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Identification, expression and characterisation 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 characterisation 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.

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; Ferroglio 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

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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 (Ferroglio et al., 2006; Hellberg et al., 2006; 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 utilising 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 standardisation 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 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 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′-GCCCTGAAAATA CAGGTTTTCTTCGTATAGTCCTGG-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 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