



Review paper

Equine insect bite hypersensitivity: What do we know?

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ABSTRACT

Insect bite hypersensitivity (IBH) is an allergic dermatitis of the horse caused by bites of insects of the genus *Culicoides* and is currently the best characterized allergic disease of horses. This article reviews knowledge of the immunopathogenesis of IBH, with a particular focus on the causative allergens. Whereas so far hardly any research has been done on the role of antigen presenting cells in the pathogenesis of IBH, recent studies suggest that IBH is characterized by an imbalance between a T helper 2 (Th2) and regulatory T cell (T_{reg}) immune response, as shown both locally in the skin and with stimulated peripheral blood mononuclear cells. Various studies have shown IBH to be associated with IgE-mediated reactions against salivary antigens from *Culicoides* spp. However, until recently, the causative allergens had not been characterized at the molecular level. A major advance has now been made, as 11 *Culicoides* salivary gland proteins have been identified as relevant allergens for IBH. Currently, there is no satisfactory treatment of IBH. Characterization of the main allergens for IBH and understanding what mechanisms induce a healthy or allergic immune response towards these allergens may help to develop new treatment strategies, such as immunotherapy.

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Abbreviations: AE, atopic eczema; *C. nubeculosus*, *Culicoides nubeculosus*; *C. sonorensis*, *Culicoides sonorensis*; DTH, delayed type hypersensitivity; IBH, insect bite hypersensitivity; IgE, Immunoglobulin E; IGHE, IgE heavy chain constant region; PAF, platelet activating factor; *S. vittatum*, *Simulium vittatum*; SIT, allergen-specific immunotherapy; T_{reg}, T regulatory cells; WBE, whole body extract.

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1. Introduction

Insect bite hypersensitivity (IBH) is the most common allergic skin disease of horses (Pilsworth and Knottenbelt, 2004) and manifests as a chronic relapsing seasonal allergic dermatitis caused by the bites of insects of the genus *Culicoides* found in various areas of the world (Quinn et al., 1983; Fadok and Greiner, 1990). Historically the first report on seasonal dermatitis in horses in France dates back to 1840 and was first published by Henry and Bory in 1937 and named “dermatose estivale récidivante du cheval”. Since that first observation, IBH has been reported worldwide with different names such as sweet itch (Baker and Quinn, 1978), summer eczema (Lange et al., 2005), Kasen (Kurotaki et al., 1994), Queensland itch (Riek, 1953a) or *Culicoides* hypersensitivity (Kleider and Lees, 1984). Beside in horses, hypersensitivity to *Culicoides* has been described in cattle and sheep (Connan and Lloyd, 1988; Yeruham et al., 1993). Several hypersensitivity reactions also exist in humans as bee or wasp venom allergy, allergic rhinitis, allergic asthma and atopic dermatitis also called atopic eczema, a disease also frequently observed in dogs (Scott and Griffin, 2001).

Clinical signs of IBH derive from intense pruritus caused by hypersensitivity reactions to bites of blood feeding insects. The disease is initially characterized by numerous papules, tufted hair, hyperesthesia, and skin sensitization followed by scratching and rubbing (Brostrom et al., 1987) (Fig. 1A). This self trauma leads to localized hair loss and excoriations which contribute to the perpetuation of secondary infections (Riek, 1953a; Kleider and Lees, 1984). Sub-epidermal edemas occur with congestion and perivascular cuffing of dermal vessels (Anderson et al., 1988). If the disease progresses and becomes chronic, it may lead to fibrosis, hypertrophy of epidermal tissue, and marked hyperkeratosis and lichenification, visible in thickening of the skin, scaling, formation of transverse ridges and folds (Fig. 1B) (Riek, 1953a). Due to the preferred feeding sites of the insects, the lesions are mostly located along the dorsal midline, at the base of the mane and tail (Townley et al., 1984; Brostrom et al., 1987), and/or along the ventral midline and, occasionally, at the ears. IBH-affected horses show seasonal manifestation with a gradual progression in severity of the signs over time. Clinical signs appear during the warmer months from spring to autumn when *Culicoides* are active and regress during the winter in the absence of exposure (Wilson et al., 2001) but in severe chronic cases, clinical signs may also persist during winter.

Histopathological analyses show that IBH lesions are characterized by mixed perivascular to diffuse cellular infiltrates consisting of mononuclear cells and, especially in acute lesions, eosinophils (Fadok and Greiner, 1990; Scott, 1990; Kurotaki et al., 1994) (Fig. 2). Increased

numbers of tryptase-positive mast cells (van der Haegen et al., 2001) and of MHC class II positive cells at the epidermo-dermal junction, presumably Langerhans cells, are found (Kurotaki et al., 1994). Furthermore, necrosis can occur in the epidermis during the chronic phase (Kleider and Lees, 1984).

1.1. Epidemiology

The prevalence of IBH varies worldwide from 3% in Great Britain (McCaig, 1973; Mellor and McCraig, 1974; Anderson et al., 1996), to 37.7% in Shire horses in Germany (Littlewood, 1998), and 60% in Queensland, Australia (Riek, 1953a). All breeds can principally be affected and the disease has been described for Quarter Horses, Thoroughbreds, Arabian horses, Warmbloods, Draft horses, Friesian horses, Shire horses and different pony breeds (Braverman, 1988; Larsen et al., 1988; Fadok and Greiner, 1990; Anderson et al., 1993; Littlewood, 1998; Steinman et al., 2003; van Grevenhof et al., 2007). Icelandic horses imported from Iceland to the European continent are, with a prevalence of >50%, particularly affected by the disease. This may well be due to the fact that they are not exposed to the antigens of biting midges early in life as *Culicoides* do not exist in Iceland (Brostrom et al., 1987; Lange et al., 2005; Bjornsdottir et al., 2006). The prevalence of IBH in offspring of Icelandic horses born on the European continent, thus exposed to *Culicoides* from the beginning of life, is around 5% and is not higher than in other breeds. Most interestingly a recent study has shown that horses imported as weanlings from Iceland to Europe and thus exposed for the first time to *Culicoides* allergens at an age of 7 to 10 months do not develop IBH more frequently than Icelandic horses born in Europe. The prevalence of IBH 5 years after import was 6% in horses imported as weanlings to Europe and thus similar to the prevalence of IBH in horses born in Europe, suggesting that in the horse the period to develop successful immune tolerance goes beyond the perinatal phase (Sommer-Locher et al., 2012). However, the prevalence of IBH is subject to considerable variations depending on the breed and, even more, on the lineage or family (Riek, 1953a; Anderson et al., 1988). The climate also has a strong influence on the prevalence of the disease. Habitats with soils of clay, heather and woody vegetation, and a warm and dry climate including low wind speeds are areas that favor the propagation of blood feeding insects and therefore the risk of development of IBH (Steinman et al., 2003; van Grevenhof et al., 2007) (Table 1). In general, clinical signs are not seen in horses before the age of 2–4 years, although younger animals are often kept together in the same environment with clinically affected horses (Wilson et al., 2001; Steinman et al., 2003; Wagner et al., 2003).

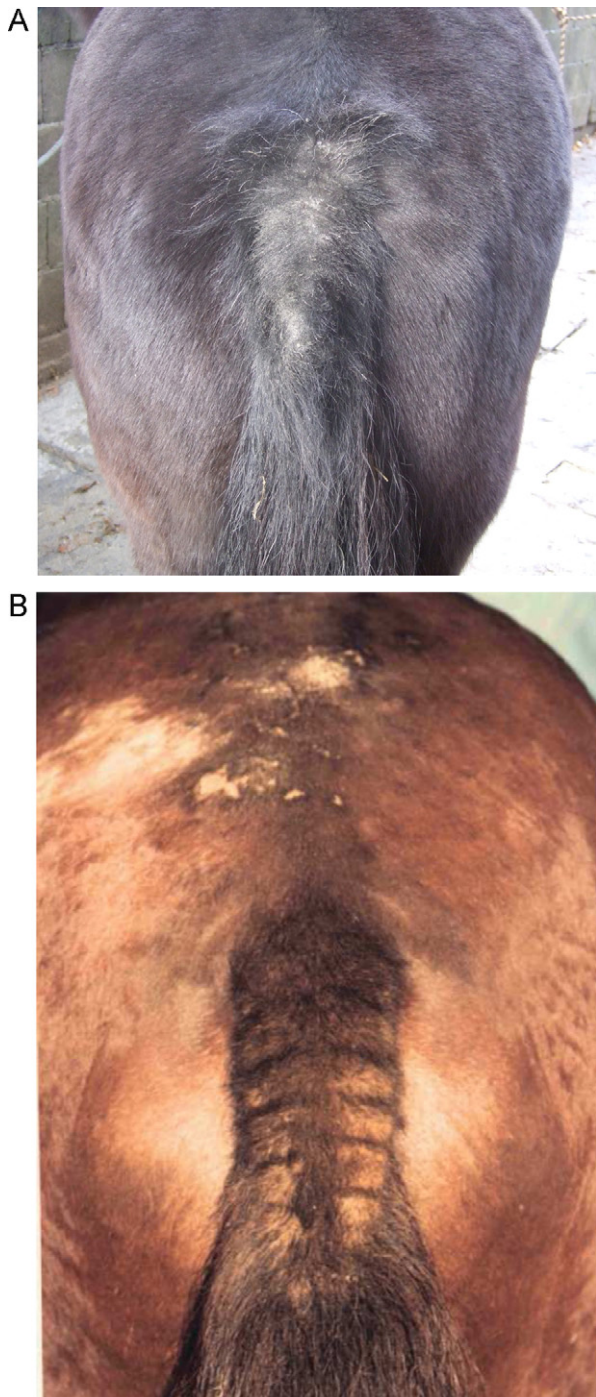


Fig. 1. Clinical signs of insect bite hypersensitivity (IBH). (A) Localized hair loss and excoriations at the tail. (B) More severe form of IBH with lichenification of the skin, a "rat-tail" and affected areas on the croup.

1.2. Etiology

IBH is a disorder of the immune system responding inappropriately to antigens, probably salivary gland proteins, of biting midges (Braverman et al., 1983; Quinn et al., 1983; Ferroglia et al., 2006; Hellberg et al., 2006). IBH

is considered to be a multifactorial disease with genetic and environmental factors contributing to onset and perpetuation of the disease (Steinman et al., 2003). IBH occurs more frequently in some horse families than in others (Marti et al., 1992) and has been associated with equine leukocyte antigens (Halldorsdottir et al., 1991; Marti et al., 1992; Lazary et al., 1994; Andersson et al., 2011). In recent studies, the heritability for IBH was estimated to range from 0.08 to 0.30 (Eriksson et al., 2008; Schurink et al., 2009, 2011).

In the early days IBH was postulated to be caused by fungi (Riek, 1954), later on by parasite larvae of the stomach (*Habronema*) and microfilariae of *Onchocerca cervicalis*, a parasite of the skin (Underwood, 1934; Datta, 1939; Kleider and Lees, 1984; Anderson et al., 1988). Other causes such as poor diet, adverse climate, unhygienic conditions, renal, hepatic or spleen disorders, lack of work (Datta, 1939; Anderson et al., 1988), feed allergy (Becker, 1964; McCaig, 1975), photosensitization (McCaig, 1975) as well as *Culicoides* bites (Riek, 1954) have been claimed to be causative agents for IBH. However, more recent studies have clearly shown that IBH is due to insect bites and particularly to bites of insects of the genus *Culicoides* (Quinn et al., 1983; Larsen et al., 1988; Halldorsdottir et al., 1989). More than 700 *Culicoides* species have been identified, of which 130 are blood feeding species (van Grevenhof et al., 2007). Beside *Culicoides* spp., *Simulium* spp. and other blood feeding insects such as *Stomoxys* and *Haematobia* were described to be of importance for IBH, because they induced positive intradermal test reactions or *in vitro* sulfidoleukotriene release more frequently in IBH-affected than in control horses (Quinn et al., 1983; Fadok and Greiner, 1990; Basalgia et al., 2006). This is most likely due to cross-reactivity between phylogenetically conserved salivary allergen structures (see below, Schaffartzik et al., 2010). During the last decade several studies have demonstrated that the salivary gland proteins of *Culicoides* are recognized by IgE of IBH-affected horses (Hellberg et al., 2006; Wilson et al., 2001, 2008). Beside in horses, hypersensitivity to *Culicoides* has also been described in cattle and sheep (Connan and Lloyd, 1988; Yeruham et al., 1993).

Although the allergens and the route of allergen encounter differ, IBH has some overlapping features with extrinsic atopic eczema (AE), an allergic skin disorder of humans. Both diseases are IgE-mediated inflammatory, chronically relapsing, non-contagious and strongly pruritic skin disorders with similar histopathological findings (Leung and Bieber, 2003; Heimann et al., 2011). Both are multi-factorial diseases based on genetic predispositions and environmental influences (Scheynius and Cramer, 2010). Predisposing factors for extrinsic AE are a primary inherited epithelial barrier defect resulting from filaggrin gene mutation and a genetic predisposition for atopy, i.e. the production of high IgE levels against environmental allergens (reviewed in Boguniewicz and Leung, 2011). The compromised barrier function leads to increased skin permeability to allergens and to activation of keratinocytes and immune cells which favor a strong Th2 biased immune response. The resulting inflammation in the skin, in particular interleukin (IL)-17-mediated responses, plays an important role in promoting systemic sensitization to

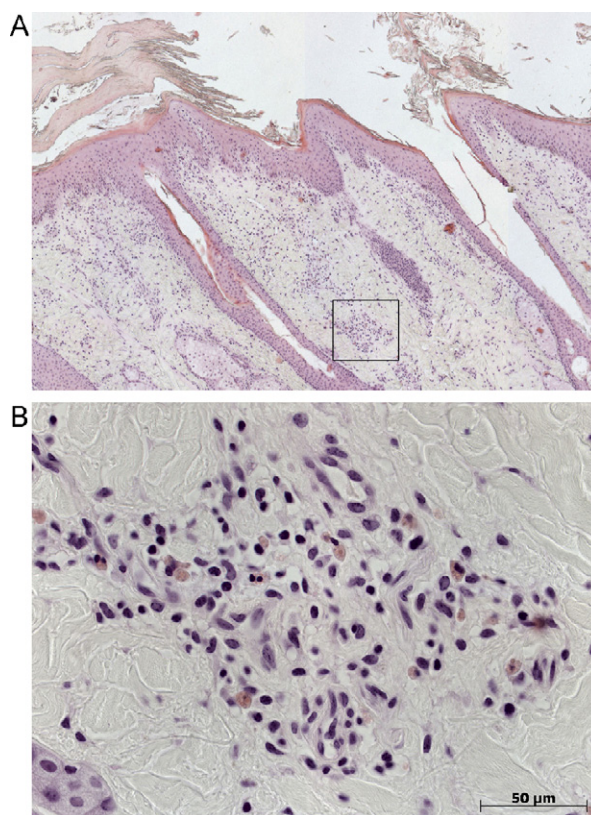


Fig. 2. HE-stained lesional skin biopsy of a horse with IBH. (A) shows hyperkeratosis and perivascular and interstitial dermatitis (original magnification 40×). (B) shows infiltration with eosinophils and mononuclear cells (original magnification 400×).

various environmental allergens and food allergy. Furthermore, patients with AD have a unique predisposition to colonization or infection by microbial organisms, most notably *Staphylococcus aureus*, which can also trigger eczematous skin responses (Darsow et al., 2004; Leung et al., 2004). Additionally, 30–80% of all AE patients are sensitized against *Malassezia sympodialis* (Casagrande et al., 2006).

The occurrence of a primary skin barrier defect in IBH has to date not been investigated but *Culicoides* induce skin barrier defects through their bites and inject various salivary proteins, including proteases. In predisposed horses this may then induce the release of cytokines from keratinocytes which favor a Th2 response similar to that which occurs in AE.

Table 1

Factors influencing the prevalence of insect bite hypersensitivity.

Impact of	Reference
Habitat	Bjornsdottir et al. (2006), van Grevenhof et al. (2007) and Steinman et al. (2003)
Climate	van Grevenhof et al. (2007)
Age	Wilson et al. (2001), Wagner et al. (2003) and Steinman et al. (2003)
Breed	Riek (1953a) and Anderson et al. (1988)
Genetic predisposition	Halldorsdottir et al. (1991), Marti et al. (1992), Lazary et al. (1994), Eriksson et al. (2008), Andersson et al. (2011) and Schurink et al. (2009, 2011)

The course of AE can be subdivided into two phases. In the acute phase, AE is induced by a type I hypersensitivity reaction in which allergen-specific T cells with a Th2-like cytokine profile and specific IgE are involved. The chronic phase is characterized by delayed type hypersensitivity reactions which might also show IgE-mediated reactions to self antigens likely to contribute to the perpetuation of the symptoms (Zeller et al., 2009). Delayed type hypersensitivity reactions have only poorly been investigated in IBH so far, but intradermal test suggest that they may play a role in some cases of IBH (see Section 3.2). The role of self-antigens in the pathogenesis of IBH is not yet known, although one study suggested that they might be involved in IBH (Althaus et al., 2004).

Even though considerable research has been dedicated to IBH in the past decades, the immunological and pathophysiological mechanisms leading to the disease are not yet fully understood.

2. Immunopathogenesis of allergic diseases

In humans, atopy is defined as the disposition to produce IgE antibodies in response to various environmental antigens, often resulting in IgE-mediated, type I hypersensitivity diseases. Individuals affected by type I hypersensitivities switch to the production of specific IgE antibodies to non-self proteins commonly

termed allergens. During the sensitization phase, antigen-presenting cells (APC) are critical in initiating and controlling allergic inflammation. APCs such as dendritic cells and cutaneous Langerhans' cells in the skin take up the antigens and traffic to draining lymph nodes while they mature and process the antigen (Lambrecht, 2001). In the lymph nodes, they present the processed allergen on their surface as peptides via the major histocompatibility complex (MHC) class II molecules to naïve T cells. Depending on the antigen receptors and co-stimulatory molecules expressed, naïve CD4⁺ T cells may differentiate into Th1, Th2, Th9, Th17, Th22 or inducible T regulatory (T_{reg}) cells. Th1 cells involved in protective responses to viruses and intracellular bacteria are induced by IFN- γ and IL-12, whereas Th2 cells which secrete IL-4, IL-5 and IL-13 are claimed to be important for protective immune responses to helminthes (Abbas et al., 1996; Biedermann et al., 2004). Furthermore, they promote allergic responses such as asthma. In atopic humans the pathogenic role of Th2 cell-mediated immune response to allergen exposure has been extensively demonstrated and allergy is regarded as a classical Th2 disease (Zhu and Paul, 2010). The Th1/Th2 paradigm mentioned above dominated allergy research for many years and was the basis for the so-called hygiene hypothesis, claiming that a hygienic environment in early childhood results in a decreased exposure to microorganisms and thus a decrease of the Th1 immune responses. Under such circumstances the immune response shifts to a Th2-biased response favoring the development of allergic diseases (Akdis, 2006). The Th1/Th2 paradigm was revisited after the discovery of T_{reg} cells, described to prevent the development of autoimmune diseases. They are considered to be a critical feature also in the context of allergy (Akdis, 2009). T_{reg} cells are subdivided into two general subsets: (a) natural T_{reg} cells expressing CD4, CD25 and the transcription factor Foxp3 and (b) inducible T_{reg} cells which produce high amounts of IL-10 and TGF- β 1. Inducible and naturally occurring T_{reg} cells play a crucial role in the maintenance of immune tolerance by regulation of antibody isotypes and suppression of pro-inflammatory cells (Akdis, 2009). There is evidence that increased levels of IL-10 and TGF- β produced by T_{reg} cells potently suppress IgE production in humans, while simultaneously increasing the production of non-inflammatory antibodies of the IgG4 and IgA isotype, respectively (Verhagen et al., 2005). Factors which influence the Th cell fate in humans might be the genotype of the individual, antigen form and concentration, the antigen-presenting cell type and its activation status, and the local cytokine milieu (Akdis et al., 1998; Tanaka et al., 2000; Mazzoni et al., 2001; Paul and Zhu, 2010; Zhu and Paul, 2010). Normal individuals generally synthesize mainly immunoglobulin of the IgG isotypes, and only small amounts of IgE. In allergic individuals, however, a Th2 over-polarization due to an increased secretion of Th2-type cytokines like IL-4 and IL-13 can induce B cells to undergo heavy chain isotype switch to IgE. Subsequently, IgE antibodies produced by short and long-living plasma B cells bind to the high affinity IgE receptor (Fc ϵ RI) on effector cells leading to sensitization of the individual. In sensitized atopic individuals, re-exposure to the sensitizing allergen induces

cross-linking of high-affinity Fc ϵ RI receptor-bound IgE on effector cells and, thus, immediate release of anaphylactogenic mediators (Gould and Sutton, 2008). Cross-linking of cell-bound specific IgE leads to degranulation of mast cells, immediate release of preformed inflammatory mediators responsible for the symptomatic, and *de novo* production and secretion of lipid mediators and cytokines. Mast cells release two kinds of mediators: preformed mediators including biogenic amines and granule macromolecules, and *de novo* synthesized mediators such as lipid derived mediators and cytokines. Biogenic amines are responsible for many biologic effects of mast cell activation. Among these, histamine is one of the most potent mediators which increases vascular permeability and stimulates smooth muscle cell contraction. Beside biogenic amines, granule enzymes and proteoglycans are released leading to tissue damage and remodeling. Upon mast cell activation, lipid mediators such as leukotrienes, platelet activating factor (PAF), and cytokines are newly synthesized. These mediators influence acute and chronic inflammatory responses. The lipid mediators such as prostaglandin D₂, E₂, leukotrienes B₄ and C₄ provoke smooth muscle contraction, increase vascular permeability and stimulate mucus secretion. Furthermore, they contribute to an activation and influx of leukocytes playing a role in the late phase response (Abraham and St John, 2010). The cytokines IL-4 and IL-13 induce IgE production. The secretion of IL-3 is critical for the development, survival and function of tissue mast cells and blood basophils. IL-5 effects eosinophil survival and activation which contribute to tissue injury (Lantz et al., 1998; Abbas et al., 2007; Murphy et al., 2007).

The inflammatory response to allergens can be subdivided into an immediate reaction occurring within five to ten minutes, a late phase reaction occurring two to four hours after allergen injection and, in some cases, delayed type hypersensitivity (DTH) (Valenta, 2002; Zhu and Paul, 2010). Upon allergen provocation histamine, prostaglandins and other preformed or *de novo* synthesized mediators promote an enhanced vascular permeability leading to leakage of plasma proteins and increased cutaneous blood flow with intravascular trapping of erythrocytes. This reaction is characterized by swelling and redness. The late phase reaction is basically caused by the accumulation of inflammatory leukocytes, including neutrophils, eosinophils, basophils and Th2 cells (Abbas et al., 2007; Murphy et al., 2007). DTH presents as an immune response in which antigen-specific effector T cells activate macrophage, thereby inducing inflammation and finally tissue injury. When an antigen penetrates the skin, it is presented by APCs to sensitized memory T cells. This subsequently leads to T cell activation, proliferation and chemokine mediated influx of macrophages, monocytes, and lymphocytes at the site of antigen exposure. These cells then secrete inflammatory cytokines, e.g. TNF- α , IL17A and IFN- γ . The endothelial cells surrounding the venules at the site of antigen exposure become leaky. In further steps fibrin is deposited, and T cells and monocytes accumulate in the extravascular tissue around the injection site. A hallmark of DTH reaction is induration and swelling (Abbas et al., 2007; Murphy et al., 2007).

3. Immunopathogenesis of insect bite hypersensitivity

3.1. Allergen sources and allergens involved in insect bite hypersensitivity

3.1.1. Sources

Several studies have identified insects of the genus *Culicoides* as a clinically relevant allergen source by demonstration of skin test reactivity and/or measurement of allergen-specific IgE levels in serum of IBH-affected horses (Anderson et al., 1993; Braverman et al., 1983; Quinn et al., 1983; Ferroglio et al., 2006; Hellberg et al., 2006). Additionally, *Culicoides* species play a crucial role as vectors of viruses such as for example Bluetongue and African horse sickness virus (Mellor et al., 2000). *Culicoides* biting midges are very small hematophagous flies measuring from 1 to 3 mm in size. They occur virtually in all large land masses ranging from the tropics to the tundra and from sea level to 4000 m with the exception of Antarctica, New Zealand and Iceland (Hesselholt et al., 1977; Mellor et al., 2000). Only female *Culicoides* (Fig. 3), having larger salivary glands than males, are bloodsuckers. During the bite procedure, they secrete their saliva into the skin. The secretions of blood feeding insects contain various pharmacologically active components to overcome the physical barriers of the host formed by epithelial, connective, and keratinized tissue. Additionally, blood feeding insects have to bypass the coagulation pathway and are therefore equipped with specific proteins to inhibit coagulation (Russell et al., 2009). Since different *Culicoides* species exist, it is likely that horses react to major antigens found in all species (Braverman et al., 1983; Halldorsdottir et al., 1989; Anderson et al., 1993). Wilson et al. (2001) and Hellberg et al. (2006) clearly demonstrated the presence of IgG and IgE antibodies recognizing *C. nubeculosus* salivary gland proteins in serum of IBH affected horses.

3.1.2. Allergens

According to the World Health Organization/International Union of Immunological Societies definition, a protein is considered to be an allergen when it elicits a specific IgE antibody response in at least five individuals (Larsen and Lowenstein, 1996). The distinction between major and minor is a functional classification: a major antigen is defined by an incidence of sensitization reaching or surpassing 50% of the population tested. Allergens are named by using the first three letters of the genus, a single letter for the species, and a number according to the priority of allergen discovery and purification (<http://www.allergen.org>).

In the past, investigations revealed that at least ten salivary protein bands from *C. nubeculosus* with molecular weights ranging from 12 to 75 kDa can bind to serum IgE of IBH-affected horses (Hellberg et al., 2006). Furthermore, immunoblot analyses also revealed the presence of IgE-binding proteins in salivary gland extract from *Simulium vittatum* (Hellberg et al., 2009). Fifty four novel protein sequences including potential allergens were identified from a *C. nubeculosus* cDNA salivary gland library and used to assign abundant protein spots in salivary gland extract

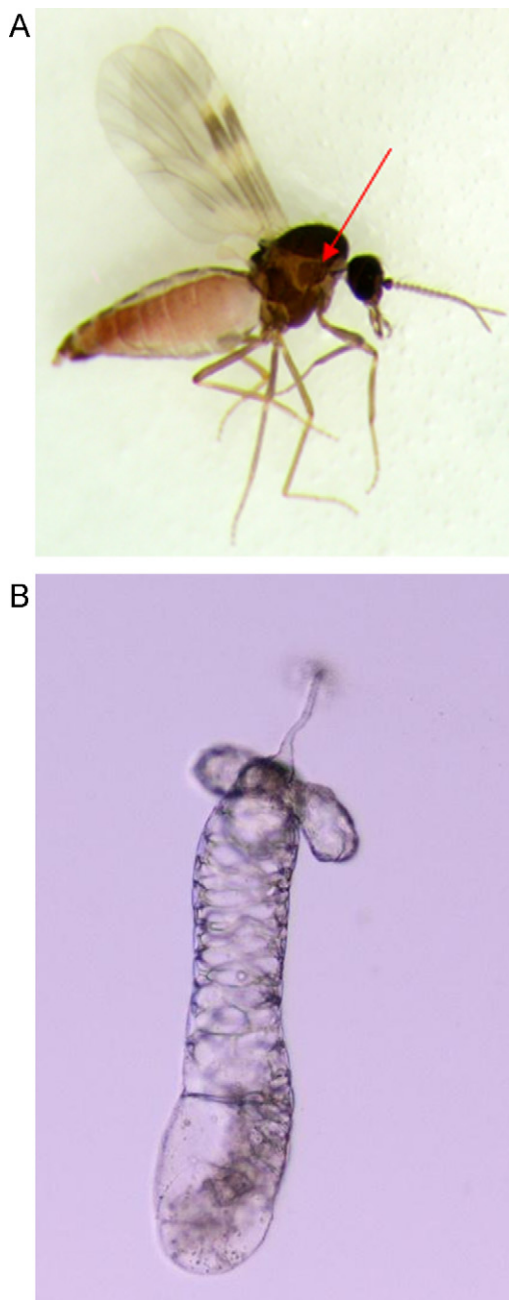


Fig. 3. (A) Female *Culicoides* (original magnification 6×, length of the wings corresponds to 2 mm). The arrow indicates where the salivary glands are situated. (B) Salivary gland of a female *Culicoides* (original magnification 40×) containing the allergens causing IBH.

by mass spectroscopy (Russell et al., 2009). Among other salivary proteins, the sequences of hyaluronidase, maltase and two serine proteases were identified, but their relevance for IBH was not assessed. The first allergen described to be relevant for IBH, a maltase of *C. sonorensis* (Cul s 1), corresponds to a protein of 66 kDa. Cul s 1 has been expressed in the baculovirus cell expression system and showed binding to serum IgE *in vitro* and skin test reactivity *in vivo* in IBH-affected horses but not in healthy

Table 2

Allergens associated with insect bite hypersensitivity which have been characterized at the molecular levels and expressed as recombinant proteins. The *Simulium vittatum* allergens are probably binding IgE from IBH-affected horses because of cross-reactivity with *Culicoides* allergens, as demonstrated for Cul n 1 and Sim v 1.

r-Allergen	GenBank accession number	aa	MW (kDa)	Homology to (NCBI accession number)	IDT	Reference
Cul s 1	Q66UC5_9DIPT	n.s.	68.6	Maltase (AY603565) ^b	pos	Langner et al. (2009)
Cul n 1	EU978899	209	25.4	Antigen 5 like Protein (ACH56843.1 ^c ; AAU06471 ^b)	pos	Schaffartzik et al. (2010)
Cul n 2	HM145950	403	46.7	Hyaluronidase (ACM40915.1) ^a	pos	Schaffartzik et al., 2011
Cul n 3	HM145951	391	44.6	Putative cysteine endopeptidase (ACM40897.1) ^a	pos	
Cul n 4	HM145952	153	17.5	Secreted salivary protein (ACM40874.1) ^a	pos	
Cul n 5	HM145953	406	45.7	Secreted salivary protein (ACM40903.1) ^a	pos	
Cul n 6	HM145954	147	16.9	Secreted salivary protein (ACM40905.1) ^a	n.t.	
Cul n 7	HM145955	186	20.9	Unknown salivary protein (AUU06522.1) ^b	pos	
Cul n 8	HM145956	603	68.7	Maltase (ACM40914.1) ^a	pos	
Cul n 9	HM145957	142	15.5	D7-related salivary protein (ACM40878.1) ^a	pos	
Cul n 10	HM145958	420	47.8	Secreted salivary protein (ACM40903.1) ^a	pos	
Cul n 11	HM145959	275	30.1	Trypsin (ACM40875.1) ^a	n.t.	
Sim v 1	EU930215	258	30.1	Antigen 5 like protein (ACH56843.1) ^c	pos	Schaffartzik et al. (2009, 2010)
Sim v 2	EU930300	83	9.6	Kunitz protease inhibitor (ACH56928.1) ^c	n.t.	
Sim v 3	EU930236	246	28	a-Amylase (ACH56864.1) ^c	n.t.	
Sim v 4	EU930294	241	26	a-Amylase (ACH56922.1) ^c	n.t.	

Cul s: *C. sonorensis*; Cul n: *C. nubeculosus*; Sim v: *S. vittatum*; IDT: intradermal skin test; n.s.: not shown; pos: positive; n.t.: not tested.

^a *C. nubeculosus*.

^b *C. sonorensis*.

^c *S. vittatum*.

controls (Langner et al., 2009). Furthermore, screening of phage surface displayed cDNA salivary gland libraries from *C. nubeculosus* revealed ten and from *S. vittatum*, six allergens associated with IBH (Table 2). The prevalence of IgE sensitization to recombinant *C. nubeculosus* salivary gland allergens analyzed by ELISA ranged from 13 to 56.5% depending on the allergen tested (Schaffartzik et al., 2011). IBH-affected horses are often sensitized against many different *C. nubeculosus* allergens, as serum IgE antibodies against a mean number of 4 out of 11 r-allergens (range=1–10 r-allergens) was found in the sera of these horses. The *S. vittatum* recombinant allergens were recognized by 20–60% of the sera of IBH-affected horses (Schaffartzik et al., 2009). *S. vittatum* also occurs in Iceland (Johannsson, 1988) where the horses do not develop clinical signs of IBH. Hence *S. vittatum* seems to play only a secondary role in the context of IBH (see below). The proteins found by phage surface display technique show sequence homology to already known salivary proteins of insects some of which having defined functions (Table 2). Some analogies were observed between salivary gland allergens and protein families inducing allergy in humans. Cul n 1 and Sim v 1 share sequence homology to antigen 5 protein of wasps representing a major allergen of vespid venoms (Schaffartzik et al., 2010). Furthermore, Cul n 2 codes for hyaluronidase which is a major allergen in bee venom allergy (Markovic-Housley et al., 2000). Cul n 9 shares sequence homology with D7-related proteins present in the saliva of female-blood sucking insects (Malafronte Rdos et al., 2003). Maltase (Cul s 1; Cul n 8) has been shown to elicit allergic reactions in humans (Horner et al., 2008). IBH is characterized by severe pruritus that is mainly due to the release of histamine from activated basophils and mast cells. All *C. nubeculosus* recombinant allergens, except for Cul n 6 and Cul n 11, where no suitable horses for skin testing could be found, elicited clear immediate type skin reactions after intradermal application in

affected horses but not in healthy controls (Schaffartzik et al., 2010, 2011). These results suggest that these recombinant allergens are indeed functionally relevant for IBH.

3.1.3. Cross-reactivity between *Culicoides* and *Simulium* allergens

S. vittatum also exist in Iceland (Johannsson, 1988) but horses living in that country do not develop clinical signs of IBH. Antigen 5 like protein from *S. vittatum* (Sim v 1) and *C. nubeculosus* (Cul n 1) share a sequence identity of about 48%. It has been demonstrated by inhibition ELISA and Western blots that they are fully cross-reactive (Schaffartzik et al., 2010). These findings may implicate that the main causative agents of IBH are allergens from *Culicoides* spp. Sensitization to *S. vittatum* is most likely secondary to *Culicoides* spp. and explainable by cross-reactive proteins from both insect species. The knowledge of cross-reactivity between antigens is important in allergic diseases including food allergy, rhinitis, conjunctivitis, bronchial asthma and atopic eczema (van Ree, 2004; Bohle, 2007; Crameri et al., 2009; Zeller et al., 2009). The phenomenon of cross-reactivity occurs when IgE antibodies, originally raised against one allergen, bind or recognize a similar protein from another source. Cloning and sequencing of allergen genes explain the molecular basis of cross-reactivity. Two types of cross-reactivity occur, one due to peptides and the other due to non-peptidic epitopes for instance sugars, typically post-translational modifications of proteins like glycosylation, called cross-reacting carbohydrate determinants (Aalberse and Crameri, 2011). In humans, cross-reactivity is not only restricted to similarity between the primary sequences of two allergens, but can also occur if a human homolog to an environmental allergen exists and is available to the immune system (Glaser et al., 2008).

3.2. IgE and IgE-mediated reactions

Various studies have shown that IgE antibodies are the hallmark of this equine allergic dermatitis (van der Haegen et al., 2001; Wagner et al., 2006; Wagner, 2009). The equine epsilon heavy chain constant region (IGHE) is localized on chromosome 24 (Wagner et al., 2004). A single gene coding for the IgE heavy chain constant region is present per haploid genome and several allelic variants of equine IGHE gene were found. Total serum IgE concentrations in horse are in the range of 84.0 µg/l, are highly variable between individual horses, and substantially higher than those observed in human sera (Wagner, 2009). It was suggested that the higher serum IgE concentrations in healthy horses are caused by generally higher parasitic loads that practically disappeared in humans living in industrialized countries. A study by Hamza et al. (2010) showed that in horses a high endoparasite burden is indeed associated with a high total serum IgE level. Because total IgE levels are strongly influenced by the degree of infection with endoparasites, they can usually not be used as indicator of atopy in horses (Scharrenberg et al., 2010) or other domestic animal, as is the case in humans. However, in horses of the Icelandic breed imported from Iceland, the total IgE concentration was significantly increased in IBH-affected horses compared to horses that did not develop IBH (Wilson et al., 2006; Wagner, 2009). This finding is probably explained by the extremely strong Th2 and IgE response against *Culicoides* allergens in IBH-affected horses born in Iceland and imported to Europe, which is usually not observed in horses born outside Iceland (Hellberg et al., 2006; Hamza et al., 2007). Various studies have shown that IBH-affected horses have serum IgE antibodies against salivary gland proteins from midges (Wilson et al., 2001; Hellberg et al., 2006; Langner et al., 2008). However, the direct and specific role of IgE in the pathogenesis of IBH has recently been confirmed by a modified Prausnitz-Küstner experiment (Prausnitz, 1921) showing that the transfer of IgE purified from serum of an IBH-affected horse to the skin of a healthy horse induces classical type I allergic reactions upon allergen challenge at the site of IgE injection (Wagner et al., 2006). Accordingly, significantly more IgE-protein and IgE-mRNA positive cells were detected in lesional skin biopsies of IBH-affected horses taken in the acute phase of the disease than in skin from healthy horses (Fig. 4) (van der Haegen et al., 2001). Moreover, intradermal injection of whole body extract (WBE) of *Culicoides* species into the skin of IBH-affected horses mimics the response to natural antigen exposure eliciting type I reactions accompanied by infiltration of T cells and eosinophils at the site of injection, directly demonstrating a relationship between *Culicoides* antigens and eczematous reactions (Foster et al., 1998; McKelvie et al., 1999).

In IBH eosinophils as well as histamine, leukotrienes and PAF seem to play a crucial role. Histamine has been detected in the plasma of IBH-affected horses during active phases of the disease (Riek, 1955). Foster et al. (1995) investigated the cutaneous inflammation and demonstrated the importance of PAF and leukotriene B₄ in IBH-affected horses. Intradermal injection of PAF leads to edema formation, as well as neutrophil and eosinophil infiltration

in the skin of affected horses during the active phase of the disease (Foster et al., 1995). In experiments with equine peripheral blood leukocytes, the release of sulfidoleukotrienes and histamine after allergen stimulation has been demonstrated (Marti et al., 1999; Langner et al., 2008; Wagner et al., 2008). 78% of IBH-affected horses but only 3% of the controls released leukotrienes after incubation with WBE of *C. nubeculosus* (Baselgia et al., 2006). Investigations on eosinophils have shown that PBMC stimulated with WBE of *Culicoides* spp. secrete a heat labile factor causing eosinophil adherence (McKelvie et al., 2001).

Various studies reported significantly more immediate type reactions in the skin in IBH-affected horses compared to controls upon allergen challenge (Halldorsdottir et al., 1989; Anderson et al., 1993; Sloet van Oldruitenborgh-Oosterbaan et al., 2009). However, positive intradermal test (IDT) reactions are also observed in a varying proportion of clinically non-allergic horses (Wagner et al., 2009). Only few studies reported delayed type reactions after intradermal allergen challenge in horses (Baker and Quinn, 1978; Ferroglio et al., 2006; McKelvie et al., 1999) but based on histopathology and cytokine profile, there is at present no evidence for DTH being a major feature of IBH. IBH resembles atopic eczema in humans which is characterized by DTH in the chronic phase and in analogy it seems to be likely that in the chronic phase of IBH DTH reactions may also be involved.

3.3. T cells

The involvement of T cells and the Th1/Th2 polarity in healthy and IBH-affected horses has been investigated both in the skin as well as in circulating or cultured PBMC. Lesional skin of IBH-affected horses contains significantly higher numbers of CD4⁺ cells but not CD8⁺ cells than skin biopsies from healthy control horses (Heimann et al., 2011). Furthermore, increased numbers of CD3⁺ and CD4⁺ T cells were observed in the skin after intradermal injections of *Culicoides* antigen (McKelvie et al., 1999). This suggests similar immunopathological mechanisms in IBH and AE as a T cell infiltrate due to an amplified recruitment of CD4⁺ Th cells is also a typical finding in AE lesions from humans (Rho et al., 2004). A further similarity between AE and IBH skin lesions is the increased expression of IL-13 which was observed in lesional and non-lesional skin of IBH-affected horses compared to controls (Heimann et al., 2011). Increased IL-13 expression has repeatedly been described in AE skin (Hamid et al., 1996; Akdis et al., 1999; Obara et al., 2002; Tazawa et al., 2004). In the study by Heimann et al. (2011), IL-4 expression was not higher in lesional skin from IBH-horses compared to skin from controls horses. This is in line with some studies in human AE which proposed that IL-13, and not IL-4, is responsible for IgE production in human AE patients (Akdis et al., 1999). This may also be explained by the stage of the disease when biopsies were taken: whereas IL-4 has a primary role in the initiation of the allergic response, IL-13 is critically involved in its effector functions (Corry, 1999). Furthermore, IL-4 seems to be expressed far more transiently than IL-13 (Pfeffer et al., 2007). Thus, as it was not known at what time following exposure to *Culicoides* bites

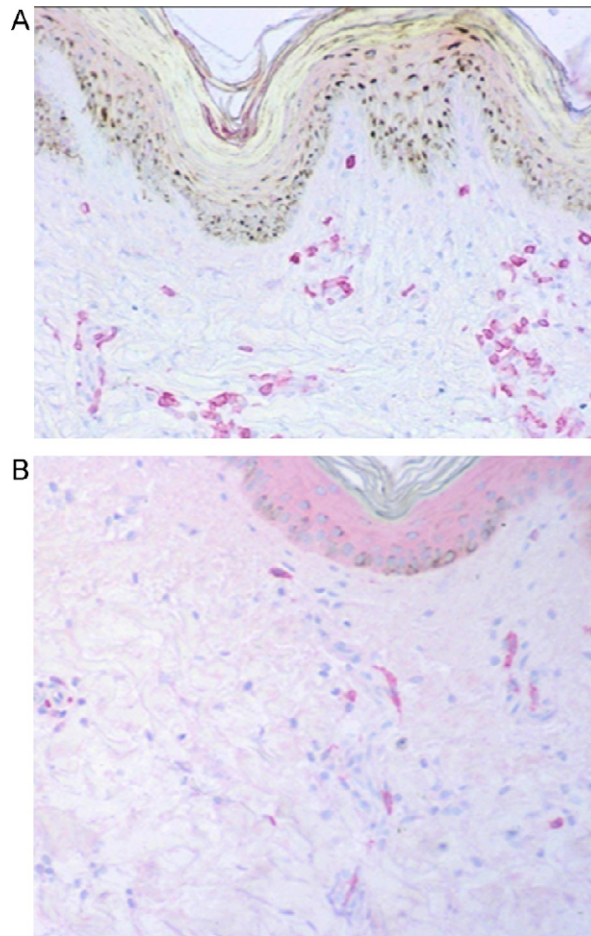


Fig. 4. Lesional skin biopsy from a horse affected with IBH (A) and from a healthy control horse (B) stained with an IgE-specific antibody developed with fast red, showing the increase in IgE-bearing cells in the subepidermal layer of the skin of the IBH horse compared to the healthy control.

the skin biopsies were taken, IL-4 mRNA expression may already have vanished while increased IL-13 expression remained detectable. In this study, the cellular source of the detected cytokines was not known. In humans it is known that beside Th2 cells, basophils, eosinophils, mast cells and NK T cells also produce IL-4 and IL-13, but this has not yet been investigated in horses (Akdis et al., 2011). No significant differences in Th2-, Th1- or T_{reg}-type cytokine mRNA expression were detected in peripheral blood from IBH-affected horses compared to controls (Heimann et al., 2011).

Conversely, *in vitro* stimulation of PBMC with *C. nubeculosus* extract induced a disease-associated increase in IL-4 mRNA expression as well as increased numbers of IL-4 protein expressing CD3+ T cells in IBH-affected compared to healthy horses during summer, i.e. when the antigen was present in the horses' environment. However, IL-4-biased responses were not observed in *C. nubeculosus* stimulated PBMCs isolated from IBH-affected horses during winter (Hamza et al., 2007). No IFN- γ production was detected in PBMC stimulated with *C. nubeculosus* extract but polyclonal stimulation of PBMC resulted in a lower production of IFN- γ by IBH-affected horses compared to controls (Hamza et al.,

2007). This is probably a consequence of the higher IL-4 levels in PBMC cultures of IBH-affected horses, which suppress IFN- γ production. These findings support the hypothesis of a Th2 polarity in IBH-affected horses (Hamza et al., 2007). Further studies revealed that Th2-type cytokine production is suppressed in healthy Icelandic horses born in Europe. Supernatant from PBMC cultures of healthy horses born in Europe and bred from imported Icelandic horses (2nd generation horses) were added to cultures of IBH-affected horses imported from Iceland (1st generation horses). A significant decrease of IL-4 production by PBMC of IBH-affected horses from the 1st generation was observed. This was probably due to IL-10 and TGF- β 1 present in the supernatant, because addition of neutralizing antibodies against IL-10 and TGF- β 1 to PBMC cultures from healthy horses stimulated with *C. nubeculosus* extract, lead to a significant increase in IL-4 levels. Moreover, addition of a combination of IL-10 and TGF- β 1 significantly decreased IL-4 production by PBMC of IBH-affected horses. Added alone, IL-10 and TGF- β 1 failed to significantly reduce IL-4 production. These finding emphasize a role for both IL-10 and TGF- β 1 in the modulation of IL-4 production (Hamza et al., 2008). Ongoing studies also suggest a role for T_{regs} in the pathogenesis

of IBH. It has recently been reported that Foxp3 is constitutively expressed by CD4⁺CD25⁺ T cells in horses (Hamza et al., 2011a). Interestingly, the proportion of circulating Foxp3⁺ cells within the CD4⁺CD25⁺ subpopulations did not differ between IBH-affected and healthy horses. In contrast, upon stimulation of PBMC with *C. nubeculosus* extract, expression of Foxp3 by CD4⁺CD25⁺ cells was significantly higher in healthy controls compared to IBH-affected horses (Hamza et al., 2011b). Such mechanisms also seem to be involved *in situ*, as skin biopsies from IBH-affected horses contain significantly lower mRNA levels of Foxp3 than skin from healthy control horses (Heimann et al., 2011).

The hygiene hypothesis was not explicitly investigated in animals, but the studies with Icelandic horses from Hamza et al. (2007, 2008) reported above suggest comparable mechanisms as observed in humans. As mentioned at the beginning, ≥50% of Icelandic horses exported as adults to mainland Europe, where *Culicoides* spp. exist, develop IBH whereas the incidence of IBH in Icelandic horses born in mainland Europe is much lower (5–10%). Icelandic horses exported from Iceland to mainland Europe are not exposed to the antigens of *Culicoides* spp. during the development of their immune system. PBMC from 1st generation horses produce more IL-4 than 2nd generation horses, even in 1st generation horses that remained free of IBH for many years after their import. This raised the question of whether a Th2 biased immune response is a characteristic of the Icelandic horses born in Iceland. A study by Hamza et al. (2010) has shown that PBMC from Icelandic horses living in Iceland produce lower IL-4 and higher IL-10 and TGF-β1 levels compared to 1st and 2nd generation horses living in Europe. Thus environmental factors seem to influence the cytokine profile in horses of the Icelandic breed. Interestingly, horses living in Iceland have a significantly higher degree of infection with endo-parasites than 1st and 2nd generation Icelandic horses living in Europe. The regulatory immune response of horses living in Iceland is thus probably due to the chronic and high endo-parasitic burden, resulting in down-regulation of a Th2 immune response, as has been reported in humans (van Riet et al., 2007). At the time of importation from Iceland, horses are de-wormed. This could be one of the factors leading to a decrease of IL-10 and TGF-β1 which may then up-regulate the Th2 cytokine production, resulting in a higher susceptibility to allergies upon contact with novel allergens (*Culicoides* antigens). Similarly in humans, antihelminthic treatment of chronically helminth infected persons has been shown to diminish IL-10 production (Araujo et al., 2000) and increase atopic reactivity to allergens (Lynch et al., 1993; Araujo et al., 2004; van den Biggelaar et al., 2004).

In summary, the immune response to allergens at the T cell level in horses resembles that known for humans, however, the research on equine T_{reg} cells is still at an early stage.

4. Current forms of diagnosis and treatment

Currently, diagnosis of type I reactions in IBH is performed by using crude WBE from *Culicoides* and sometimes *Simulium*, which have not been standardized for their allergen content (Braverman et al., 1983;

Anderson et al., 1993; Ferroglio et al., 2006; Quinn et al., 1983; Sloet van Oldruitenborgh-Oosterbaan et al., 2009). As a consequence, serological tests have a low specificity and/or sensitivity (Frey et al., 2008) and false positive reactions in intradermal tests are relatively frequent (Wagner et al., 2009), making IDT with crude *Culicoides* extract a not very reliable method for diagnostic purposes. Natural allergen extract only allows determining whether a horse is sensitized to a certain allergen source but not to which allergen it has raised specific IgE antibodies. Allergen extracts produced from natural sources are complex biological mixtures containing, beside the relevant allergenic proteins in unknown concentrations, many non-allergenic proteins, carbohydrates, and low-molecular-weight substances like histamine or immunosuppressive compounds (Schmid-Grendelmeier and Cramer, 2001). Many factors during the process of extraction and handling influence the quality of an extract such as the original source material, extraction procedure, protease content and storage conditions. Moreover, allergen extracts are difficult to standardize. To date, standardization of allergen extracts on IgE-binding is determined by biological assays which do not provide specific information about major allergen content. In terms of immunotherapy crude extract may not contain all crucial allergens in sufficient amounts to treat patients successfully, whereas the patients may become sensitized to allergens contained in the extract to which they had not been allergic before treatment (Akdis, 2006).

In the past, different approaches for treatment of IBH have been described, but, at present, the only safe and effective method is the avoidance of *Culicoides* allergens (Riek, 1953b). Avoidance or reduced allergen exposure is attempted to be achieved by mechanical protection with blankets representing the most common treatment of IBH (Olsen et al., 2010). Beside blankets horses are kept in stables in an insect-proof environment from mid-afternoon to mid-morning or relocated to other parts of the country where the occurrence of *Culicoides* is highly reduced, such as high altitude (Alps) and sea side. Insect repellents are also applied to keep blood-sucking insects away from horses. The topical pyrethrin cypermethrin has been reported to partially protect against biting flies (Papadopoulos et al., 2010).

As in human allergy, glucocorticosteroids are used for symptomatic treatment of IBH (Petersen and Schott, 2009). Severely affected horses, particularly those with marked pruritus may need anti-inflammatory treatment with systemic steroids. The disadvantages of glucocorticosteroids in long-term treatments are the toxic side-effects, e.g. immunosuppression, muscular atrophy, osteoporosis and laminitis.

Experimental approaches were conducted with histamine H₁-antagonists. The anti-histamine drugs promethazine hydrochloride and tripeleminamine hydrochloride were effective in reducing the reaction elicited by WBE of *Culicoides* (Morrow et al., 1986). Foster et al. (1998) administered locally chlorpheniramine and induced an inhibition of edema formation in response to *Culicoides* antigens. To estimate the efficacy of the non-sedative second generation histamine H₁-antagonist, cetirizine, which is widely described for an efficient treatment of allergic rhinitis,

pruritus and chronic idiopathic urticaria in humans, Olsen et al. (2010) challenged IBH-affected horses orally with this drug. However, no apparent benefit in comparison with treatment with placebo was observed (Olsen et al., 2010).

4.1. Immunotherapy

Allergen-specific immunotherapy (SIT), usually the s.c. administration of allergen extract, is effectively used in human and small animal dermatology, conferring long-term benefit. The mechanisms of action by which SIT mediates its anti-inflammatory effects are still not completely understood. However, various studies have revealed that SIT influences the response of APCs, T cells, B cells and effector cells (reviewed in Larche et al., 2006; Akdis and Akdis, 2011). SIT results in an early induction of T_{reg} cells suppressing Th2-Th1 cells and other effector cells, which can already lead to early protection against challenge. T_{reg} cells probably induce the early decrease in mast cell and basophil activity for degranulation, which cannot be explained by decreased allergen-specific IgE levels, as SIT first leads to an increase in specific IgE, followed by a very late decrease. SIT also induces production of specific IgG4 and to some extent IgG1. There is accumulating evidence that successful SIT is associated with an increase in IgG-blocking activity rather than with only high levels of IgG4 (Till et al., 2004; James et al., 2011). There are not many reports describing experimental applications of SIT in horses. However, controversial results of the efficacy have been reported for SIT with WBE of *Culicoides* spp. In an immunotherapy trial IBH-affected horses were injected subcutaneously with WBE. Weekly injections during the first year reduced the clinical signs in 90% of IBH-affected horses (Anderson et al., 1996). 37.5% of these horses were completely free from clinical signs after two years of immunotherapy, whereas 62.5% had moderately to significantly reduced clinical signs (Anderson et al., 1996). Conversely, a randomized, double-blinded, placebo-controlled clinical study did not show any improvement in the health status in IBH-affected horses after SIT over a six-month period (Barbet et al., 1990).

In conclusion and in contrast to human allergy, SIT in horses is not yet established and other long-term curative treatments do not exist.

5. Concluding remarks

The use of modern cloning strategies and molecular biology techniques has permitted the identification and characterization of *Culicoides* allergens. This will make possible new studies using pure allergens instead of crude allergen extracts and could represent a promising diagnostic tool. In addition, the findings about cross-reactive salivary gland proteins between *C. nubeculosus* and *S. vittatum* may explain why IBH-affected horses are also sensitized to *S. vittatum* salivary gland proteins although *S. vittatum* are probably not the primary sensitizing allergens. In recent years progress has also been made characterizing the immune mechanisms involved in IBH. It has been shown by various groups that IgE-mediated reactions to *Culicoides* allergens are frequently found in IBH-affected

horses and recent studies also indicate the presence of an imbalance between the T_{reg} and Th2-type immune response. Further studies are now needed to understand the role of APCs and keratinocytes in the initiation of an allergic or sound immune reaction to *Culicoides* allergens and to understand what mechanisms cause the high prevalence of IBH in horses imported from Iceland. Finally, testing by which means the balance between T_{reg} and Th2 cells can be restored together with the characterization of the major allergens for IBH could help to develop new, more effective treatment strategies for IBH, such as immunotherapy using the specific causative allergens.

Conflict of interest

None declared.

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