Published in final edited form as:

Int J Parasitol. 2009 January ; 39(2): 243–250. doi:10.1016/j.ijpara.2008.06.008.

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

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Abstract

Salivary proteins of *Culicoides* biting midges are thought to play a key role in summer eczema (SE), a seasonal recurrent allergic dermatitis in horses. The present study describes the identification, expression and clinical relevance of a candidate allergen of the North American midge *Culicoides sonorensis*. Immunoblot analysis of midge saliva revealed a 66 kDa protein (Cul s 1) that was bound by IgE from several SE-affected (SE+) horses. Further characterization by fragmentation, mass spectrometry and bioinformatics identified Cul s 1 as maltase, an enzyme involved in sugar meal digestion. A cDNA encoding Cul s 1 was isolated and expressed as a polyhistidine-tagged fusion protein in a baculovirus/insect cell expression system. The clinical relevance of the affinity-purified recombinant Cul s 1 (rCul s 1) was investigated by immunoblotting, histamine release testing (HRT) and intradermal testing (IDT) in eight SE+ and eight control horses. Seven SE+ horses had rCul s 1-specific IgE, whereas only one control animal had IgE directed against this allergen. Furthermore, the HRT showed rCul s 1 induced basophil degranulation in samples from seven of eight SE+ horses but in none of the control animals. rCul s 1 also induced immediate (7/8), late-phase (8/8) and delayed (1/8) skin reactivity in IDT on all SE+ horses that had a positive test with the whole body extract (WBE) of *C. sonorensis*. None of the control horses showed immediate or delayed skin reactivity with rCul s 1, and only one control horse had a positive late-phase response, while several non-specific late-phase reactions were observed with the insect WBE. Thus, we believe rCul s 1 is the first specific salivary allergen of *C. sonorensis* to be described that promises to advance both in vitro and in vivo diagnosis and may contribute to the development of immunotherapy for SE in horses.

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Keywords

Culicoides spp.; Summer eczema; Salivary proteins; Recombinant allergens; Immunoblotting; Histamine release test; Intradermal testing

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; Ferroglia 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 sensitized 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 hematophagous insects such as black flies (*Simulium* spp.) and mosquitoes may contribute to the allergic reaction (Marti 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 Hannover, Germany; Baselgia et al., 2006). However, these insects are likely to play a minor role in pathogenesis since SE has not been reported in regions where hematophagous insects occur, but *Culicoides* spp. are absent.

Thus far, the relevant allergens of *Culicoides* spp. are unknown. However, cellular and humoral immunoassays 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 (Ferroglia et al., 2006; Hellberg et al., 2007, Wilson et al., 2008). In addition, artificially collected *Culicoides* saliva 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 utilizing WBE of *Culicoides* have been attempted in allergic horses with varying results (Barbet et al., 1990; Anderson et al., 1996), possibly due to a lack of standardization of the extracts and a low level of relevant allergens. There might be similar explanations for the controversial IDT

results obtained when WBE of *Culicoides* spp. were used (Halldorsdottir et al., 1989; Kolm-Stark and Wagner, 2002; Ferroglio et al., 2006). 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 the present 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 to 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 1,000 g for 10 min and stored at -20 °C until assayed.

2.3. Protein electrophoresis and immunoblotting

Salivary proteins and the purified recombinant allergen were analyzed by SDS-PAGE (Laemmli, 1970) and Western blotting (Towbin, 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 Western blotting, 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 IgE detection, individual lanes were incubated with 1:10 dilutions of horse sera 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 (Immun-Star HRP, Bio-Rad, Hercules, CA) and measurement of chemiluminescence (VersaDoc 4000, Bio-Rad). Relative molecular masses (M_r) 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'-TAATTAATCATTGAGTGATAA-3' and Csm 1R: 5'-GATCAAAGATTTGAGGAA-3') designed from a nucleotide sequence of Cul s 1 (GenBank accession no. [AY603565](#)). The cDNA coding for Cul s 1 including a native signal peptide at the 5' end was amplified using a second primer set (Csm 2F: 5'-CACCATGATTCCATTTAAAAAATTAA-3' including a CACC-overhang at the 5' end and Csm 2R: 5'-GCCCTGAAAATACAGGTTTTCTTCGTATAGTCCTGG-3' carrying a protease cleavage site). The Phusion™ Polymerase (Finnzymes, Espoo, Finland) was used to create blunt end amplicons as per the manufacturer's protocol. The PCR products were analyzed 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 analyzed 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 ([His]₆)-specific antibody (Qiagen, Valencia, CA) for 1 h followed by a 1:5,000 dilution of a HRP-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 1,000 g 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 analyzed by SDS-PAGE and Coomassie 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 I 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. Maximal histamine content was obtained by boiling the blood cells for 10 min in a water bath (maximal release). The incubation of releasing buffer with washed blood cells served as a negative control (spontaneous release). Histamine concentrations were determined using a competitive RIA (LDN Nordhorn, Germany) as per the manufacturer's instructions. 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 M_r 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 characterization 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 5 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 (~3 kDa). Maximum production of the recombinant protein in both the cell lysate and the cell-free supernatant was observed at 72 h p.i. The rCul s 1 was purified from the cell-free supernatant fraction (Fig. 3A) by a one-step Co²⁺ affinity procedure. There was a considerable reduction of rCul s 1 in the flow-through, indicating the recombinant protein bound to the affinity resin, and the homogenous rCul s 1 protein was recovered by elution with 250 mM imidazole. After combining all fractions containing the recombinant protein, the BCA assay revealed that about 1 mg of purified rCul s 1 was recovered from the cell-free supernatant of 1×10^8 Sf9 cells infected with the recombinant baculovirus for 72 h. Prior to performing in vitro and in vivo studies with rCul s 1, the [His]₆ tag was cleaved by incubation with AcTEV™ protease for 6 h resulting in an untagged recombinant maltase with an M_r of about 66 kDa (Fig. 3B).

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 $\pm$ 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 $\pm$ S.E.M. at 30 min and 4 h post injection for allergic horses and control animals are summarized 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 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 the present 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 characterization 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 M_r 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 α 1-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 *C. 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 the present 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 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]₆ 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-Kuestner 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 sensitization 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 sensitization as described previously for flea-allergic dogs in flea-rich environments (Laffort-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 *C. 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.

Acknowledgments

The authors thank James Kempert, Kyle Chitty, Udo Rabe and Silke Schoeneberg for their technical assistance. This work was supported by a scholarship of the Max-Kade Foundation awarded to K.F.A. Langner, by the USDA, Medical, Veterinary, and Urban Entomology National Program, Project Number 5410-32000-014, and by NIH grant GM049734 to D.L. Jarvis.

References

- Althaus H, Mueller N, Busato A, Mellor PS, Torsteinsdottir S, Marti E. Cloning and sequencing of a cDNA expressing a ribosomal P0 peptide from *Culicoides nubeculosus* (Diptera). *Vet Immunol Immunopathol* 2004;99:99–111. [PubMed: 15113658]
- Anderson GS, Belton P, Kleider N. The hypersensitivity of horses to *Culicoides* bites in British Columbia. *Can Vet J* 1988;29:718–723. [PubMed: 17423117]
- Anderson GS, Belton P, Kleider N. Hypersensitivity of horses in British Columbia to extracts of native and exotic species of *Culicoides* (Diptera: Ceratopogonidae). *J Med Entomol* 1993;30:657–663. [PubMed: 8360890]
- Anderson GS, Belton P, Jahren E, Lange H, Kleider N. Immunotherapy trial for horses in British Columbia with *Culicoides* (Diptera: Ceratopogonidae) hypersensitivity. *J Med Entomol* 1996;33:458–466. [PubMed: 8667395]
- Barbet JL, Bevier D, Greiner EC. Specific immunotherapy in the treatment of *Culicoides* hypersensitive horses: a double-blind study. *Equine Vet J* 1990;22:232–235. [PubMed: 2209518]
- Baselgia S, Doherr MG, Mellor P, Torsteinsdottir S, Jermann T, Zurbriggen A, Jungi T, Marti E. Evaluation of an *in vitro* sulpholeukotriene release test for diagnosis of insect bite hypersensitivity in horses. *Equine Vet J* 2006;38:40–46. [PubMed: 16411585]
- Braverman Y. Preferred landing sites of *Culicoides* species (Diptera: Ceratopogonidae) on a horse in Israel and its relevance to summer seasonal recurrent dermatitis (sweet itch). *Equine Vet J* 1988;20:426–429. [PubMed: 3215168]
- Campbell CL, Vandyke KA, Letchworth GJ, Drolet BS, Hanekamp T, Wilson WC. Midgut and salivary gland transcriptomes of the arbovirus vector *Culicoides sonorensis* (Diptera: Ceratopogonidae). *Insect Mol Biol* 2005;14:121–136. [PubMed: 15796745]

- Chow LP, Liu SL, Yu CJ, Liao HK, Tsai JJ, Tang TK. Identification and expression of an allergen Asp f 13 from *Aspergillus fumigatus* and epitope mapping using human IgE antibodies and rabbit polyclonal antibodies. *Biochem J* 2000;346:423–431. [PubMed: 10677362]
- Dudler T, Chen WQ, Wang S, Schneider T, Annand RR, Dempcy RO, Cramer R, Gmachl M, Suter M, Gelb MH. High-level expression in *Escherichia coli* and rapid purification of enzymatically active honey bee venom phospholipase A2. *Biochim Biophys Acta* 1992;1165:201–210. [PubMed: 1450215]
- Eder C, Cramer R, Mayer C, Eicher R, Straub R, Gerber H, Lazary S, Marti E. Allergen-specific IgE levels against crude mould and storage mite extracts and recombinant mould allergens in sera from horses affected with chronic bronchitis. *Vet Immunol Immunopathol* 2000;73:241–253. [PubMed: 10713338]
- Fadok, VA.; Foil, CF. Equine insect hypersensitivity workshop report 2. In: von Tscharner, C.; Halliwell, R., editors. *Advances in Veterinary Dermatology I*. Baillière-Tindall; London: 1990. p. 390-394.
- Fadok VA, Greiner EC. Equine insect hypersensitivity: skin test and biopsy results correlated with clinical data. *Equine Vet J* 1990;22:236–240. [PubMed: 1976506]
- Ferroglio E, Pregel P, Accossato A, Taricco I, Bollo E, Rossi L, Trisciuglio A. Equine *Culicoides* hypersensitivity: evaluation of skin test and of humoral response. *J Vet Med A* 2006;53:30–33.
- Halldorsdottir S, Larsen HJ, Mehl R. Intradermal challenge of Icelandic horses with extracts of four species of the genus *Culicoides*. *Res Vet Sci* 1989;47:283–287. [PubMed: 2595084]
- Halldorsdottir S, Larsen HJ. An epidemiological study of summer eczema in Icelandic horses in Norway. *Equine Vet J* 1991;23:296–299. [PubMed: 1915231]
- Hellberg W, Wilson AD, Mellor P, Doherr MG, Torsteinsdottir S, Zurbriggen A, Jungi T, Marti E. Equine insect bite hypersensitivity: immunoblot analysis of IgE and IgG subclass responses to *Culicoides nubeculosus* salivary gland extract. *Vet Immunol Immunopathol* 2006;113:99–112. [PubMed: 16797724]
- Huby S, Dearman RJ, Kimber I. Why are some proteins allergens? *Toxicol Sci* 2000;55:235–246.
- Jacobson RL, Schlein Y. *Phlebotomus papatasi* and *Leishmania major* parasites express alpha-amylase and alpha-glucosidase. *Acta Trop* 2001;78:41–49. [PubMed: 11164750]
- Jones RH, Foster NM. Oral infection of *Culicoides variipennis* with bluetongue virus: Development of susceptible and resistant lines from a colony population. *J Med Ent* 1974;11:316–323.
- King TP, Hoffman D, Lowenstein H, Marsh DG, Platts-Mills TA, Thomas W. Allergen nomenclature. WHO/IUIS Allergen Nomenclature Subcommittee. *Int Arch Allergy Immunol* 1994;105:224–33. [PubMed: 7920024]
- Kolm-Stark G, Wagner R. Intradermal skin testing in Icelandic horses in Austria. *Equine Vet J* 2002;34:405–410. [PubMed: 12117115]
- Laemmli UK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685. [PubMed: 5432063]
- Laffort-Dassot C, Carlotti DN, Pin D, Jasmin P. Diagnosis of flea allergy dermatitis: comparison of intradermal testing with flea allergens and a FcεRI α-based IgE assay in response to flea control. *Vet Dermatol* 2004;15:321–330. [PubMed: 15500485]
- Langner KF, Darpel KE, Denison E, Drolet BS, Leibold W, Mellor PS, Mertens PC, Nimtz M, Greiser-Wilke I. Collection and analysis of salivary proteins from the biting midge *Culicoides nubeculosus* (Diptera: Ceratopogonidae). *J Med Entomol* 2007;44:238–248. [PubMed: 17427692]
- Langner KF, Darpel KE, Drolet BS, Fischer A, Hampel S, Heselhaus J, Mellor PS, Mertens PC, Leibold W. Comparison of cellular and humoral immunoassays for the assessment of summer eczema in horses. *Vet Immunol Immunopathol* 2008;122:126–137. [PubMed: 18155778]
- Littlewood JD. Incidence of recurrent seasonal pruritus ('sweet itch') in British and German shire horses. *Vet Rec* 1998;142:66–67. [PubMed: 9481844]
- Lorch G, Hillier A, Kwochka KW, Saville WJ, Kohn CW, LeRoy BE. Comparison of immediate intradermal test reactivity with serum IgE quantitation by use of a radioallergosorbent test and two ELISA in horses with and without atopy. *J Am Vet Med Assoc* 2001;218:1314–1322. [PubMed: 11330620]
- Marinotti O, de Brito M, Moreira CK. Apyrase and alpha-glucosidase in the salivary glands of *Aedes albopictus*. *Comp Biochem Physiol B Biochem Mol Biol* 1996;113:675–679. [PubMed: 8925436]

- Marti E, Urwyler A, Neuenschwander M, Eicher R, Meier D, de Weck AL, Gerber H, Lazary S, Dahinden CA. Sulfidoleukotriene generation from peripheral blood leukocytes of horses affected with insect bite dermal hypersensitivity. *Vet Immunol Immunopathol* 1999;71:307–320. [PubMed: 10587309]
- McDermott MJ, Weber E, Hunter S, Stedman KE, Best E, Frank GR, Wang R, Escudero J, Kuner J, McCall C. Identification, cloning, and characterization of a major cat flea salivary allergen (Cte f 1). *Mol Immunol* 2000;37:361–375. [PubMed: 11074254]
- McKelvie J, Foster AP, Cunningham FM, Hamblin AS. Characterisation of lymphocyte subpopulations in the skin and circulation of horses with sweet itch (*Culicoides* hypersensitivity). *Equine Vet J* 1999;31:466–472. [PubMed: 10596926]
- Morgan EE, Miller WH, Wagner B. A comparison of intradermal testing and detection of allergen-specific immunoglobulin E in serum by enzyme-linked immunosorbent assay in horses affected with skin hypersensitivity. *Vet Immunol Immunopathol* 2007;120:160–167. [PubMed: 17888519]
- Morris DO, Lindborg S. Determination of ‘irritant’ threshold concentrations for intradermal testing with allergenic insect extracts in normal horses. *Vet Dermatol* 2003;14:31–36. [PubMed: 12603683]
- Morrow A, Quinn PJ, Baker KP. Allergic skin reactions in the horse: response to intradermal challenge with fractionated *Culicoides*. *Zentralbl Veterinaermed B* 1986;33:508–517.
- Pearson WR, Lipman DJ. Improved tools for biological sequence comparison. *Proc Natl Acad Sci U S A* 1988;85:2444–2448. [PubMed: 3162770]
- Peng Z, Simons FE. Mosquito allergy: immune mechanisms and recombinant salivary allergens. *Int Arch Allergy Immunol* 2004;133:198–209. [PubMed: 14963366]
- Soldatova LN, Cramer R, Gmachl M, Kemeny DM, Schmidt M, Weber M, Mueller UR. Superior biologic activity of the recombinant bee venom allergen hyaluronidase expressed in baculovirus-infected insect cells as compared with *Escherichia coli*. *J Allergy Clin Immunol* 1998;101:691–698. [PubMed: 9600508]
- Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 1979;76:4350–4354. [PubMed: 388439]
- Wagner B, Radbruch A, Rohwer J, Leibold W. Monoclonal anti-equine IgE antibodies with specificity for different epitopes on the immunoglobulin heavy chain of native IgE. *Vet Immunol Immunopathol* 2003;92:45–60. [PubMed: 12628763]
- Wagner B, Miller WH, Morgan EE, Hillegas JM, Erb HN, Leibold W, Antczak DF. IgE and IgG antibodies in skin allergy of the horse. *Vet Res* 2006;37:813–825. [PubMed: 16973120]
- Wilson AD, Harwood LJ, Bjornsdottir S, Marti E, Day MJ. Detection of IgG and IgE serum antibodies to *Culicoides* salivary gland antigens in horses with insect bite dermal hypersensitivity (sweet itch). *Equine Vet* 2001;33:707–713.
- Wilson AD, Heesom KJ, Mawby WJ, Mellor PS, Russell CL. Identification of abundant proteins and potential allergens in *Culicoides nubeculosus* salivary glands. *Vet Immunol Immunopathol* 2008;122:94–103. [PubMed: 18068808]
- Xu W, Simons FER, Peng Z. Expression and rapid purification of an *Aedes aegypti* salivary allergen by a baculovirus system. *Int Arch Allergy Immunol* 1998;115:245–251. [PubMed: 9531167]

Fig. 1.

Immunoblot analysis of artificially collected saliva of *Culicoides sonorensis*. Salivary proteins were separated on 15% polyacrylamide gels and used for silver staining (G) or transfer onto polyvinylidene fluoride membranes (lanes 1 to 8). Lanes 1 to 4 were incubated with sera from allergic horses and lanes 5 to 8 with sera obtained from control animals. IgE-binding was determined by chemiluminescence after incubation with a monoclonal antibody specific for equine IgE and a horseradish peroxidase-conjugated detection antibody. Lines on the left-hand side of the figure indicate positions of molecular mass standards.

Fig. 2.

Time course of rCul s 1 expression in Sf9 cells. Cells were infected with recombinant baculovirus at a multiplicity of infection of 5 pfu/cell. Over a 5-day period, supernatants and cells were harvested at 24 h intervals. The presence of rCul s 1 in these samples was determined by immunoblotting using an anti-[His]₆-specific antibody, a horseradish peroxidase-labelled detection antibody, and chemiluminescence.

Fig. 3.

Purification of rCul s 1 and cleavage of [His]₆ tag. (A) Fractions obtained during protein purification were loaded onto 12% polyacrylamide gels and proteins were stained with Coomassie Brilliant Blue. Lane 1 (Sn): concentrated supernatant of Sf9 cells at 72 h p.i. prior to purification on Co²⁺ affinity columns; Lane 2 (Ft): flow-through containing unbound material; Lane 3-5 (E1-E3): consecutively eluted fractions containing rCul s 1. (B) AcTEVTM protease was used to cleave the affinity tag from rCul s 1. Samples were taken at 1, 2, 4 and 6 h following the addition of protease. Arrows indicate uncleaved (69 kDa) and cleaved (66 kDa) forms of rCul s 1. Arrowhead indicates the AcTEV protease.

Fig. 4.

Immunoblot analysis of rCul s 1 using sera of horses with summer eczema and control animals. The recombinant allergen was loaded onto 12% polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Lanes were incubated with sera of eight SE+ horses and eight control animals. (A) The presence of rCul s 1-specific IgE was determined by incubation with a monoclonal antibody specific for equine IgE and a horseradish peroxidase-conjugated detection antibody. (B) Specific IgG was detected by incubation with an HRP-conjugated anti-horse IgG antibody. Antibody binding was visualized using chemiluminescence.

Table 1

Mass spectrometry identification of Cul s 1.

Protein identification ^a (GenBank accession no)	Peptide sequences ^{b,c}	% Identity ^d	Observed/predicted M _r ^e
Maltase <i>Culicoides sonorensis</i> (Q66UC5_9DIPT)	²⁶ EKDWWELGNFYQVYPR ⁴¹	94	66/68.6
	⁴² SFMDSDGDGVGDLK ⁵⁵	100	
	¹²⁰ LLLDFVPNHTSDQCEWFK ¹³⁶	94	
	¹⁴² DPEYNDYYLWHPGKPNPDGG ¹⁶¹	95	
	¹⁶³ NLPPTNWVSAFR ¹⁷⁴	100	
	¹⁷⁵ SSAWEWNEERGEYYLHQFLA ¹⁹⁴	93	
	¹⁹⁵ QQPDLNYR ²⁰²	100	
	⁴¹⁵ TVDPQACTTDPNLFHAK ⁴³¹	94	

^a Proteins were identified by searching the UniProt database with peptide fragmentation data using the FASTA program (European Bioinformatics Institute, Cambridge, UK).

^b Sequence information obtained from tandem mass spectrometry. The regions of the protein to which the peptides align are noted by superscripts indicating residue number.

^c 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 reported here.

^d Identity of obtained peptide sequences to proteins from the database.

^e Observed molecular masses (M_r) in kDa estimated from gel spots and predicted M_r obtained from the database.

Table 2
Results of histamine release testing for horses with summer eczema (n = 8) and control horses (n = 8).

Preparation (µg)	SE affected horses		Control horses	
	Number of responders ^a	Average release (%) ^b	Number of responders	Average release (%)
rCul s 1 ^c	1	26.6 ± 8.1	0	5.3 ± 0.7
	0.1	31.9 ± 9.9	0	5.5 ± 0.7
	0.01	28.4 ± 8.4	0	5.4 ± 0.5
	0.001	11.8 ± 4.6	0	5.6 ± 0.6
WBE ^d	1	47.0 ± 7.3	2	7.6 ± 0.9
	0.1	9.3 ± 1.7	0	5.5 ± 0.9
	0.01	5.5 ± 2.5	0	4.9 ± 0.6
	0.001	3.8 ± 1.3	0	5.3 ± 0.8

^a Number of horses with a histamine release ≥10% of maximal release.

^b Amount of released histamine for each group of horses in % of maximal release, expressed as mean ± standard error.

^c rCul s 1, recombinant Cul s 1.

^d WBE, whole body extract *Culicoides sonorensis*.

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

Preparation (µg)	SE+ horses ^a					Control horses				
	1+	2+	3+	4+	Average diameter (mm) ^b	1+	2+	3+	4+	Average diameter (mm)
Saline					10.1 ± 0.0					10.9 ± 0.0
Histamine					20.0 ± 0.1					21.7 ± 0.1
Cul s 1 ^c	1	5	2	0	16.3 ± 0.4	3	0	0	0	10.6 ± 0.1
0.1	1	6	0	0	14.4 ± 0.8	2	0	0	0	10.3 ± 0.2
0.01	7	0	0	0	11.8 ± 0.5	1	0	0	0	9.9 ± 0.3
0.001	7	0	0	0	10.2 ± 0.1	1	0	0	0	10.2 ± 0.2
WBE ^d	0	6	1	0	15.4 ± 0.6	7	0	0	0	12.3 ± 0.3
0.1	5	2	0	0	12.9 ± 0.4	3	0	0	0	10.8 ± 0.2
0.01	7	0	0	0	11.1 ± 0.3	3	0	0	0	10.8 ± 0.3
0.001	5	0	0	0	10.2 ± 0.2	3	0	0	0	10.7 ± 0.4

^a SE+ horses, SE affected horses.
^b Diameter of skin reactions for each group of horses in mm, expressed as mean ± standard error.
^c rCul 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.

Preparation (µg)	SE+ horses ^a					Control horses			
	1+	2+	3+	4+	Average diameter (mm) ^b	1+	2+	3+	4+
Saline					5.0 ± 0.2				
Histamine					21.3 ± 0.1				
Cul s 1 ^c	1	0	3	4	1	3	1	0	0
	0.1	0	6	1	0	2	0	0	0
	0.01	4	3	0	1	2	0	0	0
	0.001	5	2	0	0	1	0	0	0
WBE ^d	1	0	1	6	1	3	3	1	1
	0.1	2	4	1	1	5	2	0	0
	0.01	4	2	1	0	4	0	1	0
	0.001	3	2	0	0	3	0	0	0

^a SE+ horses, SE affected horses.
^b Diameter of skin reactions for each group of horses in mm, expressed as mean ± standard error.
^c rCul s 1, recombinant Cul s 1.
^d WBE, whole body extract *Culicoides sonorensis*.