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Short communication

A preventive immunization approach against insect bite hypersensitivity: Intralymphatic injection with recombinant allergens in Alum or Alum and monophosphoryl lipid A

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ABSTRACT

Insect bite hypersensitivity (IBH) is an IgE-mediated dermatitis of horses caused by bites of *Culicoides* insects, not indigenous to Iceland. Horses born in Iceland and exported to *Culicoides*-rich areas are frequently affected with IBH.

The aims of the study were to compare immunization with recombinant allergens using the adjuvant aluminum hydroxide (Alum) alone or combined with monophosphoryl lipid A (MPLA) for development of a preventive immunization against IBH.

Twelve healthy Icelandic horses were vaccinated intralymphatically three times with 10 μg each of four recombinant *Culicoides nubeculosus* allergens in Alum or in Alum/MPLA.

Injection with allergens in both Alum and Alum/MPLA resulted in significant increase in specific IgG subclasses and IgA against all r-allergens with no significant differences between the adjuvant groups. The induced antibodies from both groups could block binding of allergen specific IgE from IBH affected horses to a similar extent. No IgE-mediated reactions were induced. Allergen-stimulated PBMC from Alum/MPLA horses but not from Alum only horses produced significantly more IFNγ and IL-10 than PBMC from non-vaccinated control horses.

In conclusion, intralymphatic administration of small amounts of pure allergens in Alum/MPLA induces high IgG antibody levels and Th1/Treg immune response and is a promising approach for immunoprophylaxis and immunotherapy against IBH.

1. Introduction

Equine insect bite hypersensitivity (IBH), or summer eczema, is an allergic recurrent seasonal dermatitis of horses. It is caused by bites of insects mainly of the genus *Culicoides* (biting midges) (for review Schaffartzik et al., 2012) and characterized by papules and intense pruritus affecting the feeding sites of the midges (Bröstrom et al., 1987). IBH affects all horse breeds and is found almost worldwide with the exception of places where *Culicoides* species are not indigenous, as in Iceland (Illies, 1978). However, IBH is a severe problem in Icelandic horses exported from Iceland to the European continent. Over 50% of these horses develop the disease two years or more after importing into heavily *Culicoides* infested areas (Bjornsdottir et al., 2006), while Icelandic horses foaled in Europe develop the disease with a similar prevalence as most other horse breeds (Bröstrom et al., 1987). Presently, there is no satisfactory treatment of IBH (Schaffartzik et al., 2012), and thus preventive immunization may be an attractive option for horses exported from Iceland to countries where *Culicoides* are present. In humans, prophylactic immunotherapy to prevent allergic sensitization is not yet practiced but has been considered (Valenta et al., 2012). The allergen extracts mostly used in allergen immunotherapy are not suited as they risk inducing sensitization to other proteins in the mixture. For prophylactic treatment it is essential to use well-defined pure allergens and to treat before sensitization occurs (Valenta et al., 2012). IBH in Icelandic horses is thus an interesting model for development and study of prophylactic immunotherapy

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as Iceland is free of the causative agent and the horses are only sensitized after export. Furthermore, *Culicoides* allergens have been identified and characterized at the molecular level and are available as pure recombinant proteins (Mueller et al., 2016).

According to our studies on the immune response and pathogenesis of IBH, Icelandic horses exported from Iceland to Europe develop a stronger Th2 polarized immune response than their disease status matched counterparts born in Switzerland (Hamza et al., 2007). Further studies demonstrated an imbalance between the Th2/Treg immune response in IBH, using both *Culicoides* stimulated PBMC and skin biopsies (Hamza et al., 2008, 2013; Heimann et al., 2011). These findings indicate that a preventive immunization against IBH should aim at inducing an allergen-specific Treg and/or Th1 immune response.

Due to the high cost of production and purification of recombinant proteins and the risk of side effects when using a high dose of allergens (Klimek and Pfaar, 2013) it is of great importance to have an efficient route of injection and a vigorous adjuvant to be able to use small amounts of the allergens. In a previous study we could show that when using the Th1 adjuvant IC31® (Schellack et al., 2006) intralymphatic injection gave a slightly stronger immune response than intradermal injections (Jonsdottir et al., 2015). Based on these findings and on results from immunotherapy in humans (Senti et al., 2008), it was of interest to test further this injection route with other adjuvants.

Despite being a Th2 focusing adjuvant, Alum is the classical adjuvant used in immunotherapy for humans (Moingeon, 2012). However, when T-cell-mediated immunity is necessary additional components are needed (Brewer, 2006). Monophosphoryl lipid A (MPLA) is a detoxified derivative of lipopolysaccharide (LPS) that binds to Toll like receptor 4 (TLR4) and retains most of the adjuvant capacity of LPS (Evans et al., 2003; Qureshi et al., 1982). MPLA is used in vaccine formulations and has been registered for use in humans (Casella and Mitchell, 2008). Since MPLA promotes primarily a Th1 type of response (Puggioni et al., 2005), it is being applied as an adjuvant in immunotherapy and has been shown to enhance specific IgG1 and IgG4 antibodies, and reduce allergy symptoms (Drachenberg et al., 2001; Mothes et al., 2003). The mixture of aluminum hydroxide and MPLA (AS04) is being used successfully in viral vaccines (Garçon et al., 2011).

The aim of our study was to compare the immune response induced by intralymphatic immunization with pure recombinant allergens in Alum alone or a mixture of Alum and MPLA in the search for optimal conditions for prophylactic immunotherapy against equine IBH.

2. Materials and methods

2.1. Animals

Twelve healthy Icelandic horses, 7–10 years of age, were vaccinated. In addition three healthy horses were used as controls in the skin test and six horses as control in the *in vitro* stimulation of PBMC, age 5–13 years. All horses were living in Iceland. The experiment was performed in accordance with a permit from the National Animal Research Committee of Iceland (no. 0113–16).

2.2. Vaccination and sample collection

Twelve horses were vaccinated into the submandibular lymph nodes with four *Escherichia coli*-expressed recombinant *Culicoides nubeculosus* allergens (rCul n 3, rCul n 4, rCul n 8 and rCul n 10) (Schaffartzik et al., 2011). The horses were vaccinated three times (week 0, 4, 8) with 10 μg of each allergen. The horses were randomly divided into two groups. Six horses were vaccinated with the allergens in 500 μg aluminum hydroxide gel (Alhydrogel® 2%, Invivogen) and the other six with allergens in a mixture of Alum (500 μg) and 50 μg MPLA (Avantilipids). The total volume per vaccination was 400 μL. Blood was collected by jugular venipuncture at week 0, every other week for 16 weeks, and then monthly until week 32. Serum was stored at −20 °C until used. A differential count of leukocytes from EDTA blood was carried out (Jonsdottir et al., 2015).

2.3. Production of Cul n 3 in insect cells

Cul n 3 was expressed in insect cells because pilot experiments had shown with *E. coli* derived Cul n 3 the cytokine production by PBMC was extremely low or absent, probably because this *E. coli* expressed protein precipitated in medium. The *Cul n 3* gene was amplified from a λZAPII cDNA library, made from salivary glands of *C. nubeculosus* (Schaffartzik et al., 2011), with primers (Fw: 5′- ATGCATAATTTTGCAGGGAT-3′ and Re: 5′-CGCATATGTGGTCAAAGTAG-3′) designed based on the gene sequence of Cul n 3 (GenBank Accession No. HM145951). The protein was expressed in insect cells according to Bac-to-Bac® HBM-TOPO® Secreted Expression system. The Baculovirus was amplified in Sf-9 cells and the protein expressed in High five cells.

The Bac-rCul n 3 protein was purified under native conditions with HIS-Select™ HF Nickel Affinity Gel (Sigma) in conformity with the manufacturer's protocol and dialyzed against PBS. The protein was analyzed with Coomassie blue staining (Wong et al., 2000) and detected with Cul n 3 specific polyclonal antibodies diluted 1:4000 (Schaffartzik et al., 2011) in WB (Fig. 1A) (Jonsdottir et al., 2015).

2.4. Serological tests

2.4.1. ELISA

Sera from six unvaccinated horses living in Iceland and from five IBH-affected horses living in Switzerland were included on each plate, as negative and positive controls, respectively. Allergen-specific antibody levels were determined by ELISAs as described in Jonsdottir et al. (2015) with the following changes. Plates were coated with 2 μg/mL of the recombinant allergens rCul n 3, rCul n 4, rCul n 8, rCul n 10 and Bac-rCul n 3. Sera (weeks 0, 2, 6, 10, 16) were added at a dilution of 1:5 for IgE detection, 1:10 for IgA (weeks 0, 6, 10, 24, 32) and 1:200 for IgG subclasses (weeks 0, 2, 6, 10, 16, 24, 32). Specific monoclonal antibodies were used for IgG subclasses and IgE detection as described by Jonsdottir et al. (2015) and for IgA, a monoclonal equine IgA specific antibody in a 1:250 dilution (Serotec) was applied.

2.4.2. Competitive inhibition ELISA

Pools of sera from the Alum group and Alum/MPLA group, containing the same amount of serum from each of the six horses, were used to analyze the ability of sera from vaccinated horses to block the IgE-binding of serum from an IBH-affected horse to the recombinant allergens. Pools of preimmune sera (negative control) and postimmune sera (two weeks after the third vaccination) were used in the competitive inhibition ELISA performed as described by Jonsdottir et al. (2015). The percentage of inhibition for each dilution of the pre- and postimmune serum pools was calculated.

2.5. Stimulation of PBMC and determination of cytokines

PBMC were isolated from the horses by Ficoll-Hypaque in conformity with Hamza et al. (2007) three weeks (week 11) after the 3rd vaccination, and from six unvaccinated control horses. PBMC were stimulated for 24 h (for RNA isolation) or for 4 days (for analysis of the supernatant) with ConA (10 μg/mL) as a positive control