Evaluation of the precision of intradermal injection of control substances for intradermal testing in clinically normal horses

David M. Wong, DVM, MS; Virginia A. Buechner-Maxwell, DVM, MS; Thomas O. Manning, DVM, MS; Daniel L. Ward, PhD

Objective—To evaluate the precision of intradermal testing (IDT) in horses.

Animals—12 healthy adult horses.

Procedure—IDT was performed on the neck of each horse by use of 2 positive control substances (histamine and phytohemagglutinin [PHA]) and a negative control substance. An equal volume (0.1 mL) for each injection was prepared to yield a total of 20 syringes ([4 concentrations of each positive control substance plus 1 negative control substance] times 2 positive control substances times 2 duplicative tests) for each side of the neck. Both sides of the neck were used for IDT; therefore, 40 syringes were prepared for each horse. Hair was clipped on both sides of the neck, and ID injections were performed. Diameter of the skin wheals was recorded 0.5, 4, and 24 hours after ID injection.

Results—Intra- and interhorse skin reactions to ID injection of histamine and PHA resulted in wheals of uniform size at 0.5 and 4 hours, respectively. Significant intra- and interhorse variation was detected in wheals caused by PHA at 24 hours.

Conclusions and Clinical Relevance—ID injection of histamine and PHA caused repeatable and precise results at 0.5 and 4 hours, respectively. Concentrations of 0.005 mg of histamine/mL and 0.1 mg of PHA/mL are recommended for use as positive control substances for IDT in horses. This information suggests that consistent wheal size is evident for ID injection of control substances, and variation in wheals in response to ID injection of test antigens results from a horse's immune response to specific antigens. (*Am J Vet Res* 2005;66:1341–1347)

Intradermal testing (IDT) is an accepted method for antigen detection in people and small animals and has been used to detect potential antigens leading to the development of allergic diseases.[1,2] Results of IDT have subsequently been used to develop allergen-specific immunotherapy protocols to ameliorate clinical signs in selected patients; administration of substances in accordance with these protocols has yielded positive results in people and small animals.[3-7] Intradermal testing has also been used in horses in an attempt to detect causative antigens for diseases such as **recurrent airway obstruction (RAO)** and insect hypersensitivity.[8-10] However, there have been only a limited number of studies involving IDT in horses. Furthermore, there have been conflicting results reported[8-13] with regard to the effectiveness of IDT in identifying causative antigens in horses with allergic diseases.

One of the earliest studies was published in 1964,[12] in which no differences were found in IDT results between clinically normal and RAO-affected horses. Alternatively, investigators in another study[14] stated that IDT was a diagnostic indicator of causative antigens leading to the development of respiratory hypersensitivities on the basis of differences in positive results for IDT and antigen-inhalation challenge exposure between clinically normal and RAO-affected horses. Other investigators[15] found no differences in IDT results between clinically normal and RAO-affected horses, but 1 study[13] revealed that clinically normal horses had more positive reactions during IDT, compared with the number of reactions for RAO-affected horses. These variable findings make it difficult to generate conclusive statements regarding IDT in horses and its usefulness as a diagnostic modality.

The exact reason for inconsistencies in results of IDT in horses is not known but is probably multifactorial. At a rudimentary level, the amount of wheal variation in response to ID injection of an innocuous substance (eg, saline [0.9% NaCl] solution) into the skin of a horse is not known. Similarly, the amount of wheal variation in response to ID injection of positive control substances such as histamine (immediate-hypersensitivity reaction) or **phytohemagglutinin (PHA**; cell-mediated hypersensitivity reaction) is also unknown. If a substantial amount of wheal variation in response to ID injection of the aforementioned control substances is detected, use of IDT with test antigens may preclude IDT as a reliable and precise diagnostic method for horses. For example, if ID injection of 0.0001 mg of histamine causes wheal reactions that have a substantial amount of variation in diameter in a group of clinically normal horses, the usefulness of positive wheal reactions in response to test antigens in allergic horses would be questionable at best. Other factors such as location of antigen injection, antigen selection, antigen

Received September 7, 2004.
Accepted November 29, 2004.
From the Departments of Large Animal Clinical Sciences (Wong, Buechner-Maxwell) and Small Animal Clinical Sciences (Manning), and the Research Service Laboratory (Ward), Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic and State University, Blacksburg, VA 24061-0442. Dr. Wong's present address is the Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Iowa State University, Ames, IA 50010.
Supported by the Veterinary Medical Fund and Clinical Research Grant, Virginia Polytechnic and State University.
The authors thank Dr. Robert Esch for providing materials and Dr. Zorana Ristic for technical assistance.
Address correspondence to Dr. Wong.

concentration, skin thickness, variability among horses, and IDT procedures may contribute to inconsistencies in results of IDT.

Before IDT can be used for antigen detection in diseases of horses such as RAO, validation of the repeatability of the wheals caused by injection of control substances in clinically normal horses must be established. Validation may provide information about the variability of wheal reactions to control substances. Therefore, we attempted to elucidate the amount of variation (or precision) after ID injection of positive control substances in clinically normal horses.

The purpose of the study reported here was to evaluate wheal responses after ID injection of 4 concentrations of histamine and PHA. More specifically, we compared dose-response curves for histamine and PHA among the horses tested. Furthermore, variation in wheal size in response to various concentrations of histamine and PHA was evaluated within each horse and among several horses. From this information, we determined whether there was a significant amount of intra- or interhorse wheal variation to these substances. We hypothesized that IDT would be a precise diagnostic method on the basis of minimal variability in wheals formed after ID injection of histamine, PHA, and PBS solution in clinically normal horses and that minimal variation would be evident in the dose-response curves for histamine and PHA.

Materials and Methods

Animals—Twelve adult horses (9 mares and 3 geldings) that ranged from 4 to 24 years of age were selected for use in the study. Horses were chosen 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. Breeds represented included Thoroughbred (n = 6), Arabian (3), Quarter Horse (1), Spanish Barb (1), and Saddlebred (1). 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, CBC counts, and serum biochemical analyses. Before participating in the study, all horses were maintained on pasture; vaccinated annually against equine viral rhinopneumonitis, eastern equine encephalomyelitis, western equine encephalomyelitis, tetanus, and rabies; and dewormed with ivermectin every 12 weeks. The study protocol was approved by the Virginia Tech Animal Care and Use Committee.

Housing and treatment—Horses were moved to a research facility 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 PHA[b]) were used in the study. A preliminary study was conducted by use of published[11,16-18] concentrations of positive control substances to determine the optimal concentrations of the control substances. On the basis of that preliminary study, we prepared 4 concentrations of each substance for the study reported here. Histamine concentrations were 0.005, 0.001, 0.0002, and 0.0004 mg/mL, whereas PHA concentrations were 1, 0.7, 0.4, and 0.1 mg/mL. A negative control substance consisted of PBS solution, which also was used as 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. Twenty tuberculin syringes were prepared for IDT ([4 concentrations of each positive control substance plus 1 negative control substance] times 2 positive control substances times 2 duplicative tests) for each side of the neck. Both sides of the neck were used in each horse; therefore, 40 syringes were prepared for IDT in each horse. Each group of 20 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[c] (0.5 mg/kg, IV). Injections (20 ID injections/side of the neck; 40 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 12 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.

Statistical analysis for histamine—Dose-response curves were plotted for histamine by use of a simple linear model to represent the relationship between dose (ie, stimulant concentration) and response (ie, wheal diameter). Mean wheal diameter, y-intercept, and slope were calculated for histamine. In addition, variance in wheal diameter for histamine among all concentrations was evaluated for each horse and among all 12 horses by use of a mixed-model ANOVA.[d] The time points (0.5, 4, and 24 hours) that yielded consistent wheals for histamine were determined by examination of the raw data.

Statistical analysis for PHA—Dose-response curves for PHA were plotted by use of the Chapman-Richard equation

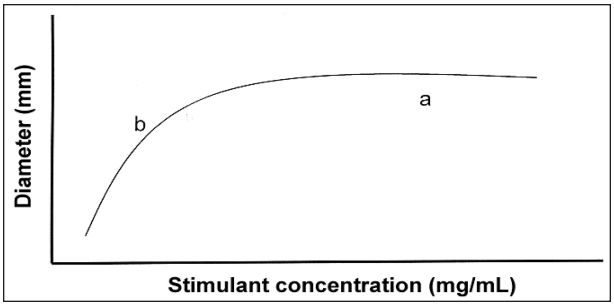

Figure 1—Diagrammatic representation of dose (ie, stimulant concentration) versus response (ie, wheal diameter) curves after ID injection of phytohemagglutinin (PHA) in clinically normal horses. Notice the plateau for wheal size (a) and rate of increase in wheal size for increasing concentrations of PHA (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 PHA (in mg/mL).

(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). The relationship between dose (ie, stimulant concentration) and response (ie, wheal diameter) was modeled to evaluate the rate of increase (which was interpreted as sensitivity to ID injection of PHA) and maximum wheal diameter (ie, plateau, which was interpreted as response to ID injection of PHA [**Figure 1**]). Mean, SD, and 95% confidence interval for the rate of increase and plateau were calculated. In addition, variance in wheal diameter of PHA was evaluated for each horse and among all 12 horses by use of a mixed-model ANOVA.[d] The time points (0.5, 4, and 24 hours) that yielded consistent wheals for PHA were determined by examination of the raw data.

Results

Results for histamine—Examination of results for CBC counts and serum biochemical analysis did not reveal significant abnormalities in any of the horses prior to initiation of the study. Results of physical examinations performed daily on all horses were within anticipated values. All horses tolerated ID injection of histamine well; only minor discomfort was observed during the procedure. None of the horses had severe reactions to any of the injections. All negative control (ie, PBS solution) injections caused wheals with a diameter of 9 to 11 mm at 0.5 hours after injection that resolved by 4 hours after injection.

On the basis of evaluation of raw data, measurable wheal responses for histamine were consistently detected at 0.5 hours after injection. All concentrations of histamine caused dose-related wheals that ranged from 9 to 22 mm in diameter by 0.5 hours after injection. Histamine at a concentration of 0.0004 mg/mL induced wheals by 0.5 hours after injection that were similar in size (9 to 12 mm in diameter) to those induced by injection of the negative control substance (9 to 11 mm in diameter). In addition, only the higher concentrations of histamine (0.001 or 0.005 mg/mL) caused wheals that were detectable at the 4-hour time point. At 4 hours after injection, histamine induced wheals that ranged from 0 to 13 mm in diameter (mean, 7 mm; median, 10 mm) for a concentration of 0.001 mg/mL and that ranged from 0 to 18 mm in diameter (mean, 13 mm; median, 14 mm) for a concentration of 0.005 mg/mL. No wheals were detected at 24 hours after injection, regardless of concentration. Therefore, wheal measurements obtained 24 hours after injection were not statistically evaluated because of the lack of a consistent response among concentrations.

A simple linear model was used to represent histamine (**Figure 2**). Mean ± SD value for the y-intercept at 0.5 hours after injection, which represented the wheal diameter of the negative control injection, was 10.1 ± 0.5 mm (95% confidence interval, 9.1 to 11.2 mm). Slope of the line revealed a mean increase of 1.16 ± 0.27 mm (95% confidence interval, 0.64 to 1.68 mm) in wheal diameter for each 1-µg increase of histamine. Calculated variance components for histamine revealed wheal-to-wheal variation within horses of 1.4 mm and variation among horses of 0.4 mm at 0.5 hours after injection (**Table 1**).

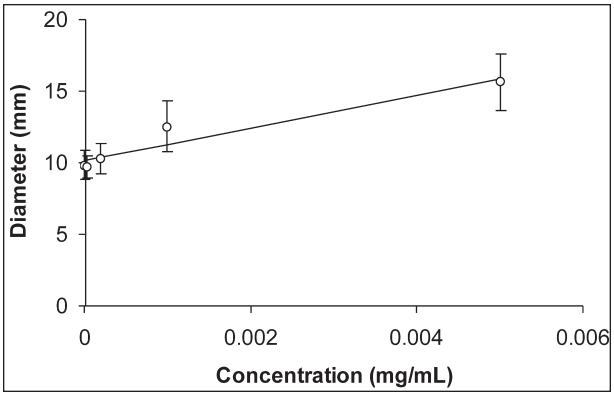

Figure 2—Mean ± SE wheal diameters for 12 horses 0.5 hours after ID injection of 4 concentrations of histamine and a negative control substance (ie, PBS solution). The line represents fit of the data (simple linear model) for histamine at 0.5 hours after ID injection for all 4 concentrations of histamine and the negative control substance.

Table 1—Mean ± SE variation in size of wheals within and among 12 horses after ID injection of histamine and phytohemagglutinin (PHA).

		PHA	
Variable	**Histamine***	**4 h†**	**24 h†**
Variation within a horse (mm)	1.4 ± 0.15	6.8 ± 0.71	43.5 ± 4.82
Variation among horses (mm)	0.4 ± 0.21	10.1 ± 4.75	42.0 ± 21.86

*Values were recorded 0.5 hours after ID injection of histamine.
†Values were recorded 4 and 24 hours, respectively, after ID injection of PHA.

Results for PHA—All horses tolerated ID injection of PHA well; only minor discomfort was observed during the procedure. None of the horses had a severe reaction to any of the injections, although all horses had signs of pain at 4 and 24 hours during digital palpation of the wheals that resulted from ID injection of PHA. Several horses had large wheals (50 to 75 mm in diameter) 24 hours after injection in response to the highest concentration of PHA (1 mg/mL). All negative control injections (ie, PBS solution) induced wheals with a diameter of 9 to 11 mm by 0.5 hours after injection that resolved by 4 hours after injection. Injection of PHA induced consistent wheal diameters 4 and 24 hours after injection. At 0.5 hours after injection, dose-related wheals ranged from 8 to 18 mm in diameter among all concentrations of PHA and were estimated to not differ subjectively from the negative control responses. Therefore, only responses measured 4 and 24 hours after injection were evaluated. Injection of PHA caused dose-related wheals that ranged from 16 to 48 mm in diameter by 4 hours after injection. At 24 hours after injection, dose-related wheals ranged from 11 to 75 mm in diameter. Most horses had greater wheal size at 24 hours after injection, compared with wheal diameter 4 hours after injection, independent of the PHA concentration injected.

The plateau at 4 hours, which represented the mean maximum diameter of wheals among concentrations, was 25.8 mm. At 4 hours after injection, mean rate of increase was 9.1 mm for each 1-mg increase of

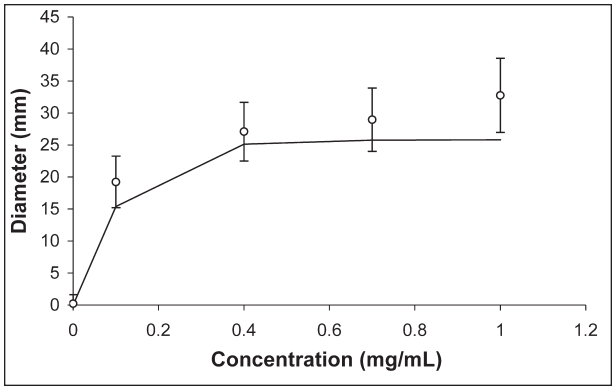

Figure 3—Mean ± SE wheal diameters for 12 horses 4 hours after ID injection of 4 concentrations of PHA and a negative control substance. The line represents fit of the data (nonlinear model) for PHA at 4 hours after ID injection for all 4 concentrations of histamine and the negative control substance.

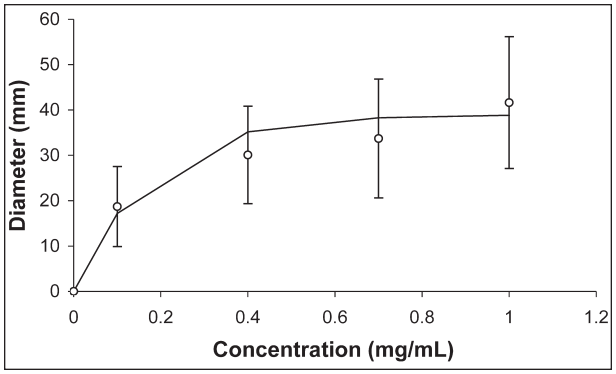

Figure 4—Mean ± SE wheal diameters for 12 horses 24 hours after ID injection of 4 concentrations of PHA and a negative control substance. The line represents fit of the data (nonlinear model) for PHA at 24 hours after ID injection for all 4 concentrations of histamine and the negative control substance.

Table 2—Mean ± SD and 95% confidence interval (CI) for plateau and rate of increase of wheal diameter 4 and 24 hours after ID injection of PHA in 12 horses.

Variable	4 h	24 h
Plateau (mm)*		
Mean ± SD	25.8 ± 5.4	38.9 ± 12.5
95% CI	15.2–36.4	14.4–63.5
Rate of increase (mm/mg)†		
Mean ± SD	9.1 ± 2.0	5.8 ± 2.2
95% CI	5.2–13.0	1.5–10.2

*Plateau represents the mean of the maximum wheal diameters induced by ID injection for all 4 concentrations of PHA and measured 4 and 24 hours after injection. †Rate of increase represents the rate at which wheal size increased with increasing concentration of PHA, as determined by wheal diameter measured 4 and 24 hours after ID injection.

PHA (**Figure 3**; **Table 2**). The plateau at 24 hours after injection, which represented the mean maximum diameter of wheals among all concentrations of PHA, was 38.9 mm. At 24 hours after injection, rate of increase was 5.8 mm for each 1-mg increase of PHA (**Figure 4**). Analysis of calculated variance components 4 hours after injection of PHA revealed wheal-to-wheal variation within horses of 6.8 mm and variation among horses of 10.1 mm. At 24 hours after injection, calculated variance components for PHA revealed wheal-to-wheal variation of 43.5 mm within horses and variation of 42.0 mm among horses (Table 1).

Discussion

It is necessary to evaluate methods for determining causative antigens in allergic diseases in horses to advance equine dermatology. In an effort to validate the relationship between positive wheal responses detected during IDT and true causative antigens, it is important to first determine whether positive wheal reactions to control substances are precise and accurate. The precision of a diagnostic test (eg, IDT) relates to the degree of variation in the specific test; variation may result from intra- and interhorse variation to ID injection of substances. In the study reported here, intrahorse variation referred to the degree of wheal variation within each horse and interhorse variation referred to the degree of wheal variation among the 12 horses. Several studies[19-22] in humans have identified intra- and interindividual variation to multiple testing procedures. However, to our knowledge, intra- and interhorse variation for IDT has not been examined.

Histamine is one of the most widely used and reliable positive control substances for IDT in various species. Histamine is a vasoactive amine that causes contraction of vascular endothelial cells and release of vascular smooth muscle relaxants. Release of histamine from cutaneous mast cells or ID injection of histamine results in leakage of plasma into surrounding tissues, which leads to wheal formation. Wheal formation reflects an immediate, nonspecific inflammatory response of the skin.[23] Wheals formed in response to ID injection of histamine reflect vascular responsiveness (vasodilation) of skin and are independent of mast cells or leukocytes within the host's skin.[23,24] Therefore, histamine does not measure the ability of mast cells to degranulate, nor does it measure the ability to mount a cell-mediated reaction in response to antigen stimulation.

Minimal intra- and interhorse variation was evident in the wheals induced by ID injection of various concentrations of histamine used in this study. Minimal variation in wheal size may have been attributable to multiple factors. An important consideration is that the response to ID injection of histamine does not depend on the body's ability to mount an inflammatory reaction. In particular, ID injection of histamine causes wheal formation regardless of the number of mast cells within a host's skin. In addition, the wheals induced by histamine injection are not dependent on cells (ie, leukocytes) that migrate to the site of injection. These factors, combined with the fact that a precise concentration and volume of histamine was administered ID, yielded relatively uniform wheal formation after ID injection of various concentrations of histamine. Interestingly, the interhorse variation in size of wheals (0.4 mm) was less than the intrahorse variation in size of wheals (1.4 mm). This may have been attributable to differences in the location of the ID injection of histamine in each horse (ie, closer or more distant from the crest of the neck), cross-reaction of histamine injection within each horse with respect to

other substances injected ID (ie, PHA), clinician error, or a combination of these factors.

Analysis of this information suggests that histamine induces consistent wheals within and among horses in response to ID injection of various concentrations of histamine by 0.5 hours after injection, as determined on the basis of minimal variance in wheal size and evaluation of the dose-response curves. Therefore, histamine is a reliable and precise positive control substance for immediate-hypersensitivity reactions. We would recommend use of a dosage of 0.005 mg of histamine/mL as a positive control substance for IDT. At this dosage, consistent wheals were evident by 0.5 hours after injection. Analysis of this information also suggests that histamine is an inappropriate positive control substance for evaluating the delayed response (4 and 24 hours after injection), at least at the concentrations used in this study.

The slope calculated for the study reported here represents the rate at which wheal size increased for increasing concentrations of histamine. Wheal size increased approximately 1.2 mm for each 1-µg increase in histamine concentration. In the horses in this study, evaluation of the dose-response curves generated for histamine revealed a linear increase in wheal size as the concentration of histamine increased. In contrast to wheals induced by ID injection of PHA, wheals induced by histamine did not reach a threshold (plateau phase) for any of the concentrations used in this study. At the time of this study, it was not known whether higher concentrations of histamine than those used would yield a threshold or plateau response (ie, whether higher concentrations of histamine [eg, 0.125 or 0.25 mg/mL] would induce similar-sized wheals, regardless of concentration). Additional studies may be required to permit use of the Chapman-Richard equation for evaluation of the histamine dose-response curve.

The y-intercept calculated for histamine, which described wheal size that would result from ID injection of the negative control substance, was 10 mm at 0.5 hours. Analysis of this information suggests that the mere ID injection of a nonreactive substance results in a transient wheal of approximately 10 mm in diameter by 0.5 hours after injection that resolves by 4 hours after injection. The ID injection of the negative control substance for PHA also resulted in formation of wheals with a mean diameter of 10 mm by 0.5 hours after injection. Applying this information to clinical use of IDT, transient small (≤ 10 mm in diameter) wheals that are evident 0.5 hours after injection should be interpreted with caution because this may represent a nonspecific dermal reaction to an injected substance. Nonspecific, small (≤ 10 mm in diameter) dermal reactions detected 0.5 hours after injection that maintain or increase in size by 4 hours after injection may be more suggestive of a positive test reaction.

Phytohemagglutinin is a lectin that stimulates growth and cell division of T lymphocytes through its mitogenic properties and induces a cell-mediated immune response mediated by T lymphocytes.[18] Injection of PHA has been used to evaluate integrity of the cell-mediated immune response in immunodeficient patients such as Arabian foals with severe combined immunodeficiency.[17,18] We used PHA in the study reported here to evaluate the cell-mediated immune response and T-cell function. Delayed (late-phase) responses have been reported[25-28] secondary to IDT with certain antigens and for various atopic conditions in multiple species.

At 4 hours, minimal intra- (6.8 mm) and interhorse (10.1 mm) variation was evident in wheals induced by ID injection of PHA. Analysis of this information suggests that horses have the potential to mount a relatively uniform cell-mediated immune response to PHA by 4 hours after ID injection. In addition, responsiveness of T cells by 4 hours after injection is relatively uniform within and among horses over a range of PHA concentrations.

Alternatively, by 24 hours after injection, a significant amount of intra- (43.5 mm) and interhorse (42.0 mm) variation was evident in wheals induced by ID injection of PHA. Analysis of this information suggests that there is a substantial amount of variation in the cell-mediated immune response within and among horses by 24 hours after ID injection of PHA. In contrast to the response after ID injection of histamine, the cell-mediated immune response induced by PHA involves a series of cellular steps, including activation of various mediators, cell-to-cell interactions, chemotaxis of lymphocytes, and cellular infiltration into the site of exposure. A moderate to high degree of intra- and interindividual variation in cell-mediated immune responses has been documented[20,29-35] in other species. Several factors have been implicated as the source of variation for the cell-mediated immune response (eg, seasonal differences in the cell-mediated immune response). Seasonal differences in maximum lymphoproliferative responses have been reported in mice (maximum lymphoproliferative response during summer and autumn)[29] and dogs (maximum lymphoproliferative response during July).[30] In addition, decreased lymphoproliferative responses have been detected as people grow older.[20,31] Other factors such as catecholamine- or steroid- (ie, stress) induced suppression of cell-mediated immunity as well as malnutrition- and hormone- (ie, progesterone or estradiol) induced suppression of cell-mediated immunity have been documented.[32-35] It is possible that any of the aforementioned factors may have contributed to the interhorse variation detected in the study reported here. However, additional studies are necessary to define factors that affect cell-mediated responses among horses.

Although there is large day-to-day variation within people for cell-mediated immunity tests, there is larger interindividual variation.[20] In the study reported here, wheals 24 hours after ID injection of PHA had a slightly greater intrahorse variation, compared with interhorse variation. Factors such as circadian variation can alter the intraindividual immune response in people and other species,[36-38] but the cause of the high intrahorse variation evident with PHA in the study reported here remains undetermined at this time.

Considering the reliance of PHA on infiltration of T cells and a delayed mechanism of action, it is not surprising that PHA did not elicit a substantial wheal

response by 0.5 hours after ID injection. However, PHA caused dose-related wheals by 4 and 24 hours after injection. After preliminary examination of the PHA data, it was evident that wheal diameter did not increase in a linear fashion (ie, wheal diameter began to plateau) with increasing concentrations of PHA. Analysis of this information suggests that at the initial low concentrations of PHA used in this study, horses had an incremental increase in wheal size. In contrast, as higher concentrations of PHA were used, the wheal size did not increase proportionately. This suggests that the cell-mediated immune response induced by PHA is limited in horses. A simple linear model was not used to generate a dose-response curve because of the observation of the plateau phase. Instead, the Chapman-Richard equation was used for the PHA data to document that the increase in wheal size induced by increasing concentrations of PHA was followed by a plateau phase at higher concentrations of PHA.

Mean diameter of the wheals induced by the higher concentrations of PHA (0.4, 0.7, and 1 mg/mL) resulted in a plateau of 25.8 mm by 4 hours after injection and 38.9 mm by 24 hours after injection. Mean rate of increase in wheal size was 9.1 mm for each 1-mg increase in PHA concentration at 4 hours after injection and 5.8 mm for each 1-mg increase at 24 hours after injection. Analysis of this information suggests that horses have a uniform potential to mount a cell-mediated immune response 4 hours after ID injection of PHA; variation in wheal size increases after 4 hours following ID injection.

Signs of pain were elicited during palpation of the wheals induced by ID injection of PHA. This may have been attributable to a degree of dermal necrosis, hemorrhage, or inflammation at the site of injection secondary to the cell-mediated response. In addition, use of the lower concentrations may have been more appropriate as a positive control substance for the late-phase (ie, cell-mediated) reaction because the higher concentrations induced larger wheals (50 to 75 mm in diameter) that coalesced with wheals of adjacent ID injections. In light of this fact, we recommend use of a dosage of 0.1 mg of PHA/mL as a positive control substance to avoid excessively large wheals. Studies[17,18] in horses that involved the use of ID injection of 50 µg of PHA have resulted in reactions at 4 and 24 hours after injection; however, precise measurements of wheal diameter were not made in those studies.

In the study reported here, ID injection of various concentrations of histamine induced minimal intra- and interhorse variation in wheal diameter by 0.5 hours after injection. Analysis of this information suggests that ID injection is a precise method of stimulant delivery and histamine is a reliable positive control substance for the immediate-phase response. Minimal variation was detected in the rate of increase and plateau for PHA at 4 hours after injection. Analysis of these collective results suggests that variation in response to ID injection of antigens, similar to results observed in other studies, is not likely attributable to intra- or interhorse variation. Alternatively, variation in wheals detected during IDT in other studies may have represented a true immune response to the specific antigen. Furthermore, the high degree of wheal variation with PHA at 24 hours after injection suggests that late-phase reactions should be interpreted cautiously. Results of this study do not provide any information regarding whether a response to ID administration of a specific antigen is evidence that the antigen is a causative factor in the development of allergic disorders in horses (eg, RAO).

a. Histamine, Sigma-Aldrich, St Louis, Mo.
b. Phytohemagglutinin from *Phaseolus vulgaris*, Sigma-Aldrich, St Louis, Mo.
c. Xylazine hydrochloride, Fermenta Animal Health Co, Kansas City, Mo.
d. PROC MIXED, SAS, version 8.01, SAS Institute Inc, Cary, NC.

References

1. Krouse JH, Mabry RL. Skin testing for inhalant allergy 2003: current strategies. *Otolaryngol Head Neck Surg* 2003;129(suppl 4):S33–S49.
2. Hillier A, DeBoer DJ. The ACVD task force on canine atopic dermatitis (XVII): intradermal testing. *Vet Immunol Immunopathol* 2001;81:289–304.
3. Halliwell RE. Efficacy of hyposensitization in feline allergic diseases based upon results of in vitro testing for allergen-specific immunoglobulin E. *J Am Anim Hosp Assoc* 1997;33:282–288.
4. Lane AP, Pine HS, Pillsbury HC III. Allergy testing and immunotherapy in an academic otolaryngology practice: a 20-year review. *Otolaryngol Head Neck Surg* 2001;124:9–15.
5. Griffin CE, Hillier A. The ACVD task force on canine atopic dermatitis (XXIV): allergen-specific immunotherapy. *Vet Immunol Immunopathol* 2001;81:363–383.
6. Nuttall TJ, Thoday KL, van den Broek AH, et al. Retrospective survey of allergen immunotherapy in canine atopy. *Vet Rec* 1998;143:139–142.
7. Willemse A, Van den Brom WE, Rijnberk A. Effect of hyposensitization on atopic dermatitis in dogs. *J Am Vet Med Assoc* 1984;184:1277–1280.
8. Jose-Cunilleras E, Kohn CW, Hillier A, et al. Intradermal testing in healthy horses and horses with chronic obstructive pulmonary disease, recurrent urticaria, or allergic dermatitis. *J Am Vet Med Assoc* 2001;219:1115–1121.
9. Halldorsdottir S, Larsen HJ, Mehl R. Intradermal challenge of Icelandic horses with extracts of four species of the genus *Culicoides*. *Res Vet Sci* 1989;47:283–287.
10. Fadok VA, Greiner EC. Equine insect hypersensitivity: skin test and biopsy results correlated with clinical data. *Equine Vet J* 1990;22:236–240.
11. Evans AG, Paradis MR, O'Callaghan M. Intradermal testing of horses with chronic obstructive pulmonary disease and recurrent urticaria. *Am J Vet Res* 1992;53:203–208.
12. Lowell FC. Observations on heaves. An asthma-like syndrome in the horse. *J Allergy Clin Immunol* 1964;35:322–330.
13. Lorch G, Hillier A, Kwochka KW, et al. Results of intradermal tests in horses without atopy and horses with chronic obstructive pulmonary disease. *Am J Vet Res* 2001;62:389–397.
14. McPherson EA, Lawson GH, Murphy JR, et al. Chronic obstructive pulmonary disease (COPD) in horses: aetiological studies: responses to intradermal and inhalation antigenic challenge. *Equine Vet J* 1979;11:159–166.
15. McGorum BC, Dixon PM, Halliwell RE. Responses of horses affected with chronic obstructive pulmonary disease to inhalation challenges with mould antigens. *Equine Vet J* 1993;25:261–267.
16. Lorch G, Hillier A, Kwochka KW, et al. Results of intradermal tests in horses without atopy and horses with atopic dermatitis or recurrent urticaria. *Am J Vet Res* 2001;62:1051–1059.
17. Hodgin EC, McGuire TC, Perryman LE, et al. Evaluation of delayed hypersensitivity responses in normal horses and immunodeficient foals. *Am J Vet Res* 1978;39:1161–1167.
18. Khalil AA, Botros BA, Kerkor M, et al. Phytohaemagglutinin skin testing of Arabian foals in Egypt. A test for detecting immune cell reactivity. *Zentralbl Veterinarmed B* 1982;29:160–163.

19. Korn EL, Dorey F, Spina CA, et al. The use of three baseline values in intervention studies: application to evaluation of immune modulation therapies. *Immunobiology* 1984;167:431–436.

20. Molls RR, Ahluwalia N, Fick T, et al. Inter- and intra-individual variation in tests of cell-mediated immunity in young and old women. *Mech Ageing Dev* 2003;124:619–627.

21. Malone JL, Simms TE, Gray GC, et al. Sources of variability in repeated T-helper lymphocyte counts from human immunodeficiency virus type 1-infected patients: total lymphocyte count fluctuations and diurnal cycle are important. *J Acquir Immune Defic Syndr* 1990;3:144–151.

22. Porzsolt F, Gaus W, Heimpel H. The evaluation of serial measurements of the NK cell activity in man. *Immunobiology* 1983;165:475–484.

23. Shipstone M, Mueller R, Bettenay S, et al. The use of compound 48/80 as a positive control in equine intradermal allergy testing. *Vet Dermatol* 1999;10:291–295.

24. Vogelnest L, Mueller R. The use of compound 48/80 and codeine phosphate as positive controls for intradermal skin testing in dogs. *Vet Dermatol* 2001;12:93–99.

25. Zweiman B, Haralabatos IC, Pham NC, et al. Sequential patterns of inflammatory events during developing and expressed skin late-phase reactions. *J Allergy Clin Immunol* 2000;105:776–781.

26. Lierl MB. Isolated late cutaneous reactions to allergen skin testing in children. *Ann Allergy Asthma Immunol* 2000;84:294–298.

27. Hill PB, Hillier A, Olivry T. The ACVD task force on canine atopic dermatitis (VI): IgE-induced immediate and late-phase reactions, two inflammatory sequences at sites of intradermal allergen injections. *Vet Immunol Immunopathol* 2001;81:199–204.

28. Dolovich J, Hargreave FE, Chalmers R, et al. Late cutaneous allergic responses in isolated IgE-dependent reactions. *J Allergy Clin Immunol* 1973;52:38–46.

29. Planelles D, Hernandez-Godoy J, Montoro A, et al. Seasonal variation in proliferative response and subpopulations of lymphocytes from mice housed in a constant environment. *Cell Prolif* 1994; 27:333–341.

30. Shifrine M, Taylor N, Rosenblatt L, et al. Seasonal variation in cell mediated immunity of clinically normal dogs. *Exp Hematol* 1980;8:318–326.

31. Murasko DM, Nelson BJ, Matour D, et al. Heterogeneity of changes in lymphoproliferative ability with increasing age. *Exp Gerontol* 1991;26:269–279.

32. Herrera LA, Montero R, Leon-Cazares JM, et al. Effects of progesterone and estradiol on the proliferation of phytohemagglutinin-stimulated human lymphocytes. *Mutat Res* 1992;270:211–218.

33. Ojo-Amaize E, Guidry A, Paape M, et al. In vitro depression of bovine lymphocyte function by treatment of cultured bovine lymphocytes with physiologic concentrations of hydrocortisone. *Am J Vet Res* 1988;49:851–855.

34. Riley-Roberts ML, Turner RJ, Evans PM, et al. Lymphoproliferative responses in diet-restricted and aging Sprague-Dawley rats. *Exp Gerontol* 1992;27:201–209.

35. Edgar VA, Silberman DM, Cremaschi GA, et al. Altered lymphocyte catecholamine reactivity in mice subjected to chronic mild stress. *Biochem Pharmacol* 2003;65:15–23.

36. Esquifino AI, Selgas L, Arce A, et al. Twenty-four-hour rhythms in immune responses in rat submaxillary lymph nodes and spleen: effect of cyclosporine. *Brain Behav Immun* 1996;10:92–102.

37. Hiemke C, Brunner R, Hammes E, et al. Circadian variations in antigen-specific proliferation of human T lymphocytes and correlation to cortisol production. *Psychoneuroendocrinology* 1995; 20:335–342.

38. Rai-el-Balhaa G, Pellerin JL, Bodin G, et al. Importance of the hour of sampling in the lymphoblastic transformation assay of sheep peripheral blood lymphocytes. *Vet Immunol Immunopathol* 1987;16:67–76.