-treated group showed a white halo of ischemia at the injection site and clearly visible tissue of a dark colour colocalized with gravitational spreading. Histologic analysis showed preserved epidermis but with the *stratum corneum* assuming a dark colour. In the dermis there were diffuse oedema, fibrin exudation, haemorrhage, necrotizing hair follicles, a marked infiltration of neutrophils, thrombosis, and myonecrosis. Forty-eight hours after venom exposure, macroscopically we noted white ischemic tissue at the injection site and the characteristic gravitational spreading of the lesion with a dark colour, that became indurated and was surrounded by erythema at the edge of the lesion. At this time histopathological changes include deep epidermal-dermal necrosis, massive haemorrhage in the dermis, necrotizing hair follicles, fibrillar exudation, a very impressive palisade of inflammatory cells (PMN) at the interface of the dermis with muscular tissue, and myonecrosis. From skelectal muscle to connective tissue a granulation tissue with new blood vessels (angiogenesis) and fibroblast proliferation indicates the beginning of the repair process. Five days post-envenomation macroscopically there was a darkened eschar at the injection site colocalized with the gravitational spreading of the lesion. Microscopically the

histologic picture showed a deep coagulative necrosis including epidermis, hair follicles, connective tissue of the dermis, and muscular tissue. At this stage the neutrophils continued to accumulate inside the lesioned areas and especially at the interface of connective and muscular tissue. The granulation tissue was present and increased compared to the 48 h-treated group, confirming the continuation of the repair process with fibrosis.

In this investigation we did not detect an infiltration of eosinophils near the site of venom exposure or eosinophilic coagulative necrosis, as described (Elston *et al.* 2000). Such disagreement could be explained by the fact that in the present investigation we studied acute lesions and all specimens for analysis were collected during the first 5 days post-venom injury and eosinophil infiltration could be a later event, since these authors (Elston *et al.* 2000) collected their materials 14 days after envenomation.

There are no previous histologic descriptions of experimental or natural dermonecrosis induced by *L. intermedia*. Comparative studies were reported for experiments using *L. reclusa* venom. Smith and Micks (1970) reported thickening of the skin, oedema, accumulation of PMN leukocytes, intravascular clotting, massive haemorrhage into the dermis and loss of integrity of blood vessel walls within the first 6 hours. Rees *et al.* (1981) also described thrombosis, vascular necrosis and neutrophil accumulation surrounding the injected region 6 hours after envenomation. By using *L. reclusa* venom and rabbit models, Strain *et al.* (1991) reported oedema, erythema, ischemia and cyanosis in the first 12 hours post-envenomation and crateriform ulcer formation and extensive purpura by 24 hours. Futrell (1992), studying the same venom under similar conditions, also reported

early accumulation of PMN leukocytes, vascular thrombosis, blood vessel wall damage and haemorrhage in the dermis in the first 3 hours post-envenomation. Pizzi *et al.* (1957), in a study of *L. laeta* venom and rabbit models, reported oedema, erythema, thrombosis, degenerative alteration of blood vessels with infiltration of leukocytes and erythrocytes into the connective tissue of the dermis in the first 24 hours of envenomation. Clinical data and biopsies from human patients after brown spider bites have shown moderate inflammatory infiltrate, thrombosis and haemorrhage (Dillaha *et al.* 1964), dermatitis, acute inflammation, induration of lesioned region, erythema, liquefactive necrosis of the epidermis and the dermis consistent with pyoderma gangrenosum (Hoover *et al.* 1990), besides necrosis of the dermis, with fibrin deposition and myonecrosis (Yiannias and Winkelmann, 1992).

The histopathological findings described during this investigation as macroscopic aspects of the lesions, the time course of dermonecrosis, features such as interstitial oedema, plasma exudation, thrombus formation inside the capillaries, vessel degeneration and a PMN leukocyte-mediated inflammatory process, open the possibility for a differential diagnosis. We hope that this insight into Loxoscelism can be useful to the pathologists in dermonecrosis ethiology studies.

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Legends

Figure 1 *Normal aspects of the skin of rabbits.*

(A) Macroscopic view of a shaved area of normal rabbit skin (open arrow). (B) Histological appearance of the epidermis and surrounding dermis (closed arrowheads) showing the presence of *stratum corneum* and keratinized cells; closed arrows show epithelial cells and open arrows depict collagen bundles from loose connective tissue (400X). (C) Details of the dermis (closed arrows) show hair follicles; white arrows point at collagen bundles from dense connective tissue (400X). (D) Appearance of normal blood vessels (closed arrowheads) showing endothelial cells from the *tunica intima*; closed arrows point at a blood vessel wall (400X). (E) Aspects of subcutaneous skeletal muscle (white arrows) show the interface of muscle fibers; closed arrowheads point at myofibrils of normal appearance (400X).

Figure 2 *Histopathological changes of the skin of rabbits 4 hours post-L. intermedia venom exposure.*

(A) Macroscopic appearance of a shaved area of the skin after venom injection

Preserved epithelial cells (closed arrows) and keratinized cells (closed arrowheads) of the epidermis, but disorganization of collagen fibers of loose connective tissue (open arrows) are present, indicating the occurrence of oedema (400X). (C) Hair follicles are preserved (closed arrowheads), but oedema is confirmed by disorganization of collagen bundles from connective tissue in the dermis (closed arrows), (200X). (D) Massive inflammatory cell accumulation within blood vessels in the dermis (closed arrows), (100X). (E) Details of a blood vessel showing inflammatory leukocytes accumulated in (white arrow) and around (closed arrows) the vessel,(630X). (F) A massive diapedesis of inflammatory cells to connective tissue of the dermis (white arrow) can be seen; the closed arrow points at the interior of the blood vessel (400X). (G) Details of the inflammatory infiltrate show neutrophils (closed arrows) as involved cells (1000X). (H) Details of dermis showing collections of leukocytes infiltrated into the connective tissue (closed arrowheads) and a profuse disorganization of collagen fibers (closed arrows), (400X). (I) Details of the dense connective tissue showing disorganization of collagen fibers (closed arrows), a collection of inflammatory cells (closed arrowheads) and a proteinaceous network diffused among collagen fibers (open arrows), (200X). (J) Positive immunohistochemical reaction using anti-fibrinogen antiserum supporting the nature of the proteinaceous network in the dermis as fibrin and evidencing plasma exudation (closed arrowheads), (430X). (K) Details of a blood vessel showing an intravascular fibrin network with adhered leukocytes (closed arrows), (630X). (L) A congested blood vessel showing intravascular clotting (closed arrowheads), collections of adhered leukocytes inside connective tissue (white arrow) and leukocytes infiltrating the surrounding connective tissue

(closed arrows), (200X). (M) Disorganization of subcutaneous skeletal muscle fibers (closed arrows) supporting the presence of muscular oedema and inflammatory infiltration (closed arrowheads), (200X).

Figure 3 *Histopathological findings of the skin of rabbits 12 hours after L. intermedia envenomation*

(A) Macroscopic appearance of a shaved area of the skin showing the beginning of the characteristic gravitational spread of the lesion; the open arrow points at the site of venom injection with haemorrhage; closed arrows point to the spreading of the lesion with swelling, haemorrhage and erythema distant from the site of venom exposure. (B) Examination of the epidermis revealed no pathologic abnormalities. Closed arrowheads point at preserved keratinized cells and closed arrows show epithelial cells; open arrows denote disrupted collagen bundles confirming oedema of loose connective tissue, (400X). (C) Inside the dermis, hair follicles reveal signals of disorganization and necrosis (open arrows), (200X). (D) Disorganization of collagen fibers (closed arrows), cellular debris and fibrin depositon are visible (open arrows) in the dermis, confirming oedema and plasma exudation, (1000X). (E) Also, a diffuse infiltration of inflammatory leukocytes is evident in the dermis, (closed arrows) (200X). (F) A dense band of leukocytes (closed arrows) is present in the dermis (100X). (G) Details of a blood vessel show necrotic thrombosis (open arrow) and a degenerated vessel wall (closed arrow) (200X). (H) Disorganization of skelectal muscle fibers showing oedema and infiltrated leukocytes in the interstitium (closed arrows) (200X). (I) A collection of leukocytes infiltrated among

muscle fibers (open arrows) as well as areas of myolysis (closed arrows) are evident (400X).

Figure 4 *Histopathological abnormalities of the skin of rabbits induced 24 hours after L. intermedia venom exposure*

(A) Macroscopically, a white halo of ischemic tissue appears at the injection site (closed arrow). The gravitational spreading of the lesion is increased compared to 12 hours and the area assumes a dark colour (white arrow). (B) The epidermis structures show some changes (white arrows), with normal epithelial cells (closed arrows), darkened *stratum corneum* and disorganized collagen fibers (closed arrowheads) (200X). (C) In the dermis, hair follicles show necrotic degeneration (closed arrowheads), and fibrin deposition and erythrocyte debris surrounding hair structures are also seen (closed arrows); infiltrated leukocytes are detected (white arrow), (200X). (D) Thrombosis (closed arrowhead), with massive accumulation of leukocytes with peripheral orientation inside blood vessels (closed arrows) and a marked diapedesis, is also noted (white arrow), (200X). (E) A palisade of inflammatory cells is evident in the dermis (closed arrows), (100X). (F) Details of neutrophils involved in the inflammatory reaction (closed arrows), (1000X). (G) Disturbed dermal collagen fibers (closed arrows), fibrin deposits (closed arrowheads) and collections of leukocytes (white arrows), (100X). (H) Analysis of subcutaneous skeletal muscle shows prominent myonecrosis (closed arrows), (200X).

Figure 5 *Histopathological features of the skin of rabbits 2 days after L. intermedia envenomatiom.*

(A) Macroscopically, the lesion shows a hardened and reddened area (closed arrow) at the site of venom injection, and the gravitational spreading also becomes indurated and dark (white arrow) with diffuse erythema (closed arrowheads). (B) Microscopic examination reveals epidermal necrosis (closed arrows) with diffuse points of dermal necrosis (closed arrowheads), (200X). (C) The hair follicles are necrotized (closed arrows) and surrounded by a massive presence of extravascular red blood cells (closed arrowheads), (200X). (D) Details of the dermis show marked haemorrhage (closed arrows), and diffuse necrotic points (closed arrowheads) in connective tissue (400X). (E) Less affected areas of the dermis immediately adjacent to the necrotic areas show disturbed collagen fibers (closed arrows), collection of leukocytes (closed arrowheads), and fibrin deposition (white arrows) (100X). (F) A very prominent palisade of leukocytes (white arrows) can be seen at the interface of the dermis (closed arrows) with subcutaneous muscle (closed arrowheads), (100X). (G) Details of the leukocyte palisade indicate the presence of neutrophil cells (closed arrowheads),(1.000X). (H) Granulation tissue appears at this time at the interface of subcutaneous muscle with the dermis (closed arrowheads), (200X). (I) Details of tissue repair show proliferation of fibroblasts (closed arrowheads) and new blood vessel formation (closed arrows); details of muscle tissue show diffuse myonecrosis (white arrows), (400X).

Figure 6 *Histological appearance of rabbit skin 5 days after L. intermedia envenomation*

(A) Macroscopic view of a dermonecrotic lesion. An eschar indurated as a reddened halo appears at the injection site (closed arrow) and a darkened eschar extends around it due to gravitational spread of lesion (white arrow). (B) An impressive necrosis of epidermis (closed arrows) and immediately adjacent dermis (closed arrowheads), (200X) is visible. (C) Debris of destroyed hair follicles are shown (closed arrows). Peripheral areas of the dermis are also completely necrotized (closed arrowheads), (200X). (D) Immediately adjacent to the leukocyte palisade (white arrows) a bed of granulation and fibrous tissue is noted (closed arrows), (100X). (E) Details of the leukocyte band indicate the presence of neutrophils (closed arrows), (1000X). (F) Details of the interface of granulation and muscle tissue showing calcifications points (closed arrows), fibrous tissue (closed arrowheads) and myonecrosis (white arrows), (100X). (G) Details of subcutaneous muscular tissue indicating massive myonecrosis (closed arrowheads) and infiltration of repairing fibroblasts (interstitial fibrosis), (closed arrows), (200X).

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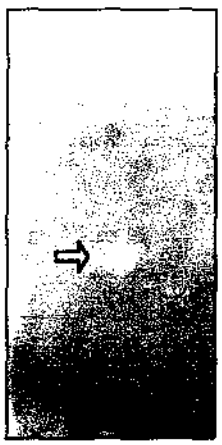

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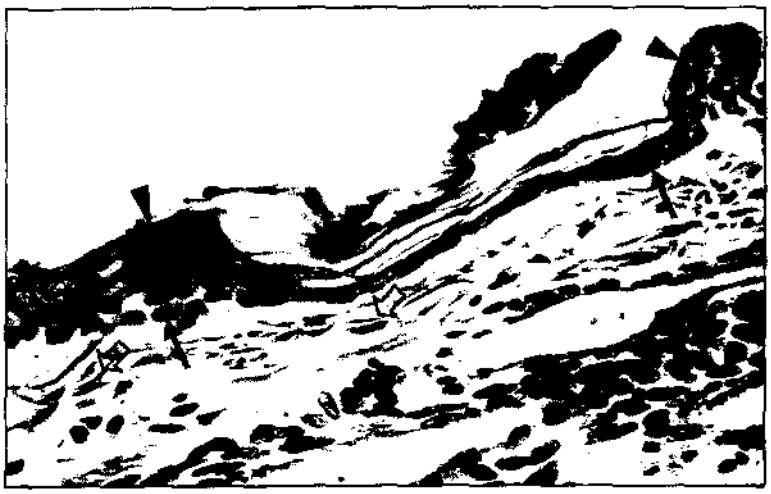

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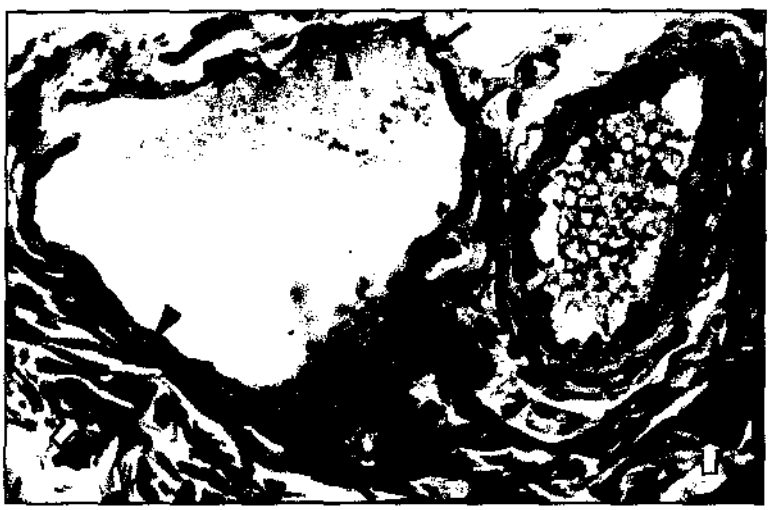

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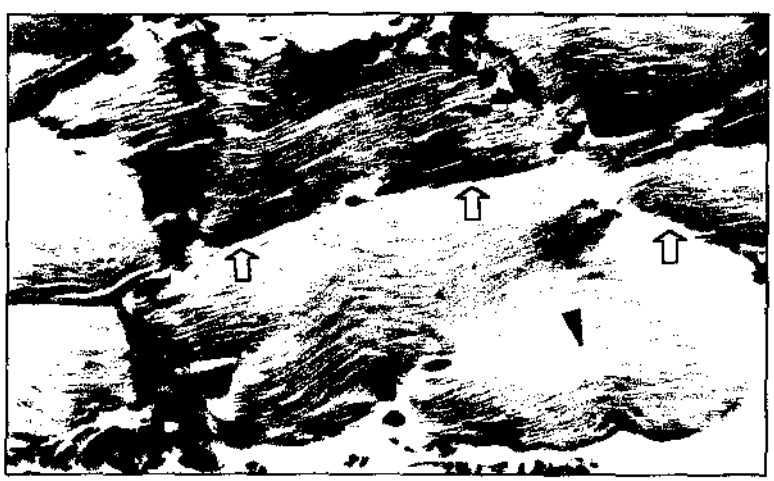

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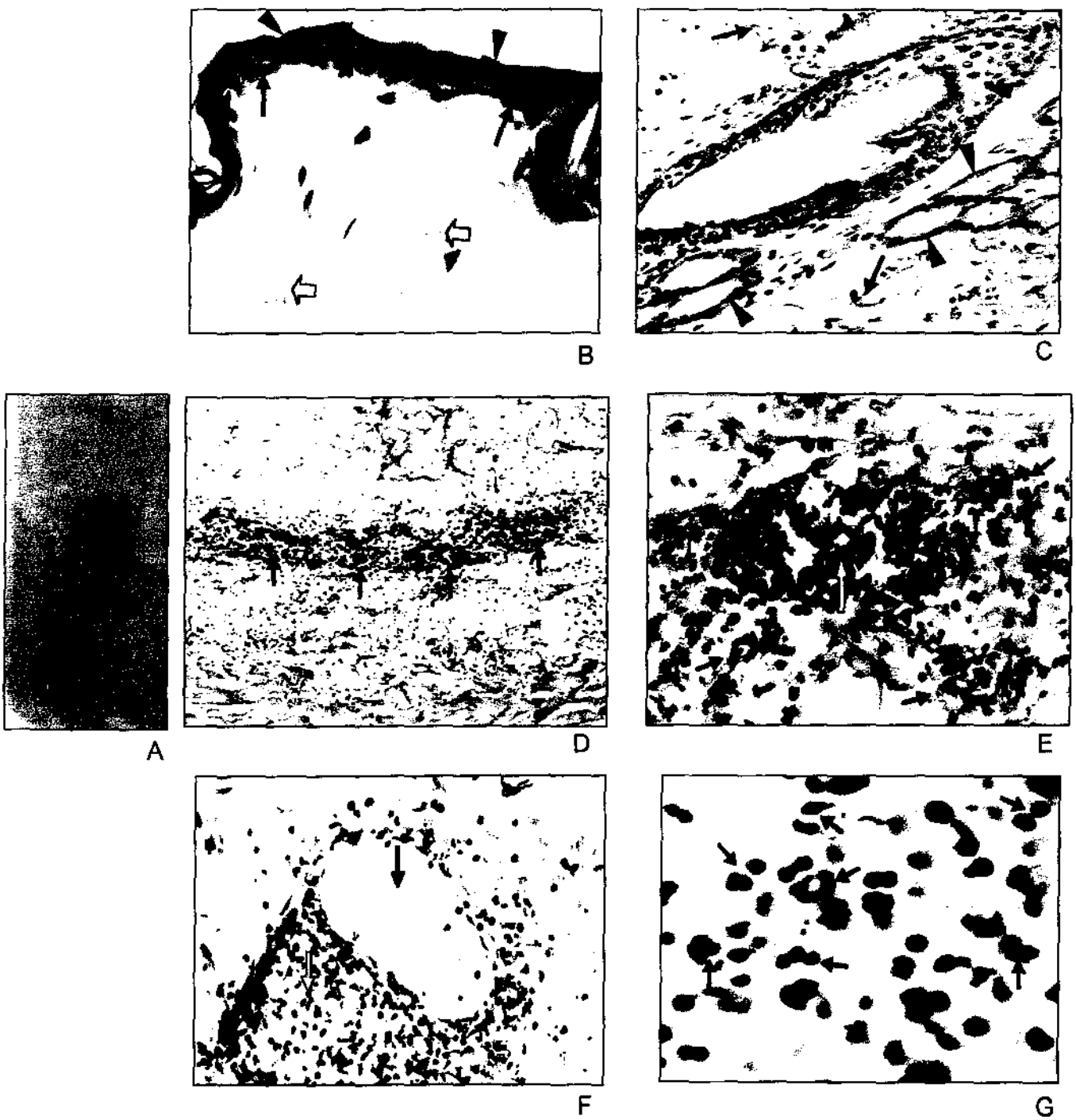
Figure 1 Ospedal *et al.*, 2002



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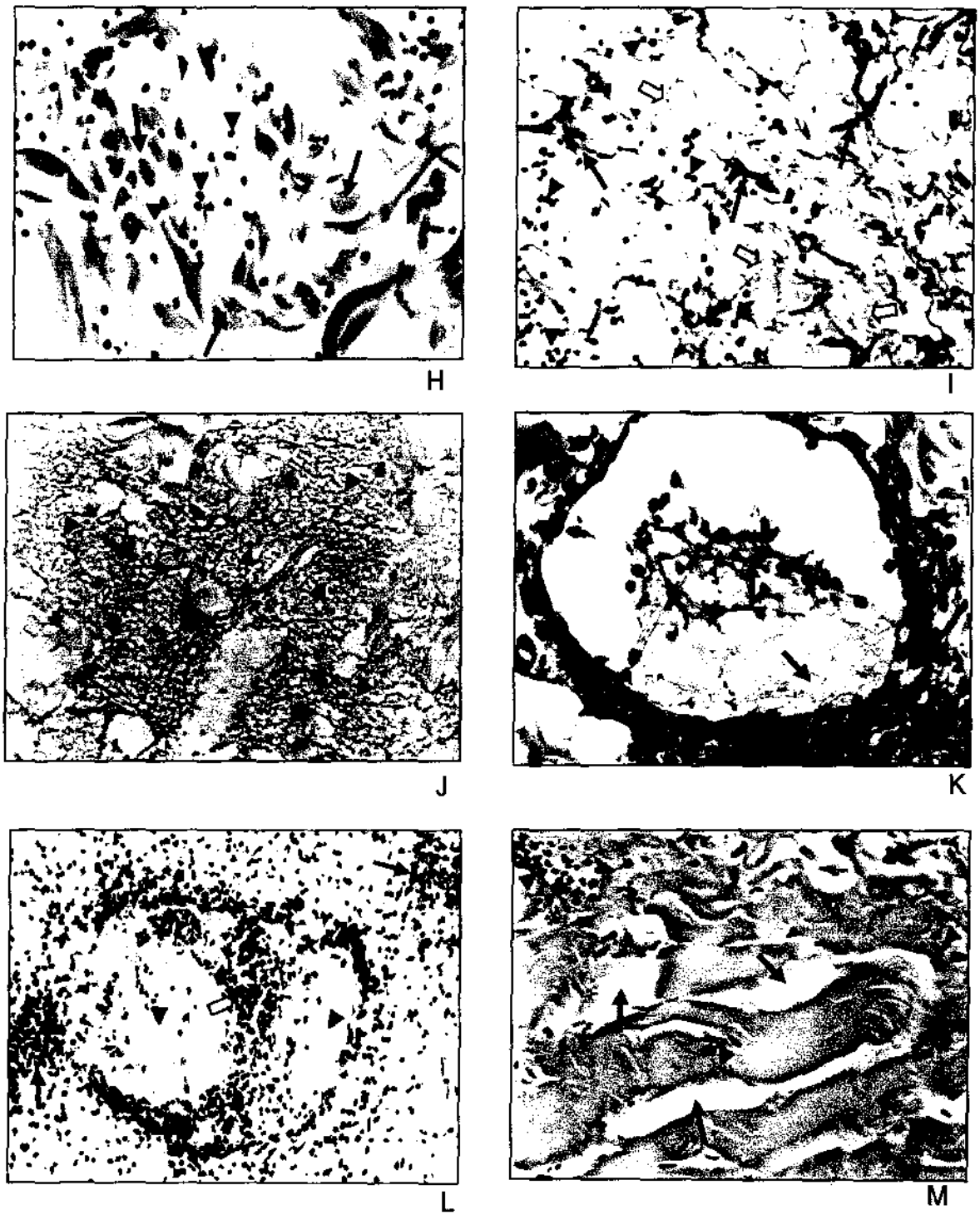

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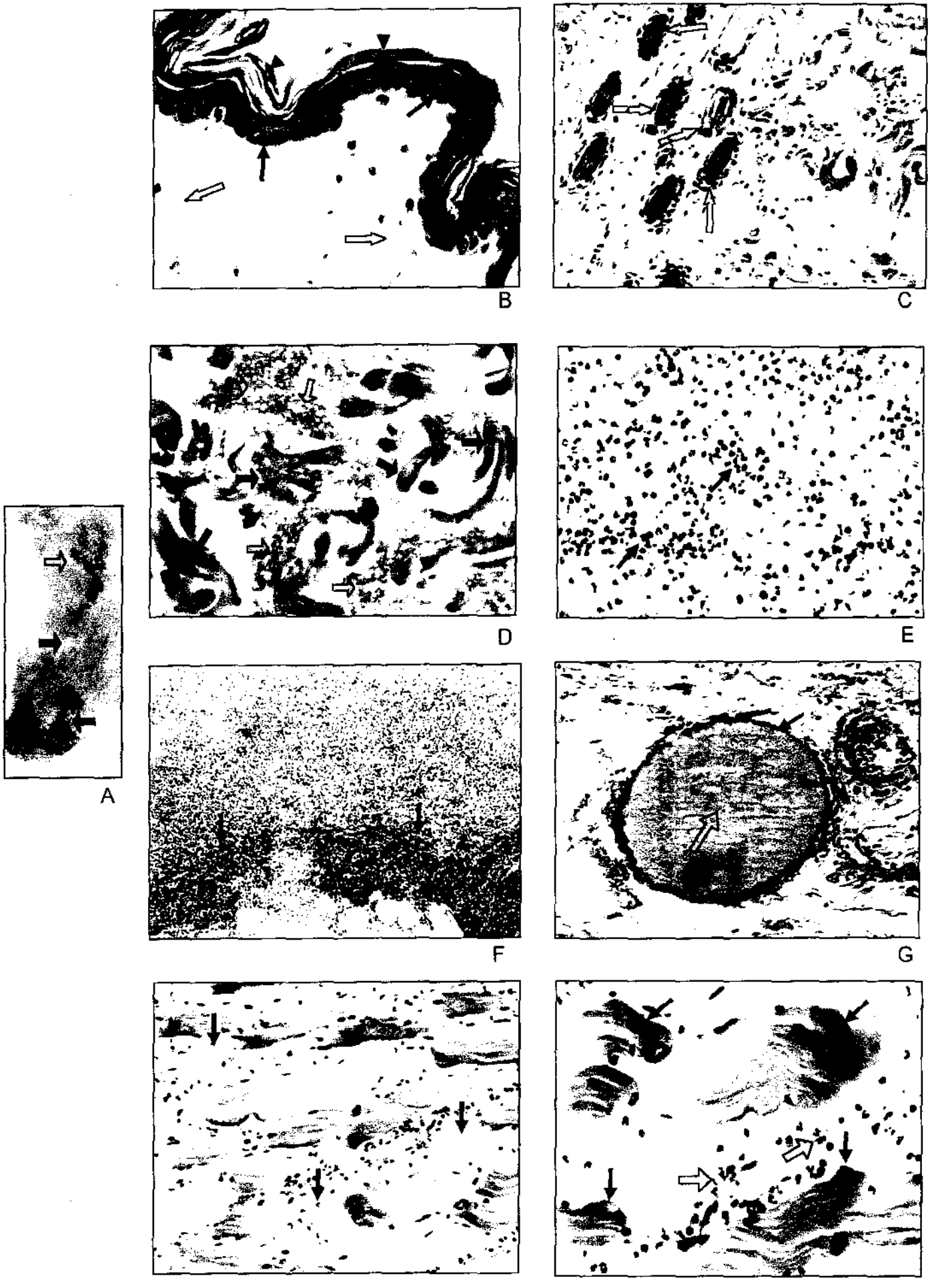

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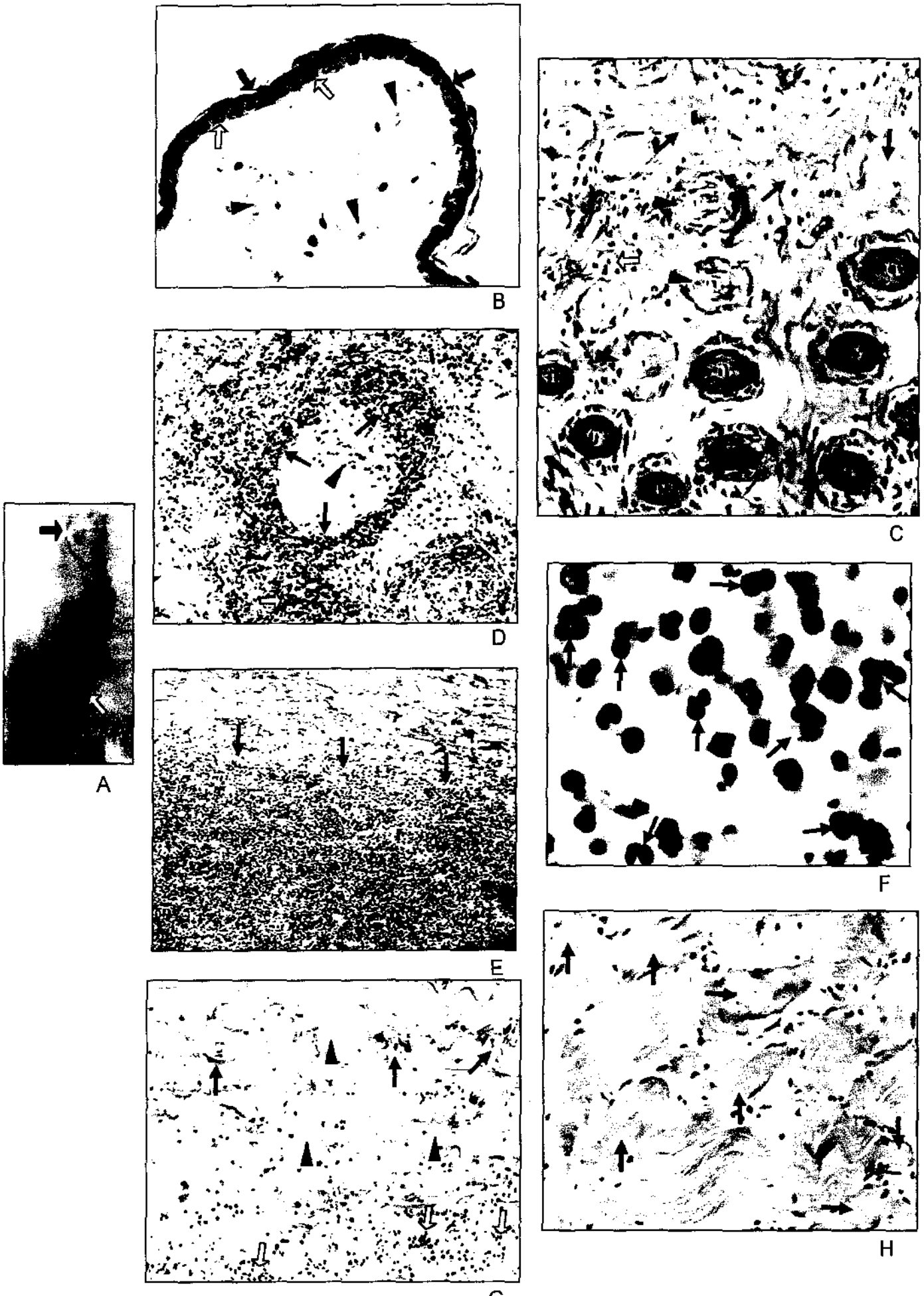

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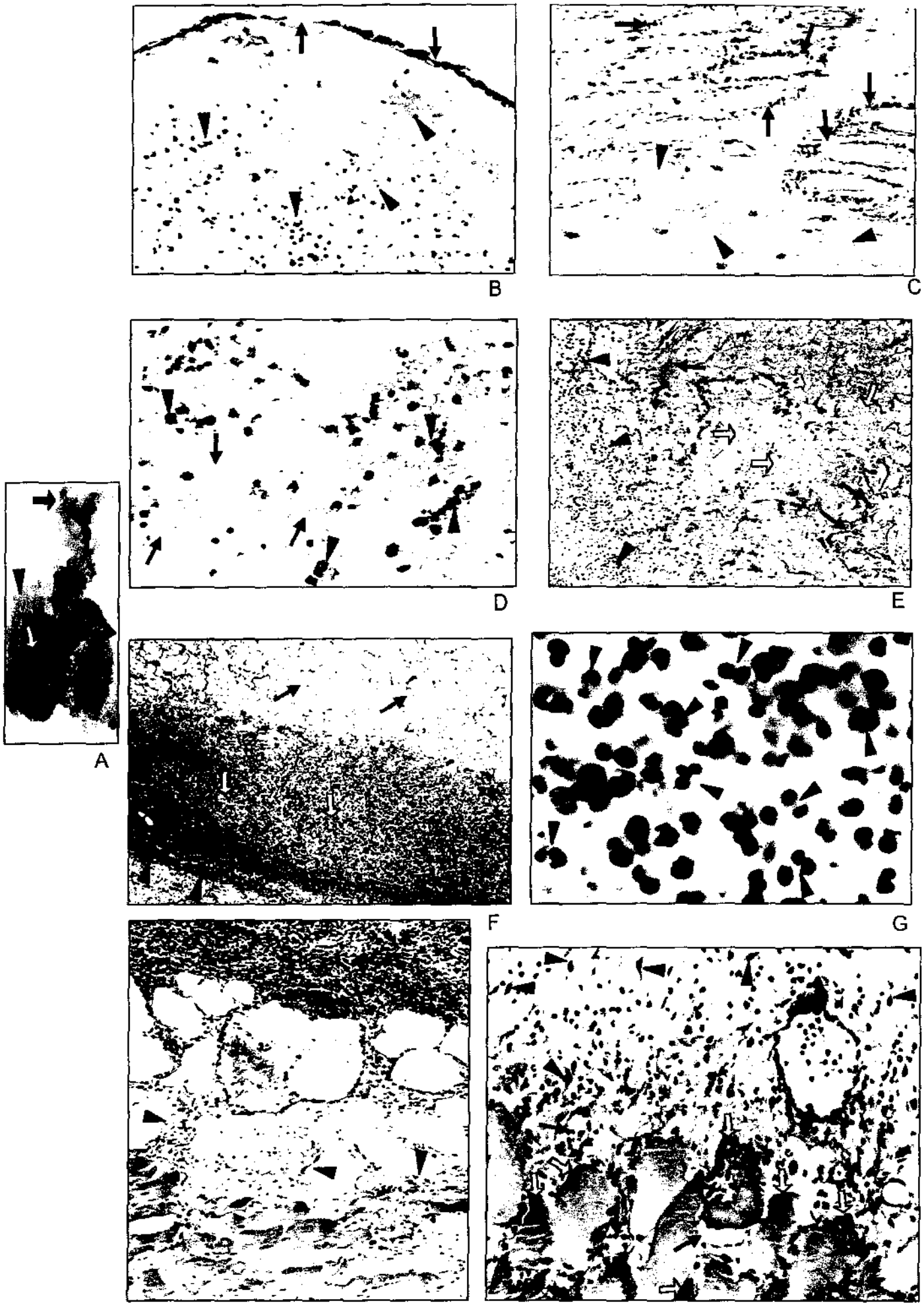

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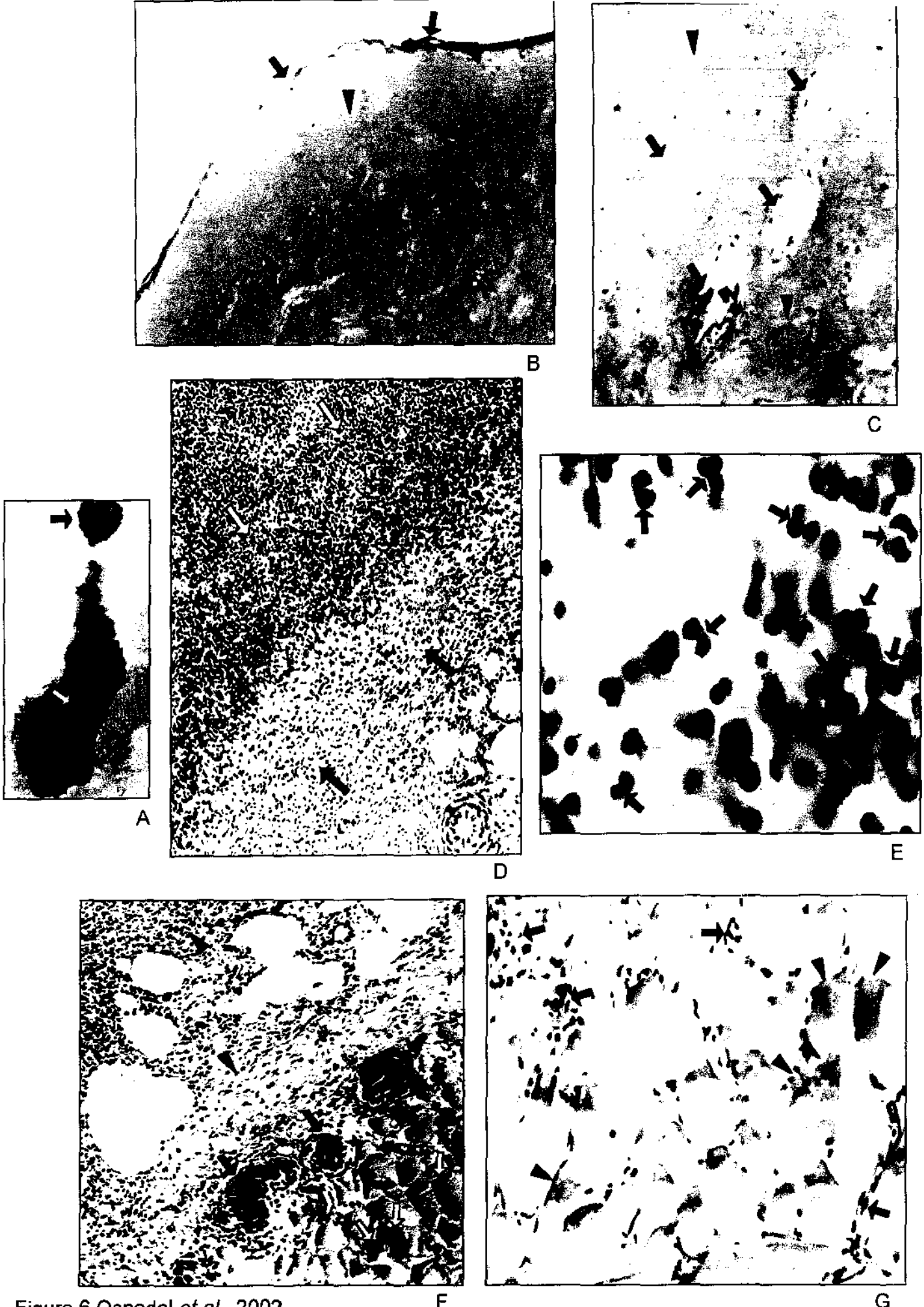

Figure 6 Ospedal *et al.*, 2002

Conclusões Finais

A partir dos experimentos e resultados mostrados neste trabalho, podemos concluir:

1. As glândulas produtoras e secretoras de veneno da aranha marrom *L. intermedia* existem aos pares por animal. Histologicamente são estruturas fusiformes, possuindo duas camadas de musculatura estriada, uma externa bastante ramificada e outra interna em contato direto com uma lâmina basal que separa o tecido muscular do epitélio secretor de veneno. As células musculares são multinucleadas, com os núcleos localizados perifericamente, têm citoplasma rico em retículo endoplasmático liso, miofibrilas e linhas Z contínuas. As células epiteliais secretoras são do tipo glandular simples, com núcleo basal e grande quantidade de eucromatina. As porções citoplasmáticas mostram sinais de intensa atividade secretora, possuem grande quantidade de interdigitações, invaginações de membranas, proeminente retículo endoplasmático rugoso, lojas mitocondriais, evidente complexo de Golgi e enorme quantidade de vesículas secretórias. A secreção é do tipo holócrina.

2. O veneno de *L. intermedia* é uma mistura complexa da proteínas e peptídeos, enriquecido em proteínas de baixa massa molecular entre 5-40 kDa, mas contendo também proteínas de alta massa molecular menos expressas.

3. No veneno de *L. intermedia* obtido por eletrochoque, existem duas serino-proteases de 85 kDa e 95 kDa, as quais são gelatinolíticas, têm pH ótimo entre 7,0 e 8,0 e podem ser ativadas por tratamento exógeno com tripsina.

4. No veneno de *L. intermedia* obtido por eletrochoque, existem duas metaloproteases, a Loxolisina A, com 20-28 kDa de massa molecular e atividades fibronectinolítica e fibrinogenolítica; e a Loxolisina B, com 32-35 kDa e atividade gelatinolítica.

5. As mesmas atividades gelatinolíticas , fibronectinolíticas e fibrinogenolíticas dependentes de metaloproteases são detectadas no veneno extrato glandular de *L. intermedia*, descartando possíveis contaminações com egesto digestivo no veneno obtido por eletrochoque. A presença de proteases também é preservada no veneno glandular de *L. laeta*.

6. O veneno de *L. intermedia* contém glicoproteínas N-glicosiladas do tipo alta-manoses e fucosiladas, mas não contém proteoglicanos. A Loxolisina B é uma glicoproteína do tipo alta-manose.

7. O veneno de *L. intermedia* apresenta atividade proteolítica e disruptiva sobre a membrana basal do sarcoma murino de EHS, degrada a entactina e o núcleo protéico do heparam sulfato proteoglicano presente nas lâminas basais, atividades estas dependentes de metaloproteases, mas não tem atividade direta sobre a laminina, o colágeno tipo IV ou glicosaminoglicanos do tipo heparam sulfato.

8. O veneno de *L. intermedia* tem atividade citotóxica direta sobre células endoteliais em cultura ou *in vivo* nos vasos sangüíneos da pele de coelhos experimentalmente expostos ao veneno.

9. Da atividade fibrinogenolítica causada pelo veneno de *L. intermedia*, o veneno parcialmente degrada as cadeias $A\alpha$ e $B\beta$ da molécula de fibrinogênio *in natura*, e significativamente degrada todas as cadeias $A\alpha$, $B\beta$ e γ do fibrinogênio desnaturado. Esta atividade fibrinogenolítica é dependente de uma metaloprotease de aproximadamente 30 kDa no veneno de *L. intermedia* e está conservada nos venenos de *L. laeta* e *L. gaucho*.

10. Da histopatologia da pele induzida experimentalmente em coelhos com veneno de *L. intermedia*, existe em nível macroscópico inchaço e hemorragia local nas primeira 4 horas, espalhamento gravitacional da lesão em 12 horas e aparecimento de escara e necrose entre 24 e 48 horas. Em

nível microscópico existe edema do tecido conectivo e mesmo muscular subcutâneo, degeneração de vasos sangüíneos, exudação de plasma, trombose, acúmulo de leucócitos do tipo PMN, com profunda diapedese na derme e mesmo musculatura adjacente, mionecrose e necrose coagulativa.

11. A bactéria anaeróbica *Clostridium perfringens* pode ser isolada do veneno e quelíceras da aranha *L. intermedia* capturadas da natureza. A associação experimental do veneno de *L. intermedia* e a bactéria *Clostridium perfringens* produz lesão dermonecrótica mais acentuada em coelhos, quando comparada à lesão induzida pelo veneno sozinho, sugerindo da participação destas bactérias como agentes potencializadores das lesões dermonecróticas.