Comparison of effects of dexamethasone and the leukotriene D4 receptor antagonist L-708,738 on lung function and airway cytologic findings in horses with recurrent airway obstruction

Jean-Pierre Lavoie, DMV; Renaud Léguillette, DMV, MSc; Kirby Pasloske, DVM, DVSc; Luc Charette, MSc; Nicole Sawyer, MSc; Daniel Guay, PhD; Terry Murphy, MSc; and Gerry J. Hickey, MVB, PhD

Objective—To evaluate whether the leukotriene (LT) D4 receptor antagonist L-708,738 is therapeutically beneficial in treating horses with recurrent airway obstruction (heaves).

Animals—12 adult horses with heaves and healthy lung lobes from 20 slaughtered horses.

Procedure—Lung lobes were used for smooth muscle tension and radioligand binding studies. Horses with heaves were given a placebo for 14 days and administered L-708,738 (n = 6; 2.5 mg/kg PO, q 12 h) or dexamethasone (6; 0.04 mg/kg, IV, q 24 h) from days 14 to 28. Pulmonary function was measured weekly for 36 days, and bronchoalveolar cells were collected on days 0, 14, and 29 for cytologic examination.

Results—Nanomolar concentrations of L-708,738 were effective at antagonizing LTD_4-induced bronchoconstriction and LTD_4-receptor binding in lung lobes. Mean peak and trough L-708,738 plasma concentrations during the treatment period were 1.54 and 0.28 μ*M*, respectively. On days 21 and 29, lung mechanics were significantly improved in the dexamethasone-treated horses but not in the L-708,738-treated horses. Neither dexamethasone nor L-708,738 had a significant effect on cytologic findings.

Conclusions and Clinical Relevance—L-708,738 was bioavailable after oral administration and sustained concentrations in plasma during the dosing period that exceeded in vitro efficacy values. However, airway function did not improve, suggesting that either drug concentrations in the lungs were subtherapeutic or that cysteinyl LT may not be important mediators of airway inflammation in heaves. Results provide the first evidence of cysteinyl LT_1 receptors in airways of horses. (*Am J Vet Res* 2002;63:579–585)

Received Apr 16, 2001.
Accepted Sep 28, 2001.
From the Department of Clinical Sciences, Faculté de Médecine Vétérinaire, Université de Montréal, St Hyacinthe, QC, Canada J2S 7C6 (Lavoie, Léguillette); Merck & Co Inc, 126 E Lincoln Ave, Rahway, NJ 07065 (Pasloske, Murphy, Hickey); and Merck Frosst, 16711 TransCanada Hwy, Kirkland, QC, Canada H9H 3L1 (Charette, Sawyer, Guay).
Supported by Merck & Co Inc, Rahway, NJ.
Results of this study were presented at the 2000 International Conference of the American Thoracic Society, May 2000, Toronto, ON, Canada.
The authors thank G. Michaud and J. Bosquet for technical assistance.
Address correspondence to Dr. Lavoie.

Recurrent airway obstruction, also known as chronic obstructive pulmonary disease and heaves, is a reversible inflammatory condition that affects the lower airways of horses. Heaves results from exposure to moldy hay and bedding and is primarily seen in countries in which horses are stabled for prolonged periods because of adverse environmental conditions. The finding that exacerbation of heaves could be provoked by exposure to dusty hay led researchers to postulate that heaves is an allergic reaction to inhaled molds and fungi. However, the immunologic events leading to airway inflammation in heaves remain ill defined.[1]

Heaves bears many similarities to features of human asthma, including bronchospasm, excess mucus production, airway hyperreactivity, and pathologic changes of the bronchiolar walls that lead to airway obstruction.[2] Of the various mediators that are known to be involved in human asthma, **leukotrienes (LT)** are considered to be among the most important. Leukotrienes are metabolites of arachidonic acid and are produced via the **5-lipoxygenase (5-LO)** enzyme and its essential cofactors, the **5-LO–activating proteins (FLAP)**. Cysteinyl LT (LTC_4, LTD_4, LTE_4) are potent bronchoconstrictors that also increase airway vascular permeability and mucus production. The LTD_4-receptor antagonists are potent inhibitors of airway inflammation and bronchoconstriction in humans with asthma.[3-6]

The role of LTD_4 in equine respiratory diseases is unknown, but results of in vitro studies suggest that cysteinyl LT may also be implicated in heaves. Results indicate that LTD_4 contracts isolated equine parenchymal smooth muscle[7] and enhances contraction of small airways after electric field stimulation.[8] The findings that inhalation of LTD_4 by horses with heaves induces bronchoconstriction[9] and that the concentration of LTC_4 in **bronchoalveolar lavage fluid (BAL)** of horses with coughing and exercise intolerance[a] is high further supports a possible role of cysteinyl LT in respiratory diseases of horses.

To our knowledge, the effects of LTD_4 receptor antagonists in horses with heaves have not been evaluated. The objective of the study reported here was to evaluate the therapeutic benefit of the LTD_4 receptor antagonist L-708,738 on airway function and inflammation in horses with heaves and compare its effects

with those of dexamethasone, a potent anti-inflammatory drug that has been proven effective for the treatment of horses with heaves.[10]

Materials and Methods

In vitro studies—Lung lobes from adult horses (n = 20) were obtained at slaughter and placed in isotonic saline (0.9% NaCl; smooth muscle tension study) solution at 4 C or in 10 m*M* HEPES-KOH at pH 7.4 (radioligand binding study) for transportation. All lungs were examined by a veterinarian and confirmed to be grossly normal.

Smooth muscle tension—Segments of bronchi (approx 5 mm diameter) were dissected as rings, cut open, and attached individually to a piece of string within 3 hours post mortem. Bronchial rings were suspended immediately in organ baths or stored overnight at 4 C in isotonic saline solution. Isometric tension changes were measured by use of a force displacement transducer. The signal was amplified[b] and acquired at a frequency of 5 Hz by use of a computerized data acquisition system[c] and analysis software.[d] Mean values for data were determined during 10-second periods throughout the experiment.

Tissues continuously received 95% O_2 and 5% CO_2 in modified Krebs bicarbonate solution at 37 C, which was renewed every 3 minutes with 2 volume changes. Indomethacin (1.4 μ*M*) was used throughout the experiment to avoid any influence from endogenously released cyclooxygenase products.[11] After setting initial muscle tension to 1 g and an equilibration time of 30 minutes, rings were challenged once with a maximal dose of carbachol (10 μ*M*) and washed for 1 hour in the presence of atropine (0.1 μ*M*) and mepyramine (1 μ*M*) to eliminate cholinergic and histaminergic influences throughout the rest of the experiment. To obtain the negative log of molar concentration of agonist producing 50% of maximal response (**effective concentration 50% [EC_{50}]**) measurements from a single cumulative **dose response curve (DRC)**, bronchial tissues from 7 horses (2 to 8 rings/horse) were treated on the day of arrival with LTD_4[e] in half log increments from 1 n*M* to 1 μ*M* at 15-minute intervals.

To calculate the concentration of the $CysLT_1$ receptor antagonist L-708,738,[e] which at equilibrium would occupy 50% of receptors in the absence of agonist (pK_B, apparent affinity constant of the antagonist for the $CysLT_1$ receptor), a first DRC was completed on overnight-stored tissues (n = 9 horses) by exposing them to cumulative doses of up to 100 n*M* LTD_4 and washing out the contraction for approximately 2 hours until baseline tone was recovered. The DRC was repeated after 10 minutes of pretreatment with vehicle (DMSO) or L-708,738 in vehicle (30 n*M*/100 n*M*). The DRC was continued until LTD_4 concentration reached 10 μ*M*.

Calculations and formulas—One hundred percent smooth muscle contraction was calculated by subtracting the actual curve baseline (mean tension) from the maximum tension achieved by the first 100 n*M* of LTD_4 of the DRC. Effective concentration 50% curves were calculated with a 4-parameter fitting routine. Potency of the agonist was expressed according to the formula:

$$pEC_{50} = -\log_{10}(\text{molar concentration } LTD_4\ EC_{50})$$

Potency of the antagonist refers to the pK_B and was expressed at each concentration tested according to the formulas:

$$\text{Dose ratio} = EC_{50}(\text{curve 2 [test]})/EC_{50}\ (\text{curve 1 [control]})$$

$$pK_B = -\log_{10}(\text{molar concentration of antagonist}) + \log_{10}(\text{dose ratio}-1).$$

If blockade of LTD_4 response induced by the antagonist was so complete that contraction with 10 μ*M* LTD_4 did not reach 50% of the first curve, the second curve (test EC_{50}) was arbitrarily set to a value > 10 μ*M*. Subsequent calculations were performed by use of 10 μ*M* test EC_{50}. Dose ratio or pK_B obtained were referred to as being greater than values observed with the present calculation.

Solutions and $CysLT_1$ antagonists—The LTD_4 stock was diluted in half-log increments in physiologic saline solution. Indomethacin was dissolved in ethanol and added to the buffer, whereas carbachol, atropine, and mepyramine were dissolved in saline solution before being added to the buffer. The L-708,738 was prepared as a 1 m*M* stock solution in DMSO and stored before use at –80 C. Final concentrations of vehicles in the bath were 0.1% for DMSO and 0.005% for ethanol.

Radioligand studies—Membrane preparations and [^{3}H]LTD_4 binding assays were performed as described for guinea pig lungs,[12] with the exception that the binding reactions were initiated by addition of approximately 100 μg of horse lung membrane protein. The [^{3}H]LTD_4-specific binding accounted for 80 to 85% of the total binding and was linear with respect to the concentration of protein in the incubation medium. Competition curves were analyzed with custom-designed software by use of a nonlinear least-squares curve-fitting routine based on a 4-parameter logistic equation to determine half-maximal inhibitory concentration (IC_{50}) values.[13]

In vivo study—All experimental procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care and were approved by the Animal Care Committee of the Faculty of Veterinary Medicine of the Université de Montréal.

Animals—Twelve adult horses with a history of heaves were used for the study. Prior to the experiment, horses were conditioned to stand in stocks wearing a facemask. A complete physical examination, thoracic radiography, upper airway endoscopy, CBC, and biochemical analyses were performed prior to the study to exclude concomitant medical conditions. Inclusion criteria were a history of chronic recurrent cough, maximal changes in transpulmonary pressure > 15 cm H_2O, neutrophil proportion > 20% in BAL fluid, and results of CBC and serum biochemical analyses within reference ranges.

The horses were stabled in the same barn for at least 3 weeks before and for the duration of the experiment. Horses were fed dry Timothy hay and sweet feed twice a day and bedded on straw. Moldy hay was finely chopped and blown in the barn once daily by use of a cutter-blower hay chopper. Management remained the same throughout the study. No other treatments were administered for at least 3 weeks prior to the experiment. Ambient temperature and humidity were monitored daily.

BAL—Horses were sedated with xylazine (0.6 to 1.0 mg/kg, IV) and butorphanol (20 to 30 μg/kg, IV). A fiberoptic flexible endoscope was passed through the nares and directed into the left lung until its tip was wedged in the bronchus. Between 50 and 100 ml of dilute (0.5%) lidocaine[f] was used to desensitize the airway mucosa. Two 250-ml boluses of sterile isotonic saline solution (37 C) were rapidly instilled into the bronchus and aspirated via the endoscope biopsy channel by use of a suction pump. Vacuum pressure in the pump ranged from 50 to 100 mm Hg. The BAL fluid was collected in silicon-treated glass vessels and kept on ice. The fluid was filtered through sterile gauze and centrifuged, and the cell pellets were resuspended in a culture medium.[g] The BAL fluid was analyzed within 1 hour after collection.

Total nucleated cells in BAL fluid samples were counted by use of a hemocytometer. Smears of the fluid were prepared

by centrifugation at 90 × g for 5 minutes and stained with a modified Wright solution. Differential counts were made on ≥ 400 cells; epithelial cells were not included in the differential count.

Respiratory mechanics—Flow rates were obtained by the use of a heated pneumotachograph[h] and associated differential pressure transducer[i] fitted to a mask placed over the horse's nose. Electronic integration of the flow signal provided **tidal volume (V_T)**. For each experiment, the system was calibrated by forcing known flows of air (0 to 8 L/s) through the pneumotachograph by use of a blower-rotameter.

Transpulmonary pressure (P_L) was obtained by use of a differential pressure transducer[j] by subtracting the esophageal pressure from the mask pressure. The esophageal pressure was measured with a balloon sealed over the end of a polyethylene catheter (inner diameter, 4.8 mm; outer diameter, 7.9 mm) placed in the distal third of the esophagus and distended with 6 ml of air. The distance between the nares and the distal third of the esophagus was visually approximated and marked on the esophageal balloon catheter. The pressure tracings were monitored and the position of the catheter modified if necessary to allow maximal **change in P_L (ΔP_L)** during a respiratory cycle and to eliminate cardiac artifacts. The length of the inserted tubing was recorded for each horse, and thereafter, the same length was used. The pressure transducer was calibrated by use of a water manometer. The signals from the transducers were amplified and passed through a digital-analog converter to a computer equipped with data acquisition and analysis software.[k] The program provides values of V_T, **minute expiratory ventilation ($\dot{V}_E$)**, **respiratory rate (f)**, **expiratory and inspiratory times (TE and TI)**, and ΔP_L for each breath. Values of **pulmonary resistance (R_L)** and **elastance (E_L)** were obtained by applying the data to the multiple regression equation for the single compartment model of the lung:

$$P_L = (E_L \times V) + (R_L \times \dot{V}) + K$$

where V is the volume, $\dot{V}$ is the airflow, and K is the transpulmonary end-expiratory pressure. The coefficients of determination for the fit of the equation to the data were calculated for each breath. The signals were sampled at a frequency of 120 Hz for 100 seconds, and all valid breaths were used for analysis.

Jugular vein catheters—Catheters were inserted percutaneously into the jugular vein by use of sterile techniques. The catheters were inspected and rinsed with heparinized saline solution (10 U/ml) twice daily.

Treatment—The experiment was conducted with a parallel design in which each group received 1 treatment (positive control or test article) after a common period of placebo (vehicle) administration. During the placebo phase of the trial, each horse received molasses (PO, q 12 h) and isotonic saline solution (10 ml, IV, q 24 h). During the drug treatment period, 6 horses received dexamethasone[l] (0.04 mg/kg, IV, q 24 h) plus molasses (PO, twice daily), and 6 horses received the LTD_4-receptor antagonist L-708,738 (2.5 mg/kg, PO, q 12 h) plus IV administration of saline solution (10 ml, once daily). The L-708,738 was first solubilized in boiling sterile water by use of a heated magnetic stir plate. When the drug was completely solubilized, it was mixed with molasses and administered orally. After oral dosing, a small amount of grain or sweet feed was offered to each horse to facilitate swallowing of the drug.

Blood sample collection—Blood samples were collected during the dosing period to measure peak and trough L-708,738 plasma concentrations. Blood samples were collected in heparinized evacuated containers, the samples were centrifuged, and the plasma was harvested. Plasma samples were stored at –20 C until analysis.

Calculation of L-708,738 in plasma—Plasma from a control horse was fortified with L-708,738 from 0.039 to 3.297 µ*M* and analyzed for L-708,738 by use of **high-performance liquid chromatography (HPLC)**. A standard curve of peak area versus L-708,738 plasma concentration was determined. The resultant standard curve was linear, with $r^2 = 0.9999$ and percentage recoveries ranging from 96 to 106%. The **signal to noise ratio (s/n)** at the 0.034-µ*M* plasma fortification concentration was 5.2, with a recovery of 102%. The s/n at the 0.067-µ*M* plasma fortification was 8.3, with a recovery of 106%. The limit of quantification (LOQ) of the assay was set at 0.07 µ*M,* whereas the limit of detection was 0.03 µ*M*. Test plasma samples from each horse were arranged so that 2 fortified standards were assayed before and 2 fortified standards were assayed after the test samples. The fortified standards ranged from 0.082 to 1.236 µ*M* of L-708,738 in plasma. The percentage recoveries ranged from 98 to 121%.

The L-708,738 assay procedure for each plasma sample was as follows: 1.1 ml of acetonitrile was added to 400 µl of fortified or nonfortified plasma in a polypropylene centrifuge tube; the sample was vortexed and then centrifuged at 2,620 × g and 1 C for 6 minutes; the supernatant was transferred to a HPLC vial, and a 25-µl aliquot was injected onto a 4.6-mm × 15-cm column[m] maintained at 35 C with a flow rate of 1 ml/min and a pressure of 53 kgf/cm^2 (751 psi). The HPLC system consisted of a system controller,[n] a liquid chromatograph pump, an auto-injector, and a column oven connected to a spectrofluorometric detector. Detection was performed by use of fluorescence with an excitation wavelength at 300 nm and an excitation emission at 400 nm. The mobile phase consisted of acetonitrile and 0.026*M* ammonium acetate buffer (pH 7) at a ratio of 60:40. A rinse phase of 50% acetonitrile was used for rinsing between injections. The retention time of the L-708,738 was 5.25 minutes.

Experimental protocol—Respiratory mechanic measurements were performed at baseline and then at weekly intervals from day 1 to day 35. Atropine[o] (0.02 mg/kg, IV) was administered on day 35, and respiratory mechanics were measured 30 minutes later. Complete blood count and plasma biochemical analyses were performed on days 0, 14, 21, and 29. Bronchoalveolar lavages were performed at baseline and on days 15 and 29. Investigators performing daily physical assessment of respiratory effort, lung function, and BAL were unaware of whether dexamethasone or the test article were being administered.

Statistical analyses—Data were determined to be normally distributed by use of the Kolmogorov-Smirnov normality test. Baseline values for respiratory function variables and BAL cell count and cytologic findings were compared between groups by use of a Student *t*-test. A value of $P < 0.05$ was considered significant. Data were further analyzed by use of a 2-group repeated-measures ANOVA incorporating group (test article or dexamethasone) and time main effects and time X group interaction effect. When a significant ($P < 0.05$) time effect or time X group interaction effect was detected, values were compared with baseline values by use of a Student *t*-test for each group separately. Results are reported as mean ± SEM.

Results

Smooth muscle tension—Bronchial tissue from only 1 horse did not respond to LTD_4 and was therefore excluded from all analyses. In preliminary experiments using fresh bronchial rings isolated from 7 hors-

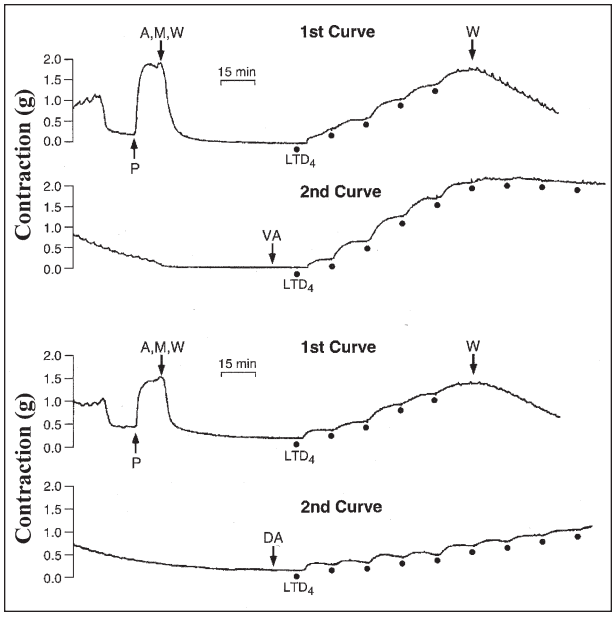

Figure 1—Simultaneous recordings of smooth muscle contraction in 2 equine bronchi from healthy horses. Top—Tissue treated with vehicle. Bottom—Tissue treated with the cysteinyl leukotriene $(LT)_1$ antagonist L-708,738. 1st Curve—After a resting period of 30 minutes, tissues were challenged with a priming (P) dose of 10 μ*M* carbachol. Blocking doses of 0.1 μ*M* atropine (A) and 1 μ*M* mepyramine (M) were added to the washing buffer and the tissue contraction was washed out (W). Cumulative doses of LTD_4 (circles) were administered, and the tissue contraction was washed out (W). 2nd Curve—After addition of vehicle (VA) or 30 n*M* L-708738 (DA), a second administration of cumulative doses of LTD_4 was performed.

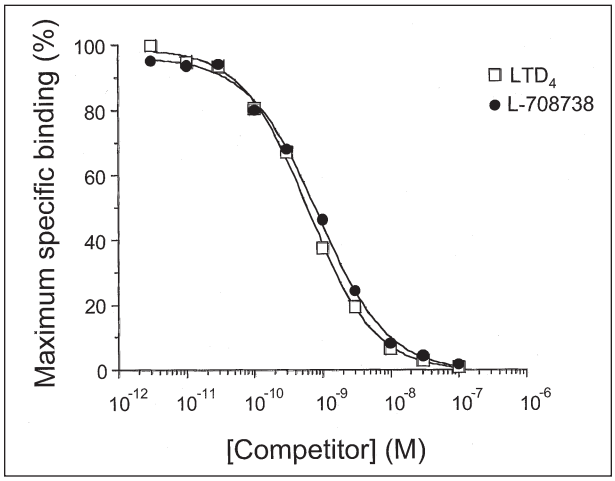

Figure 2—Titration curves of competition by LTD_4 and L-708738 for $[^3H]LTD_4$-specific binding sites in bronchi obtained from healthy horses. Curves represent results of 2 independent experiments performed in duplicate with 2 bronchial preparations.

es, the LTD_4 pEC50 was 7.8 ± 0.1 (n = 2 to 8 tissues/horse). Overnight storage of bronchi (n = 14 horses) did not significantly affect the LTD_4 response (8.04 ± 0.14; EC_{50} = 10 n*M*; n = 15 tissues/horse). Vehicle (DMSO) did not affect the LTD_4 EC_{50} (dose ratio, 0.86 ± 0.17; range, 0.4 to 3.1). Maximal contraction to the agonist DRC after DMSO increased to 117 ± 2% of pretreatment values and was probably a result of the higher LTD_4 concentration (10 μ*M*).

Table 1—Affinities of leukotriene $(LT)D_4$ and L-708,738 for $[^3H]LTD_4$-specific binding sites (50% of maximal inhibitory concentrations [IC_{50}]; mean ± SEM) in 20 horse lung membranes with (+) or without (–) 1% (vol/vol) horse serum and in guinea pig lung membranes and differentiated U937 (dU937) cell membranes.

Treatment	IC_{50} (n*M*)			
	Horse		Guinea pig	dU937†
	(–) serum	(+) serum		
LTD_4	0.62, 0.59	0.58	0.10 ± 0.01 (7)*	0.41 ± 0.04 (4)*
L-708,738	0.87, 0.42	1.35	0.31 ± 0.05 (6)	0.17 ± 0.03 (5)

*From Frey et al.[12] †Dimethylsulfoxide dU937 expresses a cysteine LT_1 receptor that resembles the human lung and human recombinant cysteine LT_1 receptors, with respect to published ligand binding constants.[12,23]

Table 2—Plasma concentrations (μ*M*) of L-708,738 after a 14-day treatment period in 6 horses with heaves

Time (h)	Type of collection	Mean ± SD
0	Pre-administration	0.0 ± 0.00
1	1 hour peak	1.17 ± 1.13
24	12 hour trough	0.18 ± 0.05
96	12 hour trough	0.27 ± 0.06
146	2 hour post-administration*	1.52 ± 1.22
168	12 hour trough	0.30 ± 0.12
264	12 hour trough	0.46 ± 0.47
312	12 hour trough	0.27 ± 0.08
313	1 hour peak	1.93 ± 0.77
336	12 hour trough	0.28 ± 0.09
338	14 hour post-administration*	0.21 ± 0.11

*At time when pulmonary function tests were performed.

The L-708,738 induced a 32-fold rightward shift of the LTD_4 response at 30 n*M* ($-LogK_B$ = 9.0 ± 0.27 [n = 4]; no shift [2]) and blocked it even further at 100 n*M* (dose ratio, ≥ 313; $-LogK_B \geq 9.5 \pm 0.14$ [3]) at concentrations that could not be totally overcome with the highest concentration of LTD_4 (10 μ*M*; **Fig 1**). Maximum contraction achieved was 59.1% ± 11.5 (n = 3). The lack of shift of the LTD_4 response in 2 tissues remains unexplained.

Radioligand binding—In equilibrium competition assays against $[^3H]LTD_4$, L-708,738 (IC_{50} = 0.87 and 0.42 n*M*), and LTD_4 (IC_{50} = 0.62 and 0.59 n*M*) competed equally well with the radioligand for specific binding to the equine $CysLT_1$ receptor (**Fig 2**). In a parallel experiment, equine serum was added to the equilibrium competition binding assay to evaluate the effect of serum proteins on the affinity of L-708,738 at equine $CysLT_1$. The affinity of the antagonist was not significantly affected by the presence of a high concentration of serum protein (305 μg) as indicated by an IC_{50} of 1.35 n*M* (**Table 1**).

Plasma L-708,738 concentrations—Mean ± SD of L-708,738 plasma concentrations after oral administration of 2.5 mg/kg twice daily for 14 consecutive days in 6 horses was determined (**Table 2**). Mean peak concentration was 1.2 μ*M* on the first day of drug administration and 1.9 μ*M* on day 13. Mean trough concentration ranged from 0.18 to 0.46 μ*M*. No individual trough concentration decreased to < 0.13 μ*M*. In preliminary studies, the plasma half-life of L-708,738 was determined to be 2 hours.

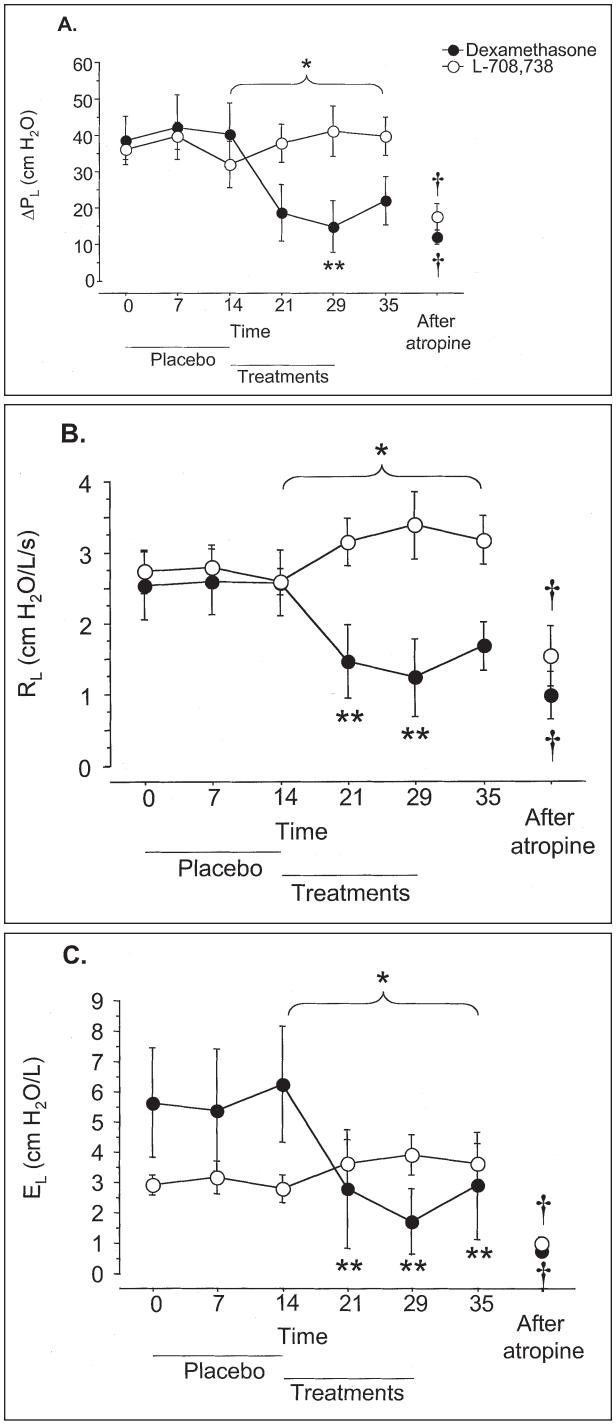

Figure 3—Maximum changes in transpulmonary pressure (ΔP_L; A), pulmonary resistance (R_L; B), and pulmonary elastance (E_L; C) in 12 horses with heaves during a placebo period (days 0 to 14), a drug treatment period (day 15 to 28), and after drug treatment (day 35). Reversibility of airway obstruction was confirmed at week 6 by administration of atropine. *Significant ($P < 0.05$) interaction between group and time main effects. **Significant ($P < 0.05$) difference from value on day 14. †Significant ($P < 0.05$) difference from values recorded prior to atropine administration (day 35).

Lung function—No significant variations in lung mechanics were observed during the placebo treatment period (**Fig 3**). There were no significant differences between groups in lung function at baseline or after the placebo period. Administration of L-708,738 did not alter lung mechanics, whereas dexamethasone administration markedly improved lung function. This lack of improvement in lung function with L-708,738 was seen even when horses had mild respiratory dysfunction (ΔP_L = 18 and 19 cm H_2O, respectively) at baseline. The airway obstruction of horses from both groups was reversible, as indicated by the positive response in lung function seen after atropine administration.

Table 3—Selected bronchoalveolar lavage variables (mean ± SEM) determined at baseline, after 2 weeks of placebo treatment, or after 2 weeks of dexamethasone or L-708,738 administration (Treatment) in horses (n = 6/group) with heaves

Treatment and variable	Baseline	Placebo	Treatment
L-708,738			
Fluid volume (ml)	194 ± 11	230 ± 24	224 ± 39
Nucleated cells (X 10^9/L)	53 ± 13	59 ± 12	66 ± 17
Neutrophils (%)	47 ± 8.1	31 ± 3.8	51 ± 8.7
Lymphocytes (%)	26 ± 4.8	42 ± 3.8	28 ± 4.8
Macrophages (%)	23 ± 4.4	25 ± 2.0	19 ± 4.7
Eosinophils (%)	1.3 ± 0.8	1.6 ± 1.1	0.9 ± 0.57
Dexamethasone			
Fluid volume (ml)	164 ± 34	201 ± 35	219 ± 40
Nucleated cells (X 10^9/L)	70 ± 44	91 ± 32	152 ± 48
Neutrophils (%)	63 ± 9	49 ± 9.7	45 ± 6.8
Lymphocytes (%)	17 ± 7.2	25 ± 5.5	33 ± 3.4
Macrophages (%)	18 ± 5.2	25 ± 6.1	21 ± 4.7
Eosinophils (%)	0.86 ± 0.60	0.25 ± 0.20	0.25 ± 0.16

BAL cytologic findings—There were no significant differences between groups in BAL volume, total cell counts, and differential cell counts at baseline, and the administration of placebos, L-708,738, or dexamethasone had no significant effect on BAL variables (**Table 3**).

Adverse effects—No adverse effects were noted during treatment with L-708,738, whereas 2 horses treated with dexamethasone developed adverse effects. One horse developed a corneal ulcer during the first week of treatment, whereas another developed lameness 1 week after treatment had ended. Both problems were mild and resolved with palliative treatment.

Clinical pathology—No abnormal changes in CBC were observed in L-708,738-treated horses, whereas 1 dexamethasone-treated horse developed a stress leukogram with neutrophilia and lymphopenia. There were no significant changes in plasma biochemical values at any time during the study.

Discussion

Results of previous studies[7-9,14] indicate that LTD_4 causes bronchoconstriction of the equine airways in vitro and in vivo. In our study, we evaluated the therapeutic benefit of the LTD_4-receptor antagonist L-708,738 for the treatment of heaves. The L-708,738 had high affinity for the LTD_4 receptor in equine bronchi and was able to antagonize with high potency (nanomolar range) in vitro the smooth muscle contractions induced by exogenous LTD_4. A 14-day course of L-708,738 administration to horses with signs of heaves provided no beneficial effect on lung function or airway inflammation. Plasma peak and trough L-708,738 con-

centrations indicated that all treated horses received adequate systemic exposure to the drug during the dosing period. Drug concentrations in all horses exceeded those required to prevent LTD_4-induced bronchoconstriction and receptor binding in the equine bronchial strip and receptor binding assays, respectively. These results suggest that LTD_4 is not an important mediator of heaves in horses or that the inflammatory cascade has inbuilt redundancy, whereby inhibition of 1 inflammatory mediator activity is not sufficient to result in clinical improvement. An alternate explanation for the lack of efficacy may be insufficient partitioning of L-708,738 into the inflamed tissue, although plasma concentrations suggest that adequate therapeutic concentrations had been reached. Quantification of drug concentrations in the lungs of treated horses was not undertaken in our study because of the invasive nature of the sampling.

The antileukotriene drugs presently available are either LT receptor antagonists or direct inhibitors of 5-LO and FLAP.[15] All 3 class of drugs have recently proven to be of benefit for the treatment of human asthma. The similarities between human asthma and heaves in horses have prompted investigators to evaluate whether these agents could also be used for the treatment of heaves. The use of a 5-LO inhibitor and a FLAP antagonist in affected horses has been investigated and led to equivocal results.[16,p] In a preliminary study in which a FLAP antagonist was administered to horses with heaves prior to allergen challenge, no improvement in airway function and inflammation was observed.[p] Similarly, administration of a 5-LO inhibitor prior to allergen challenge in affected horses did not reduce pulmonary neutrophilia and did not significantly improve their lung function.[16] However, in the 2 horses that responded severely to the challenge, there was a marked reduction in lung function associated with drug administration.[16] While these findings indicated an overall poor therapeutic benefit of these agents for the prevention of heaves, they also suggested that LT contribute in some circumstances to the airway obstruction seen in heaves.

Whereas inhibition of 5-lipoxygense will result in an overall decrease in LT production, LT-receptor antagonists provide selective inhibition of action on the basis of receptor specificity. The main receptor for cysteinyl LT in human lung tissue has been designated $CysLT_1$, previously known as the LTD_4 receptor.[17] The $CysLT_2$ receptor appears to be primarily present in vascular tissue, although there appear to be species differences in the preferential distribution of these receptors in lung tissues.[17] The in vitro radioligand binding experiments using horse lung membranes in our study provide the first evidence for the presence of $CysLT_1$ receptors in equine airways. Furthermore, the affinity of L-708,738 was comparable for the receptors of the horse and the guinea pig,[12] a species known to have a direct LT-derived airway response in vitro and in vivo. We also demonstrated that L-708,738 has high affinity for the equine $CysLT_1$ receptor in the absence and the presence of serum proteins. This latter finding is important, because binding to serum proteins can reduce the in vivo potency of therapeutics and could have contributed to the absence of any therapeutic effect of L-708,738.

Heaves is a chronic inflammatory condition that usually evolves over a period of years. Therefore, we postulated that despite the apparent poor efficacy of single doses of antileukotriene agent in acute allergen-induced bronchoconstriction and inflammation in 2 studies,[16,p] treatment extending over a number of days would be clinically more useful. Therefore, L-708,738 was administered in our study during a 14-day period to horses with heaves during continuous exposure to moldy hay. We believe that this protocol mimics field conditions in which horses are stabled all winter. Horses achieved a steady-state respiratory condition, as indicated by the lack of a significant difference in clinical scores, respiratory mechanics, and airway inflammation during the 2-week placebo control period. Although horses treated with dexamethasone (positive control) had marked improvement in their respiratory function, L-708,738-treated horses had no improvement, even when horses had mild respiratory dysfunction. Failure to respond to treatment was also not attributable to irreversible lung damage in horses in our study, as indicated by the substantial improvement in airway function after administration of atropine.

Bronchoconstriction in heaves results primarily from an increased cholinergic airway response that can be facilitated by various inflammatory mediators, including LTD_4.[8] In agreement with previous reports,[7,8] exogenous LTD_4 in absence of cholinergic or histaminic influences strongly contracted the equine airways in our study. This effect was abolished by L-708,738 in a dose-dependent manner, indicating a strong binding of the drug to the equine LTD_4 receptors. Results of in vitro studies indicate that stimulation of inflammatory cells such as eosinophils and mast cells will lead to production and release of LT, leading to smooth muscle contraction.[18] Although those cells have been implicated in asthma, their role in heaves is uncertain. Equine neutrophils, unlike equine eosinophils or human neutrophils, require the addition of exogenous arachidonic acid for LT production.[19-21] Although arachidonic acid is likely to be present in inflammatory exudates, activated equine neutrophils collected from horses with heaves do not facilitate cholinergic bronchoconstriction,[8] suggesting that their production of cysteinyl LT may not be of a great magnitude. While there is evidence of increased LTD_4 production in airway secretion of humans with asthma, there are no quantitative studies in which the concentration of LTD_4 in lung secretions of horses with heaves have been measured. The concentration of LTC_4 in respiratory secretions of horses with heaves has been shown to be similar to controls,[22] but these results need confirmation, because LTC_4 is rapidly broken down in the bronchial lumen, which may have accounted for the failure to detect differences between groups in that study.

[a]Hoffman AM, Lilly CM, Umkauf L, et al. Elevated levels of leukotriene C_4 in BAL from horses with cough and exercise intolerance [CD-ROM, abstr], in *Proceedings*. World Equine Airway Symp 1998.

[b]BUXCO Max II preamplifier unit, Model 1420, Buxco Electronics, Conn.
[c]MP100, Biopac, Calif.
[d]AcqKnowledge software (V.3.2), Biopac, Calif.
[e]Department of Medicinal Chemistry, Merck Frosst Center for Therapeutic Research, Montreal, QC, Canada.
[f]Xylocard 1, Astra Pharma Inc, Mississauga, ON, Canada.
[g]RPMI 1640 culture medium, Sigma-Aldrich Canada, Oakville, ON, Canada.
[h]Fleisch No.4, Oem Medical, Richmond, Va.
[i]Model 143PC03D, Micro switch, Honeywell, Scarborough, ON, Canada.
[j]Model HCXPM005D6V, Sensor Technics, Newport News, Va.
[k]Anadat and Labdat 5.1, RHT Infodat, Montreal, QC, Canada.
[l]Azium, Schering-Plough Animal Health, Pointe-Claire, QC, Canada.
[m]Zorbax RxC18 5u, Agilent Technologies, Wilmington, Del.
[n]System Controller SCL10A, Pump LC10AS, Autoinjector SIL10A, Column oven CTO10A, and Spectrofluorometric detector RF10A, Shimadzu Corp, Columbia, Md.
[o]Atropine Sulfate, MTC Pharmaceuticals, Cambridge, ON, Canada.
[p]Robinson NE, Boehler D, Berney C, et al. Failure of a FLAP antagonist to prevent airway obstruction in heaves-susceptible horses (abstr), in *Proceedings*. World Equine Airway Symp 1998;31.

References

1. Robinson NE. International workshop on equine chronic airway disease. *Equine Vet J* 2001;33:5–19.

2. Snapper JR. Large animal models of asthma. *Am Rev Respir Dis* 1986;133:351–352.

3. Hui KP, Barnes NC. Lung function improvement in asthma with a cysteinyl-leukotriene receptor antagonist. *Lancet* 1991;337: 1062–1063.

4. Gaddy JN, Margolskee DJ, Bush RK, et al. Bronchodilation with a potent and selective leukotriene D_4 (LTD_4) receptor antagonist (MK-571) in patients with asthma. *Am Rev Respir Dis* 1992;146:358–363.

5. Reiss TF, Chervinsky P, Dockhorn RJ, et al. Montelukast, a once-daily leukotriene receptor antagonist, in the treatment of chronic asthma: a multicenter, randomized, double-blind trial. Montelukast Clinical Research Study Group. *Arch Intern Med* 1998;158:1213–1220.

6. Hamilton A, Faiferman I, Stober P, et al. Pranlukast, a cysteinyl leukotriene receptor antagonist, attenuates allergen-induced early- and late-phase bronchoconstriction and airway hyperresponsiveness in asthmatic subjects. *J Allergy Clin Immunol* 1998;102:177–183.

7. Doucet MY, Jones TR, Ford-Hutchinson AW. Responses of equine trachealis and lung parenchyma to methacholine, histamine, serotonin, prostanoids, and leukotrienes in vitro. *Can J Physiol Pharmacol* 1990;68:379–383.

8. Olszewski MA, Robinson NE, Zhu FX, et al. Mediators of anaphylaxis but not activated neutrophils augment cholinergic responses of equine small airways. *Am J Physiol* 1999;276:L522–L529.

9. Marr KA, Lees P, Page CP, et al. Inhaled leukotrienes cause bronchoconstriction and neutrophil accumulation in horses. *Res Vet Sci* 1998;64:219–224.

10. Rush BR, Raub ES, Rhoads WS, et al. Pulmonary function in horses with recurrent airway obstruction after aerosol and parenteral administration of beclomethasone dipropionate and dexamethasone, respectively. *Am J Vet Res* 1998;59:1039–1043.

11. Jones TR, Zamboni R, Belley M, et al. Pharmacology of the leukotriene antagonist verlukast: the (R)-enantiomer of MK-571. *Can J Physiol Pharmacol* 1991;69:1847–1854.

12. Frey EA, Nicholson DW, Metters KM. Characterization of the leukotriene D4 receptor in dimethylsulphoxide-differentiated U937 cells: comparison with the leukotriene D_4 receptor in human lung and guinea pig lung. *Eur J Pharmacol* 1993;244:239–250.

13. DeLean A, Munson PJ, Rodbard D. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am J Physiol* 1978; 235:E97–E102.

14. Olszewski MA, Zhang XY, Robinson NE. Pre- and postjunctional effects of inflammatory mediators in horse airways. *Am J Physiol* 1999;277:L327–L333.

15. Barnes NC. Effects of antileukotrienes in the treatment of asthma. *Am J Respir Crit Care Med* 2000;161:S73–S76.

16. Marr KA, Lees P, Page CP, et al. Effect of the 5-lipoxygenase inhibitor, fenleuton, on antigen-induced neutrophil accumulation and lung function changes in horses with chronic obstructive pulmonary disease. *J Vet Pharmacol Therap* 1998;21:241–246.

17. Dahlen SE. Pharmacological characterization of leukotriene receptors. *Am J Respir Crit Care Med* 2000;161:S41–S45.

18. Schmidt D, Rabe KF. The role of leukotrienes in the regulation of tone and responsiveness in isolated human airways. *Am J Respir Crit Care Med* 2000;161:S62–S67.

19. Jorg A, Henderson WR, Murphy RC, et al. Leukotriene generation by eosinophils. *J Exp Med* 1982;155:390–402.

20. Asmis R, Jorg A. Calcium-ionophore-induced formation of platelet-activating factor and leukotrienes by horse eosinophils: a comparative study. *Eur J Biochem* 1990;187:475–480.

21. Lindberg A, Tornhamre S, Mugnai S, et al. Ionophore A23187-induced leukotriene biosynthesis in equine granulocytes-neutrophils, but not eosinophils require exogenous arachidonic acid. *Biochim Biophys Acta* 1998;1391:247–255.

22. Watson ED, Sweeney CR, Steensma KA. Arachidonate metabolites in bronchoalveolar lavage fluid from horses with and without COPD. *Equine Vet J* 1992;24:379–381.

23. Lynch KR, O'Neill GP, Liu Q, et al. Characterization of the human cysteinyl leukotriene CysLT1 receptor. *Nature* 1999;399:789–793.