Identification, expression and characterisation of a major salivary allergen (Cul s 1) of the biting midge Culicoides sonorensis relevant for summer eczema in horses

Kathrin F.A. Langner a,*, Donald L. Jarvis b, Manfred Nimtz c, Julia E. Heselhaus a, Linda E. McHolland b, Wolfgang Leibold a, Barbara S. Drolet d

aImmunology Unit, University of Veterinary Medicine, Biscofschoder Damm 15, 30173 Hannover, Lower Saxony, Germany
bDepartment of Molecular Biology, University of Wyoming, P.O. Box 3944, Laramie, WY 82071-3944, USA
cHelmholtz Centre for Infection Research, Mascheroder Weg 1, 38124 Braunschweig, Germany
dUnited States Department of Agriculture, Agricultural Research Service, Arthropod-Borne Animal Diseases Research Laboratory, 1000 E. University Avenue, Laramie, Wyoming 82071, USA

* Corresponding author. Tel.: +49 511 856 7241; fax: +49 511 856 7682.
E-mail address: kathrin.langner@tiho-hannover.de (K.F.A. Langner).

1. Introduction

Biting midges of the genus Culicoides are the primary cause of a seasonal recurrent allergic dermatitis known colloquially as ‘sweet itch’ or ‘summer eczema’ (SE) in atopic horses worldwide (Anderson et al., 1988; Braverman 1988; Halldorsdottir and Larsen, 1991; Littlewood, 1998). Affected animals develop strong pruritus, alopecia, excoriations and sometimes secondary bacterial or fungal infections subsequent to the insect bites. The symptoms occur at the feeding sites of Culicoides spp. along the mane, withers and base of the tail. The allergic reaction is predominantly a type-I hypersensitivity mediated by IgE and possibly IgG isotypes (Wilson et al., 2001; Hellberg et al., 2006; Wagner et al., 2006). In addition, results from skin tests revealed that the antibody-dependent reaction is occasionally followed by delayed response (type-IV hypersensitivity) of allergen-specific T cells (Fadok and Greiner, 1990; Mckelvie et al., 1999; Ferroglialio et al., 2006).

Several species of the genus Culicoides have been shown to induce SE, including Culicoides sonorensis (North America), Culicoides nubeculosus (Europe), Culicoides obsoletus (North America, Europe), Culicoides insignis (South America) and Culicoides imicola (Africa) (Halldorsdottir et al., 1989; Fadok and Greiner, 1990; Anderson et al., 1993; Kaul, S., 1998. Type-I allergies in the horse: basic development of a histamine release test. Doctoral thesis. Veterinary School Hannover, Germany). Intradermal testing (IDT) and histamine release testing (HRT) with extracts and saliva of native and foreign Culicoides spp. revealed reactivity of allergic horses to all preparations, strongly indicating the presence of species-shared antigens (Fadok and Foil, 1990; Anderson et al., 1993; Langner et al. 2008). Moreover, it has been shown that horses are sensitised only to certain Culicoides spp. suggesting also that...
species-specific allergens may be involved in SE (Halldorsdottir et al., 1989; Kolm-Stark and Wagner, 2002). In addition to Culicoides spp., other hematopoietic insects such as black flies (Simulium spp.) and mosquitoes may contribute to the allergic reaction (Martì et al., 1999; Geiben, T., 2003. Studies on summer eczema and on the influence of the immunomodulator Baypamun N® on type-I allergy in horses. Doctoral thesis. Veterinary School Hanover, Germany; Baseligà et al., 2006). However, these insects are likely to play a minor role in pathogenesis since SE has not been reported in regions where hematopoietic insects occur, but Culicoides spp. are absent.

Thus far, the relevant allergens of Culicoides spp. are unknown. However, cellular and humoral immunooassays indicate that the allergens are most likely to be found in the insects’ saliva. Immunohistochemical analysis of the head and thorax of Culicoides spp. revealed that IgE from allergic horses preferentially binds to salivary gland structures (Wilson et al., 2001). Immunoblot analysis using salivary gland extracts of the insects demonstrated several potential allergens (Ferroglio et al., 2006; Hellberg et al., 2006; Wilson et al., 2008). In addition, artificially collected Culicoides salivary has been shown to be more sensitive in allergy tests when compared with whole body extracts (WBE) of Culicoides (Langner et al., 2008).

At present, treatment of SE is restricted to insect control or symptomatic therapy, the latter often involving long-term administration of corticosteroids. Immunotherapy trials utilising WBE of Culicoides species-specific allergens may be involved in the pathogenesis of the disease. Therefore, the potential success of future immunotherapies and accurate diagnosis of SE will rely on the identification and production of specific allergens involved in the pathogenesis of the disease.

In this study, we report the identification of a 66 kDa candidate allergen in the saliva of the North American species C. sonorensis by immunoblotting, protein fragmentation, mass spectrometry and bioinformatics. Furthermore, we describe cloning of the cDNA encoding the salivary protein, expression of the candidate allergen in the baculovirus/insect cell system, and we demonstrate the ability of the recombinant protein to bind serum IgE from SE-affected horses (SE+ horses) and to elicit allergic reactions in vivo and in vitro.

2. Materials and methods

2.1. Culicoides sonorensis and saliva collection

Saliva collection and preparation of Culicoides WBE were carried out as previously described (Langner et al., 2008) using colony-reared C. sonorensis (Jones and Foster, 1974). The bicinchoninic acid (BCA) protein assay (Perbio Science, Rockford, IL) was used to determine protein concentrations in saliva preparations and WBE according to the manufacturer’s instructions.

2.2. Horses, blood and serum sampling

A total of 16 horses (ages 7–19 years) maintained in a geographic area in Northern Germany endemic for several European Culicoides species were included in the study. Eight of the horses were affected by SE and showed symptoms of the allergy during several seasons. Eight horses with no clinical evidence of SE were used as control animals. For HRT, whole blood samples were collected from all horses in Vacutainer® K2E tubes (Becton Dickinson, Heidelberg, Germany) and used within 24 h. Serum samples for immunoblotting were collected in CAT tubes (Becton Dickinson). Sera were harvested after centrifugation at 1,000g for 10 min and stored at −20 °C until assayed.

2.3. Protein electrophoresis and immunoblotting

Salivary proteins and the purified recombinant allergen were analysed by SDS–PAGE (Laemmli, 1970) and immunoblotting (Towbin et al., 1979) using 5 μg (silver staining) or 25 μg (Western blotting) of salivary protein or 5 μg of recombinant allergen per lane. Gelcode Snap Silverstain (Perbio Science) was used to stain proteins as per manufacturer’s instructions. For immunoblotting membranes were incubated for 20 min at room temperature (RT) in blocking buffer (20 mM Tris–HCl, 500 mM NaCl (pH 7.5), 5% nonfat dry milk, 0.05% Tween 20), that also served as the diluent for sera and antibodies. Subsequent incubations were done at RT. For IgG detection, individual lanes were incubated with 1:10 dilutions of serum horse for 1 h, followed by a monoclonal antibody specific for equine IgE (Wagner et al., 2003) at 1:3 dilution of cell culture supernatants for 3 h and a 1:5,000 dilution of a horseradish peroxidase (HRP)-labelled anti-mouse IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA) for 1 h. For IgG detection, membranes were incubated with 1:50 dilutions of horse sera for 1 h, followed by a 1:5,000 dilution of a HRP-labelled anti-horse IgG antibody (Jackson Immunoresearch Laboratories) for 1 h. Binding of IgE and IgG, respectively, was detected after the addition of substrate (Immuno-Star HRP; Bio-Rad, Hercules, CA) and measurement of chemiluminescence (VersaDoc 4000, Bio-Rad). Relative molecular masses (M) were estimated by comparison with a protein standard (Invitrogen, Carlsbad, CA).

2.4. Mass spectrometry and protein identification

Salivary proteins were separated as above (see Section 2.3), gels were stained with Coomassie Brilliant Blue (Sigma–Aldrich, St. Louis, MO) and the protein band of interest was excised. Protein fragmentation and analysis of obtained peptides by Electrospray Ionization Quadrupole Time of Flight (QToF)-Tandem Mass Spectrometry was done as previously described (Langner et al., 2007). Amino acid sequences were deduced from carboxy-terminal fragment ion series. The resulting sequences were used to search the protein database UniProt (European Bioinformatics Institute, Cambridge, UK) with the FASTA program (Pearson and Lipman, 1988) to identify the candidate allergen that was designated as Cul s 1.

2.5. Construction of Cul s 1 baculovirus recombinant gene expression vector

Total mRNA was extracted from 100 female sucrose-fed C. sonorensis at 24 h post feeding using the MicroPurist PolyA Kit (Ambion, Austin, TX). Reverse transcription was performed with two custom primers (Csm 1F: 5′-TAATTAATCATTTAAGTGTAAT-3′ and Csm 1R: 5′-GATGAAAGATTTGGA-3′) designed from a nucleotide sequence of Cul s 1 (GenBank Accession No. AV603565). The cDNA coding for Cul s 1 including a native signal sequence of 1.83 kb was recovered from the vector. The Phusion Polymerase (Finnzymes, Espoo, Finland) was used to create blunt end amplicons as per the manufacturer’s protocol. The PCR products were analysed on 0.5% agarose gels. A specific amplification product of ~1.83 kb was recovered from the gels, cloned into pENTR/D-TOPO (Invitrogen), and a clone containing a sequence-verified copy of the Cul s 1 gene was used to
produce a recombinant baculovirus expression vector using the BaculoDirect™ C-Term Transfection Kit (Invitrogen) as per manufacturer’s instructions. Recombinant baculovirus clones resolved by plaque purification were examined by PCR with gene-specific primers to confirm the presence and correct size of the Cul s 1 gene. Confirmed clones were further amplified on Spodoptera frugiperda (Sf9) cells in serum free medium (Sf-900 II SFM, Gibco, Carlsbad, CA) to produce working viral stocks, which were titered by plaque assay and used for subsequent expression studies. All working viral stocks were supplemented with 2% (v/v) FBS (Gibco) for storage at 4 °C.

2.6. Production and purification of rCul s 1

To examine the time-course of recombinant Cul s 1 (rCul s 1) expression, Sf9 cells were infected with the recombinant baculovirus at a multiplicity of infection (MOI) of five plaque-forming units (pfu)/cell and incubated for various time periods. Insect cells and cell-free culture media were harvested at 24, 48, 72, 96 and 120 h.p.i. The cells were lysed with 62.5 mM Tris–HCl, pH 6.8 containing 2% SDS. Samples of both the cell-free media and cell lysates were analysed by Western blotting, as described above (Section 2.3). rCul s 1 was detected by chemiluminescence after incubation of the membranes with a 1:1,000 dilution of an anti-polyhistidine (His6)-specific antibody (Qiagen, Valencia, CA) for 1 h followed by a 1:5,000 dilution of a HR- labelled anti-mouse IgG antibody (Jackson Immunoresearch Laboratories) for 1 h. For large-scale production of rCul s 1, suspension cultures of Sf9 cells were infected at a density of 1 × 10^6 cells/ml with an MOI of 5 pfu/cell. At 72 h.p.i., cell suspensions were centrifuged at 1,000g for 10 min at RT. The supernatants were harvested and concentrated using ultrafiltration units (Millipore, Billerica, MA). rCul s 1 was purified from the concentrates using cobalt affinity chromatography (Clontech, Mountain View, CA) according to the manufacturer’s instructions. Obtained fractions of purified rCul s 1 were analysed by SDS–PAGE and Coomasie Brilliant Blue staining and the elution buffer was exchanged with deionized water by ultrafiltration. The [His]6 tag was then cleaved using AcTEV™ protease (Invitrogen) as per the manufacturer’s protocol. The spent reaction was diluted in binding buffer and applied to a fresh cobalt affinity column. The flow-through containing the untagged rCul s 1 was concentrated and the binding buffer replaced by PBS by ultrafiltration. The size and purity of rCul s 1 were verified by SDS–PAGE as above and the protein concentration was determined using the BCA assay. The cleaved and re-purified rCul s 1 preparations were stored until use at 4 °C.

2.7. Reactivity of rCul s 1 with equine basophils using HRT

Basophil reactivity was determined by a modified method of Kaul (Kaul, S., 1998. Type-1 allergies in the horse: basic development of a histamine release test. Doctoral thesis. Veterinary School Hannover, Germany). Briefly, a 10-fold dilution series (ranging from 1 µg to 0.001 µg final concentration) of rCul s 1 and Culicoides WBE was prepared in PIPES buffer (AppliChem, Darmstadt, Germany), pH 7.4. Washed red and white blood cells obtained from Na–EDTA coagulation-inhibited blood were incubated with individual dilutions for 1 h at 37 °C. The reaction was stopped by incubation on ice for 20 min and the supernatant containing the released histamine was collected from each sample after centrifugation. The amount of histamine obtained for the maximal release was set at 100%, and the releases induced by samples and negative control were expressed as percentage of the maximal release. The cut-off for the discrimination between positive and negative reactions was determined as the mean of spontaneous releases plus their six-fold S.D. and equalled a histamine release of 9.4%. Thus, releases equal to or greater than 10% were considered positive.

2.8. Skin reactivity of rCul s 1 using IDT

The IDT was performed in January of 2008 in the absence of clinical SE in the 16 horses. The animals were clipped and disinfected with 70% ethanol at the dorsolateral cervical region. Recombinant Cul s 1 and Culicoides WBE as positive reference were prepared as a 10-fold dilution series in PBS ranging from 10 µg/ml to 0.01 µg/ml. Histamine (Sigma–Aldrich) was dissolved at a concentration of 0.2 mg/ml in PBS and served as positive control and PBS was used for the negative control. A volume of 100 µl of each preparation was injected intradermally using 1 ml syringes and 28 gauge needles. Skin reactions were assessed visually and by palpation at 30 min, 4 h and 24 h post injection. Evaluation was done according to the method described by Kolm-Stark and Wagner (2002). Briefly, scores ranging from 0 (reaction wheal equal to or smaller than negative control) to 4 (reaction wheal equal to or greater than positive control) were assigned to the injection sites of Culicoides preparations. Wheals with scores of 2 or greater were considered positive reactions.

3. Results

3.1. Identification of Cul s 1

Separation of the C. sonorensis salivary proteins on polyacrylamide gels revealed approximately 12 individual protein bands ranging from 90 to 14 kDa (Fig. 1). Sera from four SE+ horses and four control animals were used to detect IgE-binding proteins in the saliva (Fig. 1). Antibodies obtained from SE+ horses bound to a total of seven salivary proteins, with an individual recognition pattern for each horse. All allergic horses showed IgE-binding to...
a protein of ~66 kDa. Three SE+ horses had IgE specific for proteins of ~32 and 28 kDa and two horses had IgE specific for proteins of ~43 and 15 kDa. Other proteins detected by IgE from individual SE+ horses had M₀ of ~90 and 59 kDa. Serum IgE obtained from three out of four control horses failed to bind to any of the salivary proteins, but IgE from one control animal bound to three proteins of ~43, 32 and 28 kDa. The band intensities for the 66 and 32 kDa proteins varied between the responders.

The ~66 kDa protein was chosen for further characterisation since it was detected by IgE from SE+ horses, but not by antibodies from control animals. Tandem mass spectrometry analysis revealed eight peptides (Table 1), which indicated that this protein was a maltase (α-glucosidase) recently identified from a salivary gland cDNA library of C. sonorensis (Campbell et al., 2005). In total, the peptide sequences obtained in this analysis comprised 20.8% of the entire protein sequence of 602 amino acids. No evidence for the presence of any other proteins was found. Based upon its identification as an allergen, we designated this maltase as Cul s 1 according to the conventions of the WHO/IUIS Allergen Nomenclature Sub-Committee (King et al., 1994).

3.2. Expression and purification of rCul s 1

The BaculoDirect™ method was used to produce a clonally derived recombinant baculovirus encoding the Cul s 1 gene. Preliminary experiments in which this virus was used to infect Sf9 cells revealed that optimal rCul s 1 production was obtained using an MOI of 3 pfu/cell. The expression of rCul s 1 in Sf9 cells infected with the recombinant baculovirus for various time periods is shown in Fig. 2. Immunoblot analysis using an anti-[His]₆-specific antibody revealed a ~69 kDa protein that was detected in both the cell lysate and the cell-free supernatant. The estimated size of the protein is approximately the same as the predicted size of the C. sonorensis maltase protein (66 kDa) containing the protease cleavage site and C-terminal [His]₆ tag (Mᵣ of about 66 kDa) (Fig. 3B). With the exception of one allergic horse, all SE+ horses had IgE that reacted with rCul s 1 (Fig. 4A). The four sera of SE+ horses that revealed IgE reactivity with the native maltase (Fig. 1) also had specific antibodies to the recombinant protein. rCul s 1-specific IgE was detected in one of the sera of control animals. Immunoglobulin G with specificity for rCul s 1 was shown in sera of four SE+ horses and four control animals (Fig. 4B).

3.3. Determination of rCul s 1-specific antibodies in horse sera by immunoblotting

Sera from eight SE+ and eight control horses were evaluated for the presence of rCul s 1-specific IgE and IgG by immunoblotting. With the exception of one allergic horse, all SE+ horses had IgE that reacted with rCul s 1 (Fig. 4A). The four sera of SE+ horses that revealed IgE reactivity with the native maltase (Fig. 1) also had specific antibodies to the recombinant protein. rCul s 1-specific IgE was detected in one of the sera of control animals. Immunoglobulin G with specificity for rCul s 1 was shown in sera of four SE+ horses and four control animals (Fig. 4B).

3.4. Determination of rCul s 1 induced basophil reactivity using HRT

The HRT revealed basophil reactivity in all but one allergic horse (Table 2). All SE+ horses had a positive response with the Culicoides WBE. None of the samples from the control horses showed reactivity with rCul s 1 at any concentration, but samples from two of the control animals reacted with the highest concentration of the Culicoides WBE. The average amount of released histamine ± SEM for the two groups of horses is indicated as a percentage of the maximal release in Table 2.

3.5. Determination of rCul s 1 induced skin reactivity using IDT

Assessment of the skin test sites at 30 min post injection revealed immediate reactivity to rCul s 1 in seven of eight SE+ horses (Table 3). The same horses also had a positive response with the Culicoides WBE. None of the control animals showed a positive reaction with any of the preparations. At 4 h post injection, late-phase reactions with rCul s 1 and the WBE were seen in all SE+ horses (Table 4). One control horse showed late-phase reactivity with rCul s 1 but had no detectable rCul s 1-specific serum IgE or IgG. Several control animals had late-phase reactions with the Culicoides WBE. The individual scores and the average wheal diameter ± SEM at 30 min and 4 h post injection for allergic horses and control animals are summarised in Tables 3 and 4. Delayed reactivity at 24 h post injection with rCul s 1 and the WBE was seen in one SE+ horse. None of the control animals showed delayed reactivity with rCul s 1 but one horse of this group had a positive response.

### Table 1

<table>
<thead>
<tr>
<th>Protein Identification*</th>
<th>Peptide sequences**</th>
<th>% Identity***</th>
<th>Observed/ predicted Mᵣ****</th>
</tr>
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<tr>
<td>Maltase Culicoides sonorensis (Q66UC5_9DIPT)</td>
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<td>94</td>
<td>227TIVPDVQCTIDNLHAK131</td>
<td>94</td>
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</tbody>
</table>

* Proteins were identified by searching the UniProt database with peptide fragmentation data using the FASTA program (European Bioinformatics Institute, Cambridge, UK).
** Sequence information obtained from tandem mass spectrometry. The regions of the protein to which the peptides align are noted by superscripts indicating residue number.
*** The isobaric amino acids isoleucine (I) and leucine (L) cannot be distinguished using QToF mass spectrometry. Hence, I and L are interchangeable in the peptide sequences obtained in this analysis comprised 20.8% of the entire protein sequence of 602 amino acids. No evidence for the presence of any other proteins was found. Based upon its identification as an allergen, we designated this maltase as Cul s 1 according to the conventions of the WHO/IUIS Allergen Nomenclature Sub-Committee (King et al., 1994).
**** % Identity of obtained peptide sequences to proteins from the database.
***** Observed molecular masses (Mᵣ) in kDa estimated from gel spots and predicted Mᵣ, obtained from the database.
with the *Culicoides* WBE. Due to the absence of a wheal at the injection site of the histamine control no scores were calculated for the responses at 24 h post injection.

4. Discussion

The identification of allergens relevant to SE in horses has been the subject of many clinical and biochemical investigations (Morrow et al., 1986; Wilson et al., 2001, 2008; Althaus et al., 2004; Hellberg et al., 2006). Salivary proteins of *Culicoides* biting midges are thought to play a key role in the induction of the disease, but the precise nature of the allergens has remained unknown. The aim of this study was to identify a candidate allergen from artificially collected saliva of *C. sonorensis*. Key approaches inherent to this goal involved molecular cloning of a cDNA encoding the candidate allergen, the expression and purification of a recombinant form of the protein, and the further characterisation of the recombinant protein using in vitro and in vivo tests to determine its role in SE in horses.
Immunoblot analysis of Culicoides salivary proteins revealed seven different IgE-reactive bands indicating the presence of several allergens in the midge saliva (Fig. 1). Multiple allergens in the saliva of blood-sucking insects have been described previously for the mosquito Aedes aegypti (Peng and Simons, 2004). It has been shown that at least eight to nine of the 31 salivary proteins were bound by IgE of mosquito allergic individuals. Five of these candidate allergens have been expressed in a recombinant form and revealed strong in vitro and in vivo allergenicity. In Culicoides spp., IgE-binding proteins ranging in Mr from 200 to 6 kDa have been detected in WBE, thorax and salivary gland extracts of midges (Wilson et al., 2001, 2008; Ferroglio et al., 2006; Hellberg et al., 2006). Although horse antibodies were found to bind to a number of different proteins in the insect preparations, most studies consistently described IgE-reactive proteins of approximately 66/65, 50 and 40 kDa.

Here, we further investigated a specific 66 kDa protein that was identified as maltase, an enzyme involved in sugar meal digestion. Maltases are commonly found in the salivary glands of hematophagous insects such as mosquitoes and sand flies (Marinotti et al., 1996; Jacobson and Schlein, 2001). Recently, the maltase-like α1-glucosidase, a glycoprotein that was described to be present in high amounts in the saliva of A. aegypti, was identified as a major allergen in human hypersensitivity to mosquito bites (Peng and Simons, 2004). Genomic and proteomic analysis of different Culicoides spp. showed that maltase is one of the most abundant proteins in midge saliva along with D7-related proteins (Campbell et al., 2005; Wilson et al., 2008). In addition, the amino acid sequence of the Culicoides maltase included several glycosylation sites, a feature commonly associated with allergens (Huby et al., 2000). Due to their identification as potential allergens, the native and recombinant forms of Culicoides sonorensis maltase were re-named Cul s 1 and rCul s 1, respectively, according to the WHO/IUIS Allergy Nomenclature Sub-Committee (King et al., 1994).

A baculovirus-insect cell expression system was chosen for recombinant expression of Cul s 1 to provide posttranslational changes such as glycosylation. The importance of native glycosylation has been demonstrated for several recombinant allergens including a major allergen of the cat flea, Ctenocephalides felis and a hyaluronidase of bee venom (Soldatova et al., 1998; McDermott et al., 2000). Comparative expression of the proteins in bacteria and insect cells revealed the strongest reactivity in allergy tests for the insect cell-derived proteins. In this study, the Cul s 1 gene was introduced into a baculovirus vector that provided a [His]6 tag at the C-terminus of the recombinant protein. This enabled a one-step purification of the rCul s 1 by immobilized metal affinity.

Table 3
Results of intradermal testing for horses with summer eczema (n = 8) and control horses (n = 8) at 30 min post injection

<table>
<thead>
<tr>
<th>Preparation (µg)</th>
<th>SE+ horses</th>
<th>Control horses</th>
<th>Average diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1+ 2+ 3+ 4+</td>
<td>1+ 2+ 3+ 4+</td>
<td></td>
</tr>
<tr>
<td>Saline</td>
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<td>10.9 ± 0.0</td>
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<tr>
<td>Histamine</td>
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</tr>
<tr>
<td>Cul s 1</td>
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<td>12.3 ± 0.3</td>
</tr>
<tr>
<td>0.1</td>
<td>15.4 ± 0.8</td>
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<td>0.01</td>
<td>11.1 ± 0.3</td>
<td>10.8 ± 0.3</td>
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<tr>
<td>0.001</td>
<td>10.2 ± 0.1</td>
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<tr>
<td>WBE</td>
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<td>12.3 ± 0.3</td>
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<td>12.9 ± 0.4</td>
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<tr>
<td>0.01</td>
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<td>0.001</td>
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a SE+ horses, SE-affected horses.
b Diameter of skin reactions for each group of horses in mm, expressed as mean ± standard error.
c Cul s 1, recombinant Cul s 1.
d WBE, whole body extract Culicoides sonorensis.

Table 4
Results of intradermal testing for horses with summer eczema (n = 8) and control horses (n = 8) at 4 h post injection

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<th>Preparation (µg)</th>
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<th>Control horses</th>
<th>Average diameter (mm)</th>
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<tr>
<td>Saline</td>
<td>5.0 ± 0.2</td>
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<tr>
<td>Histamine</td>
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<td>Cul s 1</td>
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c Cul s 1, recombinant Cul s 1.
d WBE, whole body extract Culicoides sonorensis.
chromatography. Previous expression studies with allergens of mosquito saliva, honey bee venom and mould have shown that the reactivity of recombinant proteins was not affected by the tag (Dudler et al., 1992; Xu et al., 1998; Chow et al., 2000). However, to exclude the possibility of any artifacts, a protease cleavage site was introduced between the maltase sequence and the [His]$_n$ tag for cleavage from the protein prior to in vitro and in vivo experiments.

Immunoblot analysis of rCul s 1 revealed strong IgE-binding for the majority of sera obtained from SE+ horses, whereas only one control animal had rCul s 1-specific IgE (Fig. 4A). The participation of IgE in equine type-I allergies has been demonstrated recently by successful passive transfer of immediate skin reactivity to Culicoides preparations in modified Prausnitz–Küstner tests (Wagner et al., 2006). However, the discrimination of allergic and non-allergic horses based on their IgE levels often remained difficult when crude preparations were used (Eder et al., 2000; Lorch et al., 2001; Morgan et al., 2007). Our results indicate that rCul s 1 may be successfully used to diagnose SE in IgE-dependent allergy tests. By contrast, no differences between the two groups of horses were seen for rCul s 1-specific IgG detection (Fig. 4B). Similarly, the determination of IgG using recombinant mould allergens did not reveal significant differences between healthy horses with those with recurrent airway obstruction indicating lacking correlation between the serum levels of allergen-specific IgG and clinical disease in horses (Eder et al., 2000).

The HRT demonstrated the ability of rCul s 1 to induce histamine release from basophils obtained from SE+ horses (Table 2). However, one allergic horse failed to react with rCul s 1 at any concentration, but had a positive response with the WBE. This may confirm the sensitisation of SE+ horses to more than one Culicoides allergen as indicated by the multiplicity of IgE-reactive proteins in the native saliva (Fig. 1) and in salivary gland extracts (Hellberg et al., 2006; Wilson et al., 2008). By contrast to the Culicoides WBE, rCul s 1 did not induce positive reactions in the control animals. It has been shown previously in HRT and similar tests that insect WBE can provoke basophil degranulation in vitro in non-allergic horses, possibly due to lectins or cross-reactive proteins that can cause a cross-linkage of surface-bound antibodies (Baselgia et al., 2006; Langner et al., 2008). The use of pure allergen preparations as demonstrated for rCul s 1 may reduce non-specific cell activation and therefore the number of false-positive reactions in HRT.

Evaluation of the skin test sites at 30 min and 4 h revealed immediate and late-phase reactivity to rCul s 1 in SE+ horses (Table 3). Scores and/or numbers of positive reactions with both rCul s 1 and the Culicoides WBE, were typically higher at 4 h post injection than at 30 min post injection. None of the control horses had a positive response with rCul s 1 at 30 min post injection. However, one control animal showed late-phase reactivity to the recombinant allergen possibly due to subclinical sensitisation as described previously for flea-allergic dogs in flea-rich environments (LaFlort-Dassot et al., 2004). By contrast, several control horses had positive reactions with the WBE at 4 h post injection. It has been reported previously that non-allergic horses developed late-phase reactivity to a variety of allergen preparations such as insect extracts resulting in a poor discrimination of allergic horses and healthy animals (Lorch et al., 2001; Kolm-Stark and Wagner, 2002; Morris and Lindborg, 2003).

Whole body extracts contain a large number of different components that may cause inflammation in the absence of type-I-hypersensitivity mediated reactions to relevant allergens. Here, we demonstrate that rCul s 1 considerably reduced the number of false-positive results at 4 h post injection, indicating that the use of purified allergens in IDT may increase the specificity of late-phase reactions for diagnosing SE. At 24 h post injection, delayed reactivity (type-IV-hypersensitivity) to rCul s 1 was seen in one SE+ horse suggesting that rCul s 1 specific T cells may be present in individual horses. However, compared to immediate and late-phase reactions, delayed reactivity to rCul s 1 presumably plays a minor role for the induction of SE.

In conclusion, we believe rCul s 1 is the first allergen specifically associated with SE in horses to be identified, molecularly cloned and expressed in recombinant form. The purified recombinant protein had strong in vitro and in vivo allergenicity and, compared with insect WBE, provided an increased specificity in common allergy tests. In addition, our results demonstrated that rCul s 1 is a species-shared allergen. The horses used in the present study had no contact with C. sonorensis which is endemic in North America. However, all horses had been bitten by several European Culicoides species including C. nubeculosus, C. obsoletus and Culicoides pulicaris. The presence of species-shared allergens has been indicated previously for Culicoides spp. (Fadok and Foil, 1990; Anderson et al., 1993; Langner et al., 2008), and is directly demonstrated here for rCul s 1. Thus, the availability of rCul s 1 will greatly facilitate both the diagnosis of SE and the future development of immunotherapies for SE in horses.

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