ORIGINAL ARTICLE

See related commentary on pg 1284

Tetracycline Protects against Dermonecrosis Induced by *Loxosceles* Spider Venom

Danielle Paixão-Cavalcante[1], Carmen W. van den Berg[2], Rute M. Gonçalves-de-Andrade[1], Matheus de F. Fernandes-Pedrosa[1], Cinthya Kimori Okamoto[1] and Denise V. Tambourgi[1]

Envenomation by spiders belonging to the *Loxosceles* genus (brown spider) often results in local dermonecrotic lesions. We have previously shown that *Loxosceles* sphingomyelinase D (SMase D), the venom component responsible for all the pathological effects, induced the expression of matrix metalloproteinases (MMPs) in rabbits and in human keratinocytic cells. We also showed that the SMase D-induced apoptosis and MMP expression of keratinocytes was inhibited by tetracyclines. We have further investigated the ability of tetracyclines to inhibit or prevent the dermonecrotic lesion induced by *Loxosceles* venom *in vivo* and *in vitro* models. Primary cultures of rabbit fibroblasts incubated with increasing concentrations of venom or SMase D showed a decrease in cell viability, which was prevented by tetracyclines. *In vivo* experiments showed that topical treatments with tetracycline of rabbits, inoculated with crude *Loxosceles intermedia* venom or recombinant SMase D, significantly reduced the progression of the dermonecrotic lesion. Furthermore, tetracyclines also reduced the expression of MMP-2 and prevented the induction of MMP-9. Our results suggest that tetracycline may be an effective therapeutic agent for the treatment of cutaneous loxoscelism.

Journal of Investigative Dermatology (2007) **127,** 1410–1418. doi:10.1038/sj.jid.5700688; published online 11 January 2007

INTRODUCTION

Envenomation by *Loxosceles* spiders is a public health problem in Brazil and is considered the most dangerous form of araneism in the country. Systemic reactions including shock, hemolysis, renal insufficiency, and disseminated intravascular coagulation are rare. In contrast, bite reactions varying from small areas of erythema to large areas of ulceration and necrosis are frequently observed (Futrell, 1992). The bite is relatively painless and patients often are unaware they have been bitten. Mild to severe pain, beginning at 2–8 hours after envenomation, is probably due to ischemia. Erythema, with itching, swelling, and mild to severe tenderness is also observed. At this point, the patient might consult a physician (Futrell, 1992).

Treatment of the dermonecrotic lesion has been ineffective and even detrimental in some cases. A variety of treatments such as: systemic steroids, antivenom injected at lesion sites, phentolamine, heparin, chloroprofenpyridamine maleate, dapsone, hyperbaric oxygen, and other substances have been used for therapy, with little or no benefit and, in some cases, with undesirable collateral effects (Denny *et al.*, 1964; Dillaha *et al.*, 1964; Rees *et al.*, 1981; Wille and Morrow, 1988; Bitterman-Deutsch *et al.*, 1990; Allen, 1992; Barrett *et al.*, 1994; Anderson, 1997; Koh, 1998; Schenone *et al.*, 1998; Walter *et al.*, 1999; Bryant and Pittman, 2003; Mold and Thompson, 2004; Elston *et al.*, 2005; Wilson *et al.*, 2005).

The majority of the treatments have attempted to reduce the infiltration of polymorphonuclear (PMN) leukocytes, the hallmark of cutaneous loxoscelism. The PMN infiltrate, in part recruited by indirect activation of the complement system, is considered a major contributor to tissue damage (Ward and Cochrane, 1965; Smith and Micks, 1970; Pizzi, 1975; Sunderkotter *et al.*, 2001; Tambourgi *et al.*, 2005). Treatments aiming to reduce the PMN infiltrate are fraught with side effects and, in some cases, they can increase the tissue injury (Wilson *et al.*, 2005).

The low efficiency of the treatment is directly associated with the fact that the mechanism of action of *Loxosceles* venom is still not completely understood. Several studies have indicated that sphingomyelinase D (SMase D) present in the venoms of *Loxosceles* spiders is the main component responsible for the local and systemic effects observed in loxoscelism (Kurpiewski *et al.*, 1981; Tambourgi *et al.*, 1998, 2000, 2002, 2004, 2005; Fernandes-Pedrosa *et al.*, 2002; van den Berg *et al.*, 2002).

We have previously shown that the binding of the SMase D to the cell surface induces activation of endogenous metalloproteinases of the Adamalysin family (Tambourgi *et al.*, 2000, 2002; van den Berg *et al.*, 2002). Furthermore we have recently shown that *Loxosceles* SMase D induces expression of matrix metalloproteinases (MMPs) in rabbit skin

[1]*Laboratório de Imunoquímica, Instituto Butantan, São Paulo, Brazil and* [2]*Department of Pharmacology, Therapeutics and Toxicology, University of Wales, College of Medicine, Cardiff, UK*

Correspondence: Dr Denise V. Tambourgi, Laboratório de Imunoquímica, Instituto Butantan, Av. Prof. Vital Brazil, 1500, CEP 05503-900, São Paulo, Brazil. E-mail: dvtambourgi@butantan.gov.br

Abbreviations: FBS, fetal bovine serum; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocytes; SMase D, sphingomyelinase D

Received 27 June 2006; revised 5 October 2006; accepted 9 October 2006; published online 11 January 2007

(Tambourgi *et al.*, 2005), as well as, in the human HaCaT keratinocyte cell line (Paixão-Cavalcante *et al.*, 2006).

The regulation of MMPs, once they have been secreted and activated, is a complex process. Several agents have been developed to block the synthesis of MMPs, to prevent their interaction with the molecules on the cell surface or to inhibit their enzymatic activity (Engeblad and Werb, 2002). MMP-9 has been reported to play a crucial role in the transmigration of neutrophils, lymphocytes, and eosinophils (Warner *et al.*, 2001; Lee *et al.*, 2003). Overexpression of MMPs has been implicated in the pathogenesis of several diseases, such as tumor progression, atherosclerotic plaque rupture, aortic aneurisms, acute respiratory distress syndrome, and asthma (Ohnishi *et al.*, 1998; Crawford and Matrisian, 1994–95). In these diseases inhibitors of MMPs are been used therapeutically.

The tetracycline family of antibiotics, in addition to its antimicrobial properties, is also capable of inhibiting MMPs. Tetracyclines and chemically modified tetracyclines have been considered as a potential therapy for human diseases related to MMPs ((Acharya *et al.*, 2004). The mechanism of action of tetracyclines results from their ability to bind metal ions, particularly Ca^{2+} and Zn^{2+}, which are required to maintain the correct conformation and hydrolytic activity of the MMPs. Tetracyclines have also shown to downregulate the synthesis and activation of MMPs (Acharya *et al.*, 2004). In *in vitro* experiments, we have recently shown that tetracyclines can protect the human keratinocytic cell line HaCaT from *Loxosceles* venom-induced MMP-2 and MMP-9 secretion and reduction in cell viability (Paixão-Cavalcante *et al.*, 2006).

The aim of this study was to evaluate the efficacy of tetracyclines in experimental *in vivo* models of loxoscelism and the potential of this class of inhibitor as a therapeutic tool for the treatment of the dermonecrotic lesion.

RESULTS

Loxosceles intermedia venom and SMase P2 reduce cell viability

We have previously shown that *Loxosceles* venom or its main component, the SMase D, induced apoptosis in the human HaCaT keratinocyte cell line, which was associated with an increased expression of metalloproteinase 2 and 9, and that the use of metalloproteinase inhibitors, such as tetracycline, could prevent cell death and potentially control tissue destruction after envenomation. In order to verify if the same occurred with rabbit fibroblast primary cultures, cells were incubated with increasing concentrations of *Loxosceles* venom or SMase D during 3 days and the cell viability analyzed by the Alamar Blue method. Figure 1a shows that both, venom and the recombinant SMase D (P2) were able to induce loss of cell viability; the maximum percentage of death (around 60%) was reached at concentrations of 15 μg/ml. Induction of apoptosis was confirmed by DNA gel electrophoresis. DNA fragmentation was observed in samples obtained from cells treated with venom or SMase D (Figure 1c). The same pattern of degradation was observed after incubation with H_2O_2, a well-known inducer of apoptosis (Cardoso *et al.*, 2004).

In order to further investigate the possible association of loss of cell viability and MMPs expression, rabbit fibroblasts were treated with SMase D (15 μg/ml) in the presence of increasing concentrations of tetracycline, doxycycline, or minocycline. Figure 1b shows that the three tetracycline derivatives at a concentration of 50 μg/ml could fully protect the fibroblasts from the cell death induced by the venom toxin. Treatment of the cells with 50 μg/ml of tetracycline also prevented DNA fragmentation induced in rabbit fibroblast by venom and SMase D, but not by H_2O_2 (Figure 1c).

Development of skin lesion induced by *Loxosceles* venom or SMase D

Our findings of the ability of metalloproteinase inhibitor drugs, such as tetracyclines, in controlling the noxious effects of *Loxosceles* venom on the main components of the skin such as keratinocytes (Paixão-Cavalcante *et al.*, 2006) and fibroblasts is novel and of potential therapeutic significance, which prompted us to investigate the use of this inhibitor in experimental *in vivo* models of cutaneous loxoscelism.

Adult rabbits were inoculated with 5 μg of venom or SMase D and the development of the skin lesion was measured throughout a period of 48 hours. The dermonecrotic lesion induced by crude venom increased during the observed period (Figure 2a and c), reaching the maximal size at 48 hours when compared to the lesion size observed at 6 hours after inoculation. In the animals inoculated with SMase D, the dermonecrotic lesion reached its maximum size 12 hours after injection and decreased over the period of 48 hours (Figure 2b and d).

When the animals, inoculated with venom, were treated twice a day during 48 hours with lanolin cream containing tetracycline (Figure 2a) or doxycycline (Figure 2c) a significant reduction in the development of the lesions was observed. Similarly, topical application with tetracycline or doxycycline inhibited the development the SMase D-induced lesion (Figure 2b and d). Oral administration of tetracycline to venom or SMase D-injected animals was not effective in reducing the lesion (Figure 2a and b). On the other hand, oral administration of doxycycline was capable of controlling the progression of the dermonecrotic lesion in animals injected with venom but not with SMase D component (Figure 2c and d). Similar levels of lesion development inhibition were observed at 48 hours in venom-injected animals treated with doxycycline by oral or topical routes. Oral doxycycline treatment caused a statistically significant protection against SMase injection at 12 hours, however, after 24 hours a statistically significant enhancement of the lesion was observed.

Statistical analysis showed that local treatment with tetracycline and doxycycline significantly inhibited the development of the dermonecrotic lesion induced by *Loxosceles* venom and its major toxic component, the SMase D.

Histological analysis

Rabbits were inoculated with 5 μg of *L. intermedia* venom, SMase D or phosphate-buffered saline (PBS). After 6 hours of inoculation, a typical dermonecrotic lesion was detected in the envenomed animals and at this point the treatment with

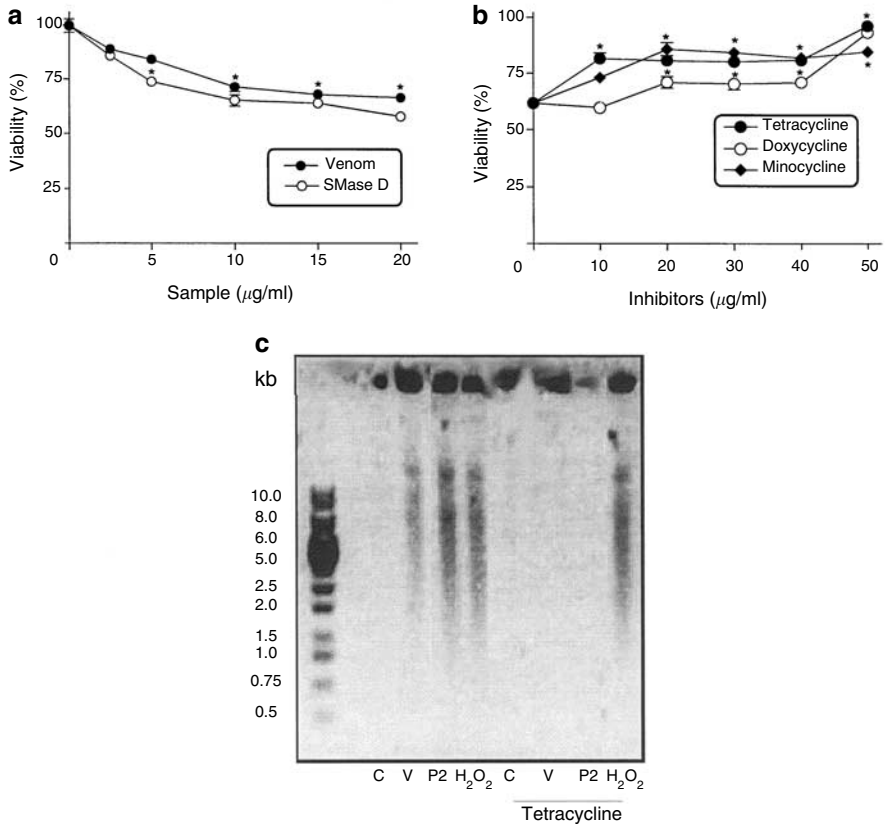

Figure 1. Effect of *L. intermedia* venom and SMase D treatment on rabbit fibroblasts. (**a**) Viability: rabbit fibroblast cell cultures were incubated with increasing concentrations of *Loxosceles* venom (○) or SMase D (●) and after 3 days of treatment, cell viability was analyzed by the Alamar Blue assay. (**b**) Effect of tetracycline, doxycycline, and minocycline on the loss of cell viability: rabbit fibroblast cell cultures were cultivated in 24-well plates in DMEM without FBS. At day zero, cells were treated with 15 μg/ml of SMase D and simultaneously incubated with different concentrations of tetracycline (○), doxycycline (▲), or minocycline (●). After 3 days of treatment the cell viability was analyzed by the Alamar Blue assay. Results are representative for two independent experiments and are expressed as the mean of triplicates ± SD. (**c**) DNA gel electrophoresis: rabbit fibroblasts, cultured in DMEM without FBS, were treated with 15 μg/ml of venom (V) or SMase D (P2) for 24 hours at 37°C in the presence or absence of tetracycline (50 μg/ml). Cells incubated with (**c**) PBS and H_2O_2 (100 μM) were used as negative and positive controls, respectively. After incubation, cells were detached, washed in cold PBS, and the DNA was isolated using Trizol reagent and analyzed by agarose gel electrophoresis.

the tetracyclines was initiated. After 48 hours, the animals were killed and skin specimens were obtained for histological examination and neutrophils quantification.

Skin samples obtained from PBS-inoculated animals showed a thin epidermis and a normal pattern for the collagenous area and muscle fibers (Figure 3a1–d1). Skin samples obtained from *L. intermedia* venom or SMase D (not shown) inoculation sites showed a thin epidermis, dissociation of the collagenous fibers owing to the edema, degeneration of muscle fibers, moderate hemorrhage in the superficial dermis and intense neutrophil infiltration in the deep dermis (Figure 3a2–d2).

Histological evaluation of the skin of the rabbits injected with venom and treated with lanolin showed at the inoculation site a general lesion profile similar to that observed in non-treated animals (Figure 3a3–d3). Histological analysis of skin sections from animals inoculated with venom and topically or orally treated with tetracycline, although revealing a similar dermonecrotic lesion profile at the site of venom injection, showed no hemorrhage in the superficial dermis (Figure 3a4–d4 and a5–d5, respectively). Moreover, no vessel damage was observed in animals inoculated with venom and topically treated with tetracycline (data not shown). Figure 3a5–c5, also shows that oral tetracycline treatment reduced the edema and prevented muscle damage. These positive drug effects at the site of venom or SMase D inoculation were not observed after topical or oral administration of doxycycline (Figure 3a6–d6 and a7–d7, respectively). In these treatments edema, muscle degeneration, hemorrhage in the superficial dermis, and intense neutrophil infiltration in the deep dermis were observed at the site of venom inoculation.

Effect of tetracyclines on PMN infiltration

Neutrophils quantification showed that both venom (Figure 3e2–e7) and SMase D (data not shown) induced migration of PMN into the deep dermis. Topical treatment with tetracycline or doxycycline reduced the number of neutrophils found in the deep dermis of venom-injected animals (Figure 3e4–e6, respectively; Figure 4a), but only

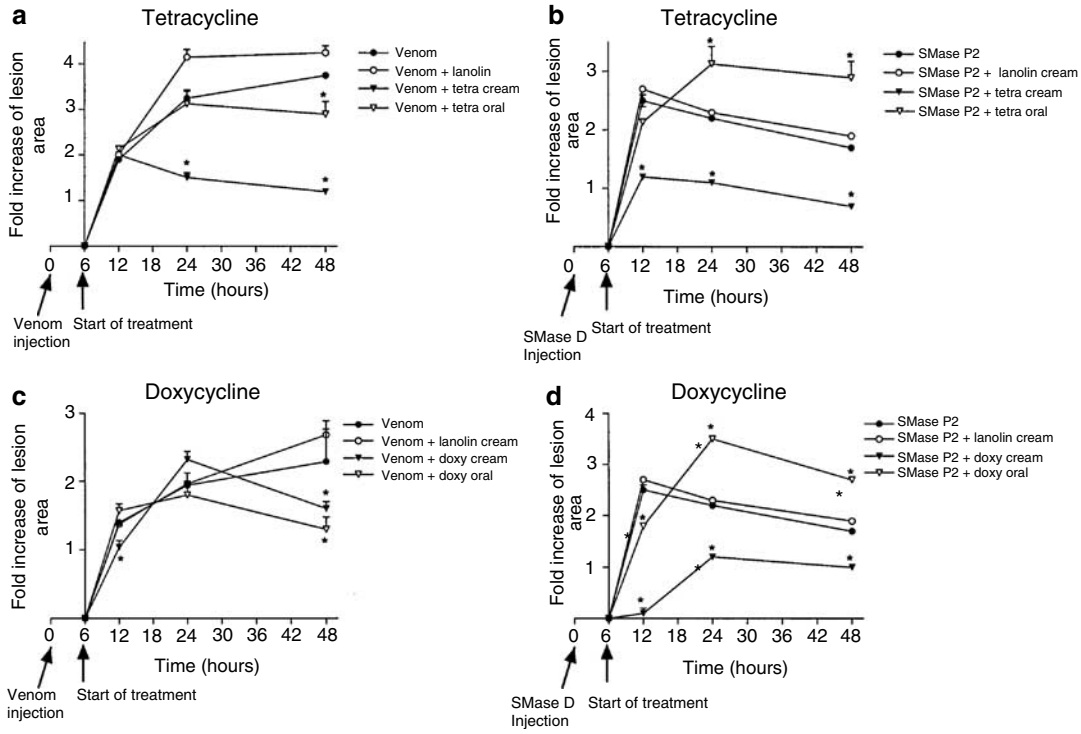

Figure 2. Protective effect of tetracyclines on the development of dermonecrotic lesion. Adult rabbits were injected intradermally with 5 μg of venom or SMase P2 from *L. intermedia* spider. After 6 hours treatments with (**a** and **b**) tetracycline or (**c** and **d**) doxycycline were initiated. The animals were treated topically (▼) with a cream containing lanolin and 5% of doxycycline or tetracycline, or orally (▽) with 15 mg/kg of the inhibitors, twice a day during 48 hours. Control animals were inoculated with venom or SMase D and not treated (●) or treated with lanolin cream alone (○). Results are representative for four independent experiments and are expressed as the mean of triplicates ± SD. The asterisks indicate values statistically different ($P<0.05$) from the controls, that is, non-treated, or lanolin cream-treated venom, or SMase D-injected animals.

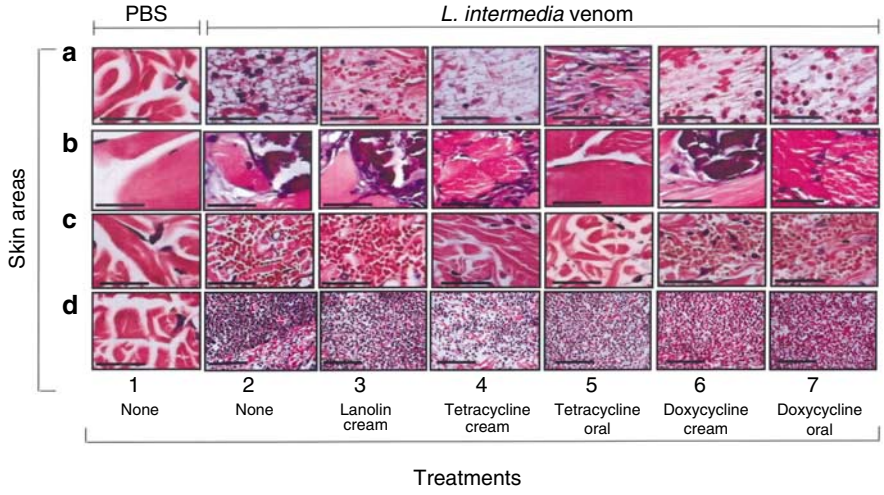

Figure 3. Histological analysis of the dermonecrotic lesion induced by *L. intermedia* venom and SMase D after MMP inhibitors treatment. Rabbits were injected with 5 μg of *L. intermedia* venom and, after 6 hours of inoculation, treated twice a day during 48 hours with tetracycline or doxycycline, as described in Materials and Methods. Control sites were injected with an equal volume of PBS. Panels correspond to skin sections from rabbits injected with (panel 1: **a**–**d**) PBS; (panel 2: **a**–**d**) *L. intermedia* venom; (panel 3: **a**–**d**) *L. intermedia* venom and treated with cream of lanolin alone or (panel 4: **a**–**d**) containing tetracycline or (panel 6: **a**–**d**) doxycycline; (panel 5: **a**–**d**) *L. intermedia* venom and orally treated with tetracycline or (panel 7: **a**–**d**) doxycycline. Skin areas are (**a**) collagenous area, (**b**) muscle fibers, superficial dermis: detail of (**c**) hemorrhage, deep dermis: detail of (**d**) neutrophil infiltration. Bar = 50 μm.

treatment with tetracycline reached statistical significance. Oral treatment of venom-injected animals with tetracyclines had no effect on PMN influx (Figure 3e5–e7; Figure 4b). Similar results were obtained for animals inoculated with SMase D and oral or topically treated with tetracyclines (data not shown).

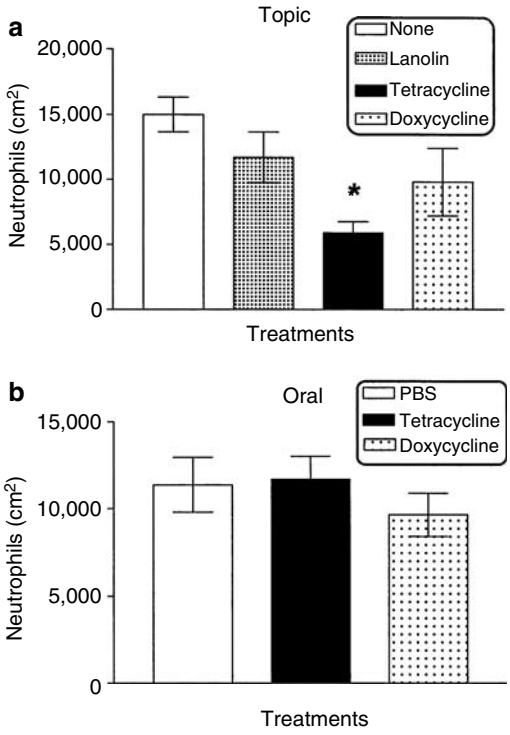

Figure 4. Determination of neutrophils number in the rabbit skin. Number of neutrophils in the histological skin sections obtained from rabbits injected with 5 μg of *L. intermedia* venom and treated twice a day with tetracycline or doxycycline as described in Materials and Methods by (**a**) local or (**b**) oral routes. Control animals were inoculated with venom and not treated or treated with lanolin cream. Neutrophils quantification was performed in histological rabbit skin sections by counting the number of cells identified by morphological criteria. Results are representative of four different experiments and represented as mean ± SD. $*P<0.05$.

Effect of tetracyclines on the MMPs expression

We have recently shown that *Loxosceles* venom and SMase D induce an increase in the expression of MMP-9 and MMP-2 in rabbit skin and the human keratinocyte cell line HaCaT (Tambourgi *et al.*, 2005; Paixão-Cavalcante *et al.*, 2006). To investigate the action of tetracyclines on MMP expression in loxoscelism, rabbit skin sections of envenomated and control animals, treated or not with tetracycline, or doxycycline, were analyzed by gelatin zymography. In skin from control rabbits only one band with gelatinolytic activity (Mr around 68 kDa; Figure 5a), was observed, which we previously identified as MMP-2 (Tambourgi *et al.*, 2005). As we have previously shown, *Loxosceles* venom induced a band with gelatinolytic activity (Mr around 90 kDa; Figure 5b), identified as MMP-9 (Tambourgi *et al.*, 2005). Topical treatment of rabbits with tetracycline or doxycycline significantly reduced expression of MMP-2 and MMP-9 (Figure 5d and e); tetracycline even completely prevented the induction of MMP-9 (Figure 5d). Oral administration of tetracycline or doxycycline did not have a large effect on expression of gelatinases. The application of lanolin cream did not prevent the expression of the MMPs in envenomated animals (Figure 5c).

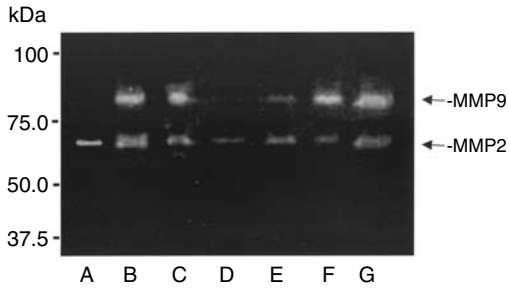

Figure 5. *Loxosceles* venom induced gelatinases expression in skin rabbit. Gelatinase activity was analyzed by zymography in skin samples obtained from animals injected with (**a**) PBS, (**b**) *L. intermedia* venom, and (**c**) topically treated with lanolin cream alone, or (**d**) containing tetracycline, or (**e**) doxycycline, or (**f**) orally treated with tetracycline, or (**g**) doxycycline. The arrows indicate gelatinase expression.

DISCUSSION

Envenomation by *Loxosceles* spiders is a well-documented cause of necrotic skin lesions in humans. We have previously shown that the Complement system plays an important role in development of cutaneous loxoscelism through C5a and membrane attack complex induced recruitment of neutrophils. Furthermore, *Loxosceles* induced expression of MMP-9, which is possibly one of the main factors involved in the pathogenesis of the cutaneous loxoscelism (Tambourgi *et al.*, 2005). We have also demonstrated that *Loxosceles* venom and the SMase D provoked apoptosis in the HaCaT immortalized keratinocyte cell line by an indirect process, which involves an augmented expression and secretion of MMP-2 and MMP-9 and that the metalloproteinase inhibitor drugs, such as tetracyclines, could control *in vitro* the noxious effects of *Loxosceles* venom associated with the dermonecrotic process (Paixão-Cavalcante *et al.*, 2006). Considering the novelty and potential therapeutic significance of these results we decided to use the metalloproteinase inhibitors in *in vitro* and *in vivo* rabbit model of cutaneous loxoscelism.

Data presented here show that venom and the recombinant SMase D P2 from *L. intermedia* spider induce reduction of rabbit fibroblast viability, which occurred by apoptosis, as loss of membrane asymmetry (data not shown) and DNA degradation were detected in these cells. In our experimental model the classic tetracycline antibiotics (tetracycline, doxycycline, and minocycline) modulated the toxic action of *L. intermedia* venom SMase D on fibroblast cells, inhibiting the DNA degradation and thus protecting cells from loss of viability.

These data together with our previous findings (Tambourgi *et al.*, 2005; Paixão-Cavalcante *et al.*, 2006) prompted us to test these inhibitors in experimentally envenomated rabbits. Results presented here show that tetracycline, applied on the area of the lesion 6 hours after venom or SMase D inoculation, largely prevented the development of the dermonecrosis. The treatment with doxycycline, although capable of controlling the increase of the lesion area, was less efficient than the tetracycline. Oral administration of tetracyclines had some beneficial effect in controlling the size of

the lesion of venom-injected animals, but this was not observed for the SMase D-inoculated rabbits. The observation that oral administration of the tetracyclines was not as effective as the topical treatment can be due to concentration of drug used. Experiments using higher concentrations of tetracyclines in oral administration of normal and envenomated animals will be conducted in further studies in order to solve this question. However, higher systemic doses of tetracycline itself may lead to toxicity.

Treatment with the tetracyclines was not able to fully block the local inflammatory process, which was already initiated before the start of the treatment. Although revealing a similar dermonecrotic lesion profile at the site of venom injection, animals treated with tetracycline in oral or topical ways showed no hemorrhage in the superficial dermis. No vessel damage was observed in envenomated rabbits topically treated with tetracycline. These positive drug effects were not observed by topical or oral administration of doxycycline. Moreover, venom-injected animals, which were treated with creams of tetracycline or doxycycline, showed reduction of neutrophil infiltration.

We have previously shown (Tambourgi *et al.*, 2005) in the rabbit model for cutaneous loxoscelism that *Loxosceles* spider venom and its SMase D induced a large influx of neutrophils, concomitant with dissociation of the collagenous fibres in the dermis. Decomplementation, using cobra venom factor, largely prevented the influx of neutrophils, whereas influx of neutrophils was also reduced in genetically C6-deficient rabbits, suggesting roles for both C5a and the membrane attack complex in the induction of dermonecrosis. However, this reduction in the neutrophil influx did not prevent the hemorrhage and the collagenous injury; whereas induction of MMP-9 was partially reduced in complement depleted rabbits, suggesting that neutrophils are not the only source of MMPs. In fact, data presented here show that fibroblasts also produce MMPs after venom/SMase contact. Moreover, *in vitro* experiments using human keratinocytic cell line HaCaT (Paixão-Cavalcante *et al.*, 2006) or primary rabbit fibroblasts cultures (Figure 1) showed that *Loxosceles* venom/SMase D induces cell death, which is associated with an increased expression/secretion of MMP-2 and MMP-9. The cell death and MMPs expression/secretion can be completely inhibited by the use of tetracyclines. These data indicate that beneficial effect of the tetracyclines involves reduction of inflammation but also of the expression/secretion of MMPs and consequently cell death.

Analysis of the action of tetracyclines on MMPs expression on an *in vivo* model of loxoscelism, revealed that MMP-2 and MMP-9 gelatinolytic activity induced by *L. intermedia* venom was significantly reduced or abrogated by topical treatment with tetracycline or doxycycline but not controlled by oral administration of the drugs. These results positively correlate with the reduction of the lesion size observed in animals treated with the lanolin cream containing tetracycline or doxycycline, indicating that the spreading of the dermonecrotic lesion may in part owing to the production of MMPs. The development of the dermonecrotic reaction in rabbit model could also be controlled by injecting the animals with venom or SMase D in the presence of 1.10-phenanthroline, but not with PMSF (inhibitor for serinoproteinase) (data not shown).

Metalloproteinases are present in healthy individuals and have been shown to be involved in various physiological and pathological processes. Regulation of metalloproteinases gene expression in normal tissues is tightly controlled to limit the biological activity. Apart from the primary function of remodelling, the extracellular MMPs have been suggested to play an important role in various physiological conditions such as fetal tissue development and post-natal tissue repair, and certain pathological conditions as periodontitis, autoimmune disorders of skin, and dermal photoaging (Kahari and Saarialho-Kere, 1997). The regulation of MMPs, once they have been secreted and activated, is a complex process that evolves a network of activation in cascade where one MMP acts as substrate for another (Johansson *et al.*, 2000). Of all the pharmacological targets, inhibition of the function of MMPs in extracellular matrix after secretion is being most actively pursued as a potential anticancer strategy. Several agents have been developed to block the synthesis of MMPs, preventing the interaction with molecules that improve their activities and direct these molecules to the cell surface or inhibit their enzymatic activity (Engeblad and Werb, 2002). Besides the ability of tetracyclines to inhibit protein synthesis by binding to the 30S subunit of bacterial ribosomes, they also have the ability to bind metal ions, which are required by enzymes to maintain its correct conformation and hydrolytic activity (Acharya *et al.*, 2004). The tetracycline derivates inhibit not only the activity but also the production of MMPs. These agents inhibit collagenases, MMP-1, MMP-3, and MMP-13, and gelatinases, MMP-2 and MMP-9, via multiple mechanisms including: blocking the activity of mature MMPs by chelation of zinc atom at the active site; interfering with the proteolytic activation of pro-MMP into their active form; reducing the expression of MMPs and protecting MMPs from proteolytic and oxidative degradation (Golub *et al.*, 1998).

Recent data, utilizing rabbits as experimental models, showed that current therapies used to treat loxoscelism like dapsone, colchicines, triamcinolone, and diphenhydramine were inefficient in controlling the lesion progression even after 7 days of treatment (Elston *et al.*, 2005) and the usual treatments used for cutaneous loxoscelism in humans can induce many collateral effects (Issekutz *et al.*, 1987; Bitterman-Deutsch *et al.*, 1990; Wille and Morrow, 1988). Tetracyclines are generally well tolerated by humans and side effects like hypersensitivity syndrome reaction, single organ dysfunction, serum sickness-like reaction as well as drug-induced lupus are rare occurrences associated with the administration of these inhibitors (Shapiro *et al.*, 1997) and side effects are less likely to occur after topical application. The use of tetracycline in the treatment of cutaneous loxoscelism, has the additional advantage that, owing to its broad spectrum antimicrobial action, it may prevent infection of the wound.

In conclusion, we have shown here that local application of tetracycline, 6 hours after envenomation with *Loxosceles* venom (the average time point after which a patient may

consult a physician), significantly reduces lesion formation and MMP-2 and MMP-9 production, suggesting that tetracycline may offer a valuable and relatively safe therapeutic agent in the treatment of cutaneous loxoscelism.

MATERIALS AND METHODS

Chemicals, reagents, and buffers

Gelatin, Triton X-100, tetracycline, doxycycline, and minocycline were purchased from Sigma (St Louis, MO). Tetracycline and doxycycline, 5% in a hydrosoluble lanolin cream were obtained from Biofórmula handle pharmacy (São Paulo, Brazil). Trizol reagent was from Invitrogen Inc. (Ontario, Canada).

Venom

L. intermedia Mello-Leitão spiders were provided by ''Laboratório de Imunoquímica, Instituto Butantan, São Paulo, Brazil''. The venom was obtained by electrostimulation by the method of Bücherl (1969), with slight modifications. Briefly, 15–20 V electrical stimuli were repeatedly applied to the spider sternum and the venom drops were collected with a micropipette, vacuum dried, and stored at −20°C. Stock solutions were prepared in PBS (10 mM Na phosphate, 150 mM NaCl, pH 7.2) at 1.0 mg/ml.

Sphingomyelinase expression

Recombinant *L. intermedia* SMase D, isoform P2 (accession number: AY304472), was expressed in *Escherichia coli* strain BL21 (DE3) as a fusion protein composed of the mature SMase with a N-terminal extension containing a 6 × histidine tag and purified as described (Tambourgi *et al.*, 2004).

Experimental animals

Adult New Zealand white rabbits weighing approximately 3 kg were obtained from ''Biotério de Animais do Instituto Butantan'', São Paulo, Brazil. The animals were maintained and used under strict ethical conditions according to the animal welfare international recommendations. All procedures with animals were carried out in accordance with institutionally approved protocols.

Cell culture and maintenance

Fibroblasts were isolated from adult male New Zealand rabbits by treatment of skin sections, obtained from killed normal animals with 0.25% trypsin in DMEM (Gibco-BRL, Gaithesburg, MD) for 1 hour. The cell suspension was filtered over sterile gauze and cells were washed twice and cultured in DMEM supplemented with 10% (vol/vol) heat-inactivated (56°C, 30 minutes) fetal bovine serum (FBS; Gibco-BRL), 100 IU/ml penicillin, and 100 IU/ml streptomycin at 37°C in humidified air with 5% CO_2. After 48 hours of culture, medium containing non-adherent cells and debris was removed and fresh medium was added.

Viability assay

The cell viability was analyzed by Alamar Blue Assay (Nakayama GR *et al.*, 1997). Briefly, rabbit fibroblasts were subcultured in 24-well plates. Cells at 50–70% confluence at passage 5 were maintained overnight in DMEM without FBS followed by incubation with different concentrations of venom or SMase P2 in the presence or absence of increasing amounts of the different metalloproteinases inhibitors, for example, tetracycline, minocycline, or doxycycline diluted in DMEM without FBS. DMEM without FBS was used as the control. After 3 days of incubation, the culture supernatants were collected and the cells were incubated with 250 μl per well of DMEM plus 10% of Alamar Blue Reagent (Biosource International, CA) for 1 hour at 37°C. Alamar Blue added to wells not containing cells was used as the background control. Supernatants of each sample (100 μl) were collected and mixed with 100 μl of water and the absorbance was measured in a spectrophotometer (Multiskan-EX, Labsystems, Finland) at 540 and 620 nm. The relative cell viability was calculated as: ((Sample $OD_{(540–620\,nm)}$−Background control $OD_{(540–620\,nm)}$)/(Control $OD_{(540–620\,nm)}$−Background OD $_{(540–620\,nm)}$)) × 100.

The Student's *t*-test was used to determine the significance of the differences between the mean values of cell viability from control and experimental samples. The minimal level of significance was considered as $P<0.05$.

Analysis of DNA fragmentation

Rabbit fibroblasts were treated with venom or SMase P2 (15 μg/ml) for 24 hours and DNA was isolated using Trizol reagent (Invitrogen Inc.), according to the manufacturer's instructions. After 5 minutes of incubation, chloroform was added and the samples were centrifuged at 12,000 r.p.m. for 15 minutes at 4°C. The interface containing DNA was collected and the DNA was precipitated, washed and solubilized in water, and analyzed by agarose gel electrophoresis. As positive and negative controls, DNA samples were obtained from cells treated with 100 μM of H_2O_2 or medium, respectively. Samples of DNA (10 μg per well) were loaded on a 1% agarose gels containing 1 μg/ml of ethidium bromide. The electrophoresis was performed for 1 hour at 90 V and the DNA visualized by UV fluorescence.

Dermonecrosis and treatment with inhibitors

Samples of 200 μl of *L. intermedia* venom or SMase D (25 μg/ml) in PBS (pH 7.2, 10 mM Na phosphate, 150 mM NaCl) were injected intradermally in the shaved back of rabbits. After 6 hours of inoculation, the animals were treated with tetracycline or doxycycline by two different schedules: (a) oral: direct administration into the mouth (15 mg/kg in H_2O) or (b) local: topical administration to the lesion area of the hydrosoluble lanolin cream containing 5% of the tetracyclines. Negative control groups were animals intradermally inoculated with PBS and after 6 hours treated as the experimental groups. Positive control groups were animals intradermally inoculated with venom or SMase D and treated or not with hydrosoluble lanolin (vehicle). The size of the lesions was measured 6, 12, 24, and 48 hours following injection. The progression of the size of the lesion was expressed as a fold increase and determined by the formula: fold increase = area of the lesion at 12, 24, or 48 hours after envenomation (in square centimeters)/lesion area at 6 hours after venom injection (in square centimeters). The experiments were repeated four times using three animals per group. After 48 hours, the animals were killed and skin specimens were obtained for histological examination.

Histological analysis

Skin samples were fixed in 10% buffered formalin solution, and then embedded in paraffin. Tissue sections were stained with hematoxylin and eosin and examined for the presence of epithelial necrosis, epithelial slough, dermal infiltrates, hemorrhage, and level of collagen dissociation in the dermis and skin muscle fiber degeneration.

Cell counting

Neutrophil quantification was performed in histological rabbit skin sections by counting the number of cells, identified by morphological criteria, in five different areas in the dermis (18 mm^2 each), using the Image Processing and Analysis System and QWin Plus Y2.8. program from Leica (Leica Microsytems, Cambridge, UK). The Student's *t*-test was used to determine the significance of the differences between the mean values of cell counts obtained for sections from control and experimental groups of rabbits. The minimal level of significance was considered as $P<0.05$.

Zymography

Gelatinase activity in supernatants of homogenized skin samples was analyzed by zymography (Kleiner and Stetler-Stevenson, 1994). Skin samples were homogenized in SDS-PAGE sample buffer at 1 g/ml in a tissue grinder, spun (1500 r.p.m. for 10 minutes) to pellet debris, the supernatants were recovered, and 20 μl of each sample were run under non-reducing conditions on a 10% polyacrylamide gel containing 1% gelatine. The gels were washed twice for 30 minutes at room temperature in 2.5% Triton X-100, and overnight incubated at 37°C in zymography buffer (50 mM Tris-HCl, 200 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij-35; pH 8.3). Gels were stained in Coomassie Brilliant Blue solution (40% methanol, 10% acetic acid, and 0.1% Coomassie Brilliant Blue).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This research was supported by grants from FAPESP, CNPq, and The Wellcome Trust.

REFERENCES

Acharya MR, Venitz J, Figg WD, Sparreboom A (2004) Chemically modified tetracyclines as inhibitors of matrix metalloproteinases. *Drug Resistence Updates* 7:195–208

Allen C (1992) Arachnid envenomation. *Emerg Med Clin North Am* 10:269–99

Anderson PC (1997) Spider bites in the United States. *Dermathol Clin* 15:307–11

Barrett SM, Romine-Jenkins M, Fisher DE (1994) Dapsone or electric shock treatment of brown recluse spider envenomation. *Ann Emerg Med* 24:21–5

Bitterman-Deutsch O, Bergman R, Friedman-Birnbaum R (1990) Brown spider bite. *Harefuah* 119:137–9

Bryant SM, Pittman LM (2003) Dapsone use in *Loxosceles reclusa* enevenomation: is there an indication? *Am J Emerg Med* 21:89–90

Bücherl W (1969) Biology and venom of the most important South American spiders of the genera *Phoneutria*, *Loxosceles*, *Lycosa* and *Latrodectus*. *Am Zool* 9:157–9

Cardoso SM, Rego AC, Penacho N, Oliveira CR (2004) Apoptotic cell death induced by hydrogen peroxide in NT2 parental and mitochondrial DNA depleted cells. *Neurochem Int* 45:693–8

Crawford HC, Matrisian LM (1994–95) Tumor and stromal expression of matrix metalloproteinases and their role in tumor progression. *Invasion Metastais* 14:234–45

Denny WF, Dillaha CJ, Morgan PN (1964) Hemotoxic effect of *Loxoscels reclusa* venom: *In vivo* and *in vitro* studies. *J Lab Clin Med* 64:291

Dillaha CJ, Jansen T, Honeycut WM, Hayden CR (1964) North America loxoscelism. *JAMA* 188:33–6

Elston DM, Miller SD, Young RJ III, Eggers J, McGlasson D, Schmidt WH *et al.* (2005) Comparation of colchicines, dapsone, triamcinolone and diphenhydramine therapy for the treatment of brown recluse spider envenomation. *Arch Dermatol* 141:595–7

Engeblad M, Werb Z (2002) New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* 2:161–74

Fernandes-Pedrosa MF, Junqueira de Azevedo IL, Goncalves-de-Andrade RM, van den Berg CW, Ramos CR, Ho PL *et al.* (2002) Molecular cloning and expression of a functional dermonecrotic and haemolytic factor from *Loxosceles laeta* venom. *Biochem Biophys Res Commun* 298: 638–45

Futrell JM (1992) Loxoscelism. *Am J Med Sci* 304:261–7

Golub LM, Lee HM, Ryan ME, Giannobile WV, Payne J, Sorsa T (1998) Tetracyclines inhibit connective tissues breakdown by multiple non-antimicrobial mechanisms. *Adv Dental Res* 12:12–26

Issekutz AC, Megyeri P, Issekutz TK (1987) Role of macrophages products in endotoxin-induced polymorphonuclear leukocyte accumulation during inflammation. *Lab Invest* 56:49–59

Johansson N, Ahonen M, Kahari VM (2000) Matrix metalloproteinases in cancer invasion. *Cell Mol Life Sci* 57:5–15

Kahari VM, Saarialho-Kere U (1997) Matrix metalloproteinases in skin. *Exp Dermatol* 6:199–213

Kleiner DE, Stetler-Stevenson WG (1994) Quantitative zymography: detection of picogram quantities of gelatinases. *Anal Biochem* 218:325–9

Koh WL (1998) When to worry about spiders bite: inaccurate diagnosis can have serious, even fatal, consequences. *Postgrad Med* 103: 235–250

Kurpiewski G, Forrester LJ, Barrett JT, Campbell BJ (1981) Platelet aggregation and sphingomyelinase D activity of a purified toxin from the venom of *Loxosceles reclusa*. *Biochim Biophys Acta* 678:467–76

Lee KS, Jin SM, Kim HJ, Lee YC (2003) Matrix metalloproteinase inhibitor regulates inflammatory cell migration by reducing ICAM-1 and VCAM-1 expression in a murine model of toluene diisocyanate-induced asthma. *J Allergy Clin Immunol* 111:1278–84

Mold JW, Thompson DM (2004) Management of brown recluse spider bites in primary care. *J Am Board Fam Pract* 1:347–52

Nakayama GR, Caton MC, Nova MP, Paranoosh Z (1997) Assessment of the Alamar Blue assay for cellular growth and viability *in vitro*. *J Immunol Methods* 26:205–87

Ohnishi K, Takagi M, Kurikawa Y, Satomi S, Konttinen YI (1998) Matrix metalloproteinase-mediated extracellular matrix protein degradation in human pulmonary emphysema. *Lab Invest* 78:1077–87

Paixao-Cavalcante D, van den Berg CW, Fernandes-Pedrosa MF, Gonçalves-de Andrade RM, Tambourgi DV (2006) Role of matrix metalloproteinases in HaCaT keratinocytes apoptosis induced by *Loxosceles* venom sphingomyelinase D. *J Invest Dermatol* 126:61–8

Pizzi T (1975) A histopathological study on necrotic arachnidism by *Loxosceles laeta*. *Bol Chil Parasitol* 30:34–6

Rees RS, Shack B, Withers F, Madden J, Franklin J, Lynch JB (1981) Management of the brown recluse spider bite. *Plast Reconstr Surg* 68:768–73

Schenone H, Saavedra T, Rojas A, Villarroel F (1998) Loxoscelism in Chile. Epidemiologic, clinical and experimental studies. *Rev Inst Med Trop Sao Paulo* 31:403–15

Shapiro LE, Knowles SR, Shear NH (1997) Comparative studies of tetracycline, minocycline and doxycycline. *Arch Dermatol* 133:1224–30

Smith CW, Micks DW (1970) The role of polymorphonuclear leukocytes in the lesion caused by the venom of the brown spider, *Loxosceles reclusa*. *Lab Invest* 22:141–4

Sunderkotter C, Seeliger S, Schonlau F, Roth J, Hallmann R, Luger TA *et al.* (2001) Different pathway leading to cutaneous leukocytoclastic vasculitis in mice. *Exp Dermatol* 10:391–404

Tambourgi DV, de Sousa da Silva M, Billington SJ, Gonçalves-de-Andrade RM, Magnoli FC, Songer JG *et al.* (2002) Mechanism of induction of complement susceptibility of erythrocytes by spider and bacterial sphingomyelinases. *Immunology* 107:93–101

Tambourgi DV, Fernandes Pedrosa MF, van den Berg CW, Gonçalves-de-Andrade RM, Ferracini M, Paixão-Cavalcante D *et al.* (2004) Molecular cloning, expression, function and immunoreactivities of members of a gene family of sphingomyelinases from *Loxosceles* venom glands. *Mol Immunol* 41:831–40

Tambourgi DV, Magnoli FC, van den Berg CW, Paul Morgan B, Araujo PS, Alves EW *et al.* (1998) Sphingomyelinase in the venom of the spider *Loxosceles intermedia* are responsible for both dermonecrotic and complement-dependent hemolysis. *Biochem Biophys Res Commun* 251:366–73

Tambourgi DV, Morgan BP, Gonçalves-de-Andrade RM, Magnoli FC, van den Berg CW (2000) *Loxosceles intermedia* spider envenomation induces activation of an endogenous metalloproteinase, resulting in cleavage of glycophorins from the erythrocytes surface and facilitating complement-mediated lysis. *Blood* 95:638–91

Tambourgi DV, Paixão-Cavalcante D, Gonçalves-de-Andrade RM, Fernandes-Pedrosa MF, Magnolia FC, Paul Morgan B *et al.* (2005) *Loxosceles* sphingomyelinase induces complement-dependent dermonecrosis, neutrophil infiltration, and endogenous gelatinase expression. *J Invest Dermatol* 124:725–31

van den Berg CW, Gonçalves-de-Andrade RM, Magnoli FC, Marchbank KJ, Tambourgi DV (2002) *Loxosceles* spider venom induces metalloproteinases mediated cleavage of MCP/CD46 and MHC I and induces protection against C-mediated lysis. *Immunology* 107:102–10

Walter FG, Bilden EF, Gibly RL (1999) Envenomations. *Crit Care Clin* 15:627–9

Ward PA, Cochrane CB (1965) Bound complement and immunologic injury of blood vessels. *J Exp Med* 121:215–34

Warner RL, Lewis CS, Beltran L, Younkin EM, Varani J, Johnson KJ (2001) The role of metalloproteinase in immune complex induced acute lung injury. *Am J Pathol* 158:2139–44

Wille RC, Morrow JD (1988) Case report: dapsone hypersensitivity syndrome associated with treatment of the bite of brown spider. *Am J Med Sci* 296:270–1

Wilson JR, Hagood CO Jr, Prather ID (2005) Brown recluse spider bites: a complex problem wound. A brief review and case study. *Ostomy Wound Manage* 51:59–66