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Studies on pulmonary and systemic *Aspergillus fumigatus*-specific IgE and IgG antibodies in horses affected with chronic obstructive pulmonary disease (COPD)

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Abstract

Inhalant exposure to *Aspergillus fumigatus* (*Asp. f.*) antigens induces marked inflammatory and immunological alterations in the lungs of horses affected with chronic obstructive pulmonary disease (COPD). In this study we investigated the role of specific allergen(s) present in *Asp. f.* on systemic and pulmonary IgE and IgG responses in control and COPD-affected horses, using an enzyme-linked immunosorbent assay (ELISA) and immunoblotting techniques. Compared with controls, horses affected with COPD had significantly higher levels of BALF IgE and IgG to somatic *Asp. f.* antigens as well as to the allergen l/a (*Asp. f.* l/a). Serum levels of IgE and IgG against these antigens did not differ between control and COPD-affected horses. Antigen specific IgE and IgG levels did not correlate between BALF and serum. Scanning of *Asp. f.* and IgE and IgG

Abbreviations: IgE, Immunoglobulin E; IgG, Immunoglubulin G; COPD, Chronic Obstructive Pulmonary Disease; *Asp. f., Aspergillus fumigatus*; r *Asp. f.* l/a, recombinant *Aspergillus fumigatus* l/a; ELISA, Enzyme Linked Immunosorbent Assay; ABPA, Allergic bronchopulmonary aspergillosis; BALF, Bronchoalveolar lavage fluid; SDS-PAGE, Sodium dodecyl sulphate-polyacrylamide gel electrophoresis; RAU, Relative antibody unit

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blots revealed bands that are recognised by both IgE- and IgG-specific antibodies. Additionally, all horses responded with BALF IgE- and IgG-specific for 93, 35, 31 and 23 kDa allergens, suggesting that these antigens are involved in the induction of airway IgE and IgG responses. These allergens may have the potential to be used as biomarkers for the diagnosis of *Asp. f.* related exacerbations of equine COPD. © 1998 Published by Elsevier Science B.V. All rights reserved.

Keywords: *Aspergillus fumigatus*; IgE; IgG; COPD; Horse

1. Introduction

Aspergillus fumigatus (*Asp. f.*) causes a wide spectrum of human lung diseases including allergic bronchopulmonary aspergillosis (ABPA) in patients with pre-existing allergic asthma (Longbottom, 1983), invasive aspergillosis (Trompelt et al., 1994; Wallenbeck et al., 1991) and hypersensitivity reactions in patients with pre-existing cystic fibrosis (Nikolaizik et al., 1995). Measurement of antibodies, such as IgE and IgG levels, is an important tool for the diagnosis of many immunological diseases and measurement of serum levels of IgE and IgG antibodies specific for *Asp. f.* is used in the diagnosis of ABPA in humans (Longbottom, 1986; Wallenbeck et al., 1991).

Chronic obstructive pulmonary disease (COPD) is the most common chronic equine respiratory disease in the Northern hemisphere where horses are stabled for long periods and are exposed to mouldy hay and straw (Dixon et al., 1995; Robinson et al., 1996). Susceptible horses can develop a pulmonary hypersensitivity to fungal antigens in hay or straw which is accompanied by inflammatory responses leading to airway obstruction. However, the precise pathogenesis of equine COPD remains speculative. In horses affected with COPD exposure to hay and straw caused pulmonary inflammation characterised by increased ratios of neutrophils, lymphocytes, particularly CD4+ T-helper cells, and raised histamine levels in bronchoalveolar lavage fluid (BALF) (McGorum et al., 1993b; McGorum et al., 1993d, d). Alteration of lung function such as minute ventilation, work of breathing and hypoxaemia have also been described (McGorum et al., 1993c).

Halliwell et al. (1993) showed increased levels of BALF IgE to *Asp. f.* in COPD-affected horses compared to control horses. However, no studies have defined a serodiagnostic marker for the putative allergen(s) of *Asp. f.* in horses affected with COPD. We hypothesised that the allergens present in *Asp. f.* preferentially stimulated IgE and IgG responses which then can be detected systemically and locally in COPD affected horses. To further understand the possible roles of specific allergen(s) present in *Asp. f.* we used a single recombinant *Asp. f.* allergen, r *Asp. f.* l/a (Moser et al., 1992) as well as soluble somatic *Asp. f.* antigens, to compare the specific IgE and IgG responses in BALF and serum in control and COPD-affected horses, using a modification of the enzyme linked immunosorbent assay (ELISA) technique used by Halliwell et al. (1993). Additionally, the range of allergens and antigens from *Asp. f.* that induce IgE and IgG responses in BALF were determined by Western Blotting.

2. Materials and methods

2.1. Subjects

16 COPD affected (6 geldings; 9 females; median age 9.6 years; range 5–24 years) and three control (1 gelding; 2 females; median age 4.5 years, range 4–19) horses of mixed breeds were used.

2.2. COPD affected horses

These horses had clinical histories of more than 2 years duration and had been diagnosed as suffering from COPD using previously described criteria (McGorum et al., 1993c). All COPD affected horses showed clinical disease within 5 hours of 'natural challenge' (exposure to hay and straw in a poorly ventilated stable), including some of the following clinical signs: coughing, hyperpnoea, double expiratory lift ('heave'), bilateral mucopurulent nasal discharge and increased or abnormal tracheal and lung sounds. Bronchoscopic examination of horses with symptomatic COPD consistently revealed abnormal accumulations of mucopurulent secretions, containing increased ratios (usually >90%) of neutrophils, in the trachea, with hyperaemia and swelling of the respiratory mucosa. Bronchoalveolar lavage fluid (BALF) cytology always comprised >5% neutrophils (McGorum, 1992). Many horses had increased intrathoracic pressure changes as assessed by an oesophageal balloon catheter (McPherson et al., 1978) and most had a PaO2 of <85 mm Hg.

All animals showed complete remission of clinical and laboratory signs of pulmonary disease at a follow up examination 1–2 months after removal from the 'natural challenge' environment to a hay and straw free environment i.e. fed silage or grass and bedded on a non-straw bedding ('controlled environment'). Horses tended to have their own individual time periods of remission (usually between 2 and 6 weeks) with the BALF cytological and bronchoscopic changes being the last parameter to revert to normal. All COPD affected horses had received multiple alterations of natural challenge and 'controlled environments' and had responded consistently as described above (McGorum, 1992).

2.3. Control horses

These horses had shown no evidence of respiratory disease for 2 years and were found to be normal on clinical, bronchoscopic, arterial blood gas tensions and pH, pulmonary mechanics and BALF cytology examinations before and after a 'natural challenge'. In all control horses, neutrophils consistently accounted for less than 5% of BALF cells.

2.4. Sample collection

BALF collection and cytological examination was carried out as described by McGorum (1992). Immediately before the horses were sedated for bronchoscopy and BALF collection, peripheral blood was collected into glass vacutainers (Becton

Dickinson, Rutherford, USA) and gently agitated to ensure thorough mixing of the anticoagulant.

2.5. Antisera

A rabbit antiserum-specific for equine IgE was used (Halliwell et al., 1993) which was cross-absorbed against somatic *Asp. f.* antigens coupled to Sepharose 4B (Pharmacia Biotech, St. Albans Herts, UK). Goat-α-rabbit IgG conjugated to alkaline phosphatase (Sigma, Poole, UK) was used to detect the rabbit-α-equine IgE. Affinity purified goat α-equine Fcγ-specific antibody (Jackson Immuno Research Laboratories West Grove, USA) was employed to detect equine IgG-specific for *Asp. f.* antigens in BALF and serum, and was followed by rabbit-α-goat IgG (H- + L-chain) alkaline phosphatase conjugate (Caltag Laboratories, South San Francisco, USA).

2.6. Enzyme linked immunosorbent assay (ELISA)

An indirect ELISA described by Halliwell et al. (1993), modified as follows, was used: 5 μg/ml solution of a somatic *Asp. f.* extract (MRC Immunology Laboratories, Penarth, UK) or 1 μg/ml of a r *Asp. f.* l/a (Swiss Institute of Allergy and Asthma Research, Davos, Switzerland) was loaded into each well of a microtitre plate (Immulon 4, Dynatech, Billingshurst, UK). Fifty microlitres of serum-dilutions or BALF-dilutions were added, as well as the standard sample, in duplicate, to the ELISA plate. The plates were then incubated at 37°C. Plates were washed and rabbit-α-equine IgE solution (1/50 dilution) or goat-α-equine IgG solution (1/2000 dilution) were added and the plates again incubated at 37°C. Plates were then washed, dried and incubated with 1/20 000 solution of goat-α-rabbit IgG, or a 1/2500 solution of α-goat IgG, both conjugated to alkaline phosphatase. Alkaline phosphatase substrate buffer was added to each well and the plates were left to develop in the dark. The resultant colour change (optical density) was measured at a wavelength of 405 nm using a programmable automated ELISA reader (MR 7500, Dynatech, Billingshurst, UK). The method of quantifying antibody levels is based on integration of the area under the antibody dilution curve, which is obtained by measuring the amount of antibody in each of eight dilutions of the test sample and a standard sample, that had been identified previously as containing a high level of allergen-specific antibody.

2.7. BALF and plasma urea determination for the standardisation of BALF concentrations

A commercial enzymatic urea nitrogen assay kit (Sigma, Poole, UK) was used, employing a modification of the technique described by Rennard et al. (1986). Serum urea concentrations were determined using a Technicon RA-XT™ (Technicon Method No. SM4-0150D91, Technicon, Dublin, Eire). Urea levels in BALF and serum were utilised to correct for dilution of BALF for individual animals described by McGorum et al. (1993a).

2.8. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Gels were cast and run using the BioRad Mini-Protean system (82 × 73 × 0.75 mm) (Bio-Rad Laboratory, Hemel Hempstead, UK), with a 10% (w/v) acrylamide concentration in the resolving gel and a 4% stacking gel.

Somatic *Asp. f.* was loaded in a 0.5 M Tris-HCl (pH 6.8), 10% SDS (BDH Lab Supplies, Merck, Lutterworth, UK), with 800 µl of glycerol (BDH Chemicals, Poole, UK), 400 µl ME (Sigma, Poole, UK) and 0.05% (w/v) bromphenol blue (BDH Chemicals, Poole, UK). Rainbow coloured protein molecular weight markers (range 220–21.5 kDa) (Amersham, Little Chalfont, UK) were included in each run. Electrophoresis was carried out at a constant 200 V (LKB Bromma, 2197 Power Supply) for 60 min. or until the dye-front had migrated to the bottom of the gel.

2.9. Western blot and detection of antibody binding

After separation of the *Asp. f.* proteins, the proteins were electrophoretically transferred on to Immobilon P (Millipore, Bedford, MA, USA) by semi-dry blot technique. The detection of IgE and IgG bindings with *Asp. f.* antigens on Immobilon P was carried out as follows: the Immobilon P blots of *Aspergillus* proteins were sliced and blocked with blocking buffer, tris-buffered-saline (TBS) containing 5% dried milk powder (Marvel; Nisa Today's, UK), followed by washing in TBS containing 0.05% Tween 20 (TTBS) (Sigma, Poole, UK). The sliced strips were incubated with dilutions of either BALF or serum. TBS buffer was used as a negative control. To detect the binding of IgE, the strips were incubated with rabbit-α-equine IgE (1 : 250 dilution) or for the detection of IgG with goat-α-equine Fc gamma (1 : 2000 dilution) followed by washing with TTBS. The strips were then incubated with goat-α-rabbit IgG conjugate (1 : 8000 dilution) or α-goat IgG conjugate (H- + L-chain) (1 : 5000 dilution) and washed with TTBS before adding substrate to visualise antigen-antibody complexes.

2.10. Densitometric and visual analysis of antigen-antibody complexes (bands)

Photographs of the immunoblots were scanned using Blackwidow Scanpro 4800 SP Laser densitometer (Devcom International, Scotland). The bands were recorded and analysed with the computer program Scan Analysis (Biosoft, Cambridge, England). To correct the density of the bands against the background, the background density of each immunoblot was recorded and substracted. Identification of each band, and comparison of bands on different plots were then performed by superimposing the plots (curves) and comparing the data (location of the bands). The location of the most frequent bands on each blot was taken as an average location value. The locations of the bands were converted into molecular weights (kDa), by using a calibration curve of the molecular weight markers, that were run on each SDS-PAGE.

To obtain a linear calibration curve, the molecular weights (220, 97.4, 66, 46, 30 and 21.5 kDa) of the marker were converted into $\log_{10}$ and plotted against the densitometer location of the markers. As the locations of the bands were known (antigen-antibody complexes), the $\log_{10}$ of the molecular weights was read from the plot and converted into natural numbers (molecular weights).

The strength of the bands was arbitrarily classified according to the net area of peaks, obtained by the densitometer:

Net area	Intensity of bands
≤1.0%	x
1.1%–10.0%	xx
10.1%–20.0%	xxx
20.1%–30.0%	xxxx
>30%	xxxxx

2.11. Statistical analysis

Differences in urea adjusted relative antibody unit (RAU) values of COPD-affected and control horses were compared for significance using the Mann-Whiney U-test. P values <0.05 were considered significant. The correlation between antibody-levels (IgE and IgG), specific to somatic and recombinant *Asp. f.*, between BALF and serum, were assessed by regression analysis, P values <0.05 were considered to show a correlation.

3. Results

3.1. Levels of Asp. f.-specific IgE and IgG in bronchoalveolar lavage fluid (BALF) and serum

IgE-levels (measured as Urea-adjusted RAUs) against somatic *Asp. f.* ($P = 0.02$) and r *Asp. f.* l/a ($P = 0.04$) (Fig. 1) in BALF from COPD horses were significantly increased

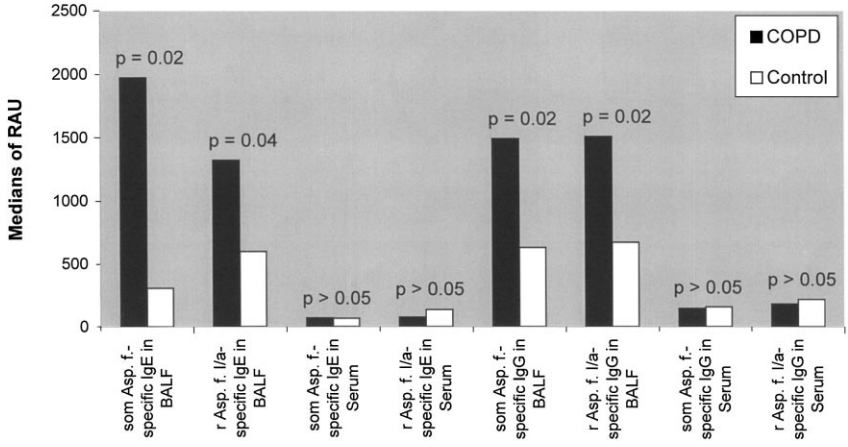

Fig. 1. Relative antibody units (RAU) of IgE- and IgG, specific for somatic *Asp. f.* (som *Asp. f.*) and recombinant *Asp. f.* I/a (r *Asp. f.* I/a) in BALF and in serum of COPD-affected and control horses were measured by ELISA. The results (median) of COPD-affected and control horses were compared.

when compared with control values. However, specific serum IgE levels in COPD affected and control horses were not significantly different ($P \geq 0.4$). The levels of IgG-specific for *Asp. f.* and r *Asp. f.* l/a in BALF from COPD horses were significantly greater than in controls ($P = 0.02$), however serum IgG levels were not significantly different ($P \geq 0.1$).

3.2. Comparison/Dissociation of the Asp. f.-specific IgE- and IgG-responses between BALF and serum

The levels of IgE-specific for somatic *Asp. f* in BALF and serum were not significantly correlated ($P = 0.7$), nor was there any correlation for r *Asp. f.* l/a ($P = 0.8$). Furthermore, no significant correlation was found between IgG levels in BALF ($P = 0.6$) and serum ($P = 1$), either for somatic or for r *Asp. f.* l/a. These results show that the antigen-specific IgE and IgG levels in the lung are independent of the systemic IgE and IgG levels, for somatic as well as for *Asp. f.* l/a allergen.

3.3. Relationship between IgE-levels and IgG-levels specific for Asp. f. in BALF and serum

In order to more closely determine the relationship between local pulmonary IgE and IgG responses to *Asp. f.*, the correlation between specific IgE and IgG levels in BALF was analysed and was found to be significantly correlated for somatic *Asp. f.*-specific IgE and IgG levels ($P = 0.002$) and for *Asp. f.* l/a-specific IgE and IgG levels ($P < 0.001$). In contrast, no significant correlation was found between IgE- and IgG-levels, specific for somatic *Asp. f.* in serum ($P = 0.1$) whereas IgE- and IgG-levels specific for r *Asp. f.* l/a in serum were highly correlated ($P < 0.001$).

3.4. Allergen(s) of a somatic Asp. f. extract that induce IgE and/or IgG response in the lung

Asp. f. antigens leading to a specific IgE and IgG response in the lung have been examined by immunoblotting. The MW of the antigen-antibody complexes were determined with the help of laser beam densitometry. In all samples multiple bands were identified (Fig. 2a, b). Fig. 2a shows 4 bands (*Asp. f.* Antigen-IgE-α-IgE-complexes) of very variable intensity but common to all horses. Their approximate MW are 93, 35, 31 and 23 kDa. Fig. 2b shows 5 bands (*Asp. f.* Antigen-IgG-α-IgG-complexes) also of varying intensity but again common to all horses, which have an approximate MW of 93, 45, 35, 31 and 23 kDa.

In order to compare the *Asp. f.* specific IgE and *Asp. f.* IgG more closely with each other and in order to find individual bands that are not uniquely detected by α-IgE antibody or by α-IgG antibody, the samples were blotted immediately next to each other for each of 16 horses. As the results from Fig. 2a and 2b suggest, the majority of immunreactive complexes were detected by α-IgE and at the same time by α-IgG as shown in Fig. 3a and b. However, several distinctive IgE-specific as well as IgG-specific antibody isotypes are recognised in all horses. Two representative blots can be seen in Fig. 3a and b.

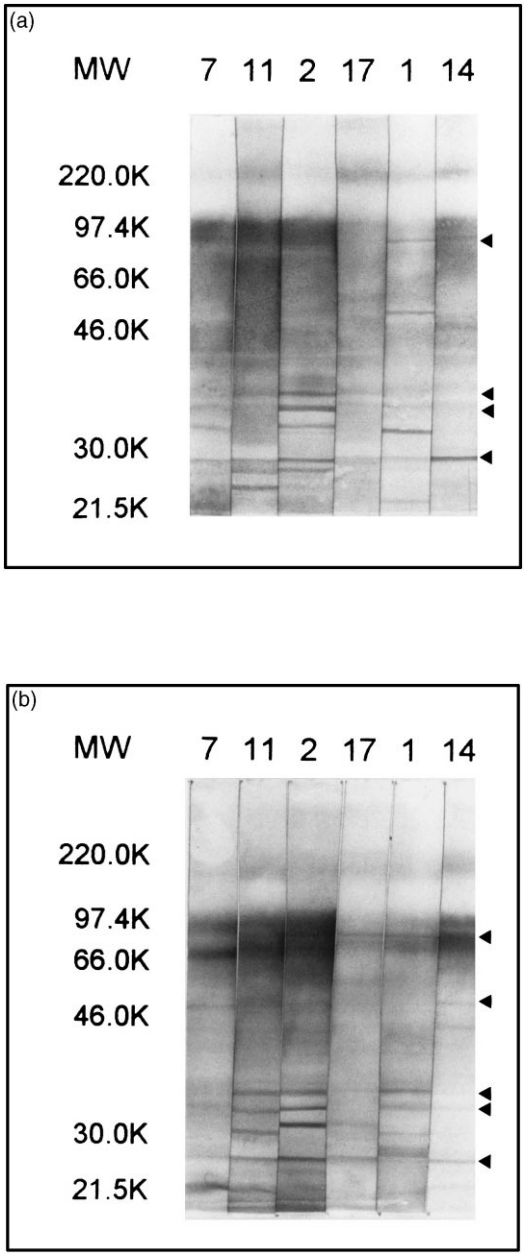

Fig. 2. Immunoblots of somatic *Asp. f.* probed with BALF antibodies from 6 horses (COPD horses: No. 7, 11, 2, 1, 14; control horse: No. 17); somatic *Asp. f.* was separated by SDS-PAGE and probed for IgE (figure 2a) and IgG (figure 2b) reactivity. Molecular weight (MW) markers are shown on the left. Horses were ranked in a declining order according to their IgE levels (RAU), obtained by ELISA - No. 7 had the highest, No. 14 had the lowest IgE reactivity. Common bands for IgE and IgG in all horses are marked by arrows.

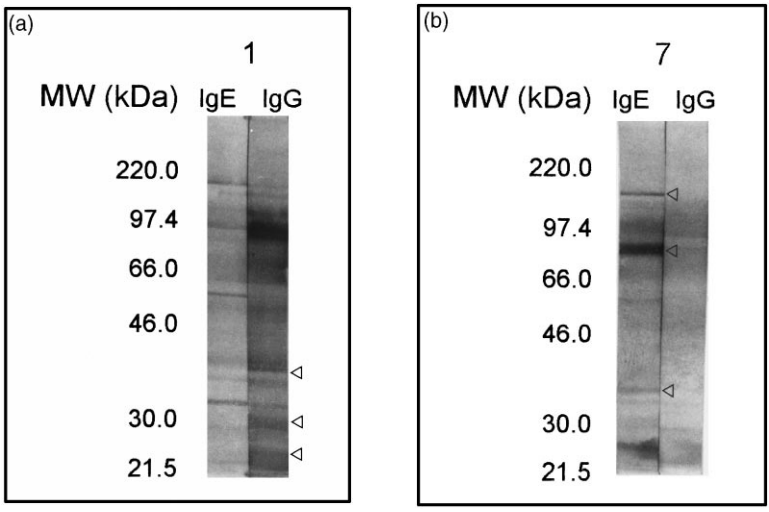

Fig. 3. b. Representative immunoblots of BALF samples from 2 horses (No. 1 in figure 3a, No. 7 in figure 3b); somatic *Asp. f.* was separated by SDS-PAGE and probed to detect IgE and IgG reactivity. Molecular weights (MW) are shown on the left. Bands that were detected only by α-IgE or α-IgG are marked by open arrows.

3.5. Differences between BALF and serum IgE reactivity patterns against Asp. f.

A comparison between BALF and serum IgE contents was carried out, to investigate which allergens commonly induce an IgE response in BALF and serum. In all BALF and serum samples multiple bands were identified (Fig. 4). It also shows that the pattern for the IgE response in BALF resembles the response in serum. Bands of 28 and 93 kDa were common to all horses examined in BALF and in serum. However, clear differences could also be seen, which are indicated by the open arrows (Fig. 4).

4. Discussion

In this study we confirm and extend the previous observations of raised levels of BALF IgE and IgG in horses affected with COPD (Halliwell et al., 1993). We examined the responses of somatic *Asp. f.* and antigen *Asp. f.* l/a on the levels of IgE and IgG in BALF and serum of COPD-affected horses. Additionally we used immunoblotting to detect the *Asp. f.* antigens leading to an IgE or IgG response. ELISA revealed that in COPD-affected horses levels of IgG and IgE in BALF, but not in serum were significantly elevated against both somatic *Asp. f.* and the recombinant *Asp. f* allergen, r *Asp. f.* l/a.

Increased serum levels of IgE and IgG have been described as a serodiagnostic marker for various human diseases (Longbottom, 1983, Wallenbeck et al., 1991, 1994). In addition, McPherson et al. (1979) described the presence of precipitating antibodies to *Asp. f.* in sera of horses with COPD and suggested that this serology could be used for the diagnosis of COPD in horses. The current results using both somatic *Asp. f.* and r *Asp. f.* l/a antigens by immunoblotting confirm the findings of Halliwell et al. (1993), but also

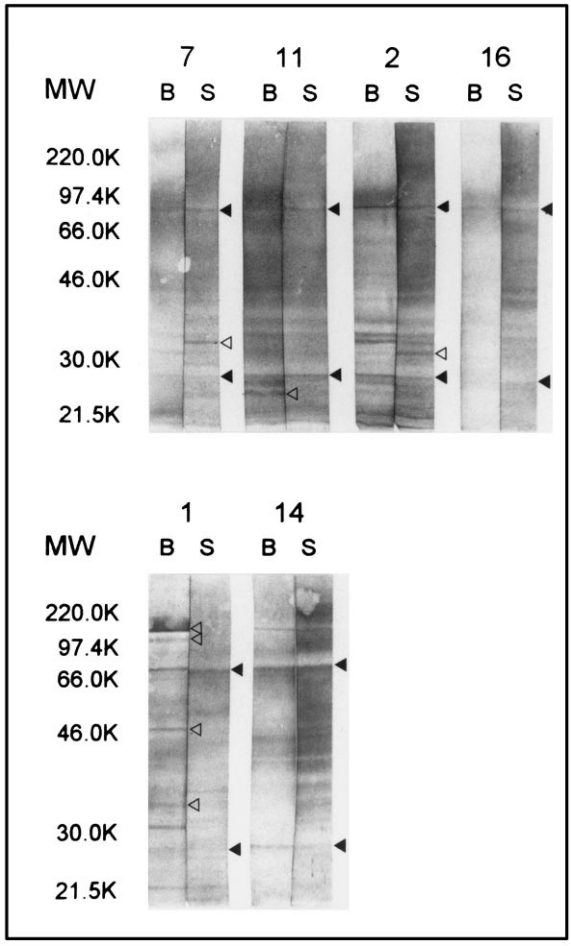

Fig. 4. Immunoblots of somatic *Asp. f.* probed with BALF antibodies from 6 horses (COPD horses: No. 7, 11, 2, 1, 14; control horse: No 16); somatic *Asp. f.* was separated by SDS-PAGE and probed for IgE reactivity in BALF and serum. Molecular weight (MW) markers are shown on the left. BALF and serum IgE detection was performed next to each other for each individual horse. Common bands in BALF and serum in all horses are marked by solid arrows, bands that were detected only in BALF or serum are marked by open arrows.

provide information that BALF but not serum antibodies are the relevant markers for the diagnosis of COPD-affected horses. Similarly in human patients, Nikolaizik et al. (1995) reported the presence of both IgE and IgG antibodies to r *Asp. f.* l/a. They also reported that the *Asp. f.* l/a allergen is involved in the pathogensis of ABPA in human patients with CF. Recombinant *Asp. f.* l/a-specific serology is a highly sensitive, specific and reliable test to identify ABPA in CF patients. Hence our results showing raised pulmonary levels of IgE and IgG against r *Asp. f.* l/a suggests that this allergen is analogous with part of the somatic allergens. Another important finding in our results, is that the systemic IgE responses do not distinguish COPD-affected horses from controls.

Using immunoblotting, we found the majority of immuncomplexes between *Asp. f.* and antibodies from BALF samples bind to both α-IgE and α-IgG, the major bands having molecular weights of 93, 35, 31 and 23 kDa. There is however one distinct IgG-specific antigen (molecular weight 45 kDa) found in all horses as well as single IgE-specific antigens or IgG- specific antigens leading to distinctive responses in individual horses. Our study demonstrating common bands of the same molecular weight on immunoblots to IgE and IgG concurs with similar findings in various human diseases such as ABPA, asthma and CF (Longbottom, 1983; Arruda et al., 1992). It is known that *Asp. f.* antigenic components react with IgE or IgG individually, as well as forming a complex with IgE and IgG antibodies (Longbottom, 1983).

When comparing *Asp. f.* specific IgE immune complexes between BALF and serum it showed that in all horses, there were systemic and pulmonary IgE responses against two prominent antigens (93 and 28 kDa) which suggests that neither is likely to be involved in the pathogenesis of COPD. It is likely, therefore, that other antigens and allergens induce the pulmonary responses associated with COPD.

In conclusion, responses to somatic *Asp. f.* and r *Asp. f.* l/a allergen were characterised by increased levels of allergen-specific IgE and IgG in BALF as measured by ELISA. However serum levels of IgE and IgG did not differ between COPD-affected and control horses, thus confirming that local and systemic responses are probably regulated independently. The finding of local pulmonary IgE in our studies as well as in the studies of Halliwell et al., 1993 suggests the involvement of an IgE-mediated type I hypersensitivity in the pathogenesis of equine COPD.

Immunoblotting revealed four common bands of molecular weights 93, 35, 31 and 23 kDa in BALF, suggesting that these four major antigens are involved in response to *Asp. f.* in COPD-affected horses. These data thus confirm the earlier results of Halliwell et al. (1993) and also suggest that the allergen *Asp. f.* l/a is a potentially useful target antigen for use in diagnostic ELISA. Importantly, the response to this antigen is upregulated in BALF and not serum and involves both IgE and IgG isotypes.

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