

Review Article

Immune responses to ectoparasites of horses, with a focus on insect bite hypersensitivity

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SUMMARY

Horses are affected by a wide variety of arthropod ectoparasites, ranging from lice which spend their entire life on the host, through ticks which feed over a period of days, to numerous biting insects that only transiently visit the host to feed. The presence of ectoparasites elicits a number of host responses including innate inflammatory responses, adaptive immune reactions and altered behaviour; all of which can reduce the severity of the parasite burden. All of these different responses are linked through immune mechanisms mediated by mast cells and IgE antibodies which have an important role in host resistance to ectoparasites, yet immune responses also cause severe pathological reactions. One of the best described examples of such pathological sequelae is insect bite hypersensitivity (IBH) of horses; an IgE-mediated type 1 hypersensitivity to the salivary proteins of *Culicoides* spp. associated with T-helper-2 production of IL4 and IL13. Importantly, all horses exposed to *Culicoides* have an expanded population of *Culicoides* antigen-specific T cells with this pattern of cytokine production, but in those which remain healthy, the inflammatory reaction is tempered by the presence of FoxP3+ CD4+ regulatory T cells that express IL10 and TGF-beta, which suppresses the IL4 production by *Culicoides* antigen-activated T cells.

Keywords immunopathology, mast cell, regulatory T cells, Th-2

INTRODUCTION

On first impression, writing a review of the immune responses of horses to ectoparasites should prove a simple task as, apart from the response to bites of midges (*Culicoides* spp.), there is a scarcity of published research in this area. However, if one begins to consider what is known about the range of species that can be counted as ectoparasites of horses, the clinical manifestations of parasite infestations and their associated pathology, a complete review of the subject rapidly becomes more challenging. Just to provide an exhaustive list and description of every ectoparasite that can be found associated with horses in all parts of the world would in itself occupy the greater part of a review article. This article will therefore be restricted to a brief discussion of the type of [immune] reactions that mammals mount against the most common ectoparasites and a closer consideration of how this applies to horses, with particular emphasis on the reaction to *Culicoides* spp. termed insect bite hypersensitivity (IBH).

The ectoparasite species associated with horses encompass a diversity of lifestyles which vary markedly in the extent and intimacy of their association with their host. Examples range from the biting louse *Werneckiella equi* an obligate parasite of the horse living its entire life on the skin surface feeding off skin flakes and debris (1, 2), through the several species of mange mite that penetrate deeper into the superficial skin layers (3), to the myriad of opportunist blood-feeding insects that only transiently visit the hosts to acquire a blood meal (4). In the course of their activities, every one of these parasites will shed secrete or excrete antigens that are capable of stimulating inflammatory, innate and adaptive immune responses.

The largest class of ectoparasite antigens to which an immune response would be expected are the antigenic components present in the saliva of blood-feeding

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arthropods. Successful blood-feeding provides the advantage to the parasite of acquiring a protein rich meal, and the ability to do so has evolved separately in several arthropod genera (4, 5). Even exceeding this diversity of blood-feeding species, the saliva of haematophagus parasites contains a remarkably wide array of pharmacologically active components to assist the parasite in its aim of acquiring a blood meal (6). To combat the threat posed by haematophagus parasites, the host species have been pressed to evolve a number of counter strategies; including behavioural responses, haemostasis, inflammation and immunity, all of which can contribute to host resistance or resilience to parasite infestations. Although traditional research approaches have treated these strategies as belonging to separate scientific disciplines, it is becoming increasingly clear that they are all mechanistically linked and interdependent on each other.

In general, all mammals have broadly similar immune/inflammatory responses to bites of haematophagus ectoparasites. The classic series of experiments carried out during the 1940s by Mellanby described the evolving inflammatory response of naïve adult human volunteers following repeated exposure to bites by the mosquito, *Aedes aegypti* (7). On first exposure, there was no immediate reaction or pruritus at the time of the bite, but after a 24-h delay, an area of erythema with a central weal developed, accompanied by pruritus which lasted for several days. Following repeated exposures over the course of a month, new bites induced an immediate hypersensitivity reaction; characterized by weal formation, erythema and pruritus developing within a few minutes of being bitten, which then progressed into a delayed response lasting several days. As the number of exposures increased, the severity and duration of the delayed reaction gradually diminished over a period of several months leaving only an immediate response that lasted a few hours. Eventually in people who had years of exposure or in individuals native to regions where *A. aegypti* occur, the immediate reaction also subsided and there was no discernable reaction to the bites. Mellanby also addressed the age-old contention by some individuals who claimed they were never bitten by mosquitoes. His experiments showed that mosquitoes would feed on every subject equally, and it was probably those whose immune system did not respond or who had a tolerant response who falsely claimed not to have been bitten (8). Various types of response analogous to all stages of these reactions are also known from clinical observation of horses exposed to biting insects (9), but overall, the majority of horses appear remarkably tolerant to bites from insects present in their native environment. Nevertheless, some individual horses show severe clinical signs associated with ectoparasites, and in such cases, the

nature and severity of the antiparasite immune response would appear to be a significant part of the problem. Most notably in the case of IBH, there is a chronic hypersensitive response to *Culicoides* bites which bears the hallmarks of the second-stage reaction described by Mellanby of immediate weal formation followed by a delayed reaction accompanied by pruritus. However, in IBH, the immune response remains stuck at this stage and fails to further develop towards a tolerant state even after prolonged exposure.

Paradoxically arthropod saliva itself contains many factors that reduce host inflammation and immune responses (5, 6); but by not reacting to the parasite or mounting the wrong type of response, the host can increase its susceptibility to arthropod-transmitted infections (10).

Anti parasite immune reactions are not just an inconvenience to the host but also have consequences for the parasite. Immunity to ticks (11) and mites (12, 13) has been shown to reduce parasite numbers in challenge experiments of pre-exposed individuals in several host parasite combinations. More subtly, the fecundity of female *Culex pipens* mosquitoes was reduced following a blood meal on a pre-exposed (immune) host compared to those fed on a naïve host (14); such costs imposed on the parasite will undoubtedly have contributed to the ongoing pharmacological arms race between the hosts immune system and parasite secretory antigens (15).

PRURITIS

One of the most obvious clinical signs induced by ectoparasites is pruritus (itching). Although pruritus and induced grooming or scratching behaviours are not usually considered a component of classical immune responses, there is evidence that parasite-induced pruritus and scratching do limit the parasite burden of cattle lice infestation (16–18) and cat fleas (19), confirming that grooming plays an important role in host resistance and resilience to ectoparasite infestations. Similarly, tick infestations can be controlled by grooming; in some cases, the grooming behaviour is innate and takes place in the absence of parasites (20), but in others, the rate of grooming is increased by the presence of parasites (21) where the immune sensation of pruritus would serve to alert the host to their presence.

Current understanding of pruritus provides several direct and indirect links between the neurobiology of pruritus and the immune response. The sensation of pruritus is mediated by a specialized population of unmyelinated slow conducting polymodal C fibres, considered to be distinct from those that transmit pain (22). These nerves have unmodified branched peripheral endings in

skin that can be stimulated to elicit itching sensations by a wide range of ligands acting on several types of receptors (23, 24). Most of the endogenous ligands that induce itching sensations also have important roles in inflammatory and immune responses; perhaps the best known examples are histamine and serotonin which are released from mast cells and can subsequently elicit itching by binding to H1, H4 or 5HT-2 receptors, respectively (25, 26). Other agonists that elicit itching include the neuropeptides substance P and endothelin-1, the lipid mediators thromboxane and leukotriene B4 (27, 28) and some members of the proteinase-activated receptor family (PAR) (29). Proteinase-activated receptor family agonists are cysteine protease enzymes act by removing a propeptide from the extracellular N-terminal of the seven-transmembrane domain of their receptor revealing a new N-terminal peptide sequence which acts as an endogenous tethered ligand for the receptor (30). PAR2 agonists include many host-derived proteases, for example, trypsin, thrombin, plasmin and factor Xa that are frequently involved in inflammatory responses to tissue damage (30). Exogenous proteinases present in venoms or plant extracts can activate PAR neuroreceptors and are also known to induce itching (31). Moreover, PAR receptor expression is not restricted to nerves; PAR family members are expressed by a variety of cell types involved in immune responses including mast cells (32). Mast cells are central to a broad range of inflammatory pathways and can influence itch responses indirectly through the release of histamine or other mediators one of which is mast cell tryptase, itself a potent activator of PAR receptors (33). However, both PAR2 and PAR4 have been shown to mediate direct activation of nerves involved in sensing pruritus independently from their effects on mast cell degranulation (29, 34). Interestingly, several insect-derived allergens also act as agonists for PAR2 (35, 36), but as yet the possible role of cysteine proteases widely present in the saliva of blood-feeding arthropods in directly activating PAR receptors thereby eliciting inflammation and itching responses of horses or other domestic animals remains unconfirmed.

In contrast, the established role of IgE in mast cell degranulation with concomitant release of histamine serotonin and mast cell tryptase provides a direct link between the immune system and pruritus. A further direct link between immune response and itching is provided by the Th2 cytokine IL-31 (37) which causes pruritus by directly activating IL31RA expressed on sensory nerves in a histamine and PAR-2 independent fashion (38). Overall, the evidence supports the view that severe pruritus represents a visible external sign of the underlying immune reaction to ectoparasites.

LICE

There are two species of louse found on horses, the biting louse *W. equi* (syn. *Damalinia equi* or *Bovicola equi*) and the sucking louse *Haematipinus asini* (2, 39), with reports indicating that the former has a higher prevalence in northern Europe (40, 41). *Werneckiella equi* lives on the skin surface where it feeds largely on skin debris, while *H. asini* feeds on blood. Apart from anaemia associated with heavy infestations of *H. asini*, the clinical manifestation of infestation with either species is reportedly very similar (9), although it would seem probable that the blood-feeding habit of *H. asini* would bring it into a closer association with its hosts immune system making it more likely to induce an immune reaction.

Macroscopic lesions including areas of focal alopecia, scaling and crusts are commonly observed, and such lesions are usually attributed to self-trauma, but there is no reason to suppose that an inflammatory or immunological response is not an important part of the pathogenesis of these lesions. There are no prior reports of detailed histological changes or of immunological responses in horses with *W. equi* infestations. However, antibodies to *Bovicola ovis* antigens have confirmed the presence of specific adaptive immune responses of sheep to biting lice and the pathogenesis of associated skin lesions (42–44). In a study of the clinical manifestations and prevalence of *W. equi* on Icelandic horses, a significant proportion of animals were infested but did not show signs, while others had severe signs but no lice were detected. Overall, there was little correlation between the numbers of lice present and the severity of clinical signs (41). However, the lesions seen on all animals improved following treatment suggesting that ectoparasites were involved in the pathogenesis of the lesions even where no lice were detected on clinical examination (45). The similarity between the macroscopic appearance of the lesions in severe cases of *W. equi* in horses (39) and the lesions seen in IBH may be an indication that some horses are sensitized to the lice, and it is this which gives rise to enhanced clinical manifestations even in cases where few parasites are detected. The complementary argument that animals in poor physical condition often have high lice burdens, implying that severe infestations can be a consequence of immune suppression leading to lowered resistance, has also been made (9). Again no experimental evidence of immune suppression in support this conjecture has been described, indeed, there is no direct evidence from horse-based studies that either type of louse induces an adaptive immune response.

Nevertheless, both species of lice found on horses can induce severe pruritus; as discussed above, itching has an important role in host defence to ectoparasites, and the

involvement of the immune system in the pathogenesis of pruritus may be taken as indirect evidence of some form of inflammatory or immunological response to lice taking place. One might consider that the movement of lice over the skin surface could cause of itching through stimulation of low modulus mechanoreceptors, but as discussed above, light touch does not usually induce itching unless there has been presensitizing of skin by inflammatory mediators (46).

To provide some preliminary evidence of the nature of the inflammatory reaction to *W. equi*, skin biopsies from infested horses were examined histologically. Figure 1 shows biopsies stained with haematoxylin and eosin, those in Figure 2 are stained with toluidine blue to highlight mast cells, and the sections in Figure 3 are stained by immunohistochemistry for IgE (Figure 3). For each stain, panel a is a sample taken from the base of the mane of a control animal on which no ectoparasites were detected and no skin lesions were present. Panel b was also sampled from the base of the mane but taken from a horse in which numerous *W. equi* were present at the site, although no gross lesions were present. Panel c is taken from a

horse that was infested with *W. equi* and had areas of alopecia with exudation on the shoulder area from which the biopsy was collected. The H&E-stained sections show remarkably little evidence of inflammatory changes between a and b, and the changes seen in section c are limited to mild oedema and thinning of the stratum spinosum with loss of overlying keratin. The toluidine blue stain revealed numerous mast cells in the normal skin sample (Figure 1a) mainly concentrated around the blood vessels, although some diffuse metachromatic staining that did not appear to be intracellular was seen immediately below the epidermis. The samples from *W. equi*-infested horses did not have noticeable increases in the number of mast cells; indeed, there were fewer cells seen in section c. However, there did appear to be a change in the distribution of the mast cells to a position immediately below the epidermis in both louse infested samples (Figure 2b,c). Immunohistochemical staining for IgE (Figure 3) confirmed the impression that there was no overall increase in IgE positive cells but that they moved away from the blood vessels to a position immediately below the epidermis. Remarkably, there was an almost complete absence of eosinophils

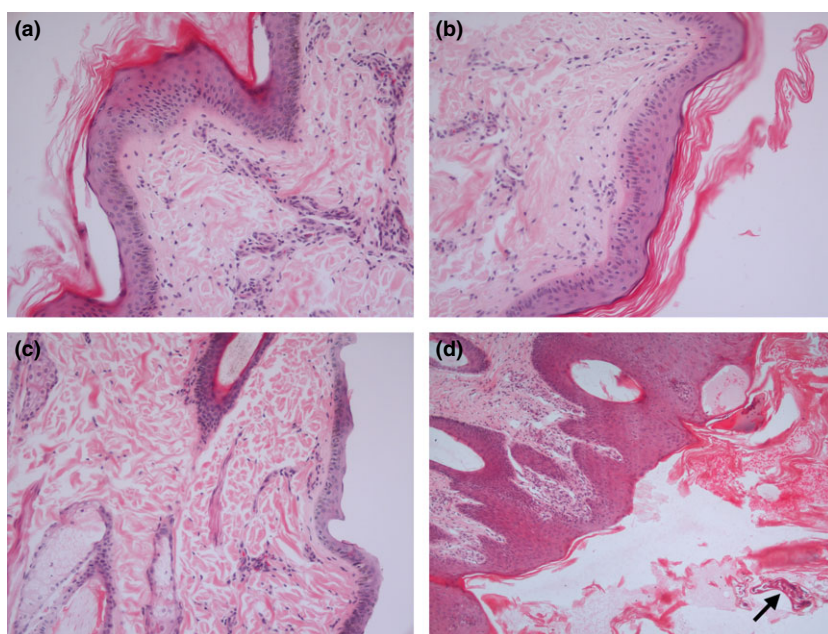


Figure 1 (a) H&E stained section ($\times 200$) of a skin sample taken from the base of the mane of a horse on which there were no macroscopic lesions and no parasites were detected. Modest numbers of cells are present in the dermis, mainly associated with blood vessels, or lying close to the epidermal boundary. (b) H&E section ($\times 200$) of skin taken from the base of the mane of a horse on which *Werneckia equi* were present at the site of the biopsy. There is little evidence of an inflammatory reaction other than a slight increase in cells close to the boundary between the dermis and epidermis. Notably no eosinophils are present. (c) H&E ($\times 200$) section of skin taken from an area showing focal alopecia and mild exudation on the shoulder of a horse on which *W. equi* were present. There is thinning of the epidermis with loss of overlying keratin, the dermis is slightly oedematous but there very few infiltrating inflammatory cells present. (d) H&E stained section ($\times 100$) of a biopsy from a horse with chronic Chorioptic mange. The remains of a mite are visible in the bottom right of the image indicated by the arrow. There is hyperplasia of the stratum spinosum with hyperkeratosis, haemorrhage and serum exudation. Increased cellularity of the underlying dermis due to infiltrating inflammatory cells is evident but surprisingly no eosinophils are present.

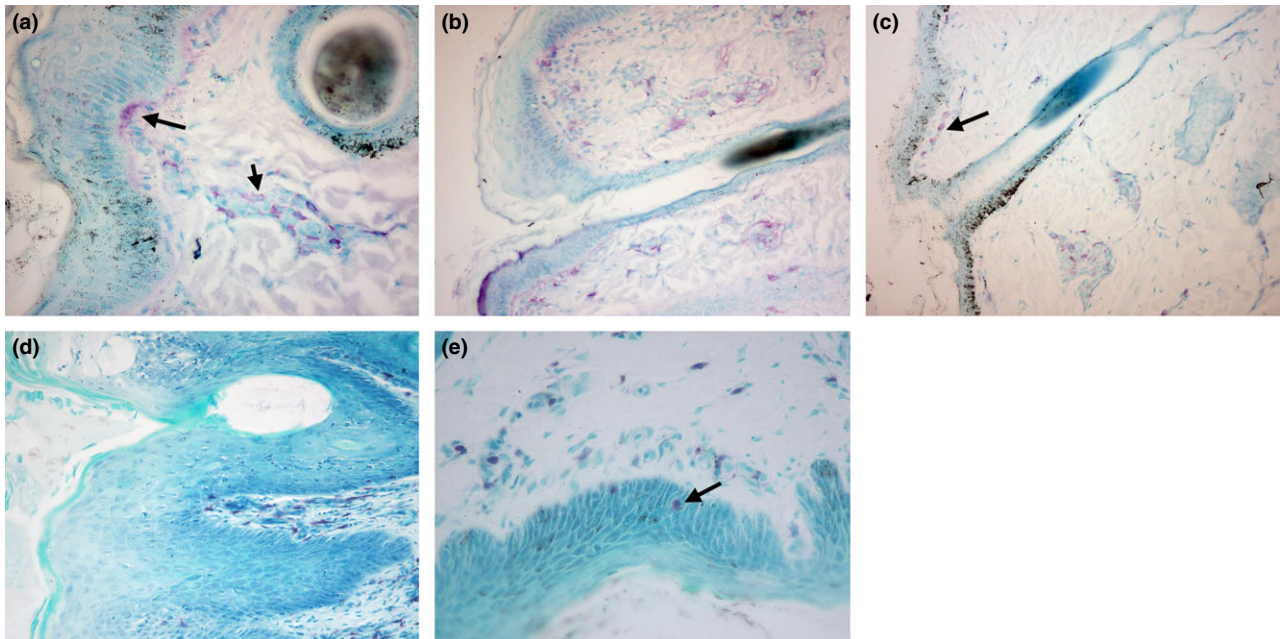


Figure 2 (a) Toluidine Blue stained section of a skin ($\times 200$) Sample taken from the base of the mane of a horse on which there were no macroscopic lesions and no parasites were detected. Metachromatic staining mast cells appear mainly around the blood vessels, some diffuse staining is also seen close to the boundary between the dermis and epidermis. (b) Toluidine Blue stained section of skin ($\times 200$). Sample taken from the base of the mane of a horse on which *Werneckiella equi* were present at the site of the biopsy. There is little difference in the numbers of mast cells compared to normal skin with polychromatic cells present close to the blood vessels, however the staining in the area adjacent to the epidermal boundary is more clearly cell associated. (c) Toluidine Blue stained section of skin ($\times 200$). Sample taken from an area of skin showing focal alopecia and mild exudation on the shoulder of a horse on which *W. equi* were present. Again there is a pattern of mast cell staining close to blood vessels and immediately below the epidermis but no obvious increase in cell numbers. (d) Toluidine Blue stained section of a skin biopsy from a horse with chronic Chorioptic mange ($\times 100$). Numerous mast cells are present in the cellular infiltrate of the dermis. (e) Toluidine Blue stained section of a skin biopsy from a horse with chronic Chorioptic mange ($\times 200$) Mast cells are also visible in the dermis providing evidence for an active inflammatory response in this condition.

in any of the samples. Eosinophils are an important characteristic of hypersensitive responses to *Culicoides* spp (47–50), and their absence in the few cases examined here suggests that there may not be an allergic inflammatory reaction to lice.

MANGE

Horses are affected by several species of mite which give rise to clinical signs of mange (3), but mange due to *Chorioptes bovis* is by far the most commonly reported (3, 51). Once again there have been no studies to determine the role of the immune system in the pathogenesis or resistance to *C. bovis* in horses. However, variations in clinical severity and response to treatment among individual horses within the same environment may be interpreted as indirect evidence for differences in host susceptibility and resistance. The well-documented susceptibility of heavy draft horses to *Chorioptic* mange is usually attributed to their feathered legs, but these same breeds have also been reported to have a high incidence of hypersensitivity to

Culicoides bites (52), which may indicate a more general breed predisposition to allergy in response to arthropod antigens. In humans, there is evidence to support immunity involving IgE as an important factor in self-cure of sarcoptic mange; yet at the same time, the pathogenesis of severe cases of crusted sarcoptic mange has been linked to an allergic response also involving IgE (53).

Figure 1(d) shows a skin biopsy from a case of chronic chorioptic mange. Compared with the control skin sample (Figure 1a), there is evidence of hyperplasia of the stratum spinosum with hyperkeratosis haemorrhage and increased cellularity of the underlying dermis. A cross section of a mite can be seen within the layer of exudate (shown by the arrow) confirming the diagnosis (Figure 1d). The cellular infiltrate in the hypodermis contains numerous mast cells shown by toluidine blue (Figure 2c,d). Once again the mast cells have moved away from the blood vessels to lie close to the epidermis, and several mast cells can be seen lying within the stratum spinosum Figure 2(d). IgE staining (Figure 3c,d) also demonstrated numerous positive cells raising the questions of whether some IgE

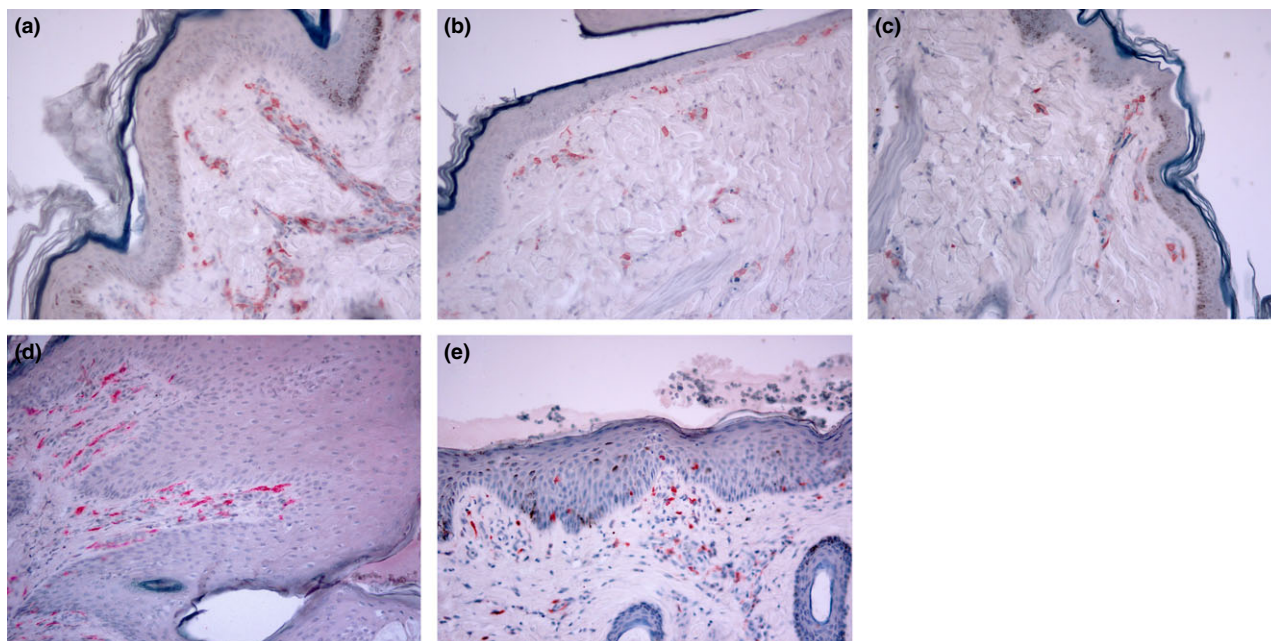


Figure 3 (a) IgE Immunoperoxidase stained section of a skin sample taken from the base of the mane of a horse on which there were no macroscopic lesions and no parasites were detected. ($\times 200$). Positive staining (red) cells are visible with a distribution similar to mast cells around the blood vessels. (b) IgE immunoperoxidase stained section of skin taken from the base of the mane of a horse on which *Werneckiella equi* were present at the site of the biopsy ($\times 200$). IgE positive cells are present close to the blood vessels, additional IgE positive cells can be seen lying immediately beneath the epidermis. (c) IgE immunoperoxidase stained section of skin taken from an area of skin showing focal alopecia and mild exudation on the shoulder of a horse on which *W. equi* were present ($\times 200$). IgE positive cells are present close to the blood vessels, additional IgE positive cells can be seen lying immediately beneath the epidermis. (d) IgE immunoperoxidase stained section of a skin biopsy from a horse with chronic Chorioptic mange ($\times 100$). Numerous IgE positive cells are present in the cellular infiltrate of the dermis. (e) IgE immunoperoxidase stained section of a skin biopsy from a horse with chronic Chorioptic mange ($\times 200$) IgE positive cells are also visible in the epidermis providing evidence for an active inflammatory response in this condition.

secreting plasma cells are present and if so what antigens the antibodies recognize. A similar migration of mast cells from connective tissue towards the epithelium of the caecum is a feature of the response of horses to the lumen-dwelling cestode *Anoplocephala perfoliata*, where there is a strong mucosal antibody response involving local production of *A. perfoliata* specific antibodies with IgE and IgG (T) antibody classes (54). The absence of eosinophils from the infiltrating leucocyte population in the skin sections from a case of chronic nonresolving *Chorioptic* mange is in agreement with the absence of eosinophils in chronic IBH lesions (50). However, it remains to be determined whether this is simply a reflection, the chronic nature of the lesion, or to what extent the absence of an eosinophil-mediated antiparasite response contributes to the prolongation of the infestation.

INSECT BITE HYPERSENSITIVITY

The bites of several insect species that feed on horses are commonly known to cause an acute circumscribed swelling

typical of type 1 hypersensitivity in some individual horses. Immediate type hypersensitivity reactions have also been experimentally reproduced by protein extracts of horse flies (*Tabanidae*), stable flies (*Stomoxys*) or mosquitoes (*Culicidae*) (55–57); but this is seldom regarded as more than a nuisance by horse owners, and as yet no follow-up immunological studies have been carried out on the nature of the equine immune response to these insects.

In contrast to the above examples, the immunology of IBH to *Culicoides* (midges) is not only well described in horses, but may well represent the best understood example of immunity to ectoparasites in any animal. The original description of this condition as an allergic response to the bites of *Culicoides* spp. was made 60 years ago in Australia by Riek (58, 59); the presence of a reagenic antibody specific for *Culicoides* antigens was subsequently demonstrated in serum of affected horses (60). During the ensuing decades, several studies in diverse continents replicated these findings and hypersensitivity of horses to *Culicoides* bites became recognized as one of the commonest skin conditions to affect horses worldwide (48, 52, 61–65).

The role of *Culicoides* as the source of the allergen causing this condition was further strengthened by the observations that IBH does not occur in Iceland where *Culicoides* are absent, although other biting insects such as *Simulium* spp. (black fly) do occur there in substantial numbers (61, 62, 66). Despite not being recognized as a disease in Iceland, the incidence of IBH is particularly high in Icelandic horses that are exported as adults to mainland Europe where they encounter *Culicoides* spp. for the first time. This reaction of the horses to their first encounter with *Culicoides* is, in many respects, similar to that originally described by Mellanby for mosquitoes in humans in that a high proportion of these horses (up to 30%) develop an immediate hypersensitivity response to intradermal challenge with *Culicoides* allergens (62, 67, 68). The analogy with mosquitoes in humans can be extended to include the observation that like people indigenous to areas of high mosquito challenge, Icelandic horses born on mainland Europe and exposed to *Culicoides* from their first year of age have a lower incidence of allergy at around 5%, which is similar to that seen in other horse breeds in mainland Europe (61, 63, 66). The reduced incidence of IBH in second-generation Icelandic horses occurs even in foals born to affected mothers who will have received *Culicoides*-specific IgE antibodies via colostrum, indicating that maternal priming by transferred antigen-specific IgE responses is not important in the aetiology IBH (69). Curiously, the incidence of IBH is lower in horses imported from Iceland during the summer, which are immediately exposed to *Culicoides*, compared to horses imported during the winter months when first exposure to *Culicoides* does not occur until the following spring (66). During this delay in exposure to *Culicoides*, there is a marked drop in total serum IgE accompanied by an increase in IL4 producing T cells but decreased numbers of IL10 and TGF- β producing regulatory T cells in peripheral blood (70, 71). One possible explanation for these changes would be that a reduction in endoparasite burden following anthelmintic treatment of imported horses has altered the balance of their immune response away from tolerance and towards the induction of hypersensitivity. The observation that environmental factors can reduce the incidence of allergic disease is similar to that posited by parts of the hygiene hypothesis in human allergic disease (72). Notwithstanding these observations which strongly support environmental factors as important in the aetiology of IBH, several studies have also demonstrated genetic factors related to the development of IBH (73–77). The extent to which genetic predisposition can be overridden by immunological or environmental factors is important; as this will have a bearing on the efficacy of future therapeutic measures.

SKIN RESPONSES TO *CULICOIDES* SALIVARY ANTIGENS

The demonstration of reagenic antibodies by intradermal skin testing with *Culicoides* extracts has been widely used to diagnose IBH, but the results have been rather variable with both false positive and false negative results reported (55, 56, 67, 68, 78–80). Factors such as the use of non-native midge species containing antigens to which the horses had not been exposed or degeneration of antigens in storage have been suggested as causes for false negative results (68, 79). Conversely, false positive results may represent innate inflammatory responses to factors present in *Culicoides* extracts (56, 57, 80) such as proteases that could directly trigger mast cell degranulation by activating PAR receptors (32). Alternatively, false positive reactions could be caused by cross-reactive carbohydrate antigens which are known to cause false positive results in human intradermal testing (81) or possibly the presence of Chitin derived from arthropod exoskeleton within the allergen extracts, which in large molecular weight form can have pro-allergenic inflammatory effects (82, 83).

IGE ANTIBODIES

Studies to formally confirm a central role for IgE antibodies in the pathogenesis of IBH had to await the development of first polyclonal and then monoclonal antibodies specific for equine IgE. The results showed a high association between the presence of IgE antibodies to salivary gland proteins and clinical signs of IBH (84–87). An example of this specificity is shown by immunohistochemistry in Figure 4, demonstrating the presence of IgE in serum from three horses with IBH binding specifically to the salivary glands of *Culicoides* spp. *Obsoletus* group Figure 4(a–c), but no IgE binding is detected control serum from a nonallergic horse Figure 4(d).

MAJOR ALLERGENS

The identity of the proteins in *Culicoides* saliva, to which horses with IBH react, has been investigated using a number of approaches. Initially, two-dimensional gel electrophoresis coupled to mass spectrometry was used to identify the major proteins present in saliva of *Culicoides nubeculosus*; from which several IgE reactive proteins were identified by Western blots using serum from IBH-affected horses (86, 88, 89). Secondly, screening a *C. nubeculosus* phage display library with allergic serum identified several cDNA sequences from which recombinant proteins were expressed (90). Encouragingly, these separate methods identified several of the same antigens using allergic horse

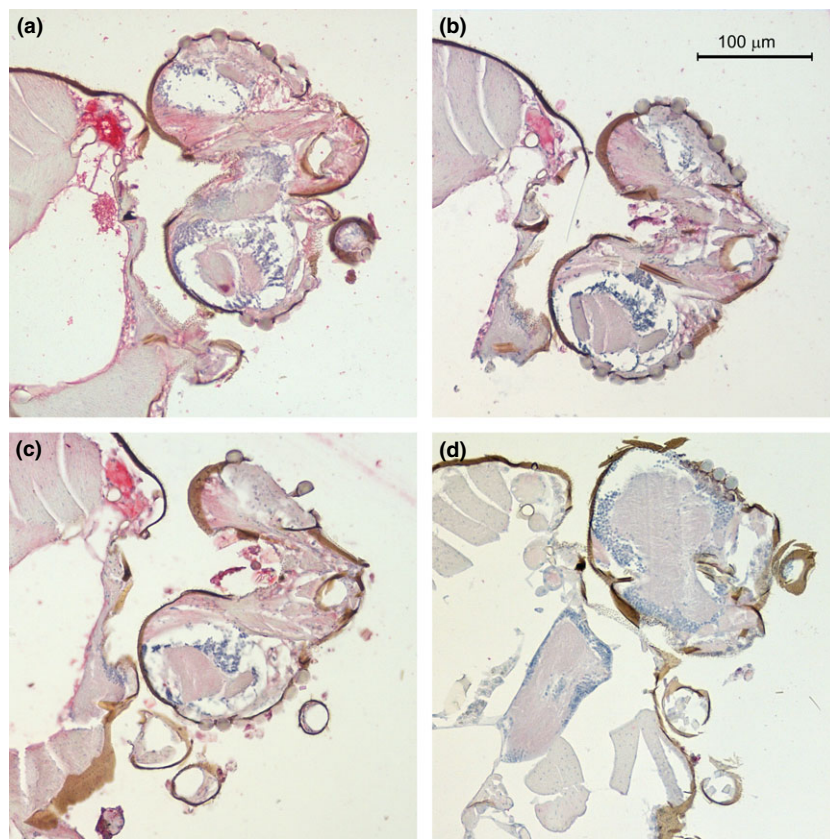


Figure 4 Detection of IgE antibodies to *Culicoides* salivary gland antigens by immunohistochemistry. Panels a, b and c Sections of wild caught *Culicoides obsoletus* group midges stained with serum from horses with insect bite hypersensitivity. Binding of IgE is shown by the red colour of the salivary glands in the upper thorax of the midges, panel d shows a control section stained with serum from a healthy horse that had been exposed to *Culicoides*. In which no IgE is detected.

serum from different parts of Europe indicating that they were major allergens. However, *C. nubeculosus* is not the most abundant species of *Culicoides* found biting horses (91, 92), and higher levels of IgE antibody are detected when extracts from *Culicoides obsoletus* are used in diagnostic testing (86, 93). This finding has been confirmed using expressed recombinant *C. obsoletus* protein antigens in an IgE specific ELISA (94, 95).

The combined studies of recombinant proteins have identified several conserved antigens in *Culicoides* saliva belonging to families of abundant insect salivary proteins such as maltase, hyaluronidase, antigen-5, D7 or Kunitz protease inhibitors (86, 89, 94, 96). Despite this progress in identifying the major IBH allergens, significant gaps remain; in particular, several *C. obsoletus* proteins of around 40kD in mass, which react with IgE in allergic horse serum, have not been identified (95), and as yet no homologues of the major *C. nubeculosus* antigens of the CNSG60, CNSG79, Cul n 3,5&10 family (88, 90) have been found in *C. obsoletus* libraries although several

candidate proteins of about this mass are present as have several *C. obsoletus* and *Culicoides pulicaris* salivary gland cDNA sequences encoding novel secretory proteins (A. D. Wilson, unpublished).

IGG ANTIBODIES

In addition to IgE antibodies, IgG antibodies to *Culicoides* salivary gland antigens are also detected, not only in the serum from IBH-affected horses but also in all horses exposed to *Culicoides* spp (84, 85, 87). The absence of anti-*Culicoides* antibodies of either isotype in the serum of native Icelandic horses that have never been exposed to *Culicoides* confirms the antigenic specificity of the response (87). This latter observation also provided the first indication that, rather than not reacting to *Culicoides* antigens, the healthy horses had developed a tolerant immune response.

All mammals studied so far have a single copy IgE gene (97), but vary in the number of their IgG genes. The

duck-billed platypus has two IgG subclasses which may represent the archetypal genotype of mammals (98), subsequently, the evolution of multiple IgG subclasses occurred separately within different mammal lineages, and consequently there are no direct homologues between IgG subclasses in different unrelated species. Within horses, four IgG subclasses were originally identified by conventional immunochemistry techniques termed IgGa, IgGb, IgGc and IgG(T) (99, 100). However, the full genome sequence has revealed there are seven equine IgG subclasses (101), these relate to the original classification as shown in Figure 5.

The subclasses of IgG antibodies to *Culicoides nubeculosus* salivary antigens detected in Western blots with monoclonal reagents recognizing IgG fractions as defined using the old classification (100) were biased towards an abundance of IgG(T), but IgGa and to a lesser extent IgGb were also present (85). IgG(T) and IgE are also prominent features of the antiparasite response of horses (54, 102–104), indicating IgG(T) class switching is favoured by Th2 responses. In support of this, IgG(T) was first identified as the antigen-binding component in the serum of horses hyperimmunized with tetanus toxoid and alum (105), an adjuvant that is known to stimulate Th2 cells in humans and mice (106). Moreover, IgG(T) is also the dominant sub-class in horses vaccinated with influenza using alum (107). Set against this, immune responses in horses to natural infection with influenza are dominated by IgGa (1) and IgGb (4/7) sub-class (107), which were also the predominant sub-class following influenza vaccination with a Th1 promoting ISCOM adjuvant (108). Taken together, the evidence supports the conclusion that IgG 2/3/5(T) probably represent a Th2 sub-class in the horse comparable to IgG1 in mouse or IgG4 in humans (109, 110).

In humans, IgG4 is strongly associated with allergies, where an increased titre of IgG4 is regarded as an indication of successful desensitisation therapy (111). The

protective function of human IgG4 is attributed partly to its ability to block allergen binding to IgE and partly to its ability to bind to low-affinity Fc γ RIIB1 (CD32) receptor on the surface of mast cells. On antigen stimulation, this transduces an inhibitory signal via an ITIM motif that increases the threshold of mast cell degranulation (112). This has in turn been related to poor interaction with Fc γ RI (CD64) on neutrophils macrophages or eosinophils and an absence of complement activation by human IgG4, which also has a more acidic pI than other IgG sub-classes (111). However, these characteristics are only partly shared by the equine sub-classes (3, 5 $\pm$ 2) that collectively constitute IgG(T) (113). Moreover, none of the IgG(T) isotypes of horses show the weak interchain bonds that permit the formation of hetero-specific antibodies described for human IgG4 (111), although this does appear to be a feature of some equine IgG4&7 molecules (113).

In horses, early studies using subcutaneous injection with an anti-IgG(T) monoclonal antibody that cross-reacted with both IgG 3/5 sub-classes were shown to cause an immediate weal response in a proportion of horses, implying that IgG(T) may play an active role in the antigen-specific degranulation of equine mast cells (84). However, later work using novel monoclonal antibodies specific for either equine IgG3 or IgG5 failed to replicate these results (80). This dichotomous finding is similar to that in rodents where polyclonal IgG antibody–antigen complexes will trigger mast cell degranulation but monomeric monoclonal antibody and antigen do not (112).

Despite the clinical relevance of elevated human IgG4 in monitoring the response to immunotherapy, more recent discussion has attributed the clinical improvement to changes in cytokine secretion by tolerogenic T cells which coincidentally provide the cytokine microenvironment necessary for suppression of IgE and elevation of IgG4 (114, 115); and at present, the exact role of equine IgG2, IgG3 and IgG5 in the pathogenesis or protection from developing IBH remains to be established.

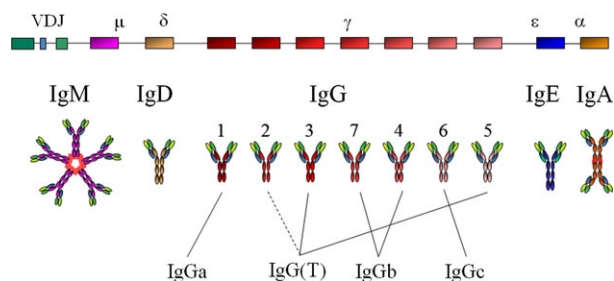


Figure 5 The immunoglobulins of the horse; showing the genomic arrangement of the heavy chain genes, the five antibody classes and seven IgG subclasses. The numbers represent the new classification of equine IgGs 1–7; their relationship to the old classification IgGa IgGb IgGc IgG(T) is depicted below.

CELLULAR IMMUNE RESPONSES IN IBH

Initial studies of the immunopathology of IBH lesions in horses reported elevated numbers of skin CD4⁺ T cells, Langerhans cells and eosinophils (47–49). Insect bite hypersensitivity-associated lesions also have higher numbers of IgE positive cells including mast cells and a population IgE mRNA expressing cells that indicate the involvement of local IgE synthesizing plasma cells (50). The increased total numbers of T cells are associated with a reduction in the proportion of CD4⁺ FoxP3⁺ regulatory cells, and the transcription patterns within skin lesions

show reduced FoxP3 and IL10 expression with increased IL13 expression, but no elevation of IL4 transcription. Significantly, no increase in IL5 transcription was detected despite the earlier evidence for increased numbers of eosinophils (116). A similar study compared transcription between samples from affected and healthy ponies taken in winter with lesional nonlesional and healthy skin samples taken during IBH season (117); the most striking result from this study was the finding of increased cytokine transcription in the skin of all horses in summer compared to winter irrespective of whether they showed signs of IBH. In both studies, there were increased numbers of skin infiltrating T cells, and cytokine transcription was not limited to Th2 cytokines with levels of Ifn transcription also detected. The ill-defined profile of skin cytokine transcription contrasts with the pattern of elevated Th2 and reduced regulatory transcription seen in antigen-specific *in-vitro* studies (*vide infra*) and may reflect skin production of cytokines from non T cells or a more generalized inflammation associated with reduced skin barrier function during the summer months that affects all horses subjected to a myriad of insect bites.

The importance of T-helper 2 cells in promoting IgE antibody class switching via production on IL-4 and IL13; and the modulation of T-helper 2 function and IgE production through the actions of regulatory cells expressing TGF-beta and IL10 have become central to understanding immunology of allergic diseases (118).

In horses with IBH, peripheral blood lymphocytes (PBL) have greater numbers of IL4 positive cells following activation by *Culicoides* antigens compared to those from healthy horses indicating a role for T-helper 2 cells (119). Further experiments demonstrated how the addition of culture supernatant from *Culicoides* antigen-activated PBL from healthy horses downregulated IL4 production by PBL from horses with IBH. Moreover, adding recombi-

nant IL10 and TGF-beta together suppressed IL4 production in cultures of PBL from horses with IBH. Conversely, the addition of neutralizing antibodies to IL10 and TGF-beta to antigen stimulated cultures of PBL from healthy horses caused an elevation in the numbers of IL4 producing T cells (120). Subsequently, higher numbers of FoxP3 CD4+ CD25+ cells were detected in *Culicoides* antigen stimulated PBL from healthy horses compared to cultures of cells from horses with IBH (121), and induced CD4+ CD25 hi T cells from healthy showed increased suppression of CD4+ T-cell responses to *Culicoides* antigens (122). Together, these results show that the key difference between healthy and IBH-affected horses is the presence of regulatory T cells, which act to limit the nascent T-helper 2 response stimulated by *Culicoides* saliva.

IMMUNOTHERAPY

Despite the long history and growing evidence confirming that IBH is a type I IgE-mediated hypersensitivity to *Culicoides*, there are as yet no effective treatments for the condition. To date, none of the attempts at allergen specific immunotherapy for IBH have been successful (123–125), although as all of these studies used a crude non-standardized extract of whole midges this is unsurprising. If allergen immunotherapy is to be successful, current scientific knowledge would indicate that it will have to stimulate regulatory T cells specific for the allergens in midge saliva. This in turn awaits the large scale production of recombinant allergens. Nevertheless, researchers should be encouraged by the progress made so far in identifying and expressing allergens from the most relevant *Culicoides* spp. and the recent demonstration that *Culicoides* salivary antigens can be used to induce T-regs in an *in vitro* model of equine IBH (122).

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