Interleukin-8 concentration and neutrophil chemotactic activity in bronchoalveolar lavage fluid of horses with chronic obstructive pulmonary disease following exposure to hay

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Objective—To analyze effects of hay dust exposure on interleukin-8 (IL-8) concentration, percentage of neutrophils, and neutrophil chemotactic activity in bronchoalveolar lavage fluid (BALF) of horses with chronic obstructive pulmonary disease (COPD).

Animals—16 healthy horses and 29 horses with COPD.

Procedure—IL-8 concentration, percentage of neutrophils, and neutrophil chemotactic activity in BALF were measured. Values were analyzed with respect to hay dust exposure. These variables were also measured in 5 asymptomatic horses with COPD after the induction of clinical signs by changing feed from silage to hay.

Results—IL-8 concentrations and chemotactic activity in BALF were greater in horses with COPD, compared with healthy horses, and greater in horses with COPD exposed to hay dust, compared with nonexposed affected horses. An increase in IL-8 concentration accompanied by an increase in percentage of neutrophils in BALF and development of clinical signs of COPD were induced in asymptomatic horses with COPD by changing feed from silage to hay.

Conclusions and Clinical Relevance—Exposure of horses with COPD to hay dust components resulted in an increase in IL-8 secretion at the bronchoalveolar surface. This chemokine may play a role in the pathogenesis of COPD, because it causes neutrophil accumulation in the bronchoalveolar space. Our results underscore the importance of eliminating dust sources for the treatment and prevention of COPD in horses. (*Am J Vet Res* 2000;61:1369–1374)

Chronic obstructive pulmonary disease (COPD) is a pulmonary syndrome in horses characterized by 3 pathophysiologic events: bronchospasm, hypersecretion of mucus, and migration of neutrophils into the airways.[1-5] These changes are responsible for the observed clinical signs such as dyspnea, abnormal respiratory sounds, exercise intolerance, cough, and nasal discharge. The clinical status of affected horses is dependent on exposure to dust, which principally originates from feeding hay.[6,7] Elimination of dust improves the clinical status of affected horses, whereas exposure to hay dust causes asymptomatic horses to become symptomatic. Bronchospasm develops, and neutrophils infiltrate into the bronchoalveolar space in such symptomatic horses.[6,8-12]

Different dust components may enhance the immunologic processes leading to COPD. Some dust components, in particular fungal antigens, may induce a type-1 hypersensitivity reaction with induction of allergen-specific IgE and IgE-mediated mast cell degranulation.[13-15] Leukotrienes and platelet activating factor secreted by mast cells may cause neutrophil infiltration.[16-18] Furthermore, immune complexes are able to attract neutrophils with or without activation of complement. High concentrations of airborne endotoxins are able to induce neutrophil infiltration and pulmonary inflammation.[a] Finally, dust components (allergen and endotoxins) may also activate macrophages or epithelial cells to secrete chemokines that specifically attract neutrophils.

Two distinct neutrophil-specific chemotactic proteins are known; **interleukin-8 (IL-8)**[19,20] and macrophage inflammatory protein-2.[21] In vitro stimulation of equine bronchoalveolar macrophages or peripheral blood mononuclear cells results in induction of expression of both of these neutrophil chemotactic agents.[22] Interleukin-8 is a potent chemoattractant and activator for neutrophils and is involved in disease processes involving neutrophils. The concentration of IL-8 in **bronchoalveolar lavage fluid (BALF)** is increased in humans with adult respiratory distress syndrome.[23] Furthermore, this chemokine is implicated in the development of chronic lung disease following respiratory distress syndrome in infants.[24,25] Increased IL-8 concentrations were also found in BALF of humans with idiopathic pulmonary fibrosis, sarcoidosis, and diffuse panbronchiolitis.[26,27] Human pulmonary IgE-mediated responses result in the rapid release of IL-8 and increased neutrophil infiltration.[28] Smokers with COPD have increased BALF concentrations of IL-8, compared with healthy, nonsmoking individuals.[29] Interleukin-8 is the major chemokine produced by airway epithelium of monkeys after ozone-induced injury[30] and can be found in BALF of healthy people after ozone inhalation.[31] We hypothesized that the inhalation of hay dust by horses would lead to secretion of neutrophil chemoattractants. This reaction may occur in horses with COPD exposed to hay dust. The

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purpose of the study reported here was to analyze the effect of hay dust exposure on IL-8 concentration and chemotactic activity in BALF of horses with COPD.

Materials and Methods

Horses—Twenty-nine client-owned horses with COPD and 16 healthy client-owned horses were used in this study. Horses were evaluated by experienced clinicians according to a standard protocol. This comprised recording of anamnestic data such as housing conditions, feeding, duration of disease, presence of cough and nasal discharge, and signs of exercise intolerance. Arterial blood samples were collected for blood gas analysis, and venous blood was collected for routine hematologic examination. Respiratory rate and sounds were analyzed with horses at rest with and without induction of tachy- and hyperpnea by administration of lobeline hydrochloride. Evidence of hypertrophy of the abdominal muscles (heave lines) and excessive respiratory efforts were also recorded. Horses were then exercised for 15 minutes, and development of a cough during exercise was noted. Respiratory rate and time necessary for respiratory rate to return to reference range after exercise were recorded. Endoscopy was performed. Detection of abnormal quantities of mucus was noted. Tracheobronchial secretions were obtained for cytologic examination. This was followed by bronchoalveolar lavage. Additional investigations were done to exclude the possibility of microbial infections of the upper airways. The diagnosis of COPD was made after confirmation of a chronic neutrophil bronchiolitis that persisted for more than 3 days associated with abnormal respiratory signs and poor performance or exercise intolerance. Inflammation of the lower airways was indicated by the accumulation of neutrophils in tracheobronchial secretions and > 5% neutrophils in BALF.

Dust exposure was evaluated after recording housing conditions. Horses fed hay were considered under high dust exposure. Horses fed silage or pellets and housed outdoors or indoors during the night only in well-ventilated stables were considered under low dust exposure.

Collection of BALF—Horses were sedated with a combination of xylazine (0.6 mg/kg of body weight, IV) and levomethadone (75 µg/kg, IV). A video endoscope (length, 1.8 m) was introduced through one topically anesthetized nostril and advanced until it wedged in a bronchus. During advancement of the endoscope, the bronchial surface was continuously anesthetized with a solution containing 1% lidocaine and 0.9% NaCl solution injected through a sterile catheter passed through the biopsy channel. Two hundred fifty milliliters of prewarmed sterile physiologic saline solution were infused through the biopsy channel into the bronchus and immediately aspirated with a suction pump into a sterile flask cooled on ice. The infusion-aspiration procedure was repeated 3 more times in the same lung segment. The fluid was centrifuged at 400 × g for 20 minutes, and bronchoalveolar cells were resuspended at a concentration of 2.5 to 5 × 10^6/ml in **minimum essential medium with Earle's salts (EMEM)**[b] containing 1% **fetal calf serum (FCS)** for use in stimulation experiments or cloning. Cell-free BALF was stored at –20 C for later determination of IL-8 concentrations and chemotactic activity.

For cell differentiation and neutrophil counting, 10^6 cells were resuspended in 1 ml EMEM containing 1% FCS. Cytological samples were made by loading 100 µl aliquots on a cytocentrifuge. Preparations were stained after May-Grünwald Giemsa. A minimum of 500 cells were differentiated under the light microscope in duplicate, and the percent of neutrophils was calculated.

Hay challenge—Five horses with COPD that had been fed silage for years (between 1 and 6 years) and housed outdoors for approximately 9 hours each day were used to evaluate change in feed on concentrations of IL-8 and percentage of neutrophils in BALF. Bronchoalveolar lavage fluid was collected before and after feed was changed from silage to good quality hay for 2 weeks. Four horses with COPD that typically received silage and were not subjected to a feed change were used as the control group. Interval between collection of BALF in the control group varied from 1 to 10 months. We did not include a group of healthy horses in this part of the study, because we found during a preliminary study that concetrations of IL-8 and chemotactic activity in BALF did not differ between healthy hay-fed horses and healthy silage-fed horses.

Induction of chemotactic activity in BAL cells—BAL cells were isolated as described . Cells were stimulated in vitro with phorbol myristate acetate[c] (200 ng/ml) and ionomycin[c] (200 ng/ml) for 18 hours at 37 C in a cell culture incubator. Supernatant was separated from the cells by centrifugation at 600 × g for 15 minutes. The supernatant was further cleared of debris by centrifugation for 10 minutes at 16,000 × g. A 1-ml aliquot of this cleared supernatant was stored at –20 C for use in the chemotactic assay.

Preparation of recombinant equine IL-8—To produce equine-specific antibodies for use in measuring IL-8 concentrations in BALF, 50 × 10^6 bronchoalveolar lavage cells from a healthy horse were stimulated in vitro for 5 hours in 20 ml of EMEM containing phorbol myristate acetate (200 ng/ml) and ionomycin (200 ng/ml). Total RNA was isolated, using a commercially available kit,[d] and eluted with 50 µl of water. Complementary DNA was synthesized from 10 µl of RNA, using murine leukemia virus reverse transcriptase free of RNase H activity,[e] and oligo-dT primers.[f] Nucleotide primers (forward, 5′-CGCGGATCCGCGGTTGTATCAAGAATTACT-3′; reverse, 5′-CCCAAGCTTTCATTCTCAGTCCTCTTTA-GAAACG-3′) were designed on the basis of the published sequence of equine IL-8[22,f] and incorporated a *Bam*HI and *Hind*III restriction endonuclease site at the 5′ and 3′ ends of the amplified fragment, respectively. These primers allowed for **polymerase chain reaction (PCR)** amplification of the sequence encoding the 74 N-terminus amino acids of equine IL-8 (after exclusion of the secretory portion of the precursor protein). The following PCR conditions were used: 94 C for 1 minute, 53 C for 1 minute, and 72 C for 1 minute for 35 cycles. The amplification product was purified with a commercially available kit[g] and ligated into a cloning vector[h] according to the manufacturers' instructions. A selected plasmid that contained the correct sequence was digested with *Bam*HI and *Hind*III to cleave the 237 base pair fragment corresponding to equine IL-8. This fragment was purified by use of agarose gel electrophoresis and a commercially available kit,[i] ligated into a bacterial expression vector[j] according to the manufacturers' instructions and transfected into *Escherichia coli*. Positive clones were selected by use of PCR followed by polyacrylamide gel electrophoresis of small-scale bacterial cultures.

Protein (ie, the 74 N-terminus amino acids of equine IL-8) for rabbit immunization was produced from 1 L of bacterial culture. The protein was extracted by sonication of pelleted E coli containing the IL-8 expression plasmid in 25 ml of buffer containing 8 *M* urea, 0.1 *M* NaH_2PO_4, and 0.01 *M* Tris, pH 8.0, followed by 3 freeze-thaw cycles in liquid nitrogen. The protein was further purified, using a commercially available kit.[k] We recovered between 6 and 9 mg of recombinant equine IL-8. Refolding was performed by use of slow dialysis against PBS solution.

The nucleotide sequence of our **recombinant equine IL-8 (rEqIL-8)** corresponded to the expected sequence,[22,f] although there were 13 and 6 extraneous amino acids at the

N- and C-termini, respectively. The recombinant protein was chemotactic for neutrophils at concentrations between 1 and 100 µg/ml.

Preparation of rabbit anti-equine IL-8 antiserum—To produce anti-equine IL-8 antiserum for use in an assay designed to measure IL-8 concentrations in BALF, 2 rabbits were immunized with 100 µg of refolded recombinant equine IL-8 in Freund's incomplete adjuvant, followed by 4 more immunizations at weekly intervals with 50 µg of protein. Serum was obtained from the ear vein 40 days after the first immunization and stored at –20 C until use.

Determination of IL-8 concentrations—A dot-blot assay was developed for determination of IL-8 concentrations. This assay had a detection threshold of 0.1 µg of rEqIL-8/ml, and the signal intensity was linearly proportional to the logarithm of the dilution of rEqIL-8 (R^2 = 0.982).

Five hundred microliters of BALF was centrifuged at 50,000 × g for 30 minutes. Two microliters of the centrifuged sample was applied in triplicate to a nitrocellulose sheet together with appropriate serial dilutions of rEqIL-8. The membrane was incubated in assay buffer (ie, PBS solution containing 1% Tween-20 and 1% bovine serum albumin) for 30 minutes at room temperature (approx 20 C), washed briefly in PBS solution, immersed in assay buffer containing rabbit anti-rEqIL-8 antiserum (diluted 1: 100 dilution), and incubated for 90 minutes at room temperature. We did not incubate a duplicate nitrocellulose sheet with preimmune rabbit serum, because results of previous experiments revealed an absence of nonspecific staining.

After washing by immersion in assay buffer 3 consecutive times for 5 minutes under agitation at room temperature, the membrane was immersed in assay buffer containing a 1:300 dilution of alkaline phosphatase-conjugated monoclonal antibody against rabbit immunoglobulins[c] in PBS solution and incubated for 45 minutes at room temperature. After washing 3 times for 5 minutes in assay buffer and a fourth time in PBS solution, the membrane was soaked in the substrate solution (one 5-bromo-4-chloro-3-indoil-1-phosphate/nitro blue tetrazolium-tablet[c] dissolved in 10 ml of distilled water) and incubated until a dark spot developed at the dot corresponding to 0.1 µg of rEqIL-8/ml (approx 1 to 15 minutes). The reaction was stopped with distilled water, and the nitrocellulose sheet was air-dried. The spots were digitalized using a scanner,[l] and concentrations in BALF samples were calculated by use of densitometry.[m] Cross-reactivity assays were performed, using rabbit anti-human IL-8 polyclonal antibody,[n] rabbit anti-ovine IL-8 polyclonal antibody,[n] and recombinant human IL-8.[n] Polyclonal antibodies to ovine IL-8 recognized rEqIL-8 (10 µg/ml) and recombinant human IL-8 (1 µg/ml). Rabbit anti-equine IL-8 antiserum cross-reacted with human recombinant IL-8; 100 µg of human IL-8/ml produced a signal corresponding to 3.5 µg of rEqIL-8/ml.

Neutrophil chemotaxis assay—Equine neutrophils were purified from blood with heparin added as an anticoagulant. Briefly, blood was diluted with an equal volume of PBS solution, underlayered with 10 ml of a ficoll-based lymphocyte separation medium,[o] and centrifuged at 400 × g for 20 minutes. The plasma and Ficoll layers containing mononuclear cells and platelets were discarded. To lyse the erythrocytes, 18 ml of water was added to the pellet, and after agitation for 2 minutes, isotonic conditions were restored by adding 10 × PBS solution. After centrifugation at 200 × g for 10 minutes, neutrophils were resuspended at a concentration of 5×10^6/ml in EMEM with 1% FCS.

For determination of chemotactic activity in BALF, 30 ml of BALF was cleared of cell debris and particles by centrifugation at 50,000 × g for 20 minutes. Ninety microliters of this fluid was added to the lower compartment of a modified Boyden chemotaxis chamber[p] in triplicate, and 100 µl of the neutrophil suspension was added to the upper compartment. For determination of chemotactic activity of our rEqIL-8, 90 µl aliquots of rEqIL-8 at different concentrations (0.1 to 100µg/ml) instead of BALF were added to the lower compartment. After 40 minutes the membrane[q] separating the upper and lower compartments was removed and stained with May-Grünwald-Giemsa solution. Chemotactic activity, expressed as **neutrophils per magnification field (nmf)**, was determined by counting the number of neutrophils attached to the lower side of the membrane, using a light microscope. For each BALF sample, mean number of cells from 5 random hpf (100X objective) was calculated.

Statistical analyses—Statistical analyses were performed using commercially available software.[r] Horses were compared with respect to their disease status and feeding regime using the Mann-Whitney U test. In the hay challenge experiment values were compared using the Wilcoxon signed-rank test. Differences were considered significant at $P \leq 0.05$.

Results

Chemotactic activity is induced in vitro after stimulation—Chemotactic activity could be induced in isolated BAL cells (**Fig 1**). This activity started to increase 1.5 hours after stimulation, reached a plateau after 7 hours and started to decrease after 18 hours of stimulation (data not shown).

Sixteen horses with COPD and 6 healthy horses were fed silage (low dust exposure), whereas 13 horses with COPD and 10 healthy horses were fed hay (high dust exposure). Interleukin-8 concentration, chemotactic activity, and percentage of neutrophils were greater in BALF of horses with COPD fed hay, compared with affected horses fed silage. Within the group of healthy controls, no significant differences were detected in percentage of neutrophils, chemotactic activity, and IL-8 concentration in BALF between horses exposed to low dust and those exposed to high dust. For this reason, horses with COPD and healthy horses could not be compared without considering dust exposure. In silage-fed horses, mean percentage of neutrophils in BALF was significantly (P = 0.015) greater in affected horses, compared with healthy horses (10.2 vs 3.3%). However, IL-8 concentration and chemotactic activity did not differ between healthy and affected horses fed silage. When only hay-fed horses were considered, values for all 3 variables were significantly greater in horses with COPD than in healthy horses (neutrophils, 23.5 vs 3.5% [$P < 0.001$]; chemotactic activity, 77.1 vs 64.0 nmf [$P < 0.001$]; IL-8 concentration, 955 vs 683 ng/ml [P = 0.04]).

Change of feed from silage to hay induced development of clinical signs in 5 asymptomatic horses with COPD. Percentages of neutrophils in BALF from these horses during silage feeding were above the generally accepted threshold of 5%, with the exception of 1 horse (4.8%). After change to hay feeding, percentage of neutrophils in all horses was > 20% (**Table 1**). Concentration of IL-8 in BALF also increased significantly in affected horses after 2 weeks of hay feeding. In contrast, in a group of 4 horses with COPD fed

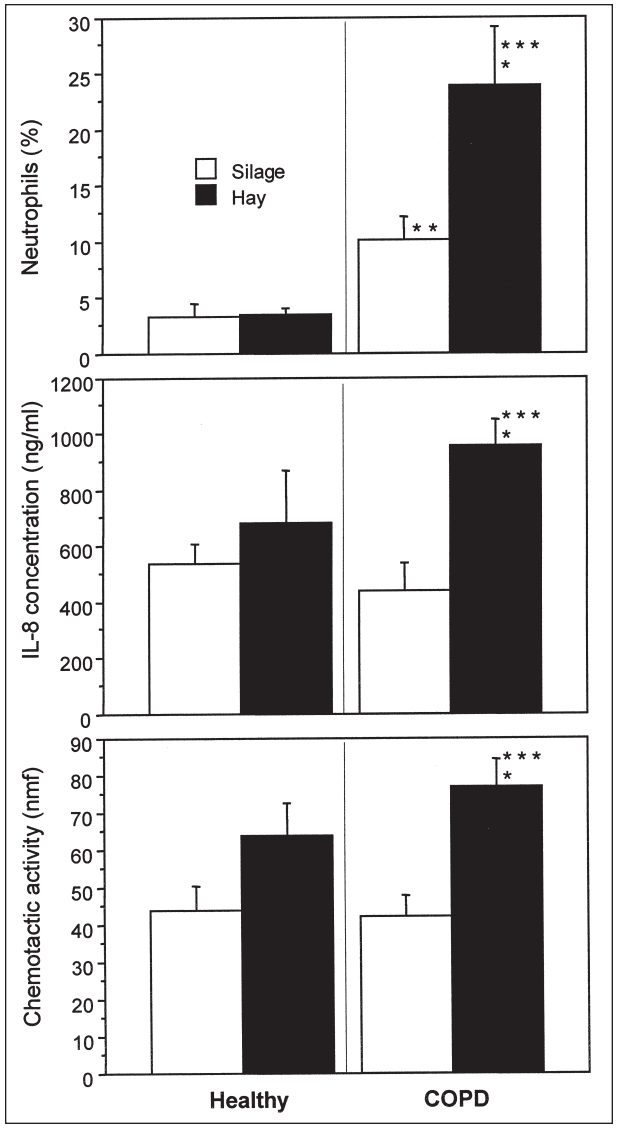

Figure 1—Mean (± SEM) percentage of neutrophils, interleukin-8 (IL-8) concentration, and chemotactic activity (neutrophils per magnification field [nmf]) in bronchoalveolar lavage fluid of 16 healthy horses and 29 horses with chronic obstructive pulmonary disease fed silage (healthy, n = 6; COPD, 16) or hay (healthy, 10; COPD, 13). *Significantly ($P < 0.05$) greater than value for affected horses fed silage. **Significantly ($P < 0.05$) greater than value for healthy horses fed silage. ***Significantly ($P < 0.05$) greater than value for healthy horses fed hay.

silage and not subjected to a feed change, neither mean (± SEM) IL-8 concentration (172 ± 21 and 180 ± 75 ng/ml) nor percentage of neutrophils (9.75 ± 1.8 and 11.0 ± 2.1%) changed significantly over time.

Discussion

Besides bronchomotor dysfunction and hypersecretion, neutrophil infiltration is an important event in the pathogenesis of COPD. In horses, neutrophil infiltration and clinical signs have been associated with hay dust inhalation.[6,7] Dust components, such as fungal spores and bacterial lipopolysaccharides, have been shown to induce an increase in neutrophils in BALF and development of clinical signs in asymptomatic horses with COPD.[6,8-11] Neutrophil migration into the bronchoalveolar space must be induced by chemotactically active substances. We induced secretion of chemotactic activity in vitro by stimulation of BAL cells.

Hay-fed affected horses had higher IL-8 concentrations in BALF than healthy horses fed hay. Furthermore, affected horses fed hay had higher IL-8 concentrations than those fed silage. Nevertheless, IL-8 concentration in BALF did not directly correlate with percentage of neutrophils or chemotactic activity. This was not surprising, because chemotactic activity in BALF fluid is not only attributable to IL-8. Leukotrienes that are liberated from mast cells during a type-1 hypersensitivity reaction may be responsible for the chemotactic activity that we found in the low molecular weight fraction. In fact, horses with COPD have increased serum IgE concentrations, compared with healthy horses.[13,15] The presence of chemotactic activity in high molecular weight fractions may have been attributable to immune complexes. In addition, some of the chemotactic activity may also be attributable to macrophage inflammatory protein-2, the second neutrophil chemoattractant detected in horses after in vitro stimulation of bronchoalveolar lavage cells.[22] Furthermore, other pathologic changes (eg, expression of adhesion molecules, metalloproteinase-induced change in permeability of the basal membrane of endothelial capillaries) are necessary to allow neutrophil infiltration.[32-34] Neutrophil infiltration may be further induced by the persistence of inhaled antigens, because inflamed lungs typically process inhaled antigens more slowly than noninflammed lungs. In addition, equine alveolar macrophages stimulated in vitro lose their ability to phagocytize yeast particles.[22,35]

Table 1—Effects of changing feed from silage to hay on concentration of interleukin-8 (IL-8) and percentage of neutrophils in bronchoalveolar lavage fluid of horses with chronic obstructive pulmonary disease (COPD)*

	Silage		Hay	
Horse No.	IL-8 (ng/ml)	Neutrophils (%)	IL-8 (ng/ml)	Neutrophils (%)
1	398	12.2	725	45.0
2	216	20.0	342	37.0
3	330	19.2	939	27.8
4	170	4.8	409	22.2
5	230	13.8	411	76.6
Mean (± SEM)	268 ± 41	14.0 ± 2.7	565 ± 115†	41.7 ± 9.5†

*Horses were asymptomatic when fed silage. Clinical signs of COPD developed during the 2 weeks horses were fed hay. †Significantly ($P < 0.05$) greater than mean value determined while horses were fed silage.

Concentration of IL-8 in BALF of hay-fed healthy horses did not significantly differ from that of silage-fed horses. This may lead to speculation that hay dust exposure of healthy horses was less intensive than that of affected horses or that the endotoxin or allergen content of hay fed to healthy horses was less, compared with hay fed to horses with COPD. Results of other studies,[9,36,37] however, indicate that a standardized allergen challenge does not induce COPD in healthy horses. Furthermore, COPD develops under conditions in which nonsusceptible horses subjected to the same antigen load remain healthy. This indicates that hay dust exposure alone cannot cause COPD. Cofactors are necessary to influence host reactivity to hay dust. A genetic predisposition has been described,[38] but other unrecognized causes should be considered. Our analysis of clinical cases of COPD (field experiment) indicated that affected horses have higher IL-8 concentrations in BALF than healthy horses, and hay-fed affected horses had higher IL-8 concentrations than silage-fed horses. The challenge experiment was performed to reinforce data obtained during the field experiment. Differences between affected horses fed hay or silage may have been attributable to factors other than type of feed. Results of the field experiment indicated that IL-8 concentrations in BALF from healthy horses were unaffected by type of feed. Thus, by changing the type of feed fed to asymptomatic horses with COPD, we induced clinical signs of disease. The appearance of clinical signs was associated with neutrophil infiltration and increased IL-8 concentrations in BALF.

Nonspecific immunologic reactions induced by inhalation of dust components that result in neutrophil infiltration appear to play an important role in the pathogenesis of COPD. In humans, IL-8 is involved in the pathogenesis of allergic respiratory diseases and can be induced by IgE-mediated responses.[28] For this reason, IL-8 is considered a regulator of type-1 hypersensitivity reactions. It remains unclear whether IL-8 in BALF of horses with COPD may also be the consequence of a type-1 hypersensitivity reaction. In contrast, it is clear that IgE-independent mechanisms can induce IL-8 secretion by bronchoalveolar macrophages.[22] Thus, IL-8 appears to represent an important substance in the pathogenesis of COPD independently of the immunologic mechanisms that enhance its secretion.

[a]Pirie RS. Changes in the BALF cytology following endotoxin inhalation: a role in the pathogenesis of COPD (abstr), in *Proceedings*. Workshop on Allergic Diseases of the Horse, 1998;10.
[b]GIBCO BRL, Basel, Switzerland.
[c]Sigma Corp SAF, Buchs, Switzerland.
[d]RNAeasy kit, Quiagen AG, Basel, Switzerland.
[e]Promega, Catalys AG, Zurich, Switzerland.
[f]GeneBank accession number AF062377.
[g]QIAquick PCR purification kit, Quiagen AG, Basel, Switzerland.
[h]LigATor, Ingenius, R&D Systems Europe Ltd, Abington, UK.
[i]QIAquick gel extraction kit, Quiagen AG, Basel, Switzerland.
[j]Vector pQE30, Quiagen AG, Basel, Switzerland.
[k]QIAexpressionist, Quiagen AG, Basel, Switzerland.
[l]Gel-Doc 2000, Bio-Rad Laboratories, Hercules, Calif.
[m]Multi-analyst software, Gel Doc 200, Bio-Rad Laboratories, Hercules, Calif.
[n]Chemicon International Inc, Juro Supply AG, Lucerne, Switzerland.
[o]Ficoll-Paque, research grade, Pharmacia Biotech, Dubendorf, Switzerland.
[p]96-well chemotaxis chamber, Neuro Probe, Sterico AG, Dietikon, Switzerland.
[q]25 × 80 mm with 5 µm pores and PVP free membranes, Nucleopore/Costar, Sterico, Dietikon, Switzerland.
[r]StatView program,versio 4.02 Abacus Concepts, Inc, Berkeley, Calif.

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