

IgE-bearing Cells in Bronchoalveolar Lavage Fluid and Allergen-specific IgE Levels in Sera from RAO-affected Horses

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Summary

Recurrent airway obstruction (RAO) is a common condition in stabled horses characterized by small airway inflammation, airway neutrophilia and obstruction following exposure of susceptible horses to mouldy hay and straw and is thus regarded as a hypersensitivity reaction to mould spores. However, the role of immunoglobulin E antibodies (IgE) in the pathogenesis of RAO is unclear. We hypothesized that the number of cells with receptor-bound IgE in bronchoalveolar lavage fluid (BALF) and IgE levels in serum would be higher in RAO-affected than in healthy horses living in the same environment. Therefore, IgE-positive (+) cells were identified by immunocytochemistry on cytospins from BALF and counted. IgE levels against the mould extracts *Aspergillus fumigatus* (*Asp. f.*) and *Alternaria alternata* (*Alt. a.*) and the recombinant mould allergen *Aspergillus fumigatus* 8 (rAsp f 8) were measured by enzyme-linked immunosorbent assay (ELISA) in the sera of seven RAO-affected and 22 clinically healthy mature horses housed in the same conventional stable environment. After correcting for the number of neutrophils, there were no significant differences in IgE+ cells on cytospins from BALF between both groups of horses (5% versus 7%, $P > 0.1$). Serum IgE levels against the mould extracts were significantly higher in RAO-affected than in clinically healthy horses [median = 119 versus 66 relative ELISA units (REU), $P < 0.05$]. Furthermore, significantly more RAO-affected than healthy horses had detectable serum IgE against the recombinant allergen rAsp f 8 (4/7 and 3/22, respectively, $P < 0.05$). Age had no significant effect on BALF cell ratios or on specific serum IgE levels. These results show that high IgE levels against mould antigens are associated with RAO under controlled environmental conditions but ranges of mould-specific serum IgE levels overlapped too much between diseased and clinically healthy animals to be of any diagnostic value. Further studies are needed to assess whether IgE-mediated reactions contribute to the pathogenesis of RAO.

Introduction

Recurrent airway obstruction (RAO) (Robinson, 2001), also known as 'heaves', is one of the most common diseases of the lower respiratory tract of mature horses housed indoors in conventional stable environment (CSE). RAO is characterized

by visibly and severely increased breathing effort because of cholinergic bronchospasm, coughing and airway hyperreactivity as well as neutrophil and mucus accumulation in the airways (McPherson et al., 1978; Dixon et al., 1995; Léguillette, 2003). A genetic predisposition for this disease has been demonstrated (Marti et al., 1991). Clinical remission is usually achieved by eliminating exposure to hay and stable dust (Schatzmann et al., 1974; Thomson and McPherson, 1984). The aetiology of the disease is thus regarded as a hyperreactivity to inhaled organic dust (reviewed in Robinson et al., 1996; Robinson, 2001) derived primarily from hay and straw. Inhalation experiments with *Faenia rectivirgula* and *Aspergillus fumigatus* (Derksen et al., 1988; McGorum et al., 1993a) showed that airway neutrophilia is induced both in controls and heaves-affected horses, but pulmonary dysfunction is observed only in affected animals. However, the full clinical severity and pathophysiological changes can only be reproduced by natural challenge in CSE (Pirie et al., 2002), suggesting that other inhaled dust components, such as endotoxins, contribute to pulmonary inflammation and dysfunction in heaves (Pirie et al., 2003).

The immunological basis of RAO remains poorly elucidated. Type I as well as type III (Lawson et al., 1979; Asmundsson et al., 1983) reactions have been suggested. Type I hypersensitivity, also referred to as immediate-type hypersensitivity, is mediated by immunoglobulin E (IgE). Allergen-specific IgE antibodies bind to high-affinity receptors present on mast cells and basophils. Allergen exposure results in IgE cross-linking with the subsequent release of various inflammatory mediators causing an early-phase inflammation often followed by a late-phase reaction. Several studies support an involvement of IgE-mediated hypersensitivity reactions in heaves: *in vitro* histamine release assays with peripheral blood leukocytes (Gerber et al., 1982; Dirschel et al., 1993) or mast cells from bronchoalveolar lavage fluid (BALF) (Hare et al., 1999) performed with crude allergen extracts suggested the involvement of IgE-mediated reactions in RAO, although release was only induced with relatively high concentrations of the extracts. Furthermore, histamine concentration in the pulmonary epithelial lining fluid of RAO-affected horses was significantly increased following exposure to CSE (McGorum et al., 1993b). Horses with RAO have higher IgE levels against *A. fumigatus* and *Micropolyspora faeni* extracts in BALF than

healthy controls (Halliwell et al., 1993; Schmallenbach et al., 1998). Although no differences were found in serum IgE levels against mould allergen extracts between RAO-affected and control horses (Halliwell et al., 1993; Schmallenbach et al., 1998; Eder et al., 2000), Eder et al. (2000) showed that horses with RAO have higher serum IgE and IgG levels against pure recombinant *A. fumigatus* allergens than control animals and that the stud-specific environment exerts a highly significant effect on allergen-specific serum IgE levels (Eder et al., 2001). In most published studies, intradermal tests with various allergen extracts correlated poorly with the clinical diagnosis (Eyre, 1972; Schatzmann and Gerber, 1972; Evans et al., 1992; McGorum et al., 1993c; Lorch et al., 2001). Immediate-type as well as late-phase or delayed reactions have been reported (Halliwell et al., 1979; Evans et al., 1992; McGorum et al., 1993c). Additionally, the immediate onset of airway obstruction, typical of a type I reaction, after exposure to allergens is rarely observed in RAO and eosinophils are only rarely found in BALF of RAO-affected horses. The role of IgE-mediated reactions in the pathogenesis of RAO is thus still controversial. Similarly, data relating to the cytokine profiles of T lymphocytes from BALF or peripheral blood of RAO horses are contradictory (Lavoie et al., 2001; Giguere et al., 2002; Ainsworth et al., 2003; Cordeau et al., 2004; Kleiber et al., 2005).

Interestingly, Lukacs et al. (1998) have demonstrated in mice, that mast cells produce ENA-78, a chemokine which can function as a potent neutrophil chemoattractant during allergic airway inflammation. This chemokine could possibly contribute to the elevated numbers of BALF neutrophils in RAO. Not only mast cells but also various other cell types such as basophils, dendritic cells, IgE-producing plasma cells and others can bind IgE through the high- or low-affinity receptors for IgE (Maurer et al., 1996; Berger et al., 1998) and are involved in allergic reactions. As there are hardly any markers available for identification of these cell types in the horse, an antibody specific for equine IgE was used to investigate IgE-bearing cell numbers in BALF.

We hypothesized that the number of cells with receptor-bound IgE in BALF and mould-specific IgE levels in serum would be increased in RAO-affected horses compared with clinically healthy horses living in the same environment. The purpose of this study was therefore to compare IgE-positive (+) cells by immunocytochemistry on cytopins from BALF and mould-specific serum IgE levels in a group of RAO-affected and clinically healthy horses housed in the same CSE. Additionally, the effect of age on these parameters was determined.

Materials and Methods

Stable environment and management conditions of horses

The Swiss National Equine Centre is stocked with over 200 Warmblood horses. They typically enter the facility at 3–4 years of age, and thereafter live permanently in a CSE. Horses are bedded on straw and housed 18–22 h/day in box stalls or tie stalls. Stable ceilings are high, >3 m. Moreover, stable ventilation and air quality are subjectively good, i.e. it is not dusty, there is no ammonia smell. A combination of good quality hay and haylage is fed three times per day along with individual rations of concentrated feed. Straw, hay and haylage are kept in dry and clean compartments separate

from the stables. All horses are used daily (except for one resting day per week) for dressage and/or show jumping, and most of them compete regularly. Primary health care is provided at the Swiss National Equine Centre, and all medical records are kept with the resident veterinarian. All horses are dewormed on a regular basis.

Horses and definitions of disease status

Initially, 35 Swiss Warmblood horses all belonging to the Swiss National Equine Centre were included in this study. Eight horses were diagnosed as suffering from RAO based on clinical records documented by the resident veterinarian of the Swiss National Equine Centre. For all horses diagnosed with RAO, disease remission upon withdrawal from hay and straw had previously been documented. One of these horses was in clinical remission at the time the study was performed and was therefore excluded. The remaining seven horses showed clinical signs of RAO at the time when the study was performed. Clinical examination was performed before (at rest: respiratory rate and lung auscultation; presence/absence of cough; presence/absence of nasal discharge), during (presence/absence of cough) and immediately after (lung auscultation; presence/absence of cough; presence/absence of nasal discharge) an exercise test consisting of walk, trot and canter for 10 min each (Table 1). A blood gas analysis was carried out. All horses were non-febrile and there was no evidence of infection based on blood count and serum chemistry profile. Bronchoscopy was performed and showed increased mucotracheal secretions in the RAO-affected horses. As recorded by the resident veterinarian, RAO-affected horses historically had all had episodes of increased abdominal breathing effort at rest when exposed to hay. Control horses had no history of lower airway disease for more than 6 months, no signs of lower airway disease such as cough, mucoid or mucopurulent nasal discharge and/or abnormal lung sounds (except mild tracheal rattles) and/or evidence of infection. Additionally, control horses with $\geq 15\%$ neutrophils in the BALF as well as one control horse with 26% eosinophils in the BALF were not regarded as suitable controls and were dismissed from the study ($n = 5$). Thus 29 horses (24 geldings and five mares; seven RAO-affected and 22 clinically healthy controls) remained in the study.

The horses came to the National Equine Centre at the age of 3 years. The RAO horses had thus been living at the National Equine Centre for a median time of 11 years (range 4–14 years) and the controls for 5 years (range 1–16 years).

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed under sedation with an α_2 -agonist (Domosedan[®], Pfizer AG, Zürich, Switzerland) in combination with a morphin derivate (L-Polamivet[®], Veterinaria AG, Zürich, Switzerland). After sedation, a BAL catheter (BIVONA Medical Technologies, Gary, IN, USA) was passed through the ventral nasal passage and the trachea and wedged in a peripheral bronchus where the balloon was inflated. Three hundred and sixty millilitres of phosphate-buffered saline (PBS), at room temperature, was infused in 120-ml aliquots and recovered by manual aspiration. On average, 52% of the instilled fluid was recovered (mean = 188 ml, range: 128–271 ml).

Table 1. Clinical status of the control ($n = 22$) and RAO-affected ($n = 7$) horses included in the study

Horse n.	Gender	Age (years)	Age group	Diagnosis	Results of clinical examination				
					Cough before/during/after exercise	Resp. rate > 16/min at rest	Nasal discharge before/after exercise	Abnormal lung sound before/after exercise	% neutrophils in BALF
1	Gelding	19	Old	Control	No	No	No	Yes [†]	0
2	Gelding	17	Old	Control	No	No	No	Yes [†]	11.2
3	Gelding	15	Old	Control	No	No	No	No	14
4	Gelding	14	Old	Control	No	No	No	Yes [†]	5.38
5	Gelding	13	Old	Control	No	No	Yes*	No	1
6	Gelding	13	Old	Control	No	No	No	No	14.2
7	Gelding	13	Old	Control	No	No	No	No	13.1
8	Gelding	14	Old	Control	No	No	No	Yes [†]	4
9	Gelding	16	Old	Control	No	No	No	Yes [†]	6
10	Gelding	11	–	Control	No	No	No	Yes [†]	2.12
11	Mare	10	–	Control	No	No	No	No	4
12	Gelding	5	Young	Control	No	No	No	No	7.51
13	Gelding	6	Young	Control	No	No	No	No	8.81
14	Mare	6	Young	Control	No	No	Yes*	No	6.34
15	Mare	6	Young	Control	No	No	No	No	12
16	Gelding	6	Young	Control	No	No	No	No	5.83
17	Gelding	5	Young	Control	No	No	No	No	7.92
18	Gelding	8	Young	Control	No	No	No	No	6.1
19	Gelding	5	Young	Control	No	No	Yes*	No	1.91
20	Gelding	5	Young	Control	No	No	Yes*	No	7.14
21	Gelding	4	Young	Control	No	No	No	No	8.5
22	Mare	6	Young	Control	No	No	No	No	0.5
23	Gelding	15	Old	RAO	Yes	Yes	No	Yes	6
24	Gelding	15	Old	RAO	No	No	Yes	Yes	14
25	Gelding	17	Old	RAO	Yes	Yes	No	Yes	71.5
26	Gelding	13	Old	RAO	Yes	Yes	No	Yes	84
27	Gelding	16	Old	RAO	Yes	Yes	Yes	Yes	64.5
28	Gelding	7	Young	RAO	Yes	No	Yes	Yes	48
29	Mare	7	Young	RAO	Yes	Yes	No	Yes	65.5

*Nasal discharge observed in the controls was of serous quality.

[†]Abnormal lung sounds in control horses were mild tracheal rattles.

All RAO-affected horses but none of the controls had a history of chronic respiratory disease.

Bronchoalveolar lavage fluid was filtered through a monolayer of surgical gauze to remove mucus, and kept on ice until processed. The viability of the cells was monitored by the trypan blue exclusion test (mean 91.6%, range 77–97%).

For differential cell counts, cytopins were prepared by centrifugation of 100 μ l of BALF for 10 min at 800 rpm. After staining with May–Grünwald–Giemsa, differential cell counts were obtained and expressed as percent of total cells by counting 200 cells using standard morphologic criteria under a light microscope. Cell counts were performed by the same person and without knowledge of the diagnoses.

Preparation of cytopins for immunocytochemistry

Cytopins used for IgE staining were prepared according to Kleiber (1996). BALF was filtered to remove the mucus, centrifuged at 150 g for 10 min at 4°C and the cell pellets were resuspended in PBS. Centrifugation was repeated and the cell pellet was resuspended in 2 ml washing solution containing 10 mM PBS, 1% Physiogel (Swiss Red Cross Blood Transfusion Service, Switzerland), 5% ethylenediaminetetraacetic acid (EDTA; Fluka Chemie AG, Buchs, Switzerland) and 0.02% sodium azide (Fluka Chemie AG). To block non-specific binding of the antibodies to Fc receptors on the BALF cells, human IgG (Globuman Berna, Serum und Impfstoffinstitut, Bern, Switzerland) was added to the cell suspension at 10 μ g/ml. The

cells were diluted to a concentration of 1×10^5 cells/ml. Cytopins were prepared by centrifuging 200 μ l of the cell suspension onto positively charged slides (Superfrost plus, Menzel-Glässer, Braunschweig). Slides were dried for 24 h at 37°C, subsequently fixed for 10 min in 2% paraformaldehyde and washed 3×5 min in PBS. After drying, slides were packed in aluminium foil and kept frozen at -80°C until use (< 6 months).

Immunocytochemistry for IgE

Cytopins were warmed to room temperature (RT) and rehydrated for 5 min in Tris buffer saline (TBS, pH 7.6). Non-specific binding sites were blocked by incubating the slides in 2% porcine skin gelatine (PSG, Fluka Chemie AG) at RT for 30 min. Thereafter, the cytopins were incubated for 2 h at 37°C with a chicken antibody (Ab) specific for equine IgE (Marti et al., 1997) diluted to a concentration of 9 μ g/ml in TBS with 2% PSG. Pre-immune chicken IgY was used at the same concentration as a negative control. Cytopins were then washed three times in TBS and an alkaline phosphatase (AP)-labelled goat anti-chicken IgG Ab (Kirkegaard and Perry Laboratories, Gaithersburg, MA, USA) was applied as developing Ab at a dilution of 1:500 in TBS, 2% PSG and 1 mg/ml levamisole (Sigma Chemical Co., St Louis, MO, USA) for 2 h at RT. Slides were washed three times with TBS and colour

processing was performed with naphtol-AS-Mx-phosphate (Sigma Chemical Co) and fast red (Chroma-Gesellschaft, Schmid GmbH + Co., Köngen, Germany) diluted in 0.1 M Tris, pH 8 after filtration through 0.45- μ m filters (Schleicher & Schnell GmbH, 37582 Dassel, Germany). Hemalum (Merck KgaA, 64271 Darmstadt, Germany) served as the counterstain. Slides were mounted with Glycergel (Dako Corporation, Carpinteria, CA, USA).

Quantification of IgE+ cells

The analysis of cytopspins was performed under a standard light microscope (Leitz, Laborlux, Leica, Germany) at $\times 400$ magnification. Positive cells were counted from a total of 2×1000 cells on two different slides. The mean value from both slides was used and expressed in % of IgE+ cells. As BALF from RAO-affected horses contains significantly more neutrophils than BALF from controls (Tables 1 and 2), we have also calculated percentages of IgE+ cells after correction for neutrophils [% IgE+ corr = number of IgE+ / (1000 cells – number of neutrophils) * 100].

Determination of allergen-specific serum IgE levels

Allergen-specific serum IgE levels were determined by enzyme-linked immunoassay (ELISA) as described previously (Eder et al., 2000, 2001). Briefly, microtitre plates were coated with *Alternaria alternata* (Alt a) or *Aspergillus fumigatus* (Asp f) extracts or with recombinant *Aspergillus fumigatus* 8 (rAsp f 8; Crameri, 1999). Non-specific binding sites were blocked with 0.2% equine serum albumin, isolated from horse plasma according to Kistler and Nitschmann (1962). Horse sera were added and serially diluted in the plate. After incubation and washing, the chicken antibody specific for horse IgE (Marti et al., 1997) was added, followed by an alkaline phosphatase-labelled goat anti-chicken IgG (Kirkegaard and Perry Laboratories). ELISA plates were subsequently developed with *p*-nitrophenyl phosphate in diethanolamine buffer. Absorbance readings were measured at 405 nm with a Molecular Devices Reader and converted to relative ELISA units (REU) for *A. fumigatus*- and *A. alternata*-specific IgE levels. For this purpose, an equine serum positive for the tested allergen was used as the reference serum (same serum as used in Eder et al., 2000) and assigned a value of 100 REU. Serial dilutions of the reference serum were used to generate a standard curve and the test sample results were calculated from the curve using an ELISA software program (SOFTmax[®] 2.31 for WindowsTM, Molecular Devices Co., Sunnyvale, CA, USA). IgE against rAsp f 8 was classified as detectable or undetectable.

Statistical analyses

Statistical analyses were carried out with the statistical software package SPSS SYSTAT for Windows, version 7.0. A preliminary analysis indicated that IgE+ cells and allergen-specific IgE levels were not distributed normally. Median and range values were thus used subsequently for descriptive purposes. The nonparametric Mann–Whitney *U*-test was used to compare the following parameters between RAO-affected ($n = 7$) and control horses ($n = 22$): age, partial pressure of oxygen in arterial blood (paO₂), % neutrophils, lymphocytes, eosinophils, mast cells and IgE+ cells in BALF. For serum

Table 2. Influence of diagnosis [recurrent airway obstruction (RAO) or control], or of age group (young: 4–8 years, old > 12 years) on different parameters [median (range)] in horses living in the same environment

	Age (years)	pO ₂ (mmHg)	% cells in bronchoalveolar lavage fluid						Serum IgE (REU*) against			N/Total (%) horses with detectable serum IgE against rAsp f 8
			Neutrophils	Lymphocytes	Macrophages	Eosinophils	Mast cells	IgE+ cells	IgE+ cells corr†	<i>Alternaria alternata</i>	<i>Aspergillus fumigatus</i>	
Diagnosis:												
RAO ($n = 7$)	15 (7–17)	95 (68–113)	65 (6–84)	12 (4–56)	14 (6–33)	0.5 (0–2.5)	4.8 (0.5–8)	3.2 (1–5.9)	5 (1.7–15.6)	112 (56–136)	119 (31–144)	4/7 (57%)
Controls ($n = 22$)	9 (4–19)	103 (84–116)	6 (0–14)	33 (19–51)	49 (8–65)	0 (0–3.5)	5.5 (1–13)	6.8 (1.4–18)	7 (1.4–18.7)	66 (32–115)	66 (24–145)	3/22 (13.5%)
<i>P</i>	0.08	≤ 0.05	≤ 0.01	≤ 0.01	≤ 0.01	ns	ns	≤ 0.05	ns	≤ 0.05	≤ 0.05	≤ 0.05
Age group:												
old ($n = 14$)	15 (13–19)	101 (68–116)	12 0–84	26 (4–56)	37 (6–59)	0.5 (0–1.5)	6 (1.5–12)	5.7 (1–13)	6.5 (3–13.2)	66 (46–120)	69 (24–130)	4/14 (29%)
Young ($n = 13$)	6 (4–8)	100 (84–113)	8 0.5–66	32 (12–48)	45 (8–65)	0 (0–3.5)	5.5 (0.5–11)	5.8 (1.4–18)	6.6 (1.4–18.7)	74 (32–136)	72 (40–145)	3/13 (23%)
<i>P</i>	≤ 0.001	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

*Relative ELISA units.

†% of IgE+ cells on BALF cytopspins after correction for numbers of neutrophils.

IgE titres against *A. fumigatus* and *A. alternata* extracts, comparison of the responses obtained for the two groups of horses was also performed using the Mann–Whitney *U*-test. Serum IgE levels to rAsp f 8 were subdivided into positive and negative results and evaluated with the Fisher's exact two-tailed test. To investigate whether age had any effect on the parameters mentioned above, the horses were grouped into two age groups: young: 4–8 years ($n = 13$, 11 controls and two RAO) and old: 13–19 years ($n = 14$, nine controls and five RAO). In order to get a clear separation between age groups, horses with ages between 9 and 12 years (two controls) were not considered for this analysis. This statistical analysis was additionally also performed with the healthy horses only (11 young horses and nine old ones). *P*-values ≤ 0.05 were considered statistically significant.

Results

Clinical signs and BALF cytology

The RAO-affected horses showed at least two clinical signs typical of RAO [coughing, increased respiratory frequency at rest ($> 16/\text{min}$), nasal discharge and abnormal lung sounds] at the time of sampling and $> 5\%$ neutrophils in the BALF (Table 1). Control horses, in contrast, at most showed one of these clinical signs, and, when present nasal discharge was serous in quality and abnormal lung sounds were mild tracheal rattles. The percentages of nucleated cells present in BALF samples are presented in Table 2. As expected, there was a significantly higher percentage of neutrophils present in the RAO-affected horses compared with controls, but, as shown in Table 1, some clinically healthy control horses also had neutrophil percentages $> 5\%$. Lymphocyte and macrophage median percentages were significantly lower in BALF from RAO-affected animals compared with controls (Table 2). There were no significant differences between both groups for mast cell and eosinophil percentages.

Age group (old: > 12 years and young: < 9 years) had no significant effect on percentages of neutrophils, lymphocytes, macrophages, eosinophils and mast cells (Table 2). Age group also had no effect on these parameters when the analysis was performed with the healthy horses only.

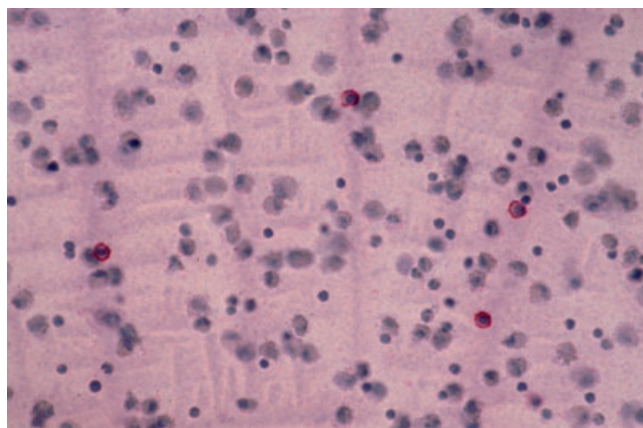


Fig. 1. Cytospin from bronchoalveolar lavage fluid stained with IgE-specific antibody developed with fast red and counterstained with Hemalum, showing few IgE-bearing cells (red).

Immunocytochemistry for IgE

Staining with the horse IgE-specific chicken Ab revealed few IgE-positive (+) cells (Fig. 1) in the cytopins from all horses, while no staining was observed with the preimmune chicken Ab (data not shown). There were significantly fewer IgE+ cells in BALF from RAO-affected horses than in controls (3.2% versus 6.8%, $P < 0.05$). However, after correcting for the number of neutrophils, there were no significant differences between both groups of horses anymore (5% versus 7%, $P > 0.1$, Table 2). Age group also had no effect on the median percentage of IgE+ cells irrespective of whether the RAO-affected animals were included in the statistical analysis or not.

Allergen-specific serum IgE levels

Determination of serum IgE levels against the crude mould extracts *A. alternata* and *A. fumigatus* showed that RAO-affected horses had significantly higher IgE levels against both mould extracts than control horses (Table 2 and Fig. 2). There were also significantly more RAO-affected horses (4/7 horses, 57%) with detectable serum IgE against rAsp f 8 than controls (3/22 horses, 13.6%). On the other hand, age group (with and without the RAO-affected horses) exerted no significant effect on IgE levels against these crude mould extracts or against rAsp f 8 (Table 2).

Discussion

The aim of the present study was to investigate whether increased levels of serum and cell-bound IgE is associated with RAO in a group of horses living in the same environment, as previous studies had shown that the stable-specific environment exerts a significant effect on antigen-specific IgE levels. We looked for IgE at two different levels: (i) in BALF, where IgE-bearing cells were stained and (ii) in serum, where antigen-specific IgE levels were measured. A preliminary study (data not shown) had revealed that detection of non-cell-bound IgE in BALF by ELISA is not possible with our anti-IgE Ab (Marti et al., 1997), probably because BALF is too dilute. Even after concentrating BALF with various techniques, IgE levels in BALF could not be measured by ELISA. This is probably because of the fact that concentrating of BALF or serum using these techniques lead to denaturation of the epitope(s) recognized by the chicken anti-horse IgE Ab, as pilot experiments had revealed (data not shown). We know from previous studies that the epitope(s) recognized by the chicken Ab are very sensitive to denaturation through heat treatment, freeze/thaw cycles, etc. (Muntwyler, 1997).

In humans with allergic rhinitis, a well-documented IgE-mediated allergic condition, immunocytochemistry has been performed in nasal brush and lavage samples. It was shown that allergen provocation, after an initial decrease of IgE-bearing cells and tryptase-positive mast cells leads to increased numbers of these cells (Juliussen et al., 1994). This study showed also that after 8–12 h, the morphology and total number of metachromatic cells outnumbered tryptase-containing cells, suggesting that these cells were mainly basophils and not mast cells. We thus hypothesized that staining of IgE protein-positive cells in BALF could indicate whether numbers of mast cells and basophils, which bind IgE through the high-affinity IgE receptor, would occur in increased numbers in

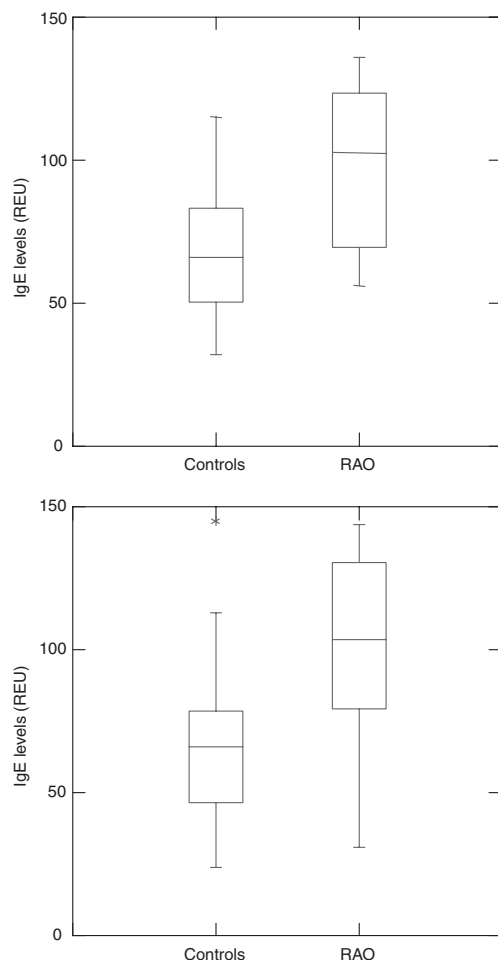


Fig. 2. IgE antibody titres in relative ELISA units (REU) against *Alternaria alternata* and *Aspergillus fumigatus* extracts in sera from eight horses with recurrent airway obstruction (RAO) and 23 healthy control horses, all living in the same environment. RAO-affected horses had significantly higher IgE levels ($P < 0.05$) than the control horses. The centre horizontal line of the box plots marks the median of the sample. The edges of the box mark the first and third quartiles. Stars indicate outside values and circles far outside values.

RAO-affected than in control horses. IgE protein-positive cells were stained specifically (no staining with preimmune chicken IgG) both in BALF from RAO-affected and control horses (Fig. 1). Only a small proportion of the BALF cells stained for IgE. This is not unexpected, as mast cells, basophils, IgE-producing plasma cells and probably also some dendritic cells are the main cell population which can bind IgE and these cells are present in low numbers in BALF. We assume that, similarly to what is known in humans (Guo et al., 1994), most of the IgE+ cells were mast cells or basophils. Double staining for IgE and the mast cell enzymes tryptase/chymase or a metachromatic stain would have allowed to investigate whether these IgE+ cells were mast cells and/or basophils. However, although these stainings were successfully performed on skin and lung tissue samples (Van der Haegen et al., 2001, 2005) they could not be used on the cytopins because of technical problems (background staining). On the cytopins, the percentage of IgE+ cells ranged from 1% to 18%. RAO horses had a significantly lower percentage of IgE+ cells than controls, but this may be explained by the high numbers of

neutrophils found in BALF of RAO horses. After correcting for neutrophil numbers, there were no significant differences between both groups of horses for percentages of IgE+ cells. This may be due to different reasons: horses with RAO may not have more mast cells, basophils and other IgE-bearing cells in their BALF than healthy control animals; this would indicate that IgE-mediated reactions are not major immunological mechanisms in the pathogenesis of RAO. This would confirm some of the studies on RAO that could not demonstrated a clear Th2-type immune response in RAO (Giguere et al., 2002; Ainsworth et al., 2003; Kleiber et al., 2005), contrarily to some other studies (Lavoie et al., 2001; Cordeau et al., 2004). Alternatively, IgE+ cells numbers in BALF may need to be compared at different times before and after provocation/elimination with mouldy hay, as an increase or decrease of IgE+ cells in BALF could be transient. This hypothesis is now supported by a recent study by Horohov et al. (2005) who showed that RAO-affected horses exhibit varying amounts of Th1 and Th2-type cytokine production in function of time. Further studies are also needed to find out which cells are the main IgE-bearing cell populations in BALF in the horse.

Interestingly, while no significant differences were found between RAO-affected and control horses for IgE+ cells in BALF, serum IgE levels against the mould extracts *A. fumigatus* and *A. alternata* were significantly higher in horses with RAO than in controls. These results are in contrast to previous findings by Eder et al. (2000), in which no significant differences were found for IgE levels against mould extracts between RAO-affected and control horses. The discrepancies between the present and the previous study can be explained by the fact that in the study by Eder et al. (2000) most of the horses were from various stables. Therefore, the environmental factors differed between horses, while the horses in the present study had all been living under the same environmental conditions for at least 1 year. An investigation with 450 Lipizzan horses from six different studs (Eder et al., 2001) had demonstrated that the stud – i.e. stable-specific environment exerts a highly significant effect on allergen-specific serum IgE levels. Nevertheless, even within the same environment, serum IgE levels against mould extracts can hardly be used for a serological diagnosis of RAO as there is still a large overlap between both groups of horses (Fig. 2). IgE antibodies against the recombinant mould allergen rAsp f 8 were also determined in the present study: 57% of the RAO-affected horses but only 13.5% of the control horses had detectable IgE against rAsp f 8 ($P < 0.05$, Table 2). These results confirm those of Eder et al. (2000) who also found significantly more often detectable IgE against this recombinant mould allergen in RAO-affected animal compared with control horses. In humans, a part of the patients with allergic bronchopulmonary aspergillosis (ABPA), but no healthy individuals nor patients sensitized to *A. fumigatus* but without ABPA, have serum IgE against rAsp f 8 (Cramer, 1999). This recombinant *A. fumigatus* allergen is a ribosomal P2 protein and thus a cytoplasmic protein. In humans, interestingly, the presence of auto-immune reactions against phylogenetically highly conserved intracellular proteins such as the ribosomal P2 protein have been described recently in severe atopic diseases (Appenzeller et al., 1999; Mayer et al., 1999). Patients with severe ABPA had high serum IgE levels and positive skin tests both to *A. fumigatus* ribosomal protein P2 and to its

human counterpart (Mayer et al., 1999). As these cytoplasmic proteins are unlikely to be accessible for antigen–antibody interactions under normal circumstances, this leads to the hypothesis that IgE autoreactivity may be a consequence of tissue damage and release of autoantigens at the site of inflammation. This finding also deserves further studies in the horse: cloning and expression of equine ribosomal proteins would be needed to determine whether autoreactivity can also be demonstrated in severe chronic cases of RAO.

We speculated that age exerts a significant effect on allergen-specific IgE, as older horses have been exposed for a longer time to environmental allergens. Our study, however, shows that in mature horses age does not exert a significant effect on allergen-specific serum IgE levels, as no significant differences were found between younger and older horses either with the mould extracts nor with the recombinant *A. fumigatus* protein.

In conclusion, serological investigations in horses living in the same environment show that high mould-specific serum IgE is associated with RAO. Furthermore, RAO-affected horses show significantly more often than controls IgE-binding to a ribosomal P2 protein from *A. fumigatus*, suggesting that similar to humans, IgE autoreactivity may occur in affected horses. This study also shows that in mature horses, age does not exert a significant effect on allergen-specific serum IgE levels. The present investigation also demonstrates that IgE+ cells can be detected in BALF, but further studies such as time-course studies under controlled allergen provocation are needed to investigate whether IgE-mediated reactions are causally involved in the pathogenesis of RAO.

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References

- Ainsworth, D. M., G. Grünig, M. B. Matychak, J. Young, B. Wagner, H. N. Erb and D. F. Antczak, 2003: Recurrent airway obstruction (RAO) in horses is characterized by IFN- γ and IL-8 production in bronchoalveolar lavage cells. *Vet. Immunol. Immunopathol.* **96**, 83–91.
- Appenzeller, U., C. Meyer, G. Menz, K. Blaser and R. Cramer, 1999: IgE-mediated reactions to autoantigens in allergic diseases. *Int. Arch. Allergy Immunol.* **118**, 193–196.
- Asmundsson, T., E. Gunnarsson and T. Johannesson, 1983: 'Hay sickness' in Icelandic horses: precipitin tests and other studies. *Equine Vet. J.* **15**, 229–232.
- Berger, P., A. F. Walls, R. Marthan and J. M. Tunon-de-Lara, 1998: Immunoglobulin E-induced passive sensitization of human airways: an immunohistochemical study. *Am. J. Respir. Crit. Care Med.* **157**, 610–616.
- Cordeau, M. E., P. Joubert, O. Dewachi, Q. Hamid and J. P. Lavoie, 2004: IL-4, IL-5 and IFN- γ mRNA expression in pulmonary lymphocytes in equine heaves. *Vet. Immunol. Immunopathol.* **97**, 87–96.
- Cramer, R., 1999: Epidemiology and molecular basis of the involvement of *Aspergillus fumigatus* in allergic diseases. In: Brakhage, A. A., B. Jahn, A. Schmidt (eds), *Aspergillus fumigatus*, Vol 2, Contributions to Microbiology, pp. 44–56. Basel, Karger.
- Derksen, F. J., N. E. Robinson, J. S. Scott and J. A. Stick, 1988: Aerosolized *Micropolyspora faeni* antigen as a cause of pulmonary dysfunction in ponies with recurrent airway obstruction (heaves). *Am. J. Vet. Res.* **49**, 933–938.
- Dirschel, P., A. Grabner and H. Buschmann, 1993: Responsiveness of basophil granulocytes of horses suffering from chronic obstructive pulmonary disease to various allergens. *Vet. Immunol. Immunopathol.* **38**, 217–227.
- Dixon, P. M., D. I. Railton and B. C. McGorum, 1995: Equine pulmonary disease: a case control study of 300 referred cases. Part I: Examination techniques, diagnostic criteria and diagnoses. *Equine Vet. J.* **27**, 416–421.
- Eder, C., R. Cramer, C. Mayer, R. Eicher, R. Straub, H. Gerber, S. Lazary and E. Marti, 2000: Allergen-specific IgE levels against crude mould and storage mite extracts and recombinant mould allergens in sera from horses affected with chronic bronchitis. *Vet. Immunol. Immunopathol.* **73**, 241–253.
- Eder, C., I. Curik, G. Brem, R. Cramer, I. Bodo, F. Habe, S. Lazary, J. Sölkner and E. Marti, 2001: Influence of environmental and genetic factors on allergen-specific immunoglobulin E levels in sera from Lipizzan horses. *Equine Vet. J.* **33**, 714–720.
- Evans, A., M. R. Paradis and M. O'Callaghan, 1992: Intradermal testing of horses with chronic obstructive pulmonary disease and recurrent urticaria. *Am. J. Vet. Res.* **53**, 203–208.
- Eyre, P., 1972: Equine pulmonary emphysema: a bronchopulmonary mould allergy. *Vet. Rec.* **91**, 134–140.
- Gerber, H., P. Hockenjos, S. Lazary, M. Kings and A. de Weck, 1982: Histamine release from equine leucocytes provoked by fungal allergens. *Dtsch. Tierärztl. Wochenschr.* **6/89**, 267–270.
- Giguere, S., L. Viel, E. Lee, R. J. MacKay, J. Hernandez and M. Franchini, 2002: Cytokine induction in pulmonary airways of horses with heaves and effect of therapy with inhaled fluticasone propionate. *Vet. Immunol. Immunopathol.* **85**, 147–158.
- Guo, C. B., M. C. Liu, S. J. Galli, B. S. Bochner, A. Kagey-Sobotka and L. M. Lichtenstein, 1994: Identification of IgE-bearing cells in the late-phase response to antigen in the lung as basophils. *Am. J. Respir. Cell Mol. Biol.* **10**, 384–390.
- Halliwel, R. E. W., J. Fleischmann, M. Mackay-Smith, J. Beech and D. Gunson, 1979: The role of allergy in chronic pulmonary disease of horses. *J. Am. Vet. Med. Assoc.* **174**, 277–281.
- Halliwel, R. E. W., P. C. McGorum, P. Irving and P. M. Dixon, 1993: Local and systemic antibody production in horses affected with chronic pulmonary disease. *Vet. Immunol. Immunopathol.* **38**, 201–215.
- Hare, J. E., L. Viel, P. D. Conlon and J. S. Marshall, 1999: *In vitro* allergen-induced degranulation of pulmonary mast cells from horses with recurrent airway obstruction (heaves). *Am. J. Vet. Res.* **60**, 841–846.
- Horohov, D. W., R. E. Beadle, S. Mouch and S. S. Pourciau, 2005: Temporal regulation of cytokine mRNA expression in equine recurrent airway obstruction. *Vet. Immunol. Immunopathol.* **108**, 237–245.
- Juliussen, S., G. Karlsson, C. Bachert and L. Enerback, 1994: Metachromatic, IgE-bearing and tryptase-containing cells on the nasal mucosal surface in provoked allergic rhinitis. *APMIS* **102**, 153–160.
- Kistler, P. and Nitschmann, H. S., 1962: Large-scale production of human plasma fractions: Eight years experience with the alcohol fractionation procedure of Nitschmann, Kistler and Lergier. *Vox Sang* **7**, 414–422.
- Kleiber, C., 1996: Phänotypisierung von T-Lymphozyten aus bronchioalveolärer Lavageflüssigkeit von chronisch lungenkranken und lungengesunden Pferden. Inaugural Dissertation, Faculty of Veterinary Medicine, University of Berne, Switzerland.
- Kleiber, C., B. C. McGorum, D. W. Horohov, R. S. Pirie, A. Zurbiggen and R. Straub, 2005: Cytokine profiles of peripheral blood and airway CD4 and CD8 T lymphocytes in horses with recurrent airway obstruction. *Vet. Immunol. Immunopathol.* **104**, 91–97.

- Lavoie, J. P., K. Maghni, M. Desnyers, R. Taha, J. G. Martin and Q. A. Hamid, 2001: Neutrophilic airway inflammation in horses with heaves is characterized by a Th2-type cytokine profile. *Am. J. Respir. Crit. Care Med* **164**, 1410–1413.
- Lawson, G. H. K., E. A. McPherson, P. Wooding, R. G. Breeze and H. M. Pirie, 1979: The presence of precipitating antibodies in the sera of horses with chronic obstructive pulmonary disease (COPD). *Equine Vet. J.* **11**, 172–176.
- Léguillette, R., 2003: Recurrent airway obstruction – heaves. *Vet. Clin. North Am. Equine Pract.* **19**, 63–86.
- Lorch, G., A. Hillier, K. W. Kwochka, W. J. Saville, C. W. Kohn and E. Jose-Cunilleras, 2001: Results of intradermal tests in horses without atopy and horses with chronic obstructive pulmonary disease. *Am. J. Vet. Res.* **62**, 389–397.
- Lukacs, N. W., C. M. Hogaboam, S. L. Kunkel, S. W. Chensue, M. D. Burdick, H. L. Evanoff and R. M. Strieter, 1998: Mast cells produce ENA-78, which can function as a potent neutrophil chemoattractant during allergic airway inflammation. *J. Leukoc. Biol.* **63**, 746–751.
- Marti, E., H. Gerber, G. Essich, J. Ouhlela and S. Lazary, 1991: On the genetic basis of equine allergic diseases. I. Chronic hypersensitivity bronchitis. *Equine Vet. J.* **23**, 457–460.
- Marti, E., P. Peveri, M. Griot-Wenk, J. Muntwyler, R. Crameri, J. Schaller, H. Gerber and S. Lazary, 1997: Chicken antibodies to a recombinant fragment of the equine immunoglobulin epsilon heavy-chain recognising native horse IgE. *Vet. Immunol. Immunopathol.* **59**, 253–270.
- Maurer, D., E. Fiebriger, C. Ebner, B. Reininger, G. F. Fisher, S. Wichlas, M.-H. Jouvin, M. Schmitt-Egenolf, D. Kraft, J.-P. Kinet and G. Stingl, 1996: Peripheral blood dendritic cells express Fcε RI as a complex composed of Fcε RIα- and Fcε RIγ-chains and can use this receptor for IgE-mediated allergen presentation. *J. Immunol.* **157**, 607–616.
- Mayer, C., U. Appenzeller, H. Seelbach, G. Achatz, H. Oberholzer, M. Breitenbach, K. Blaser and R. Crameri, 1999: Humoral and cell-mediated autoimmune reactions to human acidic ribosomal P2 protein in individuals sensitized to *Aspergillus fumigatus* P2 protein. *J. Exp. Med.* **189**, 1507–1512.
- McGorum, B. C., P. M. Dixon and R. E. Halliwell, 1993a: Response of horses affected with chronic obstructive pulmonary disease to inhalation challenge with mould antigens. *Equine Vet. J.* **25**, 261–267.
- McGorum, B. C., P. M. Dixon and R. E. Halliwell, 1993b: Quantification of histamine in plasma and pulmonary fluids from horses with chronic obstructive pulmonary disease, before and after 'natural (hay and straw) challenges'. *Vet. Immunol. Immunopathol.* **36**, 223–237.
- McGorum, B. C., P. M. Dixon and R. E. W. Halliwell, 1993c: Evaluation of intradermal mould antigen testing in the diagnosis of equine chronic obstructive pulmonary disease. *Equine Vet. J.* **25**, 273–275.
- McPherson, E. A., G. H. Lawson, J. R. Murphy, J. M. Nicholson, J. A. Fraser, R. G. Breeze and H. M. Pirie, 1978: Chronic obstructive pulmonary disease (COPD): identification of affected horses. *Equine Vet. J.* **10**, 47–53.
- Muntwyler, J., 1997: Specific antibodies against a recombinant equine IgE heavy chain fragment recognizing native horse serum IgE. Thesis, Faculty of Veterinary Medicine, University of Berne.
- Pirie, R. S., D. D. Collie, P. M. Dixon and B. C. McGorum, 2002: Evaluation of nebulised hay dust suspensions (HDS) for the diagnosis and investigation of heaves. 2: Effects of inhaled HDS on control and heaves horses. *Equine Vet. J.* **34**, 337–342.
- Pirie, R. S., P. M. Dixon and B. C. McGorum, 2003: Endotoxin contamination contributes to the pulmonary inflammatory and functional response to *Aspergillus fumigatus* extract inhalation in heaves horses. *Clin. Exp. Allergy* **33**, 1289–1296.
- Robinson, N. E., 2001: International Workshop on Equine Chronic Airway Disease, Michigan State University, 16–18 June 2000. *Equine Vet. J.* **33**, 5–19.
- Robinson, N. E., F. J. Derksen, M. A. Olszewski and V. A. Buechner-Maxwell, 1996: The pathogenesis of chronic obstructive pulmonary disease of horses. *Br. Vet. J.* **152**, 283–306.
- Schatzmann, U. and H. Gerber, 1972: Untersuchungen zur Aetiologie chronischer Lungenkrankheiten des Pferdes. *Zbl. Vet. Med. A.* **19**, 89–101.
- Schatzmann, U., R. Straub, H. Gerber, S. Lazary, U. Meister and H. Spörri, 1974: Die elimination von Heu und Stroh als Therapie chronischer Lungenkrankungen des Pferdes. *Tierärztl. Prax* **2**, 207–214.
- Schmallenbach, K. H., I. Rahman, H. H. L. Sasse, P. M. Dixon, R. E. W. Halliwell, B. B. McGorum, R. Crameri and H. R. P. Miller, 1998: Studies on pulmonary and systemic *Aspergillus fumigatus* -specific IgE and IgG antibodies in horses affected with chronic obstructive pulmonary disease (COPD). *Vet. Immunol. Immunopathol.* **66**, 245–256.
- Thomson, J. R. and E. A. McPherson, 1984: Effects of environmental control on pulmonary function of horses affected with chronic obstructive pulmonary disease. *Equine Vet. J.* **16**, 35–38.
- Van der Haegen, A., M. E. Griot-Wenk, M. Welle, A. Busato, C. von Tschanner, A. Zurbriggen and E. Marti, 2001: Immunoglobulin E bearing cells in skin biopsies of horses with insect bite hypersensitivity. *Equine Vet. J.* **33**, 699–706.
- Van der Haegen, A., F. Künzle, V. Gerber, A. Busato, N. E. Robinson, M. Welle and E. Marti, 2005: Mast cells and IgE-bearing cells in lungs of RAO-affected horses. *Vet. Immunol. Immunopathol.* **108**, 325–334.