



Production of monoclonal antibodies specific for native equine IgE and their application to monitor total serum IgE responses in Icelandic and non-Icelandic horses with insect bite dermal hypersensitivity

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Abstract

Immunoglobulin E forms a minor component of serum antibody in mammals. In tissues IgE is bound by FcεRI receptors on the surface of mast cells and mediates their release of inflammatory substances in response to antigen. IgE and mast cells have a central role in immunity to parasites and the pathogenesis of allergic diseases in horses and other mammals. This paper describes the production of several novel monoclonal antibodies that detect native equine IgE in immunohistology, ELISA and Western blotting. An antigen capture ELISA to quantify equine IgE in serum has been developed using two of these antibodies. The mean serum IgE concentration of a group of 122 adult horses was 23,523 ng/ml with a range of 425–82,610 ng/ml. Total serum IgE of healthy horses was compared with that of horses with insect bite dermal hypersensitivity (IBDH) an allergic reaction to the bites of blood feeding insects of *Culicoides* or *Simulium* spp. IBDH does not occur in Iceland where *Culicoides* spp. are absent, but following importation into mainland Europe native Icelandic horses have an exceptionally high incidence of this condition. In the present study Icelandic horses with IBDH had significantly higher total IgE than healthy Icelandic horse controls ($P < 0.05$). By contrast in horses of other breeds the difference in total serum IgE between those affected with IBDH and healthy controls was not statistically significant. Total serum IgE was also monitored in a cohort of Icelandic horses prior to import into Switzerland and for a period of 3 years thereafter. High levels of serum IgE were present in all horses at the start of the study but dropped in the first year after import. Thereafter the total serum IgE remained low in Icelandic horses that remained healthy but

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rose significantly ($P < 0.05$) in those that developed IBDH. These results support the conclusion that IBDH is a type I hypersensitivity response to insect allergens but indicate that IBDH in Icelandic horses may have a different pathogenesis from the same condition in other breeds.

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1. Introduction

Although IgE is only a minor component of serum immunoglobulin in humans and laboratory animals it has a powerful pro-inflammatory effect through its role as the antigen specific trigger of mast cell degranulation (Gould et al., 2003). Mast cell degranulation mediated by cross linking of IgE bound to cell surface FcεRI has a well-established role in anti-parasite immunity (Ahmad et al., 1991; Webster et al., 1996) and in the pathogenesis of acute allergic responses to a wide range of environmental antigens (Mediaty and Neuber, 2005; Mehl et al., 2005; Wiche et al., 2005). IgE also has antigen independent effects on mast cells. Firstly, monomeric IgE FcεRI engagement acts as a stimulus for the development and subsequent survival of mast cells (Asai et al., 2001). In addition, IgE binding stabilises and increases the expression of FcεRI, which effectively decreases the threshold of antigen concentration required to trigger degranulation (Yamaguchi et al., 1999). The cytokines released following mast cell degranulation in turn promote antibody class switching of B-cells to IgE (Pawankar et al., 1997) and favour differentiation of Th2 cells that enhanced IgE responses (Kawakami and Galli, 2002). Serum IgE can also enhance antigen presentation of allergens to T-helper cells by either FcεRI bearing dendritic cells, or FcεRII B-cells (Stingl and Maurer, 1997; van der Heijden et al., 1995). Together these effects form a positive feed back mechanism for the maintenance of IgE-mediated immune responses.

In common with other grazing animals, horses often have a heavy burden of intestinal parasites maintained by oral-faecal transmission. Horses are also affected by numbers of arthropod borne nematodes and by arthropod parasites themselves (Urquhart, 1996), all of which may stimulate IgE and mast cell mediated immune responses. In addition, allergic responses to biting insects (van der Haegen et al., 2001; Wilson et al., 2001) and to fungal antigens

(Schmallenbach et al., 1998) are important causes of disease in domesticated horses and there is increasing evidence that IgE mediated inflammatory responses are a key part of the pathogenesis of these conditions.

IBDH is an allergic dermatosis frequently seen in horses, which is caused by bites of insects, mainly *Culicoides* and *Simulium* spp. (Brostrom et al., 1987; Halldorsottir and Larsen, 1991). The allergens for IBDH, probably insect salivary gland (SG) proteins, have not yet been identified. IBDH in Icelandic horses is of particular interest as the condition does not occur in horses born and living in Iceland where *Culicoides* spp. are absent. However, following importation to continental Europe, and consequent exposure to *Culicoides* bites, up to 50% of adult Icelandic horses develop IBDH (Marti et al., 2003). In contrast, only 6–10% of the Icelandic horses born on the continent from imported parents suffer from this disease (Brostrom et al., 1987; Halldorsottir and Larsen, 1991).

In the past, research into these diseases has been hampered by a lack of widely available reagents for the detection of IgE. The problem of IgE detection in horses has now been solved by several groups each using different approaches. Elevation of local IgE in the respiratory tract of horses with respiratory disease has been demonstrated using polyclonal sera to native IgE (Halliwell et al., 1993). Chicken antibodies to a recombinant IgE heavy chain fragment have been used to demonstrate the presence of IgE antibodies, specific for *Culicoides* salivary gland antigens, in horses affected by IBDH (Wilson et al., 2001) and to fungal allergens in horses with recurrent airway obstruction (Eder et al., 2000). Others have used synthetic peptide antigens to generate polyclonal sera that detect native IgE to a range of insect and environmental antigens (Kalina et al., 2003). Most recently several monoclonal antibodies to a chimeric equine IgE Fc coupled to mouse V-region and light chains have been described (Wagner et al., 2003). In addition to these antibody reagents both the high affinity and low

affinity equine IgE Fc receptors have now been cloned (McAleese et al., 2000; McAleese and Miller, 2003; Watson et al., 2000). Here we describe another set of monoclonal antibodies raised against recombinant proteins and partially purified serum IgE that specifically recognise native equine IgE, and their use in quantifying serum IgE in healthy horses and horses affected by IBDH.

2. Materials and methods

2.1. Cloning and expression of IgE constant region products

DNA encoding constant regions C ϵ 1–4 of the equine IgE gene was PCR-amplified from mesenteric lymph node derived cDNA (Marti et al., 1997), using primers based on the published sequence (Navarro et al., 1995), which included *Eco*R I and *Hind* III restriction enzyme sites. Purified PCR product was ligated into pGEM-T easy vector (Promega, <http://www.promega.com>), then transfected into *E. coli* strain XL1-blue MRF' (Stratagene, <http://www.stratagene.com>). Plasmid was purified from selected colonies using the QIA miniprep system (Qiagen, <http://www.qiagen.com>) and the identity of the insert was confirmed by automated sequencing.

The C ϵ 1–4 encoding cDNA was recovered by an *Eco*R I/*Hind* III restriction digest and ligated into pcDNA3.1/V5-HisB vector (Invitrogen, <http://www.invitrogen.com>) in frame with the C-terminal His tag. The ligation products were transformed into *E. coli* strain XL1-blue MRF'. Plasmid DNA for eukaryotic transfections was retrieved using the HiSpeed plasmid maxi kit (Qiagen).

Cos7 cells were expanded and maintained in DMEM, containing 10% foetal calf serum (FCS), 50 U/ml penicillin, 50 μ g/ml streptomycin and 2 mM glutamine. For transfection 1.2×10^5 Cos7 cells were transferred into each well of a six-well plate (Costar, <http://www.corning.com>) in 2 ml of complete media and incubated overnight to give 70% confluent cultures. Cells were incubated with 1 μ g of pcDNA3.1bIgE in 1 ml serum free DMEM containing 6 μ L lipofectamine (Invitrogen) at 37 °C in 5% CO $_2$. After 4 h 1 ml of complete medium was added to each well, at 72 h G418 (Invitrogen) was added to a final

concentration of 500 μ g/ml. Once confluent, the transfected cells were transferred to 75 cm flasks (Costar) and expanded in complete medium containing 500 μ g/ml G418.

Cells from seven 75 cm flasks were harvested, washed with PBS then lysed by three cycles of freeze thawing in 10 ml of lysis buffer containing 300 mM NaCl, 50 mM NaH $_2$ PO $_4$, 0.05% Tween, pH 8 and 10 mM imidazole to which was added 100 μ L protease inhibitor cocktail (Sigma, <http://www.sigma-aldrich.com>). The lysate was centrifuged at $2000 \times g$ for 20 min and the supernatant incubated on ice for 1 h with 200 μ L of NiNTA agarose (Qiagen). Following incubation, the sample was spun at $500 \times g$ for 5 min and the supernatant removed. The gel was washed twice by suspension and centrifugation with 10 ml of lysis buffer containing 20 mM imidazole. After the second wash, the NiNTA agarose was suspended in a minimal volume of buffer and loaded onto a micro-spin column and dried by briefly spinning in a microfuge. The bound protein was eluted by the addition of 200 μ L of buffer containing 250 mM imidazole followed by a short spin in a microfuge. The elution was then repeated and the eluted fractions pooled.

2.2. Purification of native equine IgE

Initial partial purification of equine IgE was carried out as follows. Ten millilitres of serum from a pony with insect dermal hypersensitivity was mixed with 10 ml saturated ammonium sulphate and spun at $2000 \times g$ for 15 min. Precipitated proteins were re-dissolved in 5 ml PBS, 100 μ L aliquots were separated by gel filtration on an HR 10/50 Superdex 200 column in PBS 1% azide at a flow rate of 0.5 ml min $^{-1}$ and 0.5 ml fractions collected as described previously (Wilson et al., 2001). The IgE content of the fractions was analysed by Western blotting. Fifty millilitres of pooled fraction 10 (which contained the highest IgE content) was dialysed into 0.5 M NaPO $_4$ buffer pH 8 then passed over a protein G superpose column (Sigma) to remove contaminating IgG. To confirm the presence of IgE and the suitability of this preparation for use in screening a monoclonal antibody fusion the preparation was tested by ELISA using chicken anti equine IgE (Marti et al., 1997).

After obtaining high titre anti-IgE monoclonals from the first fusion, IgE was purified directly from

serum using an affinity column made by binding anti-IgE monoclonal antibody (4C2) to protein G superpose and cross linking the antibody with dimethyl pimelimidate HCl (DMP Pierce Perbio, <http://www.perbio.com>). IgE was bound from serum passed over the column then eluted with 0.2 M glycine buffer pH 2.5. The purity and concentration of IgE in this preparation was assayed using an Experion automated electrophoresis apparatus (Bio-Rad, <http://www.bio-rad.com>).

2.3. Western blotting

Proteins were separated in 8% polyacrylamide gels pH 8.8 with a 3% pH 6.8 stacking gel. The gels were run in 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.7 buffer, at 120 V for 1 h on a mini protean II apparatus and transferred onto PVDF membrane using a semi-dry blotter at 10 V for 30 min (Bio-Rad).

The membranes were blocked with a solution of 5% milk protein in 10 mM Tris, 150 mM NaCl, pH 7.4 (TBS), then incubated overnight with, either goat anti equine IgG, IgA and IgM (Nordic, <http://www.nordiclabs.nl>) or chicken anti-equine IgE (Marti et al., 1997) primary antibody in blocking buffer, washed in several changes of TBS 0.5% Tween, and incubated with an appropriate secondary alkaline phosphatase conjugate (Sigma) in TBS Tween for 1 h. Following three further washes in TBS Tween and rinsing in water the blots were developed with NBC/BCIP substrate (Vector Laboratories, <http://www.vector-labs.com>) in 10 mM Tris pH 9.5.

2.4. Monoclonal antibody production

Balb-C mice were immunised using a intra-peritoneal priming dose of 5 µg bacterial equine IgE Cε3-4 (Marti et al., 1997) in 100 µl RIBI adjuvant (Sigma), followed on day 10 by 10 µg of purified protein from pcDNA3.1bIgEε1-4 transfected Cos7 cells in 100 µl RIBI adjuvant ip. Five days prior to fusion mice were given a final boost with 100 µl RIBI adjuvant and 10 µg of partially purified native equine IgE or 5 µg affinity purified IgE in RIBI adjuvant. Spleen cells were fused with P3X myeloma cells using standard methods and selected in HAT media (Hudson et al., 2002). Once visible colonies of cells had become established, 50 µl of supernatant from each well was

screened for antibody production using ELISA plates coated with semi-purified equine IgE. Positive wells with O.D. greater than three times background were cloned at limiting dilution in 96 well plates. Wells still positive after two rounds of cloning were expanded for further analysis.

2.5. Immunofluorescence microscopy

Samples of equine tissue were collected immediately post-mortem then embedded in OTC compound and snap frozen in a bath of isopentane cooled on dry ice. Six micrometer thick cryostat sections were cut, placed on glass slides and air dried before fixing for 10 min in 50% acetone 50% methanol at 4 °C. Sections were re-hydrated in PBS, blocked by incubation with 1% BSA in PBS then stained with a 1:20 dilution of monoclonal antibody supernatant, followed by 1:400 dilution of goat anti-mouse IgG FITC conjugate (Sigma). The slides were mounted in Vectashield (Vector) then viewed by Leica DM RA2 fluorescent microscope. Images were captured using a Leica DC350RX camera and Leica QFluoro software (Leica microsystems, <http://www.leica-microsystems.com>).

2.6. In vitro and in vivo reversed anaphylaxis

The ability of the anti-equine IgE mAbs to induce sulfidoleukotriene (sLT) release from peripheral blood leukocytes in vitro was tested in the Cellular Antigen Stimulation Test (equine CAST[®], Bühlmann Laboratories, 4123 Allschwil, Switzerland). The CAST[®] was performed according to the manufacturers' instruction as previously described (Marti et al., 1999). Briefly, peripheral blood leukocytes (PBL) from citrate plasma were pelleted by centrifugation and re-suspended in stimulation buffer. Re-suspended PBL were incubated for 40 min at 37 °C with 0.35 µg/ml anti-equine IgE mAb (7H2, 3H10, 5A2 and 1C12) or 20 µg/ml Con-A as a positive control. As negative control purified anti-dog IgG1 mAb was used at the same concentration, buffer alone was used to determine spontaneous sLT release from PBL. The samples were then spun at 1000 × g and the sLT concentration measured by CAST[®] ELISA following the manufacturer's instructions. Additionally, the background OD of the monoclonal antibody preparations were determined in the CAST[®] ELISA.

In vivo reversed anaphylaxis was measured using the same antibody preparations. Briefly, hair was clipped from the flat surface of the shoulder and 100 μ l of a 3.5 μ g/ml solution of antibody was injected intradermally. One hundred microlitres of saline or 100 μ l of saline containing 0.17 mg/ml histamine dihydrochloride (Allergomed AG, Therwil, Switzerland) were injected as negative and positive controls. The diameter of the wheal was measured and the thickness of the skin evaluated at the injection sites after 1 and 4 h.

2.7. Preparation and biotinylation of purified anti-equine IgE

Three hybridoma clones (5A2, 3H10 and 1C12) were selected for expansion and antibody purification. Bulk cultures of hybridomas were made using “Cell Line” culture vessels (Integra Biosciences AG, <http://www.integra-biosciences.com>). Fifty millilitres of high titre supernatant from each hybridoma was dialysed against 0.1 M phosphate buffer pH 8.0, then clarified by centrifugation at $10,000 \times g$ for 20 min and passed through a 0.22 μ m filter. The supernatant was passed through a 1 ml column of protein G superose (Sigma), and bound antibody subsequently eluted with 0.2 M glycine buffer pH 2.5. The eluted antibody was immediately neutralised with 1 M Tris buffer then dialysed against PBS. Protein concentration was determined by micro Bradford assay (Pierce Perbio). Purified antibody was stored frozen in aliquots at -20°C . Five milligram of purified antibody in 1 ml volume was labelled via carbohydrate groups using toBiotin-LC-hydrazide according to the manufacturers protocol (Pierce Perbio).

2.8. Antigen capture ELISA and direct ELISA

Ninety-six well high binding polystyrene plates (Greiner Bio-One AG, <http://www.gbo.com>) were coated by overnight incubation at 4°C with 1 μ g/ml monoclonal anti-IgE 1C12 in 0.05 M carbonate buffer pH 9.6. The plates were washed with PBS 0.05% Tween pH 7.4 then blocked by incubation with 100 μ l PBS Tween 0.5% BSA at RT. Dilutions of standard or test samples were made in blocking buffer and incubated at RT for 2 h. Following washing in PBS

Tween, secondary-biotin conjugate anti-equine IgE mAb 3H10 (in some assays biotin conjugate mAb 5A2) was added and incubated for a further 2 h. Following a further wash, 100 μ l of a 1:10,000 dilution extra-avidin alkaline phosphatase was added for 30 min (Sigma). After a final wash the ELISA was developed with 1 mg/ml *p*-nitrophenyl phosphate dissolved in 0.5 M carbonate buffer pH 9.6. Plates were read when the OD 450 nm of the highest concentration of the standard curve reached 1. Duplicate standard samples of six doubling dilutions of affinity purified IgE from 100 to 3.125 ng/ml were used. The OD of the test samples were interpolated from the standard curve to give their IgE concentration in ng/ml and multiplied by the dilution factor. The concentration of the original test samples was calculated as the average of the values from those dilutions where the concentration fell within the standard curve.

Direct ELISA for the screening of the monoclonal fusion was carried out in a similar manner using partially purified equine IgE coated plates and donkey anti-mouse alkaline phosphatase conjugate (Jackson Immunolabs, <http://www.jacksonimmuno.com>). IgA and IgM antigens were a gift from D. Patel University of Bristol, School of Clinical Veterinary Science, IgG was purified by protein G chromatography. As positive controls monoclonal anti-pan equine IgG clone K1271B, monoclonal anti-equine IgA clone K1292G3 (gift from Dr. K. Haverson, University of Bristol, School of Clinical Veterinary Science) in conjunction with donkey anti mouse alkaline phosphatase, or polyclonal rabbit anti-equine IgM (Nordic) and donkey anti-rabbit alkaline phosphatase conjugate (Jackson) were used.

2.9. Assay of total IgE in normal horses and horses with IBDH

Two separate groups of horses were studied. The first group contained 122 animals blood sampled during the IBDH season (April–October) between 2001 and 2004. The horses were from different stables in Switzerland. Healthy control horses were selected randomly from the same stables where IBDH-affected horses were living. These 122 horses consisted of 65 males (mainly geldings) and 57 females, with a mean

age of 12 years (range 3–22 years). This first group comprised 73 Icelandic horses born in Iceland and imported to Switzerland (31 healthy horses and 42 horses with IBDH), 24 Icelandic horses born in Switzerland (17 healthy and 7 horses with IBDH) and 25 horses (12 healthy and 13 IBDH) belonging to various other breeds, including Shetland ponies, Arabians, Franches-Montagnes and Warmblood horses. In this first group of horses, the imported Icelandic horses had been living in Switzerland for at least 4 years.

A second group of samples were taken from a cohort of 31 horses (18 males and 13 females) prior to their export from Iceland in 2001–2002, then annually for 3 years following their import into Switzerland. During this time their health and the development of IBDH was monitored.

The diagnosis was based on the clinical signs and on the history: IBDH affected horses had pruritus, excoriations and thickening of the skin along the dorsal midline, localised mainly at the base of the mane and tail. Sometimes the ventral midline and/or the head were also affected. Furthermore, the history of pruritus was seasonal and recurrent, i.e. occurring between April and October and followed by a period of remission in winter.

The healthy control horses had no previous history of skin problems and showed no clinical signs of pruritus or cutaneous lesions at the time of sampling.

2.10. Statistical analyses

Statistical analyses were carried out using the software package Statview version 5.01 (SAS Institute Inc.).

The data from first group of 122 horses were analysed by factorial ANOVA using total IgE as the dependant variable and breed, sex, diagnosis as factors. The data from the 30 imported horses were analysed using repeated measures ANOVA, with total IgE as the dependant variable, samples from the same horse were classified as repeated measures, with diagnosis and time-point as factors. Unplanned comparisons between individual means were made using the Tukey/Kramer statistic for groups of unequal samples size at an experiment wise significance level of 5%.

3. Results

3.1. Expression and purification of Cε1-4 from Cos cells and native equine IgE from serum

A 1250 bp PCR product encoding equine Cε1-4 was sub-cloned into the pGEM-T easy vector, excised then ligated into pcDNA3.1/V5-His B. The sequence had 100% homology to the published sequence Cε^a (Wagner et al., 2001). Fig. 1 shows the Western blot analysis of 40 µl Cos cell lysate from transfected and control cell lines using a chicken anti-equine IgEε3-4 specific antibody. A band with an apparent molecular weight of 50 kD is visible in the transfected but not the control lines corresponding to the predicted molecular weight of the recombinant protein including its V-5 and 6-His tags. This same band was visible in 1 µl of eluate from the Ni-agarose affinity chromatography column. The eluate was subsequently used to immunise mice for monoclonal antibody production.

Fig. 2 shows the immunoglobulin content of a partially purified native equine IgE fraction from

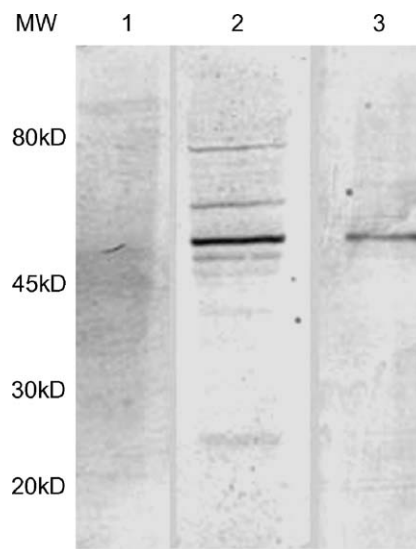


Fig. 1. Western blot of recombinant equine Fcε2-4 expressed in Cos7 cells. Lane 1: control Cos7 cell lysate. Lane 2: transfected Cos7 cell lysate containing pcDNA3.1bIgE. Lane 3: eluted protein after NiNTA purification. The blot was incubated with chicken anti-horse IgE 1:500 and goat anti-chicken alkaline phosphatase 1:1000 (Sigma) then developed with BCIP/NBT. Lanes 2 and 3 demonstrate a strong band of the approximate 50 kD close to the 47 kD calculated size of recombinant equine IgEε1-4.

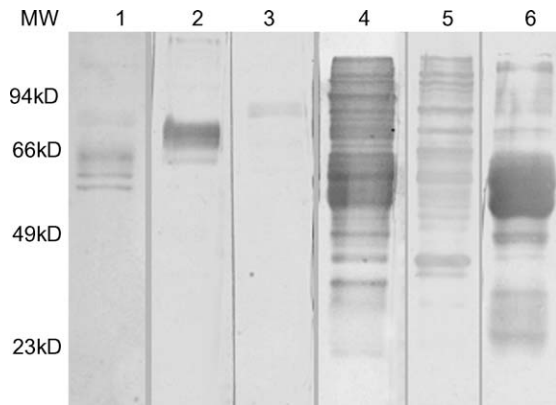


Fig. 2. Western blot of FPLC fraction 10 separated on a 12% SDS-PAGE gel run under reducing conditions, showing the immunoglobulin content of the fraction and the effect of absorption by protein G sepharose. Lanes 1–4: fraction 10 before absorption of IgG by a protein G column. Lane 1: developed with anti-horse IgA. Lane 2: developed with chicken anti-horse IgE. Lane 3: developed with anti-horse IgM. Lanes 4–6 were all developed with anti-horse IgG. Lane 4: fraction 10 prior to absorption with protein G. Lane 5: fraction 10 after absorption with protein G. Lane 6: IgG fraction eluted from the protein G column.

serum prepared as described in Section 2. An equal volume of sample was loaded into each well. After separation on 12% SDS-PAGE under reducing conditions, the blot was stained with ponceau-S and cut into strips prior to blotting with isotype specific

antibodies. In addition to IgE (lane 2) the fraction contained traces of IgA (lane 1) and IgM (lane 3). Both the IgM pentamer and the IgA (which is mainly dimeric in horse serum), are largely depleted by gel filtration due to their much higher molecular weight. The most abundant protein in this fraction is IgG (lane 4). The majority of IgG was removed by protein G affinity chromatography (lanes 5 and 6). Not all equine IgG subclasses bind to protein G with high affinity (Sheoran and Holmes, 1996), however, binding is maximal under the high pH and salt concentrations used. Following the depletion of IgG, the purity of the IgE preparation was sufficient to allow detection of IgE on ELISA plates coated with this fraction using chicken anti-C ϵ 3-4. This IgE enriched fraction was therefore used in a final immunisation and as the antigen to screen the first monoclonal fusion by ELISA.

3.2. Monoclonal antibody production

To ensure that monoclonal antibodies raised against recombinant IgE would have a high probability of recognising native equine IgE, the first fusion was screened by ELISA using plates coated with the partially purified native IgE. After primary screening the hybridomas were tested by immunohistology to confirm that they recognised putative

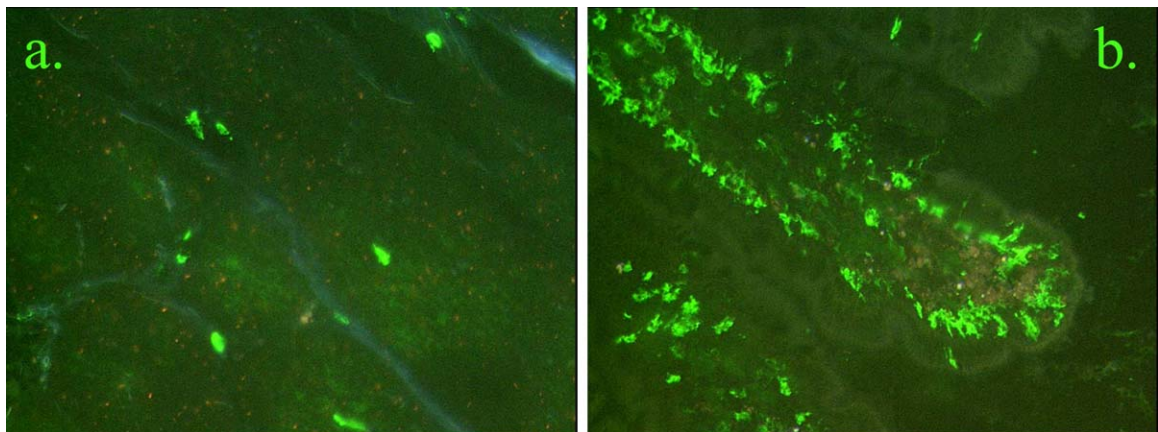


Fig. 3. Immunofluorescence of equine tissue stained with monoclonal anti-equine IgE (clone 4C2). (a) Connective tissue showing scattered positive cells. (b) Small intestinal villus with numerous positive cells lying within and immediately below the epithelium. There are several positive cells in the lamina propria and the intestinal submucosa also contained numerous positive cells (data not shown). The distribution of cells is consistent with staining of connective tissue and mucosal mast cells although antibody-producing B-cells may also be contributing to the numbers of positive cells.

mucosal and connective tissue mast cells. Fig. 3 shows the pattern of staining typical of hybridomas at this stage that were selected for cloning. Following cloning, one monoclonal antibody (4C2) was used to generate an affinity column for the purification of native IgE from horse serum. The affinity purified equine IgE was then used to screen the second hybridoma fusion and generate further monoclonal antibodies reactive with native IgE in ELISA. A total of eight monoclonal antibodies reactive with equine IgE were produced from which six were selected for further study.

3.3. Specificity of anti-IgE antibodies in ELISA

The monoclonal antibodies were tested for IgE antibody class specificity by ELISA using plates coated with preparations of equine IgA, IgM and IgG as well as affinity purified IgE. The results of four out of the eight monoclonals produced are shown in Fig. 4. The remaining four showed identical specificity of binding but have been omitted from the figure to aid clarity. None of the antibodies reacted in ELISA using plates coated with the bacterial recombinant Cε3-4 used as a priming antigen (data not shown).

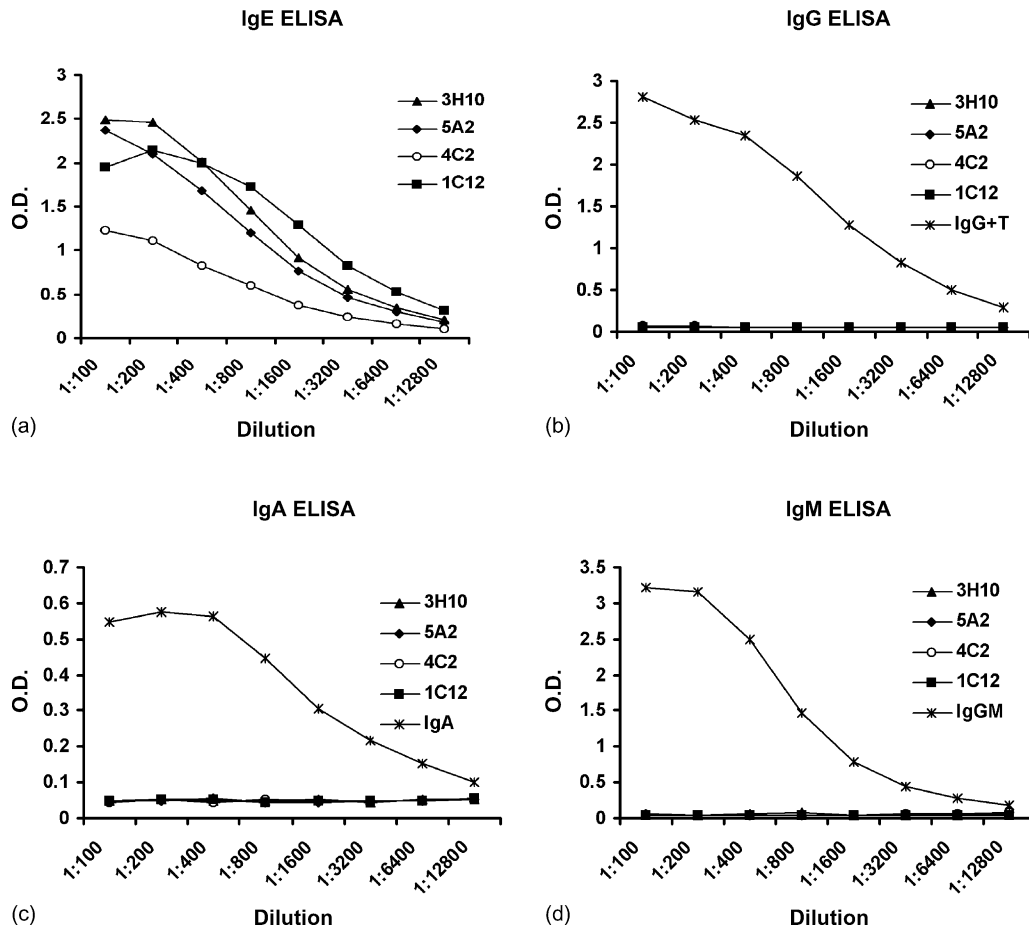


Fig. 4. Specificity of anti IgE monoclonal antibodies for immunoglobulin E in ELISA. Dilutions of monoclonal antibody culture supernatant were incubated with ELISA plates coated with purified equine immunoglobulins IgE, IgG, IgA or IgM. All anti IgE monoclonal antibodies reacted with IgE coated plates, there was no detectable reaction of any anti IgE monoclonal with ELISA plates coated with other immunoglobulin classes. Respective positive control antibodies for IgG, IgA and IgM were monoclonal anti pan IgG (clone K1271B) and IgA (clone K1292G3) and goat anti equine IgM (Nordic).

Three of the antibodies, 4C2, 3H6 and 7H2, reacted with bacterial recombinant Cε2 (Griot-Wenk et al., 2000), which was not used as an immunogen but presumably shares a conformational epitope with native IgE (data not shown). Because the protein G purified IgG may not contain all subclasses of equine IgG the data cannot rule out the possibility of an antibody cross reacting with a non-protein G binding IgG sub-class. To address this question we tested the antibodies by ELISA against equine serum fractionated by FPLC using a Superdex 200 gel filtration column. When the fractions were directly coated onto the ELISA plates, a pan anti-equine IgG monoclonal antibody detected immunoglobulin with peak concentrations in fractions 12–14. None of the anti-IgE antibodies gave any signal in directly coated plates, confirming that IgE is at relatively low abundance in horse serum and that none of the antibodies cross reacted with IgG (Fig. 5). IgE was detected in the fractions using the antigen capture ELISA with a peak centred on fraction 10. Western blotting of the fractions with chicken anti-equine IgE confirmed that this was the position of the IgE elution peak (Fig. 5).

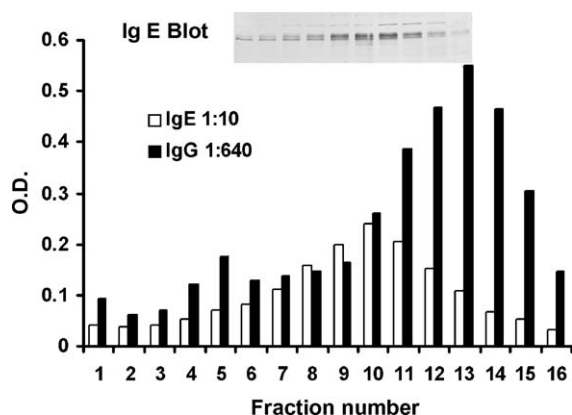


Fig. 5. Analysis of the IgG and IgE content of serum fractions eluted FPLC Superdex 200 column. IgG was detected in direct ELISA of coated plates using monoclonal K1271B anti equine pan IgG (solid bars). Some IgG was detected in all the fractions tested, peak IgG content was found in fractions 11–15, a smaller peak around fraction 5 may represent aggregated protein. None of the anti IgE monoclonals gave any signal above background where these fractions were directly coated onto ELISA plates (data not shown). IgE was detected using the more sensitive antigen capture ELISA (open bars) showing IgE eluted from the column in its predicted location slightly ahead on the IgG peak. The location of fractions containing peak IgE concentrations was confirmed by Western blot of the fractions using chicken anti equine IgE (inset panel).

3.4. Western blotting and preparation of IgE standard

All of the anti-IgE monoclonal antibodies recognised a band that ran close to the 200 kD molecular weight marker on western blots of the affinity purified IgE preparation run on a 6% gel under non-reducing conditions (Fig. 6, lanes a, b and d), only 3H10 antibody recognised a band of about 80 kD in gels run under reducing conditions (data not shown). The preparation contained several other proteins including IgG. It is likely that the IgG was bound by non-saturated IgG binding sites on the protein G of the affinity column rather than the anti IgE monoclonals. Analysis of the affinity purified preparation using the Experion automated electrophoresis apparatus gave similar results (Fig. 6, lane 1). The affinity preparation was further purified by gel filtration to remove the IgG and lower molecular weight contaminants. This yielded an IgE preparation containing 86.6 µg/ml of IgE with 99% purity (Fig. 6, lane 3). The purified IgE was diluted to 200 ng/ml in PBS 5% BSA and aliquoted as the standard for the IgE capture ELISA.

3.5. In vitro and in vivo reverse anaphylaxis

The results of the in vitro sLT release assay are depicted in Fig. 7. Spontaneous sLT release from PBL was below 150 pg/ml in all four horses, while release with Con A ranged between 520 and 1030 pg/ml. Some of the mAbs preps themselves (i.e. without addition of PBL) gave a background on the CAST[®] ELISA but this was always less than 250 pg/ml. sLT release from PBL (without subtraction of the background) was below 250 pg/ml in all four horses after incubation with the anti-dog IgG1, accordingly only values above 250 pg/ml are regarded as positive. 1C12 gave a low background reading (<130 pg/ml) with sLT release between 740 and 2350 pg/ml a value higher than that after incubation with Con A. 5A2 induced a sLT release that was higher than either Con A or 1C12 in two horses, but in the two other horses the levels of released sLT were very low. This agrees with the finding in capture ELISA where 5A2 failed to detect IgE in 30% of horses (see below). 7H2 induced consistent but low sLT release by PBL from all four horses at a

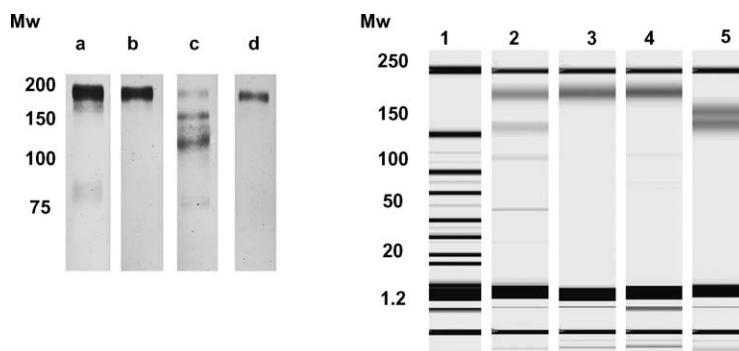


Fig. 6. Left hand panel: western blot of affinity purified IgE run under non-reducing conditions on 6% gel. (a) 3H10 anti IgE, (b) 1C12 anti IgE, (c) K1271B anti equine pan IgG and (d) 5A2 anti IgE. Right hand panel: virtual blot generated from Experion output. The black bands at the top and bottom of all lanes are “system peaks” due to buffer changes or detergent micelles. Lane 1: molecular weight standards (not all weight markers are labelled due to lack of space). Lane 2: the affinity purified IgE preparation, the top grey band is 219 kD, 71.4 ng/μl conc. and 73% of the total protein, a band of 168 kD equivalent to IgG and several smaller bands are also visible. Lanes 3 and 4: the same IgE preparation after a further purification step by Superdex G75 size fractionation. The fractions 9 and 10 were concentrated by ultrafiltration, the 219 kD band are now 99 and 98% pure with concentrations of 86.6 and 97.4 ng/μl, respectively. Lane 5: protein G purified equine IgG with two bands at 168 and 188 kD apparent molecular weight.

concentration of 0.35 μg/ml although this could be raised by increasing the concentration of antibody used (data not shown). In contrast incubation of PBL with 3H10 did not result in significant sLT release at a concentration of 0.35 μg/ml, and no release of sLT was detected even at higher concentrations of 1 and 2 μg/ml (data not shown). Similar results were obtained by reverse cutaneous anaphylaxis in vivo, i.e. no wheal and flare reactions were observed 30 min to 1 h after intra-dermal injection of 3H10 or anti-dog IgG1, while 1C12, 5A2 and 7H2 provoked skin reactions almost as great as histamine used as positive control (data not shown).

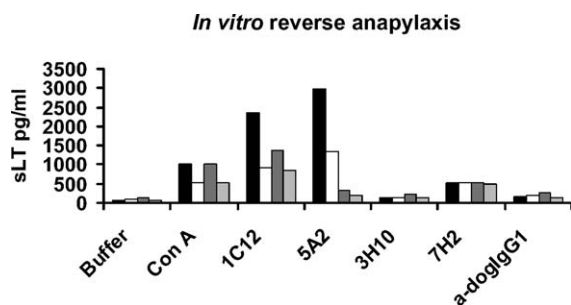


Fig. 7. Sulfidoleukotriene generation by peripheral blood leukocytes of four horses after incubation with 0.35 μg/ml purified anti-equine IgE mAbs, 20 μg/ml Concanavalin A or 0.35 μg/ml purified anti-dog IgG1 mAb as negative control.

3.6. Measurement of total IgE in normal horses and horses with IBDH

The 122 horses in first group were classified by breed and origin into three groups, Icelandic horses born in Iceland then imported into Switzerland, Icelandic horses born in Switzerland and other breeds (all born in Switzerland). Analysis of variance using total IgE as the dependent variable with the factors, breed/origin and diagnosis showed no significant overall effect of breed/origin, a highly significant overall effect of diagnosis ($P < 0.0032$) on total IgE. There was a highly significant interaction ($P < 0.0002$) between the breed/origin of the horse and the diagnosis of IBDH. Comparison of the individual means using the Tukey Kramer method showed the Icelandic horses with IBDH had significantly ($P < 0.05$) higher total IgE than the healthy Icelandic controls regardless of where they were born, but in the other breeds the IgE in the IBDH horses was not significantly different from the controls (Fig. 8).

The elevation of total IgE in Icelandic horses with IBDH was confirmed by analysis of the second group of samples taken from a cohort of horses sampled prior to their export from Iceland then for 3 years following their import into Switzerland. The health of the horses was monitored during this time. Total serum IgE was measured in the fifteen horses that had developed

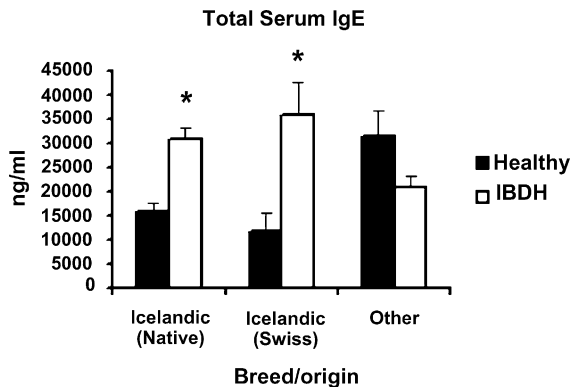


Fig. 8. Total IgE in the serum of healthy horses and horses with IBDH classified by breed and origin. Each bar shows the mean of total IgE in ng/ml, the standard error of the means are represented. Analysis of variance showed a highly significant interaction ($P < 0.001$) between breed and diagnosis. The mean total IgE in Icelandic horses was significantly higher (Tukey/Kramer, $^*P < 0.05$) in animals with IBDH compared to healthy Icelandic horses for both those born in Switzerland and those born in Iceland. In other breeds the mean total IgE in IBDH affected horses was less than that in healthy controls but the difference was not statistically significant.

IBDH by the end of the sampling period and a sample of 16 control horses selected from those that had remained healthy. In the horses included in this study, the mean time between import to Switzerland and

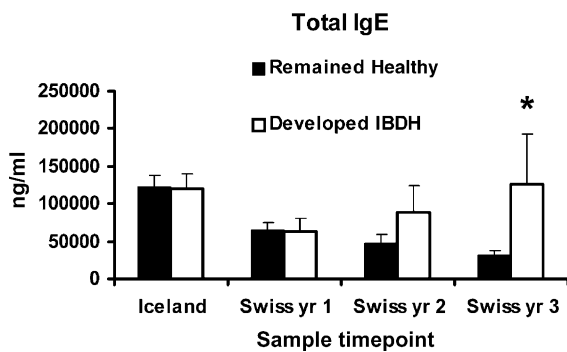


Fig. 9. Total IgE in serum of a cohort of Icelandic horses sampled before they were exported from Iceland and for 3 years after they were imported to Switzerland. The horses are divided into those that remained healthy throughout the study period ($n = 16$) and those that developed IBDH ($n = 15$). The mean serum IgE in both groups was identical at the start of the trial, by the end of the trial the IBDH group had a significantly higher total IgE (Tukey/Kramer, $^*P < 0.05$). The analysis of variance showed there was a statistically significant interaction ($P = 0.0196$) between the time-point of the sample and the diagnosis of IBDH.

onset of IBDH was 23 months after import (range 12.7–34.3 months). Repeated measures analysis of variance using total IgE as the dependant variable with time-point and eventual diagnosis as factors showed a highly significant interaction between the time-point at which the sample was taken and the diagnosis of IBDH ($P < 0.001$). These results are depicted in Fig. 9 which shows total IgE of the horses prior to export was equally high in healthy and IBDH horses. The total IgE of all horses dropped significantly in the 12 months following import. Subsequently the total IgE of horses that remained healthy continued to fall but as more of the horses in the IBDH group developed symptomatic disease the total IgE in this group rose to levels seen before export.

4. Discussion

The strategy used here to generate specific monoclonal antibodies to native IgE utilised three different sources of impure antigen to immunise the mice and a two step screening process to identify antibodies that reacted with IgE. This process has the advantage that neither pure antigens nor totally specific screening assays are required provided the desired specificity is common to all components and circumvents the problem of purifying native IgE to use as an antigen.

None of the monoclonals specific to native IgE reacted with the *E. coli* expressed Cε3-4 even though it was used as the primary immunising antigen. This agrees with the earlier finding that whereas chicken antisera did recognise a unique epitope on both recombinant Cε3-4 and native IgE, several polyclonal anti-sera from mammals immunised with this antigen did not recognise native IgE (Marti et al., 1997). The absence of mammalian antibodies that cross-react with this antigen and native IgE may be due to misfolding of the *E. coli* protein and therefore it cannot be concluded that none of the monoclonals recognise epitopes in the native Cε3-4 domains. Three of the monoclonals reacted in ELISA with both native IgE and *E. coli* expressed Cε 2, which was not itself used as an immunising antigen. Interestingly, in the ELISA against native IgE all three of these antibodies gave an OD reading approximately half that of the other antibodies (e.g. Fig. 4, 4C2). This observation could

be explained by the native folding of IgE Fc that results in an angled configuration in which the Cε2 domains are asymmetrically folded back onto Fcε3-4 (Wan et al., 2002). Thus, while epitopes in other symmetric regions of the IgE Fc may be present in duplicate (one on each heavy chain) the low binding of the antibodies to Cε 2 may be explained by their recognition of a single asymmetric epitope present only once per intact IgE molecule. Out of the eight monoclonal antibodies produced, only 3H10 detected an epitope in reducing SDS-PAGE. This antibody recognised only full-length heavy chains and did not react with either Cε3-4 or Cε2 in western blots of reduced gels, therefore it could be specific for an epitope in Cε-1, alternatively its epitope may present at the junction between Cε2 and Cε3.

In some initial antibody capture ELISA assays, the antibody 5A2 was used in conjunction with 3H10, however this combination failed to detect IgE in about 30% of horses tested (DW unpublished observation). By substituting different antibodies in the assay, this problem was determined to be due to failure of binding of 5A2. We interpret this to mean that 5A2 is specific for one of the allotypes of equine IgE (Wagner et al., 2001) although which allotype of equine IgE it recognises has not been determined. These results seem to be confirmed in the CAST[®], as this antibody induced high in vitro reversed anaphylaxis in two of the four tested horses, while it hardly induced any sLT release in the two other horses. Interestingly, 3H10, although giving comparatively high OD reading in the ELISA against native IgE, did not induce sLT release from PBL in vitro, nor cause a weal and flair responses in vivo indicating that this anti-equine IgE mAb is non-anaphylactogenic.

The concentration of IgE in healthy adult horses is up to 1000 times the reported normal range for humans, where values around 100 ng/ml are considered normal, >800 ng/ml elevated and those in excess of 4800 ng/ml indicative of hyperimmunoglobulin E syndrome (Hamilton and Adkinson, 2003). The mean concentration of IgE in serum from the first cohort of horses was 23,523 ng/ml with a range from 425 to 82,610 ng/ml. Even higher concentrations of IgE were recorded in the Icelandic horses prior to importation (mean 121,970 ng/ml). Overall the concentration of equine serum IgE in this study was similar to the values reported previously (Wagner

et al., 2003). The high levels of IgE in serum of healthy horses are most likely a result of immune response to parasites. Elevation of total serum IgE has been reported following parasite challenge of laboratory animals, sheep and cattle (Jarrett and Bazin, 1974; Miller et al., 1996; Pernthaner et al., 2005). High parasite burden may also explain the higher levels of serum IgE in Icelandic horses prior to exportation to Switzerland, as de-worming is usually not performed as frequently in Iceland as in Switzerland.

The high level of IgE in horse serum may have consequences for the pathogenesis of allergic diseases such as IBDH. The half-life of serum IgE in rats has been reported to be 12 h and in humans it has a half-life of 3 days (Tada et al., 1975; Waldmann et al., 1976). The high affinity of IgE for FcεR1 on mast cells and the slow turnover of the receptor, give cell bound IgE a correspondingly longer half-life of 14 days in humans (Geha et al., 1985). For maximum sensitivity 10% of mast cell IgE Fc receptors must be bound to IgE of a given specificity (MacGlashan and Schroeder, 2000). It follows that for mast cells to become sensitised in vivo to a specific antigen, serum IgE antibodies to that antigen would have to be present in abundance relative to all other IgE specificities for a period of several days. In human patients, where the normal IgE concentrations are frequently below 100 ng/ml, allergen specific IgE can contribute a high proportion of the total IgE (Wittig et al., 1980) causing diseases like atopic eczema in which generalised skin reactions occur in response to food allergens (Mediaty and Neuber, 2005). It would seem unlikely that allergen specific IgE can constitute the major component of total serum IgE in all horses with IBDH but this may be the case in Icelandic horses.

Icelandic horses have a known high incidence of IBDH, particularly in adult horses imported from Iceland where *Culicoides* spp. are not found (Hall-dordsottir and Larsen, 1991). We have also observed that Icelandic horses often have much more severe lesions than horses belonging to other breeds. It is not infrequent for Icelandic horses to present with lesions around the eyes, udder or preputium and on the legs but, in our experience this is rarely the case in other breeds. Contrary to an earlier study (Wagner et al., 2003) we detected a statistically significant increase in total IgE in Icelandic horses affected with IBDH, which can be accounted for by the larger sample size

in our study. If the rise in total IgE in Icelandic horses is due to insect specific IgE, then a systemic sensitisation pattern could occur and would account for the severe and extensive nature of lesions seen in horses of this breed affected by IBDH. IBDH is a polyfactorial and polygenetic condition, an association between IBDH and leukocyte antigens has been established in Icelandic horses (Halldorsdottir et al., 1991) but it is not clear how such leukocyte antigen polymorphisms could account for the observed differences in total IgE. It has been suggested that allergens themselves may act as superantigens but, as yet, direct evidence for this is lacking (Snow et al., 1996). Nevertheless some parasite antigens have been shown to promote IgE synthesis (Van der Kleij et al., 2002) and insect saliva from *Phlebotomous papatsei* causes a Th2 biased response that could stimulate B-cell class switching to IgE (Mbow et al., 1998). Further work is required to determine the exact nature of the antigens and other components in insect saliva that induce IgE responses in horses with IBDH.

The high levels of IgE in Icelandic horses prior to export from Iceland could reflect a high parasite burden. Arguments for a protective effect of parasites against allergic disease have been made for humans (Yazdanbakhsh et al., 2002). Several mechanisms have been proposed including competition of anti-parasite IgE for Fc receptor binding on mast cells, or high levels of IL10 and TGF β production by parasite induced T-reg cells (Smits et al., 2005). There is some indirect evidence that could support this view in the data from an epidemiological study documenting the incidence of IBDH following importation of Icelandic horses into Sweden. In this study the time of year at which the horses were imported had a significant effect on the incidence of disease (Halldorsdottir and Larsen, 1991). Specifically, horses that arrived in Sweden in the winter months were twice as likely to develop IBDH as those that arrived in summer. If the pattern of total IgE in those horses was the same as reported here for horses imported to Switzerland, then horses that arrived in summer would be first exposed to *Culicoides* when putative T-reg activity and their IgE levels to other (parasite) specificities competing for mast cell binding were high. Conversely, those arriving in winter would have time for their total IgE levels or parasite induced T-reg activities to decline before *Culicoides* exposure making it easier for a

Culicoides specific IgE positive feed back loop to become established. If this hypothesis is correct it follows that controlled exposure of Icelandic horses to *Culicoides* antigen prior to export from Iceland could have a protective effect against subsequent onset of IBDH.

The pathogenesis of IBDH in non-Icelandic horses is more consistent with an alternative model of compartmentalised allergic disease. In this case, allergen driven specific IgE synthesis is overall much lower, consequently sensitisation will only occur where local IgE synthesis results in high levels of allergen specific IgE antibodies in tissues that only sensitise local mast cells (Smurthwaite and Durham, 2002). Local synthesis of IgE has been documented in human patients with *Eosinophilic rhinitis* and asthma (Wilson et al., 2002). Furthermore, alterations in the local immune microenvironment following antigen challenge and mast cell cytokine release have been implicated in causing IgG producing B-cells to switch to IgE in situ at sites of allergic responses (Takhar et al., 2005). The documented accumulation of IgE mRNA expressing cells, IgE positive mast cells, eosinophils and T-cells in the lesions themselves (McKelvie et al., 1999; van der Haegen et al., 2001) are also consistent with local IgE production leading to localised skin sensitisation which does not reach levels sufficient to cause an increase in total serum IgE. This model also fits well with more common pattern of severe lesions restricted to the preferred feeding sites of *Culicoides* seen in non-Icelandic horses.

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