In vitro effects of the toll-like receptor agonists monophosphoryl lipid A and CpG-rich oligonucleotides on cytokine production by equine cells

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Abstract

Insect bite hypersensitivity (IBH) is an equine allergic dermatitis to *Culicoides* spp. antigens. Attempts at using allergen-specific immunotherapy (AIT) as a treatment for IBH have so far proven unsuccessful. Toll-like receptor (TLR) agonists can promote a shift in the immune response from the allergy-promoting T helper cell 2 (Th2) response towards a Th1, and/or regulatory response. The aim of this study was to evaluate two immunomodulatory TLR agonists in vitro as potential vaccine adjuvants for a more efficacious AIT in IBH. Peripheral blood mononuclear cells (PBMCs) from healthy and IBH-affected horses were stimulated with the TLR-agonists monophosphoryl lipid A (MPLA) or CpG-rich oligodeoxynucleotides (CpG-ODN) in the presence or absence of *Culicoides* spp. allergens. Cytokine concentrations of interferon (IFN)- α , IFN- γ , interleukin (IL)-4, IL-10 and IL-17 were quantified in the supernatants of stimulated PBMCs. MPLA induced IL-10 secretion in all horses, regardless of presence and nature of antigens, while suppressing antigen-induced production of IFN- γ , IL-4 and IL-17. CpG-ODN significantly increased IFN- α , IFN- γ and IL-4 production, but had little effect on IL-10 production. In conclusion, MPLA promotes a regulatory immune response and is therefore a promising adjuvant candidate for allergy vaccines in horses. Whilst C-class CpG-ODN is an unsuitable adjuvant for AIT, it induces IFN- γ and IFN- α , and thus may be a useful adjuvant in combination with vaccines for equine infectious or neoplastic diseases.

Keywords: Equine; Hypersensitivity; Adjuvant; Monophosphoryl lipid A; CpG-rich oligonucleotides

Introduction

Allergen-specific immunotherapy (AIT) is well-established in the therapy of human allergies, but its efficacy and practicality still need to be improved (Bousquet et al., 1998; Jutel et al., 2015). The key mechanism of AIT involves skewing the immune response away from the allergy-promoting T-helper cell 2 (Th2) type towards a regulatory T-cell (Treg) response, with subsequent induction of peripheral T-cell tolerance (Akdis et al., 2014). AIT in human beings often only leads to partial improvement of clinical signs, requires numerous injections over months or years and holds a significant risk of side effects, including anaphylaxis (Bukantz et al., 2004).

The most common and best studied immunoglobulin E (IgE)-mediated allergy in horses is insect bite hypersensitivity (IBH), a chronic seasonally recurrent dermatitis caused by bites of *Culicoides* spp. midges (Schaffartzik et al., 2012). Attempts at performing AIT in IBH-affected horses have so far been largely unsuccessful (Barbet et al., 1990; Ginel et al., 2014). Therefore, treatment of IBH is limited to allergen avoidance and symptomatic pharmacotherapy. There is a need to develop a safe and efficacious AIT protocol for horses with IBH.

The causative allergens for IBH, salivary gland proteins of *Culicoides* spp. midges, have been cloned and expressed as recombinant proteins (van der Meide et al., 2013; Mueller et al., 2016). This allows for the development of AIT protocols based on pure recombinant allergens instead of crude *Culicoides* spp. extracts. Recombinant allergens have been shown to have reduced IgE-reactivity (Karamloo et al., 2005) and are thus safer to use in AIT than allergen extracts. However, since they also exhibit a reduced T-cell immunogenicity (Wuertzen et al., 2007), co-administration of suitable adjuvants is necessary. Adjuvants enhance and polarise adaptive immune responses by engaging components of innate

immunity (Coffman et al., 2010). In this context, Toll-like receptor (TLR) agonists have been investigated in human beings and mice as potential adjuvants for allergy vaccines (Filì et al., 2014; Aryan et al., 2015).

Synthetic oligonucleotides containing CpG-motifs (CpG-ODN) act as TLR9 agonists and induce Th1 and/or Treg responses. Inhalation of gelatine nanoparticle-fused CpG-ODN had a beneficial therapeutic effect and induced a Th2 to Treg shift in horses with recurrent airway obstruction (RAO), a respiratory hypersensitivity (Klier et al. 2012, 2015). Furthermore, the non-toxic lipopolysaccharide (LPS) derivative monophosphoryl lipid A (MPLA), a TLR4 agonist, was shown to have a beneficial clinical effect in AIT of grass-pollen allergic human patients (Drachenberg et al., 2001; Musarra et al., 2010) and also in house dust mite allergy (Baldrick et al., 2001).

The aim of this study was to evaluate effects of the immunomodulators MPLA and CpG-ODN on peripheral blood mononuclear cells (PBMCs) of healthy and IBH-affected horses stimulated with the immunomodulators alone or in combination with antigens. Production of the cytokines interferon (IFN)- α , IFN- γ , interleukin (IL)-4, IL-10 and IL-17 was measured in cell culture supernatants of stimulated equine PBMCs.

Materials and methods

Horses and collection of blood samples

Blood samples were collected from the jugular vein of 12 horses (seven geldings, five mares; mean age 14.1 years, range 4-22 years) belonging to different breeds (Icelandic, Freiburger, Warmblood) using vacutainers containing sodium heparin (Vacurette, Greiner) during the *Culicoides* spp. season. To test whether the immunomodulators elicited the same effects in healthy and in IBH-affected horses, the study used six horses with a history of

clinical IBH over several years and sensitisation to *Culicoides* spp. allergens confirmed by a cellular antigen stimulation test (CAST, Bühlmann Laboratories; Baselgia et al., 2006), along with six healthy control horses.

The healthy control horses had no history of skin diseases and lived in the same environment as the allergic horses; therefore, they were also exposed to *Culicoides* spp. bites. All horses had been vaccinated yearly against equine influenza and tetanus, and were dewormed regularly. Insect exposure of all IBH-affected horses was reduced by use of blankets or insect repellents. However, some clinical signs of IBH were still apparent in unprotected body areas at time of blood collection. The study was approved by the Animal Experimental Committee of the Canton of Berne, Switzerland (approval number BE 51/13, date of approval 4 July 2013).

Isolation and stimulation of peripheral blood mononuclear cells

PBMCs were isolated by density gradient centrifugations over Biocoll $\rho = 1.077$ g/mL (Biochrom GmbH), as described by Mauel et al. (2006). PBMCs were washed twice in phosphate buffered saline (PBS) and 500,000 PBMCs were cultured in 200 μ L RPMI complete medium in a 96-well round bottom tissue culture plate (Sarstedt) for 48 h. PBMCs were stimulated with 5 μ g/mL each of the immunomodulators C-class CpG-ODN (D-SL03, InvivoGen), MPLA (from *Salmonella minnesota* R595, InvivoGen) or dexamethasone (Sigma-Aldrich), the latter as a control for immunosuppression. PBMCs were exposed to the immunomodulators alone or in combination with the antigens *Culicoides obsoletus* whole body extract (COext; Peeters et al., 2013), recombinant *C. obsoletus* 3 (Cul o 3; a major allergen for IBH; van der Meide et al., 2013) or the recall antigen tetanus toxoid (Schweizerisches Serum und Impfinstitut Bern), each at a concentration of 5 μ g/mL.

Recombinant Cul o 3 was expressed in *E.coli* and purified as described previously (Schaffartzik et al., 2011). Cells stimulated with 25 ng/mL phorbol myristate acetate (PMA) and 1 µM ionomycin (both Sigma-Aldrich) were used as positive controls, whilst cells incubated with complete medium alone served as negative controls.

Cytokine production in cell culture supernatants

After incubation, cell culture supernatants were harvested and stored at -80 °C until used. Levels of IFN- α , IFN- γ , IL-4, IL-10 and IL-17 were determined at the Department of Population Medicine and Diagnostic Sciences, Cornell University, USA, using a bead-based multiplex assay (Horse Cytokine 5-plex Assay; Wagner et al., 2009). Results were reported in pg/mL (IFN- α , IL-4, IL-10) or U/mL (IFN- γ , IL-17).

Statistical analysis

Statistical analysis was carried out using the software programme NCSS8. Descriptive statistics showed that the data were not normally distributed. Cytokine production between IBH-affected and healthy control horses were compared using the Mann Whitney *U* test. Since there were no significant differences between these two groups, the IBH-affected and control horses were pooled for subsequent analyses and results are presented for the 12 horses pooled. A non-parametric paired sample Wilcoxon (signed rank) test was used to compare cytokine responses in the presence or absence of the respective immunomodulators for each antigen. *P* values ≤ 0.05 were considered to be significant.

Results

Effect of control stimulants on production of IFN- α , IFN- γ , IL-4, IL-10 and IL-17

To investigate the effects of the TLR agonists MPLA and CpG-ODN on cytokine production of IFN- α , IFN- γ , IL-4, IL-10 and IL-17 by PBMCs, the same control conditions

were used for all immunomodulators. PBMCs incubated with cell culture medium alone served as negative controls. As expected, no IFN- α or IL-10, and negligible concentrations of IFN- γ (median 11 U/mL; range 8-19 U/mL), IL-4 (median 16 pg/mL; range 1-100 pg/mL) and IL-17 (median 3 U/mL; range 2-11 U/mL), were detected. PBMCs stimulated with PMA and ionomycin as positive controls exhibited strong induction of IL-10, IL-4, IFN- γ and IL-17, with concentrations markedly higher than measured in any of the experimental conditions (see Appendix: Supplementary Fig. 1). Significant induction of IFN- α was observed after stimulation with PMA and ionomycin, but was less pronounced than upon stimulation with the TLR9 agonist CpG-ODN (see Appendix: Supplementary Fig. 1).

Effect of MPLA on production of IFN- γ , IL-4, IL-10 and IL-17

The TLR4 agonist MPLA did not elicit secretion of IL-4, IFN- γ or IL-17 above control levels in the absence of antigen in culture (Fig. 1), and no IFN- α was induced in the presence of MPLA under any antigen condition. In the absence of antigen, MPLA strongly induced IL-10 production (median 767 pg/mL; $P < 0.01$) and addition of MPLA also significantly increased IL-10 production under each antigen condition (Fig. 1a). Median concentrations of IL-10 were similar between the different antigen conditions (Cul o 3 753 pg/mL; COext 823 pg/mL; tetanus toxoid 947 pg/mL). MPLA suppressed IL-4 production in PBMCs stimulated with COext (median without MPLA 218 pg/mL; median with MPLA 78 pg/mL; $P = 0.058$) and tetanus toxoid (median without MPLA 576 pg/mL; median with MPLA 228 pg/mL; $P < 0.05$). IL-4 production induced by Cul o 3 with MPLA (median 60 pg/mL; range 16-418 pg/mL) was similar to IL-4 production induced by Cul o 3 without MPLA (median 56 pg/mL; range 21-855 pg/mL). There was a marked reduction in IL-4 concentration evoked by MPLA in horses with high IL-4 production upon Cul o 3 stimulation (Fig. 1b). IFN- γ production induced by tetanus toxoid was significantly suppressed by MPLA (median without MPLA 94 U/mL; median with MPLA 34 U/mL; $P < 0.05$) (Fig. 1c), as well

as IL-17 production induced by Cul o 3 (median without MPLA 67 pg/mL; median with MPLA 45 pg/mL; $P < 0.05$) or tetanus toxoid (median without MPLA 465 pg/mL; median with MPLA 228 pg/mL; $P < 0.01$) (Fig. 1d).

Effect of CpG-ODN on production of IFN- α , IFN- γ , IL-4 and IL-17

In contrast to MPLA, CpG-ODN did not enhance IL-10 production by equine PBMCs (Fig. 2a). However, a significant increase in IL-4 concentration was induced by CpG-ODN in absence of antigen (median with CPG-ODN 32 pg/mL; median without CPG-ODN 16 pg/mL; $P < 0.01$) and, more clearly, in combination with the three antigens tested (median without/with CpG-ODN: Cul o 3 56/1393 pg/mL, $P < 0.01$; COext 218/623 pg/mL, $P < 0.05$; tetanus toxoid 576/1307 pg/mL, $P < 0.05$) (Fig. 2b). Likewise, antigen-induced production of IFN- γ was significantly enhanced by CpG-ODN (median without/with CpG-ODN: Cul o 3 14/918 U/mL, $P < 0.01$; COext 30/91 U/mL, $P = 0.058$; tetanus toxoid 94/906 U/mL, $P < 0.01$) (Fig. 2c). Conversely, there was no enhancement of IL-17 production by CpG-ODN (Fig. 2d). The TLR9 agonist CpG-ODN elicited marked production of IFN- α (median 12.8 ng/mL; $P < 0.01$) in the absence of antigen, as well as within each antigen condition (Fig. 2e).

Effect of dexamethasone on production of IFN- α , IFN- γ , IL-4, IL-10 and IL-17

Dexamethasone was used as a control reagent for immunosuppression. Antigen-induced production of IL-10 was not significantly affected by dexamethasone (Fig. 3a). However, co-treatment with dexamethasone resulted in significant downregulation of IL-4, IFN- γ and IL-17 production, irrespective of the nature of the antigen. IL-4 production was suppressed to baseline levels (median without/with dexamethasone: Cul o 3 56/14 pg/mL, $P < 0.05$; COext 218/24 pg/mL, $P < 0.01$; tetanus toxoid 576/14 pg/mL, $P < 0.01$) (Fig. 3b). IFN- γ production was also suppressed (median without/with dexamethasone: Cul o 3 14/11 U/mL, $P < 0.01$; COext 30/13 U/mL, $P < 0.01$; tetanus toxoid 94/11 U/mL, $P < 0.01$) (Fig. 3c).

Although concentrations of IL-17 were only slightly above baseline levels with most antigens in the absence of dexamethasone, the downregulation of this cytokine was still significant (median without/with dexamethasone: Cul o 3 67/12 U/mL, $P < 0.01$; COext 33/5 U/mL, $P < 0.01$; tetanus toxoid 465/103 U/mL, $P < 0.01$) (Fig. 3d).

Discussion

Since AIT is still largely unsuccessful in the treatment of IBH, our aim was to investigate in vitro which adjuvant would be best suited to increase the efficacy of AIT in horses. Adjuvants can be administered together with allergens, with the aim to enhance the immune response and skew it into a certain 'direction' (Pfaar et al., 2012; Filì et al., 2014). Currently, aluminium hydroxide (alum) is the most commonly used adjuvant in AIT, eliciting a depot effect with delayed allergen release. However, since alum promotes Th2 responses and IgE production (Brewer et al., 1999), it is not an ideal adjuvant for AIT. The use of immunomodulating adjuvants, such as TLR agonists, is a promising approach, not only for AIT, but also to enhance Th1 and cytotoxic T lymphocyte (CTL) responses in vaccines for tumours or infectious diseases (Vollmer et al., 2009). In the present study, we report distinctive effects of two TLR agonists on cytokine production by equine PBMCs. Stimulations were investigated with the TLR4 agonist MPLA and the TLR9 agonist CpG-ODN, each alone or in combination with antigens.

In human beings, MPLA promotes protective Th1 responses and consequently suppress Th2 responses in an IL-10 and IL-12 dependent fashion in vitro (Martin et al., 2003; Puggioni et al., 2005). In our study, we observed a marked increase in IL-10 production by equine PBMCs stimulated with MPLA. As the IL-10 concentrations induced by MPLA were similar for all antigen conditions, this effect of MPLA on equine cells seemed to be independent of the presence of an antigen or its specific nature. Additionally, IL-4 production

was significantly suppressed in PBMCs cultured with an antigen in the presence of MPLA, indicating a skewing of the immune response away from a Th2 phenotype and towards a regulatory response. This supports the findings of the study by Jónsdóttir et al. (2016), who found that production of IL-10 was increased by addition of MPLA to a vaccine containing alum-adjuvanted recombinant *Culicoides* spp. allergens administered to healthy horses in Iceland, i.e. not exposed to *Culicoides* spp.

Our in vitro study suggests that a similar effect can be expected in IBH-affected horses or healthy horses already exposed to *Culicoides* spp. The ability to promote regulatory immune responses, whilst simultaneously suppressing production of IL-4, makes MPLA a promising adjuvant candidate for use in equine allergy vaccines. The favourable results in this study are supported by findings in human medicine, where a novel allergy vaccine for allergic rhinitis (Pollinex Quattro; Drachenberg et al., 2001; Rosewich et al., 2013) using pollen allergens in combination with MPLA as adjuvant was approved for clinical use in 2009 and is currently under investigation in a phase 4 clinical trial for asthma (Sandrini et al., 2015).

Treatment with gelatine nanoparticle-fused A-class CpG-ODN has beneficial therapeutic effects in horses with respiratory hypersensitivity, in the absence of the specific allergens (Klier et al., 2012, 2015). Since a strong Th1-adjuvant activity had been reported for C-class CpG-ODN in vivo (Vollmer et al., 2004), and because of the low stability of the A-class CpG-ODN, we chose the C-class CpG-ODN D-SL03 for our experiments. Marked IFN- γ induction was observed, which confirms a previous equine study using B and C-class CpG-ODN (Liu et al., 2008). Surprisingly, we also observed enhanced IL-4 production. This is in contrast to an in vitro study, in which stimulation of equine bronchoalveolar lavage cells with A-class CpG-ODN led to a downregulation of IL-4 (Klier et al., 2011). A possible explanation for this finding is that C-class CpG motifs instead of A-class CpG-ODN were employed in the

present study. Furthermore, no significant effect of CpG-ODN on IL-10 production was observed. Therefore, since promotion of a regulatory-type immune response is crucial for successful AIT (Akdis et al., 2014), CpG-ODN D-SL03 appears to be an unsuitable adjuvant for AIT in horses.

TLR9 agonists, such as CpG-ODN, have shown promising potential as vaccine adjuvants for the treatment of cancer, as well as infectious diseases in human beings and in murine models, and preclinical studies have demonstrated the ability of CpG-ODN to enhance both cellular and humoral immune responses to various antigens (Vollmer et al., 2009). The apparent immune activation, with production of high concentrations of IFN- γ , observed in our study makes this CpG ODN a promising adjuvant candidate for treatment of tumours, such as equine sarcoid, and infectious diseases.

In contrast to the study by Hamza et al. (2007), we observed no significant difference in cytokine production between healthy and IBH-affected horses following stimulation of PBMCs with *Culicoides* spp. allergens. Whilst stronger induction of IL-4 by the allergens would be expected in atopic horses, the lack of a detectable difference in the present study could be attributed to the fact that affected horses were often protected from insect exposure by blankets. Importantly, our study shows that the same trends of enhancement or suppression of cytokine production could be observed within each group (see Appendix: Supplementary Figs. 2 and 3). The increase in IL-10 concentrations induced by MPLA reached statistical significance in the two separate horse groups ($n = 6$ in each group; see Appendix: Supplementary Fig. 2). This finding indicates that MPLA is suitable for both preventive (Jónsdóttir et al., 2016) and therapeutic AIT for IBH.

Conclusions

Due to their distinctive effects on cytokine induction in equine PBMCs, MPLA and CpG-ODN are candidate adjuvants for further clinical studies. MPLA is a potent inducer of a regulatory immune phenotype and thus a promising candidate adjuvant for AIT in allergic horses. Conversely, CpG-ODN has potential for treatment options where increased production of IFN- γ may be beneficial.

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Figure legends

Fig. 1. Production of interleukin (IL)-10 (a), IL-4 (b), interferon (IFN)- γ (c) and IL-17 (d) by peripheral blood mononuclear cells (PBMCs) cultured without antigen (No Ag) or stimulated with recombinant *Culicoides obsoletus* 3 (Cul o 3), *Culicoides obsoletus* extract (CO ext) or tetanus toxoid (Tetanus) alone, as indicated on the x-axis (light grey symbols on the left) or in combination with monophosphoryl lipid A (MPLA: dark grey symbols, on the right). Cell culture supernatants were harvested and analysed by a bead-based multiplex assay. Each symbol indicates a separate horse ($n = 12$), while red bars indicate the medians. A non-parametric paired sample Wilcoxon (signed rank) test was used to determine significant differences between stimulation with and without MPLA, as indicated by asterisks. ** $P < 0.01$, * $P < 0.05$, (*) $P = 0.058$.

Fig. 2. Production of interleukin (IL)-10 (a), IL-4 (b), interferon (IFN)- γ (c), IL-17 (d) and IFN- α (e) by peripheral blood mononuclear cells (PBMCs) stimulated with various antigens, as described in Fig. 1, alone (light grey symbols, on the left) or in combination with synthetic oligonucleotides containing CpG-motifs (CpG-ODN: dark grey symbols, on the right). Each symbol indicates a separate horse ($n = 12$) and red bars signify the medians. A non-parametric paired sample Wilcoxon (signed rank) test was used to determine significant differences between stimulation with and without CpG-ODN, as indicated by asterisks. ** $P < 0.01$, * $P < 0.05$, (*) $P = 0.058$.

Fig. 3. Production of interleukin (IL)-10 (a), IL-4 (b), interferon (IFN)- γ (c) and IL-17 (d) by peripheral blood mononuclear cells (PBMCs) stimulated with various antigens, as described in Fig. 1, alone (light grey symbols, on the left) or in combination with 5 $\mu\text{g/mL}$ dexamethasone (dark grey symbols, on the right). Each symbol indicates a separate horse ($n =$

472 12), while red bars indicate the medians. A non-parametric paired sample Wilcoxon (signed
473 rank) test was used to determine significant differences between stimulation with and without
474 dexamethasone, as indicated by asterisks. ** $P < 0.01$.

Figure 1

Figure 2

Figure 3