

Characterization of the inflammatory infiltrate and cytokine expression in the skin of horses with recurrent urticaria

Sandro Hinden*, Jolanta Klukowska-Rötzler†, Jozef Janda†, Eliane I. Marti†‡, Vinzenz Gerber§ and Petra J. Roosje*‡

*Division of Clinical Dermatology and †Department of Clinical Research–Veterinary Public Health, Vetsuisse Faculty, University of Bern, PO Box 8466, 3001 Bern, Switzerland

‡Dermfocus, Vetsuisse Faculty, University of Bern, PO Box 8466, 3001 Bern, Switzerland

§Equine Clinic, Department of Clinical Veterinary Medicine, Vetsuisse Faculty, University of Bern, PO Box 8466, 3001 Bern, Switzerland

Correspondence: Petra J. Roosje, Division of Clinical Dermatology, Department of Clinical Veterinary Medicine, Vetsuisse Faculty, University of Bern, PO Box 8466, 3001 Bern, Switzerland. E-mail: petra.roosje@vetsuisse.unibe.ch

Background – Recurrent urticaria (RU) is a common skin disease of horses, but little is known about its pathogenesis.

Hypothesis/Objective – The aim of this study was to characterize the inflammatory cell infiltrate and cytokine expression pattern in the skin of horses with RU.

Animals – Biopsies of lesional and nonlesional skin of horses with RU ($n = 8$) and of skin from healthy control horses ($n = 8$) were evaluated.

Methods – The inflammatory cell infiltrate was analysed by routine histology. Immunohistochemistry was used to identify T cells (CD3), B cells (CD79), macrophages (MAC387) and mast cells (tryptase). Expression of T-helper 2 cytokines (interleukins *IL-4*, *IL-5* and *IL-13*), a T-helper 1 cytokine (interferon- γ), IL-4 receptor α and thymic stromal lymphopoietin was assessed by quantitative RT-PCR.

Results – In subepidermal lesional skin of RU-affected horses, increased numbers of eosinophils ($P \leq 0.01$), CD79-positive ($P \leq 0.01$), MAC387-positive ($P \leq 0.01$) and tryptase-positive cells ($P \leq 0.05$) were found compared with healthy horses. Subepidermal lesional skin of RU-affected horses contained more eosinophils ($P \leq 0.05$) and tryptase-positive cells ($P \leq 0.05$) compared with nonlesional skin. There was no significant difference in infiltrating cells between nonlesional skin and skin of healthy horses. Expression of *IL-4* ($P \leq 0.01$), *IL-13* ($P \leq 0.05$), thymic stromal lymphopoietin ($P \leq 0.05$) and IL-4 receptor α ($P \leq 0.05$) was increased in lesional skin of RU-affected horses compared with control horses. Expression of *IL-4* was higher ($P \leq 0.05$) in lesional compared with nonlesional RU skin.

Conclusions and clinical importance – Analysis of cytokine expression and inflammatory infiltrate suggests that T-helper 2 cytokines, eosinophils, mast cells and presumptive macrophages play a role in the pathogenesis of equine RU.

Introduction

Urticaria (hives) is a common cutaneous reaction pattern characterized by transient wheals in the skin in humans and horses. Lesions appear suddenly and typically regress within a day, but occurrence of new lesions may persist over a longer period.

In humans, a classification of urticaria is used on the basis of its duration, frequency and causes.¹ A distinction is made between acute urticaria and chronic urticaria;

however, this is not defined in horses. In humans, urticaria is considered chronic when symptoms have been present continuously or intermittently for at least 6 weeks.^{1,2} Equine urticaria is a clinical sign with various causes. In a subgroup of horses with urticaria, persistent wheal formation occurs, while others may show recurrent episodes. Urticarial signs are usually abrogated by a favourable response to glucocorticoids or antihistamines, but lesions can recur when treatment is stopped. Lesions and their treatment make these horses often unsuitable for working purposes. Urticaria, with or without pruritus, can result from many different stimuli of immunological and nonimmunological nature.

Recently, there have been significant advances in the understanding of several molecular mechanisms underlying the cutaneous inflammation that manifests as chronic urticaria and angioedema in human medicine.³ Chronic urticaria in humans is often divided into autoimmune

Accepted 24 August 2012

Sources of Funding: This study was funded by an ESVD grant and a Swiss National Science Foundation grant (no. 310030_129837/1).

Conflict of Interest: No conflicts of interest have been declared. Part of this study was published as an abstract: Vet Dermatol 2009; 21: 210.

chronic urticaria (45%) and chronic idiopathic urticaria (55%) after exclusion of physical urticaria and urticarial vasculitis.² Autoantibodies against the high-affinity IgE receptor (FcεR1α subunit) and against IgE are associated with autoimmune chronic urticaria.² Different mechanisms eventually lead to mast cell and basophil degranulation. In lesional skin, a significantly increased expression of the interleukins IL-4 and IL-5 was reported in immunohistochemical studies in humans.⁴ Another study described an increase in cells expressing *IL-4*, *IL-5* and interferon-γ (*IFN-γ*) mRNA and concluded the presence of a T-helper 0 cytokine profile.⁵

The pathophysiology of urticaria in horses is still poorly understood, and it is currently not known whether the equine immune response shows a pattern similar to that in humans. One study reported on the clinical aspects and identification of mast cells and IgE-bearing cells by immunohistochemistry in skin biopsies of horses with a history of urticaria.⁶ This study revealed that in most of the investigated horses the clinical signs were recurring and were persistent for more than 1 week; moreover, these horses had significantly more IgE-bearing cells in the subepidermal dermis of lesional skin than the control group.⁶

Recently, thymic stromal lymphopoietin (TSLP) has been described to play a central role in T-helper 2 (Th2) differentiation in humans with atopic dermatitis and asthma.⁷ Thymic stromal lymphopoietin, produced by epithelial cells, basophils and mast cells, drives Th2 differentiation of naïve T cells through the activation of dendritic cells.⁸ Conditional overexpression of TSLP in the skin of transgenic mice resulted in atopic dermatitis-like skin disease and a systemic Th2 response.⁹ Intradermal injection of TSLP protein produced similar results.¹⁰ The equine *TSLP* gene has been characterized¹¹ and an increased expression of TSLP has been observed in bronchoalveolar lavage cells isolated from horses with recurrent airway obstruction.¹²

The primary focus of this study was to characterize the cellular infiltrate and cytokine profile in the skin of horses with recurrent urticaria (RU) further, in order to elucidate its immunopathogenesis. We used histological examination and immunohistochemistry to evaluate T cells, B cells, macrophages and mast cells. Furthermore, we used quantitative real-time RT-PCRs to determine expression of gene transcripts for Th2- and T-helper 1-type cytokines.

Materials and methods

Sample collection

The study was performed according to guidelines by the Committee on Animal Care and Use of the Canton of Bern. Eight horses with RU were recruited. Recurrent urticaria was defined as at least two periods of urticaria or recurrence of urticaria after glucocorticoid therapy. In all of these horses, urticaria-inducing factors such as drugs, exercise or physical factors (cold, heat and water) were excluded. Horses with a history and clinical signs compatible with insect-bite hypersensitivity were excluded. These eight RU horses ranged in age from 2 to 13 years. Diagnosis was based on history, clinical signs and histological reaction patterns. Skin biopsies were collected as part of the diagnostic work-up. Control samples were taken for other purposes from four healthy horses without history of dermatological problems

belonging to the equine clinic, as well as from four adult horses without skin problems from a slaughterhouse. The healthy clinic horses ranged in age from 7 to 15 years. Horses from the slaughterhouse were adult horses judged to be of comparable age based on examination of the teeth. They were examined for the presence of skin problems after death.

Six-millimetre punch biopsies were taken from lesional and nonlesional skin on the neck or trunk in horses with RU after sedation with medetomidine hydrochloride (0.007 mg/kg intravenously; Domitor; Provect Ag, Lyssach, Switzerland) in combination with butorphanol tartrate (0.025 mg/kg intravenously, Morphasol; Dr. E. Graeb AG, Bern, Switzerland) and local subcutaneous injection of lidocaine (Lidocain 2%, Streuli ad us. vet.; Streuli Pharma AG, Uznach, Switzerland). From the healthy control horses, skin biopsies were taken from comparable sites. Biopsies were cut in half and one half was fixed in 4% buffered formaldehyde solution for approximately 24 h before paraffin embedding, while the other half was submerged in RNAlater® (Sigma-Aldrich, St Louis, MO, USA) and stored at -80°C until further usage.

Histopathology and immunohistochemistry

Morphometric analysis was performed under a light microscope at ×400 magnification, using a square eyepiece reticule (0.296 mm × 0.296 mm). The dermis was subdivided into subepidermal dermis and lower dermis. The subepidermal or upper dermis was defined as the dermal part underneath the epidermis corresponding to one side of the square reticule (0.296 mm). Cells were counted in six adjacent fields. The lower dermis corresponded to an area of 2 × 6 adjacent square reticules located immediately below the subepidermal dermis.

Eosinophilic granulocytes were evaluated in sections stained with haematoxylin and eosin (H&E). The number of positive cells was calculated per square millimetre.

Prior to immunohistochemical staining of serial sections, slides were deparaffinized in xylene, fixed in 100% ethanol and left for 15 min in 3% hydrogen peroxide diluted 10 times in methanol to quench endogenous peroxidase. Antigen retrieval was adapted to the primary antibodies; CD3 was left in 0.5% trypsin/chymotrypsin for 15 min at 37°C, and CD79 and MAC387 were microwaved for 15 min in 0.1 M citric acid (pH 6.0). Slides were incubated with 5% normal goat serum for 30 min to block nonspecific binding sites. Tissue sections were rinsed in phosphate-buffered saline. Details of the primary antibodies are described in Table 1. As a secondary antibody, a peroxide-based visualization kit [Dako REAL™ Detection Systems (LSAB+), Carpinteria, CA, USA] with 3-amino-9-ethylcarbazole (Dako) as chromogen was used. After this procedure, slides were counterstained in haematoxylin and mounted with Glycergel (Dako). All described staining methods and antibodies are established for clinical diagnosis in our laboratory. Negative controls produced by omission of the first antibody were run in each staining batch. An equine lymph node served as a positive control tissue.

Extraction of RNA and real-time PCR

Total RNA from the lesional and control skin biopsies was isolated with Rneasy™ Fibrous Tissue Kit (Qiagen AG, Hombrechtikon, Switzerland) according to the manufacturer's recommendations, including homogenization of the skin samples using TissueLyser™ (Qiagen AG). The concentration of RNA was quantified spectrophotometrically at 260 nm (NanoDrop 1000; Thermo Scientific, Reinach, Switzerland).

Genomic DNA contamination was eliminated by DNase treatment included in the kit and the absence of DNA contamination was confirmed by performing quantitative RT-PCR of the ubiquitin (*UBB*) gene in the absence of reverse transcriptase. Synthesis of complementary DNA (cDNA) was performed using the Omniscript RT Kit (Qiagen AG) according to the manufacturer's protocol, and cDNA samples were stored at -20°C until analysis. To assess gene expression, quantitative real-time PCR was performed using the 7300 Real Time PCR System (Applied Biosystems, Zug, Switzerland). The SYBR Green PCR Kit (Qiagen AG) was used to quantify *IL-5*, *TSLP*,

Table 1. Details of the primary antibodies and immunohistochemical techniques

Specificity	pAb/mAb (clone)	Origin	Dilution	Incubation	Source
Human CD3e	pAb	Rabbit	1:200	2 h, room temperature	Neomarkers, Thermo Fisher Scientific, Astmoor, UK
Human CD79	mAb (HM57)	Mouse	1:25	18 h, 4°C	Dako, Carpinteria, CA, USA
Human tryptase	pAb	Rabbit	1:3000	18 h, 4°C	Professor I. Harvima, Kupio, Finland ²⁹
Human calprotectin	mAb (Mac387)	Mouse	1:400	18 h, 4°C	Dako, Carpinteria, CA, USA

Abbreviations: mAb, monoclonal antibody; and pAb, polyclonal antibody.

interleukin-4 receptor α (*IL-4R α*) and *UBB*. TaqMan Universal PCR Mastermix (Applied Biosystems, Austin, TX, USA) was used to quantify *IL-4*, *IL-13* and *IFN- γ* . Primers and probes for all genes are summarized in Table 2. The PCRs were performed in a total volume of 25 μ L, and 2 μ L cDNA was used per reaction. All samples were analysed in triplicate, and each run included a negative control containing no cDNA. Efficiencies were calculated using a relative standard curve derived from a cDNA pool of all samples. Gene expression was quantified using the $\Delta\Delta C_t$ method (User Bulletin no. 2, P/N 4303849, Applied Biosystems). Expression of each gene of interest was normalized to the expression of the housekeeping gene *UBB*.

Statistical analyses

Statistical analyses were carried out with the software package NCSS 2001 (NCSS, Kaysville, UT, USA). As the data were not normally distributed, the Mann-Whitney *U*-test was used to compare values from RU-affected horses with those from healthy control animals. The nonparametric paired Wilcoxon signed-rank test was used for comparison of paired data (lesional/nonlesional). The threshold value for statistical significance was set at $P \leq 0.05$.

Table 2. Sequences of primers and probes used for cytokine quantification by real-time RT-PCR

Gene	Primer /probe	Sequence (5'-3')	Reference
<i>IL-4</i>	Forward primer	CGTGCATGGAGCTGACTGTAG	Davis <i>et al.</i> ³⁰
	Reverse primer	CAGCCCTGCAGATTCCTTT	
	Probe	FAM-ATGCCTTTGGCCCGAAGAAC ACAGA-TAMRA	
<i>IL-4Rα</i>	Forward primer	CCAGGAACAGGCTCTCTGTC	Not published
	Reverse primer	AGGTCAACCACACGATCCTC	
<i>IL-5</i>	Forward primer	TGCTTCTGCATTGA	Davis <i>et al.</i> ³⁰
	Reverse primer	GTGTGCTA TGTCATGGGGACTT TCTACAGCA	
<i>IL-13</i>	Forward primer	CTGGAGTCCTGAGCAACGT	Ryhner <i>et al.</i> ³¹
	Reverse primer	AGGGCAGAGTTT AGTCAGCATCTT	
<i>IFN-γ</i>	Forward primer	AAGTGAACCTCA	Davis <i>et al.</i> ³⁰
	Reverse primer	TCAAAGTGATGAATGA	
	Probe	CGAAATGGATTCTGACTCCTCTTC FAM -TCGCCCAAGCTAACCTGAGGAAGC-TAMRA	
<i>TSLP</i>	Forward primer	CCACAGTAGACAG	Klukowska -Rötzel <i>et al.</i> ¹¹
	Reverse primer	TGTGGTTGAA TAGGGCTGGTGCTGACCTAC	
<i>UBB</i>	Forward primer	ATGTGAAGGCCAAGATCCAG	Bogaert <i>et al.</i> ³²
	Reverse primer	AAATCTGCATCCACCTCTTG	

Results

Histopathology and immunohistochemistry

All horses had histopathological changes compatible with a diagnosis of equine urticaria, consisting of variable dermal oedema, with a perivascular infiltrate containing eosinophils and lymphocytes (Figure 1). In many sections, there was only a minimal perivascular infiltrate.

Sections from six horses with RU and seven control horses were available for immunohistochemical evaluation. In the immunohistochemical study, only rare single cells in the epidermis were positive for the stainings performed. Thus, we did not assess the incidental positive staining in the epidermis for the statistical evaluation of the immunohistochemistry.

The results of the distribution of eosinophils and cells identified by immunohistochemical staining are listed in Table 3. In H&E-stained sections, we found a significant increase in inflammatory eosinophilic granulocyte numbers in the subepidermal (upper) and lower dermis ($P \leq 0.01$ and $P \leq 0.05$, respectively) of the lesional skin of RU horses compared with healthy control animals. Moreover, increased eosinophil numbers were also observed when comparing the subepidermal dermis of lesional with nonlesional skin from RU horses ($P \leq 0.05$).

A significant increase in tryptase-positive cells was noticed in the subepidermal dermis of lesional skin compared with nonlesional skin of RU-affected horses ($P \leq 0.05$), as well as compared with skin of healthy control horses ($P \leq 0.05$); however, no significant differences were found in the lower dermis. Likewise, the number of CD79-positive cells, i.e. B cells, in RU lesions was increased significantly only in the subepidermal but not in the lower dermis compared with skin from healthy control animals. The number of MAC387-positive cells was greatly increased in the subepidermal dermis of lesional RU skin compared with skin from healthy horses (median = 61 versus 6 cells/mm²; $P \leq 0.01$). Although not as striking, the number of MAC387-positive cells was also significantly increased in the lower dermis of RU lesions compared with the skin from healthy horses (median = 6 versus 0 cells/mm²; $P \leq 0.01$). No significant differences were observed in the number of CD3-positive cells, when comparing the different skin sites in RU-affected and healthy control horses.

Relative quantitative RT-PCR

Expression of *IL-4*, *IL-13*, *TSLP* and *IL-4R α* was significantly higher in lesional skin of RU-affected horses than in the skin of healthy control horses (Figure 2). Moreover, there was significantly higher expression of *IL-4* in lesional skin compared with nonlesional skin of RU-affected

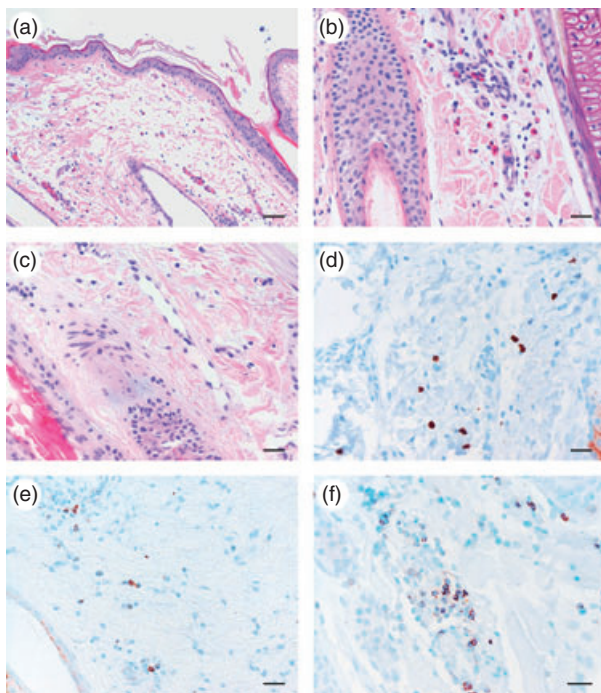


Figure 1. Skin sections of lesional skin of a horse with recurrent urticaria. (a) Overview, with oedema and a sparse dermal infiltrate. Scale bar represents 120 μ m. (b) Subepidermal oedema and scattered cells, with prominent eosinophilic granulocytes. Haematoxylin and eosin staining. (c) Perivascular infiltrate with lymphocytes. Haematoxylin and eosin staining. (d) Immunolabelling for MAC387. (e) Immunolabelling for CD79. (f) Immunolabelling for CD3. Scale bars represent 60 μ m in (b)–(f).

horses. There was no difference in expression of *IL-5* or *IFN- γ* between healthy and RU-affected horses.

Discussion

The purpose of this study was to characterize the cellular infiltrate further and to determine the cytokine pattern in the skin of horses with RU compared with healthy horses. As RU-affected horses were selected based on the clinical history and histopathological diagnosis, the histopathological findings in the H&E sections were comparable to what has been described previously.^{6,13,14} His-

tological evaluation of nonlesional skin was comparable to skin of healthy control horses. Although all horses had lesions for more than 1 week, it is likely that differences in chronicity of the lesional skin were present.

We observed a significant increase in the number of eosinophils in lesional skin of RU-affected horses compared with both nonlesional skin and skin of healthy horses. This is comparable to what is observed in acute skin lesions in equine insect-bite hypersensitivity.^{15,16} Also in human RU patients, increased numbers of eosinophils are found in lesional skin and are known to be involved in the pathogenesis of chronic urticaria.¹⁷

The significant increase of MAC387-positive cells in the subepidermal and lower dermis of lesional skin only was an interesting result. The antibody (MAC387) detects the protein calprotectin (L1) in the cytoplasm of human tissue macrophages but also in monocytes, histiocytes, neutrophils and other cells.^{18,19} In inflammatory processes in human tissue, the antibody is known to detect activated monocyte-derived macrophages in particular, and not all tissue macrophages.^{20,21} This can be explained by the fact that the monocyte/macrophage lineage is a very heterogeneous cell population, with structural and functional heterogeneity including phagocytosis, antigen processing and presentation, influenced by the local environment. A role of activated macrophages and/or dendritic cells in the immunopathogenesis of chronic idiopathic urticaria in humans is expected but not well known.^{22,23} In the veterinary field, this antibody is primarily used as a marker for macrophages in horses and other species. In a study by Parbhakar *et al.*,²⁴ alveolar macrophages stained with the MAC387 antibody in lung tissue that did not exhibit signs of inflammation. In a study on equine squamous cell carcinoma, this antibody identified macrophages but also neutrophils.²⁵ Comparison of the H&E staining with the immunohistochemistry for MAC387 revealed only few MAC387-positive cells but neither eosinophils nor neutrophils in control horses. Furthermore, in H&E-stained sections of RU-affected horses we found only incidental neutrophils, and we therefore propose that the majority of MAC387-positive cells were tissue macrophages.

We did not find any difference in numbers of T cells between skin samples of RU and control horses in this study using an antibody for CD3. However, this does not

Table 3. Median (range) of eosinophils (H&E staining) or of stained cells (immunohistochemistry) per square millimetre in the upper (subepidermal) and lower dermis in skin biopsies from seven healthy control horses and in lesional ($n = 6$) and nonlesional skin biopsies ($n = 6$) from six horses affected with recurrent urticaria

Skin biopsies	Eosinophils		Tryptase		CD3		CD79		MAC387	
	Upper dermis	Lower dermis	Upper dermis	Lower dermis	Upper dermis	Lower dermis	Upper dermis	Lower dermis	Upper dermis	Lower dermis
H	0 (0–24)	0 (0–23)	1 (0–4)	2 (1–4)	24 (3–30)	2 (1–4)	0.5 (0–2)	0.5 (0–5)	6 (3–8)	0 (0–3)
Urticaria, NL	0 (0–4)	0 (0–1)	0.5 (0–2)	1 (0–4)	13 (0–46)	2 (0–4)	2.5 (0–9)	0 (0–7)	10.5 (2–19)	1 (0–4)
Urticaria, L	171 (0–218)	51 (0–94)	4 (2–7)	3 (1–7)	9.5 (1–48)	3 (0–7)	7 (1–34)	5 (0–22)	61 (9–172)	6 (3–13)
Significance										
H versus L	≤ 0.01	≤ 0.05	≤ 0.05	n.s.	n.s.	n.s.	≤ 0.01	=0.08	≤ 0.01	≤ 0.01
H versus NL	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NL versus L	≤ 0.05	n.s.	≤ 0.05	n.s.	n.s.	n.s.	n.s.	n.s.	0.06	n.s.

Comparison of the results of the skin from healthy horses with those of lesional or nonlesional skin from urticaria-affected horses was performed using the Mann–Whitney *U*-test. Comparison of the results of lesional and nonlesional skin of individual horses was performed with the paired Wilcoxon signed-rank test. Abbreviations: H, healthy; L, lesional; NL, nonlesional; and n.s., not significant.

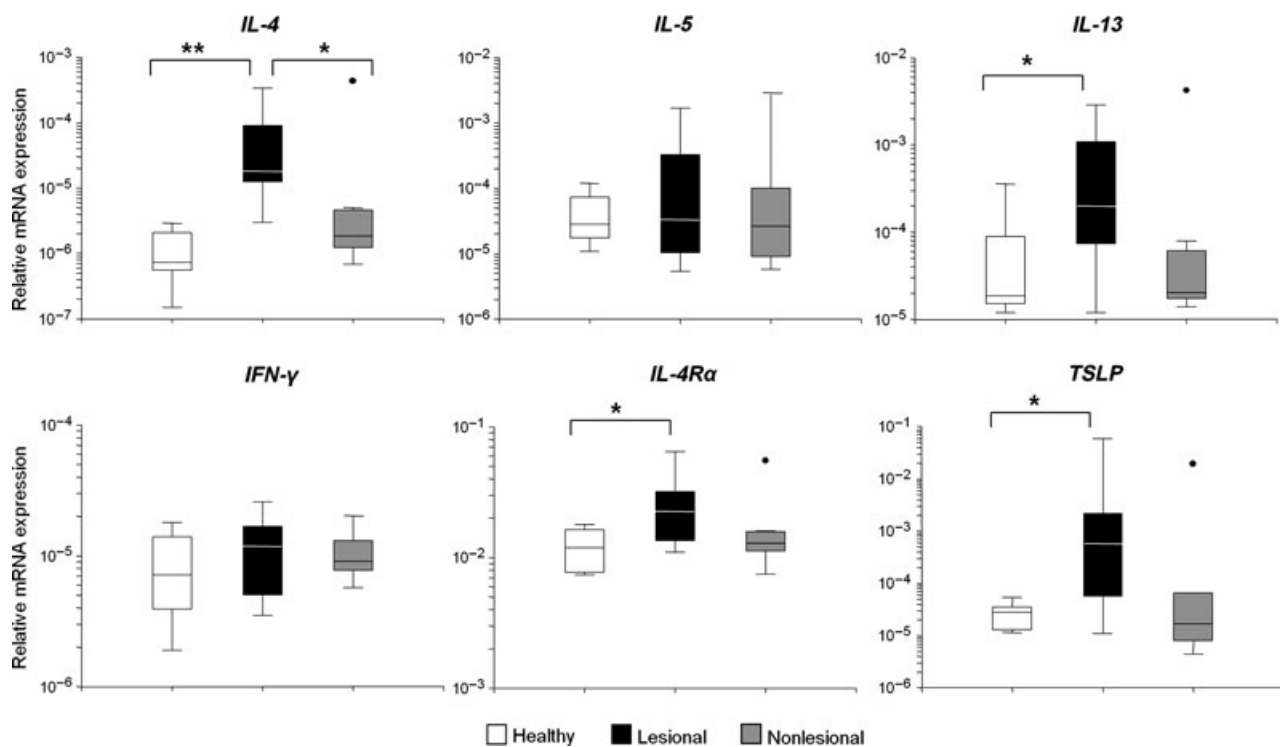


Figure 2. Cytokine expression in lesional and nonlesional skin of horses with recurrent urticaria ($n = 7$) and healthy horses ($n = 8$). Expression levels were assessed by quantitative real-time RT-PCR and normalized to the expression of the *UBB* gene. Comparison of values in the skin of healthy horses with lesional skin from recurrent urticaria-affected horses was performed using the Mann–Whitney *U*-test ($*P \leq 0.05$, $**P \leq 0.01$). Comparisons between lesional and nonlesional samples of RU-affected horses was performed using the paired Wilcoxon signed-rank test ($*P \leq 0.05$).

exclude an involvement of CD4-positive Th2 cells in RU, because the ratio between CD4-positive and CD8-positive T cells could still be altered and cells can be activated. We were not able to stain for CD4 because the available anti-equine CD4 antibody cannot be used on formalin-fixed, paraffin-embedded sections and cryosections were not available.

We observed a significantly increased number of mast cells in lesional skin of RU-affected horses compared with healthy horses with tryptase staining. This finding is in contrast to the previous study on equine urticaria, in which no difference was observed between urticaria-affected and control horses.⁶ This difference may be due to the fact that, in the present study, we excluded RU cases associated with drugs, exercise or physical factors, while this information was not available in the study by Rüfenacht *et al.*⁶ Moreover, the small sample number in the present study may have been of influence. Additional studies with well-defined cases grouped according to inducing factors could resolve the discrepancy in numbers of tryptase-positive mast cells and improve our understanding of the pathogenesis of equine RU.

Additionally, we counted an increased number of CD79-positive cells in lesional skin of RU-affected horses. This may be a bystander reaction or represent primarily a chronic inflammatory response. We did not discern plasma cells in H&E sections, and therefore we cannot comment on whether these CD79-positive cells were B lymphocytes or plasma cells. These CD79-positive cells were also reported in the dermal inflammatory infiltrate in horses with actinic keratosis and squamous cell carcinoma.²⁵

This antibody stained B cells located in lymphoid follicles of equine lymph nodes.²⁵

The results of quantitative real-time RT-PCR showed an approximately 100-fold higher *IL-4* mRNA expression in lesional RU skin compared with skin from healthy control horses, and *IL-4* expression was also significantly higher than in nonlesional skin of RU horses. In addition, there was a significantly increased expression of *IL-13*, *TSLP* and *IL-4Ra* in lesional skin of RU-affected horses compared with healthy horses, while there was no difference in the expression of *IFN-γ* between the groups (Figure 2). These results indicate that Th2 responses probably play an important role in the pathogenesis of equine RU. This is further supported by the increased numbers of tryptase-positive mast cells and eosinophils in lesional RU skin. In humans, infiltration with eosinophils is usually associated with increased *IL-5*.²⁶ In our study, we did not find increased *IL-5* mRNA in lesional RU skin samples, although they usually contained higher numbers of eosinophils. Similar findings have been observed in horses with insect-bite hypersensitivity.¹⁶ Factors other than *IL-5*, such as *TSLP*, might be responsible for eosinophil recruitment in equine allergies, given that *TSLP* is known to induce chemotactic and prosurvival effects in eosinophils.²⁷

The expression of *TSLP* was higher in lesional skin of horses with RU compared with skin of healthy control horses. This suggests that *TSLP* may play a role in the induction of Th2 responses in horses with RU. Further studies are required to identify the cellular sources and targets of this cytokine in horses. In humans and mice,

epithelial cells, mast cells and basophils have been described as major sources of TSLP.²⁸ In the present study, we observed increased numbers of mast cells, while a previous study has shown increased numbers of IgE-bearing cells in lesional skin of horses with urticaria.⁶ These cells could be one of the sources of TSLP, but also of other cytokines, such as IL-4 or IL-13.

In conclusion, the present analysis of the cellular infiltrate and cytokine expression in the skin of horses with RU indicates the involvement of Th2-mediated allergic inflammation accompanied by eosinophil, mast cell and macrophage infiltration in the pathogenesis of RU in horses.

Acknowledgements

We are grateful to Marcus Doherr for help with the statistical analyses. This study is dedicated to the memory of Christoph Prisi and Ursula Forster; we are grateful for their excellent technical assistance.

References

- Zuberbier T, Asero R, Bindslev-Jensen C *et al.* EAACI/GA(2)LEN/EDF/WAO guideline: definition, classification and diagnosis of urticaria. *Allergy* 2009; 64: 1417–1426.
- Kaplan AP, Greaves M. Pathogenesis of chronic urticaria. *Clin Exp Allergy* 2009; 39: 777–787.
- Vonakis BM, Saini SS. New concepts in chronic urticaria. *Curr Opin Immunol* 2008; 20: 709–716.
- Caproni M, Volpi W, Macchia D *et al.* Infiltrating cells and related cytokines in lesional skin of patients with chronic idiopathic urticaria and positive autologous serum skin test. *Exp Dermatol* 2003; 12: 621–628.
- Ying S, Kikuchi Y, Meng Q *et al.* TH1/TH2 cytokines and inflammatory cells in skin biopsy specimens from patients with chronic idiopathic urticaria: comparison with the allergen-induced late-phase cutaneous reaction. *J Allergy Clin Immunol* 2002; 109: 694–700.
- Rüfenacht S, Marti E, von Tscharner C *et al.* Immunoglobulin E-bearing cells and mast cells in skin biopsies of horses with urticaria. *Vet Dermatol* 2005; 16: 94–101.
- Holgate ST. The epithelium takes centre stage in asthma and atopic dermatitis. *Trends Immunol* 2007; 28: 248–251.
- Soumelis V, Reche PA, Kanzler H *et al.* Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol* 2002; 3: 673–680.
- Yoo J, Omori M, Gyarmati D *et al.* Spontaneous atopic dermatitis in mice expressing an inducible thymic stromal lymphopoietin transgene specifically in the skin. *J Exp Med* 2005; 202: 541–549.
- Jessup HK, Brewer AW, Omori M *et al.* Intradermal administration of thymic stromal lymphopoietin induces a T cell- and eosinophil-dependent systemic Th2 inflammatory response. *J Immunol* 2008; 181: 4311–4319.
- Klukowska-Rötzler J, Marti E, Bugno M *et al.* Molecular cloning and characterization of equine thymic stromal lymphopoietin. *Vet Immunol Immunopathol* 2010; 136: 346–349.
- Klukowska-Rötzler J, Marti E, Lavoie J-P. Expression of thymic stromal lymphopoietin in equine recurrent airway obstruction. *Vet Immunol Immunopathol* 2012; 146: 46–52.
- Scott DW, Miller WH. *Equine Dermatology*, 2nd edn. Missouri, MO, USA: Elsevier Saunders, 2011; 280–286.
- von Tscharner C, Kunkle G, Yager J. Immunologic diseases. *Vet Dermatol* 2000; 11: 163–178.
- van der Haegen A, Griot-Wenk M, Welle M *et al.* Immunoglobulin-E-bearing cells in skin biopsies of horses with insect bite hypersensitivity. *Equine Vet J* 2001; 33: 699–706.
- Heimann M, Janda J, Sigurdardottir OG *et al.* Skin-infiltrating T cells and cytokine expression in Icelandic horses affected with insect bite hypersensitivity: a possible role for regulatory T cells. *Vet Immunol Immunopathol* 2011; 140: 63–74.
- Cugno M, Marzano AV, Tedeschi A *et al.* Expression of tissue factor by eosinophils in patients with chronic urticaria. *Int Arch Allergy Immunol* 2009; 148: 170–174.
- Chilosi M, Mombello A, Montagna L *et al.* Multimarker immunohistochemical staining of calgranulins, chloroacetate esterase, and S100 for simultaneous demonstration of inflammatory cells on paraffin sections. *J Histochem Cytochem* 1990; 38: 1669–1675.
- Cerio R, Spaul J, Oliver GF *et al.* A study of factor XIIIa and MAC 387 immunolabeling in normal and pathological skin. *Am J Dermatopathol* 1990; 12: 221–233.
- Oshitani N, Sawa Y, Hara J *et al.* Functional and phenotypical activation of leucocytes in inflamed human colonic mucosa. *J Gastroenterol Hepatol* 1997; 12: 809–814.
- Bjerke K, Halstensen TS, Jahnsen F *et al.* Distribution of macrophages and granulocytes expressing L1 protein (calprotectin) in human Peyer's patches compared with normal ileal lamina propria and mesenteric lymph nodes. *Gut* 1993; 34: 1357–1363.
- Czarnetzki BM, Zwadlo-Klarwasser GZ, Bröcker EB *et al.* Macrophage subsets in different types of urticaria. *Arch Dermatol Res* 1990; 282: 93–97.
- Futata E, Azor M, Dos Santos J *et al.* Impaired IFN- α secretion by plasmacytoid dendritic cells induced by TLR9 activation in chronic idiopathic urticaria. *Br J Dermatol* 2011; 164: 1271–1279.
- Parbhakar OP, Duke T, Townsend HG *et al.* Immunophenotypic characterization and depletion of pulmonary intravascular macrophages of horses. *Vet Res* 2004; 35: 39–51.
- Perez J, Mozos E, Martin MP *et al.* Immunohistochemical study of the inflammatory infiltrate associated with equine squamous cell carcinoma. *J Comp Pathol* 1999; 121: 385–397.
- Kouro T, Takatsu K. IL-5- and eosinophil-mediated inflammation: from discovery to therapy. *Int Immunol* 2009; 21: 1303–1309.
- Wong CK, Hu S, Cheung PF *et al.* Thymic stromal lymphopoietin induces chemotactic pro-survival effects in eosinophils. Implications in allergic inflammation. *Am J Respir Cell Mol Biol* 2010; 43: 305–315.
- Liu YJ. Thymic stromal lymphopoietin: master switch for allergic inflammation. *J Exp Med* 2006; 203: 269–273.
- Harvima IT, Naukarinen A, Harvima RJ *et al.* Immunoperoxidase and enzyme histochemical demonstration of human tryptase in cutaneous mast cells in normal and mastocytoma skin. *Arch Dermatol Res* 1988; 280: 363–370.
- Davis MS, Malayer JR, Vandeventer L *et al.* Cold weather exercise and airway cytokine expression. *J Appl Physiol* 2005; 98: 2132–2136.
- Ryhner T, Müller N, Balmer V *et al.* Increased mucus accumulation in horses chronically affected with recurrent airway obstruction is not associated with up-regulation of CLCA1, EGFR, MUC5AC, Bcl-2, IL-13 and INF- γ expression. *Vet Immunol Immunopathol* 2008; 125: 8–17.
- Bogaert L, Van Poucke M, De Baere C *et al.* Selection of a set of reliable reference genes for quantitative real-time PCR in normal equine skin and in equine sarcoids. *BMC Biotechnol* 2006; 6: 24.

Résumé

Contexte – L'urticaire récidivant (RU) est une dermatose équine fréquente mais dont on connaît peu la pathogénie.

Hypothèses/objectif – Le but de cette étude était de déterminer le type de l'infiltrat inflammatoire cellu-

laire et le patron d'expression de cytokines de la peau de chevaux atteints de RU.

Sujets – Des biopsies cutanées lésionnelles et non-lésionnelles de chevaux atteints de RU ($n = 8$) et de peau de chevaux contrôles sains ($n = 8$) ont été testées.

Méthodes – L'infiltrat cellulaire inflammatoire a été analysé par histologie de routine. Les cellules T (CD3), B (CD79), les macrophages (MAC387) et les mastocytes (tryptase) ont été identifiées par immunohistochimie. L'expression des cytokines de T-helper 2 (interleukines *IL-4*, *IL-5* et *IL-13*), de T-helper 1 (interféron- γ), d'*IL-4* récepteur α et de lymphopoïétine stromale thymique a été évaluée par RT-PCR quantitative.

Résultats – Dans la peau subépidermique lésionnelle de chevaux atteints de RU, un nombre augmenté d'éosinophiles ($P \leq 0.01$), de cellules CD79-positives ($P \leq 0.01$), MAC387-positives ($P \leq 0.01$) et tryptase-positives ($P \leq 0.05$) a été observé en comparaison avec les chevaux sains. Les lésions subépidermiques cutanées de chevaux atteints de RU contenaient plus d'éosinophiles ($P \leq 0.05$) et de cellules tryptase-positives ($P \leq 0.05$) comparé à la peau non-lésionnelle. Il n'y avait pas de différence significative de l'infiltrat cellulaire entre la peau non-lésionnelle et la peau de chevaux sains. L'expression d'*IL-4* ($P \leq 0.01$), *IL-13* ($P \leq 0.05$), lymphopoïétine stromale thymique ($P \leq 0.05$) et d'*IL-4* récepteur α ($P \leq 0.05$) était augmentée dans la peau lésionnelle de chevaux atteints de RU comparé aux chevaux contrôles. L'expression d'*IL-4* était plus élevée ($P \leq 0.05$) dans la peau lésionnelle comparé à la peau non-lésionnelle de chevaux atteints de RU.

Conclusions et importance clinique – L'analyse de l'expression de cytokine et de l'infiltrat inflammatoire suggère que les cytokines T-helper 2, éosinophiles, de mastocytes et de macrophages présumés jouent un rôle dans la pathogénie de la RU équine.

Resumen

Introducción – la urticaria recurrente (RU) es una enfermedad común en caballos, pero se sabe poco acerca de su patogénesis.

Hipótesis/objetivo – el propósito de este estudio fue caracterizar el infiltrado celular inflamatorio y el patrón de expresión de citoquinas en la piel de caballos con RU.

Animales – se evaluaron biopsias de piel con y sin lesiones de caballos con RU ($n = 8$) y de piel de caballos control normales ($n = 8$).

Metodos – el infiltrado celular inflamatorio se analizó mediante histología básica. Se utilizaron técnicas de inmunohistoquímica para identificar los linfocitos T (CD3), linfocitos B (CD79), macrófagos (MAC387) y mastocitos (triptasa). La expresión de citoquinas de linfocitos ayudantes tipo 2 (*IL-4*, *IL-5* e *IL-13*) y de linfocitos ayudantes tipo 1 (interferon- γ), receptor α de *IL-4* y linfopoietina del estroma tímico fue investigada mediante RT-PCR.

Resultados – en la zona subepidermal de biopsias de piel con lesiones de caballos afectados por RU se observó un incremento en el número de eosinófilos ($P \leq 0.01$), células CD79 positivas ($P \leq 0.01$), células MAC387 positivas ($P \leq 0.01$) y células triptasa-positivas ($P \leq 0.05$) en comparación con la piel de caballos sanos. La zona subepidermal de las biopsias de piel con lesiones de caballos afectados con RU presentaron mayor número de eosinófilos ($P \leq 0.05$) y células triptasa-positivas ($P \leq 0.05$) comparado con zonas de piel sin lesiones. No hubo diferencias significativas entre las biopsias de piel sin lesiones y la piel de caballos sanos. La expresión de *IL-4* ($P \leq 0.01$), *IL-13* ($P \leq 0.05$), linfopoietina del estroma tímico ($P \leq 0.05$) y el receptor α de *IL-4* ($P \leq 0.05$) estuvo aumentada en las zonas de piel con lesiones de caballo afectados por RU comparado con los caballos control. La expresión de *IL-4* fue mayor ($P \leq 0.05$) en zonas de piel con lesiones comparados con zonas de piel sin lesiones en caballos con RU.

Conclusiones e importancia clínica – el análisis de la expresión de citoquinas y del infiltrado inflamatorio sugiere que las citoquinas de los linfocitos ayudantes tipo 2, eosinófilos, mastocitos y posiblemente macrófagos juegan un papel de importancia en la patogénesis de RU equina.

Zusammenfassung

Hintergrund – Wiederkehrende Urticaria (RU) stellen eine häufig auftretende Erkrankung des Pferdes dar, wobei über die Pathogenese dieser Erkrankung wenig bekannt ist.

Hypothese/Ziele – Das Ziel dieser Studie war die Beschreibung des entzündlichen Zellinfiltrates sowie des Expressionsmusters der Zytokine in der Haut von Pferden mit RU.

Tiere – Biopsien läsionaler und nicht-läsionaler Haut bei Pferden mit RU ($n = 8$) und gesunder Pferdehaut von Kontrolltieren ($n = 8$) wurden evaluiert.

Methoden – Das entzündliche Zellinfiltrat wurde mittels routinemäßiger Histologie analysiert. Die Immunhistochemie wurde eingesetzt, um T Zellen (CD3), B Zellen (CD79), Makrophagen (MAC387) und Mastzellen (Tryptase) zu identifizieren. Die Expression von Zytokinen der T2-Helferzellen (Interleukin *IL-4*, *IL-5* und *IL-13*), eines Zytokins der T1 Helferzellen (Interferon- γ), *IL-4* Rezeptor α und Thymic Stromal Lymphopoietin wurde mittels quantitativer RT-PCR untersucht.

Ergebnisse – In subepidermaler läsionaler Haut von RU-betroffenen Pferden befand sich im Vergleich zu gesunden Pferden eine erhöhte Anzahl an Eosinophilen ($P \leq 0.01$), CD79-positiven ($P \leq 0.01$), MAC387-positiven ($P \leq 0.01$) und Tryptase-positiven Zellen ($P \leq 0.05$). Die subepidermale läsionale Haut von RU-betroffenen Pferden zeigte im Vergleich zu nicht-läsionaler Haut eine höhere Anzahl an Eosinophilen ($P \leq 0.05$) und Tryptase-positiven Zellen ($P \leq 0.05$). Es bestand kein signifikanter Unterschied zwischen

den infiltrierenden Zellen der nicht-läsionalen Haut und der Haut der gesunden Pferde. Die Exprimierung von IL-4 ($P \leq 0,01$), IL-13 ($P \leq 0,05$), Thymic Stromal Lymphopoietin ($P \leq 0,05$) und IL-4 Rezeptor α ($P \leq 0,05$) war in der läsionalen Haut der RU-betroffenen Pferde im Vergleich zu den Kontrollpferden höher. Die Exprimierung von IL-4 war in läsionaler Haut im Vergleich zu nichtläsionaler RU Haut höher ($P \leq 0,05$).

Schlussfolgerungen und klinische Bedeutung – Die Analyse der Zytokinexprimierung und der Exprimierung des entzündlichen Infiltrates zeigt, dass die Zytokine der T2 Helferzellen, Eosinophile, Mastzellen und vermutlich Makrophagen bei der Pathogenese der RU des Pferdes eine Rolle spielen.

要約

背景–再発性蕁麻疹（RU）は馬で一般的な皮膚疾患であるが、その病因はほとんど知られていない。

仮説/目的–この研究の目的は、RUを生じた馬の皮膚における炎症性細胞浸潤とサイトカインの発現パターンの特徴を述べることである。

供与動物–RUを生じた馬（n=8）の病変部と非病変部ならびに健康な対照馬（n=8）から生検材料を採取し評価した。

方法–炎症性細胞浸潤は通常の組織学検査で解析した。免疫組織化学はT細胞（CD3 α ）、B細胞（CD79）、マクロファージ（MAC387）と肥満細胞（トリプターゼ）を特定するために行った。T-ヘルパー2サイトカインの発現（インターロイキンIL-4, IL-5, IL-13）、T-ヘルパー1サイトカイン（インターロイキン γ ）、IL-4レセプター α と胸腺間質性リンパ球新生因子を定量的RT-PCRで評価した。

結果–RUに罹患した馬の表皮下病変皮膚では、好酸球数（ $P \leq 0.01$ ）、CD79陽性細胞（ $P \leq 0.01$ ）、MAC387陽性細胞（ $P \leq 0.01$ ）とトリプシン陽性細胞（ $P \leq 0.05$ ）数が健康な馬と比較したときに増加していた。RUに罹患した表皮下病変を非病変部と比較するとより多くの好酸球（ $P \leq 0.05$ ）、トリプターゼ陽性細胞（ $P \leq 0.05$ ）が見られた。非病変部皮膚と健康な馬の皮膚の間に浸潤細胞の有意な差は認められなかった。IL-4（ $P \leq 0.01$ ）、IL-13（ $P \leq 0.05$ ）、胸腺間質性リンパ球新生因子（ $P \leq 0.05$ ）とIL-4レセプター α （ $P \leq 0.05$ ）の発現は、RUに罹患した馬の病変部皮膚で対照の馬と比べて増加していた。IL-4の発現は病変部で非病変部のRU皮膚より増加していた。（ $P \leq 0.05$ ）

結論と臨床的な重要性–サイトカイン発現解析と炎症性浸潤細胞はTヘルパー2サイトカイン、好酸球、肥満細胞とマクロファージと考えられる細胞が馬のRUの病因に役割を果たすことを示唆していた。

摘要

背景 – 复发性蕁麻疹（RU）是马常见的皮肤病，但发病机制知之甚少。

假设/目的 – 本研究旨在描述马RU皮肤炎症细胞浸润和细胞因子表达模式的特征。

动物 – 对RU患马病变或无病变皮肤进行活组织检查（n=8），并评估健康对照马的皮肤（n=8）。

方法 – 通过常规组织学分析炎症细胞浸润。使用免疫组化识别T细胞（CD3）、B细胞（CD79）、巨噬细胞（MAC387）和肥大细胞（类胰蛋白酶）。使用定量RT-PCR对Th 2细胞因子（白介素IL-4、IL-5和IL-13）、Th1细胞因子（ γ -干扰素）、IL-4 α 受体和胸腺基质淋巴细胞生成素表达进行评估。

结果 – 在RU马皮肤的皮下病变中，与健康马相比，发现嗜酸性粒细胞（ $P \leq 0.01$ ）、CD79阳性（ $P \leq 0.01$ ）、MAC387阳性（ $P \leq 0.01$ ）、类胰蛋白酶阳性细胞（ $P \leq 0.05$ ）数量增加。与无病变皮肤相比，RU马皮肤的皮下病变含有更多的嗜酸性粒细胞（ $P \leq 0.05$ ）和类胰蛋白酶阳性细胞（ $P \leq 0.05$ ）。细胞浸润在无病变皮肤和健康马皮肤之间没有显著差异。与对照马相比，在RU马病变皮肤中，IL-4（ $P \leq 0.01$ ）、IL-13（ $P \leq 0.05$ ）、胸腺基质淋巴细胞生成素（ $P \leq 0.05$ ）和IL-4 α 受体（ $P \leq 0.05$ ）表达增加。RU病变与无病变皮肤相比，IL-4表达更高（ $P \leq 0.05$ ）。

结论和临床价值 – 细胞因子表达和炎症细胞浸润分析表明，Th2细胞因子、嗜酸性粒细胞、肥大细胞和假定的巨噬细胞在马RU发病机制中起关键作用。