

Cloning and sequencing of a cDNA expressing a ribosomal P0 peptide from *Culicoides nubeculosus* (Diptera)

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Abstract

Insect bite dermal hypersensitivity (IBH) is an allergic dermatitis of horses caused by bites of *Culicoides* spp. and sometimes *Simulium* spp. The aim of the investigation presented here was to identify allergens causing IBH. A cDNA library expressing recombinant *Culicoides nubeculosus* proteins was screened using affinity-purified serum from an IBH-affected horse. Screening of the library resulted in identification of one immunoreactive clone. The sequence of the cDNA insert was determined and revealed a 600 bp insert with an open reading frame coding for a 78 amino acid long protein, called rCul n 1. Analysis of the deduced amino acid sequence revealed an identity of 67–78% to the C-terminal part of the 318 amino acid long ribosomal P0 protein from other *Diptera*. Furthermore, the 38 C-terminal amino acids displayed an identity of 57% with the C-terminal part of the acidic ribosomal protein P2 from *Aspergillus fumigatus*. The cDNA insert was subcloned and expressed as a [His]₆-tagged protein in *Escherichia coli* and purified using Ni²⁺-chelate affinity chromatography. The 10 kDa recombinant Cul n 1 protein bound the affinity-purified antibody fraction used for screening the expression library. Determination of IgE and IgG levels against rCul n 1 by ELISA in sera from 19 IBH-affected and 18 Swiss control horses and in sera from eight control horses living in Iceland showed no significant differences between the three groups of horses (median IgE levels = 60, 49 and 44 relative ELISA units, respectively). rCul n 1 did not induce sulfidoleukotriene (sLT) release from peripheral blood leukocytes of IBH-affected horses (*N* = 5), although sLT release was induced with the *Culicoides* whole body extract.

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Keywords: *Culicoides nubeculosus*; Recombinant proteins; Horse; Insect bite hypersensitivity

Abbreviations: AP, alkaline phosphatase; CB, chronic bronchitis; *E. coli*, *Escherichia coli*; h, hour; IBH, insect bite hypersensitivity; IgE, immunoglobulin E; IgG, immunoglobulin G; PBL, peripheral blood leukocytes; PCR, polymerase chain reaction; REU, relative ELISA units; RT, room temperature; rCul n 1, recombinant *Culicoides nubeculosus* 1; sLT, sulfidoleukotriene

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1. Introduction

Equine insect bite dermal hypersensitivity (IBH), also known as sweet itch or summer dermatitis, is a recurrent, pruritic dermatitis of the horse with seasonal occurrence due to hypersensitivity reactions to bites of insects. Affected horses suffer from severe

pruritus, alopecia and crusting along the dorsal or the ventral midline (Fadok and Greiner, 1990). Several epidemiological studies, combined with aspiration of insects from horses (Greiner, 1995) and skin tests (Fadok and Greiner, 1990) have shown that IBH is a hypersensitivity reaction to the bites of midges (*Culicoides* spp.) sometimes to black flies (*Simulium* spp.) and infrequently to other insects (Fadok and Foil, 1990). The *Culicoides* species involved in IBH vary between regions. However, as skin tests show, most horses seem to react to major antigens found in all *Culicoides* species (Braverman et al., 1983; Halldorsdottir et al., 1989; Fadok and Foil, 1990; Anderson et al., 1993). IBH can be found worldwide in areas where these insects occur. IBH has never been described in Iceland, probably because *Culicoides* spp. are not present in this region, although haematophagous black flies can be found in some areas during the summer. Interestingly, however, about 30% of the Icelandic horses imported from Iceland to continental Europe get IBH 1–7 years after importation (Brostrom et al., 1987; Halldorsdottir and Larsen, 1991).

Results from skin tests have shown that immediate type hypersensitivity reactions (type I) are often involved in the pathogenesis of IBH but that delayed type reactions (IgE-mediated) and possibly other types of allergic reactions such as delayed type (type IV) reactions may sometimes also be present (Bourdeau and Petrikovski, 1995). More recent studies, where histamine or sulfidoleukotriene release was determined after incubation of peripheral blood leukocytes with *Culicoides* or *Simulium* whole body extracts, suggest that IgE-mediated reactions play an important role in the pathogenesis of IBH (Kaul, 1998; Marti et al., 1999). Peripheral blood leukocytes from eighty per cent of IBH-affected horses release sulfidoleukotrienes in vitro after incubation with a *Culicoides* extract (Marti et al., 1999).

At present, treatment of IBH is mainly based on avoidance of the insects and on the use of corticosteroids. In two studies hyposensitisations were carried out with *Culicoides* whole body extracts: while Anderson et al. (1996) demonstrated a regression of the clinical signs in treated horses, a placebo controlled study showed no difference between horses treated with placebo and those treated with *Culicoides* whole body extract (Barbet et al., 1990).

Only whole body extracts from *Culicoides* and *Simulium* have been used so far and the specific allergens that induce IBH, probably salivary gland proteins, have not yet been identified. We also lack standardised commercially produced *Culicoides* and *Simulium* extracts. Although whole body extracts can be used successfully for some applications such as skin tests or in vitro histamine release assays, purer allergens such as salivary gland extracts or pure recombinant allergens are needed for diagnostic, therapeutic and research purposes. Whole body extracts consist of complex mixtures of a large number of proteins and glycoproteins among which the allergenic proteins represent only a small percentage of the total protein content. Furthermore, the composition of the extracts varies between preparations.

In the human field, much effort has been devoted to identifying, cloning and producing allergens of medical importance by biotechnological means (Kraft et al., 1998). Molecular technologies usually allow high-quality production in unlimited quantity. In contrast to their natural homologue, recombinant allergens tend to degrade less rapidly during extraction or storage. Furthermore, recombinant allergens allow the precise determination of the patient's individual IgE reactivity in the form of an allergogram (Laffer et al., 1996), which is useful for detailed diagnosis and for subsequent specific hyposensitisation. It has been reported though, that some recombinant allergens, compared to their corresponding natural allergens, show diminished IgE-binding (Swoboda et al., 1995), due to the existence of numerous isoforms of allergens with differing IgE-binding capacity. This finding can be taken advantage of, since hypoallergenic recombinant molecules could modulate the allergic immune response with a substantially reduced risk of anaphylactic side-effects during specific immunotherapy (Kraft et al., 1998).

Identification and biotechnological production of the allergen(s) causing IBH in the horse may thus allow a better diagnosis of this disease and open the way to new therapeutic approaches. The purpose of the following study was to identify, clone and express possible IBH allergens through the screening of a *Culicoides* expression library with sera from IBH-affected horses.

2. Material and methods

2.1. *Culicoides nubeculosus*

C. nubeculosus were kindly provided by M. Krémer, Laboratoire de Parasitologie et Pathologie Tropicale, Faculté de Médecine, 67000 Strasbourg, France. They were reared in laboratory and separated into males and females following Krémer and Lienhart (1998) before being frozen. The whole insects were stored at -70°C in a dry state until use.

2.2. Horses

Thirty-seven mares and geldings of various breeds and age groups, living in Switzerland, were examined and divided into IBH-affected ($N = 19$) and non-affected ($N = 18$) individuals. Additionally, blood samples were taken from eight horses living in Iceland, where *Culicoides* spp. are not present. The diagnosis of IBH was based on clinical signs and history. Furthermore, in 14 IBH-affected horses and in 13 controls from Switzerland, an in vitro sulfidoleukotriene release assay had been performed with the *Culicoides* extract (Marti et al., 1999), confirming the clinical diagnosis in all tested animals. In 12 cases, control horses lived on the same farm as IBH-affected animals. The remaining control horses lived in areas with an endemic prevalence of IBH, i.e. they were all exposed to bites from *Culicoides* spp.

Blood samples were taken from all horses mentioned above and serum was frozen in aliquots not later than 12 h after blood sampling and then stored at -70°C until assayed.

2.3. Protein electrophoresis and immunoblotting

The *C. nubeculosus* whole body extract and the recombinant *C. nubeculosus* protein rCul n 1 were separated by one dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on a vertical slab apparatus (MiniProtean II, BioRad Laboratories AG, Glattbrugg, Switzerland) using 12 and 15% polyacrylamide gels, respectively, run under denaturing-reducing conditions as described by Laemmli (1970). Gels were stained with Coomassie

brilliant blue G250 (Merck, Darmstadt, Germany) according to Towbin et al. (1979).

Transfer blotting of proteins from the polyacrylamide gel to microporous polyvinylidene fluoride (PVDF) membranes (Immobilon™-P^{SQ}, Millipore, Volketswil, Switzerland) was performed according to Bjerrum and Schafer-Nielsen (1986) using semidry electrophoretic transfer (Trans-Blot® SD, BioRad Lab. AG) at 15 V for 35 min. The blots were incubated for 2 h at room temperature or overnight at 4°C with sera from IBH-affected or healthy horses or with affinity-purified horse serum (see below). Sera were diluted in washing buffer (20 mM Tris, 250 mM NaCl, 0.12% Tween-20, pH 7.5) 1:4 for IgE detection and 1:25 for IgG detection. For IgE detection a chicken anti-horse IgE antibody (Marti et al., 1997) was then applied, diluted in washing buffer to a concentration of $2\text{ }\mu\text{g/ml}$, followed by an alkaline phosphatase (AP)-labelled goat anti-chicken IgG (1:1000 in washing buffer; Kirkegaard and Perry Laboratories, Gaithersburg, MA, USA). IgG Ab were detected with an AP-labelled goat anti-horse IgG, Fc-fragment-specific (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) applied at a dilution of 1:1000. Western blots of rCul n 1 were also incubated with AP conjugated to Ni-NTA (QIAGEN AG, Basel, Switzerland). The blots were developed using 5-bromo-4-chloroindolyl-phosphate (BCIP)/4-nitro blue tetrazolium chloride (NBT) in AP buffer (100 mM NaCl, 10 mM MgCl₂, 100 mM Tris, pH 9.5).

2.4. Affinity purification of horse serum on IgE-binding *Culicoides* proteins

Serum of an IBH-affected horse was affinity-purified on the IgE-binding *Culicoides* proteins, as described by Müller and Felleisen (1995). Briefly: in a first step, proteins of a crude female *C. nubeculosus* extract were separated by preparative 12% SDS–PAGE and subsequently transferred to a PVDF membrane (see Section 2.3). A flanking strip was cut from the membrane and used to perform an immunoblot with serum from an IBH-affected horse in order to locate IgE-binding antigens (see Section 2.3). Cross-strips containing the identified bands were then cut from the membrane and used as antigenic ligands for affinity purification of individual antibody fractions

from the serum. Following a three times washing step, bound immunoglobulins were released by incubation in a low pH-buffer.

2.5. Construction of a cDNA-expression library from female *C. nubeculosus*

Female *C. nubeculosus* were homogenised in Trizol (GIBCO-BRL, Life Technologies AG, Basel, Switzerland). Messenger RNA was subsequently prepared using the message makerTM kit according to the manufacturer's instructions (Gibco-BRL, Life Technologies). Briefly, double-stranded cDNA was synthesised and ligated into the *SalI/NotI* site of lambda-gt22A vector, employing the SuperScriptTM Lambda System (Gibco-BRL, Life Technologies). The primary library was amplified in *Escherichia coli* (*E. coli*) strain Y1090r- (Stratagene, La Jolla, CA, USA) after in vitro packaging using the Giga-pack Gold II packaging extracts (Stratagene). Immunoscreening was performed with horse serum, affinity-purified on IgE-binding *Culicoides* proteins, and a secondary AP-labelled goat anti-horse IgG antibody (see Section 2.3). Positive plaques were isolated and subjected to several rounds of re-screening until a pure plaque population was obtained. The phage clone expressing a cDNA that was called rCul n 1 was finally amplified according to the SuperScriptTM-Lambda System protocol (Gibco-BRL, Life Technologies).

2.6. Analysis of the rCul n 1 cDNA insert

The length of the rCul n 1 insert was determined by PCR-amplification using the conditions described by Hemphill et al. (1997) and applying primers derived from the 5'-flanking sequence on the sense strand and the 3'-flanking sequence on the anti-sense strand of the lambda gt22A vector. The cDNA sequence of the insert was obtained by direct sequencing of the PCR product by an automated sequencing service provided by Microsynth GmbH, Balgach, Switzerland. Sequences were analysed using the Genetics Computer Group (GCG) programme, Wisconsin Package Version 10.1, Madison, WI, USA. In order to establish possible relationships to previously published sequences, the sequence similarity search tool from the National Centre

for Biotechnology Information (NCBI) was used (BLAST 2.1).

2.7. Cloning, expression and purification of rCul n 1 protein

Primers were designed based on the rCul n 1 sequence. A *KpnI* and a *HindIII* restriction site were incorporated into the forward (5'-GGCGTCGACGG-TACCGCACCACACAGCATTGC-3') and reverse (5'-AACAAGCTTCTAGTGGGAAGAGACTGAG-3') primer, respectively, for unidirectional cloning. The DNA template was the phage clone expressing rCul n 1. Techniques established in previous studies were applied for cloning and expression of rCul n 1 as a histidine-tagged protein (Marti et al., 1997; Griot-Wenk et al., 1998). Briefly: the PCR product was purified with GenEluteTM Agarose Spin Columns (SUPELCO, Bellefonte, PA, USA), digested with *KpnI* and *HindIII* and ligated into the *KpnI/HindIII* cleaved vector pQE30 (QIAGEN AG) using standard protocols (Sambrook et al., 1989). The plasmids were introduced into *E. coli* M15 cells by electroporation using a Gene Pulser[®] (Bio-Rad Laboratories AG). Transformants were selected on LB-plates containing 100 µg/ml ampicillin and 25 µg/ml kanamycin (Sambrook et al., 1989).

E. coli cells containing vector pQE30 with the cloned rCul n 1, as shown by sequence determination, and repressor plasmid pREP4 were grown in 2× YT medium supplemented with appropriate antibiotics at 37 °C, following the instructions of the manufacturers. Expression was induced by addition of 2 mM IPTG followed by incubation at 37 °C for 4 h. The cells were then pelleted and lysed overnight in 6 M guanidine hydrochloride and 0.1 M NaH₂PO₄ (pH 8.0). The lysate was spun and the supernatant applied to a column packed with Ni²⁺-chelate affinity resin (QIAGEN AG). The rCul n 1 protein was eluted with an 8 M urea step gradient. For in vitro refolding, the eluted recombinant protein was dialysed twice against 0.266 mM acetic acid for 8 h at 4 °C using a molecular-porous membrane with a 3.5 kDa MW cut-off (Pierce, Socochim SA, Lausanne, Switzerland), followed by dialysis against H₂O for 8 h. Protein concentration was determined using the Bio-Rad protein assay with bovine serum albumin as standard.

2.8. Determination of rCul n 1-specific IgE and IgG antibodies by ELISA

Reactivity with rCul n 1 of the horse serum fractions used for the screening of the *Culicoides* expression library was tested by ELISA. Furthermore, in order to test the relevance of rCul n 1 for IBH, rCul n 1 –specific IgE and IgG levels were determined in sera from IBH-affected and healthy horses and in sera from horses living in Iceland.

The ELISA was performed as described in Eder et al. (2000) with the following modifications: plates were coated with 2.5 µg/ml rCul n 1 diluted in sodium carbonate-bicarbonate buffer, pH 9.4 (Pierce) overnight at 4 °C. For IgE detection, plates were blocked with 0.2% equine serum albumin in TBS for 1 h at 37 °C; horse sera were applied starting at a dilution of 1:4 and followed by three 1:1.5 serial dilutions made in the plate and incubated for 90 min at 37 °C. A chicken antibody specific for horse IgE (Marti et al., 1997) was then added at a concentration of 2 µg/ml and incubated for 2 h at RT on a shaker. An incubation with an AP-labelled goat anti-chicken IgG (Kirkegaard and Perry Laboratory) for 1 h at RT on a shaker followed. For IgG detection, coated plates were blocked with 2% porcine skin gelatine in Tris-buffered saline, pH 7.2; horse sera or the affinity-purified antibody fractions were then added to the plate, starting at a dilution of 1:50 and 1:25, respectively. As a negative control for the *Culicoides* affinity-purified horse antibodies, serum from a horse sensitised against human serum albumin (HSA), which had been affinity-purified on HSA using the same procedure, was used at the same dilutions. Two-fold serial dilution followed. For detection, an AP-conjugated goat anti-horse IgG Ab, Fc fragment-specific (Jackson ImmunoResearch Lab.) was used at a dilution of 1:1000. ELISA plates were subsequently developed with *p*-nitrophenyl phosphate (Sigma) in diethanolamine (Fluka) buffer. Absorbance readings were measured at 405 nm with a Molecular Devices reader (Sunnyvale, CA, USA). For the affinity-purified serum fractions, results were expressed as mean OD₄₀₅ of duplicate well after subtraction of background values. For measurements of rCul n 1-specific IgE and IgG levels in sera from IBH-affected horses and controls, OD₄₀₅ values were converted to relative ELISA units (REU). For that purpose, an equine serum positive for the tested

allergen was used as reference serum and assigned a value of 100 REU. Serial dilutions of the reference serum were used to generate a standard curve and the test sample results were calculated from the curve using an ELISA software programme (SOFTmax[®] PRO, version 3.0, Molecular Devices).

2.9. Testing of rCul n 1 in a sulfidoleukotriene release assay

To test whether rCul n 1 is a relevant allergen for IBH, a sulfidoleukotriene (sLT) release assay was performed as described in Marti et al. (1999). In brief, peripheral blood leukocytes (PBL) were enriched from heparin anticoagulated horse blood, washed and resuspended in HACM buffer (20 mM HEPES, 125 mM NaCl, 5 mM KCl, 0.5 mM glucose, 0.025% human serum albumin, 1 mM CaCl₂ and 1 mM MgCl₂). The PBL suspension was then incubated with Concanavalin A as stimulation control, with buffer only to determine the spontaneous sLT generation, with a *C. nubeculosus* whole body extract and with rCul n 1 (20, 10, 5 and 1 µg/ml). After an incubation of 40 min at 37 °C followed by centrifugation, the supernatants were transferred to the ELISA microtitre plate used for sLT determination according to the manufacturer's instructions using the CAST-ELISA, as commercially available from Bühlmann Laboratories AG.

2.10. Statistical analysis

A preliminary screening of serum IgE and IgG data indicated skewed distributions. It was therefore decided to use medians for descriptive procedures and non-parametric methods for hypothesis testing. Differences between groups were assessed using Wilcoxon and Kruskal–Wallis tests.

3. Results

3.1. Affinity purification of horse serum on IgE-binding protein bands from the blotted *C. nubeculosus* extract

Separation of the *Culicoides* whole body extract by SDS–PAGE and staining with Coomassie blue

revealed numerous barely distinguishable protein bands. A similar picture was produced after detection of equine IgG Ab against the *Culicoides* extract on Western blots (data not shown). Fewer bands were stained when serum IgE Ab were detected (Fig. 1, lane 1) and staining of some of these bands was much weaker or absent after heating horse serum for 5 min at 56 °C before applying it to the blot (data not shown). Such bands, or groups of bands, were selected for affinity purification of horse serum (Fig. 1, lane 1, bands shown by arrows). The four selected bands

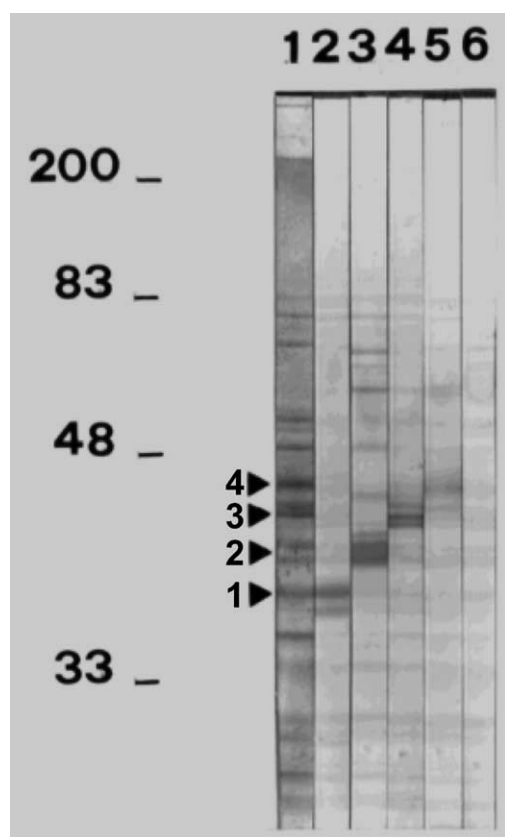


Fig. 1. Twelve percent SDS-PAGE and Western blot of a female *Culicoides nubeculosus* (*Cul. n*) whole body extract. Lane 1: Western blot incubated with serum from an IBH-affected horse, followed by a chicken anti-horse IgE Ab and developed with an AP-labelled goat anti-chicken IgG. Arrows show four *Cul. n* protein bands (1–4) that bind equine IgE and were used for affinity purification of horse serum. Lanes 2–5: reactivity of the affinity-purified serum fractions on the respective *Cul. n* bands: Western blot incubated with the affinity-purified serum fractions and developed with an AP-labelled goat anti-horse IgG. Lane 6: conjugate control. Molecular weights (in kDa) indicated at left.

corresponded to proteins with molecular weights of approximately 36, 40, 45 and 47 kDa, respectively. Incubation of the affinity-purified fractions on the Western blot showed that three of them clearly bound to the protein band on which they had been affinity-purified (Fig. 1, lanes 2–4). The antibody fraction purified on the 47 kDa proteins showed only weak binding to this band after purification (Fig. 1, lane 5). The antibody fractions that were purified on these bands were subsequently used for screening a *Culicoides* cDNA expression library and, for purposes of simplification, are called fraction 1 (fraction purified on 36 kDa proteins), 2 (40 kDa), 3 (45 kDa) and 4 (47 kDa).

3.2. Screening of the *Culicoides* cDNA-library

For each of the four purified antibody fractions, a separate screening of the *Culicoides* cDNA expression library was performed. A total of approximately 4×10^4 plaques were screened with each antibody fraction. One phage clone was isolated by repeated screening with the antibodies purified on the 36 kDa proteins (fraction 1). Screening with the other three antibody fractions did not result in the identification of recombinant plaques.

3.3. Analysis of the rCul n 1 cDNA insert

PCR amplification demonstrated that the isolated phage clone contained a cDNA insert of about 600 bp with an open reading frame coding for a 78 amino acid long protein (237 bp) followed by an untranslated sequence of approximately 350 bp and a polyA stretch (not shown). The cDNA and deduced amino acid sequence, using the codon preference of *Drosophila melanogaster*, are shown in Fig. 2. Comparison of the amino acid sequence of this open reading frame with the C-terminal sequences of the 318 amino acid long ribosomal P0 proteins from other *Diptera* showed high similarities (Fig. 3). The deduced amino acid sequence showed 78% identity with the corresponding sequence from *Sarcophaga crassipalpis*, 68% identity with *D. melanogaster* and 67% identity with *Ceratitis capitata*. Furthermore, the 38 C-terminal amino acids of rCul n 1 displayed an identity of 57% with the C-terminal amino acids of the acidic ribosomal protein P2 from *Aspergillus fumigatus*.

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(1) gca cca cac agc att gcc aat ggt ttc aag aat ctg ttg gct ttg
    A  P  H  S  I  A  N  G  F  K  N  L  L  A  L

(46) gct gca aca acc gat gtc gat tcc aag gaa gct gaa acc atc aag
    A  A  T  T  D  V  D  S  K  E  A  E  T  I  K

(91) gaa tac att aag gat cca agc aaa atc gct gct gcc gct gct gct
    E  Y  I  K  D  P  S  K  I  A  A  A  A  A  A

(136) act gca cca gct gct gaa acc aag aaa gaa gag aag aag gaa gaa
    T  A  P  A  A  E  T  K  K  E  E  K  K  E  E

(181) aag aaa gag gaa acc gaa gaa tct gat gat gat atc ggt ctc agt
    K  K  E  E  T  E  E  S  D  D  D  I  G  L  S

(226) ctc ttc cac tag
    L  F  H  *

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Fig. 2. Nucleotide and deduced amino acid sequences of the partial cDNA sequence coding for the ribosomal P0 protein from *Culicoides nubeculosus*, called rCul n 1 (sequence available from GeneBank accession number: AF314650).

3.4. Expression and purification of rCul n 1

The nucleotide sequence analysis of the insert in the vector pQE30 was consistent with the original PCR product from the positive lambda gt22 phage. The yield of the 6× his-tagged fusion protein produced by the induced *E. coli* cells after Ni²⁺-chelate chromatography was ca. 5 mg/l of medium of induced cells. After refolding, about half of the recombinant protein precipitated, resulting in a final yield of ca. 2.5 mg/l of medium of induced cells. SDS–PAGE gel analysis of the purified rCul n 1 revealed one clear band with a molecular mass of approximately 10 kDa (Fig. 4,

lane 2), confirming the size of the fusion protein predicted from the nucleotide sequence (9.9 kDa). In addition, a minor, lower molecular weight band was detected on the stained gel. The Ni-NTA AP conjugate reacted with the major protein band of 10 kDa (Fig. 4, lane 3).

The ELISA was used to assess the reactivity of antibody fraction 1 with the recombinant protein rCul n 1, using fractions 2 and 3 as negative controls. As a further control, an HSA affinity-purified antibody fraction from a horse that had been sensitised against HSA was also tested on rCul n 1. As shown in Table 1, antibody fraction 1 bound to rCul n 1 while the three

	1	10	20	30	40	50
rCul1	-----+-----+-----+-----+-----					
	APHSIANGFKNLLALAATTDVDSKERETIKYIKDPSKIAAAAAATAPAA					
C.c.	I..S.E.EF.Q.A...F.....FV.....S....					
S.c.	...N.....I...E.EF...T.....F.....					
D.m.	I...E.EF...T.....F.....S.S.-P					
Consensus	...s.....i..t..#.#f.....%.....fa...a..t..p..a					

	51	60	70	80
rCul1	-----+-----+-----+-----			
	ET--KKEEKKEEKKEETE--ESDDDIGLSLFH			
C.c.	GAGAAA.K.E.A...S.-S.E...M.FG..D			
S.c.	GA-AAA.K.E.A...S.-E.E...M.FG..D			
D.m.	AAGGAT.K.E.A..P.S.SE.E...M.FG..D			
Consensus	.a..a..k.e.a..e.s....e...n.f.g..d			

Fig. 3. Comparison of the amino acid sequences of the recombinant *Culicoides* peptide (rCul1) with the amino acid sequences of the ribosomal P0 proteins from *Sarcophaga crassipalpis* (S. c.), *Drosophila melanogaster* (D. m.) and *Ceratitidis capitata* (C. c.). The consensus sequence is also given.

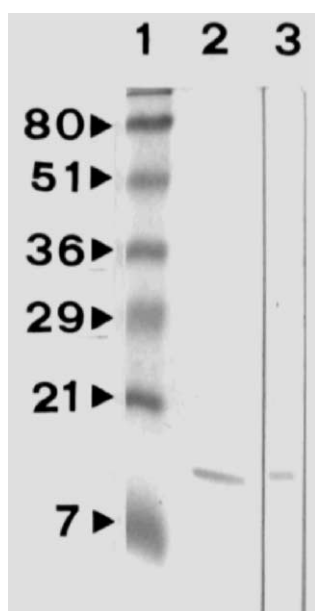


Fig. 4. Fifteen percent SDS-PAGE and Western blot of the recombinant *Culicoides* peptide called rCul n 1. Lane 1: molecular weight marker (molecular weights in kDa indicated at left). Lane 2: SDS-PAGE stained with Coomassie blue. Lane 3: Western blot incubated with Ni-NTA AP-labelled conjugate.

other fractions did not. Binding of antibody fraction 1 to rCul n 1 was, however, rather weak but clearly stronger than binding to HSA. The serum fraction that had been affinity-purified on HSA bound strongly to this antigen but not to rCul n 1, as shown in Table 1.

Table 1

Recombinant *Culicoides nubeculosus* 1 (rCul n 1)-specific IgG determination by ELISA in horse Ab fractions (fractions 1–3) that were affinity-purified on IgE-binding *Culicoides* proteins

Dilution of antibody fractions	HSA-purified fraction		Antibody fractions purified on <i>Culicoides</i> proteins:					
	rCul n 1	HSA	Fraction 1		Fraction 2		Fraction 3	
			rCul n 1	HSA	rCul n 1	HSA	rCul n 1	HSA
1:15	0.078 ^a	3.685	0.376	0.032	0.039	0.037	0.058	0.041
1:30	0.027	3.920	0.188	0.012	0.016	0.017	0.025	0.021
1:60	0.021	3.379	0.094	0.003	0.006	0.011	0.014	0.010
1:120	0.009	3.718	0.054	0	0.002	0.007	0.007	0.002
1:240	0	3.531	0.026	0	0	0.004	0	0
1:480	0	2.408	0.013	0	0	0.004	0	0
1:960	0	1.335	0.008	0	0	0.002	0	0

rCul n 1 was identified through screening of a *Culicoides* expression library with fraction 1. The same affinity-purified fractions were tested on human serum albumin (HSA) as negative control. For comparison, serum from horse, experimentally sensitised with HSA, was affinity-purified on HSA and tested in the same ELISA.

^a Optical density (405 nm) after subtraction of blank (blank was below 0.095 for all antigens).

3.5. Determination of serum IgE and IgG levels against rCul n 1

Serum IgE and IgG levels against rCul n 1 were determined by ELISA in the three following groups of horses: 19 horses suffering from IBH were compared to 18 healthy control horses living in an environment similar to that of the IBH-affected horses and to eight horses from Iceland which had not been exposed to the bites of *Culicoides* spp. (Fig. 5). The median IgE level of the IBH-affected horses was higher than the median IgE levels from the 18 controls but the difference did not reach statistical significance (60 and 49 REU, respectively, $P = 0.17$). The horses from Iceland had the lowest rCul n 1-specific IgE levels (median = 44 REU). Similar results were obtained for rCul n 1-specific IgG levels: IBH-affected horses had a median IgG level of 37, control horses from Switzerland a median of 26, and horses from Iceland a median of 17 REU. These differences were also not significant.

3.6. Testing of rCul n 1 in a sulfidoleukotriene release assay

rCul n 1 was tested in the sulfidoleukotriene release assay in five IBH-affected horses. The peripheral blood leucocytes from these five horses released sLT when incubated with the *C. nubeculosus* whole body extract but no significant sLT release could be

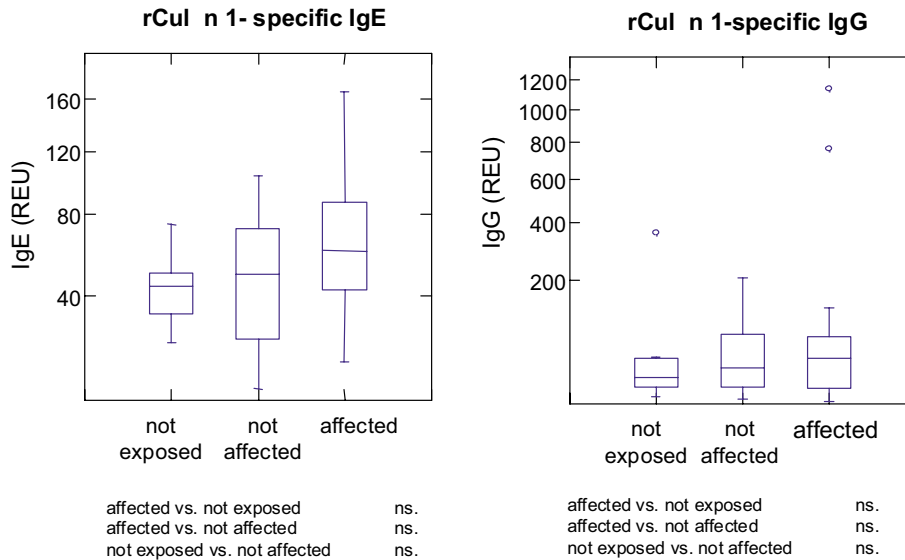


Fig. 5. IgE and IgG Ab titres in relative ELISA units (REU) against a recombinant partial ribosomal P0 protein from *Culicoides nubeculosus* (rCul n 1) in sera from 19 IBH-affected and 18 non-affected horses, and also in sera from eight horses living in Iceland, not exposed to bites of *Culicoides*. The centre horizontal line of the box plots marks the median of the sample. The edges of the box mark the first and third quartiles. Circles indicate outside values and circles far outside values.

induced by rCul n 1 in any of these five horses (sLT release < 200 pg/ml).

4. Discussion

IBH is often caused by IgE-mediated hypersensitivity reactions against bites of *Culicoides* spp. The aim of the present study was to isolate cDNA clones from a *Culicoides* expression library coding for IgE-binding proteins that could be relevant allergens for IBH. Preliminary investigations had shown that screening the library with horse serum resulted in a high background and that horse sera first needed to be affinity-purified on candidate allergens in western blots. SDS-PAGE and western blot of the *Culicoides* whole body extract showed that it consisted of a complex mixture of proteins. IgG binding proteins were so numerous that they were hardly distinguishable (data not shown), even with sera from horses living in Iceland, i.e. which should not have been bitten by these insects, suggesting that cross-reacting antibodies may be present in horse sera. Fewer *Culicoides* protein bands showed serum IgE-binding; four relatively well defined bands, which were particularly

strong with serum from one IBH-affected horse, could be identified (Fig. 1). These four bands were subsequently used for affinity-purification of the serum from this particular horse and each fraction was used to screen the *Culicoides* expression library. Screening had to be performed with an anti-horse IgG antibody because the chicken used to produce the antibody against equine IgE (Marti et al., 1997) had high antibody titres against *E. coli*, leading to high background of the affinity-purified chicken anti-horse IgE antibody on the expression library. This may be explained by the fact that the chicken was immunised with recombinant equine IgE produced in *E. coli*. Although the recombinant protein had been purified by Ni²⁺-chelate chromatography and was approximately 95% pure, the remaining contamination with *E. coli* may have been sufficient to induce high antibody titres against *E. coli* in the immunised chicken.

Only one of the four antibody fractions allowed the identification of recombinant plaques. The unsuccessful screening with the three other affinity-purified serum fractions may be due to various reasons. The proteins on which the serum fractions were affinity-purified may not have been expressed in the library, or the serum fractions may have contained antibodies

specific for glycosylated or conformational epitopes from the *Culicoides* extract which are not expressed in prokaryotic cells.

Analysis of the cDNA and deduced amino acid sequence from the isolated plaque shows a high similarity to the C-terminal part of the ribosomal P0 proteins from other *Diptera*, strongly suggesting that the isolated plaque codes for a partial P0 protein from *C. nubeculosus*. The fact that P0 proteins have a molecular mass between 35 and 38 kDa in all eukaryotes (Goswami et al., 1996), and that the serum fraction which allowed identification of the positive plaque was affinity-purified on a 36 kDa *Culicoides* protein further supports the assumption that a *Culicoides* ribosomal P0 protein was isolated from the expression library.

The acidic ribosomal P0 protein is located in the 60s subunit of eukaryotic ribosomes and forms a pentameric complex with two dimers of ribosomal proteins P1/P2 (Möller and Maassen, 1986; Liljas, 1991). This pentamer is an important structural element involved in the translocation step of protein synthesis. All P0 proteins contain three functional regions, all of which are phylogenetically well conserved. In the NH₂-terminal region a 200 amino acid long RNA-binding domain is included, as well as three clusters from positions 200 to 275, linking proteins P1 and P2 to the stalk. Consensus among the binding domain for P1 and P2 is weaker than that for the RNA-binding domain. On the other hand, the C-terminal end bears a highly conserved site involved in the interaction of the elongation factor with the ribosome (Rodriguez-Gabriel et al., 2000). rCul n 1 does not contain the RNA-binding domain, but the well-conserved C-terminal part is included in the 78 amino acid sequence of rCul n 1. Comparison of the amino acid sequence of the ribosomal P2 protein of *A. fumigatus* with rCul n 1 showed an identity of 57% over the last 38 amino acids of the C-terminus. This confirms that all three ribosomal P proteins (P0, P1 and P2) share a common linear determinant in the carboxyl-terminal amino acid sequence (Elkon et al., 1986) and that these ribosomal proteins are phylogenetically well-conserved. The ribosomal P2 protein from *A. fumigatus* is an important allergen in mould-allergic human patients (Cramer, 1998). Furthermore, a recent study suggests that some horses suffering from chronic bronchitis (CB), today called recurrent airway

obstruction (RAO), which is probably a hypersensitivity reaction to moulds in hay and straw, are also sensitised to this ribosomal protein (Eder et al., 2000). The horse whose serum was used for screening the expression library was suffering from both IBH and RAO. As earlier studies (Marti et al., 1992) had shown that horses with IBH do not suffer more frequently from RAO than horses without IBH, we did not consider this fact as a drawback for the screening. After discovering that the identified *Culicoides* clone was a ribosomal P0 protein, we measured antibody titres against the recombinant ribosomal P2 protein from *A. fumigatus* (rAsp f 8), to test whether isolation of rCul n 1 was due to crossreactive antibodies against rAsp f 8. The serum used for screening the library contained low antibody titres against rAsp f 8. However, when testing the sera from the 45 horses included in this study (see Sections 2.2 and 3.6) for IgE titres against rAsp f 8 we did not find a significant correlation between IgE titres against rAsp f 8 and rCul n 1 (data not shown). It can thus be assumed that there is no significant crossreactivity between these two recombinant peptides. This assumption is further supported by the fact that the eight horses living in Iceland, not exposed to bites of *Culicoides*, had significantly higher IgE titres against rAsp f 8 than the horses from Switzerland (data not shown). This finding is not surprising, as, due to the climate, hay is often mouldy in Iceland.

The involvement of P0 proteins in allergic diseases has not been described so far but this protein plays an important role in other immune-mediated diseases such as auto-immune diseases and, in this context, ribosomal proteins P0, P1 and P2 have been reported as capable of inducing IgG antibody responses in systemic lupus erythematosus (Elkon et al., 1985, 1986). Interestingly, the presence of auto-immune reactions against phylogenetically highly conserved intracellular proteins such as the ribosomal P2 protein or the mitochondrial manganese superoxide dismutase have been described recently in severe atopic diseases in humans (Appenzeller et al., 1999; Mayer et al., 1999). Patients with severe allergic bronchopulmonary aspergillosis had high serum IgE levels and positive skin tests both to *A. fumigatus* ribosomal protein P2 and to its human counterpart (Mayer et al., 1999). However, these cytoplasmic proteins are unlikely to be accessible for antigen-antibody interactions under

normal circumstances. This leads to the hypothesis that IgE autoreactivity may be a consequence of tissue damage and release of autoantigens at the site of inflammation. In the horse further investigations such as cloning and expression of equine ribosomal proteins would be needed to determine whether autoreactivity can also be demonstrated in severe chronic cases of IBH and RAO.

Although rCul n 1 binds equine IgG and IgE specifically (Table 1 and Fig. 5), our data gives no indication so far that the cloned and expressed *Culicoides* peptide could be a major allergen for IBH. Although horses with IBH displayed slightly higher IgE and IgG levels than control horses, the differences were not significant. Furthermore, incubation of rCul n 1 with PBL from five IBH-affected horses did not induce sLT release, although PBL from the tested horses clearly released sLT with the *Culicoides* whole body extract. Peripheral blood leukocytes from eighty per cent of IBH-affected horses but not from controls release sulfidoleukotrienes in vitro after incubation with a *Culicoides* extract (Marti et al., 1999). Unfortunately, the horse whose serum was used for screening of the expression library was no longer available to allow intradermal testing and in vitro sulfidoleukotriene and/or histamine release assays with rCul n 1. These experiments would assess whether this recombinant peptide was biologically active and could thus be an allergen responsible for IBH in only a few horses.

Ribosomal proteins are ubiquitous cytoplasmatic proteins and we would expect that the major allergens are salivary gland proteins from female *Culicoides*, as only female *Culicoides* feed on blood (James and Rossignol, 1991). Perez de Leon et al. (1994) have demonstrated strong sexual dimorphism in *Culicoides* salivary glands. Preliminary investigations with the sLT release assay, however, have shown that at least some allergens are also present in the male extract (Marti et al., 1999) and even in the larvae (data not shown), which have not yet formed fully functional salivary glands. Salivary glands have been shown to develop during the first few days after adult emergence (Perez de Leon et al., 1994). These preliminary results suggest that at least some of the allergens causing IBH (sweet itch) are not exclusively present in female salivary glands. This assumption is supported by the findings of Wilson et al. (2001): Immunohistology on

sections of fixed *Culicoides* demonstrated the presence of antibodies in horse sera which recognise *Culicoides* salivary glands, but, interestingly, staining of tissues other than salivary glands was also found with serum from some IBH-affected and from some exposed but healthy horses.

This study has led to the identification and expression of a partial ribosomal P0 protein from *C. nubeculosus*. Although, this protein does not seem to be a major allergen for IBH, it remains an interesting candidate, as ribosomal proteins have been shown to be involved in severe allergic diseases in humans. The weak reactivity of the 36 kDa affinity-purified antibody fraction with the recombinant protein suggests that cloning and expression of the complete P0 protein as well as the use of insect cell expression systems should be considered in future work. In the present study, the lack of post-translational modifications of the *E. coli*-expressed protein may be one of the reasons for the recorded, low binding. Furthermore, we cannot rule out the possibility that other IgE-binding proteins may have been included in the 36 kDa band on which the horse serum was affinity-purified. This investigation also shows that a better characterisation of the native allergens causing IBH, for example, through use of a salivary gland instead of the whole body extract, as well as monoclonal horse IgE-specific antibodies are needed to increase the chances of identifying and producing IBH allergens with recombinant technology.

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