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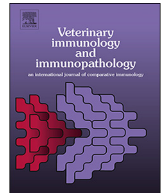
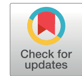


Barley produced *Culicoides* allergens are suitable for monitoring the immune response of horses immunized with *E. coli* expressed allergens



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ABSTRACT

Insect bite hypersensitivity is an allergic dermatitis of horses caused by bites of *Culicoides* midges. Sufficient amount of pure, endotoxin-free allergens is a prerequisite for development and monitoring of preventive and therapeutic allergen immunotherapy.

Aims of the study were to compare the *Culicoides nubeculosus* (Cul n) allergens Cul n 3 and Cul n 4, produced in transgenic barley grains with the corresponding *E. coli* or insect cells expressed proteins for measuring antibody and cytokine responses.

Allergen-specific IgG responses were measured by ELISA in sera from twelve horses not exposed to *Culicoides*, before and after vaccination with *E. coli*-rCul n 3 and 4. Before vaccination no IgG binding to the barley and insect cell produced proteins was detected and a similar increase in specific IgG was observed after vaccination. While IgG levels to the *E.coli* expressed proteins were higher in the post-vaccination sera, some background binding was observed pre-vaccination. *In vitro* re-stimulation of PBMC was performed for measurements of cytokines. *E. coli* expressed proteins resulted in high background in PBMC from non-vaccinated controls. The barley and insect cell expressed proteins induced similar amount of IFN-γ and IL-4 in PBMC from vaccinated horses. Barley produced allergens are promising tools for use in immunoassays.

1. Introduction

Insect bite hypersensitivity (IBH) is an allergic dermatitis of horses caused by IgE-mediated reactions to *Culicoides,* blood feeding midges, which affects between 5 and 50% of horses, depending on the breed, origin and environment (reviewed in Schaffartzik et al., 2012). The causative allergens originating from the salivary glands of the midges have been isolated and produced as recombinant proteins in *E. coli* (Peeters et al., 2013; Schaffartzik et al., 2010; Schaffartzik et al., 2011; van der Meide et al., 2013). Allergen specific immunotherapy (AIT) based on the disease causing allergens is presently the only curative treatment against allergies in human (van de Veen et al., 2017) while prophylactic immunotherapy to prevent allergic sensitization is not yet practiced although gaining attention (Valenta et al., 2012). Following prophylactic or therapeutic treatment sufficient amount of purified allergens are important for monitoring the immune response. *E. coli* is the most straight forward production system. However, the formation of inactive insolvable inclusion bodies can be a problem (reviewed in Curin et al., 2017; Jonsdottir et al., 2015). Furthermore, endotoxin contamination can lead to high background cytokine production and *E.coli* expressed proteins are thus of limited use for monitoring the cellular immune response (Jonsdottir et al., 2016). Additionally, it is beneficial to use antigens produced in a differnet expression systems that those used for the immunization, as it allows to monitor the immune response specific for the relevant antigens and not to the contaminating proteins, which even at very low concentrations (purity of the r-allergens > 95%) could still induce some immune response. Some of the *Culicoides* allergens have been expressed in insect cells, a system resulting in recombinant proteins closest to the native ones (Langner et al., 2009). However, production of proteins in insect cells is laborious and can be difficult to scale up (reviewed in Curin et al., 2017). Alternatively, barley grains are an excellent production platform for

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endotoxin-free recombinant proteins and ideal for long term storage. Recombinant proteins have been retained in barley grains for over two years without loss of function (Horvath et al., 2000). In addition, cultivation costs are low in proportion to total production costs. An efficient protein purification method using histidine tagged recombinant proteins and an inexpensive affinity purification matrix have been developed. Barley has previously been used to successfully produce other functional proteins such as bioactive growth factors that are marketed for stem-cell based research worldwide (Erlendsson et al., 2010; Magnusdottir et al., 2013).

Our goal was to investigate whether barley produced allergens could be used for evaluation of antibodies in serum and activation of cytokine production from PBMC following immunization with *E. coli* expressed allergens.

2. Materials and methods

2.1. Expression of Cul n 3 and Cul n 4

Cul n 3 and Cul n 4 were expressed in *E. coli* (*E. coli*-rCul n 3 and *E. coli*-rCul n 4), purified under denaturing conditions and refolded in H_2O in accordance with Schaffartzik et al., 2011. For long term storage the allergens were lyophilized after addition of 5% trehalose. Before immunization, the allergens were re-constituted in H_2O. The allergens were also expressed in insect cells with the Bac-to Bac® Baculovirus expression system (Invitrogen, Waltham, MA, USA) (Bac-rCul n 3 and Bac-rCul n 4) according to Jonsdottir et al., 2016, with the exception of Cul n 4 which was cloned with 6xHis-tag into the pFastBac1 vector (Life Technology, Carlsbad, CA, USA). The proteins were purified under native conditions and dialyzed against 2xPBS. For protein expression in barley grains, the cDNAs for Cul n 3 and Cul n 4 with N-terminal 6xHis-tag were codon-optimized according to barley codon usage (Genscript, George Town, Cayman Islands) and subsequently used to prepare an expression cassette under the control of a natural seed specific promoter. A natural 21 amino acid signal peptide sequence (D-hordein) was included N-terminally; this sequence is cleaved off in the cell resulting in soluble forms of the target proteins. Transgenic barley (*Hordeum vulgare* L. Cv Golden Promise) expressing Cul n 3 and Cul n 4 was generated by utilizing the *Agrobacterium tumefaciens* mediated transformation method. After milling of the grains and extraction, the proteins were purified by using an IMAC column (GE Healthcare Life Sciences, Pittsburgh, PA, USA) as the capture step and an IEX or a cation exchanger (GE Healthcare Life Sciences, Pittsburgh, PA, USA) for the subsequent polishing step. Buffer exchange was performed on the final products using 2xPBS. All proteins (1 µg) were analysed with coomassie blue staining and western blot with specific mouse polyclonal antibodies (made at the Institute for Experimental Pathology University of Iceland, Keldur) against *E. coli*-rCul n 3 and -rCul n 4 diluted 1:4000 (Jonsdottir et al., 2015; Schaffartzik et al., 2011).

2.2. Horses

Antibody measurements were performed in serum samples from twelve healthy Icelandic horses, one mare and eleven geldings (age 6–11, mean age 8), located in Iceland, *i.e.* not exposed to *Culicoides* bites. All horses were vaccinated intralymphatically on week 0, 4 and 8 with a combination of 10 µg each of *E. coli*-rCul n 3 and rCul n 4 in either 500 µg of aluminium hydroxide (alum) or 500 µg alum and 50 µg monophosphoryl lipid A (MPLA) in a total volume of 400 µL (Jonsdottir et al., 2016). Blood was taken before (week 0) and after the third vaccination (week 10) and sera collected for allergen specific total IgG and IgG subclasses determination by ELISA.

For measurement of cytokine production, three additional healthy Icelandic horses, one mare and two geldings (age 7, 10, 12), located in Iceland, were vaccinated as described above using the combination of alum and MPLA as adjuvant. At weeks 0, 6 and 10 heparinized blood was collected from the three vaccinated horses and from three non-vaccinated control horses, three geldings (age 9, 10, 11), located in Iceland, for isolation of peripheral blood mononuclear cells (PBMC). Blood was also collected for preparation of serum for antibody measurements. The experiment was carried out in accordance with a permit from the National Animal Research Committee of Iceland, no. 2016-01-03.

2.3. ELISA

The ELISA plates, 96 well flat bottom (MaxiSorp, Thermo Fisher Scientific, Waltham, MA, USA), were coated with 0.2 µg/well of the allergens: *E. coli*-, Bac- or Barley-rCul n 3 or -rCul n 4 in coating buffer (carbonate-bicarbonate buffer, pH 9.5, Sigma-Aldrich, St. Louis, MO, USA). The plates were incubated for 2 h at 37 °C and then stored at −20 °C. In each step of the ELISA 100 µL were added to each well, except in the blocking step, where 200 µL were used. The sera and the antibodies were diluted in blocking buffer (PBS containing 500 mM NaCl and 5% Tween 20 and 5% dried milk powder). Before use the plates were thawed at 37 °C and washed with high salt ELISA wash buffer (PBS containing 500 mM NaCl and 0.05% Tween 20). The washing was done after each incubation step until addition of substrate. Non-specific binding sites were blocked with blocking buffer for 1 h at 37 °C. Serum from all the vaccinated horses, diluted 1:1600 was added in triplicate and incubated for 1 h at 37 °C. Then conjugate HRP-labelled Goat anti-horse IgG (Jackson ImmunoResearch, Ely, Cambridgeshire, UK) diluted 1:7000 was added and incubated for 1 h at 37 °C. The substrate, o-phenylenediamine dihydrochloride (OPD, Dako, Santa Clara, CA, USA) and peroxide, was added and incubated in the dark for 10 min at RT. The reaction was stopped with 75 µL/well of 4N H_2SO_4 and optical density (OD) measured at 490 nm. For measurement of IgG subclasses, horse sera were incubated at a dilution of 1:800 and detected with antibodies against IgG1 (IgGa), IgG4/7 (IgGb), IgG5 (part of IgG(T)), used at a concentration of 1 µg/ml (Keggan et al., 2013) and IgG (T) (IgG3/5) (0.5 µg/ml, Serotec, Hercules, CA, USA) followed by HRP-labelled anti-mouse IgG (Jackson ImmunoResearch) as above. Optimal serum dilutions for the ELISA were chosen based on the results from a titration pilot experiment (data not shown). All sera could be tested on a single plate for each allergen. Serum with a previously determined high titer was included on every plate in twofold dilutions 1:100–1:25,600 in duplicate and used as positive control. The intra-assay coefficient of variation was below 7%.

2.4. Stimulation of PBMC and determination of cytokines

PBMC from the 3 vaccinated horses and 3 non-vaccinated controls were isolated by Ficoll-Hypaque (Hamza et al., 2007; Jonsdottir et al., 2016) before vaccination and two weeks after the second (week 6), and third vaccination (week 10). PBMC were stimulated in duplicate for 24 and 48 h with *E. coli,* Bac- and Barley-rCul n 3 and rCul n 4, 2 µg/ml (previously determined with titration, data not shown) or the two from the same expression system mixed together at a concentration of 2 µg/ml each (Jonsdottir et al., 2016). Concanavalin A (Con A 10 µg/ml) was used as a positive control and cells in medium alone as a control for spontaneous cytokine release. IL-4, IFN-γ and IL-10 were measured in cell supernatants using a fluorescent bead-based Cytokine Multiplex assay (Wagner and Freer, 2009). Correction for spontaneous cytokine release was performed on the values for the allergen and Con A stimulations. The data for IL-4 and IL-10 are shown as pg/mL and for the IFN-γ as U/mL.

2.5. Statistical analysis

Statistical analyses were carried out using the software program NCSS 10 (NCSS Statistical Software, Kaysville, UT, USA). Descriptive statistics were run and Shapiro-Wilk W test showed that the data were

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