Evaluation of systemic immunologic hyperreactivity after intradermal testing in horses with chronic laminitis

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Objective—To determine whether systemic immunologic hyperreactivity exists in horses with chronic laminitis, compared with responses for nonlaminitic horses.

Animals—7 nonlaminitic horses and 7 CL horses.

Procedure—In experiment 1, intradermal testing (IDT) was performed on 7 nonlaminitic and 7 CL horses to evaluate the response to a combination of 70 allergens at 15 and 30 minutes and 4 and 24 hours after injection. Three nonlaminitic and 3 CL horses used in experiment 1 were used in experiment 2 to determine whether histologic differences existed between the 2 groups. The H&E-stained tissue sections were evaluated on the basis of 3 criteria. For all analyses, 2-sample *t*-tests were used to determine significant differences between the groups.

Results—In experiment 1, CL horses had significantly higher total responses to IDT than nonlaminitic horses at the first 3 time periods. Also, CL horses had significantly fewer total scores of 0 than nonlaminitic horses at all time periods, except at 24 hours. In experiment 2, we did not detect significant differences between groups for any criterion.

Conclusions and Clinical Relevance—Results support the hypothesis that CL horses develop hyperreactivity to various antigenic stimuli, compared with responses for nonlaminitic horses. Therefore, the possibility that antigenic challenge may result in exacerbation of clinical signs of laminitis should be discussed with horse owners. Chronic laminitis should also be a consideration when a horse becomes lame following antigenic challenges. (*Am J Vet Res* 2003;64:279–283)

Laminitis has been recognized as a cause for lameness in horses for centuries. However, its pathogenesis remains controversial. This is especially true regarding the relationship of laminitis with systemic illness. In some studies,[1-4,a] investigators have begun to explore the association of cardiovascular, nutritional, endocrine, and renal disease with chronic laminitis.

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Anecdotal evidence suggests that horses with chronic laminitis can have exacerbations of acute laminitic episodes following exposure to antigenic challenges, such as vaccinations or environmental allergens.[1,b] Because horses that have these episodes may not have a prior history of laminitis, it is often assumed they are having an initial episode of acute laminitis. However, physical and radiographic examination of their feet reveal that a large percentage of these horses are clinically compensated patients with chronic laminitis. This observation allows the hypothesis that dysregulation of the immune system plays a role in the clinical consequences of laminitis. The purpose of the study reported here was to test the hypothesis that horses with chronic laminitis (**chronically laminitic [CL]**) have greater immunologic hyperreactivity to allergenic challenges than horses without laminitis (nonlaminitic).

Materials and Methods

Animals—Seven nonlaminitic and 7 CL horses were selected for use in the study. Horses were considered to be nonlaminitic and were included in the study when there was no known history of laminitis, physical and radiographic examination of the feet failed to reveal pathologic conditions, and results of a subjective lameness examination were negative. The 7 nonlaminitic horses consisted of 3 geldings and 4 mares that ranged from 5 to > 20 years of age. Horses represented several breeds (4 Quarter Horses, 1 Thoroughbred, 1 Appaloosa, and 1 Arabian).

All of the CL horses had naturally occurring laminitis. These horses were identified on the basis of physical and radiographic examination of the feet. The CL horses used in the study had rotation or sinking of the distal phalanx and had been affected by laminitis for at least 1 year (range, 1 to 8 years). The 7 CL horses consisted of 2 geldings and 5 mares that ranged from 6 to 17 years of age. These horses also represented several breeds (2 Quarter Horses, 1 Appaloosa, 3 Arabians, and 1 Paint). Lameness grades for the CL horses ranged from Obel grade 1 to 3. Only 1 CL horse in the study (Arabian mare; 13 years old) had a prior history of suspected allergic skin disease, and none of the nonlaminitic horses had any known history of allergic reactions. That CL mare had a single episode of urticaria on its neck and body approximately 6 months after the initial laminitic episode; however, the urticarial condition resolved 7 months before the start of the study reported here.

The study consisted of 2 separate but related experiments. Methods used in this study were reviewed, approved, and continuously monitored by the Animal Use Committee of Texas A&M University.

Experiment 1—In experiment 1, **intradermal testing (IDT)** was performed on all horses (7 nonlaminitic and 7 CL horses). Results were used to determine whether there was a

measurable difference in reactivity to various antigens between nonlaminitic and CL horses.

Seventy allergens were used for IDT. Allergens represented the following 6 classes: molds and dust (n = 17), weeds (12), trees (15), grasses (9), insects (14), and feathers, hair, and epithelia (3). These allergens comprised a standard allergen panel used clinically in diagnostic procedures. Allergens were injected at a concentration of 1,000 **protein nitrogen units (PNU)**/mL unless otherwise specified. Mold and dust allergens included *Alternaria tenius, Aspergillus fumigatus, Cephalosporium acremonium, Curvularia spicifera, Cladosporium herbarum, Epicoccum purpurascens, Fusarium solani, Helminthosporium sativum, Mucor mucedo*, *Penicillium notatum, Pullularia pullalans*, corn smut, grass smut (100 and 1,000 PNU), oat smut, and grain mill dust (10 and 100 PNU). Weed allergens included cocklebur, dock-sorrel mix, dog fennel, English plantain, firebush, lamb's-quarter, pigweed mix, ragweed mix, rough marsh elder, Russian thistle, sage mix, and western ragweed. Tree allergens included ash mix, bald cypress, box elder-maple mix, cedar-fall elm, eastern cottonwood, eastern oak, hickory mix, mesquite, mountain cedar, pecan, pine scrub, red cedar, red mulberry, Virginia live oak, and black willow. Grasses included Bahia, Bermuda, KORT grass mix (ie, Kentucky blue-June, orchard, redtop, and timothy grasses), couch-quack grass, Johnson grass, smooth brome, per rye, alfalfa, and timothy. Insects included fire ant, blackfly (62, 125, and 250 PNU), cockroach, *Culicoides* spp (5,000, 10,000, and 50,000 PNU), horsefly, mosquito (100, 100, and 1,000 PNU), moth, and black ant. Finally, feathers, hair, and epithelia included cat epithelia, sheep wool epithelia, and mixed feathers.

For IDT, each horse was sedated by administration of xylazine hydrochloride (0.25 to 0.5 mg/kg, IV, as needed) to avoid stress related to injection of the allergens. The lateral aspect on the right side of the neck of each horse was clipped and cleaned with isopropyl alcohol. A permanent black marker was used to identify injection sites. The 70 allergens were injected ID (0.05 mL/injection). We also included 2 injections of a negative-control solution (sterile saline [0.9% NaCl] solution) and 2 injections of a positive-control solution (histamine phosphate; 1:100,000 [wt:vol]). Reaction to the histamine injection was assigned a score of 4, and the reaction to injection of the saline solution was assigned a score of 0. Reactions for the test antigens were scored by comparing them to reactions for the histamine and saline solutions with regard to wheal diameter, turbidity of the wheal, and severity of erythema.[5]

In an attempt to limit subjectivity, all injections and reaction evaluations were performed by a board-certified veterinary dermatologist (CAR) who was not aware of the status (nonlaminitic vs CL) of each horse. Injection sites were evaluated 15 and 30 minutes and 4 and 24 hours after injection. At each time period, size, turbidity, and erythema of each wheal were assigned a score of 0 to 4. Scores for each of the variables were totaled at each time period to determine whether the total value of the reactions differed significantly between the CL and nonlaminitic groups. In addition, the number of low (grade of 1 or 2) and high (3 or 4) reactivity scores for each time period for nonlaminitic and CL horses was determined. These data were used to define variation in the severity of the responses between groups.

Experiment 2—Experiment 2 was conducted to define the histologic responses of nonlaminitic and CL horses to specific antigens. Three nonlaminitic and 3 CL horses used in experiment 1 were also used in experiment 2. These horses were selected because of their availability at the time of the second experiment. Nonlaminitic horses consisted of 3 mares that ranged from 5 to 22 years of age and represented 2 breeds (2 Arabians and 1 Quarter Horse). Chronic laminitic horses consisted of 3 mares that ranged from 6 to 17 years of age and represented 3 breeds (1 Arabian, 1 Quarter Horse, and 1 Paint). Lameness scores for the 3 CL horses were Obel grades 2 or 3. For each horse, a minimum of 30 days elapsed between experiments 1 and 2.

Similar to experiment 1, each horse was sedated, and the lateral aspect of the neck was prepared for IDT. For each horse, an antigen was selected that had caused a mean score of ≥ 3 by the 4-hour evaluation period in experiment 1. This antigen was injected in 4 locations on the neck, and 1 of the sites was used for collection of biopsy specimens at 30 minutes and 6, 24, and 48 hours after injection, respectively. For the biopsy procedure, horses were sedated by administration of xylazine (0.25 to 0.5 mg/kg, IV, as needed), and the biopsy technique was performed in accordance with standard methods.[6] A 6-mm biopsy punch was used. Biopsy specimens were fixed in neutral-buffered 10% formalin for 24 hours. They were then routinely processed, sectioned at a thickness of 5 µm, and stained with H&E.

Two board-certified veterinary pathologists (RWD, KMC) and a board-certified veterinary dermatologist (CAR) examined and evaluated all tissue sections. Histologic response was defined for each of 3 criteria (diffuseness of the inflammatory reaction, intensity of the inflammatory reaction, and degree of edema). Each criterion was graded on a scale of 0 to 4, with 0 representing the least degree of reactivity and 4 representing the greatest degree of reactivity (**Appendix**).

Statistical analysis—In experiment 1, the sum of the responses calculated for each horse at each time period was used for statistical evaluation. A 2-sample *t*-test was used to determine whether significant differences existed between nonlaminitic and CL horses at the observation periods. In addition, 2-sample *t*-tests were used to compare the number of low (1 or 2) and high (3 or 4) reactivity scores at each of the various periods, as well as the total number of scores of 0. Responses were considered to be significantly different at $P < 0.05$.

For experiment 2, statistical analysis was performed on each criterion used to describe the inflammatory response (ie, diffuseness, intensity, and edema). A 2-sample *t*-test was used to determine whether differences existed for each criterion between the nonlaminitic and CL horses at each evaluation period. Differences were considered significant at $P < 0.05$.

Results

Experiment 1—All nonlaminitic and CL horses had some degree of reactivity to the allergens at 15 and 30 minutes and 4 hours after injection. However, there was significantly less total reactivity in the nonlaminitic horses during these periods. At 15 minutes after injection, nonlaminitic horses had a total reactivity score (mean ± SD) of 24.9 ± 14.2 (range, 13 to 54), which differed significantly ($P = 0.007$) from the total reactivity score of the CL horses (51.6 ± 16.7; range, 33 to 69). At 30 minutes after injection, total reactivity score of nonlaminitic horses was 27.9 ± 18.1 (range, 15 to 66), which differed significantly ($P = 0.01$) from the total reactivity score of the CL horses (56.9 ± 18.2; range, 31 to 77). At 4 hours after injection, mean reactivity score for the nonlaminitic horses was 35.3 ± 17.2 (range, 13 to 61), which differed significantly ($P = 0.005$) from the total reactivity score of the CL horses (62.9 ± 11.5; range, 46 to 76). At the last evaluation period (ie, 24 hours after injection), none of the nonlaminitic horses and only 1 CL mare had delayed

reactivity to the allergens. The reactivity score was 16 for that horse at 24 hours.

The second part of experiment 1 examined whether CL horses had a higher frequency of low (scores of 1 or 2) or high (scores of 3 or 4) reactivity when compared with frequencies of nonlaminitic horses. At 15 minutes after injection, there was a significant difference in the frequency of low reactivity scores between nonlaminitic and CL horses. Nonlaminitic horses had a mean frequency of low reactivity scores of 13.8 ± 8.9, whereas CL horses had a significantly (P = 0.01) higher mean frequency of low reactivity scores (31.9 ± 13.3). Frequency of high reactivity scores did not differ significantly between groups. At 30 minutes after injection, the same pattern was evident. Nonlaminitic horses had a mean frequency of low reactivity scores of 8.9 ± 4.1, whereas CL horses had a significantly (P = 0.006) higher mean frequency of 25.4 ± 12.4. Similar to results for the evaluation period at 15 minutes after injection, the number of high reactivity scores at 30 minutes after injection did not differ significantly between groups. At 4 hours after injection, nonlaminitic horses had a significantly lower frequency of reactivity scores, compared with values for CL horses. Mean frequency of low reactivity scores differed significantly (P = 0.01) between nonlaminitic (5.7 ± 4.6) and CL (12.3 ± 4.0) horses, and mean frequency of high reactivity scores also differed significantly (P = 0.03) between nonlaminitic (8.9 ± 5.6) and CL (16.0 ± 5.3) horses.

Comparison of the frequency of scores of 0 between nonlaminitic and CL horses resulted in significant differences between groups, except at 24 hours after injection. At the initial evaluation 15 minutes after injection, mean number of scores of 0 differed significantly (P = 0.01) between the nonlaminitic (57.9 ± 9.9) and CL (39.0 ± 13.2) horses. At 30 minutes after injection, the frequencies differed significantly (P = 0.006) between nonlaminitic (60.4 ± 8.0) and CL (42.0 ± 12.1) horses. Finally, at 4 hours after injection, the frequencies differed significantly (P < 0.001) between nonlaminitic (59.4 ± 6.7) and CL (45.7 ± 4.0) horses.

Experiment 2—Experiment 2 was designed to define differences over time in the histologic response to injected antigens in nonlaminitic and CL horses. Focus of the inflammation was the subadnexal dermis into which the antigens were injected. The inflammatory reaction extended into the superficial dermis only when the inflammatory response in the deep layers of the dermis was extremely intense. Special attention was paid to changes affecting blood vessels. In some sections, many inflammatory cells were evident within the vessel walls of the deep layers of the dermis without evidence of necrosis of the vessel wall. When intensity of the mural vascular inflammation was disproportionately high, compared with the interstitial component, the lesions were classified as a nonnecrotizing vasculitis (**Fig 1**). A true necrotizing (leukocytoclastic) vasculitis was identified in only a single CL horse (**Fig 2**).

Composition of the inflammatory reaction varied with time. In biopsy specimens obtained 30 minutes after injection, the reaction was limited to scattered inflammatory cells in perivascular regions in the deep

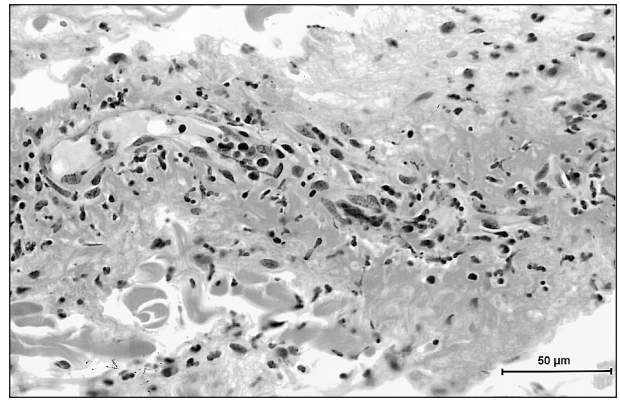

Figure 1—Photomicrograph of a skin biopsy specimen obtained from a representative horse after ID injection of a specific allergen revealing nonnecrotizing vasculitis affecting blood vessels in the deep layers of the dermis. Notice that many inflammatory cells are evident within the vascular wall, compared with the surrounding interstitium, and fibrin or necrosis is not evident. H&E stain; bar = 50 µm.

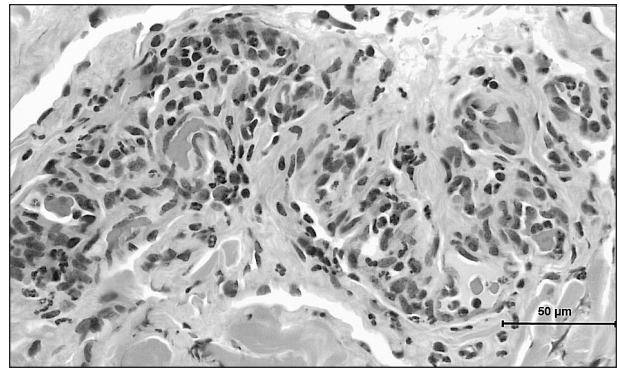

Figure 2—Photomicrograph of a skin biopsy specimen obtained from 1 chronically laminitic horse after ID injection of a specific allergen revealing necrotizing (leukocytoclastic) vasculitis involving blood vessels in the deep layers of the dermis. Notice abundant fibrin and nuclear dust within the vessel wall and edema and a mixed inflammatory-cell infiltrate composed of lymphocytes, neutrophils, and eosinophils in the surrounding tissue. H&E stain; bar = 50 µm.

layers of the dermis (ie, intensity and diffuseness were low), devoid of substantive edema (ie, edema score was low), and composed primarily of lymphocytes. The sole exception was 1 CL horse that had much more severe edema in the deep layers of the dermis with a more intense and diffuse inflammatory reaction. At 6 hours after antigen injection, the inflammatory reaction was more mixed with neutrophils, lymphocytes, and eosinophils. Edema was more intense, as was the intensity and diffuseness of the reaction. In samples obtained > 6 hours after injection, intensity of the inflammatory reaction diminished. Biopsy specimens obtained 24 and 48 hours after injection had a decrease in the number of neutrophils, leaving lymphocytes and eosinophilics as the major inflammatory cells. There were variable amounts of edema and diffuseness of the reaction. Despite variability horses, there was a pattern that CL horses had more inflammation associated with blood vessels, compared with the inflammatory reaction in nonlaminitic horses. Analysis of the data indicated that values for all 3 criteria did not differ significantly (P = 0.379) between the 2 groups of horses. In

addition, we were unable to correlate severity or duration of laminitic episodes with the intensity of responses to allergens administered during IDT.

Discussion

In experiment 1, we documented that horses with chronic laminitis have greater clinical hyperreactivity to an intradermal challenge of antigens than nonlaminitic horses. The observed hyperreactivity can be hypothesized as being coincidental to chronic laminitis, a preexisting factor that predisposed to or played an etiologic role in the development of laminitis, or a consequence or interrelation of laminitis. If consequential, the hyperimmune state can be assessed as having an innocuous or pathogenic effect.

The fact that all of the CL horses had greater overall reactivity, compared with responses for the nonlaminitic horses, indicated there is a relationship between IDT reactivity and laminitis, and it is unlikely that these results were simply coincidental to the laminitis. The diversity in age, duration and severity of illness, sex, and breed of horses, coupled with the lack of clinical evidence of a preexisting immunopathologic condition, also seem to support the contention that the hyperreactive state is a consequence associated with the laminitis rather than a preexisting condition.

Time of maximal response to ID injections of allergens (4 hours after injection) observed in experiment 1 and the primary neutrophilic infiltrate observed in biopsy specimens obtained 6 hours after injection in experiment 2 indicated that this could possibly be a type-III (immune-complex mediated) hypersensitivity.[7] However, other options would also include an increase in fragility of blood vessels associated with laminitis, as well as the antigen simply being an irritant to endothelial and extravascular tissues. Additional diagnostic testing, such as evaluation for immunoglobulin deposition in vessel walls to define the immunologic basis of the lesions and evaluation of evidence of endothelial activation by expression of E-selectin or vascular endothelial growth factor receptors, was beyond the scope of this study but would be necessary to more definitively determine the exact type of reaction that occurs. The fact that we were unable to correlate clinical changes with histologic changes is not surprising. The major clinical change at the injection sites was edema, a feature that is commonly not evident during histologic examination of tissue sections, because extravascular fluids are generally lost during tissue processing.

It is of interest that if these data do support development of a type-III hypersensitivity in horses with chronic laminitis, this finding would not be without precedence. In humans, **systemic lupus erythematous (SLE)** is described as a multifactorial autoimmune disease that is frequently found in relationship with diseases such as Raynaud's syndrome (a vascular disease characterized by intermittent episodes of digital ischemia) and various anemias.[8,9] In SLE, immune complexes are deposited in vessel walls, glomeruli, and joints via type-III hypersensitivity reactions that are manifested as vasculitis, glomerulonephritis, and arthritis.[9,10] Raynaud's syndrome is commonly associated with SLE, although it is unclear whether the 2 conditions are coincidental or causally related to each other.[11] In another study,[12] comparisons were made between Raynaud's syndrome in humans and chronic laminitis in horses with some intriguing parallel clinical signs and pathologic characteristics. In addition, SLE occurs in horses.[13] Therefore, it is interesting to speculate whether horses with chronic laminitis could serve as a model for study of the mechanistic relationship of these diseases.

It can be determined from analysis of the data in the study reported here that CL horses do develop, at some point in the disease process, a hyperreactivity to various antigenic stimuli when compared with responses for nonlaminitic horses. Even though the risk of precipitating an episode of laminitis is apparently small, the possibility that antigenic challenge may result in exacerbation of clinical signs of laminitis should be discussed with horse owners prior to administration of antigenic substances. Lastly, it would seem logical that when clinical signs of laminitis appear suddenly after administration of routine vaccinations, radiographic evaluations of the feet should be completed as soon as possible to validate any preexisting laminitic condition.

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Appendix

Criteria and scoring system used during histologic evaluation of skin biopsy specimens obtained from horses at various time periods after ID injection of specific allergens

	Grade				
Criterion	**0**	**1**	**2**	**3**	**4**
Diffuseness	No inflammation	Inflammation limited to a single focus in subadnexal dermis	Diffuse inflammation in the subadnexal dermis that involved < 50% of vessels	Diffuse inflammation in the subadnexal dermis that involved > 50% of vessels	Diffuse inflammation in the subadnexal dermis that involved > 50% of vessels with extension into the superficial dermis
Intensity	No inflammation	Minimal margination; scattered inflammatory cells around vessels; no interstitial component	Mild margination; 1 or 2 layers of inflammatory cells around vessels with a few inflammatory cells in vessel walls; mild interstitial component	Moderate margination of inflammatory cells that srarely obscured the lumen; moderate to marked inflammatory cells in vessel walls without wall necrosis (ie, nonnecrotizing vasculitis); or features of grade 2 with intense interstitial component	Marked margination of inflammatory cells that focally obscured the lumen; necrosis of vessel wall; or features of grade 3 with more intense perivascular and interstitial components
Edema	No edema	Mild separation of collagen bundles in the subadnexal dermis	More extensive separation than for grade 1, sometimes with small amounts of interstitial fibrin in the subadnexal dermis	More extensive separation than for grade 2 with clearly discernible fibrin in areas of the subadnexal dermis	Widespread separation of subadnexal collagen bundles with more abundant fibrin than for grade 3 and extension into the superficial dermis