



# Lactoferrin, a glycoprotein with immunomodulatory and mast cell stabilising properties, in skin of horses suffering from *Culicoides* hypersensitivity

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## Abstract

Lactoferrin (LF), a glycoprotein of the transferrin family with anti-bacterial and immunomodulatory properties, is expressed in various secretions and tissues. Cutaneous LF serves as a mast cell stabilising compound, modulates T cell activity and is found during IgE-mediated late phase reactions at allergen challenged sites. *Culicoides* hypersensitivity (CHS) in horses is a common IgE-mediated allergic dermatitis, characterised by an early and late phase cutaneous reaction upon allergen challenge. The aim of the study presented here was to examine whether LF mRNA expression in skin biopsies from horses affected by CHS prior to and 4 h following intradermal challenge with a commercial *C. nubeculosus* extract is modified in comparison to skin biopsies from non-affected horses. In order to obtain reliable data, real time PCR was performed and genes of interest were normalized using three different housekeeping genes,  $\beta$ -actin, GAPDH,  $\beta$ -2-microglobulin. In comparison to non-affected horses, higher variation in LF mRNA levels both prior to and post-intradermal challenge with *C. nubeculosus* extract was seen in horses affected by CHS. However, the statistical analysis demonstrated that LF mRNA expression was not significantly different between CHS affected and non-affected horses prior to intradermal challenge with *C. nubeculosus* extract. Intradermal injection of *C. nubeculosus* extract did not result in local upregulation of LF mRNA at 4 h post-injection. LF mRNA expression was therefore not significantly different pre- or post-intradermal challenge with *C. nubeculosus* extract in either group. Our data indicate that clinically normal skin of horses affected by CHS is not characterized by modified maintenance levels of LF mRNA. In contrast to human skin allergen challenged sites, LF mRNA levels in horses affected by CHS are not significantly different to that of control sites at 4 h post-injection of *C. nubeculosus* extract.

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## 1. Introduction

Lactoferrin is an 80 kD iron-binding glycoprotein of the transferrin family. It is present in a wide variety of secretions and tissues, epithelial and polymorphonuclear cells and is bound on activated blood lymphocytes (Teng et al., 1986; Pentecost and Teng, 1987; Teng, 1999; Mincheva-Nilsson et al., 1997). In addition to its bacteriostatic

and bactericidal properties, LF is involved in modulating the immune and inflammatory response by down-regulating several cytokines, such as tumour necrosis factor alpha (TNF- $\alpha$ ), interleukin-6, and granulocyte-macrophage colony-stimulating factor (Slater and Fletcher, 1987; Bellamy et al., 1992a,b; Crouch et al., 1992; Machnicki et al., 1993; Haversen et al., 2002).

In mammalian skin LF is present within the epidermis, sweat glands and hair follicles (Cumberbatch et al., 2000). Four percent of non-stimulated skin mast cells contain LF, and the proportion increases by 4-fold following

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LF incubation in humans (He et al., 2003). LF is known to be an endogenous mast cell stabilising compound that inhibits IgE-dependent release of histamine from skin mast cells with greater potency than most anti-allergic drugs (He et al., 2003). Furthermore, LF suppresses allergen-induced epidermal Langerhans cell migration (Griffiths et al., 2001) and is capable of suppressing both Th1 and Th2- cell proliferation (Moed et al., 2004). Endogenous LF, therefore, has very strong immunosuppressive properties and is thought to have a role in the down-regulation of allergic inflammation. Thus, LF levels increase progressively during the cutaneous late-phase reaction at allergen challenged sites in humans (Zweimann and von Allmen, 2000).

*Culicoides* hypersensitivity (CHS), an allergic dermatitis to the saliva of *Culicoides* midges solely or in combination with other insects, is also characterised by an early and late phase cutaneous reaction (Scott and Miller, 2003a). Skin specimens of CHS horses are characterised by an increased number of T-lymphocytes, Langerhans cells, eosinophils, as well as increased levels of IgE and leukotriene C4, D4 and E4 (Van der Haegen et al., 2001; Wilson et al., 2001; Kurotaki et al., 1994; Strothmann-Lüerssen et al., 1992; Foster et al., 1995; Baker and Quinn, 1978). Therefore, interleukin (IL)-4 induced IgE antibodies, IL-5 chemoattracted eosinophils, as well as other T helper cell subtype 2 (Th2) derived cytokines and arachidonic acid metabolites may play a pivotal role in the pathogenesis of CHS. The objective of the study presented here was to examine whether LF mRNA expression in skin biopsies from horses affected by CHS prior to and 4 h following intradermal challenge with a commercial *C. nubeculosus* extract was modified in comparison to skin biopsies from non-affected horses.

## 2. Material and methods

### 2.1. Horses

Five horses presented with a typical history and characteristic clinical signs for CHS and response to fly control. Three additional horses with no history or lesions attributable to CHS were tested at the owner's request and were included in this study. Slight cutaneous inflammation was present in affected horses, skin scrapings and cytology were normal.

### 2.2. IDT and biopsy

All horses were intradermally tested with 0.1 mg/ml histamine phosphate (positive control), isotonic buffered saline solution (negative control) and 1:1000 w/v *Culicoides* (*C.*) *nubeculosus* extract (Greer Lab. Inc., NC, USA) as standard procedure (Scott and Miller, 2003b). The challenged sites were evaluated 30 min and 4 h post injection. Results were determined by palpation of injection sites to evaluate wheal diameter and wheal turgor. The strongest skin reaction was evoked by histamine and designated

(4+) positive whereas the site challenged with buffered saline solution was termed “negative”. Skin biopsies from the negative control and the *C. nubeculosus* injected sites were taken at 4 h with a sterile 8 mm biopsy punch (Stiefel Laboratorium GmbH, Offenbach/Main, Germany) following deep subcutaneous administration of 2 ml lidocaine per site. Skin samples were divided into equal halves and one half was immediately snap frozen in liquid nitrogen while the other was placed in 10% formalin. Formalin specimens were sent as routine to Abbey Veterinary Services (Devon, UK). Their report on histopathology was descriptive.

### 2.3. RNA extraction

RNA was extracted with RNeasy® Mini Kit (Qiagen GmbH., Hilden, Germany) with proteinase K digestion and DNase I treatment according to the manufacturer protocol. Briefly, after disruption and homogenisation of 30 mg dermis plus epidermis with a sterile blade and sterile needle and syringe, samples were lysed in a guanidine-isothiocyanate-containing lysis buffer. After dilution of the lysate the sample was treated with proteinase K, and the debris pelleted by centrifugation. Ethanol was then added to the cleared lysate and RNA was bound to the RNeasy membrane. DNase I incubation mix was pipetted directly onto the RNeasy silica-gel membrane. Finally, contaminants were washed away and total RNA was eluted in RNase-free water.

### 2.4. Real-time RT-PCR

Real-time RT-PCR methodology was used to estimate the relative gene expression levels of the different genes of interest (Klein, 2002). Primers and probes for the different equine genes were designed using Primer Express software (Fa. Applied Biosystems, Foster City, USA). Where possible, the primers and probes were placed on exon–exon borders to eliminate the possibility of detection or quantification of any residual DNA. The sequences of primers and probes have been described recently (Kolm et al., 2006a,b) and are listed in Table 1. The reaction efficiency was determined using serial dilutions of cDNA (Klein et al., 2001) and ranged from 91.7% to 99.8%. The real-time RT-PCR measurements were performed on two different machines (ABI 7700, Applied Biosystems; Mx3000P, Stratagene). The 25 µl RT-PCR mixtures contained 12.5 µl 2×reaction buffer (SuperScript™III Platinum® One-Step Qunatiative RT-PCR System, Invitrogen, Austria), 300 nM of each primer, 200 nM of the fluorogenic probe, 0.5 µl of the SuperScript™III RT/Platinum® Taq Mix and 5 µl of sample. After a reverse transcription step (30 min at 42 °C) followed by a denaturation step (10 min at 95 °C), amplification was performed with 45 cycles of 15 s at 95 °C and 60 s at 60 °C. The data were analysed using the ddCt-method allowing the estimation of the relative gene expression in comparison to a house-keeping gene (Kolm et al., 2006a). Since no ideal house-keeping

Table 1  
Sequences of primers and probes of the four real-time RT-PCR assays used in this study

|                |         |                                                  |
|----------------|---------|--------------------------------------------------|
| LF             | Forward | 5'-GTG CAT CCA GGC CAT TGC-3'                    |
|                | Reverse | 5'-CAT AAT AGC GGG TTT GTG GCT T-3'              |
|                | Probe   | 5'-FAM-CCT GTG GCA GCG GAG GTC TAC CA-TAM-3'     |
| $\beta$ -2 M   | Forward | 5'-TCG TCC TGC TCG GGC TAC T-3'                  |
|                | Reverse | 5'-ATT CTC TGC TGG GTG ACG TGA-3'                |
|                | Probe   | 5'-FAM-CGT CCC GCG TGT TCC GAA GGT T-TAM-3'      |
| GAPDH          | Forward | 5'-GGC AAG TTC CAT GGC ACA GT-3'                 |
|                | Reverse | 5'-CAC AAC ATA TTC AGC ACC AGC AT-3'             |
|                | Probe   | 5'-FAM-CAT CAA CGG AAA GGC CAT CAC CAT CT-TAM-3' |
| $\beta$ -actin | Forward | 5'-CCG GGA CCT GAC GGA CTA-3'                    |
|                | Reverse | 5'-CCT TGA TGT CAC GCA CGA TT-3'                 |
|                | Probe   | 5'-FAM-TAC AGC TTC ACC ACC ACG GCC G-TAM-3'      |

gene has been described to date, the experimental plan included the design of three assays for different house-keeping genes ( $\beta$ -actin, GAPDH,  $\beta$ -2-microglobulin). The different ddCt-values obtained with the various house-keeping genes were compared and used to calculate a reliable gene expression pattern for the genes of interest.

### 2.5. Statistics

In order to identify the most reliable housekeeping genes, calculation by the computer software programme geNorm (Vandesompele et al., 2002) was performed. All other statistical evaluations were made with the statistics package SPSS 13.0. Pearson correlation analysis was used to estimate the association among the three housekeeping genes used. Data are presented as mean  $\pm$  standard deviation in relative mRNA expression within each group. Differences between paired groups (prior and post *C. nubeculosus*) were analysed with the Wilcoxon ranks signed test. Differences between unpaired groups (affected and non-affected by CHS) were analysed with the Mann–Whitney *U*-test. A *p*-value  $< 0.05$  was considered significant.

## 3. Results

### 3.1. IDT

The challenged sites of the five horses affected by CHS were 4+ positive for histamine and *C. nubeculosus*, whereas buffered saline solution did not evoke any visible or palpable changes in skin sites at 4 h. In the three horses without history or clinical signs indicative of CHS, the site challenged with histamine was 4+, whereas there was no cutaneous response to buffered saline solution or *C. nubeculosus* extract.

### 3.2. Histopathology

The skin of control horses was histologically normal at 4 h post intradermal injection of the negative control. Four hours following challenge with *C. nubeculosus*, the skin

samples of controls exhibited a slight perivascular eosinophilic infiltration of the deep and superficial epidermis.

Skin that appeared normal on visual evaluation in CHS affected horses exhibited only minimal epidermal hyperplasia and inflammation very similar to that of control horses. Histopathology from lesional but non challenged biopsy sites of CHS affected horses demonstrated marked epidermal hyperplasia, edema and dermal and perivascular infiltration of eosinophils and mononuclear cells. Apart from epidermal hyperplasia non lesional skin sites challenged with *C. nubeculosus* exhibited very similar histopathologic features to that of the lesional sites of CHS-affected horses at 4 h.

### 3.3. Choice of appropriate housekeeping genes

The three housekeeping genes,  $\beta$ -actin, GAPDH and  $\beta$ -2-microglobulin ( $\beta$ -2 M) were analysed in all samples. In order to choose the most reliable housekeeping genes, calculation by the computer software programme geNorm (Vandesompele et al., 2002), and Pearson correlation analysis were performed. The most stable housekeeping genes, as calculated by the computer software programme geNorm, were found to be  $\beta$ -actin and GAPDH. The Pearson correlation coefficient between the three housekeeping genes used was  $r = 0.792$  ( $p < 0.001$ ) for  $\beta$ -actin and GAPDH,  $r = 0.693$  ( $p = 0.004$ ) for  $\beta$ -actin and  $\beta$ -2-microglobulin and  $r = 0.737$  ( $p = 0.007$ ) for GAPDH and  $\beta$ -2-microglobulin. As the highest Pearson correlation coefficient was found between  $\beta$ -actin and GAPDH and these were also the most stable housekeeping genes according to the computer software programme geNorm, the relative expression of the genes of interest were only calculated relative to  $\beta$ -actin and GAPDH.

### 3.4. LF mRNA

LF mRNA was detectable in all samples. In comparison to non-affected horses, higher variation in LF mRNA levels both prior to and post-intradermal challenge with *C. nubeculosus* extract was seen in horses affected by CHS

(Figs. 1 and 2). The statistical analysis demonstrated that LF mRNA expression was not significantly different between CHS affected and non-affected horses prior to intradermal challenge with *C. nubeculosus* extract (LF mRNA relative to GAPDH  $p = 1.0$  and LF mRNA relative to  $\beta$ -actin  $p = 1.0$ ). Intradermal injection of *C. nubeculosus* extract did not result in an upregulation of LF transcription (Figs. 1 and 2) and LF mRNA expression was therefore not significantly different pre- or post-intradermal challenge with *C. nubeculosus* extract in either group (LF mRNA relative to GAPDH  $p = 0.89$  and LF mRNA relative to  $\beta$ -actin  $p = 0.35$  in affected horses and LF mRNA relative to GAPDH  $p = 0.29$  and LF mRNA relative to  $\beta$ -actin  $p = 0.59$  in non-affected horses).

#### 4. Discussion

Lactoferrin in horses has been detected in seminal plasma, epididymidis, colostrum, plasma, neutrophils and endometrium (Barton et al., 2004; Kikuchi et al., 2003). Based on its anti-bacterial and immunomodulatory properties, LF serves as a first line of defense and is part of the innate immune system (Kijlstra, 1991; Brock, 1995). LF transcription and translation in the skin, an organ that serves as physiologic barrier between horse and environment with immunosurveillance capability, is therefore beneficial. Cutaneous LF is associated primarily with keratinocytes, but also with lymphocytes, neutrophils and mast cells. Our hypothesis that LF mRNA expression in non-lesional and non-challenge skin sites might be modified in CHS horses is based on a number of pieces of evidence. First, LF is able to enter mast cells by a

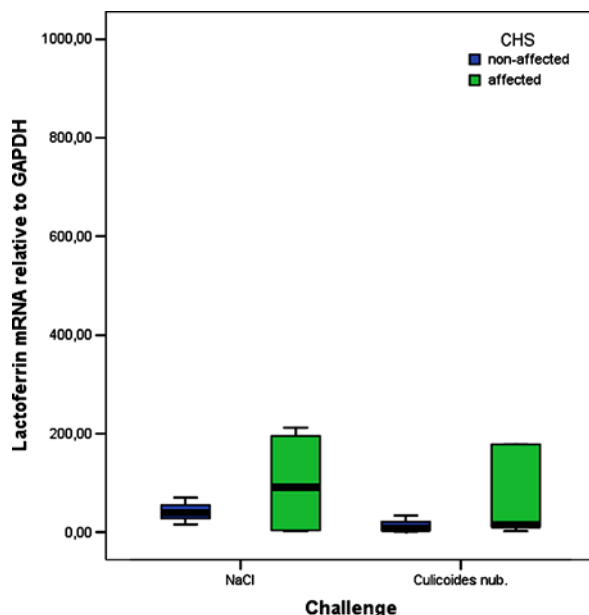


Fig. 1. Relative LF transcription normalised to GAPDH in five horses affected by CHS with positive IDT result (CHS affected) and three non-affected horses with negative IDT result (CHS non-affected) regarding *C. nubeculosus* challenged and control (NaCl) sites.

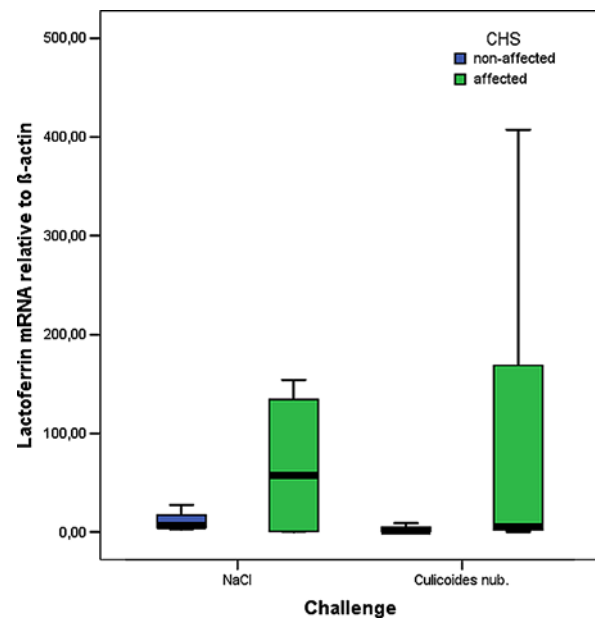


Fig. 2. Relative LF transcription normalised to  $\beta$ -actin in five horses affected by CHS with positive IDT result (CHS affected) and three non-affected horses with negative IDT result (CHS non-affected) regarding *C. nubeculosus* challenged and control (NaCl) sites.

mechanism that is still to be clarified. Pre-incubation of human skin mast cells with LF results in the inhibition of IgE-dependent histamine release by up to 50%, in tonsil mast cells by up to 30% and no inhibition in lung mast cells. The effect in skin mast cells may be mediated by interaction with mast cell proteases, in particular tryptase (He et al., 2003). Allergen specific (s-) IgE is a hallmark of CHS. The level of circulating s-IgE and IgE bound to skin cells is significantly influenced by the level of allergen exposure in horses suffering from CHS (Wilson et al., 2001; Van der Haegen et al., 2001). Histamine is released immediately after degranulation of mast cells and is responsible primarily for the cutaneous early phase reaction upon intradermal injection of *Culicoides* extract. Second, as CHS affected horses also exhibit a late phase of type I hypersensitivity reaction upon allergen challenge it is likely that T helper cells are key components in the pathogenesis of CHS (Scott and Miller, 2003). T cells express functional LF receptors that bind locally produced LF. It has been shown in humans that all major lymphocyte subsets, including alpha beta T cells, gamma delta T cells, CD8 + T cells, CD4 + T cells, B cells and NK cells, express LF receptors after activation, while un-stimulated blood lymphocytes do not (Mincheva-Nilsson et al., 1997). However, we did not find a significant difference between CHS affected and non-affected horses in non-allergen challenged skin, and, therefore, our data do not indicate that disturbances of LF, at least at the transcriptional level, might favour the allergic events in CHS.

In human skin allergen challenged sites LF levels increase progressively during the cutaneous late-phase reaction and are significantly different to that of control

sites at the fifth and sixth hour post-injection (Zweimann and von Allmen, 2000). Both late phase reaction and local inflammatory cell responses in the skin start soon after the immediate mast cell activation in IgE-mediated responses. Such inflammatory responses include leukocyte activation and mediator release, predominantly TNF- $\alpha$  (Ward et al., 2002; Griffiths et al., 2001). LF inhibits allergen-induced skin inflammation in both mice and humans, and this is likely due to suppression of local TNF- $\alpha$  production (Griffiths et al., 2001; Cumberbatch et al., 2003). Furthermore, LF is able to down regulate T cell proliferative responsiveness, chemokine receptor expression and cytokine production (Moed et al., 2004). In order to examine whether LF is also associated with anti-inflammatory processes during late phase reaction in CHS we challenged horses affected and non-affected by CHS with *C. nubeculosus* extract and examined the skin biopsies four hours post-inoculation. Previous studies reported on inconsistencies regarding potency and cross-reactivity of commercial *Culicoides* extracts (Fadok and Greiner, 1990; Kolm-Stark and Wagner, 2002). The challenged sites of the five horses affected by CHS in our study were 4+ positive for *C. nubeculosus*, whereas there was no cutaneous response to *C. nubeculosus* extract in the three horses without history or clinical signs indicative of CHS. The commercial extract used in the present study was made from the whole body of laboratory raised *C. nubeculosus* that is a European species prevailing in Austria.

We observed that LF transcription in CHS horses following allergen challenge was similar to that in non-challenged sites and was not significantly different to that in non-affected horses. It may therefore be speculated that LF does not function as an anti-inflammatory component in horse skin during the late phase reaction. Up-regulation of LF mRNA under inflammatory conditions in other equine tissues, such as the endometrium, has been demonstrated by our group (Kolm et al., 2006a). However, we only studied LF mRNA levels at 4 h post-injection during the late phase cutaneous reaction and observations on the kinetics might have revealed statistically significant differences at other time points. Furthermore, LF mRNA levels may not be correlated with LF protein levels as has been shown in other organs (Walmer et al., 1992). Finally, even if endogenous LF is not increased during the late phase reaction in CHS, this may not exclude a possible protective function of applied LF. Thus, exogenous LF may not only cause a reduction in the number of infiltrating inflammatory cells during the late phase reaction, but may also be actively taken up and stored in mast cells and inhibit IgE-dependent release of histamine (He et al., 2003). However, there might be a bias in our data due to the small sample size. Increasing the total horse number from 8 to 20 considering same power of 0.8 (single sided) and alpha of 0.05 in our study might have resulted in a statistically significant difference between CHS affected and non-affected horses prior challenge with *C. nubeculosus* (Sachs, 1999). In order to demonstrate statistical difference between prior- and post-challenge LF

mRNA expression in CHS affected horses, the sample size must have been increased to 120 and 60 for the normalisation to  $\beta$ -actin and GAPDH, respectively.

In conclusion, the present study demonstrates that LF mRNA is expressed in equine skin irrespective of whether horses suffer from CHS or not. In contrast to humans, late phase cutaneous reaction upon intradermal challenge with *Culicoides* extract at 4 h post-inoculation is not associated with increased LF mRNA levels in horses affected by CHS. Therefore, at the present time our results do not indicate that modified LF transcription is significantly implicated in the pathogenesis of CHS. However, whether horses affected by CHS might benefit from LF treatment remains to be examined.

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