BASIC INVESTIGATIONS

Intradermal Anti-Loxosceles Fab Fragments Attenuate Dermonecrotic Arachnidism

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Abstract. **Objective:** Bites from the brown recluse spider and other arachnids from the genus Loxosceles frequently induce necrotic skin lesions that can be recalcitrant to treatment and disfiguring. The authors used a rabbit model of dermonecrotic arachnidism to address the therapeutic efficacy of intradermal (id) polyclonal anti-Loxosceles Fab fragments (αLoxd Fab) raised against *Loxosceles deserta* spider venom. **Methods:** Fab fragments were prepared by papain digestion and affinity chromatography from the IgG fraction of *L. deserta* antivenom raised in rabbits. Eighteen inbred New Zealand white rabbits were assigned to six groups of three. The rabbits received *L. deserta* venom (3 μg, id) injections into each flank. Cohorts of rabbits received single id injections (at one venom site/rabbit) of 30 μg αLoxd Fab at different times (T = 0, 1, 2, 4, 8, and 12 hours) after venom injection. In each rabbit the opposite flank was left untreated. As an additional control, one group of rabbits (T = 0) received nonspecific Fab (30 μg, id) in the opposite flank. Dermal lesions were quantified as a function of time through the use of a series of digital photographs and imaging software. In addition, myeloperoxidase (MPO) activity, a measure of neutrophil accumulation, was determined in lesion biopsies. Lesion areas and MPO activities were analyzed by repeated-measures analysis of variance (ANOVA). **Results:** Lesion areas and MPO activity were markedly reduced when αLoxd Fab was administered very early after venom injections. As the interval between venom inoculation and antivenom treatment increased, the therapeutic benefit of αLoxd Fab decreased. The final time tested that demonstrated therapeutic efficacy of αLoxd Fab was T = 4 hours. Lesion attenuation was no longer apparent when αLoxd Fab was given 8 hours post inoculation. **Conclusions:** Intradermal administration of αLoxd Fab attenuates Loxosceles-induced dermonecrotic lesion formation when given up to 4 hours after venom inoculation in this rabbit model. **Key words:** Loxosceles; spider; venom; Fab fragments; arachnidism. ACADEMIC EMERGENCY MEDICINE 1999; 6: 1195–1202

MORE THAN 10,000 spider bites were reported to the American Association of Poison Control Centers (AAPCC) Toxic Exposure Surveillance System (TESS) in 1996.[1] Although bites from the brown recluse spider are rarely fatal, the manifestations of these bites are nonetheless significant. Nine hundred thirty of the brown recluse spider bites reported to TESS in 1996 resulted in minor to major morbidity.[1] Loxosceles arachnids are indigenous American spiders that possess a venom capable of causing intense dermal inflammation and disfiguring necrotic ulcers.[2,3] In some cases the bite site may resemble pyoderma gangrenosum or other neutrophilic dermatoses.[4] 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. A centrally located necrotic ulcer often forms 8–24 hours following envenomation.[2,3] Among the 13 recognized species of Loxosceles that are found in the United States, *L. reclusa*, commonly called the brown recluse spider, is the major species responsible for envenomation. Most Loxosceles envenomations occur in the south central regions of North America.[5]

Although characteristic Loxosceles arachnid-induced skin lesions were first described in the medical literature by Caveness in 1872,[6] a consistently effective treatment has yet to be demonstrated. The literature is replete with circumstantial re-

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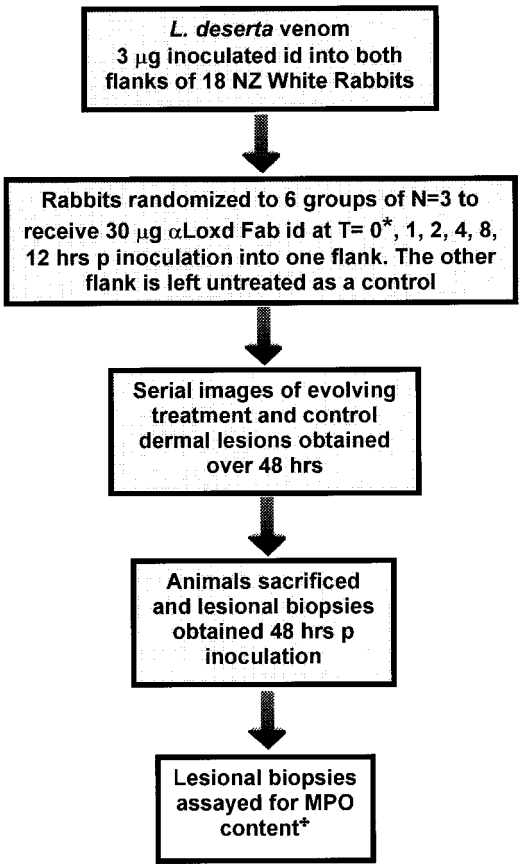

Figure 1. Schematic diagram of the investigation protocol. *L. deserta* = *Loxosceles deserta*; id = intradermal; αLoxd Fab = anti-Loxosceles Fab fragments; p = post; MPO = myeloperoxidase; IL-8 = interleukin-8.

ports and uncontrolled studies of recluse lesion therapies. Several studies attest to the potential efficacy of dapsone[7] or hyperbaric oxygen therapy,[8] but these therapies have not been studied in humans in a controlled fashion, and recent blinded animal studies have failed to reveal differences between treatment groups and their respective controls.[9,10] Literature regarding the use of polyclonal anti-Loxosceles antibody (Ab) is somewhat limited. Rees et al. administered rabbit derived anti-Loxosceles polyclonal IgG Ab intradermally (id) to rabbits 6–48 hours after envenomation with *L. reclusa* venom.[11] These authors noted decreases in Ab-treated lesion size in rabbits treated up to 24 hours after envenomation, though the difference at 24 hours was minute, and a statistical analysis was not conducted.[11] Rees et al. subsequently compared lesion sizes in human spider bite victims treated with anti-Loxosceles polyclonal IgG Ab.[12] In this investigation, lesion sizes were compared among patients treated with id Ab, those treated with dapsone and Ab, and those treated with dapsone alone.[12] No significant differences among groups of patients were observed.[12] Bravo et al. tested intradermal equine polyclonal anti-*L. laeta* Ab in rabbits inoculated with *L. laeta* venom.[13] Incubation of venom with Ab for one hour before inoculation prevented lesion development in rabbits, and lesions were attenuated when Ab treatment was delayed for one hour after venom inoculation.[13] Recently, Cole et al. reported that anti-Loxosceles polyclonal IgG Ab attenuates lesional erythema and necrosis in a rabbit model of eyelid envenomation.[14]

Fab fragments have been developed to treat a variety of conditions, including digoxin[15] colchicine,[16] tricyclic antidepressant,[17] and phencyclidine overdoses[18] as well as Crotalidae snakebites.[19,20] In order to improve safety and efficacy in humans, intact IgG Ab is cleaved with papain, yielding two antigen-binding Fabs, each with a molecular weight of approximately 50,000 daltons and one Fc fragment.[21] Since the Fc fragment does not bind antigen, and it increases the potential for hypersensitivity reactions, it is eliminated. Fabs have several advantages over intact Ab in the treatment of drug or venom toxicity. These include a larger volume of distribution, more rapid onset of action, smaller risk of adverse immunologic effects, and more rapid elimination.[21–23] We therefore investigated the hypothesis that polyclonal anti-Loxosceles Fab fragments will inhibit dermonecrotic inflammation associated with Loxosceles envenomation.

METHODS

Study Design. This was a prospective, randomized, controlled laboratory investigation examining the efficacy of Fab fragment Loxosceles antivenom in attenuating venom-induced dermal inflammation in a rabbit model. The study was approved by the University of Michigan Committee on the Use and Care of Animals. All experiments were in accord with the standards 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 Participants. Eighteen inbred New Zealand white rabbits were assigned to six groups of $n = 3$ (Fig. 1). The rabbits received *L. deserta* venom (3 μg id) into each flank. This venom dose was chosen since it resulted in reproducible (and easily measured) lesion areas in the 30–50-cm^2 range in pilot animals. The actual amount of venom deposited into prey by Loxosceles sp. spiders is unknown. The six cohorts of $n = 3$ rabbits

received single id injections (at one venom site/rabbit) of 30 μg anti-Loxosceles Fab fragments (αLoxd Fab) antivenom at different times (T = 0–12 hr) after venom inoculation. The opposite flank was left untreated as a control. An additional control of 30 μg id non-venom-specific Fab treatment immediately following venom injection (T = 0) was also included (n = 3). The dosage of 30 μg of αLoxd Fab was selected after pilot studies demonstrated consistent marked attenuation of venom-induced dermal inflammation at this selected dose.

Study Protocol. For all groups, digital photographs of evolving lesions were taken during the 48 hours following Fab administration. The images were imported into a computer, and evolving lesion areas were traced and measured using NIH imaging software. Myeloperoxidase (MPO) activity (a sensitive measure of neutrophil accumulation) was determined at 48 hours in multiple lesion biopsies obtained 0–8 cm from the site of envenomation. In addition, interleukin-8 (IL-8) tissue content was determined at the earliest time point tested (T = 0).

Antibody Production. Polyclonal IgG antibody to *L. deserta* venom (SpiderPharms, Feasterville, PA) was raised in New Zealand white rabbits. The rabbits received intramuscular *L. deserta* venom injections every three weeks for a period of five months. The IgG was purified using a modification of the method described by Goding[24] and Coulter et al.[25] Immune rabbit IgG was purified from crude serum via affinity chromatography by flowing crude serum diluted 1:3 with ImmunoPure binding buffer (Pierce, Rockford, IL) through an AffinityPak protein A-sepharose column (Pierce). The Fc portion of intact Ab binds to protein A,[24] thus allowing other serum proteins to flow through the column to be discarded. The bound IgG is eluted with ImmunoPure elution buffer, pH 3 (Pierce), and dialyzed against phosphate-buffered saline (PBS), pH 7.4. We have previously demonstrated that polyclonal IgG antibody raised against *L. deserta* venom effectively blocks dermal lesion development from both *L. reclusa* and *L. deserta* spider venoms.[26]

Fab Fragment Preparation and Purification. The procedure used to prepare Fab fragments was modified from that described by Coulter and Harris.[27] Purified IgG Ab was dialyzed against a 20-mM phosphate/10-mM ethylenediamine tetraacetic acid (EDTA) buffer, pH 7.0, and concentrated to a 20-mg/mL solution. Aliquots of purified IgG Ab were added to a digestion buffer consisting of cysteine HCl dissolved in a phosphate buffer (pH 10). Immobilized papain slurry was then added to the solution. The resultant suspension was incubated for five hours in an air shaker at 37°C to maintain constant mixing of papain and Ab. Papain was then removed using a proprietary filter separator tube (Pierce). The Fc and Fab fragments contained in the crude digest were separated using a prepacked column of immobilized protein A (Affinity-Pak). The Fab fragments were dialyzed against PBS (pH 7.4) and concentrated to 30 mg/mL. IgG and Fab concentrations were determined by spectrophotometry at 279 nm (LKB Ultrospec II Model 450, Biochrom, Cambridge, England). The αLoxd Fab molecular weight was determined to be approximately 40,000 daltons by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein molecular weight standards were obtained from Amersham (Arlington Heights, IL, RPN 756).

MPO Activity Determination. Myeloperoxidase activity, a sensitive measure of neutrophil accumulation, was measured in dermal biopsies. At the time of sacrifice, 4-mm punch biopsies were taken from predetermined distances (0–8 cm) from the inoculation site and instantly frozen with liquid nitrogen. 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) was incubated with 100 μL of 2,2′-azino-di-(3-ethylbenzthiozoline sulfonate) diammonium salt solution (ABTS substrate, Boehringer Mannheim, Indianapolis, IN) in triplicate and the maximum velocity of the substrate/MPO chromogenic reaction (Vmax) was measured by monitoring the 96-well low-protein binding flat-bottom plates (Corning Glass Works, Corning, NY) at 405 nm over a 2-minute period (BioTek ELx808 microplate reader, Bio-Tek Instruments, Inc., Winooski, VT). 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/biopsy.

IL-8 Tissue Content Determination. The enzyme immunoassay (EIA) method used for this investigation is a modification of the enzyme-linked immunosorbent assay (ELISA) procedure to detect soluble antigens.[28] Standard 96-well, flat-bottom ELISA plates (Corning Glass Works) were coated with murine monoclonal IgG_1 antibodies (courtesy Genentech, San Francisco, CA) specifically directed against rabbit IL-8. After overnight binding of the capture antibody (4 μg/mL in PBS pH 7.4), ELISA plates were incubated with blocking buffer

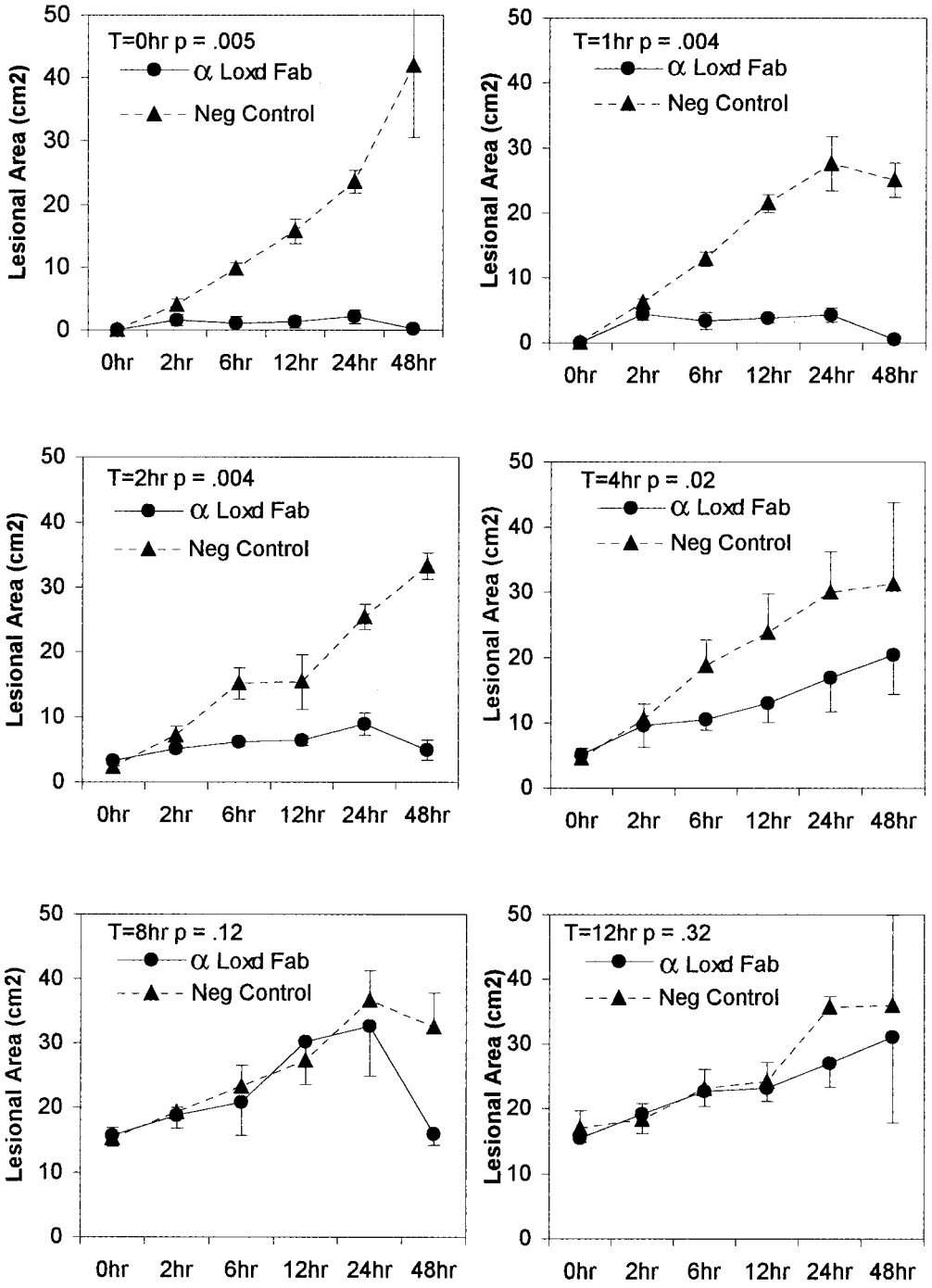

Figure 2. Fab antivenom and control lesion areas over time. Fab antivenom markedly suppressed the venom effect when administered early, with decreasing efficacy over time. Control and lesion area differences were not significant by T = 8 hr. αLoxd Fab = anti-Loxosceles Fab fragments.

[PBS pH 7.4, 1% bovine serum albumin (BSA), 0.05% Tween 20] for two hours, and washed twice (0.05% Tween 20 in PBS). Conditioned medium and standards were added in triplicate for a two-hour incubation period. Plates were washed twice with buffer followed by the addition of biotinylated chicken polyclonal IgY detection anti-rabbit IL-8 antibody 2 μg/mL. The polyclonal IgY detection antibody to rabbit IL-8 was generated by immunizing chickens with recombinant rabbit IL-8, and isolating immunoglobulin from egg yolks. Antigen specificity was confirmed by showing that the antibody bound recombinant IL-8 as well as native rabbit macrophage-derived IL-8, as assessed by radioimmunoprecipitation, Western blotting, and immunohistochemistry, and neutralized IL-8-induced neutrophil chemotaxis and calcium flux in vitro (Marks RM, Chen Y, Suarez TM, unpublished data, 1995). After the plates were washed four times with buffer, streptavidin horseradish peroxidase

1:4,000 dilution (Neutralite, 7200-05, Southern Biotechnology Associates, Inc., Birmingham, AL) was added for 30 minutes. The plates were then washed six times with EIA wash buffer, and finally exposed to 2,2′-azino-di-(3-ethylbenzthiozoline sulfonate) diammonium salt solution (ABTS substrate, Boehringer Mannheim, #1 112 422) for 35–40 minutes. Absorbance was read at 405 nm on a model EL_X808, Bio-Tek microplate reader. Interleukin-8 concentrations were calculated by four-parameter curve fitting or linear regression analysis of chemokine standard curves using KC3 software (Bio-Tek).

Data Analysis. Lesion areas, MPO activities, and IL-8 concentrations were analyzed and compared using repeated-measures analysis of variance (ANOVA).

RESULTS

Lesion Area Determination. When αLoxd Fab antivenom was administered id immediately (T = 0 hr) after envenomation, lesion development was essentially blocked (Fig. 2). At 48 hours the control lesion had reached a mean (±SD) area of 41.9 ± 11.3 cm^2, compared with a small transient erythema noted at the αLoxd Fab antivenom site, which had resolved by the end of the 48-hour study period (ANOVA p = 0.005, Fig. 2). The control results were similar at T = 0 hr using the nonspecific Fab control (results not shown). The αLoxd Fab antivenom continued to suppress the lesion areas when administered T = 1 hr up to T = 4 hr after *L. deserta* envenomation, although the longer αLoxd Fab antivenom treatment was delayed, the smaller the difference in treatment and control lesion areas (Fig. 2). At T = 4 hr the treatment lesion was attenuated with a mean ± SD area of 20.4 ± 5.9 cm^2 compared with the control mean (±SD) area of 31.2 ± 12.5 cm^2 (ANOVA p = 0.02). Mean area differences were not significant after αLoxd Fab antivenom treatment delays of T = 8 hr and T = 12 hr (Fig. 2).

MPO Activity Determination. When αLoxd Fab was administered immediately after envenomation, MPO activity was markedly attenuated. At the site of envenomation at T = 0 hr, the control lesion had a mean (±SD) MPO activity level of 3.2 ± 1.8 u/μL compared with the αLoxd Fab cohort mean (±SD) MPO activity level of 0.13 ± 0.09 u/μL (ANOVA p = 0.004, Fig. 3). As the delay in αLoxd Fab antivenom treatment post Loxosceles envenomation increased, the difference in MPO activity diminished (Fig. 3). When αLoxd Fab antivenom was administered at T = 4 hr post envenomation (after dermal erythema and induration were well under way), MPO activity was still markedly diminished throughout the Fab treatment lesion (ANOVA p = 0.01, Fig. 3). If treatment was delayed eight or more hours after envenomation, the lower mean MPO activity levels in the αLoxd Fab antivenom treatment side were no longer different (Fig. 3).

Interleukin-8 Tissue Concentration. Loxosceles venom has been found to stimulate the secretion of IL-8 by endothelial cells.[29] We determined the IL-8 dermal tissue concentrations in the T = 0 hr αLoxd Fab antivenom cohort at the end of the 48-hour study period. Lesional IL-8 tissue concentrations were lower in the αLoxd Fab antivenom treatment lesions sampled, with mean (±SD) IL-8 levels of 0.03 ± 0.02 ng/mL at the venom inoculation sites compared with a mean (±SD) of 0.21 ± 0.03 ng/mL noted on the control NS Fab envenomation sites (Fig. 4).

DISCUSSION

In this investigation, intradermally administered anti-Loxosceles Fab fragments were shown to inhibit the formation of venom-induced dermal inflammation in the rabbit model. The lesion areas and MPO activity levels were markedly attenuated when Fabs were administered early after Loxosceles envenomation. There was decreasing efficacy of the lesion attenuating effect of Fab treatment as treatment time was delayed (and dermal inflammation was well under way) after envenomation. Decreased therapeutic efficacy with delay in polyclonal IgG antivenom treatment has previously been observed in the rabbit model using intact antibody.[11,13,14] At least two hypotheses may explain the time-dependent decrease in therapeutic effect. First, if venom has spread in dermal tissue over time, an id injection of antivenom at the original envenomation site may not have physical access for complete neutralization of venom. Evidence for this hypothesis exists in an investigation recently reported by us.[30] Rabbits envenomated with a large dose (20 μg) of *L. deserta* venom developed a "double" necrotic lesion with small areas of necrosis appearing to "drip" down the side of the animal.[30] Using a modified tissue EIA assay, Loxosceles venom was detected throughout a 15-cm necrotic inflammatory dermal lesion after 24 hours.[30] Systemically administered αLoxd Fab antivenom might be one way to neutralize venom that has migrated from the envenomation site. Another contributing factor for the observed decrease in efficacy with antivenom treatment delay is that Loxosceles antivenom neutralization may be less efficacious in modifying inflammatory events once they are well under way. In this investigation, the control and

treatment lesions in the T = 8 hr and T = 12 hr cohorts had already reached a dermal inflammation area of 15 cm^2 or more prior to initiation of treatment.

Loxosceles venom has been found to stimulate the secretion of IL-8 by endothelial cells.[29] We found that αLoxd Fab administered immediately after venom inoculation inhibits tissue levels of IL-8. Biological features of IL-8, compared with other chemotactic factors, include physiologic effects on neutrophils both in vitro and in vivo.[31] In-vitro activities of IL-8 include a shape change and activation of the motile system of the neutrophil.[31] This occurs very rapidly; within 15 seconds of IL-8 addition to suspensions of neutrophils, actin microfilaments are redistributed to the subcortical region of the neutrophil.[31] In vivo, intradermal injections of human IL-8 in rabbits induce plasma exudation and neutrophil recruitment.[32] Interleukin-8 has been shown to be an important mediator

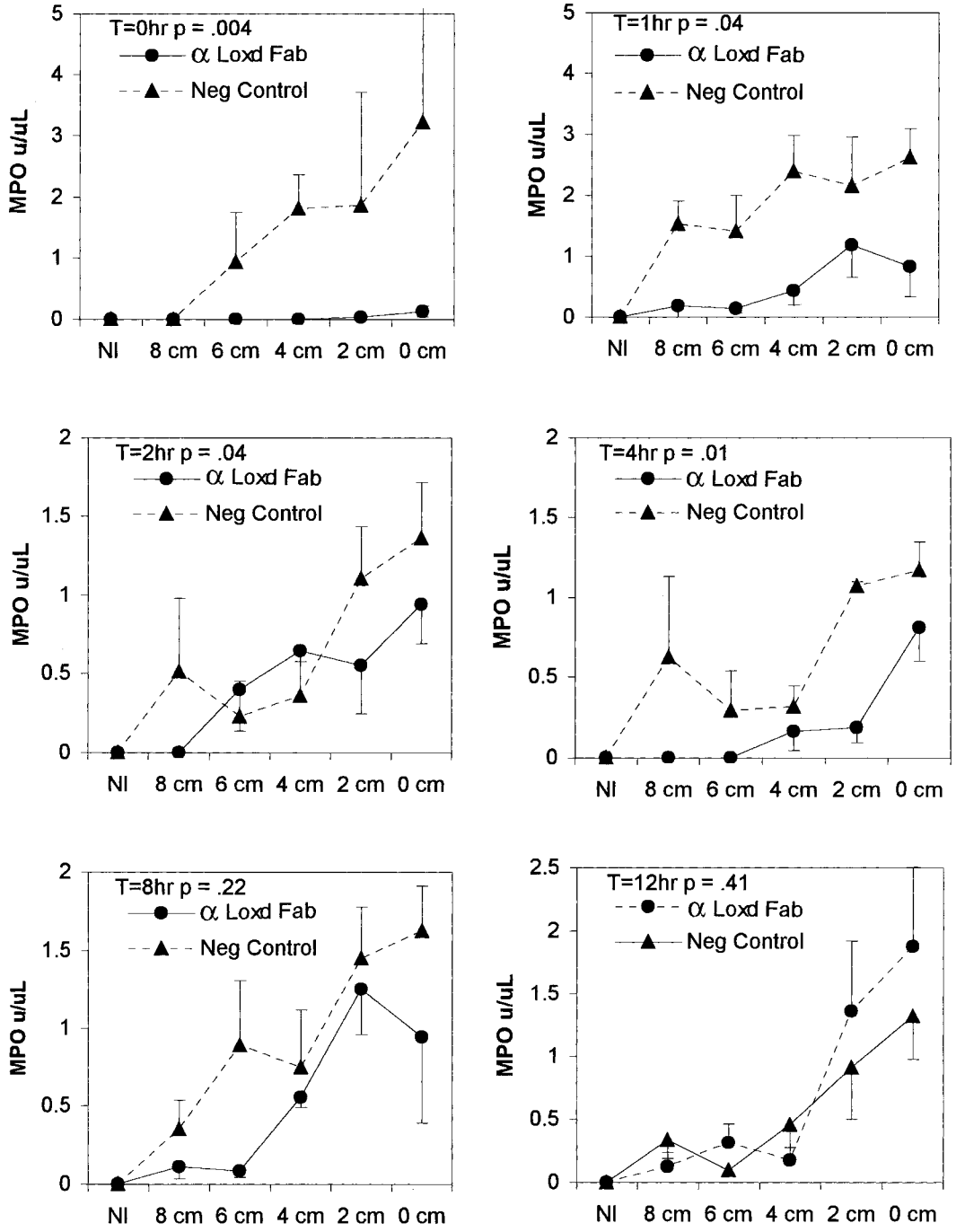

Figure 3. Fab antivenom and control myeloperoxidase (MPO) activity levels over time. Fab antivenom markedly suppressed the venom effect when administered early, with decreasing efficacy over time. Control and lesion MPO differences were not significant by T = 8 hr. 0 cm = site of Loxosceles venom inoculation. αLoxd Fab = anti-Loxosceles Fab fragments.

of other forms of tissue injury such as acid aspiration-induced lung injury and endotoxin-induced pleurisy.[33,34] Thus, the inhibition of IL-8 levels in dermal tissue may play a role in the reduced inflammation observed in envenomed animals treated with the Fab antivenom.

The therapeutic use of Fab fragments in the treatment of envenomations is a current focus of toxicologic research. Since the Fc does not bind antigen, and it increases the potential for hypersensitivity reactions, it was eliminated. Fabs have several advantages over intact Ab in the treatment of drug or venom toxicity. These include a larger volume of distribution, more rapid onset of action, smaller risk of adverse immunologic effects, and more rapid elimination.[21–23] Fabs have been developed as a safer alternative to the currently available equine-derived polyvalent crotalid antivenin. In a recent multicenter trial, Dart et al. reported that mixed monospecific crotalid antivenin ovine Fab was associated with the halt of progressive crotalid venom poisoning without adverse allergic reactions.[19] Clark et al. reported that Fabs were effective in reversing neurotoxicity associated with crotalid bites.[20] Internationally, Fabs are currently being investigated as antidotal therapy for the West African carpet viper (*Echis ocellatus*),[35] Northern Europe common adder (*Vipera berus*),[36] and a Mexican scorpion species (*Centruroides noxius Hoffmann*).[37]

Limitations and Future Questions

There are several limitations that should be noted in this investigation. One limitation pertains to its unblinded design in lesion area determination, which does not permit control for investigator bias. The MPO activity and tissue IL-8 determinations, however, were performed by a technician blinded to the source of each sample. Nonspecific Fab fragments were used as a control for the first cohort (T = 0) but were not applied in remaining cohorts. This study followed only the lesion area and MPO activity levels up to 48 hours after initiation of treatment. Thus, significant attenuating effect by αLoxd Fab antivenom after the 48-hour investigation period may have been missed, particularly in cohorts that did not receive treatment many hours after venom inoculation. In addition, the use of the same animals for both treatment and control lesions may have affected the outcome of the study. Any therapeutic αLoxd Fab injected into an inoculation site could be absorbed into systemic circulation, and attenuate the size of the control lesion on the opposite flank.

Several questions are raised by this investigation. For example, we found that id αLoxd Fab was efficacious when administered four hours post inoculation, but no longer efficacious if administered at eight hours. Thus, would larger cohorts and optimized study conditions demonstrate efficacy for longer periods of time post inoculation? Optimized study conditions might include separate control and study animals, as well as larger cohorts. Also, if venom does in fact migrate from the initial envenomation site, would parenterally administered αLoxd Fab antivenom provide better access to the venom and thus enhance neutralization? Is it possible to identify individuals who may have sustained spider envenomation prior to the development of fully expressed dermal necrosis 24–48 hours later? While further research may extend the efficacy of αLoxd Fab antivenom to an increasing period of time after envenomation, it is nonetheless likely that the antivenom would have little impact once the dermonecrotic lesion has been fully established. Finally, could αLoxd Fab antivenom be a useful therapeutic option in those individuals with the systemic form of Loxoscelism?

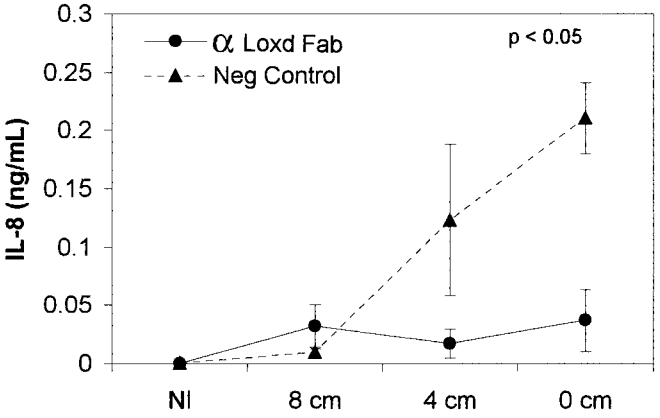

Figure 4. Tissue interleukin-8 (IL-8) concentrations 48 hours following treatment. Fab antivenom suppressed dermal IL-8 concentrations when administered immediately (T = 0) after envenomation. αLoxd Fab = anti-Loxosceles Fab fragments.

Conclusions

In this investigation using the New Zealand rabbit model, id anti-Loxosceles Fab fragments inhibited the dermal inflammation and necrosis associated with Loxosceles envenomation up to four hours post venom inoculation.

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