Direct Correlation between Diffusion of *Loxosceles reclusa* Venom and Extent of Dermal Inflammation

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Abstract. **Objectives:** Envenomation by Loxosceles species (brown recluse) spiders results in large dermal inflammatory lesions. Venom-induced dermal inflammation occurs *indirectly* via soluble mediators of inflammation. This study aimed to explore whether the anatomic extent of dermonecrotic arachnidism is due to the cascade of soluble proinflammatory mediators elicited by venom deposited at the bite site, or due to diffusion of the venom per se. **Methods:** Three New Zealand white rabbits received intradermal *L. reclusa* venom (3-μg) injections in the flank. At the time of maximum dermal inflammation (24 hr), paired 4-mm dermal biopsies were obtained in 2-cm intervals extending 0 to 12 cm from the inoculation site. Normal dermal tissue was obtained from the opposite flank to serve as a negative control. One biopsy sample from each interval was homogenized and assayed for myeloperoxidase (MPO) activity and for the presence of venom via an enzyme immunoassay (EIA). The other paired dermal biopsy was sectioned, and examined for the presence of polymorphonuclear neutrophils (PMNs) by microscopy. Lesional areas were measured using digital images imported into imaging software. **Results:** Mean ± SD lesional diameter 24 hours post inoculation measured 9.18 ± 0.64 cm. Venom was detected in biopsies 0 to 10 cm from the injection site. As expected, the highest venom concentrations were measured at the inoculation site (4.28 ± 3.9 ng/4 mm). In addition, PMNs and MPO were detected up to 8 and 10 cm from the inoculation site, respectively. Neither PMNs nor MPO was detected in tissue absent of venom (kappa = 0.88, $p < 0.001$). **Conclusions:** Loxosceles venom diffuses from the envenomation site. The extent of dermal inflammation mirrors the extent of Loxosceles venom diffusion. This observation implies that the venom itself defines the extent and magnitude of tissue injury following Loxosceles envenomation. **Key words:** Loxosceles; spider; venom; arachnidism. ACADEMIC EMERGENCY MEDICINE 2001; 8:309–314

LOXOSCELES arachnids are indigenous American spiders that possess a venom capable of causing intense dermal inflammation and disfiguring necrotic ulcers.[1,2] Among the 54 recognized species of Loxosceles that are found in North America[3], *L. reclusa*, commonly called the brown recluse spider, is the major species responsible for envenomation in the United States.[2] Clinical presentation varies from local cutaneous inflammation and necrosis to systemic loxoscelism. The bite, which initially may cause only minor discomfort, begins as an enlarging circular area of erythema and edema that extends 5–10 cm or more (in severe cases) in diameter. A centrally located necrotic ulcer often forms 8–24 hours following envenomation.[1,2]

We recently produced fragment antigen-binding (Fab) fragments that are effective in attenuating venom-induced dermal inflammation if administered intradermally within 4–8 hours following venom inoculation.[4] Since individuals bitten by Loxosceles species spiders generally seek medical attention later than this time frame, we set out to better understand the relative importance of venom diffusion versus mediator diffusion. This is an important issue from both conceptual and practical standpoints since virtually all Loxosceles antivenom investigations have used the intradermal approach.[4–8] The decreased efficacy of intradermal application of antivenom over time may be due to a rapid diffusion of venom away from the focal point of envenomation. Another possibility is that the events that result in inflammation and necrosis may be initiated rapidly following venom inoculation. In this scenario, soluble mediators of inflammation are induced and secreted by dermal cells shortly after envenomation. Once the early events leading to inflammation have begun, venom neutralization with antibody-binding fragments may occur too late to be effective. This is plausible since Loxosceles venom does not induce dermone-

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crotic arachnidism via direct effects on dermal tissue.[9] The current model summarizing the cellular and molecular basis for tissue events postulated to occur following Loxosceles envenomation is as follows (Fig. 1): after envenomation dermal endothelial and epithelial cells produce and secrete the chemokines interleukin-8,[10–12] growth-related oncogene-alpha,[11,12] and monocyte chemoattractant protein-1.[11] Endothelial cells secrete the cytokine granulocyte/ macrophage-colony-stimulating factor and express the adhesion molecule E-selectin on the cell surface.[10] Neutrophils migrate to the bite site and adhere to intercellular junctions via the E-selectin tethering protein.[10] Neutrophils are subsequently activated and degranulate, thus resulting in tissue inflammation and necrosis.[10]

In view of the indirect mechanism of Loxosceles-induced dermal inflammation, we set out to investigate the hypothesis that the immediate presence of venom is not required in the lesional area in order to induce dermonecrotic arachnidism.

METHODS

Study Design. This was a prospective, randomized, controlled, unblinded laboratory investigation examining whether the immediate presence of Loxosceles venom is required to induce dermal inflammation in the rabbit model. The study had the approval of the University of Michigan Committee on the Use and Care of Animals. All experiments were in accord with the standards outlined in the *Guide for the Care and Use of Laboratory Animals*, and were supervised by veterinarians from the Unit for Laboratory and Animal Care of the University of Michigan Medical School.

Study Setting and Animal Subjects. Inbred New Zealand white rabbits (n = 3) were used for this investigation. The white rabbit is a well-established animal model for cutaneous arachnidism because it displays local and systemic reactions similar to those of humans.[13] Rabbits were randomized by number drawing and received *L. reclusa* venom (3 μg intradermally) in the flank. This venom dose was chosen since it results in reproducible (and easily measured) lesional areas in the 30–50-cm^2 range in pilot animals. Although the actual amount of venom deposited during a native spider bite is not known, the amount of venom obtained from a spider during a single electrical stimulation during commercial milking is approximately 2–3 μg (personal communication, Charles Kristensen, SpiderPharm, Feasterville, PA).

Study Protocol. Digital photographs of the inflammatory lesions were taken 24 hours following venom inoculation and images were imported into a computer. We have observed that peak dermonecrotic inflammation occurs at the 24-hour time point in the rabbit model. Lesional areas were traced and measured using National Institutes of Health (NIH) imaging software. Animals were subsequently sacrificed and multiple-paired 4-mm dermal biopsies were obtained in 2-cm intervals 0 to 12 cm from the inoculation site. Normal dermal tissue was obtained from the opposite flank to serve as a negative control. One biopsy sample from each interval was homogenized and assayed for myeloperoxidase (MPO) content (a sensitive measure of neutrophil accumulation) and for the presence of venom via an enzyme immunoassay (EIA) developed in our laboratory, as described below. The other paired dermal biopsy was sectioned, stained with hematoxylin and eosin, and examined for the presence of polymorphonuclear neutrophils (PMNs) by microscopy. The main outcome measure was the correlation of the presence of venom with the presence markers of inflammation (neutrophils, and MPO content).

Measurements. An EIA was developed to allow measurement of tissue content of Loxosceles species venom. The EIA method used in this investigation is a modification of the enzyme-linked immunosorbent assay (ELISA) procedure described by Smith (1993).[14] Polyclonal IgG antibody to Loxosceles venom used for the EIA was raised and purified using methods previously described.[4]

Standard 96-well, flat-bottomed microtiter plates (Corning Glass Works, Corning, NY) were coated with Loxosceles polyclonal IgG antibody. After overnight binding of the capture antibody [4 μg/mL in phosphate-buffered saline (PBS), pH 7.4], plates were incubated with blocking buffer [PBS pH 7.4, 1% bovine serum albumin (BSA), 0.05% Tween 20 (Zymed Labs, San Francisco, CA)] for two hours and washed twice (0.05% Tween 20 in PBS). Loxosceles venom standards were added in triplicate and incubated for two hours. Plates were washed twice with buffer followed by the addition of the respective biotinylated rabbit polyclonal detection Loxosceles IgG antibody (4 μg/mL in PBS, pH 7.4). After plates were washed four times with buffer, streptavidin horseradish peroxidase 1:4,000 dilution (Neutralite, catalog number 7200-05, Southern Biotechnology Associates, Birmingham, AL) was added for 30 minutes. Plates were then washed six times with EIA wash buffer, and finally exposed to 2,2′-Azino-di-[3-ethylbenzthiazoline sulfonate (6)] diammonium salt crystals (ABTS, Boehringer-Mannheim Biochemica, Indianapolis, IN, #1 112 422) for 35–40 minutes. Finally, absorbance was read at 405 nm on a model EL_X808, Bio-Tek (Winooski, VT), microplate reader.

Myeloperoxidase activity, a sensitive measure

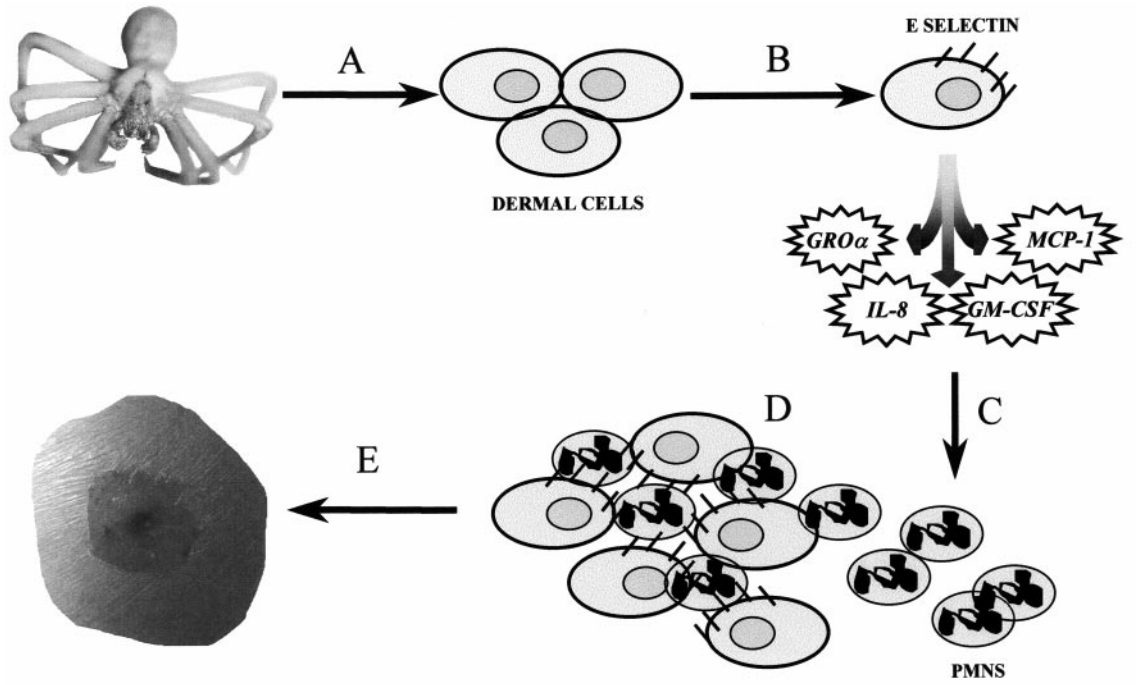

Figure 1. Schematic diagram reviewing the mechanism of Loxosceles venom-induced dermal inflammation. **A**) intradermal deposition of venom; **B**) induction of the neutrophil-tethering molecule E-selectin on endothelial cell surfaces; **C**) endothelial and epithelial cell secretion of chemokines interleukin-8 (IL-8), growth-related oncogene-alpha (GROα), monocyte chemoattractant protein-1 (MCP-1) and granulocyte/macrophage-colony-stimulating factor (GM-CSF); **D**) neutrophil migration and tethering to endothelial cell surfaces via E-selectin; and **E**) activation and degranulation of neutrophils with resultant tissue injury.[10, 11]

of neutrophil accumulation, was measured in dermal biopsies. At the time of sacrifice (24 hours following venom inoculation), multiple paired 4-mm dermal biopsies were obtained in 2-cm intervals 0 to 12 cm from the inoculation site. Normal dermal tissue was obtained from the opposite flank to serve as a negative control. The specimens were instantly frozen with liquid nitrogen and stored prior to analysis. Using a polytron tissue homogenizer (Tissue Tearor, Biospec, Bartlesville, OK), the skin biopsies were homogenized on ice in 500-μL PBS pH 7.4 containing 0.1% Tween 20. Samples were sonicated on ice and insoluble material was removed by centrifugation at 3,000 rpm for 10 minutes. Five μL of tissue extract (PBS pH 7.4 and 0.1% Tween 20) were incubated with 100 μL of 2,2′-Azino-di-[3-ethylbenzthiozoline sulfonate] diammonium salt solution (ABTS substrate, Boehringer Mannhiem) in triplicate and the maximum velocity of the substrate/MPO chromogenic reaction (V_{max}) was measured by monitoring the 96-well low-protein binding flat-bottom plates (Corning Glass Works) at 405 nm over a 2-minute period (Bio-Tek EL_X808 microplate reader). Kinetic calculations were performed using KC3 software (Bio-Tek). The MPO activity in samples was determined using a standard curve of purified MPO (Calbiochem, San Diego, CA). The MPO values are reported as units of activity/4-mm biopsy.

Data Analysis. The main goal of the analysis was in assessing the relationship between the presence of venom and the presence of markers of inflammation (neutrophils, and MPO content). We were interested in assessing the level of agreement between venom, MPO, and white blood cells, and this was measured using kappa statistics. Kappa gives a measure of agreement after accounting for the agreement by chance alone. Kappa has a maximum of 1 when agreement is perfect, and a value of zero indicates no agreement better than by chance. In addition, interrater kappa statistics were obtained between the two investigators who reviewed the same tissue biopsies for the presence of PMNs. A high interrater kappa would support high reliability in tissue PMN determination.

RESULTS

Lesional Area Determination. At 24 hours the lesions had reached a mean ± SD area of dermal inflammation and edema of 67.21 ± 17.85 cm^2 with a mean ± SD diameter of 9.18 ± 0.64 cm.

Myeloperoxidase Activity and Neutrophil Determination. Figure 2 summarizes MPO data of the 4-mm biopsies taken at the end of the 24-hour study period. Elevated dermal MPO content was detected 0–10 cm from the venom inoculation

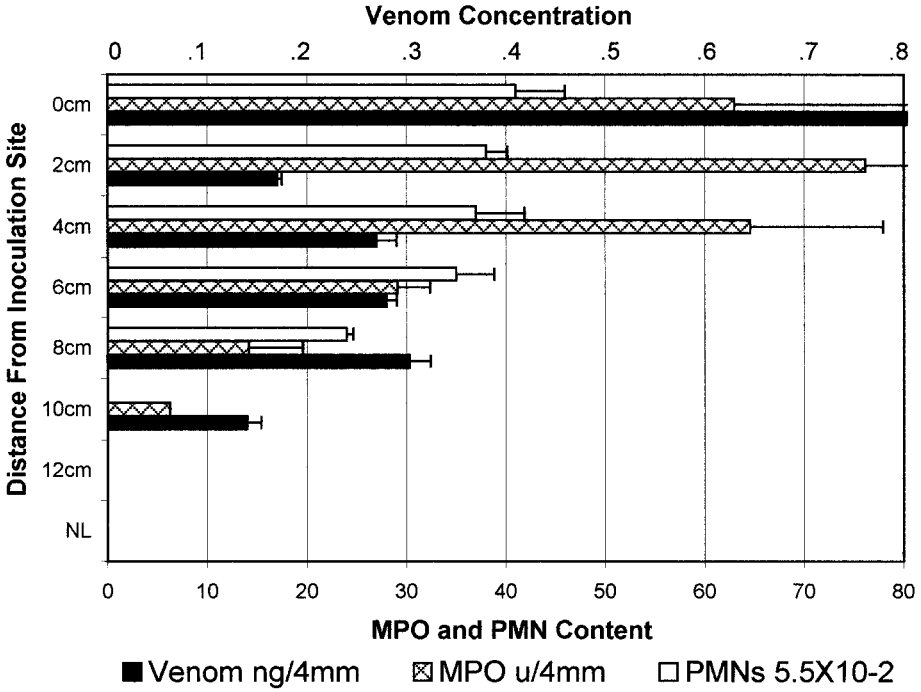

Figure 2. Myeloperoxidase (MPO), polymorphonuclear neutrophil (PMN), Loxosceles venom mean ± SD concentrations in serial dermal biopsies 0–12 cm from the inoculation site 24 hours postenvenomation. The level of agreement for the presence of the three parameters as assessed by kappa = 0.88, $p < 0.001$.

site. The highest concentration was found in dermal tissue obtained 2 cm from the venom inoculation site with a mean ± SD MPO level of 76.1 ± 22.7 u/4-mm biopsy. Myeloperoxidase was not detected in dermal tissue obtained 12 cm from the inoculation site nor in the negative control. Figure 2 summarizes the neutrophil concentrations detected in the lesions. Polymorphonuclear neutrophils were detected 0–8 cm from the inoculation site; the maximum number of PMNs was found at the inoculation site with a mean ± SD of 41 ± 4.9 PMNs/5.5 × 10^{-2} mm^2. No PMNs were seen under microscopy at 10 and 12 cm from the injection site, nor in the negative normal skin control. Interrater kappa agreement between the two investigators who reviewed tissue biopsies for the presence (or absence) of PMNs was 1.0 ($p < 0.01$).

Venom Content in Dermal Tissue. We had previously raised and purified anti-Loxosceles polyclonal IgG antibodies and developed a sensitive enzyme immunoassay to detect Loxosceles species venom. Figure 2 summarizes the venom concentrations found in dermal biopsies with our venom immunoassay. The highest venom concentrations were found at the inoculation site with a mean ± SD of 4.28 ± 3.9 ng/4-mm biopsy. *Loxosceles reclusa* venom was detected in each biopsy 0–10 cm from the inoculation site. No venom was detected at 12 cm nor in the negative control taken from the opposite flank.

Summary. Figure 2 summarizes the level of agreement between the three parameters we measured. The lesion extended just over 9.18 ± 0.64 cm in diameter. This clinical observation was reflected in our inability to detect MPO, PMNs, and migrated venom past the 10-cm dermal biopsies. Kappa gives a measure of agreement of our parameters after accounting for agreement by chance alone. The kappa value between venom and MPO was 0.91 ($p < 0.001$), that between venom and PMNs was 0.90 ($p < 0.001$), and that between all three was 0.88 ($p < 0.001$), thus demonstrating significant correlation between the parameters measured.

DISCUSSION

Using the rabbit model of dermonecrotic arachnidism, we found a significant correlation between the presence of dermal inflammation as determined by elevated concentrations of inflammatory cells and MPO content, and the immediate presence of *L. reclusa* venom in serial dermal biopsies. Tissue biopsies without detectable Loxosceles venom did not possess dermonecrotic arachnidism either by clinical exam or as determined by the lack of measurable parameters used to quantify tissue inflammation. Loxosceles venom contains the enzyme hyaluronidase, which, like some bacteria, may promote such a spread of venom through tissue.[15] Following envenomation, the venom may rapidly diffuse throughout dermal tis-

sue, inducing tissue inflammation and necrosis in a circular "wave" initiated from the focal point of venom deposition.

Given the known indirect nature of the mechanism of dermonecrotic arachnidism, we considered distant tissue signaling from the point of envenomation a plausible hypothesis for the rapid development of tissue inflammation. Loxosceles venom produces dermal inflammation and necrosis via induction and release of soluble mediators of inflammation.[10–12] This investigation demonstrates that although the physiological events leading to tissue inflammation and necrosis are indirect, the dermopathological effects of Loxosceles envenomation nonetheless require the immediate presence of venom.

We previously found evidence of venom migration in a single rabbit that was inoculated intradermally with an overwhelmingly large quantity (20 μg) of *L. deserta* venom.[16] The animal developed a large necrotic lesion at the site of inoculation, with small areas of necrosis appearing to "drip" down the side of the animal in a gravitationally dependent manner.[16] Using a modified tissue EIA, *L. deserta* venom was detected throughout the 15-cm necrotic inflammatory dermal lesion after 24 hours.[16] This finding was limited by the physiologically irrelevant amount of venom inoculated in the animal model. Although the actual amount of venom deposited during a native spider bite is not known, the amount of venom obtained from a spider during a single electrical stimulation during commercial milking is approximately 2–3 μg (personal communication, Charles Kristensen, SpiderPharm, Feasterville, PA).

Clinical Relevance

In this controlled investigation, using a relevant quantity of *L. reclusa* venom, we detected venom throughout dermal tissue, which had clinical signs of inflammation. The spread of venom away from the site of inoculation has direct clinical relevance. We previously found that intradermally administered anti-Loxosceles Fab fragments were shown to inhibit the formation of venom-induced dermal inflammation when administered early following venom inoculation in the rabbit model.[4] However, there was decreasing efficacy of the lesion attenuating effect when treatment time was delayed. Other investigators have studied intradermal administration of intact polyclonal anti-Loxosceles antibodies with varying degrees of success, but all have observed similar decreases in efficacy when delay follows venom inoculation.[5,7,8]

A rapid migration of venom away from the point of inoculation would make an intradermal administration of antivenom at the bite site a relatively inefficient means of accessing, binding, and neutralizing venom.

Limitations and Future Questions

A limitation in this study pertains to its unblinded design in MPO, PMN, and venom quantification. Unblinded parameter determination cannot control for investigator bias. We have demonstrated that the immediate presence of Loxosceles venom is required for the development of dermonecrotic arachnidism. However, several questions are raised by this investigation. Since Loxosceles venom diffuses throughout the dermis rapidly following venom inoculation, the intradermal "point" application of antivenom would not appear to be an effective means of applying antivenom. This manner of antivenom application would likely not allow antibodies effective access for venom binding and neutralization. Future studies would logically focus on whether parenteral administration or other more diffuse dispersal of anti-Loxosceles Fab antivenom might provide better access for venom neutralization. In addition, given the time-dependent factor of tissue spread of venom, is it possible to rapidly identify individuals who may benefit clinically from antivenom therapy prior to the development of fully expressed clinically characteristic dermal necrosis 24–48 hours later?

Conclusions

In this investigation using the New Zealand rabbit model, Loxosceles venom diffused throughout dermal tissue over a 24-hour period. Loxosceles-induced dermal inflammation strongly correlated with the immediate presence of venom. Although the mechanism of Loxosceles-induced inflammation is an indirect one, the immediate tissue presence of venom is nonetheless required for dermonecrotic arachnidism.

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