



Research paper

Developing a preventive immunization approach against insect bite hypersensitivity using recombinant allergens: A pilot study



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ABSTRACT

Insect bite hypersensitivity (IBH) is an allergic dermatitis of horses caused by bites of midges (*Culicoides* spp.). IgE-mediated reactions are often involved in the pathogenesis of this disease. IBH does not occur in Iceland due to the absence of *Culicoides*, but it occurs with a high frequency in Icelandic horses exported to mainland Europe, where *Culicoides* are present. We hypothesize that immunization with the *Culicoides* allergens before export could reduce the incidence of IBH in exported Icelandic horses. The aim of the present study was therefore to compare intradermal and intralymphatic vaccination using four purified recombinant allergens, in combination with a Th1 focusing adjuvant.

Twelve horses were vaccinated three times with 10 µg of each of the four recombinant *Culicoides nubeculosus* allergens. Six horses were injected intralymphatically, three with and three without IC31[®], and six were injected intradermally, in the presence or absence of IC31[®]. Antibody responses were measured by immunoblots and ELISA, potential sensitization in a sulfidoleukotriene release test and an intradermal test, cytokine and FoxP3 expression with real time PCR following *in vitro* stimulation of PBMC.

Immunization with the r-allergens induced a significant increase in levels of r-allergen-specific IgG1, IgG1/3, IgG4/7, IgG5 and IgG(T). Application of the r-allergens in IC31[®] adjuvant resulted in a significantly higher IgG1, IgG1/3, IgG4/7 allergen-specific response. Intralymphatic injection was slightly more efficient than intradermal injection, but the difference did not reach significance.

Testing of the blocking activity of the sera from the horses immunized intralymphatically with IC31[®] showed that the generated IgG antibodies were able to partly block binding of serum IgE from an IBH-affected horse to these r-allergens. Furthermore, IgG antibodies bound to protein bands on blots of *C. nubeculosus* salivary gland extract.

No allergen-specific IgE was induced and there was no indication of induction of IgE-mediated reactions, as horses neither responded to *Culicoides* extract stimulation in a sulfidoleukotriene release test, nor developed a relevant immediate hypersensitivity reaction to the recombinant allergens in skin test. IL-4 expression was significantly higher in

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horses vaccinated intralymphatically without IC31[®], as compared to horses intradermally vaccinated with IC31[®]. Both routes gave higher IL-10 expression with IC31[®].

Both intralymphatic and intradermal vaccination of horses with recombinant allergens in IC31[®] adjuvant induced an immune response without adverse effects and without IgE production. The horses were not sensitized and produced IgG that could inhibit allergen-specific IgE binding. We therefore conclude that both the injection routes and the IC31[®] adjuvant are strong candidates for further development of immunoprophylaxis and therapy in horses.

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1. Introduction

Equine insect bite hypersensitivity (IBH), also called summer eczema (SE), is an allergic recurrent seasonal dermatitis of horses. IgE-mediated, type I hypersensitivity with release of histamine and other inflammatory mediators from basophils and mast cells are often involved in IBH. However, in some cases cell-mediated, type IV hypersensitivity may also contribute to the pathogenesis. The eczema is caused by bites of insects mainly of the genus *Culicoides* (biting midges) (Fadok and Greiner, 1990; Quinn et al., 1983; Schaffartzik et al., 2012) and characterized by pruritic dermatosis affecting mainly the mane and tail area causing discomfort and often suffering in affected individuals (Bröstrom et al., 1987; Townley et al., 1984). The prevalence of the disease depends on the geographic region and exposure to insect bites. IBH is found almost worldwide, but one of the exceptions is Iceland where *Culicoides* spp. are not indigenous. However, Icelandic horses foaled in Iceland and exported to the European continent are more frequently affected than Icelandic horses or horses from other breeds born in an environment where *Culicoides* are present (Bröstrom et al., 1987; Halldorsdottir and Larsen, 1991). The frequency of IBH in horses exported as adults and not protected from *Culicoides* bites was more than 50% after two years or more in heavily *Culicoides* infested areas (Bjornsdottir et al., 2006) while only 5–10% of Icelandic horses foaled on the European continent developed the disease (Bröstrom et al., 1987; Halldorsdottir and Larsen, 1991). The reasons for this increased incidence of IBH in horses born in Iceland and exported to Europe or North America as adults are not known; environmental or epigenetic factors may contribute to this response (Marti et al., 2008). Interestingly, it has been demonstrated that the prevalence of IBH in horses imported from Iceland is influenced by the age at import, i.e. at first exposure to *Culicoides* allergens. Horses imported as weanlings at an age of 7–10 months did not develop IBH more frequently than Icelandic horses born in Europe (Sommer-Locher et al., 2012), suggesting that early life exposure to the causative allergens is required to prevent development of IBH. This may be explained by a higher capacity of young horses to develop a regulatory T cell immune response. A recent study has demonstrated that significantly higher number of functionally mature regulatory T cells can be induced *in vitro* in foals compared to adult horses (Hamza et al., 2015).

The allergens causing IBH originate in the *Culicoides* salivary glands of the blood-feeding females (Hellberg et al., 2006; Wilson et al., 2001). Different *Culicoides*

species dominate in different geographical areas and IBH-affected horses have been shown to react in skin tests to *Culicoides* extracts made from both native and exotic species (Anderson et al., 1993). The first salivary gland proteins that bind IgE from IBH affected horses to be identified and produced as recombinant proteins were derived from laboratory-produced species. These comprised one protein from *Culicoides sonorensis* (Langner et al., 2009) and eleven from *Culicoides nubeculosus* (Schaffartzik et al., 2010, 2011). These two species are not very common in Europe, but the salivary proteins from them bind IgE from IBH horses with variable frequency. Recently, seven salivary gland proteins originating from *Culicoides obsoletus*, the main midge feeding on horses in the Netherlands, have been identified and expressed (van der Meide et al., 2013).

Our findings regarding the immune response and pathogenesis in IBH suggest that Th2 type, IgE-mediated immune reactions are involved to a much stronger extent in Icelandic horses than in some other breeds, and after export there is an imbalance between Th1, Th2 and T regulatory cells (Treg) (Hamza et al., 2008, 2010, 2013; Heimann et al., 2011). It should therefore be possible to rebalance the Th1:Th2:Treg responses and restrain the inflammatory mechanisms by strengthening the Treg response specific to the allergens using immunotherapy.

In a pilot study, attempts to shift the immune response in horses toward Th1 with subcutaneous and intramuscular injections of proteins in the Th1 adjuvant Monophosphoryl-lipid A, were only partly successful (Marti et al., 2008). Therefore, both the injection method and the adjuvant part had to be reconsidered.

Data from experimental animals and from clinical trials have shown that intralymphatic allergen administration strongly enhanced specific immunotherapy. It required reduced allergen dose, shorter duration and fewer injections as compared to the classical subcutaneous method (Senti et al., 2011). Injection into the submandibular lymph nodes is however, impractical in horses and hence intradermal injections were considered as an alternative. The skin has an extended local network of several types of professional antigen presenting cells and easy access to the skin-draining lymph nodes (Combadiere and Liard, 2011).

The IC31[®] is an adjuvant that combines the antimicrobial peptide KLK₅KLK and a synthetic oligodeoxynucleotide (ODN1a). It stimulates the immune system via the TLR9/MyD88-dependent pathway. IC31[®] induces potent Th1 immune response in mice (Schellack et al., 2006) and it has been shown *in vitro* that IC31[®] modulates the cytokine profile of human dendritic cells which is important for

protection against intracellular pathogens (Lingnau et al., 2007; Szabo et al., 2013). To our knowledge, this adjuvant has not been used in horses before.

The aim of this pilot study was to obtain a first set of data on an effective injection route with a small amount of recombinant allergens in a suitable adjuvant to vaccinate horses. This could then be used as a basis for future studies on a preventive immunization strategy against insect bite hypersensitivity.

2. Materials and methods

2.1. Animals

Twelve healthy Icelandic horses aged 5–8 years were vaccinated, and three additional horses used as controls in the skin test. They were maintained according to the Icelandic animal care guidelines for experimental animals. The horses were housed and fed hay during winter and during summer they were kept out at pasture. The experiment was performed in accord with a permit from the national animal research committee of Iceland.

2.2. Vaccination and sample collection

Twelve horses nos. 1–12 were vaccinated with four recombinant *C. nubeculosus* allergens (rCul n 1, rCul n 2, rCul n 5 and rCul n 9) produced in *Escherichia coli* and purified (Schaffartzik et al., 2011). These allergens have previously been shown to be relevant allergens for IBH, as allergic horses have significantly more often serum IgE specific for these recombinant allergens than healthy control horses and they induce immediate type reaction in intradermal tests in IBH-affected horses (Schaffartzik et al., 2010, 2011). The horses were vaccinated three times (week 0, 5 and 9) with 10 µg of each r-allergen in a mixture of 136 µL. Six horses were immunized with IC31® adjuvant (250 µL), provided by Valneva Austria GmbH, Vienna and six without (250 µL PBS, instead of IC31®). The horses were assigned randomly into four groups. The veterinarian that immunized and monitored the horses was blinded to the use of adjuvant. Horses nos. 1–3 were immunized intradermally without adjuvant and nos. 4–6 intradermally with IC31®, at four different locations on the neck. Horses nos. 7–9 were immunized in the submandibular lymph nodes without IC31® and nos. 10–12 intralymphatically with IC31®.

Blood was collected by jugular venipuncture at week 0, every other week for 15 weeks, and then monthly until week 30. Serum was stored at –20 °C until used. EDTA blood was taken before, 24 h and 48 h after each vaccination. Differential count of leukocytes was carried out within 4 h from bleeding. Body temperature and site reaction, i.e. swelling of lymph nodes estimated by palpation and skin reaction measured with vernier caliper, was recorded before, immediately after, 1 h, 4 h, 24 h and 48 h after each vaccination.

2.3. Serological tests

2.3.1. Immunoblot

Immunoblots with recombinant proteins. The allergens, rCul n 1, rCul n 2, rCul n 9 (1 µg) and rCul n 5 (2 µg) were

separated by SDS-PAGE under denaturing conditions and transferred to PVDF transfer membrane (Millipore). After blocking with TBS-T (Tris buffered saline containing 0.1% Tween 20) containing extra 2% Tween 20, the membranes were cut into strips and incubated overnight (o.n.) at 4 °C on a rotator with sera diluted 1:2000 in TBS-T. The following day, the strips were washed a few times with TBS-T before incubation with rabbit anti-horse IgG-AP conjugate (Sigma) diluted 1:2000 in TBS-T. The strips were rewashed in TBS-T and bound antibodies detected with BCIP/NBT (Roche) diluted 1:50 in alkaline phosphatase buffer (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M MgCl₂, pH 9.5). As controls, strips were incubated with TBS-T alone or with protein specific antibodies (Schaffartzik et al., 2011).

Immunoblots with C. nubeculosus salivary gland extract (SGE). SDS-PAGE of *C. nubeculosus* SGE and Western blots were performed as described in Hellberg et al. (2006). Strips all derived from the same blot were incubated with the pre-immune sera and sera taken 2 weeks after the last immunization (week 11), diluted 1:30. Furthermore, for identification of protein bands in SGE corresponding to the recombinant proteins, mouse antibodies specific for the recombinant proteins were also incubated on SGE strips (Schaffartzik et al., 2011).

2.3.2. ELISA

Antibody levels (IgG subclasses and IgE) in sera of immunized horses (weeks 0, 2, 7, 11, 19 and 30) were measured by ELISA. Sera from five unvaccinated horses living in Iceland were added as negative controls and sera from five IBH-affected horses of the Icelandic breed living in Switzerland with high IgE to the recombinant allergens were included on each of the plates as positive controls. All samples were tested in duplicates. In all steps 100 µL were added to each well except for the blocking step and for the phosphatase substrate, where 200 µL/well were added. ELISA plates (Thermo Scientific) were coated with recombinant allergens (rCul n 1, rCul n 2, rCul n 5 or rCul n 9) (Schaffartzik et al., 2010, 2011), 2 µg/mL, diluted in 0.2 M Carbonate-Bicarbonate buffer, pH 9.4 (Thermo scientific) for 2 h at 37 °C. Plates were then washed with 150 mM NaCl (Merck) and 0.05% Tween 20 (Sigma). Non-specific binding sites were blocked with 5% dried milk powder and 5% Tween 20 in PBS (Calbiochem®), for 1 h at 37 °C. The sera (weeks 0, 2, 7, 11, 19, 30) were diluted in blocking buffer, 1:5 for IgE and 1:200 for IgG subclass detection and incubated overnight (o.n.) at 4 °C. After washing, monoclonal antibodies specific for equine IgE (1 µg/mL, clone 3H10 (Wilson et al., 2006)) or for equine IgG1 (1 µg/mL CVS45), IgG1/3 (1 µg/mL clone 159 (Goodman et al., 2012)), IgG4/7 (1 µg/mL; CVS 39), IgG5 (1 µg/mL; clone 416 (Goodman et al., 2012)) IgG6 (1 µg/mL, clone 267 (Goodman et al., 2012)) or IgG(T) (Serotec) were added to the plate and incubated for 2 h at RT on a shaker. After washing alkaline-phosphatase-conjugated goat-anti mouse IgG with minimal cross-reactivity to horse serum proteins (Jackson ImmunoResearch) diluted 1:2000 in blocking buffer was added to each well and incubated for 1½ h at RT on a shaker. After a final wash, the plates were developed with a solution of a 1.5 mg/mL phosphatase substrate (Sigma) in 10% diethanolamine (Fluka), pH 9.8.

Absorbance was measured at 405 nm after 2 h for IgE and 30 min for the IgG subclasses. Analysis of the ELISA results was performed as described in [Peeters et al. \(2013\)](#). Briefly, after blank correction on the optical density (OD₄₀₅) values the correction factor for each plate were calculated by using the average OD values of the positive control samples (sera from horses living in Switzerland and from two samples of the immunized horses that were included on each plate). Further analyses of the data were performed using the corrected OD₄₀₅ values.

2.4. Competitive inhibition ELISA

The ability of sera from horses vaccinated intralymphatically with IC31® (i.l.+ group) to block IgE-binding of serum from IBH-affected horses to the recombinant allergens was tested.

The ELISA plates were coated and then blocked as described in Section 2.3.2. The sera from the i.l.+ group, week 0 (as negative control) and week 11 were serially twofold diluted from 1:5 to 1:40 and 50 µL/well were added in duplicates to the plates. After 1 h incubation at 37 °C, 50 µL/well of serum from an IBH-affected horse with high IgE to the respective allergens was added at a final dilution of 1:5. Plates were incubated at 4 °C o.n. after washing, anti IgE was added followed by an alkaline-phosphatase-conjugated goat-anti mouse IgG with minimal cross-reactivity to horse serum proteins (Jackson ImmunoResearch) and developed as described in Section 2.3.2. Percentage of inhibition for each dilution of the week 0 and week 11 sera was calculated with the following formula:

$$\% \text{ Inhibition} = 100 - \left(\frac{\text{OD of IBH serum after inhibition with serum from immunized horse}}{\text{OD of IBH serum}} \times 100 \right)$$

2.5. Stimulation of PBMC

Before immunization (week 0) and 2 weeks after the 3rd immunization (week 11), PBMC were isolated by density gradient centrifugation as described by [Hamza et al. \(2007\)](#) using Ficoll-Hypaque procedure. PBMC, 2×10^6 in 2 mL, were cultured with ConA, as positive control (10 µg/mL), or 10 µg/mL of rCul n 1, rCul n 2, rCul n 5 or rCul n 9 or were left unstimulated for 24 h.

All cell cultures were performed in RPMI medium with glutamax (Invitrogen) supplemented with horse serum (10%), penicillin (100 U/mL), streptomycin (100 µg/mL), non-essential amino acids (1%), MEM vitamins (100 µM), sodium pyruvate (1 mM), 2-mercaptoethanol (50 µM). After 24 h PBMC were harvested, lysed and homogenized in RA1 lysis buffer and stored at –80 °C until they were processed for total RNA isolation.

2.6. Total RNA isolation, reverse transcription of RNA and QRT-PCR

Total RNA isolation was performed using a commercial kit (Macherey-Nagel) according to the manufacturer's protocol. RNA concentration was measured by spectrophotometry (NanoDrop® ND-1000). cDNA synthesis

was performed using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. Briefly, 0.69–1.84 µg of total RNA and random hexamers were incubated at 65 °C for 5 min and subsequently chilled on ice. Then, 5× reaction buffer, dNTPs, RiboLock RNase inhibitor and RevertAid reverse transcriptase were added and incubated for 5 min at 25 °C and 50 min at 42 °C. Reaction was stopped by incubating for 5 min at 70 °C.

cDNA was stored at –80 °C until used for subsequent quantification of mRNA coding for IFN-γ, IL-4, IL-10 and FoxP3 using TaqMan procedure and the same primers and probes as in [Heimann et al. \(2011\)](#). All samples were run blind by the operators for qRT-PCR in order to exclude bias by the operator. Duplicate cDNA samples were amplified for 45 cycles (15 s at 95 °C and 60 s at 60 °C) in an AB7300 real-time PCR instrument (Applied Biosystems, CA, USA) using Maxima Probe qPCR Mastermix (Fermentas). Cycle threshold (Ct) values of cytokine gene expression were normalized to the 18s rRNA content (ΔCT) as described in [Lanz et al. \(2013\)](#). No significant difference was detected in any of the measured parameters in the un-stimulated cells between the groups of horses. Therefore, the number of transcript copies in each sample was calculated in relation to un-stimulated cells according to $(2^{-\Delta\Delta CT})$ Method. The values obtained after immunization (week 11) were expressed in relation to the values obtained before immunization, i.e. week 11/week 0 and named relative IFN-γ, IL-4, IL-10 or FoxP3 mRNA expression.

2.7. Sulfidoleukotriene release test

Before vaccination and six weeks after the last vaccination, a sulfidoleukotriene (sLT) release test was performed according to [Baselgia et al. \(2006\)](#). In short, ACD-B anticoagulated blood samples (Vacuette) were mixed, and 20 min later, after sedimentation of the erythrocytes, the leukocyte-rich plasma was collected, transferred into a propylene tube and centrifuged at $130 \times g$ for 10 min at RT. The supernatant containing most of the platelets was discarded and the pellet resuspended in 3 mL of stimulation buffer (Bühlmann Laboratories AG). Resuspended leukocytes were incubated at 37 °C with *C. nubeculosus* extract (2 and 6 µg/mL) prepared from laboratory-bred *C. nubeculosus* as described by [Marti et al., 1999](#) ([Marti et al., 1999](#)) or concanavalin A (ConA, 20 µg/mL) used as a positive control or with buffer alone to determine spontaneous sLT release. After 40 min incubation, plates were centrifuged at $1000 \times g$ at 4 °C and the supernatant transferred into a new 96-well microtiter tissue culture plate and kept at –20 °C until assayed. sLT determination was carried out using the CAST 2000-ELISA following the manufacturer's instruction (Bühlmann Laboratories AG). For all further evaluation

values of the net stimulation were used, i.e. the spontaneous sLT release was subtracted from the values obtained with ConA or with *C. nubeculosus* extract, as performed previously (Baselgia et al., 2006). The cut offs defined in a previous study (Baselgia et al., 2006) were used to define a positive test result.

2.8. Intradermal test

Intradermal tests were performed six months after the first vaccination on vaccinated horses and on three healthy control horses, as described in Schaffartzik et al. (2011). The recombinant allergens (rCul n 1, 2, 5 and 9) were diluted to a concentration of 10 µg/mL and 1 µg/mL in dH₂O or PBS depending on the solubility of the protein. The skin test area, in the lateral neck (opposite site to the vaccination) was clipped and 100 µL of the recombinant allergens, of dH₂O and PBS used as negative controls, and of 0.2 mg/mL histamine diluted in dH₂O or PBS as positive control, were injected intradermally. Diameters of the wheal reaction at the injection sites were measured after 30 min.

2.9. Statistical analyses

The statistical analyses on the IgG subclass and cytokine data were performed using the statistical software program NCSS 8 (NCSS Statistical Software, 329 North 1000 East, Kaysville, UT 84037, USA). Descriptive statistics were run on the IgG subclasses and IgE ELISA results. Shapiro Wilk test showed that after log transformation of the data, IgG(T) levels were normally distributed (Shapiro Wilk $W \geq 0.99$, $p > 0.05$). According to the Shapiro Wilk W test, the log-transformed IgG1, IgG1/3, IgG4/7 and IgG5 antibody levels were strictly taken still not normally distributed (Shapiro Wilk $W \geq 0.96$, $p < 0.01$). However, because there was no severe violation of normality according to the visual evaluation of the normality plots, and because the repeated measures ANOVA is robust to minor violations of multivariate normality (http://www.ats.ucla.edu/stat/sas/library/repeated_ut.htm), repeated measures ANOVA was performed for determination of the effects of the administration route (intradermal versus intralymphatic) of the adjuvant (with or without IC31®) on antibody levels in function of time (week 0 to week 30) and of the 4 r-allergens (rCul n 1, 2, 5 and 9) used for immunization on these variables. Separate models were run with levels of IgG1, IgG1/3, IgG4/7 and IgG5 as outcome variables, respectively. Interaction terms of significant variables were also included in the models. Mauchly's test was used to check for the assumption of circularity of within-subject covariance matrices. Because this assumption was not always fulfilled, the Geisser–Greenhouse correction was used for the within-subject F test. $p \leq 0.05$ was considered significant. For significant factors in any of the repeated measures ANOVA models, significant subgroup differences were identified with a pairwise multiple comparison test with a Bonferroni correction of the significant level.

IgG6 and IgE values could not be analyzed with the repeated measures ANOVA because of severe violation

of normality, even after log-transformation (Shapiro Wilk $W < 0.91$, $p < 1 \times 10^{-10}$).

As even after log transformation the cytokine data were not normally distributed, the non-parametric ANOVA Kruskal–Wallis multiple-Comparison Z-Value Test was used to compare cytokine (IFN-γ, IL-4, IL-10) and FoxP3 mRNA expression by stimulated PBMC from horses of the four immunization groups (immunized in lymph node with [i.l.+] or without [i.l.–] or intradermally with [i.d.+] or without [i.d.–] IC31®). The Bonferroni test was used to correct for multiple comparisons. On each occasion $p \leq 0.05$ was considered significant.

A linear mixed effect model was used to compare the mean diameter (from the four different injection locations) of skin reaction in RStudio (version 0.98.1091, <http://www.rstudio.com/>). The model used adjuvant, time and vaccination as categorical variables and horse as a random factor. The horses received the allergens with or without adjuvant, the skin reaction was measured at five different time points post injection and the horses were vaccinated three times.

3. Results

3.1. Clinical examination

The immunization was well tolerated, no rise in temperature, local swelling or soreness up on palpation of submandibular lymph nodes was observed. Diameter of skin reaction was measured at five different time points (0, 1, 4, 24 and 48 h) post injection of allergens alone or with IC31®. Four hours after injection the reaction was significantly greater in the horses that received the allergens in IC31® ($p = 0.002$) but significant difference was not observed at other time points (data not shown). Leukocyte count was within normal range following vaccinations (data not shown).

3.2. Specific antibody response of vaccinated horses

3.2.1. Total IgG response

The total IgG response two weeks after the third vaccination was measured by immunoblot of the allergens used for vaccination. Horses that were vaccinated with the recombinant allergens in IC31® responded much more strongly than those vaccinated with allergens alone. This was seen regardless of whether they were injected intradermally or intralymphatically. There was no clear difference between the two injection routes (supplementary data, Table S1 and Figure S1).

Supplementary table and figure related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vetimm.2015.05.002>.

3.2.2. IgG subclass response

Sera from vaccinated horses, taken before, two weeks after each of the three vaccinations and after the skin test were tested for IgG subclass responses against rCul n 1, rCul n 2, rCul n 5 and rCul n 9 in ELISA.

The repeated measures ANOVA showed that immunization with 10 µg of each of the four r-allergens resulted

in a highly significant increase in antibody levels (Fig. 1), although the time course and degree of the increase varied depending on the IgG subclass and the specific r-allergen (Table 1 and Fig. 1). For IgG1 and IgG1/3 subclasses, a significant increase was already observed two weeks after the 1st immunization. These antibody levels increased further after the 2nd and 3rd immunization, and then decreased until week 19, where they were significantly lower than at week 7 and 11, i.e. two weeks after the 2nd and 3rd immunization, respectively (Table 1). The intradermal test performed at week 28 boosted the antibody response. At week 30, the IgG1 and IgG1/3 response reached similar levels as at week 7 and 11, except for Cul n 9. The repeated measures ANOVA further revealed that use of IC31® resulted in significantly higher IgG1 and IgG1/3 levels, while there was only a tendency for intralymphatic compared to intradermal immunization to induce a higher IgG1/3 response ($p=0.07$). Although an increase in IgG1 and IgG1/3 levels was observed for all four r-allergens, the antibody responses to rCul n 9 and rCul n 5 were significantly higher than to rCul n 1 (IgG1, IgG1/3) and rCul n 2 (IgG1/3).

For IgG4/7, the results were similar but not as clear as for the two subclasses described above. Even before immunization relatively high (mean OD value 0.68–1.48) values were obtained by ELISA with the four rCul n allergens. Immunization nonetheless induced an increase in IgG4/7 levels, but conversely to IgG1 and IgG1/3, this increase was only statistically significant at week 7, i.e. two weeks following the 2nd immunization. As for IgG1 and IgG1/3, IgG4/7 levels also decreased until week 19 and then increased following the IDT (Fig. 1), but these variations were not as clear as for IgG1 and IgG1/3 and did not reach statistical significance. Immunization with adjuvant again resulted in a higher, although not significant ($p=0.07$), antibody response; nor did the immunization route influence the IgG4/7 response significantly. The different r-allergens also induced different IgG4/7 levels ($p=0.052$), with again rCul n 5 and rCul n 9 resulting in higher titers than rCul n 1 and Cul n 2.

The measured IgG5 and IgG(T) levels were lower than the IgG1, IgG1/3 and IgG4/7 levels, but a significant increase following the 2nd immunization (week 7) could be observed and the increase and decrease of IgG5 and IgG(T) levels followed a similar pattern as the other IgG subclasses. However, for IgG5 and IgG(T) no significant effect of adjuvant or administration route could be observed in the repeated measures ANOVA, although intralymphatic immunization with IC31® led to the highest IgG5 and IgG(T) responses (Fig. 1). The strongest IgG5 response was seen against rCul n 9, which was significantly higher than against the three other r-allergens. For IgG(T), rCul n 5 and rCul n 9 induced the highest response (Table 1).

IgG6 antibody levels were very low and no increase could be observed following immunization (data not shown).

3.2.3. IgE response

Sera from vaccinated horses taken before, two weeks after each of the three vaccinations, and after the skin test, were tested for IgE response against rCul n 1, rCul n 2,

rCul n 5 and rCul n 9 by ELISA. No IgE production could be detected at any time point (supplementary data, Figure S2). The OD values of IBH affected horses, used as positive controls, were between 2 and 2.8.

Supplementary figure related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vetimm.2015.05.002>.

3.3. Inhibition of allergen-specific IgE-binding by sera from horses vaccinated intralymphatically with IC31®

The sera from the horse group with the strongest IgG responses, i.e. those vaccinated intralymphatically with the allergens in IC31® were tested for their IgE blocking capacity. While preimmune sera led to no or only marginal inhibition of IgE-binding, addition of the postimmune sera resulted in a mean inhibition of 54.4–18.4%, depending on the allergen tested (Fig. 2).

3.4. Binding of allergen-specific IgG antibodies from vaccinated horses to *C. nubeculosus* salivary gland extract

Sera taken before immunization and two weeks after the last vaccination were tested on salivary gland extract from *C. nubeculosus* by immunoblotting. IgG1 from vaccinated horses bound to proteins of the correct size, namely Cul n 1 and Cul n 9, as shown by comparison with the binding of mouse antibodies specific for the corresponding r-proteins (Schaffartzik et al., 2011). Binding to Cul n 2 and Cul n 5 cannot be distinguished because of the similar size of these two protein bands. The immunoblot also showed that IgG binding of post-vaccination sera to Cul n 1 and Cul n 9 was usually stronger in the IC31® vaccinated group compared to horses vaccinated without IC31®. This difference was especially clear for the Cul n 1 protein (Fig. 3).

3.5. Cytokine and FoxP3 mRNA expression of the vaccinated horses

The relative cytokine expression by stimulated PBMC was compared between the four immunization groups. Testing of the results for the single r-allergens or stimulant showed no significant differences in the One-Way ANOVA for any of the cytokines or FoxP3. As this was probably due to the small number of horses per group, the data of the 4 r-allergens was then pooled to enhance the power of the analyses (Fig. 4). No significant differences between the immunization groups could be found for relative IFN- γ and FoxP3, while IL-4 expression was significantly higher in the i.l.– group compared to the i.d.+ group. Interestingly, horses immunized with IC31® either intralymphatically or intradermally had a significantly higher IL-10 expression than those immunized intradermally without IC31® (i.d.–). IL-10 expression in the i.l.+ group was also higher than in the i.l.– group, but the difference did not reach statistical significance (Fig. 4).

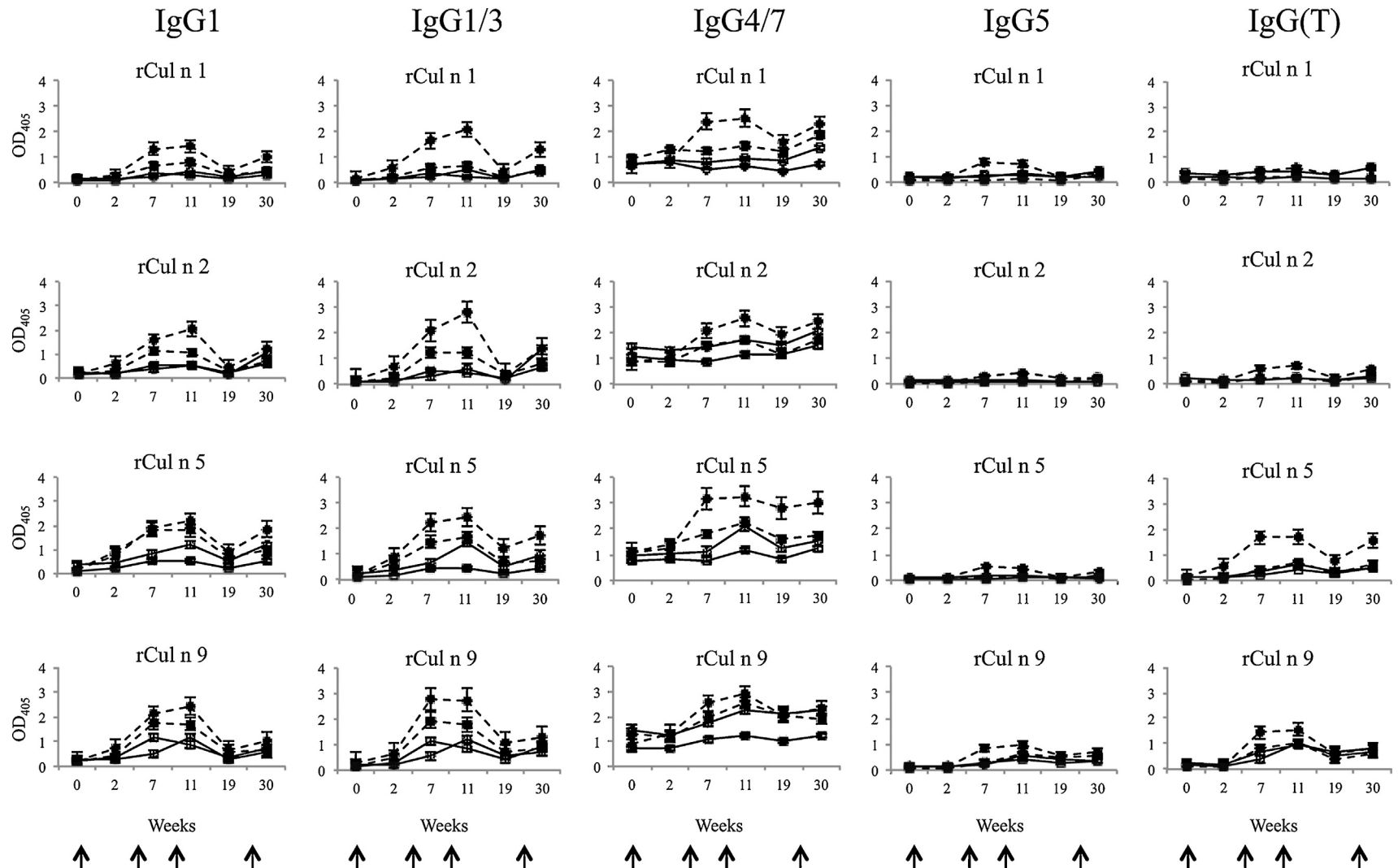


Fig. 1. Time course of IgG subclass response measured by ELISA. Corrected OD mean values and standard error for the three horses in each group at different time points. Intradermal injection without adjuvant (—□—), intradermal injection with IC31® adjuvant (—■—), intralymphatic injection without adjuvant (—○—), intralymphatic injection with IC31® adjuvant (—●—). The first three arrows indicate the vaccination time points and the last the intradermal test.

Table 1Factors influencing the IgG subclass responses following immunization of horses with recombinant *Culicoides nubeculosus* (Cul n) allergens. Results of the repeated measures ANOVAs.

	log IgG1		log IgG1/3		log IgG4/7		log IgG5		log IgG(T)	
	Mean (SE)	p value	Mean (SE)	p value	Mean (SE)	p value	Mean (SE)	p value	Mean (SE)	p value
Adjuvant (IC31®)										
No	2.49 (0.06)	$p \leq 0.01$	2.47 (0.06)	$p \leq 0.01$	2.99 (0.06)	$p = 0.07$	2.00 (0.13)	ns	2.38 (0.08)	ns
Yes	2.79 (0.06)		2.81 (0.06)		3.17 (0.06)		2.11 (0.13)		2.42 (0.08)	
Localization										
Intradermal	2.57 (0.06)	$p = 0.17$	2.55 (0.06)	$p = 0.07$	3.03 (0.06)	ns	1.94 (0.13)	ns	2.31 (0.08)	ns
Intralymphatic	2.70 (0.06)		2.74 (0.06)		3.12 (0.06)		2.17 (0.13)		2.48 (0.08)	
Allergen										
Cul n 1	2.47 (0.04)	$p \leq 0.001$ ^a Cul n 5, Cul n 9	2.50 (0.04)	$p \leq 0.0001$ ^a Cul n 5, Cul n 9	2.93 (0.06)	$p = 0.052$	2.06 (0.08)	$p \leq 0.001$ ^a Cul n 9	2.37 (0.05)	$p \leq 0.01$
Cul n 2	2.60 (0.04)		2.55 (0.04)		3.10 (0.06)		1.94 (0.08)		2.22 (0.05)	
Cul n 5	2.75 (0.04)	Cul n 1	2.74 (0.04)	Cul n 1, Cul n 2	3.14 (0.06)		1.86 (0.08)	Cul n 9	2.47 (0.05)	Cul n 5, Cul n 9
Cul n 9	2.73 (0.04)	Cul n 1	2.78 (0.04)	Cul n 1, Cul n 2	3.15 (0.06)		2.37 (0.08)	Cul n 1, 2, 5	2.53 (0.05)	Cul n 2
Week after 1st immunization										
W0	2.19 (0.04)	$p \leq 0.00001$ ^a W2-30	2.09 (0.04)	$p \leq 0.000001$ ^a W2-30	2.88 (0.03)	$p \leq 0.000001$ ^a W7, 11, 19, 30	1.70 (0.07)	$p \leq 0.000001$ ^a W7, 11, 19, 30	2.06 (0.05)	$p \leq 0.000001$ W7, 11, 19, 30
W2	2.46 (0.04)		2.44 (0.04)		2.95 (0.03)		1.74 (0.07)		2.04 (0.05)	
W7	2.87 (0.04)	W0, 7, 11, 30	2.89 (0.04)	W0, 7, 11, 30	3.11 (0.03)	W7, 11, 19, 30	2.15 (0.07)	W7, 11, 19, 30	2.54 (0.05)	W7, 11, 19, 30
W11	2.94 (0.04)	W0, 2, 19	2.97 (0.04)	W0, 2, 19	3.22 (0.03)	W0, 2	2.38 (0.07)	W0, 2	2.71 (0.05)	W0, 2
W19	2.51 (0.04)	W0, 2, 19	2.55 (0.04)	W0, 2, 19	3.11 (0.03)	W0, 2	2.14 (0.07)	W0, 2	2.43 (0.05)	W0, 2, 11
W30	2.85 (0.04)	W0, 7, 11, 30	2.91 (0.04)	W0, 7, 11, 30	3.21 (0.03)	W0, 2	2.23 (0.07)	W0, 2	2.62 (0.05)	W0, 2, 11
Interactions										
Adjuvant*week		≤ 0.001		≤ 0.001		≤ 0.05		≤ 0.05		≤ 0.0001
Allergen*week		≤ 0.00001		≤ 0.000001		0.07		≤ 0.0001		≤ 0.00001
Localization*week		ns		≤ 0.05						
Localization*week*allergen										

^a Significantly different from ... in Bonferroni corrected (all-pairwise) multiple comparison test.

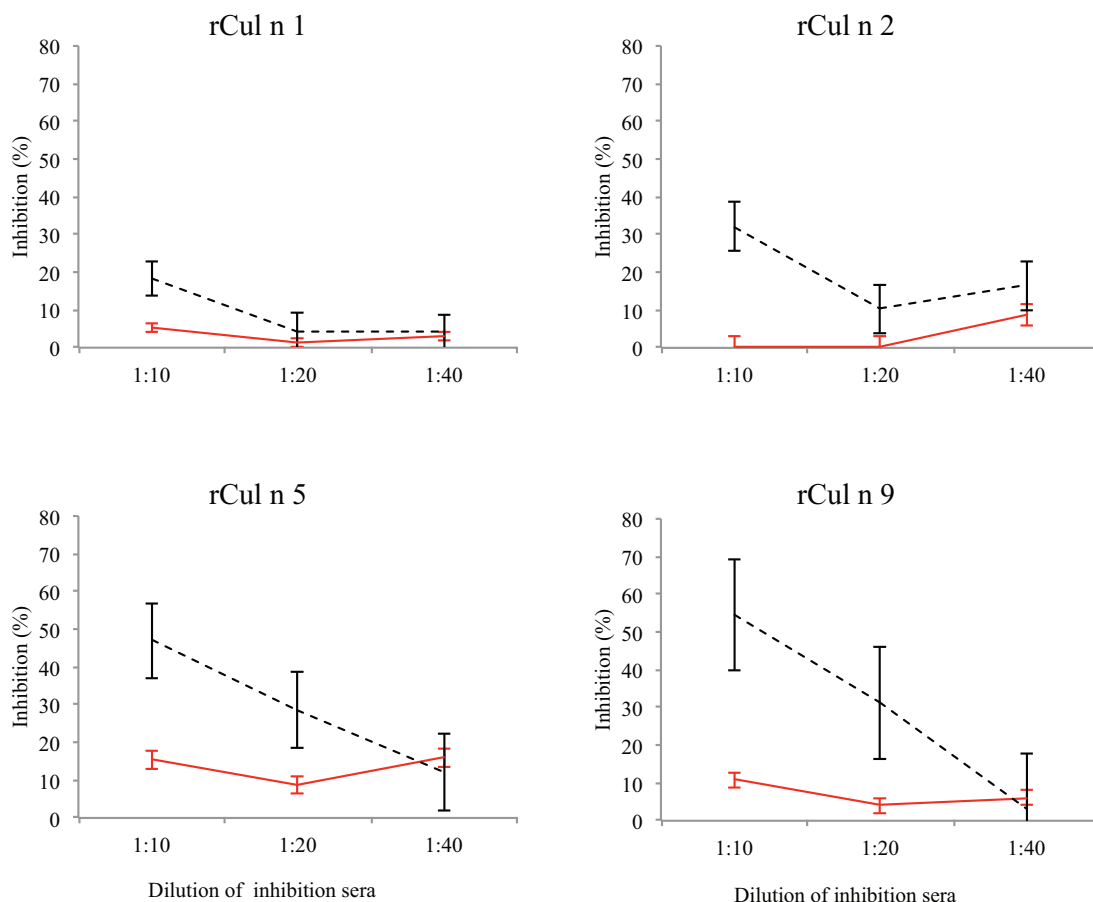


Fig. 2. Inhibition of IgE binding to allergens with sera from i.l.+IC31® vaccinated horses. Binding of IgE from serum of an IBH positive horse to rCul n 1, rCul n 2, rCul n 5, rCul n 9 was inhibited by postimmune sera (week 11) of i.l.+IC31® vaccinated horses. Mean % inhibition by pre- (whole red line) and post- (broken black line) vaccination sera from horses nos. 10–12, diluted 1:10–1:40 applied to an ELISA plate coated with allergens, prior to adding serum from an IBH positive horse at a 1:5 dilution.

3.6. *In vitro* sulfidoleukotriene release following *C. nubeculosus* extract stimulation of leukocytes

Sensitization to the recombinant allergens was investigated with *in vitro* sulfidoleukotriene release test

prior to vaccination and six weeks after the 3rd vaccination. Sulfidoleukotriene production was detected after stimulation of PBL from all horses with Con A, used as positive control, but not after stimulation with the four recombinant allergens independent

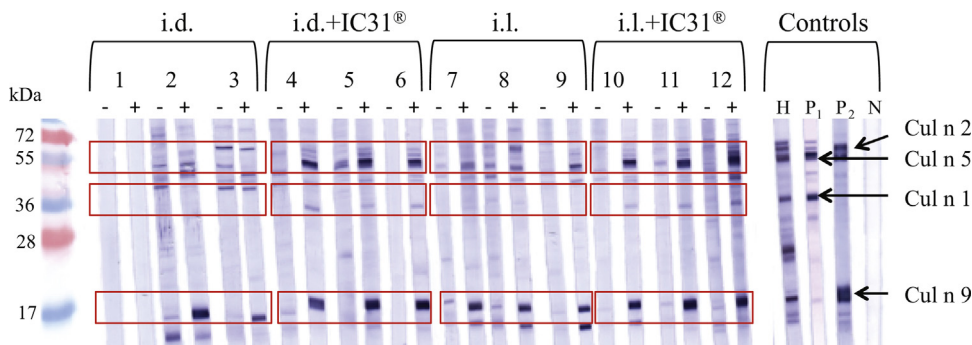


Fig. 3. Binding of IgG1 serum antibodies from vaccinated horses (1–12) to the relevant allergens (Cul n 1, Cul n 2, Cul n 5, Cul n 9) in *C. nubeculosus* salivary gland extract (SGE) separated by SDS-PAGE. PVDF membrane strips incubated with pairs of serum (1/30 dilution), week 0 (–), two weeks after the third vaccination (+). The control strips incubated with; serum (1/5 dilution) from IBH-affected horse for detection of IgE (H), specific polyclonal mouse antibodies (1/500 dilution) against Cul n 1 and Cul n 5 (P₁), Cul n 2 and Cul n 9 (P₂), TBS-T as negative control (N). Horses vaccinated: 1–3 intradermally without adjuvant (i.d.), 4–6 intradermally with IC31® (i.d.+IC31®), 7–9 intralymphatically without adjuvant (i.l.), 10–12 intralymphatically with IC31® (i.l.+IC31®).

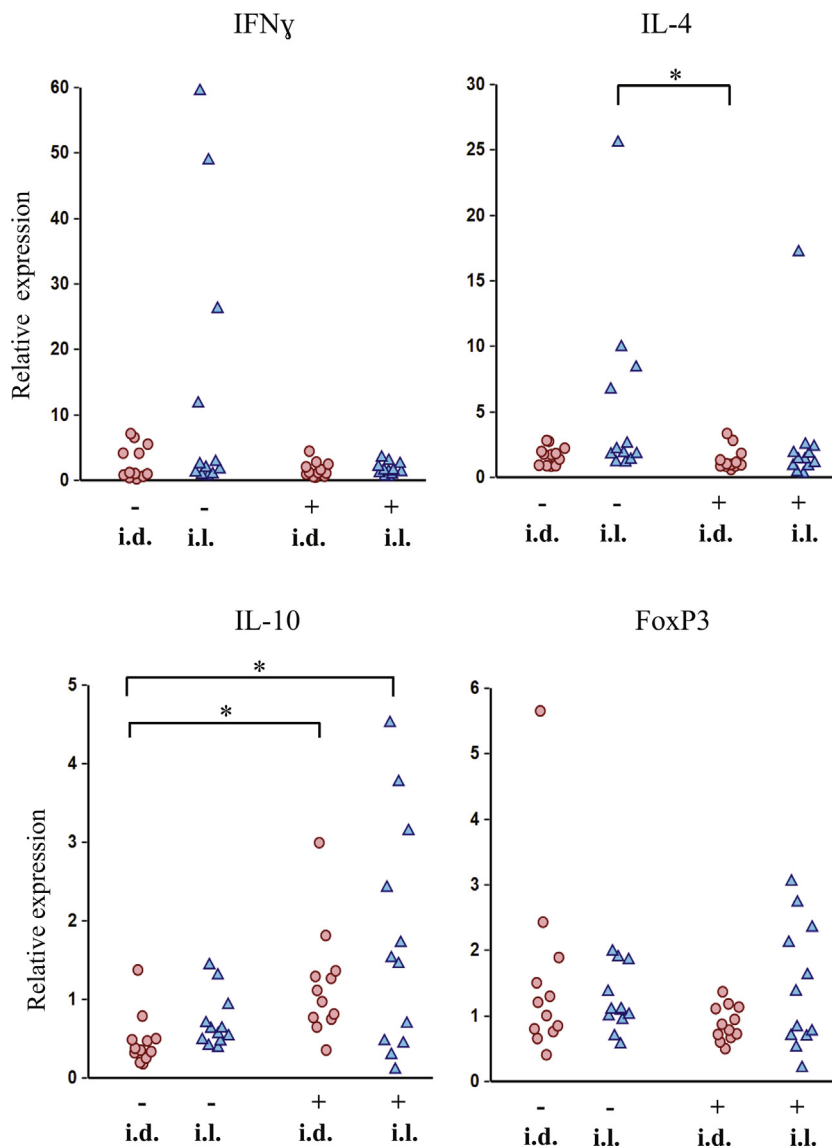


Fig. 4. Relative cytokine and FoxP3 expression after *in vitro* stimulation of PBMC. Expression levels were assessed by quantitative RT-PCR and normalized to 18S rRNA using the ddCt method. Comparison between vaccination groups was performed with One-Way ANOVA (* $p \leq 0.05$).

of injection site and injection material (data not shown).

3.7. Intradermal test (IDT)

The ability of the recombinant allergens to induce type I hypersensitivity reactions in the vaccinated horses and three healthy controls was tested *in vivo* by IDT. All horses responded strongly to histamine but did not react to any of the allergens 30 min post-injection (data not shown).

4. Discussion

IBH is a severe problem in Icelandic horses exported to the European continent, but does not occur in horses living in Iceland, because the causative agent of this allergy,

Culicoides spp., are not endemic in Iceland (Schaffartzik et al., 2012). This provides an opportunity for an experimental setup for developing a preventive vaccination strategy against IBH. Horses could be vaccinated in Iceland, where they are not exposed to the allergens, and then transported to highly infested *Culicoides* areas where at least 50% of them would be expected to develop IBH (Björnsdóttir et al., 2006).

Allergen specific immunotherapy using subcutaneous injections has been practiced in humans for treatment of allergies for over 100 years (Fitzhugh and Lockey, 2011). This has also been tried in IBH-affected horses using whole body extract of *Culicoides*, with contradictory results (Anderson et al., 1996; Barbet et al., 1990; Ginel et al., 2014). Whole body *Culicoides* extract can be expected to contain various substances not relevant for IBH, and therefore it is

difficult to determine the dosage and frequency of injections needed to standardize the treatment. Today many of the causative allergens of IBH have been identified and produced as recombinant proteins. This provides the opportunity to develop an allergen specific immunotherapy against IBH (Langner et al., 2009; Schaffartzik et al., 2011; van der Meide et al., 2013).

Immunotherapy as presently practiced, using subcutaneous injection of increasing doses of allergen over a very long period of time (years), has low compliance in humans and probably also in horses. A more practical and efficient immunotherapy is thus required.

In the present study we have tested two injection (intralymphatic and intradermal) methods to raise an immune response that could protect against IBH. The intralymphatic route has been shown in humans to need shorter time, less protein and fewer injections, and was therefore an obvious choice (Senti et al., 2009). We have compared the intralymphatic to the intradermal injection route. Epicutaneous immunization has been shown to be more effective than subcutaneous or intramuscular applications and required 5 to 10-fold less antigen (Briggs et al., 2000; Kenney et al., 2004; Redfield et al., 1985).

We used four allergens originating from the salivary glands of *C. nubeculosus*, Cul n 1 (antigen 5-like protein), Cul n 2 (hyaluronidase), Cul n 5 (unknown protein) and Cul n 9 (D7-related protein). Three (Cul n 1, 2 and 9) of the allergens are known salivary proteins from other insects. Antigen 5-like protein is a major allergen in vespid venoms (King and Spangfort, 2000) and hyaluronidase in bee venom (Soldatova et al., 1998). D7 related proteins that belong to the superfamily of pheromone/odorant binding proteins are widespread in blood-sucking Diptera and are immunogenic in humans (Calvo et al., 2006; Doucoure et al., 2013; Valenzuela et al., 2002). The recombinant proteins were produced and purified from *E. coli* (Schaffartzik et al., 2010, 2011). They were injected either alone or with IC31[®] adjuvant that induces a Th1 type response in mice (Olafsdottir et al., 2012; Pattacini et al., 2012) and in humans when used with *Mycobacterium tuberculosis* antigens (van Dissel et al., 2010).

In our study, the administration of 3 doses of only 10 µg of each recombinant allergen in combination with IC31[®] gave a strong IgG antibody response with both injection routes. The intralymphatic application resulted in a somewhat stronger antibody production than intradermal injection, detected both by WB (Figure S1) and ELISA (Fig. 1). This IgG response was without induction of IgE antibodies and consequently also no sensitization of the horses as demonstrated with an *in vitro* sLT release assay with *Culicoides* extract and by intradermal tests. Ideally, an IDT and sLT release assay should be performed at the same time on a horse allergic to *Culicoides* proteins as positive controls. However, as horses in Iceland are not exposed to bites of *Culicoides* and are not sensitized to these allergens, this could not be done. Nevertheless, IDT and sLT release assays have been and are performed with the same proteins by our group in Switzerland (Baselgia et al., 2006; Schaffartzik et al., 2011).

The antibodies generated after vaccination bound not only to the r-proteins (Figure S1), but also to the respective

proteins extracted from the salivary glands of *C. nubeculosus*. This was more pronounced in horses that received the r-allergens in IC31[®] (Fig. 3).

Horses have seven IgG subclasses (Wagner et al., 2004) and antibodies have been raised against all of them (Keggan et al., 2013). The IgG subclasses differ in function, with IgG1, IgG3, IgG4 and IgG7 being the equine immunoglobulins that bind complement (Lewis et al., 2008) and are important in protection against viral and bacterial infections (Goodman et al., 2012; Lopez et al., 2002; Nelson et al., 1998; Sheoran et al., 1997; Soboll et al., 2003; Svansson et al., 2009). They are therefore the IgG subclasses which it is vital to raise in Th1-mediated vaccinations. The antibody response measured with IgG(T) specific antibody, which binds to both IgG3 and IgG5, is on the other hand not associated with protective immunity in viral infections (Goehring et al., 2010; Goodman et al., 2012; Soboll Hussey et al., 2011), but has been linked to sensitization (Wagner, 2006). This suggests that the proposed association of IgG(T) with allergic conditions is more due to the IgG5 than IgG3 subclass detected by the anti IgG(T) antibodies.

The antibody response data are presented without subtracting the pre-vaccination values to show the background response that is especially pronounced for IgG4/7. This is true for the twelve vaccinated horses and the five controls, but slightly lower for rCul n 1 than the other three (Fig. 1). The IgG4/7 antibodies represent the most prevalent isotype in horse serum and are important for secondary immune responses and in latent infections, whereas IgG1 is mainly produced in primary infections and is rather short lived (Goodman et al., 2012; Svansson et al., 2009; Wagner, 2006). The only biting insects known to bite mammals in Iceland are *Simulium vittatum* (Johannsson, 1988). However, it has been shown that the antigen 5-like proteins from *S. vittatum* (Sim v 1) and *C. nubeculosus* (Cul n 1) share IgE-binding epitopes (Schaffartzik et al., 2010) and 50% of IBH-affected Icelandic horses also react to salivary gland extracts of *S. vittatum* (Baselgia et al., 2006). Therefore the response of the untreated horses could be due to IgG4/7 cross-reactivity to the salivary gland proteins from *S. vittatum*.

A repeated ANOVA was performed because it allowed analysis of the effects of the different factors on antibody levels as function of time, and also enhanced the power of the analysis. We are aware that the number of horses per group in this pilot study was very small, but statistically significant differences are probably relevant. This analysis showed that for all tested IgG subclasses there was a significant increase in antibody levels following immunization. The IgG1 and IgG1/3 response occurred very early, as antibody levels were already significantly higher than in the preimmune sera two weeks after the 1st immunization but they also decreased quite rapidly. This is in accordance with previous studies (Goodman et al., 2012; Svansson et al., 2009; Wagner, 2006). Immunization with the r-allergens also induced antibodies of the IgG4/7 and IgG5 but a significant increase in these IgG subclasses was only seen two weeks after the 2nd immunization.

The repeated measure ANOVA also showed that the use of IC31[®] lead to a higher antibody production. The increased response was mainly seen for IgG1 and IgG1/3

and was statistically significant. IgG4/7, IgG5 and IgG(T) levels were also higher in the horses immunized with IC31[®] but the difference did not reach statistical significance.

Antibodies were induced against all four r-allergens, at least in the horses immunized with IC31[®]. However, there were significant differences in the level of the responses between the r-allergens. Overall, rCul n 5 and rCul n 9 induced a better antibody response than rCul n 1 and rCul n 2 (Table 1). The antibodies generated against rCul n 5 and rCul n 9 also displayed the best IgE-blocking activity (Fig. 2). Interestingly, Figs. 1 and 3 suggest that the effect of IC31[®] was stronger for the weaker antigens. Repeated immunization without IC31[®] resulted in an increase in Cul n 9 and Cul n 5 specific antibodies, but hardly any increase in Cul n 1 and Cul n 2 specific antibodies. This is particularly well illustrated for Cul n 1 in Fig. 3. After the 3rd immunization IgG antibodies specific for the 35-kDa protein band corresponding to Cul n 1 can only be detected in the sera of the horses immunized with IC31[®]. This is independent of the immunization route. Use of IC31[®] seems especially important to improve the antibody response against weak antigens. Table 1 and Fig. 1 also suggest that the time course of the antibody response is influenced by IC31[®], as shown by significant interaction between the adjuvant and the week (Table 1). Fig. 1 shows that the peak of the antibody response is nearly reached after the 2nd immunization with IC31[®] while the response without IC31[®] is usually still low after 2nd immunization and only increases, if at all, after the 3rd vaccination. Finally, intralymphatic immunization leads to a higher antibody response than intradermal immunization. This difference was weaker than the effect of IC31[®] and did not reach the level of significance, probably because of the small number of horses included. An interesting observation is that the intradermal test boosted the antibody response (Fig. 1 and Table 1).

Due to the small group sizes, the cytokine expression data from the PBMC stimulation with the four r-allergens was pooled for statistical analysis. No difference between the groups was seen in IFN- γ and FoxP3 expression. IL-4 expression was significantly higher in horses vaccinated intralymphatically without adjuvant as compared to those that received the allergens in IC31[®] intradermally. Most interestingly, horses that were vaccinated with the adjuvant using either route had higher IL-10 expression than those immunized intradermally without IC31[®] (Fig. 4). Although this indicates that vaccination with IC31[®] favors Th1 and T regulatory responses, a larger group of horses is needed in order to obtain more significant results. In addition, purified r-allergens produced in *E. coli* tended to give background in stimulations of PBMC taken from both pre-vaccination and control horses. Allergens produced in insect cells should be evaluated and also an earlier time point for stimulation than two weeks after vaccination.

Effective immunotherapy in humans elicits an increase in IL-10 production leading to an increase in allergen-specific IgG4 and often a decrease in IgE. The IgG4 antibodies then block IgE binding to allergens, inhibit IgE facilitated allergen presentation to T cells and allergen-induced boost of memory IgE production (Cavkaytar et al., 2014; Focke-Tejkl et al., 2014; Larche

et al., 2006; Ozdemir et al., 2011). We tested the sera from the horse group with the strongest IgG response, i.e. those vaccinated intralymphatically with the allergens in IC31[®], for IgE blocking capacity. Indeed these sera could inhibit the binding of IgE from an IBH horse (Fig. 2).

In summary, vaccination of horses previously not exposed to the allergens, both intralymphatically and intradermally with r-allergens in IC31[®] adjuvant, was well-tolerated and induced immune responses without IgE production. The horses produced IgG that could inhibit r-allergen-specific IgE-binding. Furthermore, the use of IC31[®] led to increased production of the regulatory cytokine IL-10. We therefore conclude that both injection routes and the IC31[®] adjuvant are strong candidates for further development of immunotherapy in horses.

Conflicts of interest

Andreas Meinke is an employee of Valneva Austria GmbH.

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