Original article

Time-dependent changes of cytokines mRNA in bronchoalveolar lavage fluid from symptomatic Recurrent Airway Obstruction-affected horses

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Abstract

During an 18 day test, we measured the cytokine mRNA expression (Interleukin-1β [IL-1β], Interleukin-8 [IL-8], Interferon-γ [IFN-γ], Tumor Necrosis Factor-α [TNF-α]) of cells from bronchoalveolar lavage fluid [BALF] in five horses previously diagnosed with RAO, before and during challenge exposure, and after the desensitization phase which involved dexamethasone treatment and environmental modification. Simultaneously, the same cytokine mRNA expression of cells from BALF in four asymptomatic RAO-affected horses maintained outdoors was analyzed. An evident respiratory distress was observed in the challenge group within 3 days, with a significant overexpression of IL-8 and TNF-α mRNA on the ninth day. The pharmacological and environmental desensitization provided a down regulation of all the cytokines. No statistical modification characterized the cytokine kinetics of the asymptomatic horses maintained outdoors. A comparison for each time point of the cytokines between the exposed and unexposed horses showed no significant differences. The study suggested that a standardized exposure protocol and sampling time in experimental studies of RAO is mandatory for a correct comparison of the results obtained by different Authors. However, the absence of significant changes between the exposed and unexposed horses could depend on the lack of the sample uniformity since the evolution of the disease represents a continuum from a healthy to a pathological condition.

Key words: horse, respiratory tract, Recurrent Airway Obstruction, PCR assay, cytokine

Introduction

Recurrent airway obstruction is an equine respiratory disease common in the northern hemisphere where airway obstruction is the consequence of a natural challenge exposure. The disease, characterized by airway inflammation, airway obstruction and bronchial hyper-responsiveness resulting in airway remodeling, is induced by the inhalation of airborne dust and endotoxins present in stalls (Pirie et al.

2003). The inflammatory response is due to the release of inflammatory mediators including histamine, serotonin, leukotrienes, thromboxane and 15-HETE but, in spite of the number of studies carried out on the pathogenesis, to date, the exact role of the cytokines during the induction, maintenance and switching off phases has not been completely clarified. In particular, no unique indication about the cytokine cascade in the development of RAO has been reported.

Analogously, to date, no accepted recommendations exist for standardized exposure protocols and sampling times in experimental studies of RAO (Marti et al. 2007).

The aim of this study was to evaluate the kinetics of the bronchoalveolar lavage mRNA of 4 cytokines (IL-1β, IL-8, IFN-γ, TNF-α) during the induction of the inflammatory process in RAO-affected horses, when they are moved from a pasture to a moldy stall, as well as during the clinical stage of the disease and the desensitization phase, comparing them, step by step, with the bronchoalveolar lavage mRNA of the same cytokines obtained at the same experimental time points from four asymptomatic RAO-affected horses maintained outdoors.

In order to obtain a complete profile, absolute quantification of the mRNA of the cytokines was performed at 6 experimental time points over 18 days.

Materials and Methods

Animals

Nine saddle horses (three males and six females, 22.4±3 years of age, weighing 430±30 kg [949±66 lb]), not having the same bloodlines, were used in the study. The horses were selected on the basis of their history and clinical diagnosis of RAO. The anamnesis reported the development of airway obstruction with exhibition of spontaneous coughing, mucopurulent nasal discharge, wheezing and increased expiratory effort when the animals were housed in a barn, on unchanged straw, eating hay, and their subsequent recovery when pastured.

The clinical diagnosis of RAO was based on the findings of respiratory distress, pulmonary neutrophilia (neutrophils >25% in the bronchoalveolar lavage) and a maximal transpulmonary pressure (ΔP_{plmax}) > 15 cm H_2O (measured as pressure difference between peak inspiration and peak expiration, using an esophageal catheter positioned at the level of the mid-thoracic esophagus)[a] when housed in stalls and exposed to hay, without modification of the blood count and serum biochemistry parameters over the reference values. During the 6 months prior to the study, the animals were kept in a pasture, vaccinated and dewormed, and showed no clinical signs of RAO.

The day before the test, in order to confirm the asymptomatic status, all horses were again subjected to pleural pressure measurement, showing values of ΔP_{plmax} less than 4 cm H_2O.

The study was approved by the Ethics and Scientific Committee of the *Alma Mater Studiorum* – University of Bologna and subsequently submitted to the Italian Ministry of Health. All experimental procedures were carried out in accordance with European legislation regarding the protection of animals used for experimental and other scientific purposes (Council Directive 86/609/EEC).

Experimental protocol

The method of exacerbation of disease in RAO-affected horses namely stabling the horses in a natural challenge environment, was also set based on the previous studies of Ainsworth et al. (2002), Giguère et al. (2002) and Ainsworth and Cheetam (2010).

The sample was randomly divided into two groups: Group A (5 horses) constituted the challenge group and Group B (4 horses), which was left in a pasture, was used as the control group.

The horses in Group A were moved from the pasture to poorly ventilated stables with the doors and air vents closed, on unchanged straw, eating hay; once a day, hay and moldy straw were shaken in front of the horses' heads in order to increase the environmental dust.

A clinical examination of the Group A horses was performed once a day and a bronchoalveolar lavage was executed at nine o'clock in the morning before the challenge (T1) and three (T2), seven (T3) and nine (T4) days after the beginning of the trial. After the bronchoalveolar lavage at T4, the horses were treated with dexamethasone (0.1 mg/kg [0.045 mg/lb] IM once a day for 2 days)[b], and the indoor environment was modified by changing the straw and opening the windows in order to reduce the environmental dust, as previously indicated by Giguère et al. (2002).

Additional bronchoalveolar lavages were executed at the eleventh day (T5) and the eighteenth day (T6).

At the same experimental times (T1, T2, T3, T4, T5, T6), without environmental modification, the horses in Group B also underwent clinical examination and a bronchoalveolar lavage was collected. Analogous to the horses in Group A, the horses in Group B were also treated with dexamethasone at the same dosage (0.1 mg/kg [0.045 mg/lb] IM once a day for 2 days)[b] after the BAL of the ninth day (T4).

The choice of the experimental intervals was made setting 48 h as the lower limit since previous studies of Sweeney et al. (1994) had demonstrated that a neu-

trophilic inflammatory response occurs within the lavage lung, and can be detected if subsequent lavages are performed within 48 h. The longest interval, seven days, (from T5, the eleventh day, to T6, the eighteenth day) was used for the last sampling with the aim of demonstrating the persistence of the post-treatment cytokine modifications.

Each bronchoalveolar lavage was performed after tranquilization with an IV injection of acepromazine maleate (2 mg/100 kg [0.907 mg/100 lb])[c] and detomidine HCl (1 mg/100 kg [0.454 mg/100 lb])[d], using a BAL catheter.[e]

A 210 mL aliquot of pre-warmed (37-38ºC [98.6-100.4ºF]) sterile isotonic saline solution, followed by 30 mL of air, was infused and re-aspirated (using a suction pump) into a glass vessel which was kept on ice (Mazan and Hoffman 2003).

The correct execution of BAL was demonstrated by the constant presence of surface foam indicating the presence of surfactant.

Each sample was processed within 15 min of collection. The fluid was filtered by means of sterile gauze and centrifuged at 4ºC [39.2ºF] at 2000xg for 10 min; the cell pellets were washed twice in phosphate-buffered saline and the sample was then frozen in liquid nitrogen until RNA extraction.

After the T2 and T6 withdrawals, indirect measurement of the pleural pressure was performed in the horses of both Groups using the device for measuring the pulmonary pressure oscillations.[a]

Cytospin slides of BALF at T1 and T4 were prepared and stained with May-Grünwald-Giemsa. For each sample 300 cells were counted under high magnification, classified as alveolar macrophages, lymphocytes, neutrophils, eosinophils or mast cells and expressed as percentage of total count.

RNA extraction and cDNA synthesis

Total cellular RNA was extracted by Trireagent according to the manufacturer's protocol.[f]

All RNA samples were treated with DNAase I in order to remove any trace of genomic DNA contamination.[g] Briefly, 1 U of DNAase I was mixed with 9 μL of total extracted RNA, incubated for 30 min at 37ºC [98.6ºF] and then inactivated for 5 min at 95ºC [203ºF].

cDNA strands were generated using 5 μL of RNA into a reaction buffer with MgCl$_2$ 5mM, 10x RNA PCR Buffer 1X, dNTP mixture 1 mM, 0.25 units/μL of Reverse Transcriptase AMV[h] 1 unit/μL of RNase Inhibitor, random hexamers 2.5 μM in a final volume of 20 μL of reaction. The retro transcription was carried out for 10' at 30ºC [86ºF], 15' at 45ºC [113ºF] and 2' at 99ºC [210.2ºF].

Quantitative real-time PCR analysis

The Rotor Gene 3000 was used for the amplification and data collection.[i] Primers for the gene cytokines (β actin, IL-1β, IL-8, IFN-γ, TNF-α) were designed using the software Oligo[j] based on published sequences (Table 1). The beta actin gene was used as an internal housekeeping gene to normalize the variation of cell numbers in the bronchoalveolar lavage fluid. The beta actin primers were designed based on the partial equine mRNA sequence as for Giguère and Prescott (1999).

Messenger RNA quantification for each target gene was achieved by constructing standard curves using cDNA of known concentrations of plasmid in which we inserted the target gene by means of a cloning reaction. The standard curves consisted of serial dilutions of the PCR amplicon corresponding to the gene of interest. At least six standard curve samples were included in each run. All concentrations of target gene cDNA were calculated relative to their respective standard curves.

A real time PCR assay using the Syber Green method was carried out in a final volume of 25 μl containing 1X of SYBR PREMIX Ex Taq Takara, 200 nM of each of the forward and reverse primers, 1X of Rox Reference Dye and 2 μL of cDNA. The cycling parameters were as follows: 10 min at 95ºC [203ºF] for polymerase activation followed by 40 cycles of 15 sec at 95ºC [203ºF], 15 sec annealing (β actin 56ºC [132.8ºF], IL-1β 55ºC [131ºF], IL-8 55ºC [131ºF], IFN-γ 55ºC [131ºF] and TNF-α 58ºC [136.4ºF]) and 20 sec at 72ºC [161.6ºF]. The signal was acquired on the FAM channel (multichannel machine) (source, 470 nm; detector, 510 nm; gain set to 5) with the fluorescence reading taken at the end of each 72ºC [161.6ºF] step.

To distinguish specific from non-specific products and primer dimers, a melting curve was obtained after amplification by holding the temperature at 72ºC [161.6ºF] for 12 sec followed by a gradual increase in temperature to 95ºC [203ºF] at a rate of 0.1ºC/sec [32.18ºF/sec], with the fluorescence signal acquisition mode set to step.

All the data are expressed as the ratio of the copies of the target gene and the copies of the housekeeping gene β actin, as endogenous control that is constitutively expressed in cells (Bowles et al. 2002).

Statistical analysis

In every group, the ratio between cytokine mRNA and corresponding β actin of the different steps were analyzed by an analysis of variance (Fisher's test) with repeated measures and an LSD test as a post hoc test.

Table 1. Real Time PCR Primers designed with Oligo software according to published gene sequences.

Target Gene	Primers forward (5'-3')	Primer reverse (5'-3')	Frame (bp)	Reference sequence Genbank code
β actin	CTGGCACCACACCTTCTACAACGAG	TCACCGGAGTCCATCACGA	214	AF035774.1
IL-8	TCTCTTGGCCGTCTTCCTG	CCGTTGACGAGCTTTACAA	195	AY184956.1
IFN-γ	GTGTGCGATTTTGGGTTCTTCTA	TTGAATGACCTGGTTATCT	235	D28520.1
IL-1β	GAGGCAGCCATGGCAGCAGTA	TGTGAGCAGGGAACGGGTATCTT	257	D42165.1
TNF-α	AGCTCTCCTGGGCACCTAC	TGGGGTTCGGAGGGGTGTTAC	205	AF503366.1

In order to compare the cytokine mRNA/corresponding β actin results between Group A and Group B for each experimental time point, a Student's *t* test for unpaired data was applied.

Differences were considered significant when *P*-values were less than 0.05.

All statistical analysis was performed by use of a software package.[k]

Results

Clinical signs

The horses in Group A showed evident respiratory distress within three days (T2) from the beginning of the trial, including flaring nostrils, serous nasal discharge, coughing and abdominal lift.

Measurement of the ΔP_{plmax}, obtained after T2, showed a mean value of 32.2±9.8 cm H_2O.

The clinical signs decreased quickly after environmental modification and therapy with dexamethasone carried out after the bronchoalveolar lavage at T4, and a clinical condition similar to the pre-trial stage was recorded at T5 and at T6.

Table 2. Bronchoalveolar lavage fluid cytology in horses affected by RAO during challenge exposure (Group A) and maintained in pasture (Group B), at T1 (pre-trial value) and T4 (day 9).

		T1 (pre-trial value)	T4 (day 9)
Macrophages %	Group A	57.1 ± 7.2	18.4 ± 5.8
	Group B	52.4 ± 3.5	57.2 ± 6.3
Lymphocytes %	Group A	39.2 ± 7.3	39 ± 3.6
	Group B	42.3 ± 2.4	40.2 ± 6
Neutrophils %	Group A	2.8 ± 1.5	42.5 ± 3.9
	Group B	3.8 ± 1.6	1.7 ± 1.8
Eosinophils %	Group A	0.6 ± 0.4	0.1 ± 0.1
	Group B	1.1 ± 0.4	0.8 ± 0.4
Mast Cells %	Group A	0.3 ± 0.3	/
	Group B	0.4 ± 0.2	0.1 ± 0.2

After T6 withdrawal, the ΔP_{plmax} showed a mean value of 4.3±0.8 cm H_2O.

No clinical modification was observed in the horses of Group B.

In this group, the ΔP_{plmax} of the horses was 2.6±0.4 cm H_2O after T2 withdrawal and 2.5±0.5 cm H_2O after T6 withdrawal.

Cytological features of BALF at T1 and T4 in the horses of Group A and Group B were reported in Table 2.

Cytokine mRNA expression

Table 3 reported the mean values and standard deviations of the ratio between the cytokine mRNA and the corresponding β actin, respectively, of Group A and Group B, related to the pre-trial value (T1), at the third day from the beginning of the trial (T2), at the seventh day from the beginning of the trial (T3), at the ninth day from the beginning of the trial (T4), after desensitization, at the eleventh day from the beginning of the trial (T5) and at the eighteenth day from the beginning of the trial (T6), nine days after desensitization.

Analyzing cytokine movement in Group A during the trial, no significant modification was evidenced for IL-1β and IFN-γ mRNA, but a significant increase was evidenced for IL-8 mRNA at T4 with respect to T1 (*P*<.01), to T2 (*P*<.01), to T3 (*P*<.01), to T5 (*P*<.01) and to T6 (*P*<.01), and for TNF-α mRNA at T4 with respect to T1 (*P*<.05), to T3 (*P*<.05), to T5 (*P*<.01) and to T6 (*P*<.01).

No significant change was demonstrable in any cytokine of Group B during the various phases of the trial. A comparison of each time point of the cytokines of Group A with respect to the cytokines of Group B showed no significant differences.

Discussion

In the past ten years, several studies have been carried out in order to better explain the influence of

Table 3. Group A and Group B: cytokine mRNA/corresponding β actin.

		T1 (pre-trial value)	T2 (day 3)	T3 (day 7)	T4 (day 9)	T5 (day 11)	T6 (day 18)
IL-1β	Group A	0.4449 ± 0.4692	1.3942 ± 2.9187	0.0902 ± 0.1171	0.1127 ± 0.1343	0.0148 ± 0.0256	0.0332 ± 0.0232
	Group B	0.4903 ± 0.3363	0.2916 ± 0.2134	0.0517 ± 0.0065	0.0962 ± 0.1149	0.0672 ± 0.0999	0.0899 ± 0.0844
IL-8	Group A	0.3229 ± 0.3452^{B}	0.7972 ± 0.6641^{B}	0.1243 ± 0.2385^{B}	1.6242 ± 1.2206^{A}	0.4357 ± 0.4948^{B}	0.1390 ± 0.0911^{B}
	Group B	0.3861 ± 0.2583	0.2765 ± 0.2250	0.0317 ± 0.0285	0.2423 ± 0.2540	0.1249 ± 0.0851	0.1824 ± 0.2925
IFN-γ	Group A	0.0007 ± 0.0009	0.0004 ± 0.0006	0.0031 ± 0.0065	0.0003 ± 0.0006	ND	0.0005 ± 0.0006
	Group B	0.0004 ± 0.0003	0.0008 ± 0.0015	0.0001 ± 0.0001	0.0003 ± 0.0002	0.0001 ± 0.0001	0.0004 ± 0.0007
TNF-α	Group A	1.0309 ± 0.4046^{b}	1.2975 ± 1.2264	0.5750 ± 0.7094^{b}	3.0087 ± 2.6328^{aA}	0.0734 ± 0.0775^{B}	0.3203 ± 0.2907^{B}
	Group B	1.0332 ± 0.3376	0.8955 ± 0.1302	0.6462 ± 0.1009	0.7676 ± 0.0188	0.8348 ± 0.7347	0.0005 ± 0.0001

ND: not detected.
Different letters between the different experimental points of the same cytokines indicate a significant difference (lower case letters = p <.05; upper case letters = p <.01)

different cytokines in the development of RAO. However, these studies differed in their extreme variability in relation to the environmental treatment, the time of analysis in function of RAO development, the cytokines which had to be analyzed and the substrate analyzed, contributing to the lack of agreement between results reported by different Authors.

For example, Lavoie et al. (2001) have studied cytokine mRNA expression from bronchoalveolar lavage cells without modification of environmental conditions, while subsequent analyses were carried out by Kleiber et al. (2005) before and 48 h after stabling, by Debrue et al. (2005) before and after 35 days of continuous exposure to moldy hay, and by Riihimäki et al. (2008a) after two days of challenge and after remission when horses had been in the pasture for 2 months. Three subsequent analyses were carried out by Ainsworth et al. (2003) before the trial and after housing in a poorly ventilated dusty stable for one day and five weeks, respectively and another three by Cordeau et al. (2004) before the trial and after housing in a stable for one and nine days, respectively and, finally, five subsequent analyses were done by Ainsworth et al. (2006) before the trial and 1, 14, 35 and 49 days after challenge exposure.

Moreover, as regards the number of cytokines analyzed, numerous studies have focused on only a single cytokine (Franchini et al. 2000, Debrue et al. 2005), two cytokines (Laan et al. 2005) or three cytokines (Bowles et al. 2002, Cordeau et al. 2004) while other Authors have investigated more extensive profiles of different immunomodulatory proteins (Giguère et al. 2002, Ainsworth et al. 2003, Kleiber et al. 2005, Ainsworth et al. 2007, Riihimäki et al. 2008a, 2008b).

It is also worth noting that another possible element of discrepancy between the results of previous studies on cytokines with respect to our study is represented by the biological material analyzed for the extraction of cytokine mRNA.

In fact, some Authors examined the mRNA from the BALF lymphocytes (Horohov et al. 2005, Kleiber et al. 2005), macrophages (Laan et al. 2005) and mononuclear cells (Ainsworth et al. 2007, Reyner et al. 2009) while other Authors (Franchini et al. 2000, Lavoie et al. 2001, Bowles et al. 2002, Giguère et al. 2002, Ainsworth et al. 2003, Debrue et al. 2005) studied the cytokine network of all BALF cells. Moreover, it is necessary to realize that, in addition to the BALF cells, the bronchial epithelium is also capable of producing the cytokines involved in the development of acute RAO as reported by Bureau et al. (2000) and by Ainsworth et al. (2006), although a strict correlation between cytokine expression in BALF cells and in bronchial tissue has not been demonstrated (Riihimäki et al. 2008a).

In our study, we chose to analyze the mRNA cytokines extracted from BALF cells, excluding the mRNA cytokines from biopsies of the bronchial tissue.

The absence of research standardization was correctly pointed out at the 3rd Havemeyer workshop on allergic diseases where it was reported that "... no accepted recommendations exist for standardized exposure protocols and sampling time in experimental studies of RAO...", making comparison between studies difficult (Marti et al. 2007).

Our study design was adopted in order to quantify the mRNA value of 4 cytokines (IL-1β, IL-8, IFN-γ, TNF-α) from BALF cells over a period of eighteen days which was compatible with the wellbeing of the animals and could be considered appropriate for investigating the kinetics of the relevant cytokines involved in the pathogenesis of RAO.

We chose to exclude IL-17 mRNA from the cytokines investigated as its contribution to the course of RAO is already known (Debrue et al. 2005, Ainsworth et al. 2006).

The simultaneous BAL sampling in the asymptomatic horses allowed the exclusion of the natural fluctuations of cytokines and the influence of repeated bronchoalveolar lavages on the cytokine levels.

Before discussing the results of the trial, it is necessary to point out some elements that must be

taken into account in order to correctly interpret the results; in the first place, one must remember that the group was made up of horses with different bloodlines which were carriers of RAO in different degree, and who could have had varying cytokines response. Furthermore, induction of the exacerbation was carried out, following what had previously been reported in the literature, with a natural method, maintaining all the horses in the trial in the same environment, a condition which cannot exclude the summation of an additional element of variability. Finally, the times fixed for the sampling were chosen in such a way as to reduce the influence of the manual handling on the cytokine population, maintaining a frequency adapted to adequately design the movement of the cytokine in the course of the exacerbation and defervescence.

Examining the kinetics of cytokine mRNA in the horses of Group A, IL-8 and TNF-α mRNA showed statistical differences during the trial.

The increase in the cytokine mRNA expression of IL-8 in cells from the BALF appeared only after 9 days from the beginning of challenge, confirming that this cytokine is not involved in the first phase of RAO exacerbation, as reported by Ainsworth et al. (2003) who found no changes in the IL-8 expression in the BALF cells at 24 hours. Our results are in substantial agreement with those of Giguere et al. (2002) who reported a significant increase in IL-8 expression in the BALF cells after 5 days (median time) of natural challenge exposure. Since we desensitized the RAO affected horses at day 9 post challenge (T4) we could not determine if this cytokine continued to be up-regulated after this time. Nevertheless, other Authors demonstrated that, in the absence of a desensitization, the over-expression of IL-8 mRNA is maintained for the duration of the exposure (Ainsworth et al. 2003, 2006). Thus, it is possible to affirm that once IL-8 mRNA levels rose after approximately 5-9 days post challenge, this up-regulation was maintained in RAO-affected horses continuously, without significant fluctuations, as long as the stimulus persisted, since we observed elevated mRNA levels after 9 days and significant up-regulation had previously been demonstrated after 14, 28, 35 and 49 days (Ainsworth et al. 2003, 2006). On this basis, it is likely that, while the persistence of a large number of neutrophils in the airways during RAO may be related to increased expression of IL-8 mRNA, it is likely that their recruitment during the acute phase is due to other mechanisms.

Similarly to our findings for TNF-α mRNA, Giguère et al. (2002) observed an increase in the exacerbation phase in BALF of horses with RAO after exposure to moldy hay, and Laan et al. (Laan et al. 2005, 2006), noted an increase in the *in vitro* production of TNF-α by equine alveolar macrophages of RAO-affected horses. To our knowledge there are no studies which investigate on the expression of these cytokines for a period of time longer than that of our study. Since in our trial a desensitization was carried out after 9 days (T4), we cannot say exactly how long the up-regulation of TNF-α persists in challenged RAO-affected horses, but data from our study indicate that this molecule exhibits its pro-inflammatory role together with IL-8 and not during the first days of RAO crisis but instead in a later phase. Nevertheless, it should be noted that TNF-α and IL-8 are both expressed by equine neutrophils (Joubert et al. 2001) and macrophages (Laan et al. 2006) of RAO-affected horses but not by bronchial tissue (Riihimäki et al. 2008c) or by BALF lymphocytes (Kleiber et al. 2005).

Unlike what has been reported by some authors, in our study, we did not record significant changes of IL-1β and IFN-γ mRNA during the challenge exposure.

Regarding IL-1β our findings are in contrast with those reported by Giguère et al. (2002) who found an increase in IL-1β mRNA in the exacerbation phase (5 days after exposure) in RAO-affected horses. Nevertheless we stressed that the differences were statistically significant in only one of two trials reported in the work and, furthermore, the significance was assessed for $P \leq 0.1$.

Other studies on IL-1β expression have been carried out *in vitro*. Laan et al. (2006) found significant increase of IL-1β mRNA in equine pulmonary macrophages, but not constantly, at 6 and 24 hours when stimulated by *Escherichia coli* derived lypopolysaccharide or hay dust suspension.

However in another study, it has been evidenced that IL-1β mRNA from pulmonary mononuclear cells isolated from RAO-affected horses exposed to dusty hay was not greater than in healthy horses (Ainsworth et al. 2007). On the other hand, equine bronchi passively sensitized with serum from animal suffering from RAO, showed a significant increase in IL-1β mRNA (Matera et al. 2005). Our data suggest that IL-1β seems not to be involved in RAO development after three days of challenge, but in the light of other studies it is not possible to exclude its earlier involvement.

The lack of time-dependent changes we found in IFN-γ mRNA were not in agreement with the results of previous study. Giguère et al. (2002) and Ainsworth et al. (2003) stressed that an increase in IFN-γ mRNA was characteristic of the BALF of horses in dusty stables. An increase in IFN-γ mRNA was also recorded by Horohov et al. (2005) in the BALF of horses which had been kept outdoors in the summer and had summer pasture-associated obstructive airway disease. Moreover, Cordeau et al. (2004) reported a statistical reduction of IFN-γ mRNA in bronchoalveolar lymphocytes after 9 days of continuous moldy hay exposure, while Kleiber et al. (2005)

showed no statistical change in IFN-γ mRNA extracted from BALF CD4 and CD8 T lymphocytes in RAO-affected exposed to hay/straw.

It is hard to explain whether the reduction of all the cytokines we found in Group A after the administration of dexamethasone and environmental modification, in the desensitization phase, are attributable to the first factor rather than the second.

In fact, Giguère et al. (2002) reported no statistical decrease in IL-1β, IL-8, and IFN-γ, (unlike TNF-α) mRNA expression of RAO-affected horses treated with inhaled fluticasone propionate during the post-exacerbation period.

The interpretation of the mRNA level of the cytokines in the BALF cells of the control group (Group B) is easier in that it showed no statistical modification of the experimental times. This could indicate that the cytokine population in asymptomatic RAO-affected horses does not vary over time and BALF collection using a BALF catheter with intervals ranging from three to eight days is not capable of influencing the mRNA cytokine population.

Note that the administration of dexamethasone[b] 0.1 mg/kg [0.045 mg/lb] IM once daily for two days appeared to cause no change in the titres of mRNA of the cytokines of Group B horses at T5 and T6 when compared to previous values.

Moreover, it is necessary to point out that no statistical differences were observed at the same experimental time between the cytokine value of Group A and that of Group B; this can be justified for the pre-trial value (T1) because the horses in Groups A and B were classified randomly from the same population, and at T5 and T6 because the administration of dexametasone associated with an environmental modification was capable of reducing the increased cytokine values of Group A, reducing them to values more or less similar to the Group B values.

It is more difficult to explain the absence of statistical differences between the cytokine values of Group A vs. Group B during the experimental times T2, T3 and T4, even if the mean values of Group B are constantly maintained below the values of Group A.

One explanation for IL-1β, IL-8 and TNF-α mRNA, may be due to the small number of subjects in both groups and the lack of uniformity of the extent of the disease which in turn could determine the development of non-homogeneous values of the cytokine mRNA, meanwhile the number of animals cannot be used as an explanation for lack of significant differences for IFN-γ because the numbers do not appear to change over time or from unexposed horses.

Certainly the inclusion in our study for another group of clinically healthy horses kept outdoors might provide better interpretation of these results, even if although the literature is not unanimous on this point.

In fact comparing the results of BAL cytokines of asymptomatic RAO-affected horses with those of healthy subjects, some authors (Giguère et al. 2002, Ainsworth et al. 2003) showed values statistically different, whereas in other studies (Ainsworth et al. 2006, Riihimäki et al. 2008a) no variation between two groups was detected.

Analyzing the overall results we obtained, it is possible to affirm that a coordinated involvement of the following cytokines (IL-8, TNF-α) corresponds to the clinical development of RAO in susceptible horses. In particular, a consensual overexpression of both IL-8 and TNF-α, which could have synergic chemotactic action on the neutrophils, manifested on the ninth day. Finally, it is worth noting that the desensitization caused prompt clinical improvement which coincided with the down regulation of all the cytokines investigated which then remained at basal levels until the end of the observation period (9 days post-desensitization).

As already reported in our study, the results we observed and the timing in relation to the onset of symptoms do not correspond fully with those obtained by several Authors.

In summary, several factors may be called upon to explain the differences in our results as compared to those of other studies regarding the profile and kinetics of these cytokines during the development of RAO: 1) the absence of standardization in experimental time 2) the impossibility of defining the presence or absence of RAO as a dichotomized clinical status and 3) the strong interrelationship between the different cytokines, their rapid activation and rapid disappearance.

In conclusion, it is correct to affirm that the evolution of the pathology represents a continuum from the healthy to the pathological condition and not a qualitative condition from absence of disease to disease. Consequently, it is possible that the variations expressed by the values of the cytokines represent a different level of disease in different horses.
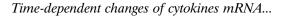
Legend

[a] Venti-Graph PG 100/REC, Boeringer Inghelheim, Germany.

[b] Desashock, Fort Dodge Animal Health SpA, Netherlands.

[c] Prequillan, FATRO SpA, Italy.

[d] Domosedan, Pfizer Italia Srl, Italy.

[e] Kruuse, Worldwide Veterinary Supplier, Denmark.

[f] Sigma-Aldrich, St.Luis, MO, USA.

[g] Gibco Invitrogen BRL, Carlsbad, CA, USA.

[h] Takara bio Inc, Japan.

[i] Corbett Research, Sydney, Australia.

[j] Molecular Biology Insights inc., W. Cascade, CO, USA.

[k] Statistica for Windows, StatSoft inc, Tulsa, OK, USA.

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