Comparison of results for intradermal testing between clinically normal horses and horses affected with recurrent airway obstruction

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Objective—To evaluate differences in response to ID injection of histamine, phytohemagglutinin (PHA), and *Aspergillus* organisms between clinically normal horses and horses with recurrent airway obstruction (RAO).

Animals—5 healthy adult horses and 5 adult horses with RAO.

Procedure—Intradermal testing (IDT) was performed on the neck with 2 positive control substances (histamine and PHA) and a mixture comprising 5 *Aspergillus* species. Four concentrations of each test substance plus a negative control substance were used. Equal volumes (0.1 mL) of each test substance were prepared to yield 15 syringes ([4 concentrations of each test substance plus 1 negative control substance] times 3 test substances) for each side of each horse (ie, 30 syringes/horse). Intradermal injections were administered; diameter of wheals was recorded 0.5, 4, and 24 hours after injection.

Results—Hypersensitive responses to ID injection of histamine were detected 0.5 hours after injection, and a delay in wheal formation after ID injection of *Aspergillus* mixture 24 hours after injection was detected in RAO-affected horses but was not observed in clinically normal horses. No differences were detected between the 2 groups after ID injection of PHA.

Conclusions and Clinical Relevance—RAO-affected horses are hypersensitive to histamine, suggesting that RAO is associated with a heightened vascular response to histamine. Higher concentrations of *Aspergillus* mixture may be needed to detect horses that are sensitive to this group of antigens. Wheal reactions to *Aspergillus* may be a delayed response, suggesting that IDT results should be evaluated 0.5, 4, and 24 hours after ID injection. (*Am J Vet Res* 2005;66:1348–1355)

Recurrent airway obstruction (RAO) is a common respiratory disease that affects horses and is characterized by intermittent periods of airway obstruction. Clinical signs include wheezing, coughing, exercise intolerance, tachypnea, and increased respiratory effort.[1,2] This disease has no breed or sex predilection and commonly affects middle-age to older horses (> 7 years old). It is believed that RAO is an allergic hypersensitivity disease that develops when horses are exposed to various antigens (eg, *Micropolyspora faeni*, *Aspergillus fumigatus*, and *Thermoactinomyces vulgaris*) located in a horse's environment and fodder.[3-5] Diagnosis of RAO is based on clinical signs and evaluation of bronchoalveolar lavage fluid. Although these diagnostic tests may confirm RAO, they do not specifically identify causative antigens involved in airway hypersensitivity. The pathophysiologic characteristics and treatment of RAO are thoroughly discussed elsewhere.[1,2] Interestingly, most current treatment modalities involve palliation of clinical signs rather than identification of causative antigens and treatment of the underlying disease process.

Intradermal testing (IDT) is a diagnostic tool in humans and small animals that allows more precise identification of causative antigens in allergic patients.[6-8] Intradermal testing is useful because it exploits cutaneous mast cells as a reflection of mast cell reactivity in other body systems (eg, the respiratory tract). Therefore, when a particular antigen causes degranulation of cutaneous mast cells, the same antigen may also cause degranulation of mast cells in other organ systems. Once causative antigens are identified, selected patients can be treated with allergen-specific immunotherapeutics in an attempt to decrease the patient's dependence on corticosteroids and ameliorate the frequency and severity of clinical signs.

Techniques used during IDT in people and small animals have been extrapolated to horses in an attempt to identify causative antigens involved in horses with RAO. Allergen-specific immunotherapeutic protocols have also been extrapolated for use in equine patients, but results of IDT and allergen-specific immunotherapy in horses have been inconsistent.[4,5,9-16] The exact reasons for the ambiguous results of IDT in horses have not been defined. Some of the inconsistencies in other studies of IDT in horses may be attributable to the fact that appropriate antigens have not been identified, appropriate dosages of antigens have not been determined, appropriate evaluation time points after ID injections have not been established, and differences in response to IDT antigens between clinically normal and RAO-affected horses have not been adequately defined.

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To establish IDT in horses as a valid diagnostic tool, it is important to define appropriate IDT techniques and dosages specific for horses and to characterize differences in response to ID injection of positive control substances (ie, histamine) and causative antigens (ie, *Aspergillus* organisms) between clinically normal and RAO-affected horses. The objective of the study reported here was to determine whether RAO-affected horses in remission would have a hypersensitive response (ie, would have an exaggerated immune response to a foreign agent) after ID injection of 2 positive control substances. An additional objective of this study was to determine whether there were differences in response to ID injection of *Aspergillus* organisms, a potential causative antigen in RAO horses, between clinically normal and RAO-affected horses. Furthermore, we attempted to determine the appropriate dose and time points at which to evaluate ID injections of an *Aspergillus* mixture in horses. We hypothesized that RAO-affected horses would have a hypersensitive response for the formation of wheals after ID injection of positive control substances, compared with the response in clinically normal horses. We also hypothesized that RAO-affected horses would develop positive wheal reactions after ID injection of *Aspergillus* mixture that would not be observed in clinically normal horses.

Materials and Methods

Animals—Five healthy adult Thoroughbred mares that ranged from 6 to 24 years of age were selected for use in the study. Horses were selected on the basis of a lack of historical and clinical evidence of allergic disease. The horses were part of a university teaching herd and had not had evidence of allergic disease for at least the past 3 years. All horses were examined within 1 week before onset of the study and assessed as clinically normal on the basis of results of physical examination.

Five adult RAO-affected horses (2 mares and 3 geldings) that ranged from 6 to 23 years of age were selected for use in the study. These horses were selected on the basis of historical and clinical evidence of RAO. Horses with RAO had been donated to our facility because of a chronic history of RAO. There were 2 Thoroughbreds, 1 Tennessee Walking Horse, 1 Paint, and 1 Appaloosa.

Recurrent airway obstruction was confirmed several months before commencement of this study; RAO was confirmed on the basis of results of physical examination, CBC count, serum biochemical analysis, arterial blood gas analysis, analysis of bronchoalveolar lavage fluid, and inhalation challenge with moldy alfalfa hay. At the time of the study reported here, all RAO-affected horses had been in remission for at least 4 months and did not have signs of respiratory compromise.

Before participation in the study, all horses (clinically normal and RAO-affected) were maintained on pasture; vaccinated annually against equine rhinopneumonitis, eastern equine myeloencephalitis, western equine myeloencephalitis, tetanus, and rabies; and dewormed with ivermectin every 12 weeks. The study was approved by the Virginia Tech Animal Care and Use Committee.

Housing and treatment—Horses were moved to a research facility on the day before commencement of IDT to acclimatize to the facility (day 1). Horses were housed separately in 4 × 4-m box stalls with access to separate outdoor paddocks; horses were fed grass hay ad libitum and had continuous access to water. Intradermal testing was initiated on day 2 of the study, and horses were returned to their pasture environment on day 3. Physical examinations were performed on the horses on each of the 3 days they were at the research facility.

Preparation of test substances and IDT—Two positive control substances (histamine[a] and **phytohemagglutinin**[b] **[PHA]**) and an environmental antigen (*Aspergillus* mixture[c]) were used in the study. Concentrations of histamine and PHA used in this study were selected on the basis of results of another study[17] conducted by our laboratory group. In contrast to that study, the study reported here used lower concentrations of PHA to avoid excessively large wheals and slightly higher concentrations of histamine in an attempt to reach a plateau for wheal response.

Four concentrations of histamine (0.02, 0.004, 0.0008, and 0.00016 mg/mL) and PHA (0.5, 0.2, 0.08, and 0.032 mg/mL) were prepared. *Aspergillus* mixture was selected for use because it was isolated from moldy hay that had been used to induce clinical signs of RAO in the affected horses before this study (clinical signs of RAO were detected within 3 days after exposure to moldy hay as evidenced by tachypnea, increased respiratory effort, and wheezes or crackles heard during auscultation of the lungs). Concentrations of *Aspergillus* mixture were selected on the basis of studies[13,16,18] that involved IDT of horses; concentrations used were 4,000, 2,000, 1,000, and 500 **protein nitrogen units (PNUs)**/mL. We used PBS solution as a negative control substance; PBS solution was also used as the diluent during preparation of the positive control substances.

An equal volume (0.1 mL) for each injection was placed in tuberculin syringes with an attached 27-gauge, 3/8-inch needle. Fifteen tuberculin syringes were prepared for IDT ([4 concentrations of each test substance plus 1 negative control substance] times 3 test substances) for each side of the neck. Both sides of the neck were used in each horse; therefore, 30 syringes were prepared for each horse. Each group of 15 syringes was placed in random order in a syringe tray as determined by a computer-generated randomization chart. Each horse was assigned a separate randomization chart, but the same randomization chart was used for both sides of the neck of a specific horse.

Intradermal testing was performed on all horses at the same time (June). Hair was clipped (No. 40 clipper blade) from a 12 × 6-cm rectangle on both sides of the lateral aspect of the neck of each horse. A permanent marking pen was used to draw a grid within the clipped area on each side of the neck; the grid consisted of 20 squares (10 squares horizontally and 2 squares vertically). Each horse was then sedated by administration of xylazine hydrochloride[d] (0.5 mg/kg, IV). Injections (15 ID injections/side of the neck; 30 ID injections/horse) were administered simultaneously on both sides of the neck by 2 clinicians. The same 2 clinicians (VABM, TOM) performed all ID injections on all 10 horses and were not aware of the contents of each syringe. Measurements of the wheals that developed in response to each injection in each horse were obtained 0.5, 4, and 24 hours after injection by a board-certified veterinary dermatologist (TOM) who was not aware of the substance injected at each site on the grid.

After wheal measurements were obtained at 24 hours, 0.5 mL of lidocaine hydrochloride was injected SC and skin biopsy specimens (6 mm in diameter) were collected from all horses from the area of the ID injection of *Aspergillus* mixture (4,000 PNUs/mL). Simple interrupted sutures were inserted to appose the skin of the biopsy defects, and specimens were placed in neutral-buffered 10% formalin for 24 hours. Specimens were then embedded in paraffin, sectioned, mounted on glass slides, and stained with H&E in a standard

manner. Biopsy sections were subsequently examined by use of light microscopy. A subjective comparison of infiltration of inflammatory cells at the site of ID injection of *Aspergillus* mixture was made between clinically normal and RAO-affected horses.

Statistical analysis for histamine and PHA—Dose-response curves for histamine and PHA were plotted by use of the Chapman-Richard equation (also known as the natural growth model), $y = a(1 - [e^{-bx}])$, where y is the size of the wheal (in millimeters), a is the plateau for wheal size, b is the rate of increase in wheal size for increasing concentrations of PHA, and x is the concentration of PHA (in mg/mL). Specifically, the relationship between dose (ie, concentration of histamine or PHA) and response (ie, wheal diameter) was modeled to evaluate the rate of increase (which was interpreted as sensitivity to ID injection of histamine or PHA) and the maximum wheal diameter (ie, plateau, which was interpreted as response to ID injection of histamine or PHA) for the clinically normal and RAO-affected horses. Hypersensitivity was defined as a greater rate of increase or plateau of the dose-response curve generated from results of ID injection of histamine or PHA in RAO-affected horses, compared with results for clinically normal horses (**Figure 1**).[19] Variables estimated by use of the dose-response models were subjected to analysis by use of an ANOVA.[e] The ANOVA was used to test for significant ($P < 0.05$) differences in the rate of increase and plateau between clinically normal and RAO-affected horses.

Statistical analysis for *Aspergillus* mixture—The *Aspergillus* data did not fit the Chapman-Richard equation; therefore, a simple linear model was used to examine the relationship between concentration of antigen and response (ie, wheal diameter) after ID injection. Variables estimated by use of the simple linear models were subjected to analysis by an ANOVA.[e] The ANOVA was used to test for effects of RAO on the slope. Specifically, the slope of the line was evaluated to identify significant ($P < 0.05$) differences in response after ID injection of *Aspergillus* mixture between clinically normal and RAO-affected horses.

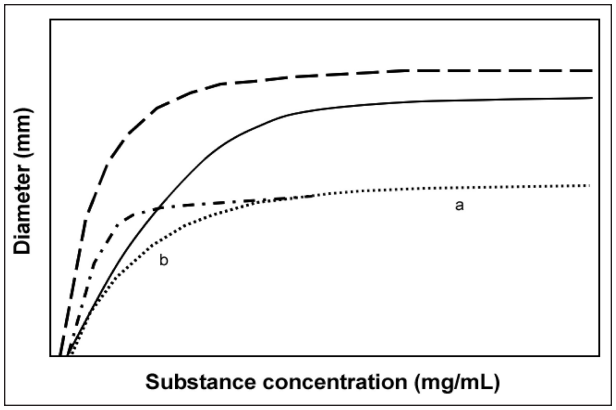

Figure 1—Diagrammatic representation of dose (ie, stimulant concentration) versus response (ie, wheal diameter) curves after ID injection of histamine or phytohemagglutinin (PHA) in a clinically normal horse and a horse with a hypersensitive response. Notice the plateau for wheal size (a) and rate of increase in wheal size for increasing concentrations of the injected substance (b). Values were plotted by use of the Chapman-Richard equation: $y = a(1 - [e^{-bx}])$, where y is the size of the wheal (in millimeters) and x is the concentration of the injected substance (in mg/mL). The wheal response to ID injection of histamine or PHA for a clinically normal horse (dotted line) differs from that for a horse with a hypersensitive response, which may have a greater rate of increase for wheal formation (dotted and dashed line), a greater plateau (solid line), or both (dashed line).

Results

Results for histamine and PHA—Results of daily physical examinations were within expected limits. All horses tolerated IDT with histamine and PHA well with only minor discomfort observed during the procedure. No severe reactions to the injections were detected; however, digital palpation of wheals induced by injection of PHA at 4 and 24 hours after ID injection elicited signs of pain. All negative control (ie, PBS solution) injections induced wheals of 8 to 12 mm in diameter by 0.5 hours after injection that resolved by 4 hours after injection.

On the basis of examination of the raw data, results for histamine were evaluated at 0.5 and 4 hours after ID injection. At 24 hours after injection, there were no wheals for any of the injections of histamine, regardless of concentration. We evaluated results for PHA at 4 and 24 hours after ID injection. Injection of PHA induced wheals that ranged from 8

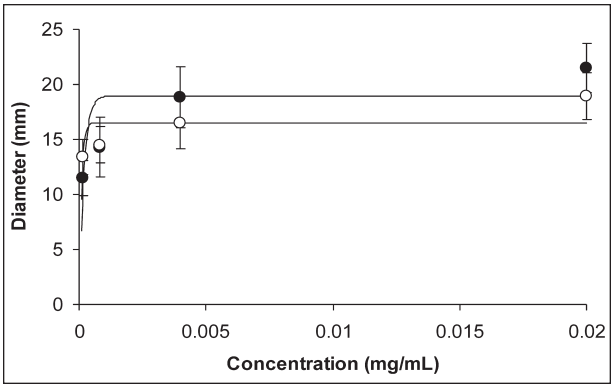

Figure 2—Mean ± SE wheal diameters for 5 clinically normal horses (open circles) and 5 horses with recurrent airway obstruction (RAO; solid circles) 0.5 hours after ID injection of 4 concentrations of histamine. The RAO-affected horses had a significantly ($P = 0.023$) greater rate of increase for wheal formation, compared with values for the clinically normal horses. The fit of the data is indicated for all 4 concentrations of histamine in clinically normal (bottom line) and RAO-affected (top line) horses.

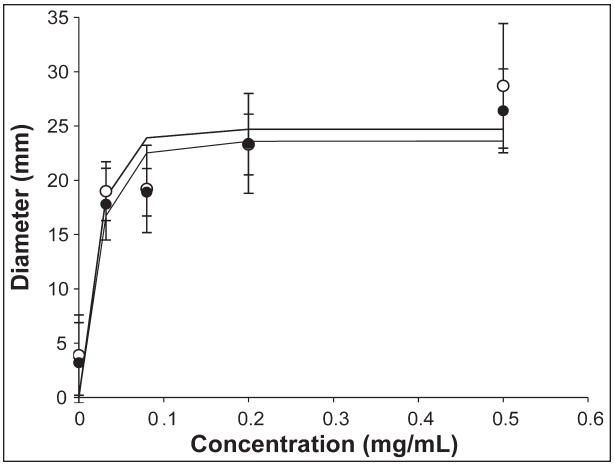

Figure 3—Mean ± SE wheal diameters for 5 clinically normal horses (open circles) and 4 horses with RAO (solid circles) 4 hours after ID injection of 4 concentrations of PHA. Values for rate of increase or plateau did not differ significantly ($P > 0.05$) between the 2 groups of horses. *See* Figure 2 for remainder of key.

Table 1—Mean ± SE rate of increase in wheal formation and plateau 0.5 and 4 hours after ID injection of histamine in 5 clinically normal horses and 5 horses with recurrent airway obstruction (RAO).

	0.5 h		4 h	
Group	Rate of increase* (mm of increase/μg)	Plateau† (mm)	Rate of increase* (mm of increase/μg)	Plateau† (mm)
Clinically normal	5.4	19.0	3.6	18.1
RAO-affected	10.8‡	16.5	4.2	17.0

*Rate of increase represents the rate at which wheal size increased with increasing concentration of the injected substance, as determined by wheal diameter measured 0.5 and 4 hours after ID injection. †Plateau represents the mean of the maximum wheal diameters induced by ID injection of all 4 concentrations of the substance and measured at the indicated time points after injection. ‡Within a column, value differs significantly ($P = 0.023$) from the value for the clinically normal horses.

Table 2—Mean ± SE rate of increase in wheal formation and plateau 4 and 24 hours after ID injection of phytohemagglutinin in 5 clinically normal horses and 4 horses with RAO.

	4 h		24 h	
Group	Rate of increase* (mm of increase/μg)	Plateau† (mm)	Rate of increase* (mm of increase/μg)	Plateau† (mm)
Clinically normal	42.9	24.7	59.0	28.9
RAO-affected	38.6	25.6	45.4	31.1

See Table 1 for key.

to 14 mm in diameter by 0.5 hours after injection; these values did not differ subjectively from the size of wheals induced by injection of the negative control substance (8 to 12 mm in diameter). A nonlinear model (ie, Chapman-Richard equation) was used to plot results for histamine and PHA (**Figures 2 and 3**). At 0.5 hours after injection, a significant ($P = 0.023$) difference in the rate of increase for histamine was detected between clinically normal and RAO-affected horses (ie, RAO-affected horses had a greater rate of increase, compared with the rate of increase for clinically normal horses). This difference in the response to ID injection of histamine between clinically normal and RAO-affected horses was not evident at 4 hours after injection (**Table 1**).

Two concentrations of PHA were incorrectly labeled in 1 horse and provided erroneous results; therefore, data for that horse were removed from the evaluation of PHA at 4 and 24 hours after injection. We did not detect significant differences between clinically normal and RAO-affected horses at 4 and 24 hours after injection of PHA (**Table 2**).

Results for *Aspergillus* mixture—All horses tolerated IDT with *Aspergillus* mixture well with only minor discomfort observed during the procedure. We did not observe severe reactions to any of the injections. All negative control injections induced wheals of 9 to 11 mm in diameter by 0.5 hours after injection that resolved by 4 hours after injection.

On the basis of examination of the raw data, measurable wheal responses for *Aspergillus* mixture were evaluated at 0.5, 4, and 24 hours after injection; dose-response curves were plotted for *Aspergillus* at these time points. Data for *Aspergillus* did not fit the Chapman-Richard equation; therefore, a simple linear model was used to represent results for injection of the *Aspergillus* mixture (**Figure 4**). *Aspergillus* data obtained at 0.5 and 4 hours after injection were subjected to analysis by use of an ANOVA, but no significant differences were detected between clinically normal and RAO-affected horses. However, none of the clinically normal horses had wheals 24 hours after injection, whereas 4 of 5 RAO-affected horses had measurable wheals 24 hours after injection of the highest concentration of *Aspergillus* mixture (**Table 3**). Therefore, *Aspergillus* (concentration of 4,000 PNUs/mL) had an effect on RAO-affected horses at 24 hours after injection that differed significantly($P = 0.002$) from the effect observed for clinically normal horses.

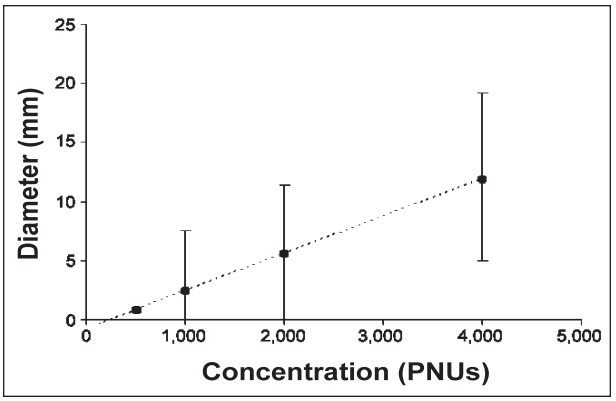

Figure 4—Mean ± SE wheal diameters for 5 horses with RAO 24 hours after ID injection of 4 concentrations of *Aspergillus* mixture. The dashed line represents fit of the data at 0.5 hours after ID injection for all 4 concentrations of *Aspergillus* mixture. No wheal responses were detected in clinically normal horses. PNUs = Protein nitrogen units.

Microscopic examination of skin biopsy specimens collected from the injection sites of the highest concentration of *Aspergillus* mixture at 24 hours after injection revealed mild edema of the deep dermis and a mixed perivascular inflammatory response consisting of macrophages, lymphocytes, eosinophils, and a few neutrophils in the RAO-affected horses. The clinically

Table 3—Mean ± SE rate of increase in wheal formation and plateau 0.5, 4, and 24 hours after ID injection of *Aspergillus* mixture in 5 clinically normal horses and 5 horses with RAO.

	0.5 h		4 h		24 h	
Group	Intercept* (mm)	Slope† (mm of increase/μg)	Intercept* (mm)	Slope† (mm of increase/μg)	Intercept* (mm)	Slope† (mm of increase/μg)
Clinically normal	9.6	0.0001	1.6	0.001	NA	NA
RAO-affected	9.3	0.0002	1.4	0.002	0	0.003‡

*Intercept represents the mean of all wheals (total, 10 wheals) induced by ID injection of the negative control substance (ie, PBS solution) at all indicated time points for 5 clinically normal horses and 5 RAO-affected horses. †Slope represents the rate at which wheal size increased with increasing concentration of *Aspergillus* mixture. ‡Within a column, value differs significantly ($P = 0.002$) from the value for the clinically normal horses.

NA = Not applicable because there was no response evident 24 hours after injection in the clinically normal horses.

normal horses had minimal inflammation with a few perivascular neutrophils.

Discussion

It is not known whether clinical signs of RAO in horses result from an immediate-hypersensitivity response, cell-mediated (ie, delayed) response, or a combination of the 2. Therefore, positive control substances that reflect the immediate and cell-mediated immune responses were used in the study reported here. Histamine is a commonly used positive control substance for IDT that mimics antigen-induced histamine release from mast cells seen in type I hypersensitivity.[20] Intradermal wheal formation secondary to histamine injection is attributable to its ability to increase local vascular permeability and leakage of plasma into the surrounding tissues.[20,21]

In the study reported here, a difference was detected in the rate of increase between clinically normal and RAO-affected horses at 0.5 hours after ID injection of histamine. In particular, the rate at which the wheals induced by ID injection of histamine increased was more rapid in RAO-affected horses, which suggested that RAO-affected horses were hypersensitive to ID injection of histamine, compared with the response of clinically normal horses. This difference in the rate of increase was not detected 4 hours after ID injection of histamine. In 1 study,[22] investigators documented increased numbers of mast cells and basophils in bronchoalveolar lavage fluid and increased concentrations of histamine in pulmonary epithelial lining fluid obtained from RAO-affected horses 5 hours after challenge with hay, whereas in another study,[23] other investigators reported that the sensitivity of basophils was increased in RAO-affected horses. Analysis of the results of those studies suggests that RAO-affected horses have increased numbers of mast cells and basophils and increased sensitivity to specific antigens. Analysis of the results for the study reported here suggests that RAO-affected horses have increased tissue sensitivity to exogenous administration of histamine. Poeple with atopic dermatitis reportedly[24-26] are more sensitive to ID injection of histamine, compared with the response for clinically normal humans; however, this is not a universal finding, and this topic remains controversial among human dermatologists.

Phytohemagglutinin, a potent mitogen, was used to evaluate cell-mediated immunity and T-cell function. Delayed or late-phase reactions have been detected 24 hours after ID injection of various antigens in people and have also been observed in various other species with atopic conditions.[27-29] Phytohemagglutinin did not cause significant differences in the rate of increase or plateau between clinically normal and RAO-affected horses. This information supports results of another study[23] in which no differences were found between clinically normal and RAO-affected horses for in vitro stimulation of lymphocytes. In addition, evaluation of this information suggests that clinically normal and RAO-affected horses react in a similar manner after ID injection of a mitogen and that the nonspecific cell-mediated immune system is similar for each of these groups of horses.

The Chapman-Richard equation was used to evaluate results for histamine and PHA. This model was chosen to determine the increase in wheal size induced by increasing concentrations of histamine and PHA. A simple linear model was not used because, for the higher concentrations of histamine and PHA used in this study, the concentration-response curve had limited growth and plateaued after a certain concentration was exceeded. Analysis of this information suggests that at the initial (lower) concentrations of histamine and PHA, horses had an incremental increase in wheal size. In contrast, as higher concentrations were used, the wheal size reached a threshold and did not subsequently increase substantially. This type of statistical analysis has been used in other biological systems (eg, growth of fish, timber, and tumors) that are characterized by limited growth.[19]

In the study reported here, no significant differences were detected for intercept or slope between clinically normal and RAO-affected horses when evaluating results for the *Aspergillus* mixture 0.5 and 4 hours after injection. However, 4 of 5 RAO-affected horses did have wheals (range, 12 to 22 mm in diameter) 24 hours after injection with the highest concentration (4,000 PNUs/mL). We did not detect wheal reactions in the clinically normal horses at the corresponding time and concentration. Interestingly, the RAO-affected horses did not have significant differences from the clinically normal horses 0.5 and 4 hours after ID injection of 4,000 PNUs of *Aspergillus*/mL, time points at which IDT results are commonly evaluated. This information supports results of a study[10] in which approximately 5.5% of positive reactions during IDT were detected 24 hours after injection of RAO-affected horses. Additionally, another study[18] revealed

that most delayed (ie, 24 hours after injection) reactions were associated with reactions evident at earlier time points (0.5 and 4 hours) and < 1% of all delayed reactions were not preceded by a reaction at an earlier time point. Therefore, analysis of the information from the study reported here suggests that results of IDT in RAO-affected horses should be assessed 24 hours after injection as well as 0.5 and 4 hours after injection. Furthermore, the reaction to ID injection of *Aspergillus* may be a delayed-type or late-phase reaction. This is supported by a study[5] in which investigators found that reactions to ID injection of *Aspergillus* in RAO-affected horses resulted in a dual hypersensitivity response (ie, a weak initial response shortly after injection followed by an Arthus-type response 4 to 8 hours later). Interestingly, microscopic examination of biopsy specimens obtained 24 hours after ID injection of the *Aspergillus* mixture in the study reported here revealed cells associated with a delayed hypersensitivity response, including macrophages, lymphocytes, eosinophils, and a few neutrophils, in the RAO-affected horses that were not observed in the clinically normal horses.

In other studies[13,16,17] on IDT in horses, investigators have used a maximum dose of 0.05 to 0.1 mL of *Aspergillus* mixture at a concentration of 1,000 PNUs/mL. Analysis of results for the study reported here suggests that higher doses of this antigen must be used for ID injection to achieve a wheal response in RAO-affected horses. Although excessively high concentrations of antigen may elicit false-positive dermal responses, evaluation of our results suggests that an *Aspergillus* concentration of 4,000 PNUs/mL is not excessive on the basis of the fact that no wheals were detected in the clinically normal horses for this concentration.[4,30] Therefore, *Aspergillus* concentrations of 4,000 PNUs/mL or higher may be most appropriate for use in identifying RAO-affected horses that are sensitized to *Aspergillus* organisms.

The exact reason that 1 of 5 RAO-affected horses did not have a positive IDT reaction after injection with the *Aspergillus* mixture is not known. *Micropolyspora* organisms may have been the antigen that caused clinical signs of RAO in this horse during challenge exposure to hay. It is also possible that results of IDT may not be an indicator of causative antigens in all RAO-affected horses. This is supported by the findings in other studies[4,5] in which investigators detected differences between positive IDT results and results for inhalation challenge with *A fumigatus* in RAO-affected horses. Another group of investigators suggested[30] that the lack of correlation between bronchial and dermal reactivity may be attributed to local production of allergen-specific IgE within the respiratory tract or skin or regional heterogeneity in responsiveness of mast cells.

As mentioned previously, *Aspergillus* organisms were selected as a potential causative antigen because they were isolated from moldy hay used to induce clinical signs of RAO in the affected horses prior to the study reported here. Ideally, an inhalation challenge study with *Aspergillus* organisms in the horses used in this study would have provided more substantial evidence of the role of *Aspergillus* organisms for inducing respiratory compromise in the RAO-affected horses; however, this was not performed. It has been suggested in other reports[4,5] that *Aspergillus* and *Micropolyspora* organisms are involved in the pathogenesis of RAO, as determined on the basis of inhalation challenge and hay exposure.

Another major limitation of this study was the small sample size. A comparison of IDT responses in a larger number of RAO-affected and clinically normal horses would provide more convincing evidence of the role of *Aspergillus* organisms as a causative antigen and better evaluation of the involvement of delayed hypersensitivity responses in the evaluation of IDT in horses.

Intradermal testing has been used extensively in human medicine to identify causative antigens in various allergic disorders (eg, asthma, insect hypersensitivity, and allergic rhinitis)[6] and has subsequently been extrapolated to equine medicine in an attempt to identify causative antigens in RAO-affected horses. However, many of the studies[4,5,9-13,17] involving IDT in horses have provided contradictory results. In a study[12] conducted in 1964, IDT was performed in 6 clinically normal and 6 RAO-affected horses; IDT involved use of 11 antigens, including a hay dust extract made of dust from a sample of hay that induced clinical signs in RAO-affected horses. In that study, ID injection of hay dust extract induced reactions in the clinically normal and RAO-affected horses that peaked at 4 hours. In the study reported here, the *Aspergillus* mixture induced wheals in both groups of horses 0.5 and 4 hours after injection, whereas only the RAO-affected horses had wheals 24 hours after injection. Furthermore, *Aspergillus* organisms were specifically isolated from the hay used for the challenge exposure and induced wheals in 4 of 5 RAO-affected horses 24 hours after ID injection, which were not observed in the clinically normal horses. Differences in results may have been attributable to the fact that specific antigens for the study reported here were isolated from hay used for challenge exposure, whereas an assortment of nonspecific antigens was used in that other study.[12] Use of nonspecific antigens and the possibility that inadequate concentrations of antigen were administered in that study[12] may have resulted in a nonspecific or inadequate response in clinically normal and RAO-affected horses; the author of that study subsequently concluded that there were no differences between clinically normal and RAO-affected horses with regard to IDT.

In another study,[5] investigators thoroughly compared IDT and inhalation challenge with *M faeni* and *A fumigatus* as potential causative antigens in RAO-affected horses. That study revealed that significantly more RAO-affected horses had skin sensitivity to *M faeni* and *A fumigatus*, compared with responses for clinically normal horses. In the study reported here, we detected positive skin wheals 24 hours after ID injection of the *Aspergillus* mixture in RAO-affected horses. In contrast, investigators in the aforementioned study[5] observed the maximum response at 4 hours after injection. Although those authors reported a satisfactory correlation between positive IDT results and results of inhalation challenge with *M faeni*, poor correlation was

reported for *A fumigatus* (positive skin response was as likely for horses with a negative response to inhalation challenge as for horses with a positive response to inhalation challenge). Thus, they concluded that IDT results may correlate with true causation for some antigens (*M faeni*) but not others (*A fumigatus*). Positive results for inhalation challenge with *Aspergillus* organisms in the study reported here would have provided stronger evidence that *Aspergillus* spp were a causative antigen in the RAO-affected horses. However, the findings of that other study[5] may partially explain the reason that 1 of 5 RAO-affected horses did not react to ID injection of *Aspergillus* mixture in our study.

Several investigators have reported[9-11] a pattern for greater numbers of positive IDT reactions in RAO-affected horses, compared with results for clinically normal horses. However, a reliable distinction between the 2 groups could not be established for comparison of specific antigens.[10] Investigators in 1 study[10] used 58 allergens to compare IDT results between a group of 6 RAO-affected horses and 8 clinically normal horses. They found that both groups of horses had positive reactions for IDT, but only 3% of possible allergens resulted in significant differences in positive reactions between the 2 groups. Many factors may have been associated with the lack of response between the 2 groups; however, the test antigens selected and antigen concentrations used may have contributed to the lack of divergence between the 2 groups. Notably, no difference between the groups was observed 0.5, 4, or 24 hours after ID injection of *Aspergillus* mixture (1,000 PNUs/mL).[10] This finding was also documented in another study[11] in which *Aspergillus* mixture (1,000 PNUs/mL) induced positive reactions in clinically normal horses and horses with RAO, recurrent urticaria, and allergic dermatitis. As mentioned for the study reported here, higher doses of *Aspergillus* mixture (4,000 PNUs/mL) were needed to elicit a positive IDT reaction in the RAO-affected horses. This reiterates the need for additional investigations into appropriate dosages of test allergens for use in IDT of horses. Alternatively, *Aspergillus* organisms may not have been the cause of RAO in a group of RAO-affected horses tested in another study.[10] In that study and the study reported here, wheals were detected on clinically normal horses 0.5 and 4 hours after injection; however, none of the clinically normal horses in either study had positive wheal reactions 24 hours after ID injection of *Aspergillus* mixture, thus suggesting that wheals evident 24 hours after ID injection of *Aspergillus* mixture (4,000 PNUs/mL) may have a greater potential to be involved with development of clinical signs of RAO.

In contrast to the greater number of positive reactions found in RAO-affected horses in the aforementioned studies, another group of investigators[17] found that clinically normal horses had a greater number of positive reactions (32 positive reactions/horse), compared with results for RAO-affected horses (23 positive reactions/horse). The authors of that study speculated that clinically normal horses had a greater number of reactions as a result of more prolonged environmental exposure (on pasture for three fourths of the year), compared with exposure for RAO-affected horses (on pasture for less than half of the year), which led to sensitization to a greater number of inhalant pollen allergens for the clinically normal group. Interestingly, the RAO-affected horses in the study reported here were kept on pasture throughout the entire year. Although we did not administer a battery of antigens in our study, the RAO-affected horses did respond to 1 group of antigens (*Aspergillus* mixture), whereas the clinically normal horses did not. We cannot comment on the relevance of amount of time on pasture and effects on IDT.

Review of a small selection of studies related to IDT in horses reveals inconsistent findings when the results are evaluated collectively. Variations in study design and IDT procedures may be associated with these inconsistencies. Moreover, extrapolation of IDT procedures, antigen selection, antigen dosage, and IDT evaluation time points may result in a poor correlation between human and equine patients. Consequently, techniques currently used for IDT of horses need to be reevaluated.

Analysis of results of the study reported here suggests that RAO-affected horses may have exaggerated responses to ID injections, compared with responses of clinically normal horses, as evidenced by the hypersensitive response of RAO-affected horses to histamine. This study also documents the need to establish appropriate dosages for test antigens used specifically for IDT of horses. Furthermore, additional investigations of the time points at which clinicians evaluate injection sites during IDT are warranted because delayed (24 hours) wheal responses may be suggestive of causative antigens.

a. Histamine, Sigma-Aldrich, St Louis, Mo.
b. Phytohemagglutinin from *Phaseolus vulgaris*, Sigma-Aldrich, St Louis, Mo.
c. *Aspergillus* mix, Greer Labs, Lenoir, NC.
d. Xylazine hydrochloride, Fermenta Animal Health Co, Kansas City, Mo.
e. SAS system, version 8.01, SAS Institute Inc, Cary, NC.

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