MARCIA HELENA APPEL

PRODUÇÃO DE FERRAMENTAS BIOLÓGICAS E ESTUDO DE PROTEÍNAS DERMONECRÓTICAS RECOMBINANTES DE ARANHA MARROM *Loxosceles intermedia*

Tese de Doutorado apresentado como requisito parcial para a obtenção de título de doutor ao Programa de Pós-Graduação em Biologia Celular e Moleculardo Departamento de Biologia Celular do Setor de Ciências Biológicas da Universidade Federal do Paraná.

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SUMÁRIO

INTRODUÇÃO

As aranhas são animais que dentro da classificação taxonômica pertencem ao grupo dos artrópodes terrestres, classe dos aracnídeos, a qual é a segunda no número de espécies, sendo que as aranhas compõem seu maior subgrupo (RUPPERT E BARNES, 1996). Existem descritas mais de 40.000 espécies de aranhas e provavelmente 100.000 a serem descritas, porém com apenas 3 famílias – Theridiidae, Sicariidae e Ctenidae – reconhecidas como perigosas. Além do mais existem apenas três gêneros *Atrax*, *Lactrodectus* e *Loxosceles* associados à mortalidade humana (ESCOUBAS *et al*., 2000; RASH E HODGSON, 2002).

Há dois grupos principais de aranhas: o Migalomorfa (aranha primitiva ou aranha "trapdoor") cuja quelícera se projeta à frente partindo do cefalotórax enquanto as presas posicionam-se para baixo. O outro grupo é o das Araneomorfa, no qual as representantes possuem as quelíceras posicionadas verticalmente e que conjuntamente com as presas se movem lateralmente como pinças (RASH E HODGSON, 2002); a aranha marrom se insere neste grupo.

Do ponto de vista morfológico, possuem o corpo dividido em dois segmentos, o cefalotórax (união entre cabeça e tórax) e o abdômen; quatro pares de patas; apresentam quelíceras e pedipalpos e podem possuir até oito olhos (RUPPERT E BARNES, 1996).

As espécies mais perigosas de aranhas são encontradas no grupo das Araneomorfas: *Lactrodectus* (viúva negra – Theridiidae), *Loxosceles* (aranha violino ou aranha marrom – Sicariidae), *Phoneutria* (aranha banana – Ctenidae), as quais são responsáveis por muitos casos de envenenamento grave e registros de óbito. Muitas outras famílias são consideradas perigosas – Segestriidae, Agelenidae, Salticidae, Gnaphosidae, Thomisidae, Heteropodidae, Clubionidae e

Lycosidae – embora a maioria das notificações de envenenamentos não seja adequadamente documentada. Notificações relacionadas com acidentes envolvendo estas espécies devem ser cuidadosamente consideradas à luz do polimorfismo natural da síndrome causada por este tipo de envenenamento e a incerteza da identificação taxonômica (ESCOUBAS *et al*., 2000).

Aranhas do gênero *Loxosceles*

Para as aranhas do gênero *Loxosceles*, a organização das glândulas de veneno segue a arquitetura geral das glândulas de veneno dos demais grupos, com epitélio secretor é revestido por uma membrana basal e rodeado por uma espessa musculatura (JUNQUA E VACHON, 1968; BÜCHERL, 1972; FOIL *et al*., 1979). Um exame mais apurado das glândulas de veneno de *L. intermedia* mostra que as mesmas têm duas camadas de fibras musculares estriadas, uma externa e uma interna que toca na estrutura mais interior que separa a região muscular da região epitelial das glândulas de veneno. Este epitélio é simples glandular secretor, com células mononucleadas com os núcleos alinhados perifericamente próximos ao tecido que separa as células epiteliais das musculares. O citoplasma das células epiteliais é arranjado lado a lado, contendo projeções para o lúmen das glândulas, o que sugere hiperatividade. O lúmen das glândulas é rico em vesículas secretoras contendo veneno (DOS SANTOS *et al*., 2000).

As aranhas do gênero *Loxosceles* pertencem à família Sicariidae, subordem Araneomorphae, ordem Araneae, classe Arachnida e filo Arthropoda (RUPPERT E BARNES, 1996; SOERENSEN, 1996).

As aranhas deste gênero recebem também a denominação popular de aranhas marrons por seu colorido uniforme que varia de marrom claro até o marrom escuro. Variações nesta coloração podem ajudar NA identificação de algumas espécies como a *L. gaucho,* que apresenta mancha clara ou *L. laeta,*

com mancha escura no cefalotórax. Estas são aranhas de pequeno porte, possuem comprimento corporal variando de 8 a 15 mm e suas patas medem de 8 a 30 mm e seis olhos brancos brilhantes reunidos sobre o cefalotórax, em três grupos de dois, em semicírculo. O cefalotórax é baixo, não ultrapassando em altura o abdômen (BÜCHERL, 1972). Os machos diferenciam-se das fêmeas por terem corpo menor e pernas relativamente mais longas do que as fêmeas.

De hábitos noturnos, são sedentárias e não agressivas. Preferindo a escuridão, algumas vivem sob pedras, troncos de árvores, restos vegetais, telhas e tijolos empilhados. Com hábitos intradomiciliares podem ser encontradas atrás de quadros e móveis, no meio de roupas, livros, caixas de papelão e outros objetos. As teias são irregulares semelhantes a algodão esfiapado. As aranhas marrons são carnívoras alimentando-se de pequenos insetos e se reproduzem com facilidade, mesmo em ambientes desfavoráveis BÜCHERL E ROSENFELD, 1954; HITE *et al*., 1960; GAJARDO-TOBAR, 1966; SCHENONE E LETONJA, 1975; LUCAS, 1988; FUTRELL, 1992). Distribuindo-se cosmopolitamente podem estar presentes em ambientes com temperaturas entre 8 e 43°C. Os acidentes com aranhas marrons ocorrem principalmente durante as estações mais quentes do ano, primavera e verão (SCHENONE E LETONJA, 1975). Ambos os sexos são venenosos e podem sobreviver até 276 dias sem alimento (GERSTCH E ENNIK, 1983; FUTRELL, 1992).

Ao contrário de outros animais peçonhentos, como ofídios, escorpiões e outros quelicerados, a aranha marrom não é agressiva. A maior parte dos acidentes loxoscélicos (nome dado ao acidente provocado pela picada da aranha marrom) ocorre devido à compressão do animal, inadvertidamente, contra a pele no ato de vestir-se, calçar-se, enxugar-se ou durante o sono (SUAREZ *et al.,* 1971; LUCAS, 1988; FUTRELL, 1992).

Venenos loxoscélicos

As proteínas dos venenos de aracnídeos incluem tanto neurotoxinas de alta massa molecular quanto enzimas. Contudo, com exceção de venenos altamente necróticos como o de *Loxosceles*, nos quais enzimas já foram bem caracterizadas, a presença de enzimas em venenos de aranhas deve ser vista com cautela. Proteases, hialuronidases, esfingomielinases, fosfolipases e isomerases foram descritas em venenos destes animais (SCHANBACHER *et al.,* 1973). Porém, em muitos dos casos deve-se suspeitar de contaminação do veneno por saliva ou egesto digestivo, em particular quando o veneno é colhido por eletrochoque. No gênero *Loxosceles* a presença de proteases foi detectada no extrato de glândula descartando-se a contaminação por egesto digestivo e comprovando a existência de tais moléculas ativas biologicamente (DA SILVEIRA *et al*., 2002). Muitas toxinas de alta massa molecular já foram descritas em veneno de aranhas, como por exemplo, do gênero *Latrodectus* (viúva negra); a alta neurotoxicidade deste veneno, tanto para invertebrados quanto para vertebrados, é creditada à presença de uma família de proteínas com massa molecular em torno de 110 kDa, chamada latrotoxinas (HOLTZ E HABENER, 1998).

GRISHIN (1999) divide quimicamente as moléculas de baixa massa molecular de venenos de aranhas em dois grupos maiores: as toxinas poliamínicas e as polipeptídicas, estas últimas são subdivididas por suas características funcionais e moleculares. Estas toxinas são representadas por dois grupos majoritários. O primeiro grupo compreende peptídeos relativamente pequenos, os quais podem interagir com canais iônicos das membranas excitáveis. O segundo cobre neurotoxinas de mais alta massa molecular que se limita a agir sobre componentes de receptores da membrana pré-sináptica e intensificando a secreção dos neuromediadores.

Como constituintes orgânicos e inorgânicos estes venenos contêm íons e sais (Ca^{2+}, Na^{+}, K^{+}, Mg^{2+}, Cl^{-}), ácidos livres (cítrico, lático, diidrofenilacético), glicose, aminoácidos livres, aminas biogênicas (espermicina, espermidina, putrescina, cadaverina) e neurotransmissores (glutamato, aspartato, GABA, histamina, dopamina, serotonina, epinefrina, epinina) (WELSH E BATTY, 1963; CHAN *et al.,* 1975). O papel destes constituintes é desconhecido embora possam potencializar a ação de neurotoxinas em alguns casos. Podem também representar produtos de degradação de outros componentes do veneno. Outra classe química, as acetilpoliaminas, foi caracterizada pela primeira vez em venenos de aranhas (ARAMAKI *et al.,* 1986; TOKI *et al.,* 1990). Apesar de algumas poliaminas poderem ser tóxicas para vertebrados, por administração intracraniana direta, as mesmas parecem ter essencialmente atividade inseticida e são responsáveis pela rápida paralisia observada durante a predação. Esta paralisia é mediada por bloqueio rápido e reversível da junção neuromuscular do inseto, onde o glutamato é o principal neurotransmissor. ESCOUBAS *et al.* (1997) mostraram a presença destes compostos no veneno de tarântulas da América do Sul.

Quando se trata da presença de polipeptídeos nas toxinas de aranhas, a vasta maioria apresenta massa molecular entre 3.000 Da e 8.000 Da, altamente reticulados por várias pontes dissulfeto (ESCOUBAS *et al.,* 2000). O alto número de resíduos de cisteínas é o responsável pela formação de pontes dissulfeto intramoleculares. O número destas pontes nestas toxinas varia de 6 a 14. Como regra geral, as toxinas contendo o mesmo número de pontes apresentam homologia estrutural, mas diferem nas suas características funcionais. Recentemente, uma família de toxinas inseticidas (LiTx×1; LiTx×2; LiTx×3) de baixa massa molecular (5,6-7,9 kDa) foi identificada no veneno de *Loxosceles intermedia* (DE CASTRO *et al*.,2004).

Muitas neurotoxinas de aranhas afetam a função de diferentes canais de cálcio, servindo como perfeitas ferramentas de pesquisa em experimentos eletrofisiológicos e/ou bioquímicos. Poucas toxinas são conhecidas com atividade sobre canais de sódio e potássio (GRISHIN, 1999).

Em combinação com as poliaminas, os peptídeos parecem representar o principal arsenal tóxico das aranhas. Até os dias de hoje, mais de 60 toxinas peptídicas já foram descritas. Aparentemente com ação sobre vários canais iônicos de membrana. Os peptídeos podem bloquear a liberação dos neurotransmissores por afetarem a exocitose das vesículas pré-sinápticas e induzirem modificações anormais da transmissão sináptica resultando em paralisia flácida. Alguns peptídeos podem também provocar paralisia excitatória resultante da atividade paroxística induzida pela polarização excessiva. A atividade das toxinas pode também ser específica para alguns grupos zoológicos, por exemplo, podem ter ação sobre insetos e não ter ação nenhuma sobre os mamíferos (ESCOUBAS *et al.,* 2000).

O veneno loxoscélico é essencialmente protéico, com ação enzimática ou tóxica e pode ser comparado a alguns venenos de cobras, onde os estudos demonstraram a presença abundante de enzimas em sua composição (MARTINEZ-VARGAS, 1987). Entre estas enzimas foi descrita uma esfingomielinase de 32-35 kDa, com inúmeras isoformas dentro do veneno, constituindo uma família de esfingomielinases, envolvida na agregação plaquetária e dermonecrose (KURPIEWSKI *et al*., 1981; FUTREL,1992, PALUDO *et al*., 2006). Outras moléculas de 33, 34 e 35 kDa foram descritas associadas com atividades nocivas do veneno (GEREN *et al*., 1976; BARBARO *et al*., 1992; TAMBOURGI *et al*., 1995; FEITOSA *et al*., 1998; VEIGA *et al*., 1999). A presença de hialuronidases pode ser potencializadora de outros componentes do veneno, no sentido de facilitar a penetração destes em vários compartimentos celulares e tecidos, porém

a função exata da maioria das enzimas existentes ainda necessita de elucidação. 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). MACHADO *et al*., (2005) conseguiram descrever, nos venenos de *L. gaucho, L. intermedia e L. laeta*, 8 isoformas da loxnecrogina (30-35 kDa) usando técnicas de proteômica e com o veneno fracionado identificaram 11 isoformas da loxnecrogina com massa molecular em torno de 30-32 kDa.

Quanto à natureza bioquímica do veneno, a glicosilação de suas proteínas é uma das mais importantes modificações pós-traducionais. Ela precede ou atua na incorporação destas proteínas nos vários compartimentos citoplasmáticos e organelas, assim como na sua ativação funcional ou secreção. A glicosilação também determina grande parte das propriedades funcionais e estruturais, conferindo as proteínas funções biológicas essenciais e direcionando sua movimentação transcelular (DAMJANOV, 1987).

Estudando o perfil oligossacarídico do veneno de *L. intermedia* e associando-o as funções desempenhadas por esta toxina determinou-se também que a capacidade de agregação plaquetária e as atividades fibrinogenolíticas do veneno são independentes dos resíduos de açúcares presentes nas moléculas; no entanto, as atividades gelatinolítica e dermonecrótica são dependentes de glicolisação (VEIGA *et al*., 1999).

Sabe-se que a ação tóxica do veneno das aranhas é resultante do efeito combinado (efeitos tóxicos sinérgicos) de todos os componentes nele presentes (GEREN *et al*., 1976).

BARBARO *et al*. (1996) estudaram a atividade biológica das três espécies de aranhas do gênero *Loxosceles* encontradas no sul do Brasil e reconheceram o

veneno de *L. intermedia* como sendo o mais letal para camundongos (DL_{50} 0,48 mg/kg), seguido do veneno de *L. gaucho* (DL_{50} 0,74 mg/kg) e finalmente, do veneno de *L. laeta* (DL_{50} 1,45 mg/kg).

No veneno de *L. reclusa* foi identificada uma hemolisina termo-lábil, cálcio dependente e de massa molecular aproximada de 19 kDa que, em contato com eritrócitos de carneiro fornece, como produto de degradação, uma esfingomielina hidrolisada por atividade tipo esfingomielinase D, comprovando a atividade lítica direta do veneno sobre o eritrócito (FORRESTER, 1978). FUTRELL *et al*. (1979), por outro lado, sugeriram que a esfingomielinase D não tem papel primário na reação hemolítica "in vivo", visto que a reação lítica direta com eritrócitos de coelho e humano foi insignificante.

REES *et al*. (1984), usando fração de veneno parcialmente purificado (S_3) de *L. reclusa,* obtiveram uma intensa resposta inflamatória localizada, consideraram a possibilidade de que esta fração não difunde do local da injeção, mas é adsorvida às membranas celulares "in situ". A alta afinidade de interação da fração S_3 com as membranas celulares não foi limitada às membranas de eritrócitos, mas estava presente nos vários sistemas de membranas testados. Os autores sugerem a possibilidade de que exista um receptor de membrana da classe II para fração S_3. O receptor pode ser a esfingomielina, presente em altas concentrações na parte externa da membrana do eritrócito. Existem evidências de que cátions divalentes são necessários para a ativação da esfingolisina D.

O estudo das atividades enzimáticas do veneno da glândula de *L. reclusa* mostrou que este é desprovido de atividade de colagenase, dipeptidase, acetil colinesterase, fosfodiesterase, ribonuclease A e desoxirribonuclease. As atividades encontradas foram de estearase e hialuronidase, que foi apontada como responsável pelo aspecto difuso da lesão observada no quadro clínico e experimental do envenenamento pela aranha marrom (WRIGHT *et al*., 1973).

A determinação da seqüência amino terminal do componente dermonecrótico do veneno de *L. gaucho* (massa molecular 35 kDa), obtido pela purificação em SDS-PAGE, seguido pelo eletroblotting em membrana de PVDF, sugere um alto grau de homologia quando comparado à seqüência amino terminal da toxina correspondente no veneno de *L. reclusa* (MOTA E BARBARO, 1995).

A trombocitopenia decorrente de uma agregação plaquetária induzida pelo veneno, bem como o quadro hemorrágico e a coagulação intravascular disseminada que surgem em alguns acidentados, representam fenômenos dependentes de moléculas da matriz extracelular, tais como a fibronectina plasmática e o fibrinogênio (WILLIANS *et al*., 1983; RUOSLAHTI, 1988; VEIGA *et al*., 1996) e, mais uma vez, tais transtornos podem ser atribuídos às enzimas presentes no veneno com atividades proteolíticas sobre estes substratos (FEITOSA *et al*., 1998) como bem demonstrado nos quadros hemorrágicos produzidos nos acidentes por ofídios (BARAMOVA *et al*., 1989; HITE *et al*., 1992).

A insuficiência renal aguda, com hemoglobinúria e proteinúria, decorrente do acidente com aranha marrom, representa ações potenciais de constituintes presentes no veneno (alguns constituintes até o momento desconhecidos ou com mecanismos de ação obscuros) sobre o tecido renal e mais uma vez parte da matriz extracelular renal (com ênfase especial sobre a membrana basal glomerular que atua na fisiologia renal como uma barreira seletiva, determinando uma ação filtrante entre o sangue e a formação da urina). A ação de constituintes moleculares encontrados no veneno da aranha marrom sobre membranas basais poderia explicar alguns efeitos nocivos presentes no loxoscelismo, com ênfase nos processos hemorrágicos (membrana basal subendotelial venosa) e na insuficiência renal aguda (membrana basal glomerular) (FUTRELL, 1992).

Tentando dar suporte científico a esta hipótese FEITOSA *et al*., (1998) identificaram e caracterizaram duas metaloproteases de invertebrados ligantes de

gelatina, uma delas com atividade fibronectinolítica e fibrinogenolítica parcial (proporção fibrinogênio/veneno – 100:1) provavelmente envolvidas nos distúrbios hemostáticos desencadeados pelo veneno de *Loxosceles intermedia*.

Resultados obtidos por microscopia eletrônica e imunohistoquímica mostram uma destruição perceptível da membrana basal. Aparentemente o veneno de *L. intermedia* não tem atividade sobre laminina ou colágeno tipo IV. No entanto, o veneno degrada o núcleo protéico de heparan sulfato proteoglicano e hidrolisa entactina, molécula que age como ponte de organização da membrana basal, pois liga laminina, colágeno tipo IV e heparan sulfato proteoglicano (VEIGA *et al*., 2000b). Estudos histológicos e ultramicroscópicos em rim de camundongos injetados intraperitonealmente com veneno de aranha marrom mostraram hialinização de túbulos proximal e distal, eritrócitos acumulados em espaço de Bowman's, colapso glomerular, bolhas e vacúolos nas células epiteliais tubulares, edema intersticial e depósito de material eosinofílico no lúmem do túbulo, citotoxicidade em célula epitelial glomerular e endoltelial, mudanças em membrana basal (LUCIANO *et al*., 2004). CHAIM *et al*., 2006 com auxilio de uma proteína dermonecrótica recombinante (LiRecDT1) mostrou indução direta de dano sobre rim de camundongo desta esfingomielinase, incluindo edema glomerular e necrose tubular, além de alterações morfológicas e de viabilidade em células estabelecidas em cultura MDCK (epitélio renal).

Os problemas de hemostasia sistêmica causados pelo envenenamento por aranhas do gênero *Loxosceles* são pouco entendidos, embora sejam uma das causa de óbito em acidentados. O veneno de aranha marrom provoca coagulação intravascular disseminada, causando oclusão da parede de vasos de animas de laboratório e humanos expostos ao veneno (REES *et al*., 1984; FUTRELL, 1992). O veneno pode causar prolongamento do tempo de tromboplastina parcial e no tempo de protrombina (REES, 1988). O veneno da aranha marrom induz

alterações nos vasos sangüíneos com ocorrência de bolhas subendoteliais, vacúolos e degeneração de paredes de vasos sangüíneos em coelho como modelo experimental (VEIGA *et al*., 2001a). Uma molécula protéica de 30kDa com ação fibrinogenolítica foi identificada no veneno de *L. intermedia* (ZANETTI *et al*., 2002). Estudos realizados com células endoteliais de aorta de coelho estabelecidas em cultura (CLPs) e veneno de *L. intermedia* mostraram ligação direta de toxinas sobre a superfície celular e matriz extracelular, além de indução de alterações morfológicas como retração das células, desadesão homofílica e aumento de projeções filopódicas. Também pode ser observada desorganização de pontos de adesão focal e microfilamentos de actina sem que houvesse mudança na viabilidade das células, somando-se a estes eventos alterações no perfil matricial de fibronectina sintetizado pelas células e na adesão celular à fibronectina, sugerindo que as alterações vasculares induzidas pela picada se devem a ação direta das toxinas do veneno sobre a superfície endotelial e estruturas adesivas envolvidas na estabilidade dos vasos sanguíneos, efeitos estes que indiretamente levam a ativação de leucócitos e plaquetas, coagulação intravascular disseminada e aumento de permeabilidade vascular. Estudo dos efeitos do veneno de *L. intermedia* sobre células da medula óssea e sangue periférico de coelhos mostrou que as células vermelhas não são afetadas, contudo as eritrocitárias nucleadas sofreram decréscimo transitório depois da exposição ao veneno. A depressão no número de megacariócitos e a trombocitopenia mostraram forte correlação com as mudanças histopatológicas observadas em biópsias obtidas de pele de coelho. Alterações na celularidade e nos neutrófilos da medula óssea foram fortemente correlacionados com aquelas observadas no sangue periférico e pele. A trombocitopenia e neutropenia no sangue periférico foram correlacionadas à depressão da medula óssea, podendo ser conseqüência de uma extensiva migração de plaquetas e neutrófilos para a região da

dermonecrose ou de um efeito transitório do envenenamento (DA SILVA *et al*., 2003).

Loxoscelismo ou Acidente Loxoscélico

Loxoscelismo é a denominação do quadro clínico provocado em indivíduos picados por aranhas do gênero *Loxosceles*. Como o desenvolvimento do quadro dermonecrótico não está bem elucidado e os sintomas que podem aparecer são variados, desde a gravidade (leve, moderada ou grave) ao tipo de lesão que pode ocorrer (sistêmica ou local), várias discussões sobre o assunto têm surgido, principalmente envolvendo o reconhecimento da lesão como efetivamente provocada por picada de aranha marrom. Estudos epidemiológicos devem ser levados em conta durante o diagnóstico da picada, por exemplo, pacientes de regiões endêmicas com lesões de pele típicas constituem um forte indício de envenenamento, já para o paciente de regiões com baixa incidência do aracnídeo podem-se buscar também outras causas (WENDELL, 2003; HOGAN *et al*., 2004).

Os acidentes se relacionam aos hábitos adotados pela aranha marrom, por isso tendem a ocorrer com maior freqüência em pessoas do sexo feminino, e os locais mais atingidos são as regiões proximais dos membros inferiores e superiores e no tronco, o que caracteriza o acidente como doméstico e ocasionado principalmente pelo ato de defesa da aranha ao ser comprimida contra o corpo do indivíduo, durante o sono ou mesmo no momento de vestir-se (RIBEIRO *et al.*, 1993).

O quadro clínico provocado por acidentes envolvendo as aranhas do gênero *Loxosceles* pode ser de dois tipos: o quadro cutâneo ou dermonecrótico (84 - 97% dos casos) e o quadro cutâneo-visceral ou sistêmico (3-16% dos casos) (Ministério da Saúde, 1998). O desenvolvimento de um quadro ou outro, ou de ambos e a gravidade do acidente vai depender de alguns fatores relacionados

com a espécie da aranha (Ministério da Saúde, 1998), sexo do animal (OLIVEIRA *et al.,* 1999), seu estágio de desenvolvimento (ANDRADE *et al.,* 1999), quantidade de veneno inoculada, assim como a idade (SEZERINO *et al.,* 1998) e características genéticas do indivíduo acidentado (BARRETO *et al.,* 1985), bem como o estado nutricional, local da picada, susceptibilidade ao veneno e o tempo que este indivíduo leva para procurar um tratamento adequado (GAJARDO-TOBAR, 1966; SCHENONE *et al*., 1989; BARBARO *et al*., 1994).

O quadro cutâneo caracteriza-se por dermonecrose no local da inoculação do veneno (REES *et al.,* 1984). A picada inicial, por ser pouco dolorosa, geralmente passa despercebida pelo paciente, porém após 2 a 8 horas, a dor pode variar de moderada a severa e é descrita como dor local do tipo “queimação” ou ardência, podendo ser acompanhada por prurido, edema, eritema, sensação de mal-estar geral e, em alguns casos, febre. Em seguida, pode surgir uma lesão de 1 a 30 cm de diâmetro, circundada por halo vermelho e uma zona pálida, denominada placa marmórea (RODRIGUES *et al*., 1986; FUTRELL, 1992). Após 3 a 5 dias do acidente pode ocorrer acúmulo de leucócitos polimorfonucleares, necrose e formação de abscesso (SMITH E MICKS, 1970; FUTRELL, 1992). Em alguns casos a lesão cutânea necrótica evolui em 2 a 6 semanas, com formação de uma escara de difícil cicatrização e pode dar origem a seqüelas deformantes de importância (PIZZI *et al*. 1957). O ferimento crônico produzido pela picada da aranha marrom apresenta vasculite mediada por leucócitos, que pode produzir lesões como piodermia gangrenosa (REES *et al*., 1985). Esta lesão pode ter como agente sinérgico, acentuando sua gravidade, a presença de microorganismos provenientes das quelíceras, que no momento da picada injeta-os concomitantemente com o veneno. Um agente importante de infecção secundária à picada é o *Clostridium perfringens*, bacilo gram positivo anaeróbio (MONTEIRO *et al.,* 2002).

Nos casos em que ocorre formação de úlcera necrótica ou mancha gangrenosa de difícil cicatrização e a cura não se completa em menos de um mês, no local, como seqüela, permanece cicatriz que pode ser desfigurante ou pode até mesmo causar prejuízo funcional. Nesses casos, uma cirurgia reparadora pode, às vezes, solucionar tais problemas (FUTRELL, 1992). Em 5% dos casos, principalmente na face, ocorre uma forma edematosa que não é necrótica, sendo caracterizada por extenso processo flogístico (BARBARO *et al*., 1992).

Por razões óbvias, não foram executadas biópsias consecutivas do desenvolvimento do loxoscelismo cutâneo em humanos. Porém, as mudanças histopatológicas informadas incluem edema e espessamento do endotélio dos vasos sangüíneos, presença de células inflamatórias, vasodilatação, coagulação intravascular, degeneração da parede dos vasos sangüíneos, hemorragia dérmica e até mesmo subcutânea (SMITH E MICKS, 1970; FUTRELL, 1992).

Em estudos realizados em coelhos, com veneno de *L. laeta* e *L. reclusa*, as amostras histopatológicas mostraram após 3 horas, o acúmulo de leucócitos polimorfonucleares ao redor de vênulas e eritrócitos extravasculares sugerindo perda da integridade vascular. Nas arteríolas, foi verificado somente edema de células endoteliais. Após 6 horas, havia edema da derme e epiderme, infiltração de leucócitos polimorfonucleares nas paredes das vênulas, vasodilatação, coagulação intravascular, hemorragia volumosa subcutânea, e até mesmo no músculo, necrose, vacuolização das paredes das arteríolas e destruição da integridade das mesmas. Após 48 horas, o infiltrado de leucócitos polimorfonucleares continua crescendo (SMITH E MICKS, 1970; FUTRELL, 1992). O estudo histopatológico detalhado da dermonecrose induzida por veneno de *L. intermedia* em coelhos mostra aparecimento de injúria tecidual a partir de 4 horas após a injeção do veneno chegando a um pico máximo de dano tecidual, inclusive com lise de tecidos mais profundos que a derme (mionecrose e necrose

coagulativa) em 5 dias, com aparecimento de tecido conjuntivo de reparo (OSPEDAL *et al.,* 2002), além de lesão de vasos sangüíneos com aparecimento de bolhas no endotélio, fibrinogenólise e trombose (VEIGA, *et al*., 2001a; VEIGA, *et al.,* 2001b; ZANETTI *et al*., 2002).

O loxoscelismo cutâneo-visceral ou sistêmico ocorre com menor freqüência (3 – 16% dos casos) e é observado apenas nos casos mais graves. As primeiras manifestações aparecem após 24 horas e os sintomas incluem, além da reação local, astenia, febre, episódios eméticos, alterações sensoriais, cefaléia, insônia e nos casos mais graves ocorrem convulsões e coma. Pode também ocorrer prurido generalizado e petéquias (MARTINEZ-VARGAS, 1987; SCHENONE *et al*.,1989; FUTRELL, 1992; BRAVO *et al*., 1993).

Nos casos de gravidade ainda maior, as alterações no quadro hematológico incluem anemia hemolítica, agregação plaquetária causando trombocitopenia (BASCUR *et al.,*1982) e coagulação intravascular disseminada (DENNY *et al.,* 1964) as quais podem determinar diminuição do hematócrito, aumento da bilirrubina indireta e icterícia (SCHENONE E SUAREZ, 1978; REES *et al.*, 1988; FUTRELL,1992). Outras conseqüências decorrentes do envenenamento incluem alterações vasculares nos pulmões, fígado e rins (PIZZI *et al*., 1957; LUNG E MALLORY, 2000).

Uma das alterações funcionais mais graves, sendo a principal causa de morte nestes acidentes (Ministério da Saúde, 1998) é a insuficiência renal aguda (IRA), a qual se caracteriza por hemoglobinúria e hematúria, e em casos extremos pode levar a obstrução da luz tubular (IRA oligúrica ou não oligúrica) (WASSERMAN E ANDERSON, 1984; SCHENONE *et al*., 1989; FUTRELL, 1992; SEZERINO, 1998; LUNG E MALLORY, 2000).

Esta síndrome não tem ligação alguma com sexo e idade do paciente, estação do ano e seriedade da lesão cutânea. Não tem relação entre o tamanho e

o tipo da lesão cutânea e o grau do comprometimento visceral. A reação sistêmica não é necessariamente proporcional à reação local e vice-versa, uma vez que os sintomas sistêmicos podem desenvolver-se antes de alguma reação local poder ser notada. A evolução dos sintomas está relacionada à quantidade de veneno inoculada, localização da picada e a condição imunológica do paciente (MORÁN *et al*., 1981; CICARELLI *et al*., 1983/84; HEREDIA *et al*., 1989).

A compreensão molecular do loxoscelismo ainda é bastante incompleta. O mecanismo pelo qual os venenos loxoscélicos causam a dermonecrose está sob investigação. PIZZI *et al*. (1957) fez o primeiro estudo sistemático e detalhado das lesões histológicas produzidas experimentalmente pelo veneno de *Loxosceles laeta*. O estudo das lesões locais, em cobaias e coelhos, demonstrou a existência precoce de intensas alterações vasculares, caracterizadas essencialmente por vasoconstrição inicial das arteríolas e posteriormente, por vasodilatação acentuada. A parede dos vasos da área afetada foi intensamente comprometida, apresentando fenômenos degenerativos e trombóticos. Nas primeiras horas relataram a formação de um intenso edema com extravasamento das células sangüíneas, inclusive eritrócitos. O estudo histológico da lesão local demonstrou alterações parecidas com as descritas no fenômeno de Arthus. Segundo os autores a necrose se explicaria pelas marcantes alterações vasculares, provocadas por um fenômeno semelhante aos anafilactóides de caráter local.

PIZZI *et al*. (1957) salientaram ainda, que a intensidade das lesões locais esteve, em geral, em relação inversa com a severidade das lesões da outros órgãos (rim, pulmão, S.N.C., etc.) e com a gravidade do envenenamento. Com isso, inferiram que as importantes alterações vasculares locais, assim como a grande intensidade do edema, poderiam ser fatores que impediriam uma rápida ação sistêmica do veneno. Células inflamatórias (especialmente polimorfonucleares) acumuladas no local do envenenamento parecem exercer

alguma atividade nesta função (PATEL *et al*., 1994). Estudos conduzidos com a depleção de leucócitos PMN, anterior a injeção do veneno de *Loxosceles reclusa*, resultam em inibição completa da hemorragia e redução marcante de edema e necrose sugerindo que estes leucócitos são críticos no desenvolvimento da lesão pelo veneno (SMITH E MICKS, 1970).

De acordo com SMITH E MICKS (1970), a infiltração leucocitária precoce dos vasos sangüíneos no local da picada, implicando em injúria de vasos sangüíneos, foi considerada como patologia primária para a perda da pele. Em estudos histopatológicos por MACHADO *et al*. (1978/79) foram encontradas lesões degenerativas e necróticas características comprometendo glândulas sudoríparas. Estudos experimentais histológicos realizados por vários autores relatam edema difuso com extravasamento de leucócitos e eritrócitos de intensidade variável, necrose de extensão variável da epiderme, acompanhada de vacuolizações das células da camada basal. Os vasos sangüíneos de pequeno porte apresentam necrose, arterite e trombose. Alterações locais dos vasos sangüíneos variaram desde simples ectasia a fenômenos degenerativos de suas paredes. Por vezes houve o predomínio de lesões hemorrágicas e degenerativas do tecido conjuntivo, adiposo e muscular (PIZZI *et al*., 1957; GAJARDO-TOBAR, 1966; MACHADO *et al*., 1978/79).

O quadro dermonecrótico avançado, que é a manifestação mais característica do loxoscelismo, com morte celular e destruição dos constituintes intercelulares (matriz extracelular) na região lesionada, representa potencialmente a presença de atividade enzimática do veneno, tendo sido até o presente momento, demonstrada a existência de metaloproteases, hialuronidase, e serino-proteases (FORRESTER *et al*., 1978; KURPIEWISKI *et al*., 1981; REKOW *et al*., 1983; FEITOSA *et al*., 1998; VEIGA *et al*., 1999; VEIGA *et al*., 2000a).

Efeitos de ativação plaquetária induzidos pela toxina da aranha marrom na microcirculação podem ser responsáveis pela trombose vascular, isquemia tecidual e perda da pele na picada clínica (SMITH E MICKS, 1970). Além disso, componentes plasmáticos também parecem ser requeridos para efeitos nocivos do veneno (REES *et al*., 1988). O componente amilóide P presente no plasma parece participar na ativação da agregação plaquetária provocada pelo veneno (GATES E REES, 1990). A ativação da via alternativa do sistema complemento também parece participar dos efeitos nocivos do veneno (TAMBOURGI *et al*., 1995; TAMBOURGI *et al*., 2000). Por outro lado, tanto as atividades dermonecróticas como as hemorrágicas podem estar diretamente associadas com enzimas proteolíticas presentes no veneno, as quais têm a capacidade de degradar fibronectina, fibrinogênio, entactina e heparam sulfato proteoglicano de células endoteliais (FEITOSA *et al*., 1998; VEIGA *et al*., 1999; VEIGA *et al*., 2000a; VEIGA *et al*., 2000b; VEIGA *et al*., 2001a; VEIGA *et al*., 2001b).

O tratamento do loxoscelismo ainda é de grande preocupação, já que não se estabeleceu um protocolo medicamentoso eficiente. O uso de esteróides sistêmicos não se mostrou eficaz na diminuição da infiltração leucocitária polimorfonuclear ou do tamanho da lesão em experimentos controlados em animais. Com exceção do envenenamento sistêmico, eles provavelmente não têm função terapêutica. Além do mais, a injeção dos mesmos, intralesionalmente, pode aumentar o edema e a pressão no local de inoculação contribuindo para a necrose tecidual (FUTRELL, 1992). É sabido que o veneno loxoscélico pode ficar retido no local da picada por um período de tempo, particularmente em região de tecido gorduroso. Então, se o antiveneno tem um papel, poderia ser não de prevenção do aparecimento da lesão, mas na remoção do veneno residual e favorecendo a cicatrização periférica a picada. O aquecimento do local da inoculação do veneno não pode ser imediatamente creditado a própria ação do veneno ou infecção

secundária, então em Abril de 1986, KING E REES recomendaram um protocolo a ser seguido para tratamento de picadas severas de aracnídeos e correlacionados; a primeira medida terapêutica é a administração de eritromicina ou cefalosporina, ainda recomendaram (1) dapsona (se excluída deficiência em G6PD); (2) antibióticos; (3) gelo e elevação do local; (4) evitar atividade física exagerada; (5) evitar calor, cirurgia imediata e aspirina, além de exames laboratoriais adicionais para identificar qualquer sinal de complicações sistêmicas (FUTRELL, 1992). A administração de antibióticos do grupo da penicilina G pode ser útil para prevenir instalação de microorganismos associados à lesão, como por exemplo, *Clostridium perfringens* (MONTEIRO *et al.,* 2002) O tratamento com câmara hiperbárica de oxigênio e soroterapia também são procedimentos que podem ser cogitados (HOGAN *et al*., 2004).

O Ministério da Saúde através do Manual de diagnóstico e tratamento de acidentes por animais peçonhentos (2001) preconiza o seguinte:

Tratamento específico – Soroterapia: as recomendações para utilização do antiveneno dependem da classificação de gravidade (leve, moderada e grave).

Outros – a) Corticoterapia: embora não existam estudos controlados, tem sido utilizada a prednisona por via oral na dose de 40 mg/dia para adultos e em crianças 1 mg/kg/dia durante, pelo menos, cinco dias.

b) Dapsone (DDS): tem sido testada, em associação com a soroterapia, como modulador da resposta inflamatória para redução do quadro local, na dose de 50 a 100 mg/dia via oral por duas semanas aproximadamente. Embora pouco freqüente, em face de risco potencial da Dapsone em desencadear surtos de metemoglobinemia, o paciente deve ser acompanhado do ponto de vista clínico-laboratorial durante o período de administração dessa droga.

Suporte – Para as manifestações locais:

- Analgésicos, como dipirona (7 a 10 mg/kg/dose);

- Aplicação de compressas frias auxiliam no alívio da dor local;
- Antisséptico local e limpeza periódica da ferida são fundamentais para que haja uma rápida cicatrização. A úlcera deverá ser lavada cinco a seis vezes por dia com sabão neutro, e compressas de KMn04 - 1:40.000 (um comprimido em quatro litros de água) ou água boricada 10% aplicados por cinco a dez minutos duas vezes ao dia;
- Antibiótico sistêmico (visando à cobertura para patógenos de pele), havendo infecção secundária;
- Remoção da escara deverá ser realizada após estar delimitada a área de necrose, que ocorre, em geral, após uma semana do acidente;
- Tratamento cirúrgico pode ser necessário no manejo das úlceras e correção de cicatrizes.

Para as manifestações sistêmicas:

- Transfusão de sangue ou concentrado de hemácias nos casos de anemia intensa;
- A prevenção da IRA deve ser tentada em todo paciente acidentado por animal peçonhento, não sendo um procedimento exclusivo para casos de loxoscelismo. É realizada pela administração precoce do antiveneno específico, tratamento da hipotensão arterial, do choque e manutenção de um estado de hidratação adequada. Considera-se que este último objetivo é alcançado quando o fluxo urinário é de 1 ml a 2 ml/kg/hora nas crianças e 30 a 40 ml/h nos adultos. Os pacientes que, apesar da administração de líquidos em quantidade satisfatória, permaneçam em oligúria ou anúria, devem ser medicados com furosemida por via venosa (1 mg/kg/dose na criança; 40 mg/dose no adulto). A diurese osmótica pode ser tentada com a administração venosa de solução de manitol a 20% (5 ml/kg de peso na criança e 100 ml no adulto). Feito o diagnóstico de IRA secundária a acidentes por animais peçonhentos, o paciente deve ser encaminhado para

tratamento especializado. O tratamento não difere daquele realizado na IRA de outras etiologias. Os envenenamentos que levam a mionecrose com possível NTA (necrose tubular aguda) de tipo hipercatabólico deverão ser encaminhados para tratamento dialítico o mais precocemente possível.

Estudos realizados com soro anti-loxoscélico comercial produzido pelo CPPI e o soro antiaracnídico produzido pelo Instituto Butantan ambos no Brasil foram capazes de neutralizar o aparecimento de dermonecrose induzida por venenos loxoscélicos de diferentes procedências geográficas, sugerindo que estudos devem ser realizados para a viabilização de produção de um único soro anti-loxoscélico mundial (BARBARO *et al*., 2005).

Epidemiologia

O primeiro caso documentado de loxoscelismo ocorreu em 1879 no Tenessee (EUA). Contudo dados mais concretos foram colhidos no Chile (meados dos anos 1950) e depois no Brasil seguido dos Estados Unidos da América. Estes relatos associam a picada da aranha marrom com lesões dermonecróticas. A grande sinantropia das aranhas aproximou-as dos seres humanos levando a um aumento dos casos de envenenamento e chegando a ser considerado um problema de saúde pública no Brasil, Chile e Estados Unidos da América (SCHENONE 1998; SEZERINO *et al.,* 1998; NICHOLSON E GRAUDINS, 2003)

Os acidentes loxoscélicos no Brasil começaram a ser reconhecidos em 1954, por BÜCHERL e ROSENFELD. Posteriormente, outros trabalhos como os de FURLANETTO (1961) e FURLANETTO *et al.* (1962) contribuíram para caracterização do gênero e produção de soro antiveneno específico.

Entre 1990 e 1993, no Brasil foram registrados 17.785 acidentes araneídicos onde 36,6% atribuídos à aranha marrom (6.512 casos), sendo 6.224 ocorrências na região sul (segundo dados do Ministério da Saúde), tornando o

loxoscelismo o mais importante dos casos de envenenamento envolvendo aranhas.

O loxoscelismo tornou-se um problema de grande interesse para a área médica e de saúde pública no Estado do Paraná, pois os índices de acidentes vêm aumentando de forma alarmante. Outro fator preocupante é a elevada infestação domiciliar da aranha da espécie *L. intermedia*.

No Brasil, o Estado do Paraná tem sido o que mais notifica esses acidentes. Eles correspondem a mais de 50% daqueles provocados por aracnídeos notificados ao Mistério da Saúde do Brasil.

No Paraná o loxoscelismo, comparado com outros acidentes toxicológicos provocados por animais peçonhentos, ocupa o primeiro lugar com 58,1% dos casos (dados fornecidos pelo Centro de Epidemiologia do Paraná), e em particular, a cidade de Curitiba e Região Metropolitana são consideradas, pelas autoridades sanitárias, como áreas endêmicas para este tipo de ocorrência. Números como os registrados entre 1988 e 1989 de 595 casos (que corresponderam a 62,2% das notificações de acidentes causados por *Loxosceles spp*) e de 923 casos entre 1989 e 1990 (perfazendo 77,4% dos acidentes registrados em todo território nacional), com incidência maior nos períodos mais quentes do ano, justificam esta classificação. O registro de casos em Curitiba apenas no ano de 1994 foi de 2428 acidentes com 3 pacientes indo a óbito (Secretaria de Estado da Saúde) tornando de extrema importância qualquer avanço no sentido de adquirir conhecimento tanto para prevenir, diagnosticar e tratar os acidentes, quanto para compreender e caracterizar os mecanismos de ação deste veneno visando sua aplicabilidade biotecnológica.

Segundo dados obtidos através da Secretaria de Saúde do Estado do Paraná, em 1990 foram diagnosticados 526 casos e no ano seguinte houve um aumento, chegando a 1.111 casos. Já em 1992, o número de casos diminuiu, com

1.057 casos, porém em 1993 o número de casos voltou a subir, sendo registrados 2.148 casos. Em 1994 foram diagnosticados 2.428 casos e em 1995 foram 2.722 casos. Já em 1996 o número de casos ficou em 2.898 e em 1997 foram aproximadamente 2.456 casos (comunicação pessoal da Secretaria de Estado da Saúde do Paraná). Entre 1993 e 2001, a incidência de picadas de aranha marrom em Curitiba foi de 1,4 casos por 1000 habitantes e 23% das picadas foram na região da coxa, 16,7% no tronco, 14% nos braços e 13% nas pernas. A maioria dos casos foi ameno, apenas 1% sendo considerados severos (Secretaria de Saúde de Curitiba, Paraná, Brasil, 2002). Marques-da-Silva e Fischer (2005) estudaram a casuística sobre loxoscelismo na Paraná, analisando 20.620 casos de acidentes registrados em apenas oito anos (entre 1993 e 2000). Estes casos estavam distribuídos em 175 municípios paranaenses (43,9% dos municípios). Marcantemente, 92,61 % dos casos foram registrados na região metropolitana de Curitiba e se concentraram nos períodos mais quentes do ano. O local mais freqüente de picada em habitantes tanto da zona rural como urbana é membro inferior, seguido por membro superior, pé, mão, tronco e cabeça.

Dados colhidos junto ao SESA/ Centro de Saúde Ambiental do Estado do Paraná mostram que nos casos de loxoscelismo registrados entre 1995 e 1999 as alterações sistêmicas mais freqüentes foram tontura (21%), sonolência (19%), sede (20%), “rash” cutâneo (19%), náusea (18%), mal estar (25%) e cefaléia (30%).

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, justificando a tentativa de 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, por exemplo.

Organização dos resultados

Nesta tese apresentaremos 6 isoformas de toxinas dermonecróticas que estão sob investigação. O estudo das cinco primeiras já gerou artigos científicos publicados ou submetidos em revistas internacionais (anexo) e uma sexta isoforma tem sua clonagem detalhada no corpo desta tese.

OBJETIVOS

Objetivo geral

Avaliação biológica de toxinas presentes no veneno da aranha marrom *L. intermédia.*

Objetivo específico

- Clonagem de diferentes isoformas da toxina dermonecrótica do veneno da aranha marrom *L. intermedia*,
- Expressar em modelos heterólogos (bactérias) diferentes isoformas de toxinas dermonecróticas presentes no veneno da aranha marrom,
- Avaliar propriedades biológicas das diferentes isoformas de toxinas dermonecróticas obtidas por clonagem e expressão
- Avaliar o nível de homologia entre as diferentes isoformas isoladas com o auxílio de anticorpos

MATERIAIS E MÉTODOS

MATERIAIS

Os sais utilizados foram adquiridos da Merck (Darmstadt, Alemanha), a agarose foi adquirida da Invitrogen (Carlsbad, CA, EUA), o ágar-ágar da Sigma (St. Louis, MO, EUA). O marcador de números de pares de bases “Gene Ruler 100 pb DNA Ladder Plus” e o X-Gal foram adquiridos da empresa Fermentas (Vilnius, Lituânia). O brometo de etídio da Promega (Madison, WI, EUA). Para confecção dos meios de cultura foram usados triptona e extrato de levedura da HiMedia (Mumbai, índia). A amplicilina e o DEPC (dietillpirocarbonato) usados foram adquiridos da USB (Cleaveland, OH, EUA). IPTG e o TRIzol são da Invitrogen (Carlsbad, CA, EUA).

MÉTODOS

Extração de RNA total a partir de glândula produtora de veneno

O RNA total foi extraído de aproximadamente 200 glândulas produtoras de veneno de *L. intermedia* com o auxílio do reagente TRIzol, que baseia-se no método descrito por CHOMEZYNSKI, 1993. Após a precipitação com isopropanolol e lavagem com etanol 75 %, o RNA foi redissolvido com água DEPC-tratada e quantificado por espectrometria a 260 nm. O rendimento total foi de 37 µg.

5’RACE (Rapid Amplification of cDNA Ends) para amplificação da porção 5’ do cDNA da proteína LiRecDT6

A partir de fragmento de cDNA da proteína LiRecDT6 obtido e seqüenciado de biblioteca de cDNA da glândula de *L. intermedia* foram construídos oligonucleotídeos gene – específicos que reconhecem o cDNA da LiRecDT6.

Todas as concentrações citadas se referem à concentração final dos componentes das soluções, e geralmente segue recomendação dos reagentes utilizados para confecção das reações.

Transcrição reversa e geração da dupla fita de cDNA

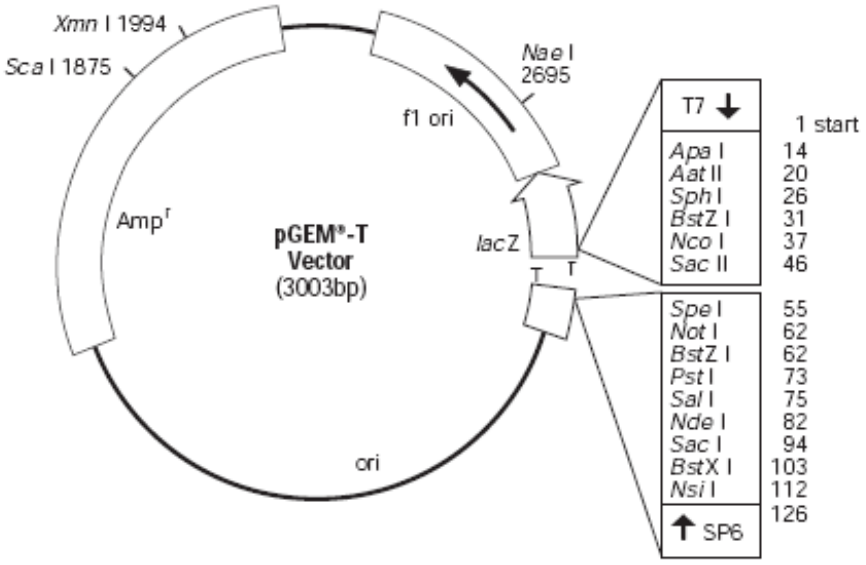
Em tubo para reação de PCR (reação em cadeia da polimerase) foi adicionado GSP1 ("Primer" gene-específico 1) (0,2 µM); RNA total (1 µg) numa reação com volume final de 20 µl com água DEPC - tratada (adicionando 0.1 % a água e homogeneizado por 2 h, seguindo para autoclavação por 45 min destruindo o DEPC por hidrólise e evaporação). Este tubo foi incubado a 70 °C por 5 min para desnaturação do RNA e imediatamente colocado em gelo. A ele foi adicionado tampão para transcriptase reversa (1X); $MgCl_2$ (2,5 mM); dNTPs (mistura de nucleotídeos trifosfato: A,T,C,G - 0,4 mM) (Promega, Madison WI, EUA); inibidor de RNase (20U)(RNaseOUT –Invitrogen). Esta mistura foi bem homogeneizada e incubada a 42 °C por 1 min (anelamento do oligonucleotídeo). Acrescentou-se em seguida 200 U da enzima transcriptase reversa (Promega, Madison WI, EUA) e novamente a mistura foi levada ao termociclador (My Cycler – Thermal Cycler BioRad, Hercules, CA, EUA): 25 °C/ 5 min; 60 min a 42 °C. A reação foi parada a 70 °C/ 15 min. Para adição de cauda homopolimérica foi necessária a retirada dos dNTPs excedentes da etapa anterior. Para isso foram realizados dois ciclos repetidos de precipitação seletiva do cDNA com auxílio de acetato de amônio (2,5 M), seguindo-se a adição de etanol absoluto gelado à amostra nos volumes (3+1), com agitação vigorosa e incubação a temperatura igual ou inferior a – 20 °C por 30 min. Para separação do precipitado centrifugou-se a amostra a 4 °C 20.000 x g por 30 min tornando possível a retirada do sobrenadante. Novamente, lavou-se o sedimento formado agora com etanol 70 % (500 µl) gelado sem agitação. Imediatamente a amostra foi centrifugada a 4 °C,

20.000 x g por 5 min. Secando o sedimento para retirada total de etanol da amostra precipitada livre de dNTPs. Este sedimento foi ressuspenso em 16,5 µl de água qualidade para biologia molecular, adicionou-se tampão para Terminal desoxinucleotidil Tranferase (TdT) (1X); dATP (0,2 mM). Incubado por 2 min a 94 °C. Colocado imediatamente em gelo e adicionado 20U de TdT, incubado a 37 °C por 10 min e inativada a enzima aquecendo a 65 °C por 10 min. No final desta etapa obtive-se um cDNA simples fita com cauda 5' gene específica e cauda 3' homopolimérica (adenina). Com este produto pudemos fazer uma reação de PCR para gerar a dupla fita. Para esta reação utilizou-se tampão para *Taq* DNA polimerase (1 X); $MgCl_2$ (1,5 mM); dNTPs (0,2 mM); GSP2 (oligonucleotídeo gene-específico 2 – 0,4 µM); $(dT)_{17}$Adaptor-primer (oligonucleotídeo adaptador para cauda homopolimérica – 0,4 µM); cDNA (5 µl da reação anterior para adição de cauda homopolimérica); *Taq* DNA polimerase (1,25 U/ 50 µl) para um volume final de reação de 50 µl. Esta mistura foi incubada em termociclador num protocolo a seguir: 1 ciclo a 94 °C/ 2 min; 35 ciclos a 94 °C/ 30 s – 50 °C/ 30 s – 72 °C/1 min; 1ciclo 72 °C/ 10 min; 1 ciclo de espera a 4 °C/ infinito.

O produto de PCR foi analisado em gel de agarose 2 % com brometo de etídio (0,5 µg/ ml) em tampão TAE (Tris base 40 mM/ acetato 20 mM/ EDTA 1 mM) e corrida eletroforética em cuba horizontal a 5 V/ cm. O gel foi visualizado e a imagem foi registrada com auxílio de aparelho de análise de imagens Chemidoc – XRS e software Quantity One – SW (BioRad). O gel de agarose foi recortado com auxílio de bisturi no local de visualização da banda correspondente ao produto desejado de PCR (dupla fita de cDNA). E dele foi extraído com auxílio de reagentes apropriados de extração cDNA em gel (Quick Gel Extraction Kit Invitrogen, Carlsbad, CA, EUA).

Ligação do cDNA ao vetor A – T

O cDNA extraído do gel foi ligado a um vetor do tipo A – T. Foi usado vetor pGEM-T (Vector System I, Promega, Madison WI, EUA) (50 ng); tampão ligase (1X); T4 DNA ligase (3 U/ µl); inserto (produto da extração do gel de agarose diluído em 8 µl de água); um volume final de reação de 20 µl. A ligação ocorreu durante 16 h a 4 °C.



Precipitação do plasmídeo

O plasmídeo foi precipitado com uma solução de acetato de sódio pH 6,0 (0,3 M) e mais 3 partes de etanol absoluto gelado, homogeneizado com auxílio de vortex e deixado à temperatura igual ou inferior a -20 °C por 30 min, centrifugado a 4 °C, 20000 x g por 30 min. O sobrenadante foi retirado e o sedimento obtido foi lavado com etanol 70 % gelado e centrifugado a 4 °C por 5 min. Nova lavagem com retirada da totalidade do etanol. O sedimento foi completamente seco para eliminação total do etanol. O plasmídeo foi ressuspenso em 5 µl de água e homogeneizado por cerca de 10 min com auxílio de vortex.

Transformação bacteriana por eletroporação do plasmídeo

A cepa bacteriana DH5α eletrocompetente (30 µl) foi eletroporada em eletroporador (Gene Pulser X-Cell, BioRad) para entrada do plasmídeo com inserto de estudo (1 µl) na bactéria. Esta deve ser colocada em meio SOC (triptona 20 g/l; extrato de levedura 5 g/l; NaCl 0,5 g/l; KCl 2,5 mM; $MgCl_2$ 10 mM, $MgSO_4$ 10 mM, glicose 0,2 M) (969 µl) a 37 °C por 1 h (período de recuperação).

Plaqueamento em ágar LB das bactérias transformadas

As bactérias recuperadas foram plaqueadas em meio LB ágar (triptona 10 g/l, extrato de levedura 5g / l, cloreto de sódio 10 g/ l, agar-ágar 15g / l) suplementado com ampicilina (100 µg/ ml) previamente tratadas com IPTG (100 mM – 100 µl) e X-GAL (20 mg/ ml – 40 µl). O plaqueamento foi feito em duas placas uma recebeu 100 µl da cultura de bactérias em SOC e a outra recebeu os 900 µl restantes que foram centrifugados e ressuspensos em 150 µl do próprio meio SOC. Cada uma das placas com a suspensão bacteriana foi friccionada com alça de Gauss (estéril) até completa absorção (secagem) do líquido. As placas foram incubadas a 37 °C (em incubadora tipo BOD 411 D, Nova Ética, Campinas, Brasil) por 16 h.

PCR de colônia

Nas placas com as bactérias cresceram basicamente dois grupos de colônias: azuis (sem inserto) e brancas (com inserto). Foram escolhidas 26 colônias brancas, uma levemente azul e uma azul para replaqueamento e para confecção de PCR de colônia. Cada uma das colônias escolhidas foi recolhida com auxílio de palito de madeira estéril, parte do material foi colocado em tubo de 0,2 ml e outra parte replaqueada para uma placa marcada por quadriculado

numerado (“master plate”). As colônias sofreram amplificação do material plasmideal: Tampão *Taq* DNA polimerase (1 X); dNTPs (0,2 mM); $MgCl_2$ (1,5 mM); oligonucleotídeo GSP2 (0,2 mM); oligonucleotídeo Adaptor Primer (0,2 mM); *Taq* polimerase (1,25 U/50 µl). Esta mistura foi incubada em termociclador num protocolo a seguir: 1 ciclo a 95°C/5 min; 35 ciclos a 95°C/30s – 50°C/30s – 72 °C/1 min; 1ciclo 72 °C/10 min; 1 ciclo de espera a 4 °C/ infinito. O produto foi analisado em gel de agarose 2 %. E o resultado foi visualizado e registrado em aparelho de captura de imagem Chemidoc.

Pré-inóculo para processamento de seqüenciamento

A partir da placa “master plate” os clones escolhidos são repicados com auxílio de pinça e palitos de madeira estéreis para um pré-inóculo líquido (ausência de agar-ágar) LB/amp (100 µg/ ml) e incubado por 16 h a 37 °C (em incubadora 430 RDB tipo “shaker” Nova Ética, Campinas, Brasil) em tubo de 5 ml com tampa de rosca. Cada cultura (com crescimento saturado) foi centrifugada (14000 x g por 1 min) a temperatura ambiente e se obteve um sedimento bacteriano consistente. De cada um dos centrifugados bacterianos foi feita a extração do plasmídeo com auxílio de reagentes Miniprep da Invitrogen (Pure Link Quick Plamid Miniprep Kit, Invitrogen).

PCR de seqüenciamento

Para reação de amplificação do cDNA para sequenciamento foi usado o “DYEnamic ET Terminator Cycle Sequencing Kit” (GE Healthcare, Piscataway, NJ, EUA): molde (0,15 pM). Cada clone gera duas reações para sequenciamento (oligonucleotídeos: SP6 e T7) uma, no sentido sense e outra no antisense dependendo do sentido que o inserto entrou. O sequenciamento foi realizado em seqüenciador automático ABI Prism 377 (Applied Biosystems, Foster City CA,

EUA) em gel de sequenciamento em parceria com a Escola Paulista de Medicina (Departamento de Bioquímica – UNIFESP).

Precipitação do produto de sequenciamento

Adicionou-se acetato de sódio/ EDTA (1,5 M/0,25 M) (1/10 do volume inicial da reação) em seguida etanol absoluto gelado (3 X o volume da reação), homogeneizou-se bem e incubou-se em gelo por 10 min. Seguiu-se centrifugação 4 °C por 20 min, 20.000 x g. Retirando-se o sobrenadante e lavando o sedimento com etanol 70 % gelado (500 µl) só passando o líquido pelo sedimento sem homogeneizar, centrifugou-se por 10 min 20.000 x g a 4 °C. Desprezou-se completamente o sobrenadante e secou-se o sedimento em estufa 60 °C por 15 min, retirando-se completamente o etanol que pode interferir no sequenciamento.

Desenho de oligonucletídeos sense e antisense específicos para cópia do cDNA da proteína LiRecDT6

Com base no resultado do sequenciamento foram desenhados oligonucleotídeos específicos sense e antisense para a amplificação do cDNA da LiRecDT6.

Algumas regras básicas foram seguidas para o desenho dos oligonucleotideos sense e antisense. O oligonucleotideo devia ter em torno de 20 bases (17 – 30) distribuídas de forma homogênea ao longo da seqüência. Quanto maior o oligonucleotideo, maior sua especificidade, porém aumenta a Tm (temperatura média de desnaturação do duplex). O custo para confecção também depende do número de bases. Abaixo de 17 bases fica comprometida a especificidade. Quanto à natureza das bases, procurou-se ter em torno de 40 – 60 % de C (citosina) e G (guanina). Procuramos quando possível manter na extremidade 3’ do oligonucleotídeo as bases C ou G que favorecem a

estabilização da ligação no pareamento com o cDNA-alvo. Os oligonucleotídeos foram escritos sempre no sentido 5' – 3' tanto para os sense como para os antisense. Os oligonucleotídeos foram confeccionados pela empresa Bioneer (Coréia).

Obteção do cDNA para reação com a Pfu DNA polimerase

Para a obtenção do cDNA foi realizada uma reação com RNA total extraído de glândula produtora de veneno (1 µg); ao tubo de reação foi adicionado oligonucleotídeo do tipo dT (0,5 µg/ µl) e água (DEPC-tratada) para totalizar 5 µl de volume final. Incubou-se a solução por 5 min a 70 °C (desnaturação da simples fita de RNA) e imediatamente foi colocada em gelo. Foi adicionado ao tubo em gelo tampão para transcriptase reversa (1 X); $MgCl_2$ (2,5 mM); dNTPs (mistura de nucleotídeos trifosfato: A,T,C,G - 0,4 mM) (Promega, Madison WI, EUA); inibidor de RNase (20 U) (RNaseOUT –Invitrogen). Esta mistura foi bem homogeneizada e incubada a 42 °C por 1 min (anelamento do oligonucleotídeo). Acrescentou-se em seguida 200 U de enzima transcriptase reversa (Promega, Madison WI, EUA) e novamente a mistura foi levada termociclador: 25 °C/5 min; 60 min a 42 °C. A reação foi parada a 70 °C/15 min.

PCR com *Pfu* DNA polimerase e oligonucleotídeos LiRecDT6 específicos

Para amplificação do segmento completo da LiRecDT6 com alta fidelidade foi usada uma polimerase de alta fidelidade a *Pfu* DNA polimerase (Promega). Para reação com a *Pfu* DNA polimerase usamos: cDNA (originário da reação de transcriptase reversa) (10 % do volume final), tampão para *Pfu* DNA polimerase com Mg (2,0 mM) (1X), dNTPs (0,2 mM), oligonucleotídeo gene específico P6 sense (0,5 µM), gene específico P6 antisense (0,5 µM), Pfu DNA polimerase (1,25 U/ 50 µl). A reação foi colocada no termociclador com início pré-aquecido a 95 °C

seguindo-se os ciclos: 95 °C/2 min (1 ciclo); 95 °C/30 s, 47 °C/30 s, 72 °C/3 min (40 ciclos); 72 °C/5 min (1ciclo); 4 °C ciclo de espera.

Extração com fenol, clorofórmio e álcool isoamílico e precipitação do cDNA

O produto da reação anterior apresenta-se sem a adição de adenina terminal, pois a *Pfu,* diferentemente da *Taq* DNA polimerase, não apresenta a atividade de adição de adenosina terminal. Para adição da adenina, o que facilita a ligação ao vetor pGEM-T, é necessária a precipitação do produto para retirada da *Pfu* que se estiver presente vai retirar a adenina, pois tem atividade exocluclease de correção (daí a alta fidelidade desta enzima). A amostra teve o volume elevado com TE (tampão Tris base 10 mM pH 8,0/ EDTA 1 mM) e foi adicionado para a precipitação fenol:clorofórmio:álcool isoamílico (25+24+1), misturou-se até criar uma emulsão, centrifugou-se 3 min a 12.000 x g. Coletou-se a fase aquosa (superior) desprezando-se a fase orgânica. Para retirada de resíduos de fenol foi feita uma nova emulsificação agora apenas utilizando clorofórmio. Para precipitação adicionou-se 1/10 do volume recolhido na fase anterior de acetato de sódio 3 M pH 6,0 e 3 volumes de etanol absoluto gelado, misturando por inversão. A mistura ficou por 30 min a – 20 °C (ou menos). Seguindo-se centrifugação a 4 °C a 20.000 x g por 30 min. Desprezou-se o sobrenadante e lavou-se o sedimento com etanol 70 % gelado (500 µl) procedeu-se centrifugação novamente por 5 min, 20.000 x g a 4 °C. Retirou-se o sobrenadante completamente e secou-se o sedimento. Para adição da adenina terminal a reação teve volume final de 50 µl, as concentrações finais foram: dNTPs (0,2 mM), tampão *Taq* (1 X), $MgCl_2$ (1,5 mM) e *Taq* DNA polimerase (1,25 U/50 µl). A reação foi levada para o termociclador: 72 °C/15 min (1ciclo). O produto foi submetido novamente à extração fenólica e precipitação.

Processamento do produto obtido

O produto obtido foi ligado a plasmídeo pGEM-T como já descrito. O plasmídeo com o inserto foi precipitado e eletroporado em bactéria DH5α, que foi processada e plaqueada. As colônias para sequenciamento foram escolhidas após análise de gel de agarose com produto de PCR de colônia. As colônias com produto plasmideal adequado tiveram os plasmídeos extraídos e processados seguindo posteriormente para sequenciamento. Tudo como já foi descrito anteriormente na metodologia.

Desenho de oligonucleotídeos para proteína LiRecDT6 madura

Para desenho deste oligonucleotídeos foi analisada a estrutura da LiRecDT6 obtida do primeiro sequenciamento (5' RACE). Procurou-se desenhar oligonucletídeos que anelassem a porção 5' madura da LiRecDT6 (sem peptídeo sinal) sentido sense e a porção 3' (região códon finalizador – "stop codon") sentido antisense. Estes oligonucletídeos foram associado a sítios de restrição, *Xho*-I para o oligonucleotídeo sense e *Bam*-HI para o antisense. Aqui também foram seguidas as regras já mencionadas.

RESULTADOS

Oligonucletídeos GSP-1, GSP-2, Adaptor Primer e (dT)17-Adaptor Primer

GSP-1: 5’ GCC AGA TGT GTT CTT TAT CTT GC 3’ Tm= 54,1 °C
GSP-2: 5’ CAT CAT AAG CAG TAG AAG CGC TC 3’ Tm= 55,1 °C
Adaptor primer: 5’ CGG TAC CAT GGA TCC TCG AG 3’ Tm= 56,6 °C
(dT)17 Adaptor primer: 5’ CGG TAC CAT GGA TCC TCG AGT TTT TTT TTT TTT TTT TV 3’ Tm= 60,0 °C

Figura 1: representação dos oligonucleotídeos usados durante a reação com a transcriptase reversa e amplificação por PCR

5’RACE (Rapid Amplification of cDNA Ends) para amplificação da porção 3’ do cDNA da proteína LiRecDT6

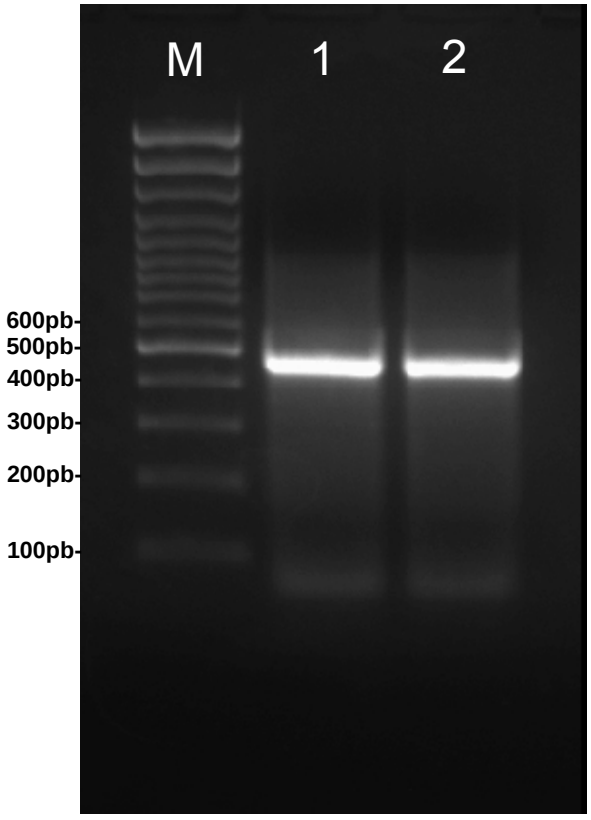


Figura 2: Gel de agarose 2 %. A coluna M mostra o marcador de número de pares de base. As colunas 1 e 2 representam a amostra de cDNA obtido por reação de 5’ RACE proveniente da reação com transcriptase reversa (RT-PCR).

PCR de colônia

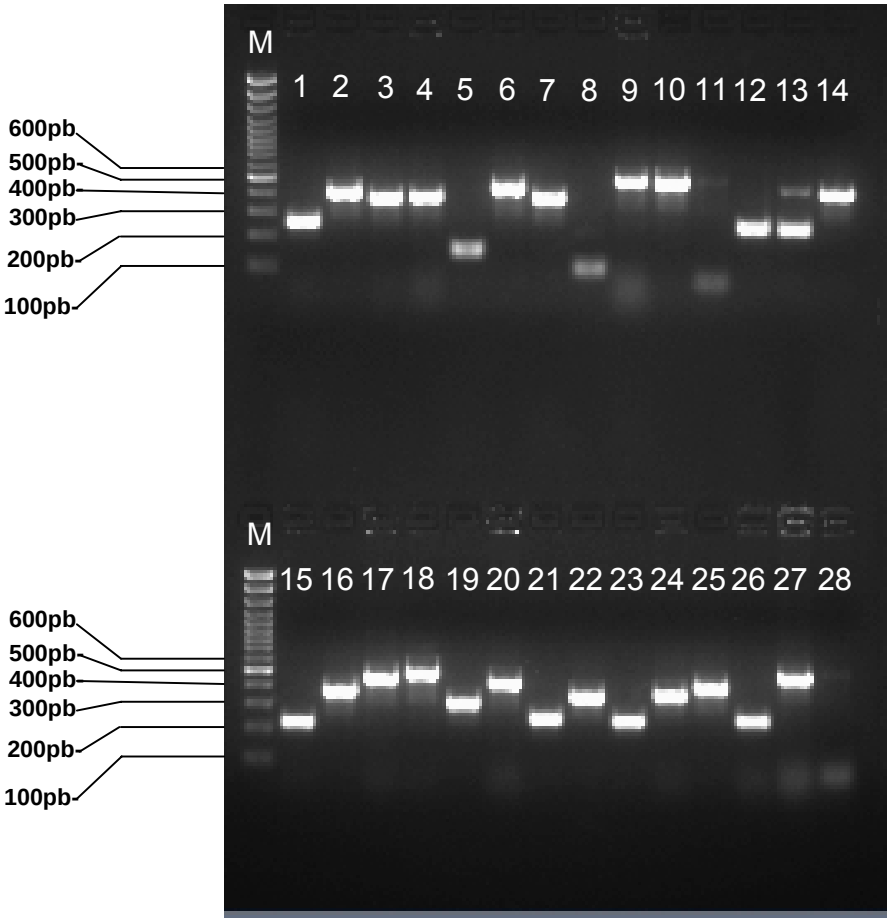


Figura 3: Gel de agarose 2 %. As colunas M representam os marcadores de número de pares de base. As demais colunas representam o perfil do inserto presente nos plasmídeos inseridos nas colônias isoladas de bactérias transformadas. As colônias escolhidas para dar continuidade às reações foram: 2, 6, 14, 17, 18 e 27.

Sequenciamento do Produto da Reação 5'RACE e Alinhamento dos clones seqüenciados

```
P6_RACE_CL6       ------------------------------------------------------------
P6_RACE_CL17      ------------------------------------------------------------
P6_RACE_CL18      ---ACTTATATTTGATCAGAAGGAAAATCAATTTCAGCATGTTGTGTTTCTTTGTATTAT 57
P6_RACE_CL2       ATCACTTATATTTGATCAGAAGGAAAATCAATTTCAGCATGTTGTGTTTCTTTGTATTAT 60
P6_RACE_CL27      ATCACTTATATTTGATCAGAAGGAAAATCAATTTCAGCATGTTGTGTTTCTTTGTATTAT 60


P6_RACE_CL6       ---------GTGGGACCGTCCTCCTAGAGGGAGCAGACATTGATGAAATCGAACGTGCAG 51
P6_RACE_CL17      -----------GGGACCGTCCTCCTAGAGGGAGCAGACATTGATGAAATCGAACGTGAAG 49
P6_RACE_CL18      TCTTCTGTTGTGGGACCGTCCTCCTAGAGGGAGCAGACATTGATGAAATCGAACGTGAAG 117
P6_RACE_CL2       TTTTCTGTTGTGGGACCATCCTCCTAGAGGGAGCAGACATTGATGAAATCGAACGTGCAG 120
P6_RACE_CL27      TCTTCTGTTGTGGGACCGTCCTCCTAGAGGGAGCAGACATTGATGGAATCGAACGTGCAG 120
                            ****** ************************** ********** **


P6_RACE_CL6       ACAAGCGCAGACCAATATGGAATATGGGTCATATGGTGAATGCCGTTTATCAGATTGACG 111
P6_RACE_CL17      ACAAGCGCAGACCAATATGGAATATGGGTCATATGGTGAATGCCGTTTATCAGATTGACG 109
P6_RACE_CL18      ACAAGCGCAGACCAATATGGAATATGGGTCATATGGTGAATGCCGTTTATCAGATTGACG 177
P6_RACE_CL2       ACAAGCGCAAACCAATATGGAATATGGGTCATATGGTGAATGCCGTTTATCAGATTGACG 180
P6_RACE_CL27      ACAAGCGCAGACCAATATGGAATATGGGTCATATGGTGAATGCCGTTTATCAGATTGACG 180
                  ********* ************************************************


P6_RACE_CL6       AATTCGTGGACCTTGGGGCAAATGCCATTGAAACAGACGTCACCTTTACCAAAAGCGCAA 171
P6_RACE_CL17      AATTCGTGGACCTTGGGGCAGATGCCATTGAAACAAACGTCACCTTTACCAAAAGCGCAA 169
P6_RACE_CL18      AATTCGTGGACCTTGGGGCAAATGCCATTGAAACAGACGTCACCTTTACCAAAAGCGCAA 237
P6_RACE_CL2       AATTCGTGGACCTTGGGGCAAATGCCATTGAAACAGACGTCACCTTTACCAAAAGCGCAA 240
P6_RACE_CL27      AATTCGTGGACCTTGGGGCAAATGCCATTGAAACAGACGTCACCTTTACCAAAGGCGCAA 240
                  ******************** ************** ***************** ******


P6_RACE_CL6       ACGCAGAGTACACCTACCACGGAGTTCCTTGCGACTGCCACAGGTGGTGCAAGAAGTGGG 231
P6_RACE_CL17      ACGCAGAGTACACCTACCACGGAGTTCCTTGCGACTGCCACAGGTGGTGCAAGAAGTGGG 229
P6_RACE_CL18      ACGCAGAGTACACCTACCACGGAGTTCCTTGCGACTGCCACAGGTGGTGCAAGAAGTGGG 297
P6_RACE_CL2       ACGCAGAGTACACCTACCACGGAGTTCCTTGCGACTGCCACAGGTGGTGCAAGAAGTGGG 300
P6_RACE_CL27      ACGCAGAATACACCTATCACGGAGTTCCTTGCGACTGCCACAGGTGGTGCAAGAAGTGGG 300
                  ******* ******** ******************************************


P6_RACE_CL6       AATACGTCAATGACTTCTTGAAAGCATTGCGCCGAGCCACAACCCCTGGCGATGCAAAGT 291
P6_RACE_CL17      AATACGTCAATGACTTCTTGAAAGCATTGCGCCGAGCCACAACCCCTGGCGATGCAAAGT 289
P6_RACE_CL18      AATACGTCAATGACTTCTTGAAAGCATTGCGCCGAGCCACAACCCCTGGAGATGCAAAGT 357
P6_RACE_CL2       AATACGTCAATGACTTCTTGAAAGCATTGCGCCGAGCCACAACCCCTGGCGATGCAAAGT 360
P6_RACE_CL27      AATACGTCAATGACTTCTTGAAAGCATTGCGCCGAGCCACAACCCCTGGCGATGCAAAGT 360
                  ********************************************** **********


P6_RACE_CL6       ATCGGAGTCAGCTGATCCTGGTAGTGTTCGATTTGAAAACGGATTATCTGACCGCTTCTA 351
P6_RACE_CL17      ATCGGAGTCAGCTGATCCTGGTAGTGTTCGATTTGAAAACGGATTATCTGACCGCT-CTA 348
P6_RACE_CL18      ATCGGAGTCAGCTGATCCTGGTTGTGTTCGATTTGAAAACGGATTATCTGACCGCTTCTA 417
P6_RACE_CL2       ATCGGAATCAGCTGATCCTGGTTGTGTTCGATTTGAAAACGGATTATCTGACCGCTTCTA 420
P6_RACE_CL27      ATCGGAATCAGCTGATTCTGGTTGTGTTCGATTTGAAAACGGATTATCTGACCGCTTCTA 420
                  ****** ********* ***** ********************************* ***


P6_RACE_CL6       CTGCTTATGATGA------------- 364
P6_RACE_CL17      CTGCTTATGATG-------------- 360
P6_RACE_CL18      CTGCTTATGATGA------------- 430
P6_RACE_CL2       CTGCTTATGATGA------------- 433
P6_RACE_CL27      CTGCTTATGATGAATCACTAATGCGG 446
```

Figura 4: Alinhamento das seqüências obtidas foi feito através de ferramentas existentes no site: http://www.ebi.ac.uk/clustalw/. Os asteriscos representam homologia de bases entre os diversos insertos da LiRecDT6 nos clones bacterianos sequenciados. O sequenciamento do inserto do clone bacteriano 14 não foi aproveitado, pois não houve boa leitura.

Alinhamento das seqüências das proteínas dermonecróticas clonadas

```
P1                  ------------------------------------TCATGTTGCCGTACATTGTCCTAG 24
P2                  ------------------------------------------------------------
P4                  ------------------------CGGCTACCTCCACCATGTTACTGCACATTGCCTTGA 36
P6_5'RACE_CL27      ATCACTTATATTTGATCAGAAGGAAAATCAATTTCAGCATGTTGTGTTTCTTTGTATTAT 60
P3                  ----------------------------------CACCATGTTGCTCCCCGCTGTCATAT 26
P5                  --------------------GCTTTACCTTCACCAAGATGCAATTATTCATTATTTTGT 39
P6_3'               ------------------------------------------------------------

P1                  TATTGGGGTGTTGGAGCGTCTTGTCCCAGGCTGCTCAAACAGATGATGAAGAACGCGCAG 84
P2                  ------------------------------------CAGATGTTGAGGAACGCGCGG 21
P4                  TCTTAGGGTGTTGGAGCGTCTTTTCTGAGGGTGCTGAAACAGATGTTGCGGAACGTGCAG 96
P6_5'RACE_CL27      TCTTCTGTTGTGGGACCGTCCTCCTAGAGGGAGCAGACATTGATGGAATCGAACGTGCAG 120
P3                  CCTTTATCGTATATGCTGTCTTCCTCCAGGAAGCTAATGGGCATGCTGCAGAAAGGGCTG 86
P5                  GTCTTGCTGGATCAGCTGTGCAGCTTGAGGGTACTGAACTTGACGGAGTTGAACGTGCAG 99
P6_3'               ------------------------------------------------------------

P1                  GTAATCGTCGGCCTATATGGATCATGGGGCACATGGTAAATGCCATCGGTCAGATAGACG 144
P2                  ATAAACGTCGACCCATATGGATCATGGGCCACATGGTGAACGCCATCGCTCAGATAGACG 81
P4                  ATGGTCGCCGGCCTATATGGAACATGGGACACATGGTTAACGGCATTTGGCAGATTGACC 156
P6_5'RACE_CL27      ACAAGCGCAGACCAATATGGAATATGGGTCATATGGTGAATGCCGTTTATCAGATTGACG 180
P3                  ACAGCCGGAAGCCAATTTGGGATATAGCGCACATGGTAAACGATCTTGGGCTGGTGGACG 146
P5                  ATAATCGCCGTCCTATATGGAATATAGCACATATGGTCAACGATAAGGGTCTCATAGACG 159
P6_3'               ------------------------------------------------------------

P1                  AGTTCGTGAACCTTGGAGCAAACTCCATCGAAACAGACGTGTCTTTCGATGACAATGCCA 204
P2                  AGTTCGTGAACCTTGGAGCAAATTCCATCGAAACAGACGTGTCTTTCGATGACAATGCCA 141
P4                  AATTTGTGGACCTTGGAGTCAATTCCATCGAGTTTGACATAAACTTTGACAAAAATGGCA 216
P6_5'RACE_CL27      AATTCGTGGACCTTGGGGCAAATGCCATTGAAACAGACGTCACCTTTACCAAAGGCGCAA 240
P3                  AATATTTAGGTGATGGCGCGAACGGCCTTGAGTTAGACGTAGCTTTTACAGCTGATGGGA 206
P5                  AATATTTGGACGATGGAGCAAACAGTGTTGAATCTGACGTATCATTCGACTCTAACGGAA 219
P6_3'               ------------------------------------------------------------

P1                  ATCCTGAGTATACTTATCACGGCATTCCATGTGATTGTGGAAGGAATTGCAAGAAATATG 264
P2                  ATCCTGAGTACACTTATCACGGCATTCCATGCGATTGTGGAAGGAGTTGTCTGAAATGGG 201
P4                  AACCCGTCTACACATACCACGGGGTTCCTTGCGATTGCTTCAGGAGCTGCCTGAACTGGG 276
P6_5'RACE_CL27      ACGCAGAATACACCTATCACGGAGTTCCTTGCGACTGCCACAGGTGGTGCAAGAAGTGGG 300
P3                  CTGCTGATAAGATGTATCACGGAGTACCATGTGACTGTTTCAGAAGCTGTACGCGAACAG 266
P5                  AGCCGGAGAAAATGCTTCATGGATCTCCTTGTGACTGTGGTAGAAGTTGCAAAAGGCAGA 279
P6_3'               ------------------------------------------------------------

P1                  AGAATTTTAACGATTTTCTGAAAGGTCTCCGAAGCGCCACAACACCTGGTAATTCAAAGT 324
P2                  AGAATTTCAACGATTTTCTCAAAGGCCTGCGAAGTGCCACAACACCCGGTAATGCAAAGT 261
P4                  AATACTTCGGCGAATTTTTGACAGCTCTGCGACATCGGACAACGCCTGGTGACAAATTGT 336
P6_5'RACE_CL27      AATACGTCAATGACTTCTTGAAAGCATTGCGCCGAGCCACAACCCCTGGCGATGCAAAGT 360
P3                  AGGGATTCACCAAGTACATGGACTATATACGTCAACTAACGACACCAGGCAACAGCAAGT 326
P5                  TGAGTTTCGCCGACTATCTAGATTATATGCGGCAGTTGACCACTCCTGGTGATCCAAAGT 339
P6_3'               ------------------------------------------------------------
P1                  ATCAGGAAAAACTGGTCTTAGTCGTGTTCGACTTAAAGACAGGTAGCCTCTACGATAATC 384
P2                  ATCAGGCAAAATTAATCCTAGTTGTTTTCGACTTAAAAACGGGTAGTCTCTACGATAATC 321
P4                  ACAAAGAGAAGCTAATTCTATTTGTCTTTGATATGAAAACGAATAGTCTTTATGACAATC 396
P6_5'RACE_CL27      ATCGGAATCAGCTGATTCTGGTTGTGTTCGATTTGAAAACGGATTATCTGACCGCTTCTA 420
P3                  TCAAAAGTCAACTTATATTGTTAATAATGGACCTGAAACTGAATGGCATCGAACCGAATG 386
P5                  TTCGTGAGAATCTAATATTAGTTATGCTGGATTTAAAACTGAAAAAACTTTCATCGGAGC 399
P6_3'               ------------------------------------------------------------

P1                  AAGCCAACGACGCCGGAAAGAAATTGGCGAAGAATCTCTTACAACATTACTGGAACAATG 444
P2                  AAGCCAACGAAGCCGGAAAGAAATTGGCGAAAAATCTCCTAAAGCATTACTGGAACAATG 381
P4                  AGGCATACCAAGCTGGTGTAAACATGGCAACGGATATCTTTAAATACTACTGGAACAATG 456
P6_5'RACE_CL27      CTGCTTATGATGA-ATCACTAATGCGG---------------------------------- 446
P3                  TTGCGTATGCGGCAGGAAAAAGCGTTGCGGAGAAACTTTTGAGCGGCTACTGGCAGAACG 446
P5                  AAGCGTACTCGGCCGGGCAGGAAGTAGCAAGTCAGATGTTGGATAAATACTGGAAACGAG 459
P6_3'               ------------------------------------------------------------

P1                  GCAATAATGGTGGAAGAGCATACATAGTGTTATCGATCCCAGACCTTAATCATTATCCAC 504
P2                  GCAATAATGGTGGAAGAGCATACATAGTGTTATCGATCCCAGACCTTAATCATTATCCAC 441
P4                  GTCAAAATGGTGGAAGGGCATACTTCATATTATCAATACCCAACCTCAATCATTATGACT 516
P6_5'RACE_CL27      ------------------------------------------------------------
P3                  GAAAAAGTGGAGCTAGGGCATATATAGTACTTTCCCTGGAAACCATCACCCGACCAAATT 506
P5                  GTGAAAGCGGAGCGAGGGCTTATATTGTGTTGTCAATACCCACTATCACGCGGGTCACGT 519
P6_3'               ------------------------------------------------------------
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```
P1                  TGATTAAAGGATTCAAAGACCAGCTTACAAAGGACGGACACCCAGAGTTGATGGACAAAG 564
P2                  TGATTAAGGGATTCAAAGACCAGCTTACACAGGACGGACACCCAGAGTTGATGGACAAAG 501
P4                  TGATAAAAGGATTTAGAGAAACGATTACAAAGAAAGGCCATCCAGAGTTAATGGAGAAAG 576
P6_5'RACE_CL27      ------------------------------------------------------------
P3                  TCATAAGCGGTTTCAGAGATGCGATTAAAGCGAGTGGACACGAGGAGTTATTCGAAAAAA 566
P5                  TTGTAAATGGCTTTTACGATAAACTTCACAGCGAAGGCTTTGACCAATATAGGGAGAAAG 579
P6_3'               ------------------------------------------------------------

P1                  TTGGACACGACTTCTCCGGAAACGACGACATCGGCGACGTTGGAAAAGCTTACAAGAAAG 624
P2                  TTGGACATGACTTCTCCGGAAACGACGCCATCGGTGACGTTGGGAATGCTTACAAGAAAG 561
P4                  TAGGGTATGACTTCTCTGCGAATGACAATATCCCAGATGTGGAGAAAGCCTACGGAAAAG 636
P6_5'RACE_CL27      ------------------------------------------------------------
P3                  TAGGTTGGGATTTCTCTGGCAACGAGGACTTGGGCGAGATCCGCAGAGTTTACCAGAAAT 626
P5                  TTGGTGTCGATTTCTCAGGTAACGAGGACCTTGAAGACACAGGTAAAATTTTGAAATCGC 639
P6_3'               ------------------------------------------------------------

P1                  CAGGAATAACTGGCCATATTTGGCAGAGCGATGGTATCACCAACTGTTTACCACGTGGCC 684
P2                  CCGGAATATCCGGCCATGTGTGGCAGAGCGACGGTATTACCAACTGTTTACTGCGTGGTC 621
P4                  TCGGAGTAACAGATCATGTGTGGCAAAGCGACGGTATAACCAATTGCATAGCACGGGGTC 696
P6_5'RACE_CL27      ------------------------------------------------------------
P3                  ACGGAATCGAGGATCACATTTGGCAAGGCGACGGTATCACCAACTGTTTGCCACGAGGGG 686
P5                  GGGATATCCTCGACCACATTTGGCAAAGTGATGGCATTACTAATTGCCTATTTAGAATCA 699
P6_3'               ------------------------------------------------------------

P1                  TTAGTCGTGTGAACGCAGCTGTGGCAAACAGAGAT---TCCGCAAACGGATTCATTAACA 741
P2                  TTGATCGTGTAAAGCAAGCTATTGCAAACAGAGAT---TCCGCAAACGGATTCATTAACA 678
P4                  TCAGTCGAGTGAAAGAAGCTGTAAAAGAGAGAGAC------AGCGGTGGAGTCATTAACA 750
P6_5'RACE_CL27      ------------------------------------------------------------
P3                  ACTATCGTCTGACAGAAGCTATGAAAAAGAAAAACGATCCCAACTACAAATACACTTTAA 746
P5                  TGAAGCGTCTAAAGGCGGCGATAAGGAAAAGGGAT------TCGAATGGATACATGGTTA 753
P6_3'               ------------------------------------------------------------

P1                  AAGTGTACTACTGGACAGTGGACAAGCGCTCAACGACCAGAGATGCACTTGATGCTGGAG 801
P2                  AAGTGTACTACTGGACAGTGGACAAGCGCGCAACGACCAGAGATGCACTTGATGCCGGAG 738
P4                  AAGTCTACATTTGGACTATAGACAAATTCTCTTCAACGAGAGATGCACTTGATGCTGGCG 810
P6_5'RACE_CL27      ------------------------------------------------------------
P3                  AAGTCTACACCTGGAGCATTGACAAAGAGTCTTCTATCCGAAATGCACTAAGACTTGGCG 806
P5                  AAGTTTACACCTGGAGCGTGGACAAGTACACAACAATGAGGAAAGCACTCCGCGCGGGAG 813
P6_3'               ------------------------------------------------------------

P1                  TTGACGGCATAATGACCAACTACCCGGATGTTATCACTGATGTTCTCAACGAAGCCGCAT 861
P2                  TTGATGGTGTAATGACCAATTACCCAGATGTAATTACTGATGTCCTCAACGAATCCGCTT 798
P4                  TTGACGGCATAATGACCAACTACCCATATGTACTAAATGACGTCCTAAAAGAAGGAGCTT 870
P6_5'RACE_CL27      ------------------------------------------------------------
P3                  TAGATGCTGTAATGACTAATTATCCAGCACGTGTAAAAAGCATCTTGAGAGAGAGTGAAT 866
P5                  CTGATGGAATGATAACCAATTTTCCTAAACGACTTGTGTCAGTGCTCAACGAACGTGAAT 873
P6_3'               ---GCTGAAAGAAAACGACTTTA--AAGGCAAATTCAGAATGGCCACATACAATGATAAC 55

P1                  ACAAGAAGAAATTCCGAGTTGCCACATACGACGAAAATCCATGGGTGACATTCAAGAAAT 921
P2                  ACAAGAATAAATTTAGAGTTGCTTCATACGAGGACAATCCTTGGGAGACATTCAAGAAAT 858
P4                  ACAAAAATAAATTCAGAATGGCCACATACGAAGACAACCCTTGGGTGACCTTCAAAGCAT 930
P6_5'RACE_CL27      ------------------------------------------------------------
P3                  TCTCTGGCACACACAGGATGGCAACATACGATGATAATCCCTGGCAGA-------AGTAA 919
P5                  TCTCTGGAAAATTTAGATTGGCTACTTACAATGACAACCCATGGGAAA-----GATACAC 928
P6_3'               CCGTGGGAAACGTTTAAGTAGTAAATTGAGATCAAGAGGCCCAG---------AGTATGT 106

P1                  AAATTCTGC----AGGTTGATTGTGGAAAAACACATGGCAATCTGGATTTCACGATTTTC 977
P2                  AAATTCTGC----CGGTTGGTTCTGTGCAAACTCATACCAAACTGAATTTCTCGGTTTTC 914
P4                  AAAGTACGTTTAAAGGCCGATCTTCTAGGGACAATTATCGATGCGGATTTCTGCAAACGT 990
P6_5'RACE_CL27      ------------------------------------------------------------
P3                  AATCTGTGCACCAAAATGACTGACTGCAAGGAATTTTTCAAAGCCGATGTATTAAAGAAA 979
P5                  AGGTTGAAT-----AGGTATAATTCAGGGAGATTTTTACGCTATAGCAAATGAATTTTTA 983
P6_3'               AAAATCAGG-----CGCAATACTTTAGAAGCG-------GATGTCGAAGAAGCAAATCCA 154

P1                  ATTGAACTTTGTTGAAAAACCAATTTGATGCGAAAAACTAAAAACTATGCACTATGGAAG 1037
P2                  ATAGAACTTTGTTGGAA--CCAAATTGATACA-----------------CACTATGGATG 955
P4                  TTATAGAAGTTTTCATAGACTATTACTATATGTGTAAAGAATATTTATCTATTGCCAAAG 1050
P6_5'RACE_CL27      ------------------------------------------------------------
P3                  TAATATAGAGCTCAAAAAAAAAAAAAAA-------------------------------- 1007
P5                  TTCTTTATTATTTCTTAAGTCTCTCTGGAGCCT---------ATGTTCTTGTTACA---T 1031
```

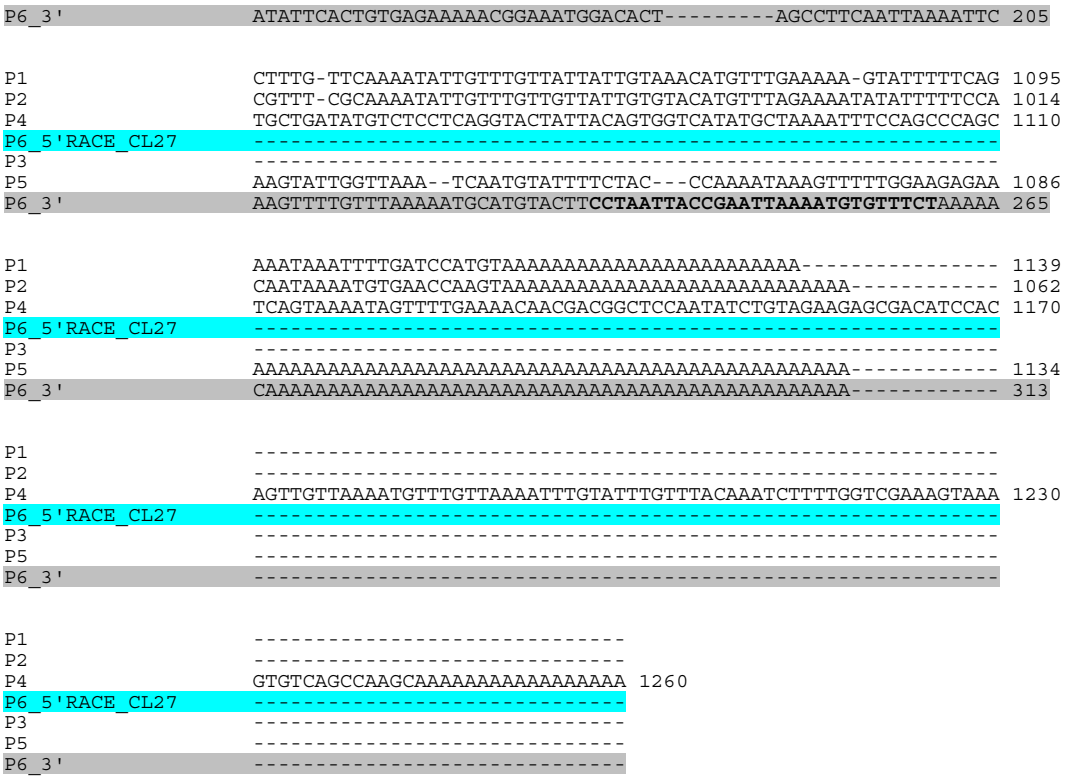


Figura 5: As linhas representam o pareamento por homologia das diversas seqüências de bases para cada uma das proteínas dermonecróticas rastreadas e clonadas da biblioteca de cDNA de *L. intermedia*. A linha em azul claro mostra o pareamento do fragmento da LiRecDT6 obtido pela reação de 5'RACE e em cinza está representado o fragmento original da biblioteca, que serviu de base para a confecção dos oligonucleotídeos GSP-1 e GSP-2 (destacado em negro). Temos representado em vermelho a metionina inicial da seqüência codificante da proteína, em azul escuro (relevo) o códon da alanina que marca o início da proteína madura e em verde o códon de parada de transcrição, representando, portanto a região codificante da proteína madura do códon da alanina até o códon de parada. Destacada em azul mais intenso está a região de pareamento do oligonucleotídoes gene específico da região codificante da proteína madura. Alinhamento obtido através do site: http://www.ebi.ac.uk/clustalw/.

Desenho de oligonucletídeos sense e antisense específicos para cópia do cDNA da proteína LiRecDT6

Para a confecção dos oligonucleotídeos descritos abaixo foi feita análise das extremidades 5' e 3' da seqüência codificante da LiRecDT6 obtida da reação 5"RACE e da biblioteca, buscando-se um local para assentamento dos oligonucleotídeos mais específico para a seqüência alvo e que divergisse das demais já que existe grande homologia entre as seqüências (isoformas). Esta análise levou à confecção dos seguintes oligonucleotídeos:

Li RecDT6 Oligo Específico Sense: 5' ATC ACT TAT ATT TGA TCA GAA GGA AAA TC 3' Tm= 51,9°C

Li RecDT6 Oligo Específico Antisense: 5' AGA AAC ACA TTT TAA TTC GGT AAT TAG G 3' Tm= 52,3°C

Figura 6: representação gráfica dos oligonucleotídeos desenhados com auxílio de ferramentas do site: www.idtdna.com.

PCR com *Pfu* DNA polimerase e oligonucleotídeos LiRecDT6 específicos

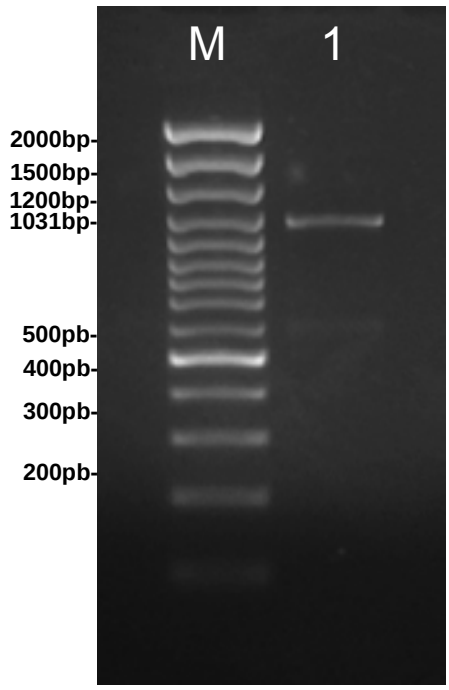


Figura 7: A coluna M representa os marcadores de número de pares de bases e a coluna 1 representa o produto de PCR obtido a partir do cDNA (proveniente da reação com transcriptase reversa), com o emprego da *Pfu* DNA polimerase como enzima de transcrição e os oligonucleotídeos desenhados especificamente para as porções 5' e 3' da LiRecDT6.

PCR de colônia e oligonucleotídeos LiRecDT6 específicos

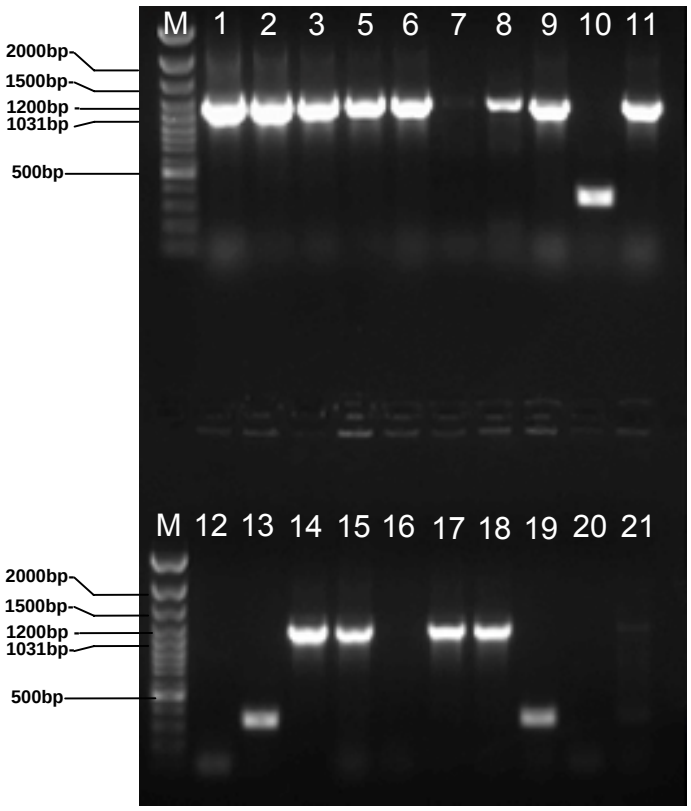


Figura 8: As colunas M mais uma vez representam os marcadores de número de pares bases. As demais colunas representam o perfil do inserto produzido com os oligonucleotídeos específicos (Li RecDT6 Oligo Específico Sense e Li RecDT6 Oligo Específico Antisense) e com enzima de transcrição de alta fidelidade (*Pfu* DNA polimerase) presente nos plasmídeos inseridos nas colônias isoladas de bactérias transformadas. As colônias escolhidas para dar continuidade às reações foram: 14,15 e 17, 18.

Desenho de oligonucleotídeos para proteína LiRecDT6 madura

Para desenho dos olígonucleotídeos que pareiam com as regiões codificantes da proteína madura (a partir da alanina até o códon de parada) foi

analisada a seqüência obtida da biblioteca de cDNA e reação de 5'RACE procurando manter alta especificidade.

Li RecDT6 OLIGO ESPECÍFICO SENSE PROTEÍNA MADURA (*Xho*-I)

5' CCG CTC GAG – GCA GAC AAG CGC AGA CCA ATA 3' Tm= 67,7°C

Li RecDT6 OLIGO ESPECÍFICO SENSE PROTEÍNA MADURA (*Bam*-HI)

5' CG GGA TCC – CTA CTT AAA CGT TTC CCA CGG GT 3' Tm= 65,4°C

Figura 9: representação gráfica dos oligonucleotídeos específicos para a proteína madura. Os primeiros nucleotídeos na região 5' até o traço representam as regiões para as enzimas de restrição especificadas entre parênteses.

Seqüência aminoacídica do cDNA da LiRecDT6

ATCACTTATATTTGATCAGAAGGAAAATCAATTTCAGC**ATG**TTGTGTTTCT

M L C F

TTGTATTATTCTTCTGTTGTGGAACCGTCCTCCTAGAGGGAGCAGACATTG

F V L F F C C G T V L L E G [A] D I

ATGAAATCGAACATGCAGACAAGCGCAGACCAATATGGAATATGGGTCATA

D E I E H A D K R R P I W N M G H

TGGTGAATGCCGTTTATCAGATCGACGAATTCGTGGACCTCGGGGCAAACG

M V N A V Y Q I D E F V D L G A N

CCATTGAAACAGACGTCACCTTTACCAAAAGCGCAAACGCAGAATACACCT

A I E T D V T F T K S A N A E Y T

ACCACGGAGTTCCTTGCGACTGCCACAGGTGGTGCAAGAAGTGGGAATACG

Y H G V P C D C H R W C K K W E Y

TCAATGACTTCTTGAAAGCATTGCGCCGAGCCACAACCCCTGGCGATGCAA

V N D F L K A L R R A T T P G D A

AGTATCGGAGTCAGCTGATCCTGGTTGTGTTCGATTTGAAAACGGATTATC

K Y R S Q L I L V V F D L K T D Y

TGACCGCTTCTACTGCTTATGATGCTGGAAAGGACTTTGCAAAGAGGCTAC

L T A S T A Y D A G K D F A K R L

TACAACACTACTGGAACGGAGGCAGTAACGGTGGAAGAGCATACATAATAT

L Q H Y W N G G S N G G R A Y I I

TATCCATCCCGGACCTTGCCCATTATAAATTTATAAACGGGTTTAAGGAAC

L S I P D L A H Y K F I N G F K E

AACTTAAAACCCAAGGTCATGAAGACTTGCTGGCAAAAGTAGGCTATGACT

Q L K T Q G H E D L L A K V G Y D

TTTGGGGGAATGAAGACCTAAGCTCGACTCGCGCTGCCTTTCAAAAAGCTG

F W G N E D L S S T R A A F Q K A

GAGTTCAAGATAAAGAACACATCTGGCAGAGTGATGGTATCACAAACTGTT

G V Q D K E H I W Q S D G I T N C

GGCTCCGTACTCTTAAACGCGTGAGAGAAGCTGTGGCAAACCGAGATTCGT

W L R T L K R V R E A V A N R D S

CCAATGGATATATCAACAAAGTGTATTACTGGACTGTAGACAAATATGCAT

S N G Y I N K V Y Y W T V D K Y A

CCGTCAGAGACGCAATCAATGCTGGTGCCGATGGCATAATGACGAACTATC

S V R D A I N A G A D G I M T N Y

CGAATGTTATTGTCGACGTGCTGAAAGAAAACGACTTTAAAGGCAAATTCA

P N V I V D V L K E N D F K G K F

GAATGGCCACATACAATGATAACCCGTGGGAAACGTTTAAGTAGTAAATTG

R M A T Y N D N P W E T F K *

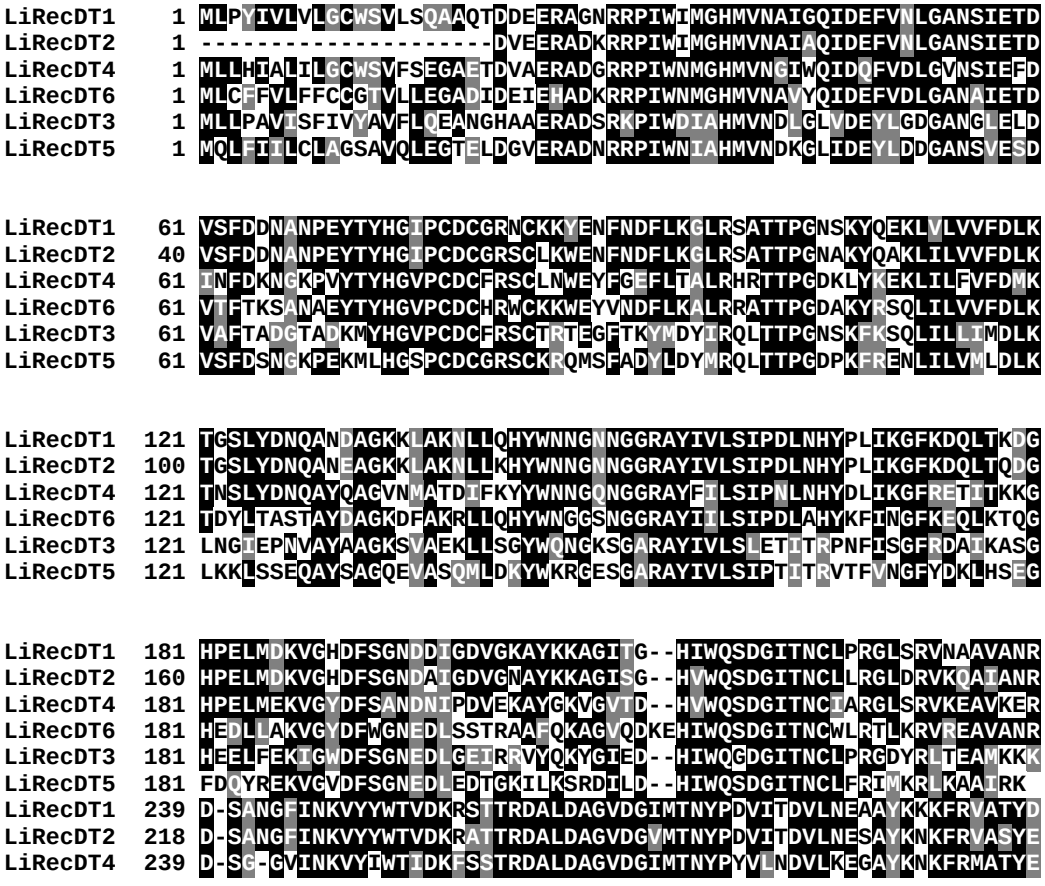
ATATCAAGAGGCCCAGACTATGTAAAATCAGGCGAAATACTTTAGAAGCGGAT

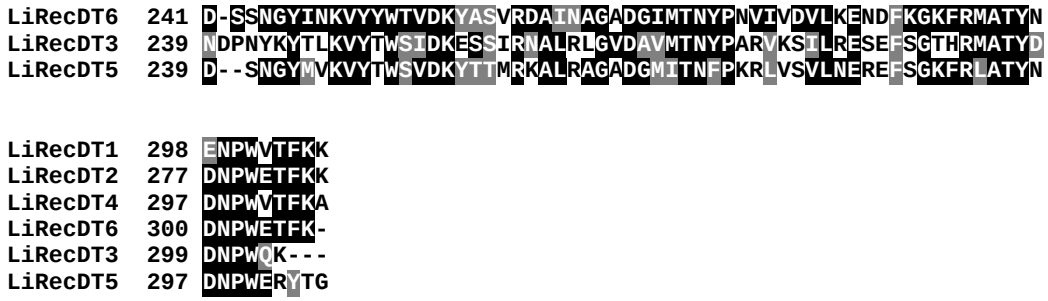
GTCGAAGAAGCAAATCCAATATTCACTGTGAGAAAAACGGAAATGGACACTGG

CCTTCAATTAAAATTCAAGTTTTGTTTAAAAATGCATGTACTTCCTAATTACCGA
ATTAAAATGTGTTTCTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAA

Figura 10: LiRecDT6 toxina dermonecrótica clonada a partir da biblioteca de cDNA da glândula produtora de veneno de *L. intermedia*. Representação da seqüência nucleotídica e dedução da seqüência aminoacídica com 307 aminoácidos. Em negrito está o códon da metionina inicial e a metionina inicial. O asterisco representa o códon de parada (TAG) e no quadrado está a alanina inicial da proteína madura.

Alinhamento das seqüências aminoacídicas das isoformas da toxina dermonecrótica





	LiRecDT1	LiRecDT2	LiRecDT3	LiRecDT4	LiRecDT5
LiRecDT6	64%	67%	46%	59%	50%

Figura 11: análise do múltiplo alinhamento das seqüências aminoacídicas preditas a partir dos cDNA das 6 isoformas das LiRecDT. As identidades aminoacídicas estão representadas em negro. As substituições conservativas estão em cinza. O quadro mostra a percentagem absoluta de identidade entre a LirecDT6 e as outras cinco isoformas estudadas. O alinhamento foi realizado com ferramentas do programa Clustal W.

Filograma

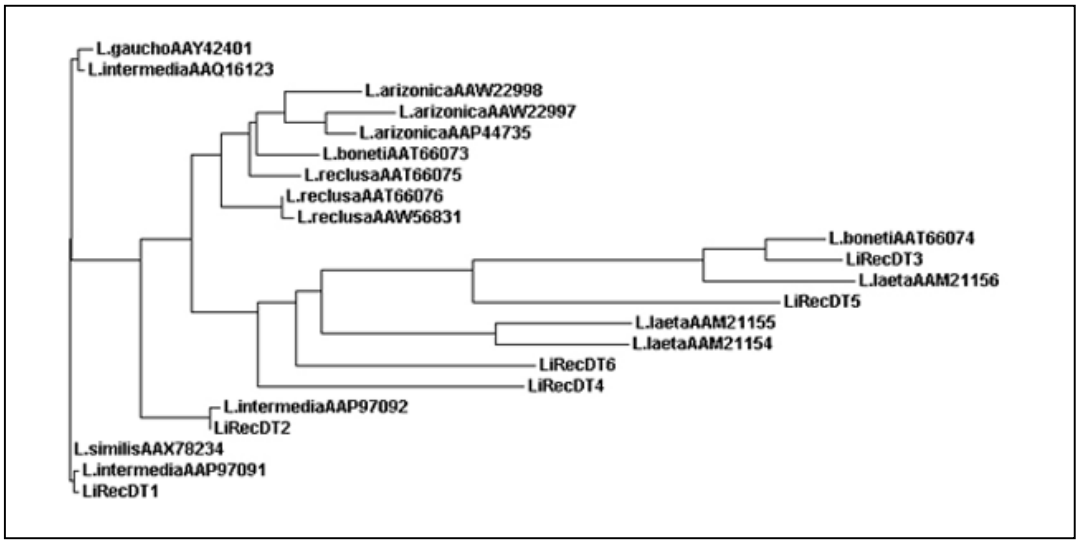


Figura 12: filogenia das toxinas dermonecróticas clonadas estabelecida com base nas seqüências depositadas no GenBank e na seqüência obtida para LiRecDT6 (figura 10). A árvore foi construída com auxílio do programa ClustalW.

DISCUSSÃO

Com os avanços em biologia molecular foi possível estudar proteínas de venenos isoladamente. A clonagem e expressão de proteínas em bactérias possibilitaram a avaliação individualizada de toxinas que têm atividades muitas vezes sinérgicas entre elas e estão na mistura protéica que forma os venenos de aranhas. A aplicação biotecnológica destas toxinas isoladas é alvo dos estudos recentes. Uma mistura de plasma normal e veneno de *L. reclusa* foi descrita por mimetizar a presença de um anticoagulante lúpico servindo de controle positivo deste teste (MCGLASSON *et al*., 1993). Uma proteína dermonecrótica funcional de veneno de *L. laeta* foi descrita por PEDROSA *et al*., 2002. Uma proteína recombinante de *L. intermedia* (LiD1) não tóxica foi usada para produção de anti-soro (que reconheceu a proteína nativa) e para imunização de coelhos e camundongos posteriormente submetidos ao contato com o veneno total. Esta imunização preveniu o aparecimento dos efeitos tóxicos do veneno (KALAPOTHAKIS *et al*., 2002, ARAUJO *et al*., 2003)

Neste trabalho buscou-se a clonagem de uma sexta isoforma da enzima com atividade esfingomielinásica (LiRecDT) chamada LiRecDT6. As cinco anteriores foram clonadas e expressas e suas atividades biológicas estão sob estudo, recebendo denominação de LiRecDT 1, 2, 3, 4 e 5. (CHAIM *et a*l., 2006; DA SILVEIRA *et al*., 2006a; DA SILVEIRA *et al*., 2006b). Esses dados mostram a existência de uma família de proteínas dermonecróticas intra-espécie no veneno de *L. intermedia* (seis até o presente momento), comprovando dados anteriores realizados por análises proteômicas e bioquímicas os quais mostraram a presença de diferentes isoformas da toxina dermonecrótica no veneno de *L. gaucho* (MACHADO *et al*., 2005). Outras proteínas loxoscélicas com diferentes atividades estão também sob investigação, mostrando que o leque de estudo, com a aquisição deste tipo de tecnologia, é de grande valor. Estas toxinas clonadas,

expressas e com as atividades biológicas sendo investigadas são fruto do trabalho do grupo de Matriz Extracelular e Biotecnologia de Venenos da UFPR e do Departamento de Bioquímica da UNIFESP/EPM.

O ponto de partida para pesquisa da presença de uma sexta isoforma de proteína dermonecrótica foi um fragmento, apresentando homologia com outras proteínas dermonecróticas descritas, que foi seqüenciado a partir de biblioteca de cDNA produzida na UNIFESP/EPM (Da Silveira *et al*., 2006a, b). O fragmento omitia a porção 5'. Para tentar elongar totalmente o fragmento foi realizada a técnica de 5'RACE (SAMBROOK E RUSSEL, 2001) que consiste na confecção de oligonucleotídeos antisense específicos para o fragmento 3' conhecido. Os oligonucleotídeos específicos devem interagir com a região mais próxima da porção 5'. O molde utilizado de início foi o RNA total extraído da glândula de veneno de *L. intermedia*. A primeira etapa consiste na transcrição reversa do RNA correspondente à LiRecDT6, objetivando esta meta usamos o oligonucleotídeo GSP-1 e obtivemos o cDNA. Para que fosse possível a confecção do complementar foi necessário a adição de uma cauda homopolimérica (cauda de adenina) ao cDNA obtido. Para obter a segunda fita foi usado um oligonucleotídeo com 17 timinas e uma região adaptadora - (dT)17adaptor primer – tendo como local de pareamento a cauda homopolimérica (figura1). É importante que este oligonucleotídeo tenha logo após a sua seqüência homopolimérica uma degeneração de 3 nucleotídeos (V= A, C, G, mas não T) que inviabilize a ligação do oligonucleotídeo numa região qualquer da cauda homopolimérica do cDNA, mas que permita a ligação as primeiras 17 adeninas imediatamente em seguida do término da seqüência correspondente ao RNA que gerou o cDNA. Para amplificação do produto em dupla fita por PCR foi usado um segundo oligonucleotídeo gene específico (GSP-2) e o (dT)17 adaptor primer. A razão para a utilização de um segundo oligonucleotídeo gene específico é a tentativa de

aumentar ainda mais a especificidade durante a amplificação, pois sabendo que estávamos buscando uma sexta isoforma poderíamos amplificar um produto muito parecido indesejado que por ventura estivesse presente e em maior concentração. Obtivemos a amplificação de um fragmento de aproximadamente 400pb, que era um tamanho dentro do esperado para o produto desejado. A figura 2 mostra a intensidade de emissão luminosa captada das amostras com boa amplificação (anelamento adequado pelos oligonuceotídeos propiciando a amplificação), porém ainda não temos a informação da especificidade de anelamento. O produto de PCR foi extraído do gel para que fosse clonado ao plasmídeo pGEM-T que é um plasmídeo comercial usado para clonagem de produtos de PCR gerados por *Taq* DNA polimerase, mas muito útil para sequenciamento de cDNA. Após a ligação do plasmídeo e inserto a construção foi introduzida em bactéria eletrocompetente DH5α. A bactéria eletrocompetente é uma bactéria tratada com sucessivas lavagens com água ultrapura deixando-a absolutamente livre de íons. O motivo pelo qual é necessária a ausência de íons é que o meio de abrir caminho para a construção plasmideal através da membrana das bactérias é a colocação de ambas sob um campo elétrico que permeabiliza por alguns milisegundos as membranas bacterianas e na presença de íons pode haver condução elétrica levando ao aparecimento de descarga elétrica forte que destrói as bactérias. A permeabilização, mesmo que rápida das membranas, faz com as bactérias tenham sua viabilidade prejudicada. Para minimizar estes problemas, imediatamente após a eletroporação das bactérias, elas são colocadas em meio SOC, que é um meio rico em glicose e íons o que permite a reestabilização das membranas e recuperação das bactérias. Porém a principal função desta incubação é permitir à bactéria que recebeu o vetor expressar a resistência ao antibiótico, característica obtida através desta aquisição. Depois de uma hora, as bactérias resistentes a ampicilina podem ser plaqueadas em meio sólido para que

se obtenham colônias isoladas. Nestas placas só crescem as bactérias que têm o plasmídeo de interesse, pois uma das características deste plasmídeo é trazer uma região de resistência bacteriana a antibiótico, no caso resistência a ampicilina. Neste plaqueamento também é introduzido o IPTG (isopropil-tio-β-D-galactopiranosideo) e o X-Gal (5'Bromo-4-cloro-3-indolil-β-D-galactopiranosideo). O IPTG é um análogo da lactose não metabolizável que induz os promotores do tipo lac, tanto no cromossomo da bactéria quanto no plasmídeo, levando à transcrição do fragmento lacZ, presente no plasmídeo e o X-Gal, que é um substrato cromogênico para a β-galactosidase, é usado para identificar aquelas bactérias cujo plasmídeo efetivamente tem o inserto. Isso é possível porque as bactérias que possuem plasmídeos sem inserto ligado mantêm a capacidade de α-complementação e assim produzir β-galactosidase ativa (gene lac-Z intacto). Nas colônias, que possuem o inserto, está presente o gene é interrompido e não há a metabolização do X-Gal e as colônias apresentam cor branca, o que não ocorre com as outras que ficam azuis.

Vinte e seis colônias brancas, uma levemente azul e uma efetivamente azul foram escolhidas para a amplificação por PCR. Na figura 3 podemos notar que a amplificação foi bem sucedida para as colônias brancas (têm inserto), enquanto que no caso da levemente azul e azul a amplificação não foi bem sucedida (colunas 11 e 28). Os oligonucleotídeos escolhidos para esta amplificação foram o gene-específico (GSP-2) e o adaptor primer, mostrando amplificação boa, mas apresentando problemas com a especificidade, conforme observado pela variedade de tamanho de produtos de amplificação no gel de agarose. Para a etapa seguinte era necessário escolher algumas colônias que tiveram inserto no tamanho que foi estimado para o fragmento 5' que era objetivo de estudo. Escolhemos as colônias 2, 6, 14, 17, 18 e 27. Estas colônias bacterianas foram colocadas em crescimento em meio líquido (pré-inóculo) durante 16 horas para

atingir a saturação (grande quantidade de bactérias). Destas bactérias foram extraídos os plasmídeos com auxílio de reagentes apropriados. O plasmídeo purificado de cada uma das colônias isoladas escolhidas foi submetido à reação de amplificação para sequenciamento do inserto. Para esta PCR foram usados como oligonucleotídeos o T7 e o SP6 que estão presentes na seqüência original do plasmídeo (não pertence ao inserto) e estão ladeando a região de inserção do cDNA de estudo. Cada plasmídeo purificado gera duas reações de sequenciamento, uma no sentido de leitura do T7 e outra do SP6 que são complementares, mas estão separadas para análise individual de cada uma delas. O produto desta reação foi enviado a UNIFESP/EPM para sequenciamento. O resultado do sequenciamento dos diversos plasmídeos purificados foi analisado com ajuda dos programas Genetyx-Mac 7.3 e EditView. O resultado foi alinhado para comparação de homologia dos diversos fragmentos 5' produzidos tentando buscar o que estava mais completo e que realmente era a amplificação da LiRecDT6. Todos os seqüenciados tiveram grande homologia mostrando que representavam o mesmo fragmento. Apenas o produto do clone 14 não teve leitura adequada, por problemas com a reação de sequenciamento. A figura 4 mostra os clones seqüenciados e alinhados. A homologia entre eles está destacada por asteriscos. A homologia mostra que os clones são praticamente idênticos indicando a amplificação do mesmo cDNA. O clone 27 foi escolhido para análise na seqüência por ser o mais representativo deles. Sendo assim o clone 27 foi alinhado às seqüências existentes das outras cinco isoformas disponíveis e mais o fragmento 3' da LiRecDT6 (da Silveira comunicação pessoal). Para tal foram usadas as ferramentas existentes no site: http://www.ebi.ac.uk/clustalw. Com as seqüências alinhadas foi possível a construção de oligonucleotídeos específicos para a porção 5' e 3' da seqüência alvo. Para isto foram analisadas as regiões 5' e 3' que divergissem das outras isoformas. Isso foi feito para se obter

maior especificidade. Também, para confecção destes oligonucleotídeos, seguiram-se regras que favorecessem seu anelamento ao molde. Uma destas características é a temperatura de desnaturação do oligonucleotídeo do molde (Tm ou Melting Temperature), que não deve ser muito alta, pois dificultaria a desnaturação da dupla fita formada na primeira etapa dos ciclos de amplificação no PCR, e nem muito baixa para que houvesse um mínimo de estabilidade de ligação evitando inespecificidade. Tampouco deve formar estruturas chamadas "hairpin" que tenham Tm próximos ou superiores ao Tm do oligonucleotídeo em relação ao molde. Estas formações são dobras (grampos) com pareamentos entre os pares de bases do próprio oligonucleotídeo. Outras interações indesejadas são as que podem ocorrer entre dois oligonucleotídeos sense e que podem acontecer entre o sense (homodímero) e o antisense (heterodímero). Se forem inevitáveis estas interações, elas devem ter Tm baixo e não podem ser estáveis na região 3'. O número de bases deve ficar em torno de 17-20 com 40-60% de C ou G. A figura 6 mostra a seqüência de bases escolhidas com seus respectivos Tm. Na figura 5 a região de anelamento dos oligonucleotídeos está representada em negrito nos fragmentos 5' e 3' da LiRecDT6.

Com os oligonucleotídeos confeccionados especificamente para as regiões 5' e 3' da LIRecDT6 foi dada continuação ao trabalho buscando agora amplificar a totalidade da seqüência alvo. Para que isso fosse alcançado foi necessário usar uma polimerase de alta fidelidade, a *Pfu* polimerase. A *Taq* polimerase insere um erro a cada 1000 bases inseridas, o que torna a amplificação de uma seqüência de mais de 1000 bases inviável em termos de fidelidade e além do mais, tais erros inseridos podem levar à geração de um códon de parada no produto amplificado. A *Pfu* DNA polimerase tem a mais alta taxa de fidelidade de todas as polimerases e coloca 500 bases por minuto de velocidade de elongação, porém alguns problemas técnicos surgem de uma propriedade exonucleásica 3'-5' da *Pfu* DNA

polimerase, que pode levá-la a degradar os oligonucletídeos. Para minimizar este problema toda a reação em que esta enzima está presente, deve até o instante de ir para o termociclador, ficar em gelo. O termociclador deve estar programado para começar a reação de PCR em “hot start”, o primeiro ciclo da reação inicia-se pré-aquecido à 95°C. O molde desta reação foi obtido a partir do RNA total extraído da glândula produtora de veneno. O RNA total foi submetido à reação com a transcriptase reversa juntamente com um oligonucleotídeo do tipo dT, que permite a transcrição de todos os RNAm presentes na amostra em cDNA. Em seguida, o cDNA foi colocado em presença de oligonucleotídeos específicos para a LiRecDT6 e *Pfu* DNA polimerase. A figura 7 na coluna 1 mostra um produto amplificado com tamanho em torno de 1200pb (tamanho esperado) e de intensidade baixa, o que também era esperado já que o número de cópias desse gene dentro do nível de expressão das demais é menor. Como amostra, no gel foi aplicado 1/10 (5 µl) do produto obtido pelo PCR. O restante, após confirmação da amplificação com produto dentro dos parâmetros esperados, foi submetido à extração orgânica e precipitação para retirada da *Pfu* DNA polimerase da reação. Esta etapa era necessária para que fosse possível colocar uma adenina na extremidade 3’. A *Taq* DNA polimerase tem esta propriedade de adenina terminal transferase, já *Pfu* DNA polimerase não. Sua presença na etapa de adição faria que através de sua propriedade de exonuclease 3’-5’ ela retirasse a adenina recém colocada que é importante para a fase de ligação com o plasmídeo. Após este tratamento o produto está pronto para ser ligado ao plasmídeo pGEM-T pelas razões já descritas. O produto da ligação foi eletroporado para bactéria eletrocompetente DH5α. As bactérias foram plaqueadas em ágar LB/amp (acrescido de IPTG e X-Gal) e colocadas em incubadora a 37 °C por 16 h. Dezoito colônias isoladas brancas e duas azuis foram escolhidas para repique em placa mãe e foram submetidas à PCR de colônia. Os oligonucleotídeos escolhidos foram

os LiRecDT6 Oligo Específico sense e antisense para se manter a especificidade na amplificação. Para amplificação de inserto em plasmídeo a *Taq* DNA polimerase pode ser usada apesar do tamanho do inserto ser maior que 1000pb. A figura 8 mostra o resultado do PCR de colônia, com produto bem amplificado e de tamanho esperado com 1200pb. As colônias 14, 15, 17 e 18, mais representativas, foram escolhidas. Estas colônias foram submetidas a crescimento em meio LB/amp líquido durante 16 h a 37 °C (até a saturação). Os plasmídeos gerados de cada crescimento bacteriano são purificados com alto grau de pureza para ser submetido à reação de sequenciamento. O produto para sequenciamento foi encaminhado a UNIFESP/EPM. Com o resultado deste último sequenciamento foi possível se obter o cDNA completo da LiRecDT6 que perfaz 307 aminoácidos na sua estrutura protéica predita por este cDNA clonado (figura 10). A figura 10 também tem em destaque em negrito a metionina inicial que sinaliza o começo da proteína e a alanina inicial no quadrado onde começa a proteína madura. O códon de parada está marcado pelo asterisco sinalizando o fim da proteína. Na figura 11 podemos observar as 6 isoformas da LiRecDT alinhadas conforme suas seqüências aminoacídicas preditas por cDNA. Com este alinhamento e pela análise da filogenia (figura 12) podemos avaliar a homologia entre elas. A LiRecDT6 é mais parecida com a LiRecDT4 tanto pela filogenia como pela análise dos aminoácidos.

CONCLUSÃO

Com os resultados obtidos durante o doutorado pudemos:

Publicar 3 artigos científicos em revistas internacionais:

1. Artigo de revisão:

Brown spider and loxoscelism

Autores: Paulo Henrique da Silva, Rafael Bertoni da Silveira, **Marcia Helena Appel**, Oldemir Carlos Mangili, Waldemiro Gremski, Silvio Sanches Veiga.

Revista: Toxicon 44: 693-709, 2004

2. Artigo de revisão:

Insights into brown spider and loxoscelism

Autores: **Marcia Helena Appel**, Rafael Bertoni da Silveira, Waldemiro Gremski, Silvio Sanches Veiga.

Revista: Invertebrate Survival Journal 2:152-158, 2005

3. Artigo regular

Molecular cloning and functional characterization of two isoforms of dermonecrotic toxin from *Loxosceles intermedia* (brown spider) venom gland

Autores: Rafael Bertoni da Silveira, Romine Bachmann Pigozzo, Olga Meiri Chaim, **Marcia Helena Appel**, Juliana Luporini Dreyfuss, Leny Toma, Oldemir Carlos Mangili, Waldemiro Gremski, Carl Peter Dietrich, Helena Bonciani Nader, Silvio Sanches Veiga.

Revista: Biochimie 88: in press

Conseguimos submeter um artigo a revista internacional:

4. Artigo submetido:

Two novel dermonecrotic toxins from brown spider (*Loxosceles intermedia*) venom: from cloning to functional characterization

Autores: Rafael Bertoni da Silveira, Romine Bachmann Pigozzo, Olga Meiri Chaim, **Marcia Helena Appel**, Dilza Trevisan Silva, Juliana Luporini Dreyfuss, Leny Toma, Carl Peter Dietrich, Helena Bonciani Nader, Silvio Sanches Veiga, Waldemiro Gremski

Revista: Biological Chemistry submetido Abril/2006

Com as revisões pudemos compilar dados dos últimos 14 anos, onde avanços significativos na compreensão do loxoscelismo e aspectos moleculares dos venenos de aranhas do gênero *Loxosceles* puderam ser melhor esclarecidos.

Nos outros dois artigos, onde por técnicas de biologia molecular, publicamos resultados de clonagem e expressão de 5 isoformas da toxina dermonecrótica (LiRecDT1, 2, 3, 4 e 5). Demonstramos atividades biológicas de cada uma delas (dermonecrose, infiltração leucocitária, permeabilidade vascular, agregação plaquetária, atividade esfingomielinásica) além evidenciar a presença desta família de proteínas intra-espécie no veneno de *L. intermedia*. Esta família apresentou homologia em suas seqüências nucleotídicas e aminoacídicas, além destas toxinas terem epítopos conservados que foram reconhecidos por anticorpos policlonais.

5. Neste trabalho se mostrou a existência de uma sexta isoforma da toxina dermonecrótica (LiRecDT6) presente no veneno da aranha marrom (*L. intermedia*), que foi seqüenciada e clonada e que em breve estará sob investigação de suas atividades biológicas.

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ANEXOS

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Artigos científicos publicados

Review:

Brown spider and loxoxcelism

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Review

Brown spiders and loxoscelism

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Abstract

Accidents caused by brown spiders (*Loxosceles* genus) are classically associated with dermonecrotic lesions and systemic manifestations including intravascular haemolysis, disseminated intravascular coagulation and acute renal failure. Systemic reactions occur in a minority of cases, but may be severe in some patients and occasionally fatal. The mechanisms by which *Loxosceles* venom exerts these noxious effects are currently under investigation. The venom contains several toxins, some of which have been well-characterised biochemically and biologically. The purpose of the present review is to describe some insights into loxoscelism obtained over the last ten years. The biology and epidemiology of the brown spider, the histopathology of envenomation and the immunogenicity of *Loxosceles* venom are reviewed, as are the clinical features, diagnosis and therapy of brown spider bites. The identification and characterisation of some toxins and the mechanism of induction of local and systemic lesions caused by brown spider venom are also discussed. Finally, the biotechnological application of some venom toxins are covered.

Keywords: Loxoscelism; Brown spider; Venom

Contents

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1. Introduction

Bites from the *Loxosceles* genus (brown spiders) cause several clinical manifestations, especially necrotic skin degeneration and gravitational spread, renal failure and haematological disturbances (see Section 8). These spiders have a world-wide distribution and accidents have been described in America, Europe, Asia, Africa and Oceania (see Section 3). Brown spider venom has a highly complex composition, containing many different toxins. Some of these have been well-described biochemically and biologically, providing insights into the mechanism by which the venom causes its deleterious effects (for details, see Section 5). Numerous histopathological findings and host cellular changes triggered by the venom have been described in recent years and we now have good information about the cellular inflammatory response evoked after envenomation, the extent and time-dependency of reactions after envenomation and the effect of venom on endothelial cells, keratinocytes and tissue structures, such as basement membranes, as well as blood cellular alterations (discussed in Section 4). Clinical features, diagnosis and therapy have also been extensively studied in recent years. In the light of recent investigations into the effects of brown spider venom, some authors have reporting the design of immunologically based laboratory tests for the diagnosis of brown spider bite (see Section 9). Although little progress has been made in establishing an efficient protocol for therapy of loxoscelism in the past 10 years (see Section 10), biotechnology has been widely applied to the study and use of brown spider venom and the first products based on venoms or toxins have been described. Monoclonal antibodies and recombinant toxins are now available which may greatly enhance our knowledge of loxoscelism and perhaps provide some direct pharmaceutical applications (see Section 11). This review focuses on the last 10 years of literature data on loxoscelism.

2. Biology of the brown spider

Spiders of the genus *Loxosceles* (see Fig. 1) range from 8 to 15 mm in body length and their legs measure 8–30 mm. Their colour varies from a pale brown (*L. laeta*) to a dark chocolate (*L. gaucho*). A characteristic structure of this genus is the cephalothorax which resembles a violin (Futrell, 1992). These spiders can live for 3–7 years (Andrade et al., 2000). *Loxosceles* spiders have six eyes arranged in pairs with one anterior dyad and another lateral dyad on each side; this disposition of the eyes has been described as the best means of identifying the brown spider (Vetter and Visscher, 1998). These spiders are sedentary and nocturnal (Andrade et al., 1999). They build irregular webs that look like cotton thread (Futrell, 1992). The silk ribbons of *L. laeta* are organised in two sheets, a lower and an upper. The former is in contact with the substratum and the latter forms an exit hole for the spider and it is attached to the underside of objects. The sheets are composed of a meshwork of silk ribbons, which are anchored to surfaces by attached plaques derived from the pyriform glands. The silk ribbons are spun from glands which are homologous to the major ampullate gland of orb web spiders (Knight and Vollrath, 2002). All the ribbons in the web have the same width, suggesting that the web may consist entirely of this single type of silk ribbon structure. This ribbon morphology is unique among spiders (Coddington et al., 2001). The web glands are composed of a separate transverse zone, a duct and a spigot. The duct is short and the spigot is a highly elongated slit. The spinning mechanism in *Loxosceles* may be more ancestral than that in orb web spiders (Knight and Vollrath, 2002). The composition of the web silks of mature female *L. arizonica* and *L. laeta* is very similar and rich in glycine and alanine (Coddington et al., 2001). These spiders can withstand temperatures ranging from 8 to 43 °C and can survive for several days or even months without food and water (Futrell, 1992). Sandidge (2003) observed that *L. reclusa* prefers dead scavenged prey to live prey.

Fig. 1. Brown spider (*Loxosceles intermedia*) (magnification 2×).

According to the author, this may explain how immense populations of these spiders flourish even in adverse conditions. The brown spider is not aggressive, is retiring and prefers to live in dark areas (Futrell, 1992; Málaque et al., 2002). *L. intermedia* females produce more venom than the males and this may be related to their greater length and weight (see Fig. 1). Female venom causes a more severe dermonecrotic reaction in rabbits (Oliveira et al., 1999). *Loxosceles* venom is used to paralyse insect prey and also as a defence mechanism. The total venom volume is minute (about 4 μl) and contains 65 to 100 μg of proteins (Sams et al., 2001a). A 35 kDa dermonecrotic toxin named F35 protein (Tambourgi et al., 1995) is a toxic component of *L. intermedia* venom and starts to appear in its fully active form in third instar spiderlings, being absent in extracts of eggs or first and second instar spiderlings (Andrade et al., 1999).

The venom glands of *L. intermedia* are composed of two layers of striated muscle fibres, one external and the other internal in contact with an underlying structure that separates muscular cells from the epithelial cells of the venom glands, which lie internally on the secretory epithelium, a simple glandular epithelium. The epithelial cells are arranged side by side and send out projections to the lumen of the glands, which is rich in secretory vesicles containing venom. The morphology and cellular appearance of the venom glands suggest a holocrine mechanism controlling venom secretion. The basal lamina that separates the secretory epithelial cells of the glands from the muscular cells contains glycoproteins, glycosaminoglycan sulfated residues, laminin and entactin (Santos et al., 2000).

The genital female tract of *L. intermedia* contains a pair of elongated, sac-like ovaries, located in the ventral face of the abdomen among the digestive tract and silk glands. The mature oocyte reaches the uterus by travelling along the ovarian and oviduct lumen, rather than detaching from the pedicle, and is then liberated into the hemocoel. In *L. intermedia*, it is suggested that, after the oocyte enters the ovarian lumen, the pedicle cells are engulfed by, and appear to get lost among, the many folds of the retracting proteic band and the thinner basement membrane. Fertilisation may occur during the transit of the oocyte inside the uterus while it is covered by the viteline membrane and the granules of the future chorion. These coats permit sperm contact and probably act as a species-specific barrier. Oogenesis appears to be a continuous process in the mature *L. intermedia* female, and no regionalization of the ovary is seen (Morishita et al., 2003).

3. Epidemiology of the brown spider

Loxoscelism is a term used to represent accidents involving spiders of the genus *Loxosceles* and has been reported in South America (Ministry of Health, Brazil, 1998; Sezerino et al., 1998; Schenone, 1998), North America (Futrell, 1992; Escalante-Galindo et al., 1999), Europe (Nicholson and Graudins, 2003), Africa (Futrell, 1992; Nicholson and Graudins, 2003), the Middle East and some parts of Asia (Nicholson and Graudins, 2003), Israel (Borkkan et al., 1995; Cohen et al., 1999) and Australia (Young and Pincus, 2001; Nicholson and Graudins, 2003). In the USA, the range of *L. reclusa* extends from southeastern Nebraska to southernmost Ohio and south into Georgia and most of Texas; the spider is rarely found outside this range (Vetter and Bush, 2002a). *L. deserta* and *L. arizonica* inhabit Arizona, Nevada, New Mexico, Texas, Utah and Southern California. *L. rufescens* is found along the Gulf of Mexico coast (Sams et al., 2001a). Only *L. laeta* has been verified in Canada, being found in Vancouver, British Columbia and Ontario (Vetter and Bush, 2002a).

In Brazil, seven species have been reported, most of them in the South and South-east regions (Sezerino et al., 1998), but three (*L. intermedia*, *L. gaucho* and *L. laeta*) have

been mostly implicated in human envenomation (Málaque et al., 2002). *L. similes* has only been described in the State of Mato Grosso do Sul (Andrade et al., 2001). The brown spider is the only spider implicated in necrotizing-haemolytic syndrome (Sezerino et al., 1998). In the present decade, an extensive outbreak of *Loxosceles* envenomation and a high rate of intra-domiciliary infestation have been reported in Brazil (Ribeiro et al., 1993). Loxoscelism, in Brazil, was responsible for 36% of the 17,781 spider bites notified to the Ministry of Health between 1990 and 1993 (Sezerino et al., 1998). Loxoscelism is particularly prominent in the metropolitan area of Curitiba, Parana State, Brazil, with about 2000 cases per year (Málaque et al., 2002). *L. intermedia* is the predominant brown spider in the urban environment of Parana and Santa Catarina (southern states of Brazil) (Fischer, 1994; Mattosinho et al., 1997; Andrade et al., 2000). Accidents involving *Loxosceles* genus spiders represent around 4% of the total number caused by venomous animals in Argentina (Roodt et al., 2002a). Although the fertility of *L. laeta* is greater than that of *L. intermedia*, as the former has a greater total number of eggs and number per egg sac, it has been suggested that the expansion of *L. intermedia* (described above) may be due not to a great reproductive rate, but to environmental alterations in the South region of Brazil (Andrade et al., 2000).

In a retrospective study (Sezerino et al., 1998) carried out in Florianopolis, Santa Catarina State, Brazil between January 1985 and December 1995, 487 suspected cases were found, 267 of which fulfilled the criteria for inclusion in the study; 66.7% of these occurred during the warmest months of October–March and 80.5% occurred in the coastal region of the state, and 21.7% of the patients were getting dressed, 17.2% were sleeping and 15.4% were performing other activities inside the house. The average age of the patients was 25.1 years. Málaque et al. (2002) studied 359 cases of loxoscelism between January 1985 and December 1996 at the Butatan Institute, Sao Paulo, Brazil. The results are very similar to those described in the retrospective study of Sezerino et al. (1998), the difference being that 51 (14%) patients brought the spider with them and 28 were classified as *L. gaucho*, 5 as *L. laeta* and 18 as non-classified *Loxosceles*. In a restrospective study (1955–1995) of 1384 patients of probable spider bite or insect sting, Schenone (1996) reported that the main incidence of loxoscelism occurred in the warmest month. In 17.7% of cases, the spider was identified as *L. laeta*. In Curitiba, between 1993 and 2001, the incidence of brown spider bites was 1.4 cases per 1000 habitants and 23% of the bites were in the thigh, 16.7% in the trunk, 14% in the arm and 13% in the lower leg. Most of the cases were mild, only 1% being severe (data obtained from the Health Secretary, Curitiba, Parana, Brazil, 2002). Good housekeeping and care during dressing and before sleeping, especially when using bed clothes, can be effective in preventing accidents. Although spiders are arachnids and not insects, insecticides are effective in reducing the brown spider population, but re-infestation is a continuous problem and the toxic side effects for humans have to be considered (Forks, 2000).

4. Histopathology and cellular changes after exposure to brown spider venom

Clinical data for, and biopsies of, human patients after brown spider bites show an inflammatory infiltrate, thrombosis, haemorrhage, dermatitis, acute inflammation, induration of the lesioned region, erythema and liquefactive necrosis of the epidermis and dermis consistent with pyoderma gangrenosum (Futrell, 1992; Yiannias and Winkelmann, 1992). The histopathological findings described in experimental animal models using venom from different *Loxosceles* sp support the above mentioned pathological data. Histopathological findings of the effect of *L. intermedia* venom on rabbits were described by Ospedal et al. (2002). These authors described intravascular fibrin network deposition and thrombosis of the dermal blood vessels, degeneration of the blood vessel wall and infiltration and aggregation of inflammatory cells. The skeletal muscle was markedly infiltrated by neutrophil leukocytes and muscular oedema was present, with myonecrosis of some myofibrils. Destruction of the epidermis, a massive haemorrhage and necrosis of surrounding collagen near the epidermis were also described. Studying mice injected with *L. reclusa* venom, Sunderkötter et al. (2001) described local haemorrhage after 6 h accompanied by blistering of the ear skin. Histopathology showed a vasculitis reaction after 2 h of exposure. Histopathological findings 14 days after envenomation of rabbits with brown recluse spider venom were described by Elston et al. (2000). The major finding in the eschar of the inoculated rabbits was a mixed inflammatory cell infiltrate, coagulative tissue necrosis and vasculitis. All animals demonstrated a well-delineated zone of eosinophilic staining as 'mummified' coagulative necrosis of the epidermis and dermis, and a dense band of neutrophils bordered the zone of necrosis. The histological analysis of some organs from mice after envenomation with various doses of *L. intermedia* venom revealed remarkable alterations confined to the kidney. Acute tubular necrosis was seen in several nephrons, accompanied by deposition of eosinophilic material inside the proximal and distal renal tubules (Tambourgui et al., 1998a). Renal biopsies from *L. intermedia* venom-treated mice showed hyalinisation and erythrocytes in the Bowman's space, glomerular collapse, tubular epithelial cell cytotoxicity and deposition of eosinophilic material within the tubular lumen. Ultrastructural studies showed glomerular epithelial and endothelial cell cytotoxicity, alterations of the basement membrane and tubular epithelial cell degeneration (Luciano et al., 2004).

L. intermedia venom has a noxious effect on the endothelium of vessels, as shown by vessel instability, cytoplasmic endothelial cell vacuolisation and blebs in

biopsies of the skin of rabbits (Veiga et al., 2001a; Zanetti et al., 2002). This endothelial cell cytotoxicity was proved using rabbit aorta endothelial cells in culture, as *L. intermedia* venom treatment led to loss of cell adhesion to the culture substrate and the shedding of proteoglycans from the extracellular matrix and cell surface into the medium (Veiga et al., 2001a). Experiments using human umbilical vein endothelial cells (HUVEC) treated in culture with *L. reclusa* venom showed a potent endothelial cell agonist activity of the venom, which induced endothelial cell expression of E-selectin and the release of interleukin-8 and granulocyte macrophage colony-stimulating factor, resulting in a dysregulated inflammatory response (Patel et al., 1994). *L. deserta* venom induces the expression of vascular endothelial growth factor (VEGF) in human keratinocytes (Desai et al., 2000), suggesting that keratinocyte-derived VEGF may contribute to the vasodilation, oedema and erythema seen in brown spider envenomation. Exposure of HUVECs to *L. deserta* venom induces the production of interleukin-8, growth-related oncogene α and monocyte chemoattractant protein-1 via an NF-kB-dependent pathway (Desai et al., 1999; Gomez et al., 1999a). Primary cultures of human keratinocytes exposed to 100 ng/ml of *L. gaucho* venom release TNF-α into the medium after 6 h (Málaque et al., 1999).

Brown spider venom can disrupt basement membrane structures. This was demonstrated by the activity of *L. intermedia* venom on the murine tumour EHS (Engelbreth-Holm-Swarm) basement membrane, which was degraded and fragmented (Veiga et al., 2000a). The venom seems to have no activity on purified type IV collagen and laminin, but displays hydrolytic activity for entactin and heparan sulphate proteoglycan, two important constituents of basement membranes (Veiga et al., 2000a, 2001a,b). Double staining immunofluorescence using antibodies against type IV collagen or laminin and antibodies against venom toxins and confocal microscopy demonstrated the deposition and binding of venom toxins along the tubular and glomerular basement membrane of the kidney of mice exposed to *L. intermedia* venom (Luciano et al., 2004); ultrastructural analysis further demonstrated the collapse and destruction of glomerular basement membrane structures.

Cellular alterations in the bone marrow and peripheral blood caused by *L. intermedia* venom in rabbits have been studied (Silva et al., 2003). There were changes in the number of nucleated red cells, which initially showed a significant decrease, then recovered to normal values 10 days after venom administration. Marrow depression of megakaryocytes correlated with the thrombocytopenia in the peripheral blood observed at the beginning of envenomation, and the platelet count and number of megakaryocytes returned to normal after 10 days. Neutropenia in the peripheral blood, low neutrophil counts in the bone marrow and low leukocyte counts were consequences of marrow depletion, which may reflect an extensive neutrophil influx to the tissues. No changes were observed in eosinophil numbers. Fig. 2 depicts the histopathological findings evoked in rabbits by *L. intermedia* venom.

5. Identification and characterisation of various toxins in brown spider venom

Several enzymes, including alkaline phosphatase, 5′ ribonucleotide phosphohydrolase and hyaluronidase, have been identified in *L. reclusa* venom, but none of these produce necrotic lesions in experimental animals (Futrell, 1992). A biochemically well characterised component of *L. reclusa* venom is sphingomyelinase D (32 kDa), which, in laboratory animals, can produce necrotic lesions, haemolysis of red blood cells and platelet aggregation, resulting in death (Futrell,1992). Sphingomyelinase D exists in four active forms, each of which hydrolyses sphingomyelin and releases choline and *N*-acylsphingosine phosphate (Futrell, 1992).

L. gaucho, *L. laeta* and *L. intermedia* venoms have very similar SDS gel electrophoretic profiles. The major *L. laeta* bands are located between 32 and 30 kDa, while those in *L. gaucho* and *L. intermedia* are between 35 and 33 kDa (Barbaro et al., 1994a; Mota and Barbaro, 1995). Citrate is found in the venom of *L. reclusa* at a concentration of 1.5% based on dried venom solid (Fenton et al., 1995). The toxins of the venoms of *L. gaucho*, *L. laeta* and *L. intermedia* can be separated into three major fractions (A, B and C) and the dermonecrotic and lethal activities are found only in fraction A (higher molecular mass component) (Barbaro et., 1996b). The protein in fraction A has a molecular mass of about 35 kDa in *L. gaucho* and *L. intermedia* and 32 kDa in *L. laeta*. The high percentage identity of the N-terminal sequences of dermonecrotic toxins from *L. gaucho*, *L. intermedia* and *L. laeta* strongly suggests that they are similar proteins (Barbaro et al., 1996a, 1997). A 30 kDa fraction was purified from the venom of *L. gaucho* and two isoforms further characterised. These were called Loxnecrogin A (31.4 kDa) and Loxnecrogin B (31.6 kDa), both of which induce necrosis in rabbits, but the lesions are smaller than those produced by the total 30 kDa fraction (Cunha et al., 2003), showing that more than one protein is involved in the dermonecrotic mechanism. Loxnecrogin A shows a high similarity to recombinant LiD1 protein from *L. intermedia* (Kalapothakis et al., 2002; Cunha et al., 2003). Phospholipase D from *Loxosceles* venom resembles phospholipase D from *Corynebacterium*, both being basic proteins with similar isoelectric values and molecular masses (Bernheimer, 1996). Tambourgi et al. (1995) purified a fraction from *L. intermedia* venom named F35. This fraction has a molecular mass of 35 kDa on SDS-PAGE, but seems to be composed of three isoforms, which were named P1, P2 and P3. P1 and P2 have sphingomyelinase activity and can induce in vivo the effects seen with whole spider venom, including complement-dependent haemolysis and dermonecrosis, while P3 is completely inactive

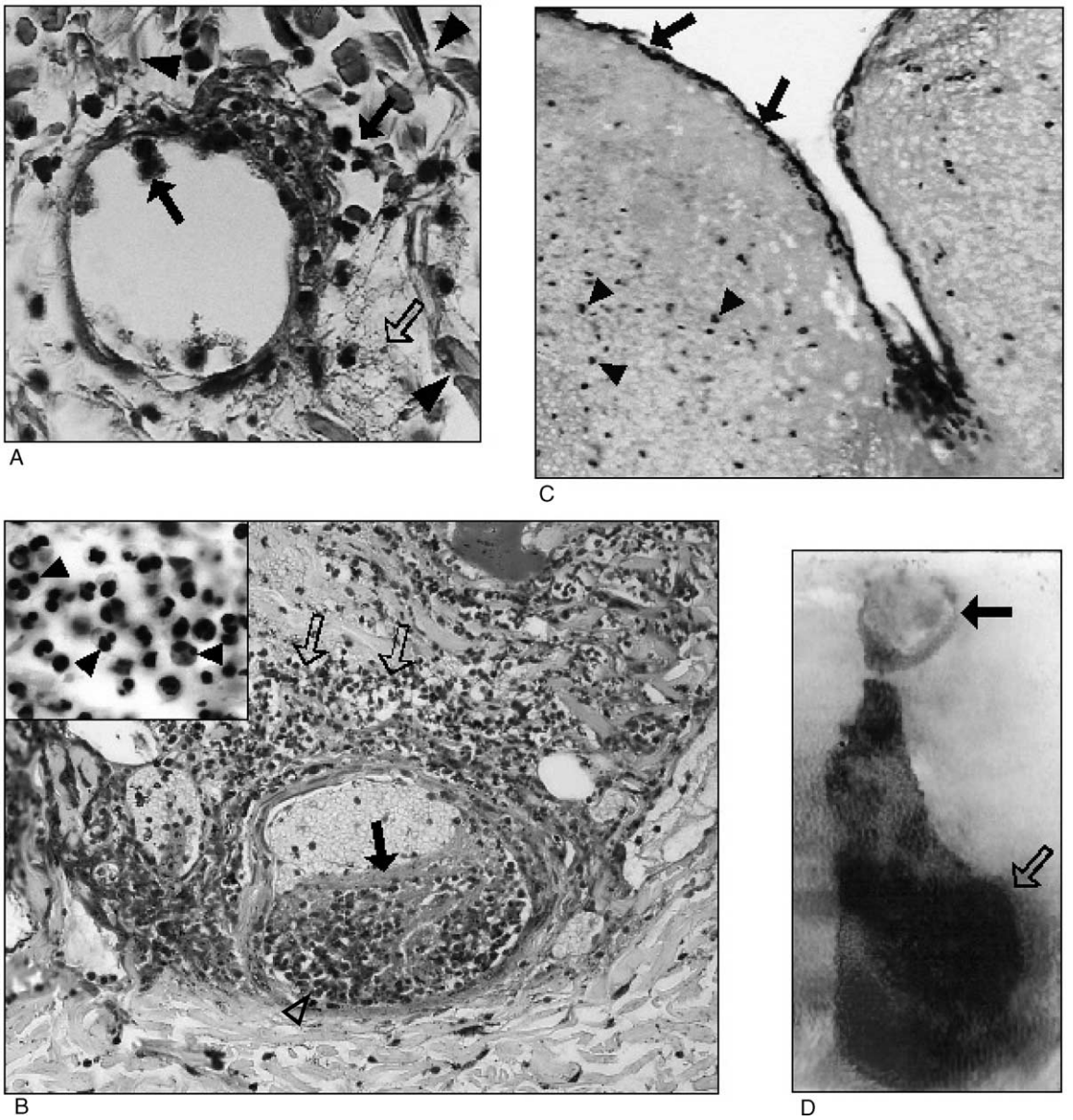

Fig. 2. Histopathological changes in the skin of rabbits after brown spider venom exposure. All panels show results from a rabbit injected with 40 μg of crude brown spider venom protein. (A) Blood vessel and connective tissue of the skin of a rabbit 30 min after exposure to brown spider venom. The black arrows indicate leukocytes interacting with endothelial cells of the blood vessel or infiltrating connective tissue. The white arrow indicates a proteinaceous network of fibrin in the dermis and the black arrowheads show profuse disorganisation of collagen fibres (magnification 1000×). (B) Details of a blood vessel of the skin of a rabbit 8 h after exposure to the venom showing inflammatory leukocytes accumulated in (white arrowhead) and around (white arrows) the vessel. The black arrow indicates intravascular clotting (magnification 200×). The inset shows details of the cellular inflammatory infiltrate represented by neutrophils (magnification 1000×). (C) Microscopic examination of the skin of a rabbit 2 days after brown spider envenomation. The black arrows indicate epidermal necrosis and the black arrowhead diffuse points of dermal necrosis (magnification 200×). (D) Macroscopic visualisation of dermonecrosis in the skin of a rabbit at 24 h after injection with venom. The black arrow indicates the site of venom injection and the white arrow gravitational spreading (hallmark of envenomation).

(Tambourgi et al., 1998b). A rapid assay for monitoring the lipolytic activity of brown recluse spider venom and toxin has been developed and the optimal reaction conditions determined (Merchant et al., 1998). These authors studied the venom- and toxin-mediated hydrolysis of yolk sphingomyelin in Triton X-100 or lysophosphotidylcholine micelles using phosphorous-31 nuclear magnetic resonance spectroscopy and found that an increased overall reaction rate

was observed not only with increased temperature, but also with decreased ionic strength. The presence of divalent calcium ions was found to be necessary for hydrolytic activity, but only in catalytic amounts (less than 1 mM).

Two metalloproteinases named Loxolysin A (20–28 kDa) and Loxolysin B (32–35 kDa) have been described in *L. intermedia* venom. The former has fibronectinolytic and fibrinogenolytic activity and the latter is gelatinolytic (Feitosa et al., 1998). The action of *L. intermedia* venom on fibrinogen shows a partial fibrinogenolytic activity on intact fibrinogen and a complete cleaving effect on the denatured molecule, supporting the idea of a conformation-dependent susceptibility of native fibrinogen. In the presence of 12.5 μg of venom, hydrolysis of the A α chains is first seen between 30 min and 1 h after venom exposure, with progressive proteolysis with time, whereas the B β chain begins to be cleaved after 4 h of venom treatment. This fibrinogenolytic activity is blocked by EDTA and 1,10-phenanthroline, suggesting that a divalent metal ion is critical for enzyme activity (Feitosa et al., 1998; Zanetti et al., 2002). This fibrinogenolytic effect and metalloprotease-dependent activity is seen in venom from *L. intermedia*, *L. laeta*, and *L. gaucho,* and a 30 kDa molecule with fibrinogenolytic activity has been purified from *L. intermedia* venom (Zanetti et al., 2002). The proteolytic activity of *L. intermedia* venom obtained directly from the venom glands was compared with that of venom obtained by electroshock (Silveira et al., 2002). A protein profile study of the two preparations showed that, in both cases, the venom is a mixture of proteins with a very similar electrophoretic profile, especially in the region of low molecular mass proteins (20–45 kDa), and that both showed the same fibronectinolytic and fibrinogenolytic activities. *L. intermedia* and *L. laeta* gland venom extracts have a similar level of metalloprotease-dependent proteolytic activity (gelatinolytic) (Silveira et al., 2002). *L. intermedia* venom shows basic aminopeptidase and prolyl-dipeptidyl aminopeptidase IV activities, analysed by fluorometric assay using naphthylamide substrate (Gasparello-Clemente and Silveira, 2002). Two 85 and 95 kDa serine proteases identified in *L. intermedia* venom have gelatinolytic activity in the pH range of 7.0–8.0, and act on casein to a lesser extent than on gelatin, but do not hydrolyse haemoglobin, immunoglobulin G, bovine serum albumin, laminin or fibrinogen (Veiga et al., 2000b). These proteases may be involved in the deleterious activities of the venom.

The oligosaccharide profile of *L. intermedia* venom shows asparagine-linked high mannose, complex type structures and N-linked fucosylated molecules. One serine/threonine-linked *N*-acetyl-galactosylated protein was detected. The venom contains no glycosaminoglycan, proteoglycan, galactose or sialic acid residues as complex structures (Veiga et al., 1999). Table 1 summarises the molecules described in the venom of *Loxosceles* sp. spiders.

Table 1
Molecules described in the venom of *Loxosceles sp.* spiders

Molecule	*Loxosceles* sp	Molecular mass	Activity	References
Alkaline phosphatase	*L. reclusa*	Not described	Not described	Futrell (1992)
Hyaluronidase	*L. rufescens*, *L. reclusa*	32.5 kDa	Digests hyaluronic acid	Futrell (1992) and Young and Pincus (2001)
5′ Ribonucleotide phosphohydrolase	*L. reclusa*	Not described	Not described	Futrell (1992)
Sphingomyelinase D	*L. reclusa*	30–32–35 kDa	Necrotic lesion	Futrell (1992) and Barbaro et al. (1994a, 1996a,b, 1997)
	L. rufescens		Platelet aggregation	
	L. gaucho, *L. laeta*		Haemolysis	Mota and Barbaro (1995)
	L. intermedia			Tambourgi et al. (1995)
Loxnecrogin A	*L. gaucho*	31.4 kDa	Necrosis in rabbits	Cunha et al. (2003)
Loxnecrogin B	*L. gaucho*	31.6 kDa	Necrosis in rabbits	Cunha et al. (2003)
LiD1 recombinant protein	*L. intermedia*	31.4 kDa	Sphingomyelinase D family without dermonecrotic activity, but with antigenic activity	Kalapothakis et al. (2002) and Cunha et al. (2003)
Loxolysin A	*L. intermedia*	20–28 kDa	Fibronectinolytic, fibrinogenolytic	Feitosa et al. (1998)
Loxolysin B	*L. intermedia*	32–35 kDa	Gelatinolytic	Feitosa et al. (1998)
Serine protease	*L. intermedia*	85 kDa	Gelatinolytic	Veiga et al. (2000b)
Proteases	*L. intermedia*	Not described	Hydrolysis of entactin, heparan sulphate proteoglycan and basement membrane	Veiga et al. (2000b, 2001a,b)
Metalloproteases	*L. rufescens*	Broad range	Caseinolytic, gelatinolytic, fibrinogenolytic	Young and Pincus (2001)

6. Mechanisms of local and systemic lesions caused by brown spider venom

The mechanisms by which brown spider venom causes local and systemic lesions are under investigation, but are currently unknown. In addition, there are no rational explanations why mice and rats do not develop dermonecrosis, while humans, guinea pigs and rabbits do. *L. deserta* venom causes a large necrotic lesion in swine and the size of the lesion is dose-dependent (Hobbs and Yealy, 1994). Some noxious activities of the venom can be attributed to proteolytic toxins, which degrade fibrinogen, fibronectin, entactin and heparan sulphate proteoglycan and disrupt basement membrane structures, resulting in local haemorrhage and gravitational spreading of the cutaneous lesions and systemic pathogenesis involving disseminated intravascular coagulation and renal failure (Feitosa et al., 1998; Veiga et al., 1999, 2000b, 2001a,b; Luciano et al., 2004). There is considerable evidence for the role of neutrophils in the dermonecrosis caused by the venom of *Loxosceles* spiders (for details, see Smith and Micks, 1970; Futrell, 1992) and this was proved by histopathological findings in animal models (Elston et al., 2000; Ospedal et al., 2002) and biopsies of human patients (Yiannias and Winkelmann, 1992). Neutrophil participation and the inflammatory response seem to be dependent on an endothelial cell agonist effect triggered by the venom which leads to an indirect and dysregulated neutrophil activation involved in dermonecrosis (Patel et al., 1994). The venoms from *L. gaucho*, *L. laeta* and *L. intermedia* have similar dermonecrotic activity (Barbaro et al., 1996a); in mice, the most lethal is that from *L. intermedia* and the least toxic that from *L. laeta*. The venoms of these three species have very low levels of caseinolytic and phospholipase A_2 activities even when large amounts are used, and have low myotoxicity (mouse model) and no direct haemolytic activity. *L. gaucho* venom causes increased degradation of red blood cell ghost protein 3 in patients with either the cutaneous or viscerocutaneous forms (Barretto et al., 2003); this is not inhibited by *N*-ethylmaleimide (a cysteine protease inhibitor) or phenylmethylsulphonyl fluoride (a serine protease inhibitor), but is inhibited by ethylenediaminetetraacetic acid (a metalloprotease inhibitor).

Bravo et al. (1993) concluded that *L. laeta* venom has a direct haemolytic action on red blood cells which is calcium- and complement-dependent, but antibody-independent. Tambourgi et al. (2000) studied the mechanism of induction of complement-dependent haemolysis by *L. intermedia* venom and proposed that the sphingomyelinase activity of the toxins induces the activation of an endogenous metalloproteinase, which then cleaves glycophorins, making the erythrocytes susceptible to lysis by human complement. They also compared the phospholipase activities of *Corynebacterium pseudotuberculosis* and *L. intermedia* venom and found that they have similar phospholipase D activity and that the purified phospholipase D induced similar clinical symptoms to those induced by envenomation (Tambourgui et al., 2002). Using the ECV304 cell line, van den Berg et al. (2002) showed that *L. intermedia* venom toxins induce cleavage of the complement regulator membrane co-factor protein (MCP/CD46) and the major histocompatibility complex I molecule from the cell surface by activation of a metalloproteinase of the adamalysin family and that this reduced MCP expression, resulting in an increased resistance to complement-mediated lysis.

Monteiro et al. (2002) isolated and identified many different bacteria, including *Clostridium perfringens*, from the fangs and venom of *L. intermedia* and compared the dermonecrotic lesions produced by venom alone and venom plus *C. perfringens*. The results showed that the combination resulted in a striking increase in the size of the dermonecrotic lesion, suggesting a role for *C. perfringens* in the severe dermonecrotic picture.

7. Immunogenicity of Loxosceles spider venom

Loxosceles venom is a mixture of different proteins. *L. intermedia* venom is enriched in proteins with low molecular masses in the range of 5–40 kDa and, to a lesser extent, proteins of high molecular mass (60–850 kDa), (Veiga et al., 2000b). The venom of *L. gaucho* is enriched in low molecular mass proteins (Barbaro et al., 1992). Because of its proteic nature, *Loxosceles* venom can induce antibody production. A characterisation of the antigenic cross-reactivity of *L. reclusa* and *L. deserta* venoms suggested that the venoms contain similar proteins, which show greater than 90% amino acid sequence identity and marked antigenic cross-reactivity (Gomez et al., 2001a). Antibodies in antisera raised against *L. gaucho* toxins in rabbits and horses also bound to *L. laeta* and *L. intermedia* toxins (Barbaro et al., 1996a), suggesting some antigenic conservation in these three venoms; the electrophoretic profile and immunoblotting of the proteins from these three venoms showed that almost all venom proteins, including the main 33–35 kDa component of *L. gaucho* venom responsible for the dermonecrotic and lethal activities, were recognised by the antisera. When the venom of each species of *Loxosceles* was incubated with these antisera, the dermonecrotic and lethal activities of each venom was completely neutralised (Barbaro et al., 1994a, 1996a).

Monoclonal antibodies produced against the 35 kDa dermonecrotic component of *L. gaucho* neutralised the dermonecrotic and lethal activities of the whole venom. The results of this study showed low cross-reactivity with heterologous toxins and that the antibodies failed to neutralise the toxic activities of *L. laeta* and *L. intermedia* venom, suggesting that different epitopes are present in the main proteins responsible for the toxic activity of *Loxosceles* venom (Guilherme et al., 2001). *L. gaucho* venom has an immunological adjuvant effect in rabbits which is

associated with high molecular mass components, which trigger inflammatory activity (Barbaro et al., 1994b; Mota and Barbaro, 1995).

The horse anti-arachnid antiserum from the Butantan Institute (Sao Paulo, Brazil), which is raised against a mixture of venoms from *Loxosceles gaucho*, *Phoneutria nigriventer*, *Tityus serrulatus* and *T. bahiensis*, did not inhibit human red blood cell haemolysis by *L. laeta* venom in vitro, and inhibition of the development of a skin necrotic lesion in rabbits was time-dependent (Bravo et al., 1994). On the other hand, Fab fragments of polyclonal antibodies raised in rabbits using *L. deserta* venom have been used therapeutically and injection within 4 h after envenomation inhibits dermal inflammation and necrosis in experimental animal models (Gomez et al., 1999b). A specific horse anti-*L. intermedia* antivenom was produced and compared with the horse polyvalent anti-arachnid antiserum from the Butantan Institute in order to evaluate their ability to block the development of the dermonecrotic lesion and the lethal activities of the venom; both kinds of antivenom were efficient in neutralising these actions, but the anti-*Loxosceles* antivenom was more efficient (Braz et al., 1999). In Argentina, a monovalent horse anti-*L. laeta* antiserum raised against spider venom gland homogenates was developed and its ability to neutralise both the toxic effect in mice and dermonecrotic activity in rabbits was at least as high as that of the anti-arachnid antivenom from the Butantan Institute (Brazil) or the anti-*Loxosceles* serum from the National Institute of Health (Peru) (Roodt et al., 2002b).

8. Clinical features of brown spider bites

Brown spider bites give rise to a classical well known picture of necrotic skin lesions with gravitational spreading that is the hallmark of envenomation. Futrell (1992) gave a clinical description of the bite of spiders of the genus *Loxosceles*, stating that the initial bite is relatively painless and the patient is often unaware that he has been bitten. Pain, varying from mild to severe, begins at 2–8 h. There may be small puncta and transient erythema with itching, swelling and mild to severe tenderness. This is followed by the appearance of a bleb or blister (12–24 h) surrounded by a halo of ischemic tissue and the bleb may become haemorrhagic. Over the following few days, the necrotised lesions become a fixed dull blue-violet colour, with a characteristic gravitational spread and, consequently, the blue area increases. An eschar may form at this time, roughly between 3 and 7 days. Over the following week, the area becomes indurated and the central area hardens. The eschar may drop off, leaving an ulcer that heals after varying lengths of time (6–8 weeks), but which sometimes requires skin grafting. Similar descriptions were made by Schenone (1996), Sezerino et al. (1998) and Málaque et al. (2002). Systemic involvement is much less common than cutaneous involvement and occurs in a minority of cases. It is generally mild and self-limited, although, in some cases, it may also be the cause of death, usually associated with renal failure, disseminated intravascular coagulation and intravascular haemolysis (Futrell, 1992; Sezerino et al., 1998). Brown spider venom manifestations vary from mild erythema to extensive necrosis and are influenced by the victim's health, degree of obesity, location of the bite and other factors (White et al., 1995; Barbaro et al., 1996a; Sams and King, 1999; Sams et al., 2001b; Vetter and Bush, 2002a). While erythema, pain and macule at the bite site are the most common local symptoms of a *L. reclusa* spider bite, fever is the most common systemic symptom. Bites on the extremities are more common and more severe (Clowers, 1996). The extension of the dermal inflammation seen in the necrotic arachnidism induced by *L. reclusa* venom is proportional to the amount of venom in the bite site. *Loxosceles* venom induces a inflammatory mechanism which is indirect, so venom diffusion defines the extent and magnitude of the dermonecrotic lesion (Gomez et al., 2001b). Cutaneous loxoscelism with an oedematous predominance occurs in about 4% of loxoscelism cases involving *L. laeta* (Schenone, 1998). Severe systemic loxoscelism occurs in far fewer than 1% of cases of focal necrosis of the skin induced by *L. reclusa* venom (Anderson, 1998). Brown spider bites can have nephrotoxic effects. The clinical and laboratory features observed in victims can include haemoglobinuria, haematuria and proteinuria (Futrell, 1992; Williams et al., 1995; Lung and Mallory, 2000). The pathological effect of the venom on the kidney may reflect haematological disturbances, such as intravascular haemolysis and disseminated intravascular coagulation, which are systemic manifestations of envenomation (Futrell, 1992; Williams et al., 1995; Lung and Mallory, 2000). Nevertheless, in mice exposed to *L. intermedia* venom, Luciano et al. (2004) were able to show direct binding of venom toxins to renal structures, suggesting that *Loxosceles* venom toxins act as direct and potentially nephrotoxic agents. Because of their biochemical and physicochemical properties, such as a positive charge and low molecular mass size, venom toxins can act as 'planted antigens' along the kidney structures, evoking nephrotoxicity.

A retrospective study was carried out on 111 patients in the Vanderbilt University Hospital (Nashville, Tennesee, USA) between May 1993 and October 1995 (Wright et al., 1997). The inclusion criteria for this study were the presence of a lesion consistent with brown spider bite and the identification of the spider or the patient reporting seeing a spider. Eighty-one percent of wounds involving the leg showed central discoloration at the bite site and 37% showed necrosis. Skin grafting in patients with necrotic lesion was rarely required. Sixteen of the patients (14%) complained of feeling ill and six (5%) were admitted to the hospital. Most patients did not have severe systemic involvement. Haemolytic anaemia was seen in one patient

and haemolysis and coagulopathy in another (Wright et al., 1997). In an observational retrospective study of 11 paediatric patients (Escalante-Galindo et al., 1999), there was a relationship between lesion severity and the time delay before the beginning of treatment. As reported by Cacy and Mold (1999), in the period between April 1996 and August 1998 in the Oklahoma Physicians Research Network (Oklahoma, USA), loxoscelism (*L. reclusa*) was more common in women (18–65 years), pain was a common sign and most of the bites were in the extremities. Erythema was always present, 40% of the patients developed necrosis, and some systemic signs and symptoms, such as nausea (7%), rash (5%), fever and fatigue (3%), were also described. A retrospective study was carried out in Israel between 1988 and 1997 on 11 patients hospitalised after *Loxosceles* spider bites (Cohen et al., 1999). All patients presented dermonecrotic lesions with haemorrhagic bulla and necrotic ulcers, six showed systemic involvement with an elevated body temperature, diarrhoea (only one patient), vomiting, hypotension, and a diffuse petechial rash. Three patients presented inguinal lymphadenitis. Sams et al. (2001b) carried out a retrospective study between 1987 and 1993 on 19 documented cases diagnosed as brown recluse spider envenomation. The extremities were most often affected and the main symptoms were pain and localised erythema at the bite site. Eleven patients (58%) had skin necrosis and six had areas of necrosis larger than 1 cm^2. The time to healing ranged from 5 days to more than 17 weeks.

In order to investigate intravascular haemolysis, Morena et al. (1994) performed a haptoglobin assay on 19 patients with the cutaneous form of loxoscelism and found no decrease in haptoglobin, demonstrating that these patients did not show haemolysis. Haemolytic anaemia was described in a 12-year-old female after a *L. reclusa* bite (Murray and Seger, 1994). The patient presented fever, headache, nausea and vomiting, and maculopapular rash and erythema were seen at the bite site. Haemoglobin levels were decreased and the haematocrit was 24%, indicating haemolysis as a systemic reaction to the venom. A 38-year-old man was bitten on his left upper extremity by a *L. reclusa* and presented a thrombocytopenia (17,000 platelets/μl that reverted in 4 days (110,000/μl) (Young, 1994). Two cases of *L. reclusa* loxoscelism were described by Williams et al. (1995); one was associated with extensive tissue necrosis, severe intravascular haemolysis and fulminant disseminated intravascular coagulation and the other with severe haemolysis and a positive direct antiglobulin test for IgG and complement. Leung and Davis (1995) described another case of systemic envenomation with haemolysis following a *L. reclusa* spider bite. Goto et al. (1996) described a 7-year-old boy with progressive cervical soft tissue swelling, necrosis and airway compromise after a spider bite in the neck; the spider was in his bed and was identified as *L. reclusa*. Bey et al. (1997) described a 13-year-old girl who developed shock following *L. arizonica* envenomation; the spider was identified by an entomologist as a female *L. arizonica*. The patient developed a cutaneous lesion and, after 15 h, the clinical signs were hypotension, tachycardia and delayed capillary refill. Yosef et al. (1998) described a 28-year-old man with a mutation in factor V (Factor V Leiden) and in the gene coding for methyl tetrahydrofolate reductase, both predisposing the patient to thrombogenic events, who was bitten by a brown spider and presented an extensive necrotic lesion, suggesting that these genetic mutations may be predisposing factors for severe necrosis induced by loxoscelism, but more research is required to confirm this. Lung and Mallory (2000) described a 7-year-old boy who presented systemic symptoms of viscerouscutaneous loxoscelism with no evidence of haemolysis, but with haematuria in the first 24 h, and who showed a high anti-streptolysin-O title and decreased C3 levels. Jarvis et al. (2000) described an 8-year-old girl who was bitten in the left eye by *L. reclusa*, resulting in periorbital erythema and oedema. The treatment included canthotomy and cantholysis and administration of methylprednisolone, dexamethasone, dapsone, erythromycin ointment, cefazolin and hyperbaric oxygen (HBO). A 17-year-old woman and a 4-year-old boy were admitted to the Hospital Vital Brazil and Hospital das Clinicas (Sao Paulo, Brazil) after *Loxosceles* bites (França et al., 2002). Both presented viscerocutaneous loxoscelism with severe oedema, erythema and dermonecrosis at the bite site, rhabdomyolisis and acute renal failure with elevated creatine kinase levels. A 3-week-old infant developed a sudden onset jaundice and unexplained haemolysis in the absence of the classically described necrotic cutaneous lesion probably caused by an *L. reclusa* bite (Hostetler et al., 2003), illustrating the importance of considering loxoscelism in the differential diagnosis of massive haemolysis, particularly in endemic areas.

9. Diagnosis of brown spider bites

The diagnosis of loxoscelism is rarely based on the identification of the spider, as the patients do not bring the spider, but on epidemiological and historical findings or clinical signs and symptoms (Wright et al., 1997; Vetter, 1999; Málaque et al., 2002) and the exclusion of other aetiologies (Sezerino et al., 1998). The diagnosis of recluse spider bites purely on the basis of clinical examination is always difficult and speculative. The diagnosis should be performed taking into account the natural geographic distribution and epidemiology of brown recluse spiders in North America. Brown recluse spider bites have been misdiagnosed because several of these medical reports have originated from regions of non-endemicity (Vetter, 1999; 2000; Nishioka, 2001; Vetter and Barger, 2002; Vetter and Bush, 2002a,b; Vetter et al., 2003). *Erythema migrans* is a lesion characteristic of Lyme disease (localised infection) and is very similar to the lesion of necrotic arachnidism,

and may mimic the systemic symptoms of loxoscelism. Osterhoudt et al. (2002) reported that a 9-year-old boy with Lyme disease was misdiagnosed as having been bitten by a brown recluse spider. Lyme disease may be considered as a differential diagnosis principally in regions in which *Ixodes* spp ticks are highly prevalent. Vetter and Bush (2002c) described a case of chemical burn misdiagnosed as a brown recluse spider bite. The patient was from Nevada City, California, where there are no populations of brown recluse spiders.

Nevertheless, based on basic research and histopathological findings from animal models and from clinical data, which present a typical profile of inflammatory cells (Futrell, 1992; Yiannias and Winkelmann, 1992; Ospedal et al., 2002), in case of doubt, physicians requiring a confirmatory diagnosis can use biopsies from lesioned skin. Barrett et al. (1993) developed a passive haemagglutination inhibition test specifically diagnosing *L. reclusa* envenomation using a collection of exudates taken from skin lesions of guinea pigs. According to the authors, the test sensitivity was 90% as late as 3 days after venom injection and the specificity was 100% compared to other spider species. An ELISA sandwich test for the detection of *Loxosceles* sp venom proteins has been developed, which, in addition to identifying mice injected with *L. intermedia*, *L. gaucho* or *L. laeta* venom, can also detect circulating antigens in the sera of patients envenomed by *L. intermedia* (Chávez-Olórtegui et al., 1998). Miller et al. (2000) reported a novel *Loxosceles*-specific competitive enzyme immunosorbent assay using a rabbit anti-*Loxosceles* venom polyclonal antiserum and used it to examine a dermal biopsy and hairs plucked from a skin lesion of a patient bitten by *L. deserta*. Using this technique, the authors reported that the venom was detected in the analysed samples for up 4 days after envenomation. A similar ELISA method has been used for the detection of *Loxosceles* species venom. The authors postulated that it may have clinical applications in the detection of *Loxosceles* venom in hair, aspirate and biopsy specimens (Gomez et al., 2002; Krywko and Gomez, 2002). Although these laboratory tests have potential for clinical application, they require more clinical correlation and statistical analysis and unfortunately are not commercially available.

10. Therapy of brown spider bites

A variety of treatments have been described for loxoscelism. Phentolamine, heparin and other substances, such as topical nitro-glycerine, cyproheptadine and HBO, have been used for therapy, but research on these therapies is inconclusive and their use is not recommended (Futrell, 1992; Wendell, 2003). The established therapy is dapsone, acetylsalicylic acid, antibiotics (erythromycin and cephalosporins), ice and elevation, avoidance of strenuous activity and heat and, when necessary, surgery. Early surgical excision has not been shown to be of benefit and, in most cases, delays healing (Futrell, 1992; Merigian and Blaho, 1996; Goddard, 1998). In Curitiba, Brazil, in which there were 3400 cases of brown spider bites between 1995 and 2001, prednisone, dapsone and antiserum therapy are used. Anti-*Loxosceles* antiserum is only administered in severe cases of viscerocutaneous loxoscelism. When the spider is identified as *L. intermedia* or *L. laeta*, prednisone treatment must be initiated (even in cases with no lesion), the patient must be monitored during the first 36 h and serum therapy may be used if the patient develops the severe form of envenomation. For severe cutaneous loxoscelism, dapsone in association with antiserum is indicated (data obtained from the Health Secretary, Curitiba, Parana, Brazil, 2002), but there is little evidence to support the effectiveness of anti-*Loxosceles* antivenom, especially against local effects. Systemic envenomation studies in animals and humans have demonstrated that the antivenom neutralises the deleterious effects of the venom. In countries in which the antivenom was introduced, paediatric mortality has been reduced (Isbister et al., 2003). The effectiveness of *Loxosceles* antivenom in the treatment of necrotic arachnidism resulting from the bite of recluse spiders is less clear, mainly due to the late presentation of victims (Nicholson and Graudins, 2003). The effectiveness seems to be time-dependent. Experimentally, anti-*Loxosceles* venom antibodies used within 4 h reduce *Loxosceles*-induced dermonecrotic lesions in rabbits (Gomez et al., 1999). Barrett et al. (1994) concluded that dapsone therapy is more effective than either electric shock or no therapy for *L. reclusa* envenomation in guinea pigs. An eyelid dermonecrotic lesion model was developed in rabbits using *L. reclusa* venom, the severity of the lesion being venom dose-dependent (Cole et al., 1995). When the rabbits were divided into three groups, which were treated with steroids, dapsone or antivenom, the best response was seen with dapsone or antivenom, with a decrease in erythema and necrosis. When animals were given combined treatment of steroids plus dapsone, steroids plus antivenom and dapsone plus antivenom, the dapsone plus antivenom group showed the best response, with a marked reduction in erythema and necrosis. Dapsone therapy is recommended by the Kentucky Regional Poison Center (KRPC, Louisville, Kentucky, USA) for all suspected cases of brown recluse spider envenomation. A retrospective study was conducted at the KRPC in 1994 and 38 patients with proven brown recluse spider envenomation were identified (Roos et al., 1995). In the 31 patients treated with dapsone, no haemolytic anaemia or methaemoglobinemia was reported and no complications of dapsone were identified. According to the authors, dapsone administration may decrease the need for surgical wound excision. Escalante-Galindo et al. (1999) reported that, in 11 paediatric patients, treatment with

dapsone and/or paracetamol and dicloxacilin was successful; one patient required surgical treatment, but no systemic loxoscelism occurred. Dapsone can cause a dose-dependent haemolytic anaemia. Rapid or severe haemolysis is not seen with dapsone unless the patient is deficient in glucose-6-phosphate dehydrogenase (G6PD), when a rapid and severe haemolysis is seen during the first week of therapy (Murray and Seger, 1994). Colchicine is another putative medicine currently being studied because of the potential for haemolysis with dapsone, especially in patients with G6PD deficiency (Elston, 2002), but there are no clinical data supporting its use in loxoscelism and more studies are required.

If the signs of the viscerouscutaneous form do not appear within 24 h, cutaneous loxoscelism may be the diagnosis and may have a good prognosis. Patients with symptoms of viscerocutaneous loxoscelism have a good prognosis if they survive for 48 h and are treated with antihistamines and corticosteroids for cutaneous loxoscelism and with corticosteroids for viscerocutaneous loxoscelism (Schenone, 2003). HBO therapy is controversial for loxoscelism, some studies reporting it to be effective, while others do not. Phillips et al. (1995) concluded that HBO did not decrease the severity of rabbit skin lesions resulting from *L. deserta* envenomation, and Hobbs et al. (1996) and Hobbs (1997) reached the same conclusion using swine and *L. reclusa* envenomation. Merchant et al. (1997) examined the effect of high pressure oxygen on the catalytic action of *L. reclusa* venom on sphingomyelin and concluded that the venom components or toxins responsible for sphingomyelinase D activity were not affected by HBO therapy under the test conditions. Beilman et al. (1994) used guinea pigs exposed to *L. deserta* venom and concluded that HBO resulted in decreased lesion sizes when compared to control and dapsone-pretreated animals. Similar results were described when the venom of *L. reclusa* was injected into rabbits and the skin lesions treated with HBO within 48 h; HBO therapy reduced skin necrosis and the wound was significantly smaller (Maynor et al., 1997). A 19-year-old man who was bitten on the glans penis by a brown spider brought the spider with him to the William Beaumont Army Medical Center, Houston (Broughton, 1996). The patient received intravenous diphenhydramine, methylprednisolone, calcium gluconate and famotidine, and oral dapsone treatment was begun immediately. After 24 h, HBO treatment was given twice daily for 5 days. Skin necrosis was avoided and no surgical intervention was required.

Topical nitro-glycerine is reported to prevent skin necrosis from brown recluse spider bites. Lowry et al. (2001) tested this hypothesis and concluded that it did not prevent skin necrosis and could increase inflammation and serum creatine phosphokinase levels. The results of their study did not support the use of topical nitro-glycerine in the treatment of *L. reclusa* envenomation and suggested that systemic toxicity could be increased.

11. Biotechnology of brown spider venom toxins.

McGlasson et al. (1993) have described a product named ARACHnase (Hemostasis Diagnostics International Co., Denver, CO, USA). This is normal plasma containing *L. reclusa* venom which mimics the presence of a lupus anticoagulant and may provide a positive control for lupus anticoagulant testing. The Butantan Institute, Sao Paulo, Brazil, produces anti-arachnid antiserum using a mixture of venoms from *L. gaucho*, *P. nigriventer*, *T. serrulatus*, and *T. bahiensis,* which also reacts with *L. intermedia*, and *L. laeta* toxins. The CPPI (Production Center of Immunobiologic Products, Parana, Brazil) has also produced an anti-*Loxosceles* antiserum using *L. intermedia* venom which is able to neutralise some noxious activities of *Loxosceles* venom. The National Institute of Health (Peru) produces anti-*Loxosceles* antiserum against *L. laeta* venom. All of these antisera have been used as bioproducts for serum therapy (Barbaro et al., 1994a, 1996a; Roodt et al., 2002; Health Secretary, Curitiba, Parana, Brazil, 2002). Guilherme et al. (2001) produced monoclonal antibodies against the 35 kDa dermonecrotic toxin of *L. gaucho* which neutralise the dermonecrotic effect and lethal activities of *L. gaucho*, but not those of a heterologous venom. M.H. Appel and O.M. Chaim (personnel communication), working at the Federal University of Parana, have prepared more than 30 hybridomas for the production of monoclonal antibodies to different *L. intermedia* toxins. Monoclonal antibodies against brown spider venom toxins are not only useful tools for clinical applications, but can also be used as reagents for the affinity purification or affinity screening of venom toxins, which may greatly enhance our knowledge of loxoscelism over the next few years. Molecular biological approaches have also been used to study and analyse brown spider venom toxins. Using recombinant venom proteins, scientists can generate enough material for research, diagnosis and treatment. The study of toxins is particularly difficult, as the amounts of venom collected are often limited. In this respect, the cloning and expression of recombinant toxins should be a very useful tool for better structural and functional studies on the mechanisms of loxoscelism. Recent studies reporting the cloning of venom toxins also offer potential biotechnological tools. Pedrosa et al. (2002) reported the cloning and expression of a functional dermonecrotic and complement-dependent haemolytic factor obtained from a cDNA library of *L. laeta* spider venom glands. By sequencing of aleatory clones obtained from the cDNA library, they identified at least three containing different inserts coding for proteins with a high similarity to known N-terminal sequences of *L. intermedia* sphingomyelinases.The entire sequences of the cDNAs for these three clones have been obtained and deposited at GenBank under Accession numbers AY 093599, AY093600 and AY093601. The longest cDNA cloned was used for expression of a mature *L. laeta* protein in a bacterial system. The recombinant toxin was expressed

as a fusion protein of approximately 32 kDa with a 6× His-tag at the N-terminus. The recombinant *L. laeta* protein exhibits similar biological properties to whole venom or purified sphingomyelinases, but has a decreased ability to bind to the erythrocyte surface. Kalapothakis et al. (2002) reported the cloning and expression of what they called LiD1, a protein present in the venom of *L. intermedia*. LiD1 was cloned from an expression cDNA library of *L. intermedia* spider venom glands and the partial cDNA sequence is available at GenBank under Accession no. AY340702. The deduced amino acid sequence reveals a mature protein belonging to a family of proteins with dermonecrotic activity. The aim of the study was to generate a fused nontoxic recombinant protein to be used for the production of an *L. intermedia*-specific antiserum. LiD1 was expressed as a β-galactosidase fusion protein, which was recognised by anti-dermonecrotic protein antibodies and was able to generate antibodies against native dermonecrotic proteins present in *L. intermedia* venom. This LiD1 recombinant protein was used in a study (Araujo et al., 2003) in which it was used to pre-immunise rabbits and mice and was found to neutralise the in vivo lethal and dermonecrotic effects of *L. intermedia* whole venom. The authors raised the possibility of using non-toxic recombinant proteins for the production of alternative neutralising antibodies and for the development of a vaccine. GenBank also contains three different sequences of exons of sphingomyelinase D obtained from genomic DNA of *L. arizonica* that are available under Accession Nos. AF512954, AF512955 and AF512956. The complete sequence of the cDNA for sphingomyelinase D from *L. arizonica* is also available under Accession No. AF512953.

12. Future prospects

Venom toxins can be used to investigate molecular and cellular mechanisms, as models for the design of new drugs and directly for therapeutic or diagnosis use. The development of recombinant *Loxosceles* toxins in the near future should provide insights into the understanding and putative applications of venom-derived molecules for use in pharmacology, medicine and other biological sciences.

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Review:

Insight sinto brown spider and loxoscelism

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Review

Insights into brown spider and loxoscelism

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Abstract

Loxosceles is a genus of cosmopolitan spiders comprising several species, and popularly known as brown spiders or brown recluses. Brown spider bites can cause dermonecrotic lesions and systemic reactions known as loxoscelism. Systemic effects are less common but may be severe or even fatal in some patients. Systemic manifestations include intravascular hemolysis, disseminated intravascular coagulation and acute renal failure. A rapid diagnosis and an understanding of the venom's molecular activity are crucial for satisfactory treatment. Mechanisms by which venoms exert their deleterious effects are under investigation, and searches are underway for diagnostic envenomation assays. Molecular biology is being used to produce quantities of several of the most important venom molecules and has contributed to the study and understanding of their mechanisms of action.

Key words: brown spider; loxoscelism; venom; recombinant toxins; dermonecrosis

Introduction

More than 40,000 spider species exist, with probably 100,000 to be described, but only 3 taxa are recognized as dangerous, namely Theridiidae, Loxoscelidae and Ctenidae. Moreover, only the genera *Atrax*, *Lactrodectus* and *Loxosceles* are associated with human deaths (Escoubas *et al.*, 2000; Rash and Hodgson, 2002). Early European tales during the Middle Ages linked injuries or illness to spider bites (Schienle *et al.*, 2005). For example the tarantula bite was associated with a disease (tarantism) for which the cure was a frenetic dancing for 3-4 days. This energetic dance, called tarantella, is now a typical Italian dance (Isbister, 2004). Today, as a consequence of mistaken diagnoses of spider bites, scientists are looking for methods to characterize and identify spider bites and their manifestations as well as to better understand the biological and molecular mechanisms of envenomation.

The genus *Loxosceles* (variously known as the brown spider, brown recluse, fiddleback, or gaucho spiders) is important in these studies because of its commonness in and around human dwellings. Their bite is characterized by dermonecrosis and systemic effects known as loxoscelism (Hogan *et al.*, 2004).

The first case of documented loxoscelism occurred in 1879 in Tennessee. However, consistent data traced back about 50 years ago and were collected in Chile, then other observations were made in Brazil followed by the United States. These reports linked brown spider bite with necrotic skin lesions (Macchiavello, 1947; Atkin *et al.*, 1958; Sams *et al.*, 2001). Spiders' habits have caused a close association with humans, and the number of bites is increasing and has become a public health problem in Brazil, Chile and the United States (da Silva *et al.*, 2004). Most bites occur during sleep or dressing, and women are bitten more often than men. Thighs, trunk, hands and arms are more often bitten (Hogan *et al.*, 2004).

Loxosceles spiders

Loxosceles spiders are known as violin (fiddleback) spiders due to a characteristic violin

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shape on their cephalothorax (Futrell, 1992). They are also known as brown spiders because their colour varies from a pale (*L. laeta*) to a dark brown (*L. gaucho*). *Loxosceles* body length ranges from 8 to 15 mm with legs measuring from 8 to 30 mm (da Silva *et al.*, 2004). They are sedentary and nocturnal (Andrade et al., 1999) with a lifetime of 3 – 7 years (Andrade *et al.*, 2000). Brown spiders have three pairs of eyes (an important characteristic useful to identify the genus) (Vetter and Visscher, 1998). They build irregular, cottony webs (Futrell, 1992) and normally prefer dead scavenged prey rather than live preys (Sandidge, 2003). They can survive months without food or water and withstand temperatures ranging from 8 °C to 43 °C. They are not aggressive and prefer dark dry places (Futrell, 1992; Málaque *et al.*, 2002; Vetter and Barger, 2002; da Silva *et al.*, 2004). The sexes produce venom with differences in volume, toxicity and compounds proportion (Oliveira *et. al.*, 1999). Comparative analysis of sex and species in *L. laeta* and *L. intermedia* venom showed some biological activities (complement-dependent hemolysis and dermonecrosis) more prominent in venom from female spiders, especially from *L. laeta* (Oliveira *et al.*, 2005).

Epidemiology

Loxosceles spiders can be found distributed all over the world. In North America, the most important species are *L. reclusa*, *L. deserta*, *L arizona*, *L. rufences* (United States and Mexico) and *L. laeta* (Canada) (Sams *et al.*, 2001; Vetter and Bush, 2002a). Europe, Africa, Middle East, some parts of Asia, Israel, and Australia are hosts to some *Loxosceles* species (Futrell, 1992; Borkkan *et al.*, 1995; Young and Pincus, 2001; Nicholson and Graudins, 2003).

In Brazil, seven species have been described but three are the most implicated in human bites *L. intermedia, L. gaucho and L. laeta* (Sezerino *et al.*, 1998). From 1990 to 1993, the Brazilian Ministry of Health received 17.781 reports of spiders' bites, of which 36 % were due to *Loxosceles* (Sezerino *et al.*, 1998). In the metropolitan area of Curitiba, in the state of Parana (southern Brazil) about 3.000 brown spider bites are reported annually (Málaque *et al.*, 2002). In a retrospective study in Florianopolis, in the state of Santa Catarina, Brazil, 487 suspected cases of brown spider bites were found, 267 of which fulfilled the criteria for inclusion in the study (Sezerino *et al.*, 1998). In 359 cases of loxoscelism between January 1985 and December 1996 at Butantan Intitute, São Paulo, Brazil, 14 % of patients captured the spiders so that 28 were classified as *L. gaucho*, 5 as *L. laeta* and 18 as non-classified *Loxosceles* (Málaque *et al.*, 2002). More bites occur in warmer months (Schenone, 1996). In Curitiba, from 1998 to 2001 the incidence of *Loxosceles* bites was 1.4 cases per 1,000 habitants. Of these, 23 % were in the thigh, 16.7 % in the trunk, 14 % in the arm and 13 % in the lower leg. Only 1 % of cases were severe (Health Secretary, Curitiba, Parana, Brazil, 2002).

Pathophysiology of Loxoscelism

Dermonecrosis is the hallmark of loxoscelism (Fig. 1). Histopathology and clinical data are obtained from biopsies of human patients after brown spider bites. Rabbit skin artificially injected with *Loxosceles* venom is used for more controlled investigation since this animal model reproduces human skin lesions that follow envenomation (Ospedal *et al.*, 2002). Systemic effects, such as renal failure, are less common and are usually reproduced in mouse (Luciano *et al.*, 2004). Observation of human skin biopsies showed an inflammatory infiltrate, thrombosis, hemorrhage, dermatitis, erythema, induration of affected area and liquefactive necrosis of the epidermis and dermis consistent with pyoderma grangrenosum (Futrell, 1992; Yannias and Winkelmann, 1992). Symptoms in an experimental study in rabbits showed that after 4 h oedema, hemorrhage, 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 leucocytes) in the dermis, and subcutaneous muscular oedema all occur. Over the following hours and up to 5 days after envenomation, the changes progressed to a massive neutrophil infiltration into the dermis and even into subcutaneous muscle tissue, destruction of blood vessels, thrombosis, hemorrhage, myonecrosis, and coagulative necrosis on the 5th day (Ospedal *et al.*, 2002). Neutrophil participation and the inflammatory response seem to be dependent on an endothelial cell agonist effect triggered by the venom that leads to an indirect and dysregulated neutrophil activation involved in dermonecrosis (Patel, 1994). Envenomation of rabbit skin with *L. reclusa* venom after 14 days results in a mixed inflammatory cell infiltrate, coagulative tissue necrosis, vasculitis and a dense band of neutrophils bordering the zone of necrosis (Elston *et al.*, 2000). *L. intermedia* venom damaged vessel endothelia, as shown by vessel instability, endothelium cell vacuolization in biopsies of rabbit skin (Veiga *et al.*, 2001a; Zanetti *et.al.*, 2002). *In vitro* experiments on rabbit aorta endothelium cell cultures showed cytotoxicity of *L. intermedia* venom associated with loss of cell adhesion to the culture substrate and the shedding of proteoglycans from the extracellular matrix and cell surface into the medium (Veiga *et al.*, 2001a). In human umbilical vein endothelial cell (HUVEC) cultures treated with *L. reclusa* venom, agonist activity ensued, inducing endothelial cell expression of E-selectin and the release of interleukin (IL)-8 and granulocyte macrophage colony-stimulating factor, resulting in dysregulated inflammatory response (Patel *et al.*, 1994). HUVEC exposed to *L. deserta* venom produced IL-8, growth-related oncogene-α and monocyte chemoattractant protein-1 via an NF-κB- dependent pathway (Desai *et al.*, 1999; Gomez *et al.*, 1999). *L. deserta* venom induces the expression of vascular endothelial growth factor (VEGF) in human keratinocytes, suggesting that keratinocyte-derived VEGF may contribute to vasodilatation, oedema and erythema in brown spider envenomation (Desai *et al.*, 2000). Primary cultures of keratinocytes exposed to 100 ng/ml of *L. gaucho* venom release tumour necrosis factor (TNF)-α into the medium after 6 h (Málaque *et al.*, 1999).

Mice injected with *L. reclusa* venom developed local hemorrhage after 6 h accompanied by blistering of the ear skin (Sunderkötter *et al.*, 2001).

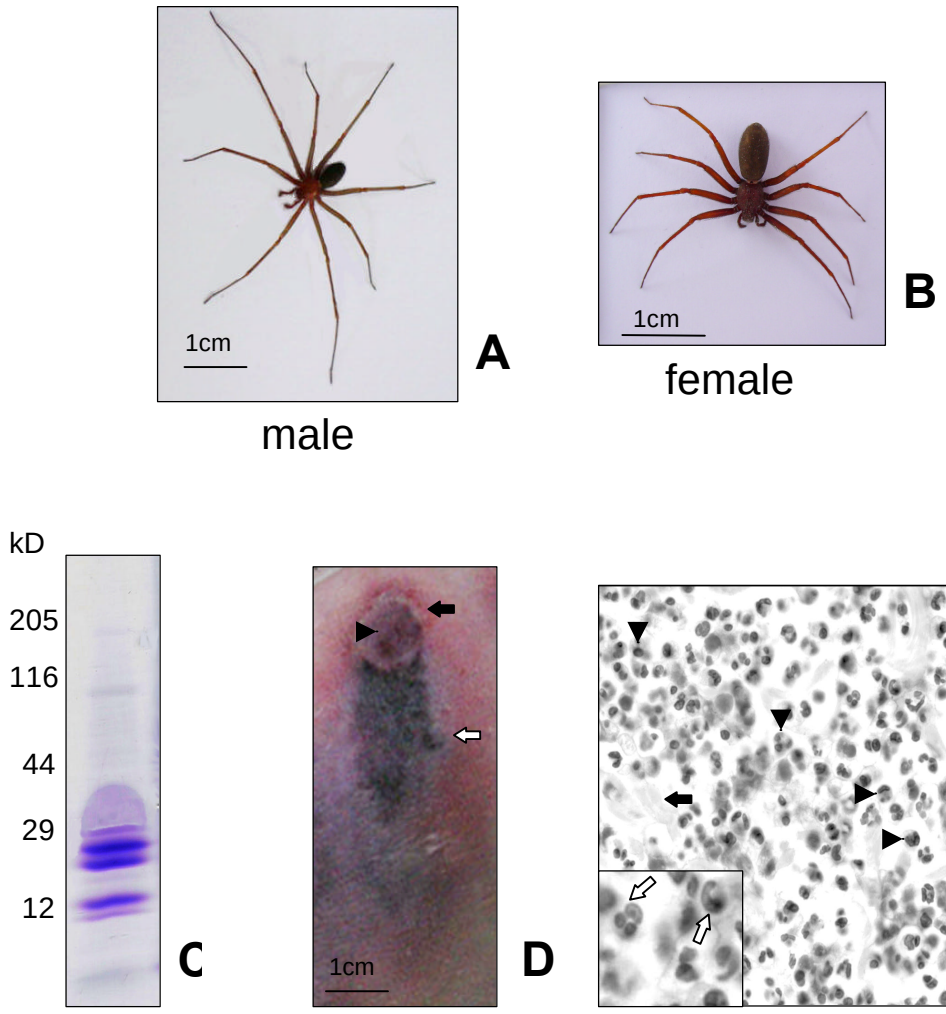

Fig. 1 Cellular and molecular aspects of brown spider and loxoscelism. (A) *Loxosceles intermedia* (brown spider) male. (B) *L. intermedia* (brown spider) female. (C) SDS-PAGE 3-20 % venom profile stained by Coomassie blue dye. (D) Dermonecrotic lesion on rabbit skin after 24 h post-*L. intermedia* venom (10 µg) exposure. Arrowhead indicates the site of venom injection with characteristic black and white eschar named marble plate. Black arrow points an erythema surrounding the lesion and white arrow shows the gravitational spreading of lesion (a hallmark of dermonecrotic loxoscelism). (E) Microscopical view of dermonecrotic lesion showing inflammatory leukocytes accumulated in the connective tissue (arrowhead) and disorganization of collagen fiber and oedema (black arrow) (magnification 400X). The inset shows inflammatory cells of the infiltrate represented by neutrophils (white arrow) (magnification 1.000X).

Histopathology showed a vasculitis reaction 2 h after exposure. The microscopical analysis of some mouse organs injected with different doses of *L. intermedia* venom revealed remarkable kidney alterations. Acute tubular necrosis accompanied by deposition of eosinophilic material inside the proximal and distal renal tubules was seen in several nephrons (Tambourgi *et.al.*, 1998). Mouse kidneys, treated with *L. intermedia* venom showed hyalinisation and erythrocytes in the Bowman's space, glomerular collapse, tubular epithelial cell cytotoxicity and deposition of eosinophilic material within the tubular lumen (Luciano *et al.*, 2004). Confocal microscopy observations of double staining immunofluorescence against type IV collagen or laminin and *L. intermedia* venom showed that toxins deposit and bind along the tubular and glomerular basement membrane of mice kidneys. Ultrastructural observations showed glomerular epithelial and endothelial cell cytotoxicity, the collapse and destruction of glomerular basement membrane and tubular epithelial cell degeneration. The basement membrane is a target for brown spider venom, as shown administrating *L. intermedia* venom to murine tumor Engelbreth-Holm-Swarm (EHS), which is rich in basement membrane molecules. *L. intermedia* venom degraded and fragmented the basement membrane (Veiga *et.al.*, 2000a). Venom

displays hydrolytic activity for entactin and heparan sulphate proteoglycan, two important constituents of basement membranes, while having no apparent activity on purified type IV collagen and laminin (Veiga *et al.*, 2000a, 2001a,b).

In the bone marrow and peripheral blood cells, *L. intermedia* initially causes a decrease in the number of nucleated red cells, bone-marrow depression of megakariocytes with thrombocytopenia in peripheral blood and decrease of platelet count (da Silva *et al.*, 2003). Neutropenia in peripheral blood and low neutrophil counts were observed as consequence of bone-marrow depletion, which may reflect an extensive neutrophil influx to the tissues. Eosinophils are apparently unaffected.

Brown spider venom toxins

L. intermedia and *L. laeta* have different protein patterns of glycosylation and the same is between sexes of the same species (Oliveira *et al.*, 2005). Hemolytic and dermonecrotic activities have been described for *L. similes* venom. Sphingomyelinase D molecules, with molecular mass ranging from 30 to 35 kDa and having hemolytic, necrotic and platelet aggregation activity were found in *L. reclusa*, *L. rufescens*, *L. gaucho*, *L. laeta* and *L. intermedia* venoms (Futrell 1992; Barbaro *et al.*, 1994; Mota and Barbaro, 1995; Tambourgi *et al.*, 1995; Barbaro *et al.*, 1996a,b, 1997). Three sphingomyelinase D isoforms were purified from *L. boneti* venom (Lb1, Lb2 and Lb3). Only Lb1 and Lb2 had dermonecrotic activity (Ramos-Cerrillo *et al.*, 2004). An alkaline phosphatase was described in *L. reclusa* venom (Futrell, 1992). Hyaluronidase (32.5 kDa) was found in *L. refescens* and *L. reclusa* (Futrell, 1992; Young and Pincus, 2001). *L. deserta*, *L. gaucho*, *L. intermedia*, *L. laeta* and *L. reclusa* venoms contained an enzyme of similar molecular size (44 kDa), which digested hyaluronic acid (Barbaro *et al.*, 2005). A 5'-ribonucleotide phosphohydrolase was found in *L. reclusa* venom (Futrell 1992). Loxnecrogin A (31.4 kDa) and Loxnecrogin B (31.6 kDa) with necrotic activity on rabbit skin were found in *L. gaucho* venom (Cunha *et al.*, 2003). *L. intermedia* has a range of proteases described in its venom: Loxolysin A (20-28 kDa) with fibronectinolytic and fibrinogenolytic activity; Loxolysin B (32-35 kDa) with gelatinolytic activity (Feitosa *et al.*, 1998); a serin protease (85 kDa) with gelatinolytic activity (Veiga *et.al.*, 2000b) and proteases able to hydrolyse entactin, heparan sulphate proteoglican and basement membrane (Veiga *et al.*, 2000b, 2001a,b). *L. rufescens* also has a broad molecular range of caseinolytic, gelatinolytic and fibrogenolytic metalloproteases (Young and Pincus, 2001). To test whether proteases in *L. intermedia* venom were due to natural constitution and not a digest fluid contamination, da Silveira *et al.*, (2002) compared the proteolytic activity of the venom obtained directly from venom glands with that obtained by electroshock. Both protein profiles showed very similar electrophoretic and enzymatic characteristics.

At present, a new generation of molecules developed through cloning techniques is under study. *L. intermedia* LiD1 recombinant protein (31.4 kDa) is a sphingomyelinase D family molecule without dermonecrotic activity but with antigenic activity (Kalapothakis *et al.*, 2002). *L. laeta* recombinant protein (33 kDa) is a sphingomyelinase isoform able to degrade sphingomyelin (Pedrosa *et al.*, 2002). This recombinant protein induced complement susceptibility, release of glycophorins and had dermonecrotic activity. *L. intermedia* recombinant protein (LiRecDT, 34 kDa) has dermonecrotic activity and was able to directly induce nephrotoxicity in mice (Chaim *et al.*, 2005). *L. laeta* recombinant phospholipase D generated lysophosphatidic acid and was hemolytic (Lee and Lynch, 2005).

Clinical features, diagnosis and treatment of brown spider bites

Diagnosis of loxoscelism is rarely based on spider identification and therefore clinical features, epidemiological and historical findings must be well known (Wright *et al.*, 1997; Vetter, 1999; Málaque *et al.*, 2002). Lesion recovery improves once the patient is treated. However, brown recluse bites have been misdiagnosed in North America because they occurred in regions of non-endemicity (Vetter, 1999; Nishioka, 2001; Vetter and Barger, 2002; Vetter and Bush, 2002a,b; Vetter *et al.*, 2003). A typical necrotic skin lesion begins soon after the spider bites the victim, followed by gravitational spreading (da Silva *et al.*, 2004). The bite is painless, hence the patient is often unaware that he has been bitten (Futrell, 1992), and the delay between the bite and when the victim pursues help makes the treatment less effective. From mild to severe pain begins 2-8 h after the bite. At the bite a small puncture wound may appear, associated with transient erythema with itching and swelling and mild to severe tenderness (Futrell, 1992; da Silva *et al.*, 2004). Blebs or blisters appear (12-24 h), may become hemorrhagic, and surrounded by a halo of ischemic tissue. In the following days, necrotized lesions become a dull blue-violet, the area of the gravitational spread turns blue, and the size of the blue area increases. Within three to seven days an eschar may form, after which the lesion hardens. The eschar may drop off leaving an ulcer that may require a skin graft (Schenone, 1996; Sezerino *et al.*, 1998; Málaque *et al.*, 2002; da Silva *et al.*, 2004).

Success of therapy depends upon a correct and rapid diagnosis, the volume of the venom injected, and the patient susceptibility to the venom (Futrell, 1992; da Silva *et al.*, 2004). Phentolamine, heparin, topical nitro-glycerine, cyproheptadine and hyperbaric oxygenation have been used for therapy, but the efficacy of these therapies is inconclusive and their use is not recommended (Futrell, 1992; Wendell, 2003; da Silva *et al.*, 2004). The established therapy is dapsone, acetylsalicylic acid (aspirin), antibiotics (erythromycin and cephalosporin), ice and elevation, avoidance of strenuous activity and heat and, when necessary, surgery. Early surgical excision has not been shown to be effective and often delays healing (Futrell, 1992; Merigian and Blaho, 1996; Goddard, 1998, Monteiro *et al.*, 2002; da Silva *et al.*, 2004). Serum anti-*Loxosceles* venom is used only in severe cases and effectiveness is doubtful especially against local manifestation. Systemic envenomation studies in

animals and humans have demonstrated that antivenom neutralizes the deleterious effects of the venom and reduces paediatric mortality (Isbister *et al.*, 2003). Effectiveness of antivenom to prevent dermonecrotic lesions seems to be time dependent and usually patient looks for medical help 4 h after the bite when lesions is already established (Ospedal *et al.*, 2002; Nicholson and Graudins, 2003). Some local and systemic noxious activities of the venom are attributed to proteolytic toxins that degrade fibrinogen, fibronectin, entactin and heparan sulphate proteoglycan and disrupt basement membrane structures, thereby causing local hemorrhage, gravitational spreading, disseminated intravascular coagulation and renal failure (Feitosa, *et al.*, 1998; Veiga *et al.*, 1999, 2000b, 2001a,b; Luciano *et al.*, 2004; Chaim *et al.*, 2005).

Biotechnological products from brown spider venom

Recently developed technologies are being used to produce biotechnological products from *Loxosceles* venom. ARACHnase (Hemostasis Diagnosis International Co., Denver, CO, USA), normal plasma containing *L. reclusa* venom, mimics a lupus anticoagulant and may provide a positive control for anticoagulant testing (McGlasson *et al.*, 1993). An antiserum against venoms of *L. gaucho*, *Phoneutria nigriventer*, *Tityus serrulatus*, and *Tityus bahiensis* that reacts with *L. intermedia* and *L. laeta* toxins is produced by The Butantan Institute, São Paulo, Brazil. The CCPI (Production Center of Immunobiologic Products, Parana, Brazil) has also produced antiserum using *L. intermedia* venom that is able to neutralize some activities of *Loxosceles* venom. *L. laeta* antiserum is produced by the National Institute of Health (Peru) (da Silva *et al.,* 2004). These antisera have all been used as bioproducts for serum therapy (Roodt *et al.,* 2002; Barbaro *et al.*, 1994; 1996a; Health Secretary, Curitiba, Parana, Brazil). Guilherme *et al.* (2001) produced monoclonal antibodies recognising *L. gaucho* venom toxins, which were able to neutralize the dermonecrotic effect and lethal activities of this species venom but not those of heterologous venoms.

Monoclonal and polyclonal antibodies are not only powerful tools for neutralizing the effects of venom; they are also useful for research. They can be used to purify toxins from venom by affinity chromatography. They can be used on location of specific toxins on cell and tissue treated with venom toxins. Immunofluorescence techniques such as confocal microscopy and flow cytometry are modern techniques based on antibody specific binding.

In contrast to the collection of snake venom, spiders provide very little venom, which limits the ability to study spider venom toxins. Protein cloning techniques are helping to solve this problem. After cloning it is possible to have milligrams of the same protein thereby improving the quality of research work and allowing more controlled experimental studies. Today several spider venom recombinant proteins are under investigation most of which are in the sphingomyelinase protein family (Kalapothakis *et al.,* 2002; Pedrosa *et al.,* 2002; Chaim *et al.,* 2005).

Future perspectives

Toxins from *Loxosceles* spiders are a group of proteins with a great range of different activities. Each toxin may be used to investigate molecular and cellular effects of venom. Also each of these proteins is a putative molecular model for drug design and to develop knowledge on some effects not yet fully understood such as the inflammatory reaction of dermonecrosis and platelet aggregation.

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Molecular cloning and functional characterization of two isoforms of dermonecrotic toxin from *Loxosceles intermedia* (Brown spider) venom gland

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GenBank data deposition information for *Loxosceles intermedia* cloned cDNAs are: LiRecDT1, DQ218155; LiRecDT2, DQ266399 and LiRecDT3, DQ267927

Abstract

Brown spider (Genus *Loxosceles*) bites are normally associated with necrotic skin degeneration, gravitational spreading, massive inflammatory response at injured region, platelet aggregation causing thrombocytopenia and renal disturbances. Brown spider venom has a complex composition containing many different toxins, of which a well-studied component is the dermonecrotic toxin. This toxin alone may produce necrotic lesions, inflammatory response and platelet aggregation. Biochemically, dermonecrotic toxin belongs to a family of toxins with 30–35 kDa characterized as sphingomyelinase-D. Here, employing a cDNA library of *Loxosceles intermedia* venom gland, we cloned and expressed two recombinant isoforms of the dermonecrotic toxin LiRecDT2 (1062 bp cDNA) and LiRecDT3 (1007 bp cDNA) that encode for signal peptides and complete mature proteins. Phylogenetic tree analysis revealed a structural relationship for these toxins compared to other members of family. Recombinant molecules were expressed as N-terminal His-tag fusion proteins in *Escherichia coli* and were purified to homogeneity from cell lysates by Ni^{2+} chelating chromatography, resulting in proteins of 33.8 kDa for LiRecDT2 and 34.0 kDa for LiRecDT3. Additional evidence for related toxins containing sequence/epitopes identity comes from antigenic cross-reactivity using antibodies against crude venom toxins and antibodies raised with a purified dermonecrotic toxin. Recombinant toxins showed differential functionality in rabbits: LiRecDT2 caused a macroscopic lesion with gravitational spreading upon intradermal injection, while LiRecDT3 evoked transient swelling and erythema upon injection site. Light microscopic analysis of skin biopsies revealed edema, a collection of inflammatory cells in and around blood vessels and a proteinaceous network at the dermis. Moreover, differential functionality for recombinant toxins was also demonstrated by a high sphingomyelinase activity for LiRecDT2 and low activity for LiRecDT3 as well as greater in vitro platelet aggregation and blood vessel permeability induced by LiRecDT2 and residual activity for LiRecDT3. Cloning and expression of two recombinant dermonecrotic toxins demonstrate an intraspecific family of homologous toxins that act in synergism for deleterious activities of the venom and open possibilities for biotechnological applications for recombinant toxins as research tools for understanding the inflammatory response, vascular integrity and platelet aggregation modulators.

Keywords: Brown spider; Venom; Dermonecrotic toxin

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1. Introduction

Brown spider (*Loxosceles* genus) commonly causes several clinical problems, especially necrotic lesions, gravitational spreading, hematological disturbances and renal failure [1,2]. Brown spiders are found worldwide and spider bites have been reported from all continents [1–5].

Crude venom has a complex composition, with a mixture of different proteic toxins. Some of these have been well described biologically and biochemically and the mechanisms by which *Loxosceles* spider venom causes its deleterious effects are currently under investigation. Sphingomyelinase-D, metalloproteases, serine proteases and hyaluronidases are among the enzymes that have been identified in *Loxosceles* venom [1,2,6–9]. Dermonecrotic toxin (sphingomyelinase-D) is the best-known molecule found in different Brown spider venoms. Previous studies identified sphingomyelinase-D as contributing to the major toxic effect of spider bites, including red blood cell lysis [10], platelet aggregation [11] and the typical dermonecrotic lesions of brown spider bites [1,2]. Sphingomyelinase-D toxin has also been found in the venoms of *L. gaucho* [12], *L. intermedia* [13], *L. rufescens* [14] and *L. laeta* [15].

Biochemical analysis of native purified sphingomyelinase-D toxin from *L. reclusa* shows this molecule to have four, immunologically cross-reactive isoforms that hydrolyze sphingomyelin and release choline and *N*-acylsphingosine phosphate [1]. Three native isoforms of sphingomyelinase-D have been found in *L. intermedia* [13]. Two isoforms of dermonecrotic toxins, Loxnecrogin A (31.4 kDa) and Loxnecrogin B (31.6 kDa), were found in *L. gaucho* [16]. Thus, the sphingomyelinase-D family in Brown spider is recognized by its very similar SDS gel electrophoretic profiles and high percentage identity of the N-terminal sequences of toxins from *L. gaucho*, *L. intermedia* and *L. laeta* [2,15]. At least 11 isoforms for these toxins in *L. gaucho* were found by proteomic approaches to the study of Brown spider venom [17] and the identity of sphingomyelinase-D toxins in *Loxosceles* venom was finally demonstrated by cloning and molecular analysis. Several genomic studies have found dermonecrotic toxins in this way. Cisar et al. [18] previously screened from a genomic library of *L. reclusa*, a venom toxin related to dermonecrotic (sphingomyelinase) molecule. Kalapothakis et al. [19] cloned and expressed a recombinant toxin called LiD1 from *L. intermedia* venom gland, which shows a high similarity to Loxnecrogin A from *L. gaucho*. Pedrosa et al. [20] cloned and expressed a functional dermonecrotic toxin obtained from a cDNA library of *L. laeta* spider venom gland. This recombinant toxin has high similarity to N-terminal sequence of *L. intermedia* sphingomyelinase-D. Binford et al. [21] reported three cDNA sequences from venom-expressed mRNAs from *L. arizonica*. Results pointed similarities with sphyngomyelinases from other *Loxosceles* species. Chaim et al. [22] cloned and expressed a functional dermonecrotic toxin from *L. intermedia* venom gland that was able to reproduce dermonecrosis, inflammatory response and renal disturbances.

Here we describe the cloning, expression, purification and functional evaluation of two new isoforms of the *L. intermedia* dermonecrotic toxin.

2. Methods

2.1. Reagents

Polyclonal antibodies to *L. intermedia* crude venom toxins and dermonecrotic toxin (LiRecDT1) were produced in rabbits following [23,24]. Hyperimmune IgGs were purified from serum using a mixture of Protein-A and Protein-G Sepharose beads (Amersham Biosciences, Piscataway, USA) as recommended by the manufacturer. Crude venom from *L. intermedia* was extracted from wild-caught spiders following Feitosa et al. [6]. Evans Blue dye from Vetec (São Paulo, Brazil) and Formamide from Merck (Darmstadt, Germany) were used.

2.2. cDNA library construction

The venom gland cDNA library was constructed following Chaim et al. [22]. Briefly, venom gland mRNAs from two hundred adult *L. intermedia* spiders were purified using the FastTrack 2.0 mRNA Isolation Kit (Invitrogen, Carlsbad, USA). The cDNAs were then synthesized using the SuperScript Plasmid System with Gateway Technology for cDNA Synthesis and Cloning (Invitrogen), cloned to *Not* I/*Sal* I pre-cut pSPORT1 vector and transformed into *Escherichia coli* DH5α cells. Transformants were selected on LB (Luria–Bertani) agar plates containing 100 μg/ml ampicillin.

2.3. cDNA library screening

Randomly chosen colonies (approximately 50 clones) were inoculated in LB broth containing 100 μg/ml ampicillin, grown overnight at 37 °C (with aeration) and recombinant plasmids were purified using QIAprep Spin Miniprep Kit (QIAGEN, Valencia, USA). The cloned cDNAs were sequenced on both strands using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, UK). Reactions were analyzed by ABI 377 automatic sequencer (Applied Biosystems). T7 promoter and SP6 promoter were primers used for sequencing. The cDNA sequences were analyzed and the putative protein products from these sequences were used to search the GenBank protein databases at NCBI [25].

2.4. Recombinant protein expression

cDNAs encoding for putative mature dermonecrotic proteins LiRecDT2 and LiRecDT3 were amplified by PCR. The forward primers used were: 5′-CCGCTCGAGGCGGATAAACGTC GACCCAT-3′ for LiRecDT2 and 5′-CCGCTCGAGGCTGA CAGCCGGAAGCCAAT-3′ for LiRecDT3. The reverse primers

were: 5′-CGGGATCC**TTA**TTTCTTGAATGTCTCCC-3′ for LiRecDT2 and 5′-CGGGATCC**TTA**CTTCTGCCAGGGAT TAT-3′ for LiRecDT3. The forward primers were constructed with *Xho* I restriction sites at 5′ ends (underlined) plus the sequence related to the first six amino acids of mature protein. The reverse primers were designed to contain *Bam*H I sites (underlined) and the stop codon (bold). PCR products were digested with *Xho* I and *Bam*H I restriction enzymes and gel purified using PerfectPrep Gel Extraction Kit (Eppendorf, Hamburg, Germany). These amplified and digested sequences were then subcloned into pET-14b (Novagen, Madison, USA) digested with the same enzymes. The correct constructs were confirmed by sequencing. The recombinant constructs were expressed as fusion proteins, with a 6x His-Tag at the N-terminus and a 13 amino acid linker including a thrombin site between the 6x His-Tag and the mature protein. The expression constructs were transformed into *E. coli* BL21(DE3)pLysS competent cells and plated on LB agar plates containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Single colonies of both LiRecDT2 and LiRecDT3 constructs were inoculated into LB broth (100 μg/ml ampicillin and 34 μg/ml chloramphenicol) and grown overnight at 37 °C. These cultures were diluted 1:100 into 1 l fresh LB broth/ampicillin/chloramphenicol and incubated at 37 °C until the $OD_{550\ nm} = 0.5$. Recombinant expression was induced by the addition of IPTG (isopropyl β-D-thiogalactoside) to a final concentration of 0.025 mM and cells were incubated for 3.5 hours at 30 °C (with vigorous shaking). Cells were harvested by centrifugation (4000 × *g*, 7 min), resuspended in 40 ml of extraction buffer (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 10 mM imidazole, 1 mg/ml lysozyme) and frozen at –20 °C overnight.

2.5. Protein purification

Cell suspensions were thawed and additionally disrupted by six cycles of 10 s sonication at low intensity. Lysed materials were centrifuged (20,000 × *g*, 20 min) and the supernatants were incubated with 1 ml Ni^{2+}-NTA agarose beads for 1 hour at 4 °C (with gentle agitation). The suspensions were loaded into a column and the packed gel was exhaustively washed with the appropriate buffer (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 20 mM imidazole) until the OD at 280 nm reached 0.01. The recombinant protein was eluted with 10 ml of elution buffer (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 250 mM imidazole) and 1 ml fractions were collected and analyzed by 12.5% SDS-PAGE under reducing conditions. Fractions were pooled and dialyzed against phosphate buffered saline (PBS).

2.6. Animals

Adult Swiss mice weighing approximately 25–30 g and adult rabbits weighing approximately 3 kg from the Central Animal House of the Federal University of Paraná were used for in vivo experiments with crude venom and recombinant toxins. All experimental protocols using animals were performed according to the "Principles of laboratory animal care" (NIH Publication n° 85-23, revised 1985) and "Brazilian Federal Laws", and ethical committee agreement number 126 of Federal University of Paraná.

2.7. In vivo studies on rabbits

For the evaluation of the dermonecrotic effect, 10 μg of crude venom, and of purified recombinant dermonecrotic toxins (LiRecDT1, LiRecDT2 and LiRecDT3), diluted in PBS were injected intradermally into a shaved area of rabbit (n=3 per toxin treatment). Animals (all treatments were maintained under identical conditions during the experiment) were examined for dermonecrosis at 4 and 24 hours after injection [8]. As a control, a purified recombinant venom toxin not related to dermonecrotic toxin and with no dermonecrotic effects but obtained under the same conditions as dermonecrotic toxins was used to test for bacteria contamination or an unspecific inflammatory reaction. Rabbits were used in experiments for dermonecrosis because this animal model reproduces skin lesions very close to those seen in accidents with human [1,2,38].

2.8. Gel electrophoresis, immunoblotting and ELISA

Protein content of samples was determined by the Coomassie Blue method (BioRad, Hercules, USA) following Bradford [26]. For protein analysis, 12.5% SDS-PAGE were carried out under reduced conditions following Laemmli [27] and for protein detection, gels were stained with Coomassie Blue dye. For immunoblotting, proteins were transferred to nitrocellulose filters overnight following Towbin et al. [28] and immunostained with hyperimmune purified IgG, which reacts to dermonecrotic toxin LiRecDT1, or antibodies against crude venom toxins (as described in Section 2.1). Molecular mass markers were acquired from Sigma (St. Louis, USA). Immunoassays used ELISA-antibody capture assays as follows crude venom or purified recombinant toxins (10 μg/ml) were bound to the plate bottoms (Nunc MaxiSorp, Roskilde, Denmark) for 2 hours at room temperature in humid conditions. Plates were washed with PBS and the remaining sites for protein binding on the plates were saturated with blocking buffer (3% BSA-PBS) for 2 hours at room temperature. After washing the plates with PBS primary antibodies (against crude venom toxins or against LiRecDT1) were incubated for 2 hours at room temperature and with secondary antibodies conjugated to horseradish peroxidase for 1 hour at room temperature and the colorimetric reaction was developed using OPD [23].

2.9. Histological methods for light microscopy

Rabbit skin was collected from animals anesthetized with ketamine (Agribands, Paulinia, Brazil) and acepromazine (Univet, São Paulo, Brazil) and then fixed in "ALFAC" fixative solution (ethanol absolute 85%, formaldehyde 10% and glacial acetic acid 5%) for 16 hours at room temperature. After fixa-

tion, samples were dehydrated in a graded series of ethanol before paraffin embedding (for 2 hours at 58 °C). Then, thin sections (4 μm) were processed for histology. Tissue sections were stained by hematoxylin and eosin (HE) [29].

2.10. Sphingomyelinase activity assay

Sphingomyelinase activity was determined by the ability of samples to hydrolyze the sphingomyelin analogue, TNPAL-sphingomyelin (Sigma), following Gatt et al. [30] as follows 10 and 20 μg of samples (crude venom or recombinant toxins) were mixed with 30 nmol of TNPAL-sphingomyelin diluted in 0.2% Triton X-100, 500 mM Tris–HCl buffer (pH 7.4) with 20 mM $MgCl_2$. The reaction lasted for 2 h at 37 °C and was terminated by adding 2-propanol/heptane/5 M H_2SO_4 (40:10:1, v/v), followed by water and *n*-heptane. After phase extraction, a portion of the upper organic phase was removed and the reaction product, TNPAL-sphingosine, was measured spectrophotometrically at 330 nm. One unit of activity is defined as the amount of enzyme that is able to hydrolyze 1 nmol of TNPAL-sphingomyelin/h at 37 °C. Specific activity is given as units of enzyme activity per milligram of protein. Experiments were performed in triplicates and values given are the mean ± S.E.M.

2.11. Platelet aggregation assay

Human platelet-rich plasma was obtained by differential centrifugation from fresh human blood drawn into acid-citrate-dextrose. Platelet aggregations in the presence of crude venom or recombinant toxins (10 μg/ml) were recorded at 37 °C at a stirring rate of 1000 rpm using a Chrono-log Whole Blood Aggregometer, Havertown, PA, USA following Plow et al. [31].

2.12. Measurement of vascular permeability

Change in capillary permeability was based on the leakage of plasma protein-bound dye into the extravascular compartment of the skin [32]. Evans Blue dye (Vetec) diluted in saline was administered intravenously (30 mg/kg of mice) 5 min prior to sample injections. Crude venom and recombinant toxins (10 μg) were injected intradermally into dorsal skin of mice ($N = 5$). At 30 min thereafter, the animals were anesthetized as described above, sacrificed and the dorsal skin was removed for visualization of dye extravasation. Subsequently, the patches of skin were excised and incubated in 2 ml of formamide at room temperature for 5 days after which the absorbance of the resulting supernatant was measured at 595 nm. The same experimental conditions were used with control group except that animals received only the vehicle (PBS) rather than venom or recombinant toxins. Mice were used in experiments of vascular permeability because this animal model does not develop dermonecrosis and local hemorrhage following brown spider venom exposure, an event that could mask vascular permeability interpretation [1,2].

2.13. Statistical analysis

Statistical analyses were performed using analysis of variance (ANOVA) post Tukey test for average comparisons GraphPad InStat program version 3.00 for Windows 2000. Statistical significance was established at $P < 0.05$.

3. Results

3.1. Molecular cloning of two dermonecrotic toxin isoforms from L. intermedia venom gland

By sequencing random clones of a cDNA library from *L. intermedia* venom gland, two different cDNAs encoding for dermonecrotic toxins were isolated, as found by protein-BLAST searches. The putative protein products from these cDNAs were named LiRecDT2 and LiRecDT3 (we previously cloned a dermonecrotic toxin isoform that we named LiRecDT1; [22]). Sequence analysis revealed a partial coding region for the putative signal peptide (underlined) and a complete mature protein product of 280 amino acids for the 1062 bp cDNA of LiRecDT2 (Fig. 1A) and a complete 1007 bp cDNA sequence for LiRecDT3 (Fig. 1B), which includes a 26 amino acids signal peptide (underlined) [33] and a 278 amino acids mature protein. The cDNAs encode a mature protein of theoretical 31.291 Da and p*I* 6.42 for LiRecDT2 and a mature protein of theoretical 31.471 Da and p*I* 6.27 for LiRecDT3.

3.2. Amino acid sequence comparison and structural relationships among cloned dermonecrotic toxins

A protein-BLAST search using GenBank database revealed that both LiRecDT2 and LiRecDT3 are homologous proteins to other dermonecrotic toxins from a variety of *Loxosceles* species. This homology was investigated by a multiple sequence analysis of the putative protein products of LiRecDT2, LiRecDT3 and LiRecDT1 ([22]; Fig. 2A). LiRecDT2 is most homologous to LiRecDT1 sharing 89% sequence identity, while LiRecDT3 is 45% homologous to both LiRecDT1 and LiRecDT2. The phylogenetic tree analysis for *L. intermedia* recombinant dermonecrotic toxins and other cloned cDNAs revealed a close structural relationship for LiRecDT1 and LiRecDT2 compared to other members of dermonecrotic toxin family, while LiRecDT3 is more similar to dermonecrotic toxins from *L. laeta* (AAM21156) and *L. boneti* (AAT66074) (Fig. 2B).

3.3. Expression, purification and immunological cross-reactivity of recombinant dermonecrotic toxins

Recombinant toxins were expressed as N-terminal 6x His-tag fusion protein in *E. coli* BL21(DE3)pLysS cells and puri-

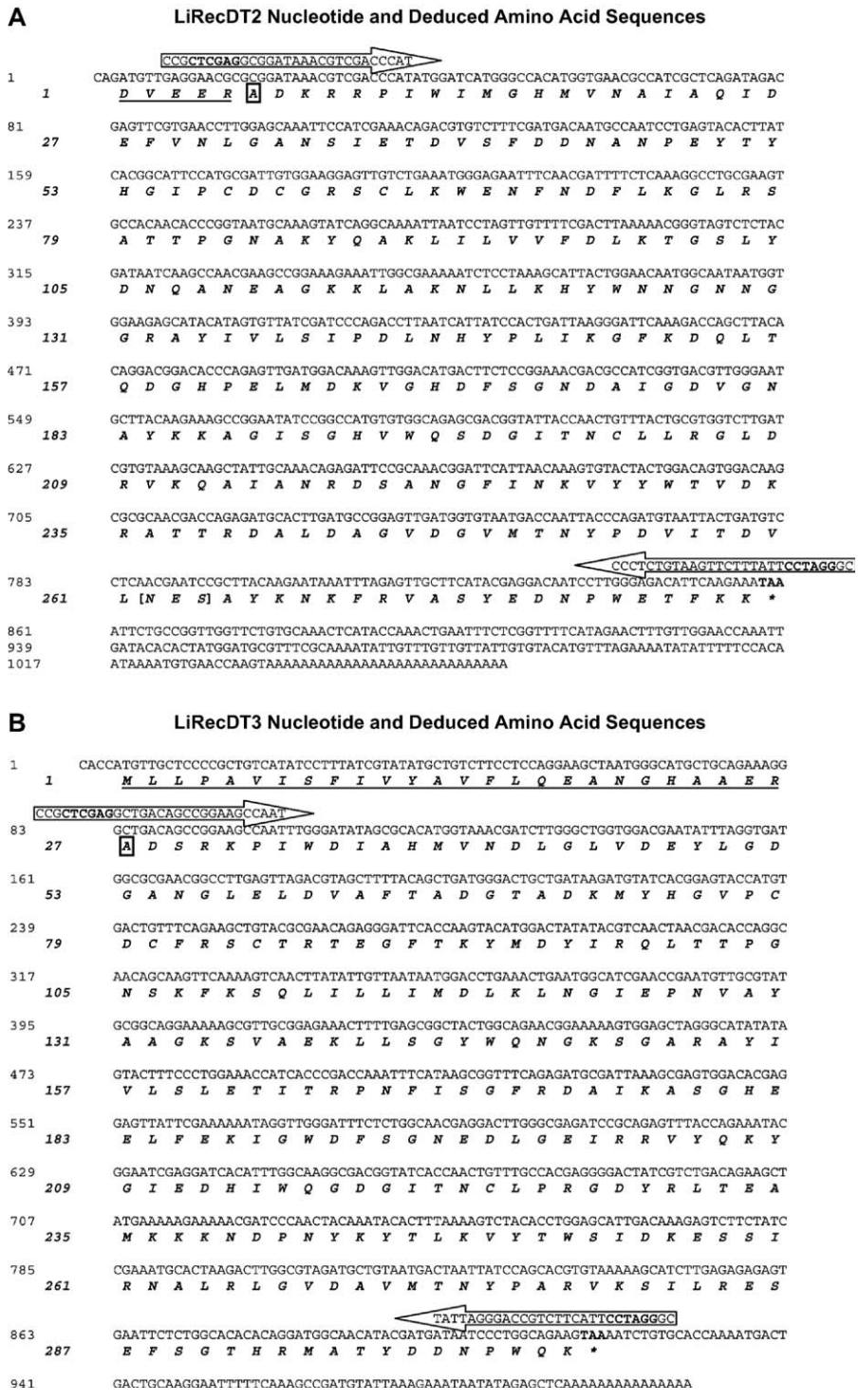

Fig. 1. Molecular cloning of recombinant dermonecrotic toxin LiRecDT2 and LiRecDT3 from *Loxosceles intermedia* venom gland cDNA library.
Nucleotide and deduced amino acid sequences of cloned cDNA for LiRecDT2 (A) and for LiRecDT3 (B) from *L. intermedia* venom gland. The predicted signal peptides are underlined in the protein sequences. Annealing positions for primers used for subcloning into the expression vector are indicated by arrows. Alanine (in the box) is the first amino acid of mature protein with 280 amino acids for LiRecDT2 and 278 Amino acids for LiRecDT3. Asterisks indicate stop codons (TAA) in bold for both proteins. Brackets mark a potential *N*-glycosylation site in LiRecDT2.

fied from soluble fraction of cell lysates by Ni^{2+}-chelating chromatography resulting in 3.0 mg/l for LiRecDT2, 2.7 mg/l for LiRecDT3 (Fig. 3). LiRecDT2 has 33.8 kDa and LiRecDT3 34.0 kDa. Differences between deduced molecular mass and SDS-PAGE mobility for recombinant proteins are consequence of His-tag fusion peptide. Additionally, in order to strengthen the idea of related toxins as evidenced by their nucleotide sequences and alignment, we produced polyclonal antisera against crude venom toxins and LiRecDT1 and checked for antigenic cross-reactivity with recombinant toxins

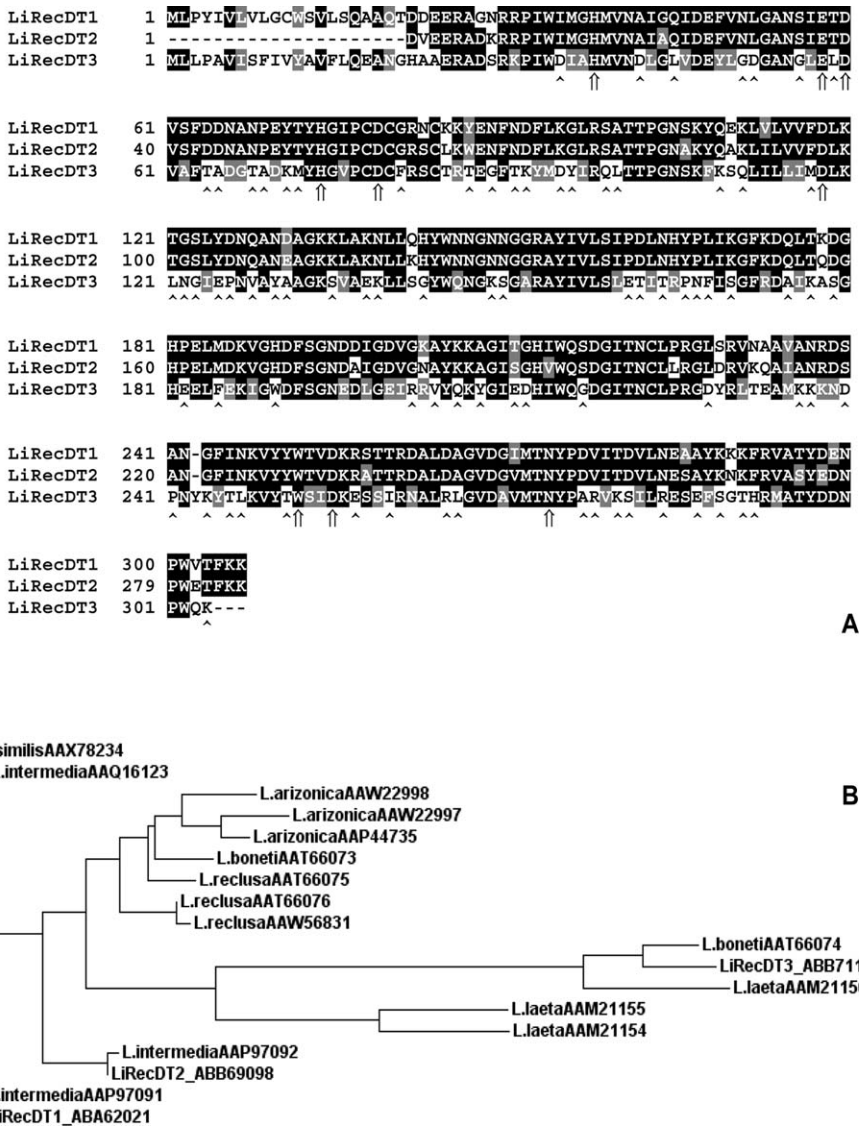

Fig. 2. Multiple alignment analysis of the cDNA-deduced amino acid sequences for the recombinant toxins LiRecDT1, LiRecDT2 and LiRecDT3.
Phylogenetic relationship of LiRecDT1, LiRecDT2 and LiRecDT3 toxins to other dermonecrotic toxin family members. Sequences were aligned using the CLUSTAL W program (www.ebi.ac.uk/CLUSTAL). Amino acid identities are shaded in black. Conservative substitutions are in gray, arrowheads point to different amino acids for LiRecDT3 compared to conserved amino acids for LiRecDT1 and LiRecDT2 and arrows point to amino acid residues involved in sphingomyelinase catalysis (A). Phylogeny of the cloned dermonecrotic toxin members based on sequence data from GenBank and from Fig. 1A. The tree was constructed with the program CLUSTAL as described above (B).

by immunoblot (antibodies that recognize denaturation-resistant epitopes on the antigen) and antibody capture assay-ELISA (antibodies that recognize non denatured epitopes on the antigen). Both antibodies for crude venom toxins and for LiRecDT1 cross-reacted with recombinant toxins corroborating that crude venom contains similar proteins to recombinant toxins and that toxins show sequence/epitopes identity and antigenic cross-reactivity (Fig. 4).

3.4. Dermonecrotic and inflammatory reaction induced by recombinant toxins

Dermonecrotic macroscopic lesions and light microscopic histological analysis of biopsies from skin that received crude venom or different purified recombinant toxins showed that recombinant toxins were similar in their effects to natural toxins (Fig. 5A and B). Animals injected with crude venom showed a halo of ischemic and necrotic tissue surrounded by erythema at the injection point and gravitational spread of lesion. LiRecDT1 and LiRecDT2 caused reactions very similar to those of crude venom with dermonecrotic lesions at the injection point, erythema and gravitational spreading. LiRecDT3, on the other hand, did not cause apparent necrosis at the injection site, but rather transient swelling and erythema 4 hours after toxin exposure but without spreading of lesion with the time. For LiRecDT3, experiment was repeated but now using 20 μg and 40 μg of purified toxin and animals were accompanied for 5 days. There was just erythema and swelling but without dermonecrosis (data not shown). Light microscopic

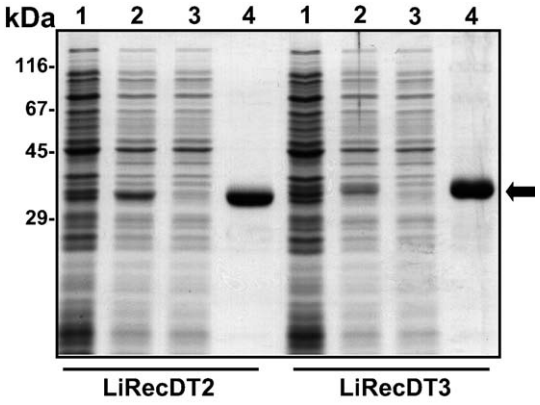

Fig. 3. Expression and purification of recombinant dermonecrotic toxins analyzed by 12.5% SDS-PAGE under reducing conditions and Coomassie blue dye staining. Lanes 1 show *Escherichia coli* BL21(DE3)pLysS collected by centrifugation and resuspended in Laemmli sample buffer prior to 3.5 hours induction with 0.025 mM IPTG. Lanes 2 and 3 show the supernatant of cell lysates induced with 0.025 mM IPTG obtained by freeze thawing and sonication in extraction buffer before and after affinity chromatography using Ni^{2+}-NTA agarose column. Lanes 4 show purified eluted recombinant proteins through Ni^{2+}-NTA agarose column. Arrow indicates LiRecDT2 and LiRecDT3 localization. Molecular protein mass standards are shown on the left.

analysis of skin biopsies exposed to crude venom and all recombinant toxins (24 hours after exposure) showed very similar histopathological reactions, including diffuse edema of the skin and a massive collection of inflammatory cells in and around blood vessels (indicative of functionality for recombinant dermonecrotic toxins; [1,2]).

3.5. Sphingomyelinase activity of recombinant toxins

Based on the fact that native brown spider dermonecrotic toxins have sphingomyelinase activity (as previously discussed) and with the aim of demonstrating such as functionality for recombinant isoforms, we used colorimetric reactions for sphingomyelinase activity comparing crude venom to recombinant toxins. All recombinant isoforms contained sphingomyelinase activity (Fig. 6). Crude venom, LiRecDT1 and LiRecDT2 have similar levels of sphingomyelinase activity compared to LiRecDT3, with lower activity. Sphingomyelinase activity was concentration-dependent and such results strengthen the idea of functionality for recombinant toxins.

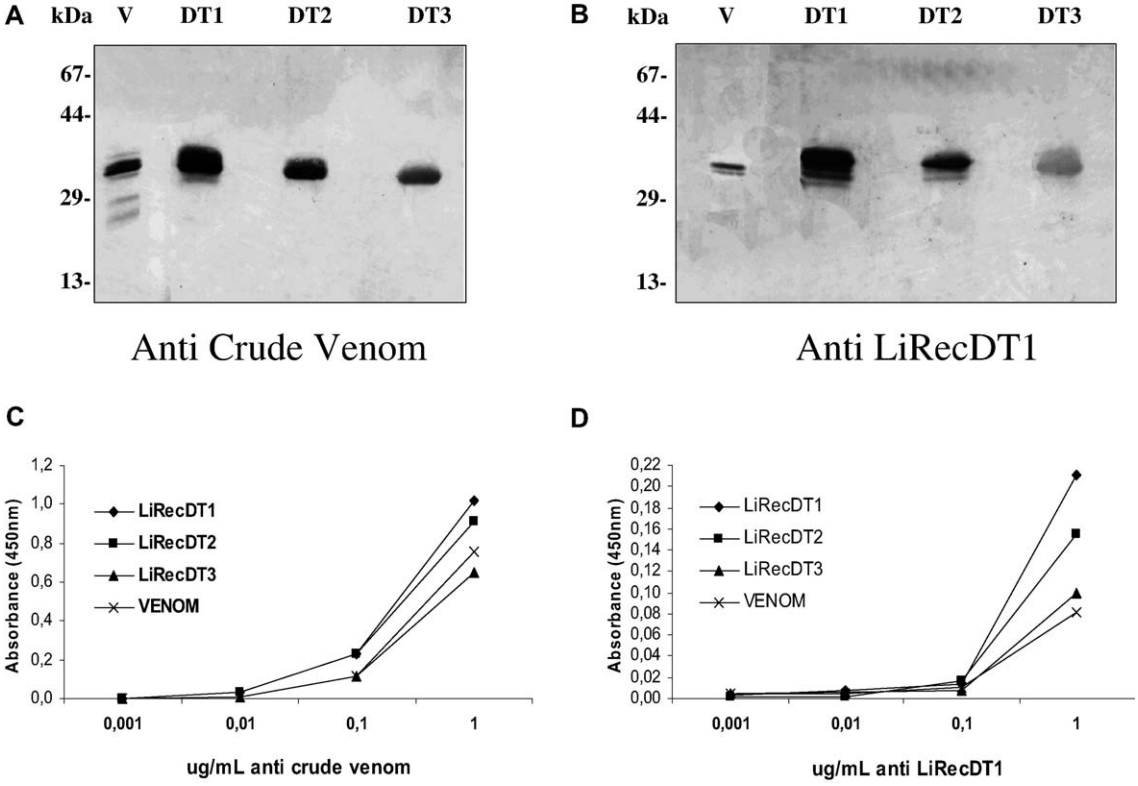

Fig. 4. Comparative immunological cross-reactivity of crude venom and recombinant toxins.
Loxosceles intermedia crude venom (V) and purified recombinant toxins LiRecDT1 (DT1), LiRecDT2 (DT2) and LiRecDT3 (DT3) at concentration of 2.5 μg were separated by 12.5% SDS-PAGE under reducing conditions, transferred onto nitrocellulose membranes that were exposed to purified antibodies against crude venom toxins (A) or antibodies to LiRecDT1 (B). Molecular mass markers are shown on the left of figure. Also, antibody capture assays (ELISA) were carried out using crude venom and recombinant toxins LiRecDT1, LiRecDT2 and LiRecDT3 (10 μg/ml) immobilized on a solid phase. Primary purified antibodies against crude venom toxins (C) and LiRecDT1 (D) at indicated concentrations (abscissa) were incubated for 2 hours at room temperature and reaction developed as described in materials and methods. In both cases (immunoblotting and ELISA) cross-reactivity occurred among antibodies to crude venom toxins and recombinant toxins or antibodies to LiRecDT1 with both recombinant toxins and venom toxins.

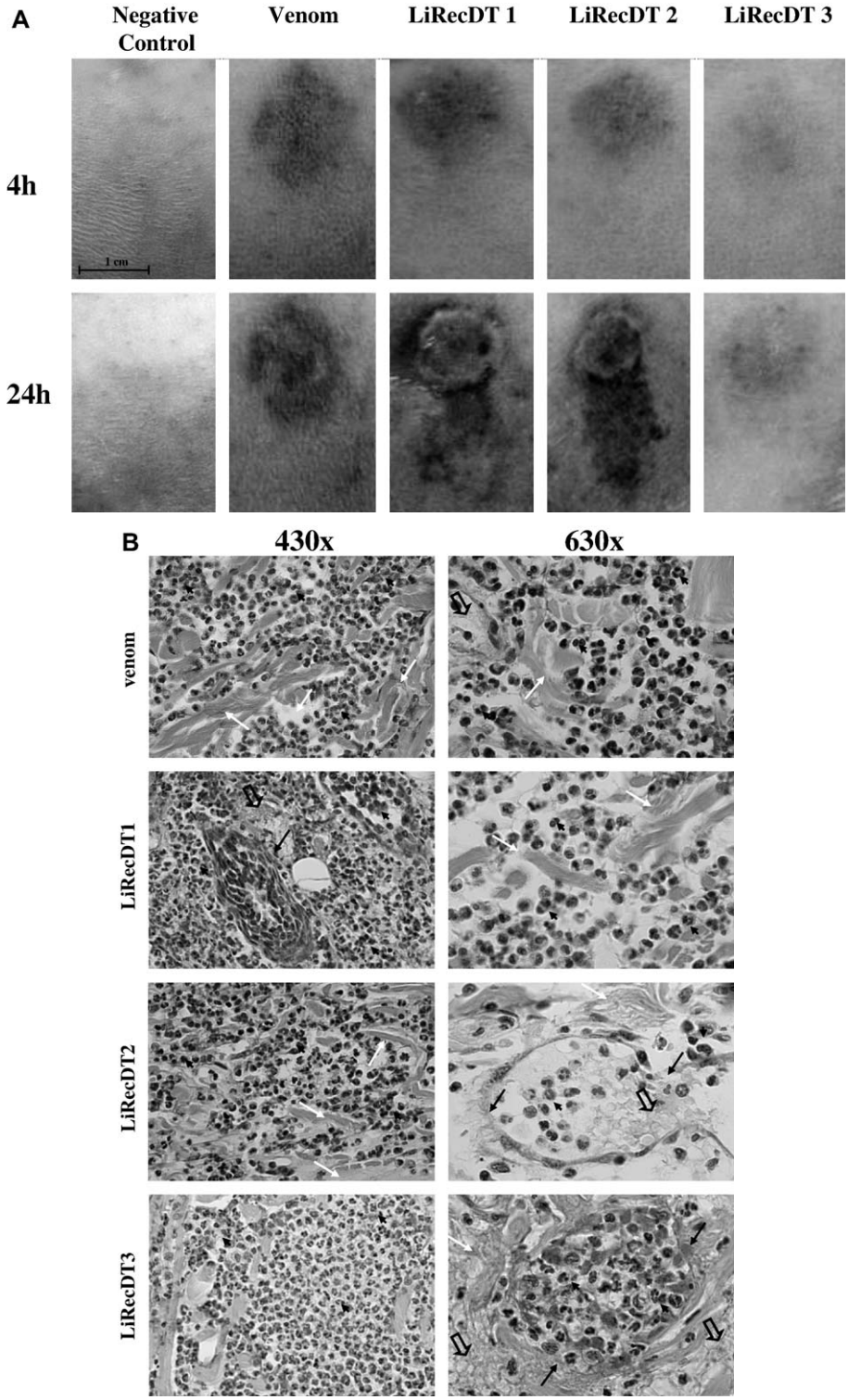

Fig. 5. Macroscopic and histological changes of rabbit skin exposed to crude venom and recombinant toxins. Macroscopic dermonecrosis is visible in rabbits intradermally injected with 10 μg of crude venom and 10 μg of purified recombinant toxins LiRecDT1, LiRecDT2, LiRecDT3 and a recombinant toxin without dermonecrotic activity (negative control). Lesions were noted at 4 and 24 hours following injection (A). Histopathology of rabbit skin 24 hours following toxin injection. Light microscopic analysis of sections of dermonecrotic lesions stained with HE. Closed arrows indicate disruption of blood vessel walls, and open arrows indicate fibrin network deposition, closed arrowheads indicate a massive inflammatory response with the presence of neutrophils and white arrows indicate disorganization of collagen fibers and dermal edema (B).

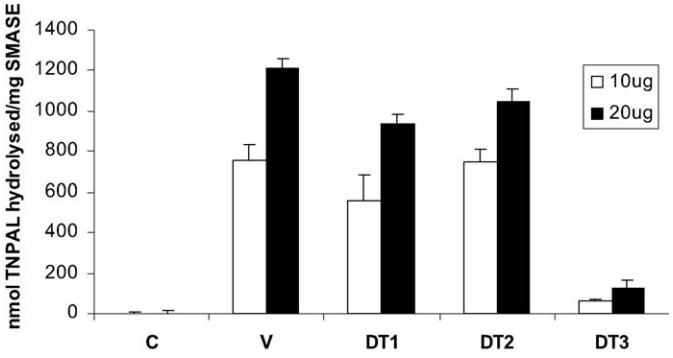

Fig. 6. Comparative sphingomyelinase activity of crude venom and recombinant toxins. Sphingomyelinase activity of crude venom (V) or LiRecDT1 (DT1), LiRecDT2 (DT2) and LiRecDT3 (DT3) was evaluated by trinitrophenylamino lauroyl-sphingomyelin hydrolysis at 37 °C for 2 hours; the product of reaction was determined colorimetrically at 330 nm. PBS was used as Control (C) to solubilize venom or recombinant toxins. Values given are the average ± S.E.M.

3.6. In vitro platelet aggregation induced by recombinant toxins

Brown spider venom is remarkable by inducing platelet aggregation, an event related to thrombocytopenia and thrombosis of the dermal blood vessels. Such as venom activity is attributed to dermonecrotic toxins that when purified from crude venom of different *Loxosceles* species can produce platelet aggregation in vitro [1,2]. With the objective of corroborating functionality for recombinant toxins, we compared their ability to induce platelet aggregation with that of crude venom. As shown in Fig. 7, we can observe that recombinant dermonecrotic toxins LiRecDT1 and LiRecDT2 induced platelet aggregation similarly to crude venom whereas LiRecDT3 just displayed a residual platelet aggregation activity.

3.7. Effect of recombinant dermonecrotic toxins on the blood vessel permeability

It is known that the crude venom of *Loxosceles* spider promotes blood vessel instability, edema and hemorrhage at the bite site of patients who have had accidents and experimental animal models [1,2]. To test the relation between venom-induced vessel histopathology and dermonecrotic toxins as well as to strengthen the idea of functionality for recombinant toxins, Miles assay (vessel permeability) was used in mice. Crude venom and recombinant toxins induced vessel permeability (Fig. 8). Crude venom caused diffuse leakage of dye compared to recombinant toxins that caused a sharper extravasation near the injection point. LiRecDT1 and LiRecDT2 stimulated similar and greater activity when compared with LiRecDT3.

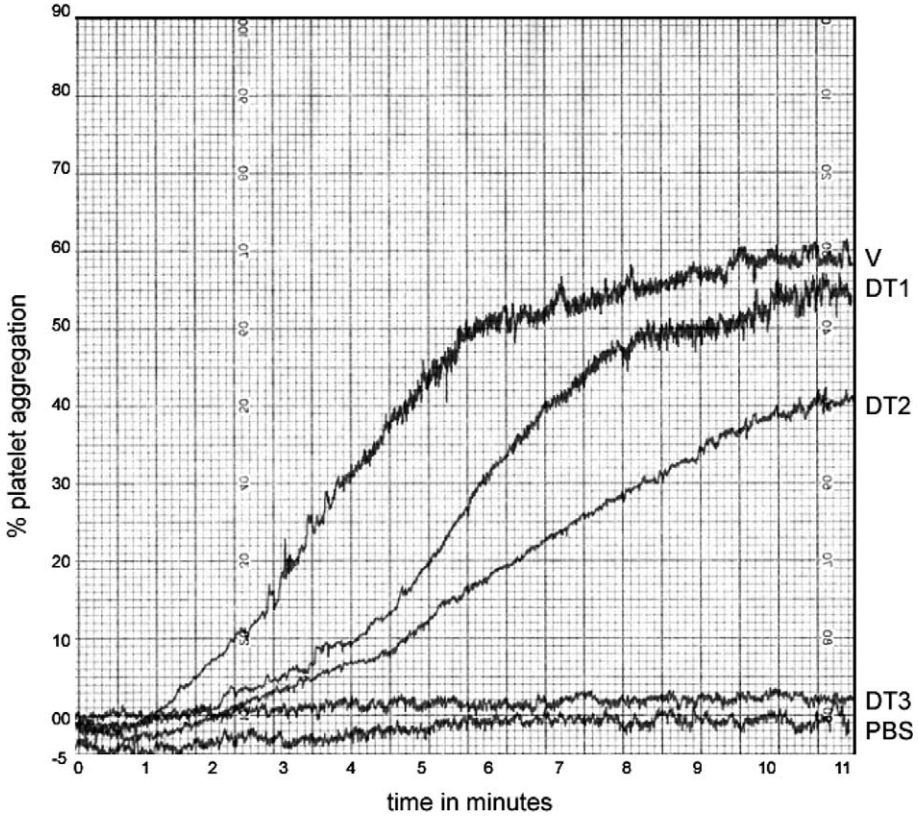

Fig. 7. Effect of crude venom and recombinant toxins on platelet aggregation.
Platelet-rich plasma was incubated with crude venom (V) or LiRecDT1 (DT1), LiRecDT2 (DT2) and LiRecDT3 (DT3) (10 μg/ml) or PBS (Control). Aggregation was monitored by measuring the light transmittance during 10 min by an aggregometer. The abscissa represents the time in minutes and the ordinate is percent platelet aggregation.

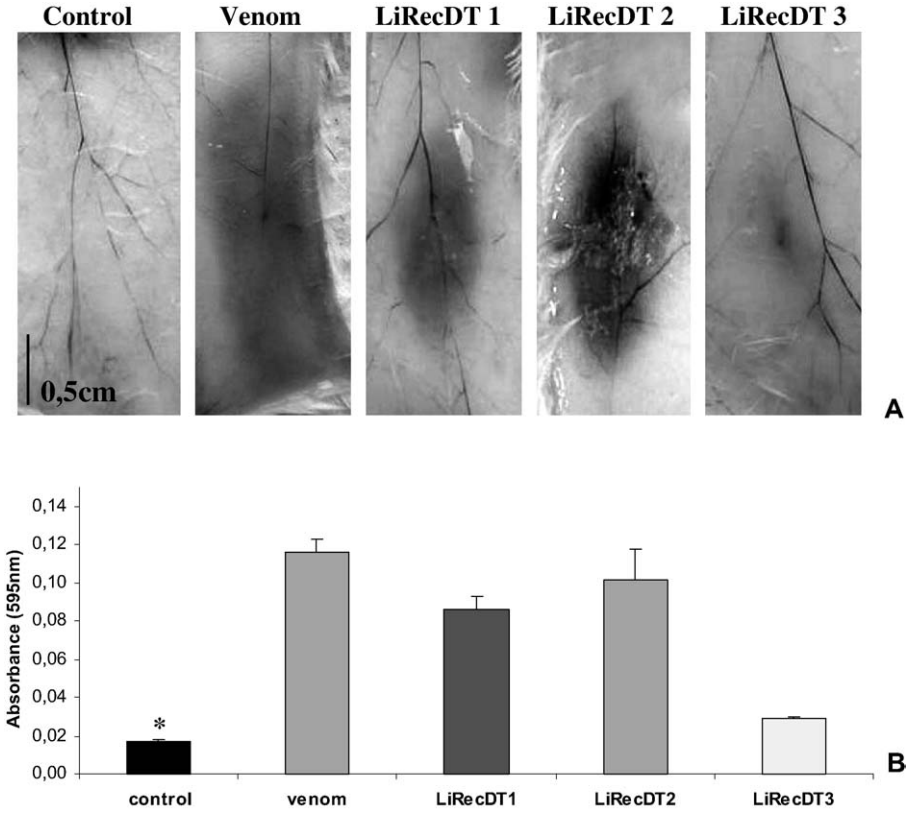

Fig. 8. Effect of crude venom and recombinant toxins on induction of microvascular permeability of skin vessels. Mice were given intradermal injections (10 μg) of crude venom or LiRecDT1, LiRecDT2 and LiRecDT3 or PBS (control for baseline permeability level). Photographs show extensive Evans blue dye leakage after venom or LiRecDT1 and LiRecDT2 exposure compared to minimal vessel permeability induced by LiRecDT3 or PBS (A). Evans blue leakage was determined spectrophotometrically at 595 nm (B). All graphs represent averages of five experiments ± S.E.M. $*P \leq 0.001$.

4. Discussion

Brown spider (*Loxosceles*) bites cause well known necrotic skin lesions with gravitational spreading. Although less commonly, systemic involvements, such as renal failure, disseminated intravascular coagulation and intravascular hemolysis, have been also reported after accidents [1,2].

The literature includes ample evidence that venom from different species of Brown spiders contain similar toxins. Polyclonal antibodies for *L. gaucho* venom also bound to *L. laeta* and *L. intermedia* toxins, including the dermonecrotic toxin at region of 33–35 kDa. This suggests antigenic conservation in these three venoms [15]. Antibodies to *L. gaucho* venom also neutralized the dermonecrotic and lethal activities of three *Loxosceles* species [15,34]. Immunological and biochemical analysis of *L. reclusa* and *L. deserta* venoms showed antigenic cross-reactivity and biochemical homologies for some venom proteins, with greater than 90% amino acid sequence identity [35]. Preliminary immunological data were supported by subsequent descriptions and purification of different native isoforms of dermonecrotic toxins in *Loxosceles* spider venom [1, 12,14–16]. Finally, through a proteomic approach with two-dimensional electrophoresis, Edman sequencing and mass spectrometry eleven isoforms of dermonecrotic toxin in *L. gaucho* venom were found [17], supporting the idea of a family of homologous dermonecrotic toxins in *Loxosceles* spider venom. Apparently each toxin isoform causes noxious activities and the effect induced by crude venom is a family synergism.

Here, we cloned, expressed, purified and tested the functionality of two new recombinant toxins from *L. intermedia* venom gland. Based on cDNA-predicted amino acid sequence, we acknowledged these two venom toxins that belong to the brown spider dermonecrotic toxin family. We named these different recombinant isoforms as LiRecDT2 and LiRecDT3, since we had previously identified and cloned LiRecDT1 [22]. Their primary sequences include a signal sequence and a mature protein similar to those of other brown spider dermonecrotic molecules. Several lines of evidence support LiRecDT2 and LiRecDT3 as dermonecrotic molecules. The nucleotide and deduced amino acid sequences are similar to those of other members of the family (15, 18, 20, 21, 22). The molecular mass and the isoelectric point calculated from deduced amino acid sequences of mature proteins are very similar to those described for other dermonecrotic toxins [17]. In addition, LiRecDT2 and LiRecDT3 caused skin injuries and evoked a massive inflammatory response when intradermally injected in the rabbit skin [1,2,5]. Moreover, immunological studies also suggested a structural similarity between LiRecDT2 and LiRecDT3 with LiRecDT1 and these toxins reacted through immunoblot and ELISA analysis with antibodies

raised against crude venom. Additional evidence for LiRecDT2 and LiRecDT3 as members of brown spider dermonecrotic toxins comes from their platelet aggregating activity [1,2]. Finally, biochemical studies demonstrate the sphingomyelinase activity of LiRecDT2 and LiRecDT3, which also strongly suggests that these recombinant toxins are closely related members of the dermonecrotic (sphyngomyelinases) toxin family [1,2].

Protein sequence analysis of dermonecrotic toxins from different *Loxosceles* species showed three major subfamilies for these molecules. A subfamily composed of *L. similes* (AAX78234) and *L. intermedia* (AAQ16123) toxins, a subfamily which includes *L. intermedia* (AAP97091) and LiRecDT1 (ABA62021) toxins and a major subfamily including dermonecrotic toxins from *L. arizonica*, *L. reclusa*, *L. laeta*, *L. boneti*, LiRecDT2 and LiRecDT3. The analysis demonstrates that LiRecDT2 (which triggers dermonecrosis, gravitational spreading, massive inflammatory response, platelet aggregation with strong sphingomyelinase activity) is very similar to other *L. intermedia* dermonecrotic toxins including LiRecDT1. On the other hand, LiRecDT3 that induced edema, erythema and inflammatory response but not dermonecrotic lesion and has low platelet aggregation and sphingomyelinase activity was similar *L. boneti* and *L. laeta* toxins. However, no biological function has been described for any of these last toxins.

By applying a selective affinity chromatography (Ni^{2+} column), LiRecDT2 and LiRecDT3 could be specifically eluted in a pure form as visualized by SDS-PAGE. Characterization of the antigenic cross-reactivity by immunoblotting and ELISA of LiRecDT2 and LiRecDT3 compared amongst themselves and to crude venom or to LiRecDT1 suggested that crude venom contains similar proteins, which show antigenic sequence identity to recombinant dermonecrotic toxins and there was a marked antigenic cross-reactivity for LiRecDT1, LiRecDT2 and LiRecDT3. This cross-reactivity supports antigenic conservation in these three toxins. Additionally, antisera raised against crude venom toxins produced a high cross-reactivity with recombinant toxins when compared to crude venom itself, suggesting that epitopes present in dermonecrotic toxins are strong antigenic determinants.

Recombinant toxins showed differential functionality as observed by dermonecrosis of rabbit skin. LiRecDT2 showed similar activities to those evoked by crude venom and LiRecDT1 causing erythema, swelling and a violaceous halo of ischemic tissue at the injection point 4 hours after injection. Twenty-four hours after injection the lesion showed diffuse erythema, swelling, the ischemic area grew with gravitational spread of the lesion. On the other hand, with 4 hours post-LiRecDT3 exposure there was merely a transient erythema and swelling and at 24 hours there was no increase in damage, without dermonecrosis or gravitational spreading. Histological analysis 24 hours after injection with crude venom, LiRecDT1 and LiRecDT2 showed similar histopathologies with diffuse edema, fibrin exudation and marked diffuse infiltration of inflammatory cells (with neutrophils prevalent) into the dermis. Interestingly, while having lower dermonecrotic activity LiRecDT3 caused similar histopathology with respect to the inflammatory response with diffuse infiltration of leukocytes into the dermis. Considerable evidence supports the role of PMN leukocytes in cutaneous lesions caused by the Brown spider venom. When neutropenia is experimentally induced in rabbits previously to venom injection, the dermonecrotic lesion is inhibited [36]. Additionally, histological descriptions of experimental or natural dermonecrosis induced by Brown spider venom have shown massive inflammatory infiltrates at the venom exposure site [2,37,38]. Finally, inflammatory response and neutrophil participation in dermonecrotic lesions seem to be caused by venom activity upon endothelia, which leads to an indirect and disregulated neutrophil activation [39]. Variation in dermonecrotic and sphingomyelinase activities, platelet aggregation and vessel permeability may be related to structural differences among toxins, perhaps in the function of important amino acid substitutions (Fig. 2A). Interestingly, despite immunoblotting and ELISA results that pointed some antigenic conservation for these recombinant toxins, it is clear that LiRecDT3 showed less cross-reactivity when reacted with antibodies against crude venom toxins or LiRecDT1, suggesting that different epitopes (and structural conformation) are present in this toxin. Nevertheless, the LiRecDT3 ability to cause a massive inflammatory response without dermonecrotic lesions suggests that other, currently unknown, cellular or molecular components must be involved in such activities. Probably, the residual activity of LiRecDT3 (such as phospholipase activity, vascular disturbs and increasing vessel permeability, together with antigenic nature) make this toxin effective to induce inflammatory response at injection site but the total number of recruited cells during this event is not enough to cause skin necrosis, but only inducing edema and erythema as described in Fig. 5.

Although no direct correlation between sphingomyelinase activity and dermonecrotic effect is known, we can speculate on some correlations for such effects. This hypothesis is strengthened by the fact that sphingomyelinases can generate lysophosphatidic acid that is known to induce several pathological responses, including inflammation and platelet aggregation [1,2,40]. Interestingly, while LiRecDT1/LiRecDT2 and LiRecDT3 have different activities, the amino acid residues involved in catalysis or metal ion coordination important for sphingomyelinase activity [41] are conserved (arrows Fig. 2A). A putative explanation for differences described could be that amino acid residues share high sequence homology for LiRecDT1 and LiRecDT2 but not with LiRecDT3 (arrow heads Fig. 2A). For instance, there are several important substitutions in amino acid residues neighboring amino acids of catalytic site comparing LiRecDT3 and LiRecDT1/DT2. There is an aspartic acid residue (negative charged and hydrophilic) at position 9 of mature protein in LiRecDT3 instead of isoleucine (hydrophobic) in LiRecDT1/DT2. Identically, there is an aspartic acid at position 16 of LiRecDT3 replacing residue of alanine (nonpolar) in LiRecDT1/DT2. A glycine residue (nonpolar and small) at position 30 of LiRecDT3 substitutes serine (polar) in LiRecDT1/DT2. A leucine residue (hydrophobic) at position 33 of LiRecDT3 substitutes threonine (polar) in LiRecDT1/DT2. Methionine (nonpolar) at position 46 of LiR-

ecDT3 replacing threonine residues (uncharged and polar) for LiRecDT1/DT2. A leucine residue (95) in LiRecDT3 substitutes threonine in LiRecDT1/DT2. There is a glutamic acid residue at position 231 (negative) for LiRecDT3 replacing arginine (positive) in LiRecDT1/DT2 and respectively residues of alanine (251) nonpolar and arginine (252) positive in LiRecDT3 replacing aspartic acid (negative) and valine (nonpolar) for LiRecDT1/DT2. In some way these residues could participate in the stabilization and organization of catalytic site or synergistic domains of toxins explaining differences in their functionality.

The mechanism by which *Loxosceles* venom causes gravitational spreading of dermonecrosis is under investigation. Some speculate that the participation of hyaluronidases in the venom plays a role in the gravitational spreading [1,2]. Hyaluronidases and proteases have been detected in the venom of many different animals (snakes, caterpillars, scorpions, bees and spiders; [42,43]). These enzymes apparently are spreading factors that act by degrading hyaluronic acid and other extracellular matrix constituents, and then facilitate the diffusion of other venom toxins. Additionally, metalloproteases described in the venom [2,6,44,45] could also be involved in dermonecrotic spreading. These proteases degrade fibronectin, entactin and basement membrane thereby causing blood vessel instability and venom toxin spreading. Nevertheless, since purified LiRecDT1 and LiRecDT2 evoked gravitational spreading (Fig. 5A), these toxins alone may cause the spreading of skin lesions. However, we cannot rule out proteolytic and hyaluronic acid lytic toxins as playing a role in such effects. Additionally, diffuse Evans blue permeability after venom exposure compared to more concentrated effect of recombinant toxins (Fig. 8) suggests the participation of venom spreading factors acting in synergism with dermonecrotic toxins. Based on described results, apparently we can speculate on the lack of correlation between dermonecrosis and vascular permeability. Mice do not developed dermonecrosis but have increased vascular permeability following dermonecrotic toxin exposure. Apparently by inducing inflammatory response and leukocyte infiltration into the dermis of mice [2,47], dermonecrotic toxin could increase vessel permeability as shown in Fig. 8. In addition, a direct toxin effect upon endothelia causing endothelial cell activation and cytotoxicity [39,45,46] could generate increase in vessel permeability by causing disturbs of blood vessel wall.

The study of brown spider toxins is particularly difficult due to the small quantities of toxins that can be collected [2]. Thus, cloning and expression of biologically important venom toxins (as is the case of sphingomyelinases) is a very useful tool for structural and functional research on the mechanisms of toxic activities. Additionally, biologically active recombinant toxins also offer biotechnological possibilities, such as substitute antigens for production of immunologic products and antisera or as tools for investigating molecular mechanisms in the cell biology for instance as inflammatory response activators, blood vessel permeability modulators and platelet aggregating agonists.

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Artigos científicos submetidos

Artigo:

Two novel dermonecrotic toxins from brown spider (*Loxosceles intermedia*) venom: from cloning to functional characterization

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Two novel dermonecrotic toxins from Brown spider (*Loxosceles intermedia*) venom: from cloning to functional characterization.

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GenBank data deposition information for *L. intermedia* cloned cDNAs is:
LiRecDT4, GenBank accession no. **DQ431848**; LiRecDT5, GenBank accession no. **DQ431849**.

Abstract

Loxoscelism (the condition produced by the bite of brown spiders) has been reported worldwide, but especially in warmer regions. Clinical manifestations include skin necrosis with gravitational spreading while systemic loxoscelism may include renal failure, hemolysis and thrombocytopenia. The venom contains several toxins, of which the best biochemically and biologically studied is the dermonecrotic toxin, a sphyngomyelinase. Purified toxin induces cutaneous and systemic loxoscelism, especially necrotic lesions, hematological disturbances and renal failure.

Herein, we describe cloning, heterologous expression and purification of two novel isoforms of the dermonecrotic toxin: LiRecDT4 and LiRecDT5. The recombinant proteins stably expressed in *Escherichia coli* cells were purified from culture supernatants in a single step using Ni^{2+}-chelating chromatography producing soluble proteins of 34kDa (LiRecDT4) and 37kDa (LiRecDT5). Both proteins were recognized by whole venom serum antibodies and by a specific antibody to dermonecrotic toxin. Also, recombinant isoforms with lipase activity induced experimental skin lesions and caused a massive inflammatory response in rabbit skin dermis. Nevertheless, recombinant toxins displayed differential activities of platelet aggregation, increase in vascular permeability as well as not caused death in mice. These characteristics in combination with functional studies illustrates that a family of dermonecrotic toxins exists, and includes two novel members that are useful for future structural and functional studies. They will also be useful in biotechnological ends, for example, as inflammatory and platelet aggregating studies and antigens for serum therapy source.