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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.

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; Halldordsottir 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; Halldordsottir 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 $KLKL_5KLK$ 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