UNIVERSITY OF
LIVERPOOL

Complement and Endotoxins in Equine Colic

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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July 2008

Contents

Acknowledgements

My first acknowledgement is to The Horse Trust (aka The Home of Rest for Horses), who not only funded this scientific project but also funded my residency during which time the preliminary work to this project was carried out.

Professor Martin Leuwer and Professor Gernot Marx provided the initial inspiration and support for this project. Dr Jacqui Matthews fostered and built on that beginning, and made me realise that I wanted to develop further as a researcher. I thank them all for their contribution.

The Faculty of Veterinary Science facilitated my project by allowing me to take research leave from my post and I am grateful for that support. My colleagues within the Division of Veterinary Anaesthesia have supported me in many ways, not least Alex Dugdale, whom encouraged me to undertake the project despite it being a difficult time for our Division.

During the project I have received help from many people but Di Isherwood, Dr Susan Bell, Dr Lisa Macrory, and Dr Anne Vaughan-Thomas deserve particular mention for their patience and guidance. I really benefited from working within such a helpful group of people.

I am indebted to Nicola Swift for helping to adapt and validate the endotoxin assay to work on equine plasma samples.

My supervisors, Professor Stuart Carter and Professor Christopher Proudman have each supported me with insightful and constructive comments throughout the project and the production of the thesis. I can never thank each of them enough. Stuart in particular has given almost constant encouragement and advice and helped to maintain motivation during periods when the project was not going as I had expected. I could not have asked for a better supervisor.

Finally, I want to thank my wife Avril for her patience, love and support. I have had to deal with some personal challenges during the project, and it is to her credit that the impact of these challenges on the project was minimal.

Mark Senior, July 2008.

Structured abstract

Colic (abdominal pain) is a relatively common disease of horses ranging from mild, transient episodes to a life-threatening syndrome that requires surgical intervention. Equine intestinal abnormalities that involve local stasis are often accompanied by marked proliferation of Gram negative enteric bacteria and breakdown of the mucosal barrier resulting in the transmural and transvascular migration of bacteria and their endotoxins into the circulation (endotoxaemia). Even if the original cause of colic is corrected or resolved, the presence of endotoxaemia can result in significant morbidity and mortality in horses. This project aimed to characterise a specific equine complement component, *complement 1 esterase-inhibitor* (C1-inh) because it is considered a key inhibitor of the complement, coagulation and contact activation pathways. These pathways are pivotal in the aetiopathogenesis of the inflammatory response and disease syndrome observed in equine colic-derived endotoxaemia. The project also aimed to measure levels of endotoxin in the plasma of clinical cases presented to the hospital and correlate these levels to morbidity and mortality outcomes.

A preliminary study identified an antibody against human C1-inh that showed apparent excellent cross-reactivity and more importantly, selectivity to equine C1-inh. Work was undertaken to establish whether the same antibody or others would show adequate cross-reactivity and selectivity to equine C1-inh to allow the development of an immunological based assay for this protein in plasma. Lapine antisera raised against custom peptides designed from the amino acid sequence for equine C1-inh were also assessed. Cross-reactivity and specificity were evaluated to be poor in all antibodies tested.

Attempts were made to purify C1-inh from equine plasma to use as a standard and for antibody generation. Attempts were also made to purify equine kallikrein and equine pro-kallikrein to utilise in inhibition assays to detect equine C1-inh. Chromatographical techniques to purify proteins were reviewed and techniques were selected that could be replicated in our laboratory. The purification attempts were not successful.

Molecular biological techniques were employed to produce data which would provide structural and functional information on equine C1-inh. Results from these studies provided evidence that C1-inh exists in the horse, and is produced in several cell types. The full nucleotide sequence for the protein was determined and the amino acid sequence derived. This information was used to generate a 3D model of the protein which was compared to a model of the human protein. From the 3D model of equine C1-inh protein, likely sequences acting as immunogens were identified. Custom conjugated peptides were made that were then incubated in rabbits to raise polyclonal antisera for use to detect native equine C1-inh in equine samples. These antisera were tested by ELISA and Western blotting. Quantitative real-time PCR was used to assess if there were differences in gene expression for equine C1-inh and other complement components in different normal and pathological tissues. No differences were found in these experiments.

The Limulus Amoebocyte Lysate (LAL) assay is able to detect all LPS and is based on an enzyme found in blood cells of the Horseshoe Crab called amoebocyte lysate. A kinetic chromogenic version of the LAL assay was developed to detect LPS in plasma samples from clinical cases. This part of the work also sought to investigate the effect of a colic treatment (polymixin B) on LPS levels in horses. Three hundred and seventy-five plasma samples from 247 horses were tested. LPS was detected in 29% of horses presenting with colic. Horses that had detectable LPS were significantly more likely to die whilst in the hospital. Whilst there was evidence that polymixin B treatment resulted in a decrease in detectable LPS in samples, there was no evidence that polymixin B treatment decreased mortality in colic cases.

Introduction

Complement

The complement system is a complex protein cascade involved in early non-specific defence mechanisms; the innate immune response (Sim and Tsiftsoglou 2004, Gigli and Austen 1971, Walport 2001a,b). So far, around 25 soluble and 10 cell surface proteins have been identified which interact to facilitate recognition, opsonisation and clearance (by phagocytosis) of invading micro-organisms and diseased host cells. The complement system also interacts with cells of the adaptive immune system influencing adaptive immunity (Walport 2001a,b).

The complement system recognises targets through multiple weak interactions between carbohydrate and/ or charged residues on target surfaces and complement recognition proteins. There are three recognition pathways which utilise overlapping recognition mechanisms, thus generating 'redundancy' within the complement system. These pathways are named the classical pathway, the lectin pathway and the alternative pathway. They share a common terminal pathway.

Recognition proteins from each pathway bind to targets causing activation of a series of serine protease proenzymes, which in turn activate atypical serine proteases which lead to activation of the major opsonisation protein of the system, complement protein 3 (C3). Activated C3 covalently binds to the target surface and is recognised by phagocytic cell receptors.

Classical pathway

The recognition protein of the classical pathway is C1q which binds to the Fc portion of complexed IgG which has previously specifically attached to antigenic epitopes on foreign (or self) molecules. Binding of C1q activates the serine proteases C1s and C1r, which circulate with C1q as the complex $C1qr_2s_2$. Activated $C1qr_2s_2$ then cleaves complement component C2 and component C4 leading to the formation of the complex C4b2a, which cleaves complement component C3 into C3a and C3b. C3b is a pivotal component of complement and initiates the terminal pathway (see below).

Lectin pathway

The recognition protein of the lectin pathway is mannose-binding lectin (MBL), which recognises the neutral carbohydrate clusters commonly found on microbial surfaces. More recently, a group of lectin-like proteins called ficolins have also been demonstrated as being

able to act as lectin pathway recognition proteins (Holmskov et al. 2003).The binding of MBL causes the activation of MBL-associated serine proteases 1 and 2 (MASP1 & 2), which then cleave C2 and C4 leading to the formation of C4b2a, C3b and the initiation of the terminal pathway (see below).

Alternative pathway

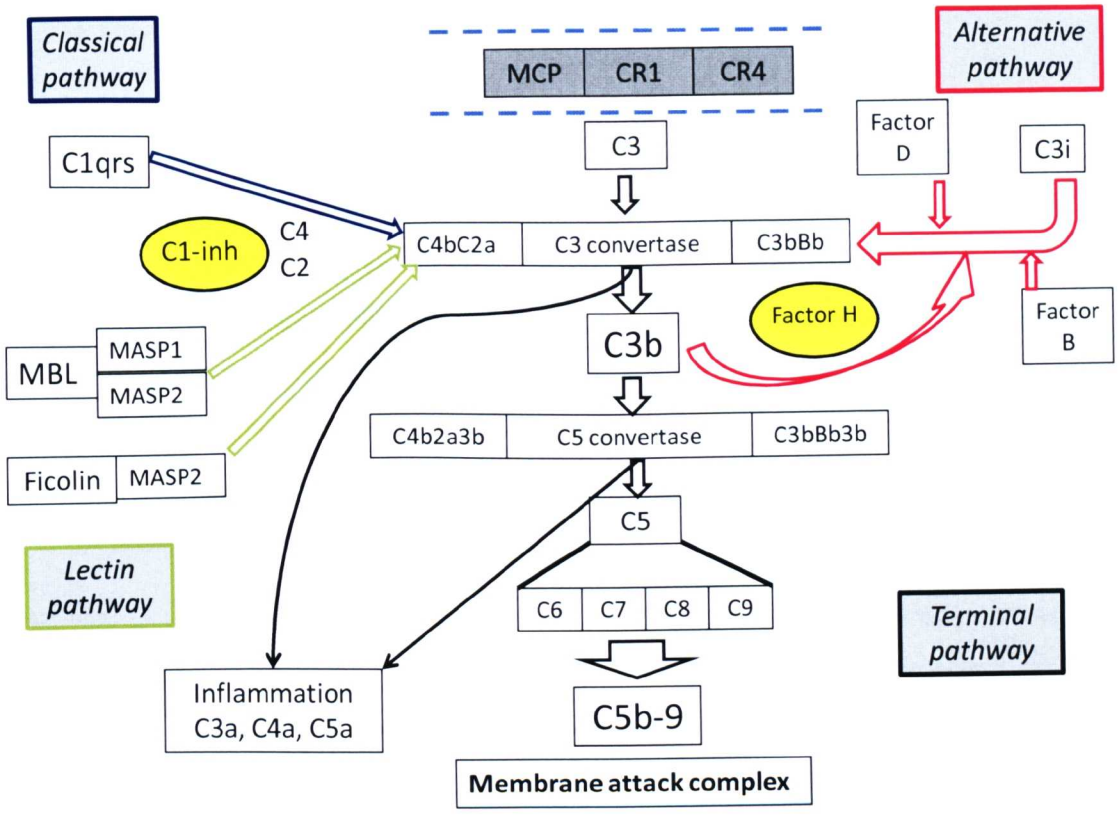
The alternative pathway recognises surface clusters of both neutral and charged carbohydrates which are commonly found on pathogens. Essentially, there is continuous spontaneous hydrolysis of C3 to form $C3.H_2O$ or C3i. C3i forms a complex with protease factor B to form C3iB. C3iB is then cleaved by serine protease factor D to form the C3iBb complex. In the C3iBb complex the hydrolysed C3 is then broken down to C3a and C3b. C3b then can bind to any nearby cell or macromolecular surface. The bound C3b then continues to bind more factor B, and forms a C3 convertase (via factor D) called C3bBb. The C3bBb then continues to convert more C3 into C3b producing strong opsonisation. If C3b binds to host cell surfaces it is prevented from binding any factor B by factor H, thus preventing further reaction.

Terminal pathway

Surface bound C3b and its breakdown product iC3b are opsonins for phagocytes. Complement receptors CR1, CR2 and CR4 on the surface of phagocytic cells recognise bound C3b and promote adhesion of the target to the phagocytic cells. C3b and C4b2a also complex to form a C5 convertase (C4b2a3b), which cleaves C5 and initiates the assembly of the membrane attack complex (MAC), consisting of complement components C5 to C9. The MAC forms pores in the lipid bilayers of target cells promoting cell lysis. Breakdown products of the complement cascade also have other proinflammatory roles. C3a, C4a and C5a are potent anaphylotoxins. C5a also acts as a chemotactic factor for neutrophil migration.

Figure 1: schematic representation of the complement system cascade.

Grey shaded boxes = membrane associated regulators. Yellow shaded ovals = fluid phase regulators. MCP = membrane co-factor protein. C1-inh = C1-inhibitor



The main physiologic roles of complement are summarised below (Walport 2001a);

Host defence against infection

Opsonisation: covalently bound C3 and C4 fragments

Chemotaxis/ leukocyte activation: anaphylatoxins (C5a, C3a and C4a)

Inflammation: anaphylatoxins (C5a, C3a and C4a)

Lysis of bacteria and cells: Membrane attack complex (C5b-9)

Interface between innate and adaptive immunity

Augmentation of antibody response: C3b and C4b binding of immune complexes or antigens/ C3 receptors on B-lymphocytes

Enhancement of 'memory': C3b and C4b binding of immune complexes or antigens/ C3 receptors on dendritic cells

Disposal of unwanted host cells

Clearance of immune complexes and apoptotic cells: C1q/ covalently bound C3 and C4 fragments

Recently, evidence has emerged to suggest that the complement system has a number of important wider roles within the body. There is evidence that complement may play a role in bone and cartilage development in the fetus, in tissue regeneration, haematopoiesis and vascular development (Mastellos and Lambris 2002). Complement has also been shown to be important in neuronal physiology and diseases such as Alzheimer's, multiple sclerosis and epilepsy (Kirschfink and Mollnes 2003, Aronica et al. 2007). It is clear that the complement system is an important biological system and warrants scientific investigation to elucidate its role in health and disease.

The complement system is an extremely robust and effective host defence mechanism against foreign substances and micro-organisms (Kirschfink and Mollnes 2003). As a result, it exists in a wide variety of animal species and is relatively well conserved across mammalian species, although species differences do exist (Gigli and Austen 1971). Complement is finely regulated so that activation is focused on fulfilling the roles summarised above and deposition of complement on normal cells and tissues is limited. When these regulatory mechanisms are overwhelmed or break down then the complement system may cause injury or disease to the host animal. The complement system has been shown to play a prominent role in various autoimmune (systemic lupus erythematous, rheumatoid arthritis) and inflammatory (systemic inflammatory reaction syndrome (SIRS), ischaemia-reperfusion syndrome, graft rejection) diseases (Kirschfink and Mollnes 2003). Overwhelming activation of the complement cascade can cause life-threatening tissue damage through inflammation and an animal may develop a state of uncontrollable disseminated intravascular coagulation.

Increased levels of C3 have been associated with SIRS and sepsis (Sungurtekin et al. 2006) in humans and in endotoxaemia in mice (Wang et al. 1998). C5 has been shown to be closely linked to mortality in response to *E.coli* or *E.coli* LPS infusion in mice (Hollman et al. 2008). Studies in various models of severe inflammation have demonstrated organ-protective effects following treatment with molecules targeted at modulating the complement cascade (reviewed by Morgan and Harris 2003). Substances that regulate complement may be useful in

regulating inflammation. C1-inhibitor is one such regulator of complement, but also has a number of other important anti-inflammatory functions (see below). It is likely that the activation/ regulation of complement plays a vital role in inflammation in horses and especially in the inflammatory response to endotoxaemia, and the study of the equine complement system is vital in understanding this inflammatory process, but has so far received relatively little attention.

Endotoxin

Lipopolysaccharides (LPS) are essential components of the outer membrane of all Gram negative bacterial cell walls and are major determinants of virulence in pathogenic species (Poxton 1995). Structurally, LPS is made up of three distinct components; lipid A, core oligosaccharide and an O-polysaccharide chain consisting of long chains of repeating polysaccharide units. The lipid A component is the toxic moiety and is very highly conserved across many Gram negative bacterial species. The core oligosaccharide component is genus specific. The O-polysaccharide chain is species or serotype specific. Mutants of some enterobacteria have lost the O-polysaccharide region of their LPS and are said to produce a rough form (R-form) of LPS as opposed to the O-polysaccharide containing smooth form (S-form). Some species of Gram negative bacteria are capable of producing both S and R-forms of LPS, and in these species the R-form is called lipooligosaccharide (LOS) (Poxton 1995). If LPS enters into a host animal, it is termed endotoxin, where it acts as an extremely potent stimulator of inflammation.

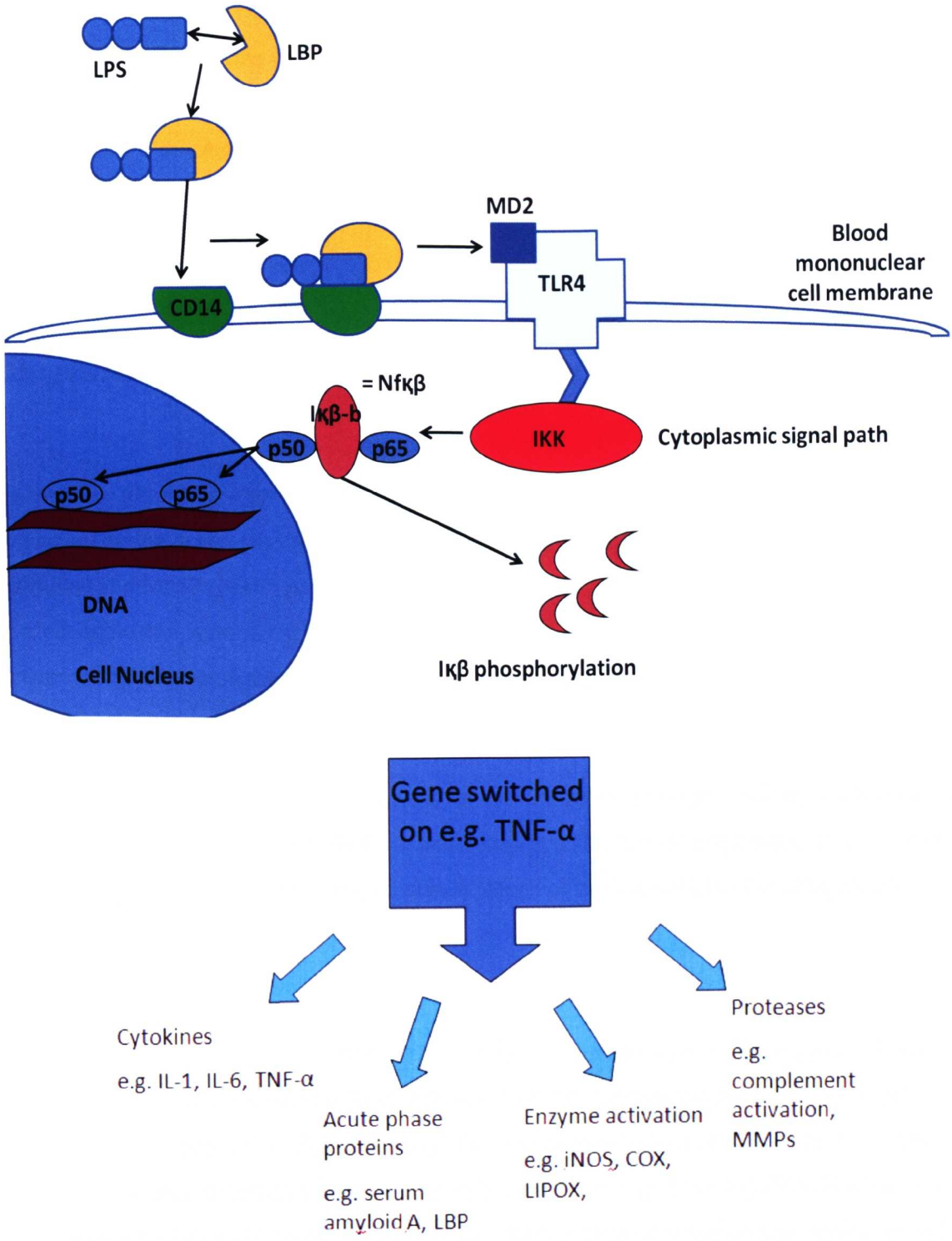
LPS can originate from any of the diverse species of Gram negative bacteria and enter into the body of an animal as whole or fragments of cell wall released when bacteria multiply or die. LPS from different Gram negative bacterial species are not equally pathogenic in various host organisms; some LPS may be pathogenic in one host species but not in another species (Lohmann et al. 2003). This fact highlights an important point; that it is not the LPS itself that causes disease but rather the host inflammatory response to the LPS.

Inflammatory responses to LPS

If LPS liberated by Gram negative bacteria enters the blood of an animal, it binds to an acute phase plasma protein called lipopolysaccharide binding protein (LBP). The LBP orientates the LPS so that the toxic lipid A moiety is exposed. This is important as exposure of the lipid A moiety allows the LBP-LPS complex to interact with white blood cell membrane bound or soluble cluster of differentiation antigen 14 (mCD14 or sCD14). The mCD14/ LBP-LPS complex then interacts with myeloid differentiation protein 2 (MD2) on toll-like receptor 4 (TLR4) which is also present on the cell membrane of white blood cells (e.g. mononuclear cells). TLR4 is a transmembrane receptor complex, which, after recognition of the mCD14/ LBP-LPS complex induces the production of nuclear factor κβ (NFκβ) through the phosphorylation of inhibitory κβ (Iκβ) via cytoplasmic signal pathways. NFκβ contains transcription factors (e.g. p50, p65), which pass into the nucleus of the cell and activate genes that result in a pro-inflammatory state e.g. upregulation and production of pro-inflammatory cytokines (e.g. IL-6, Tumour necrosis factor-α) and acute phase proteins (e.g. serum amyloid A, LBP, activated protein C), enzyme activation (e.g. cyclooxygenase, lipoxygenase, nitric oxide synthase) and protease activation/ production (e.g. complement, matrix metalloproteinases). If the pro-inflammatory response is of sufficient magnitude and/ or remains unchecked then physiological disturbances, tissue damage and organ dysfunction result which can cause significant morbidity and mortality (Frevert et al. 2000, Bryant et al. 2003, Knapp et al. 2003, Sykes and Furr 2005, Werners et al. 2005).

Figure 2: schematic representation of the inflammatory response to LPS in blood.

See text for explanatory notes. iNOS = inducible nitric oxide synthase, COX = cyclooxygenase, LIPOX = lipoxygenase, MMPs = matrix metalloproteinases, IL-1 = interleukin 1, IL-6 = interleukin 6, TNF-α = tumour necrosis factor-α



Gram negative bacteria are ubiquitous in the environment and can live in close association with animals without normally causing disease (e.g. within the gastro-intestinal (GI) tract). Indeed some animals, including the horse, rely on the presence of Gram negative bacteria in their GI tract to help to digest the plant material the animal ingests. It is normal for small amounts of LPS to be adsorbed from the GI tract of horses, but this LPS is removed from the portal circulation by Kuppfer cells in the liver (Mathison and Ulevitch 1979) and therefore LPS does not normally enter the circulation in sufficient amounts to cause an inflammatory reaction. However, any disease process resulting in the increased absorption of LPS into the circulation can overwhelm the LPS removal mechanisms, leaving LPS to circulate freely and trigger a pro-inflammatory state.

Complement 1 inhibitor (C1-inh) [C1s inhibitor, C1 inactivator]

C1 inhibitor (C1-inh) is a **se**rine **p**rotease **in**hibitor (serpin) that inactivates several different important serine proteases involved in inflammation and coagulation: C1r, C1s (classical pathway) and MASPs (lectin pathway) in the complement system, Factor XIIa and kallikrein in the contact activation system, Factor XIa and thrombin in coagulation, and tissue plasminogen activator and plasmin in the fibrinolytic system (Caliezi et al. 2000, Cicardi et al. 2005).

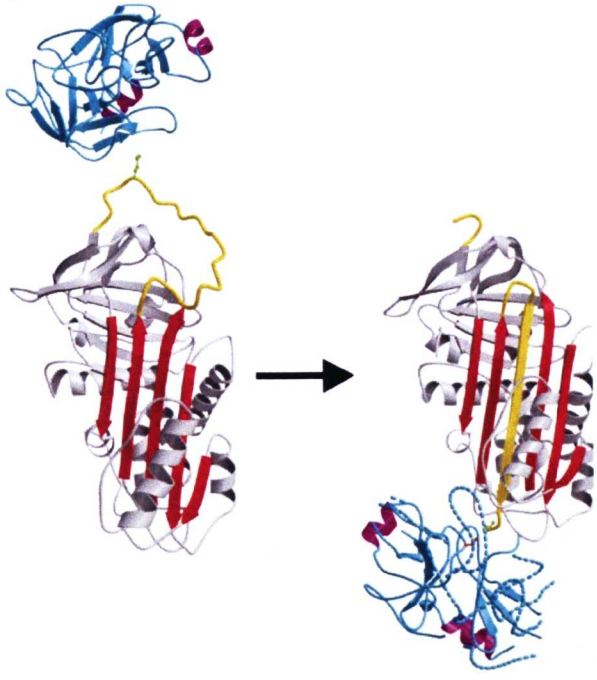
C1-inh has been studied in detail in humans for over 25 years, as people deficient in this molecule develop a condition known as hereditary angioedema, a life threatening disease associated with complement-driven swelling of internal organs. Much of the information regarding C1-inh is based on studies in man.

Human C1-inh is a heavily glycosylated (~30%), 478 amino acid single chain polypeptide, with an apparent Mw ~105 kDa (assessed by analytical ultracentrifugation and polyacrylamide gel electrophoresis) (Bock et al. 1986), although the calculated Mw is 78 kDa (Perkins et al. 1990). C1-inh shares a similar amino acid homology of its C-terminal (serpin domain) with other members of the serpin protein superfamily which confers a similar 3D structure characterised by three β sheets and an exposed reactive loop. Like other serpins, C1-inh blocks the activity of its target proteases by a 'suicidal' mechanism, i.e. it forms an irreversible covalent bond with the target protease meaning neither molecule can react with any other substrate. The reactive site acts as a

pseudo-substrate for the target proteases; after the target protease binds to C1-inh, breaking the covalent bond between the reactive site residues, C1-inh acts as a "mousetrap" swinging the target protease to the other pole of the C1-inh molecule, forming a covalent bond with the protease and inactivating it. The remainder of the reactive site inserts as a fourth strand in the β sheets, effectively closing the door of the trap (Cicardi et al. 2005) (see below).

Figure 3: Schematic illustrating the formation of the complex of serpin and protease.

Ribbon depictions of a serpin [α1-antitrypsin] (lower molecule) and a protease [trypsin] (upper molecule) [left], and of the resulting complex showing the shift and full insertion of the cleaved reactive-centre-loop into the central β-sheet [right]. Regions of disordered structure in the complexed trypsin are shown as interrupted coils projecting from the structure. [taken from Huntington et al. 2000]



C1-inh-substrate complex is an equimolar, denaturation resistant complex with a covalent bond forming between the inhibitor and its substrate that induces a conformational change in both proteins. Interestingly, the N-terminal (non-serpin) domain of C1-inh has no homology to the other serpins or any other known protein (Bos et al. 2002, Cicardi et al. 2005), and is not essential for the formation of the serpin-protease complex (Coutinho et al. 1994). However, the N-terminal region has been shown to be important for plasma clearance of C1-inh (de Smet et al. 1993) and for interactions with endotoxin (Davis et al. 2007, Zhang et al. 2007) and selectins (Cai and Davis 2003, Cai et al. 2005).

Activation of the contact and/or complement system has been shown to occur in patients with septic shock and is associated with poor outcome (Nuijens et al. 1989, Caliezi et al. 2000,

Sungurtekin et al. 2006). Purified C1-inh was introduced as a replacement therapy for hereditary angioedema over 25 years ago and there is now convincing evidence from animal and human studies that administration of C1-inh may also be beneficial in conditions where over-activation of the complement, contact or coagulation systems is important (Caliezi et al. 2000, Cicardi et al. 2005). Purified C1-inh has been tested as a therapeutic agent in cases of sepsis, myocardial infarction and cytokine-induced vascular leak syndrome (reviewed by Caliezi et al. 2000). Initial studies in sepsis patients showed an increase in *cleaved inactive C1-inh (iC1-inh)* compared with healthy volunteers and that the extent of plasma C1-inh proteolysis and iC1-inh levels were positively correlated with mortality (Nuijens et al. 1989). Subsequently, C1-inh has been used in human patients with beneficial results, as evidenced by a reduction in vasopressor therapy required in critically ill people (Hack et al. 1992, Marx et al. 1999, Caliezi et al. 2002) and double-blind controlled studies are now being performed to establish whether C1-inh treatment will reduce mortality or morbidity in sepsis.

Equine Colic

Background

Colic is a general term used to describe the symptoms of abdominal pain in horses. Colic is a relatively common disease of horses and ranges from mild, transient episodes to a life-threatening syndrome that requires surgical intervention. Strangulating obstruction of intestine is accompanied by marked proliferation of Gram negative enteric bacteria and breakdown of the mucosal barrier. This results in the transmural and transvascular migration of bacteria and their endotoxins into the circulation (endotoxaemia). Even if the original cause of colic is corrected or resolved, the presence of endotoxaemia can result in significant morbidity and mortality in horses (Proudman et al. 2002a,b).

Endotoxaemia induces release of acute phase proteins and cytokines that drastically alter the horse's physiological status and can result in severe cardiovascular and gastrointestinal dysfunction, complement activation, organ failure and death (Moore 1988, Sykes and Furr 2005, Werners et al. 2005). Endotoxaemia also stimulates clotting disorders (coagulopathy) and intravascular formation of thrombi, thereby reducing tissue perfusion and when diffuse, leads to multiple system organ failure and death. Endotoxin can initiate this cascade through various mechanisms, which ultimately lead to activation of the intrinsic and extrinsic coagulation cascades (Moore 1988). The results of several studies indicate that the severest

forms of colic are associated with plasma alterations suggestive of a hypercoagulative state (Prasse et al. 1993, Monreal et al. 2000, Proudman et al. 2002b). For example, in a long-term survival study on surgical colic cases, development of signs indicative of a hypercoaguable state were significantly linked to reduced survival (Proudman et al. 2002b). In a separate study where 233 colic cases were examined, non-surviving horses with the most severe forms of intestinal ischaemia had pathological changes interpreted as hypercoagulative (Prasse et al. 1993). Recent research (French et al. 2002, Proudman et al. 2002b) has indicated that particular clinical signs associated with endotoxaemia (i.e. elevated heart rate and presence of a hypercoaguable state) are significantly linked to survival of horses that have undergone colic surgery.

The horse's inflammatory response to endotoxaemia is responsible for the severity of disease. Unfortunately, other than clinical examination, there are currently no methods that accurately quantify the degree of endotoxaemia in equine colic. Such methods would be helpful as prognostic tools. Moreover, there are few recognised treatments for endotoxaemia that specifically affect the outcome: once horses develop endotoxaemia, there is little in the way of intervention therapies that can be instituted that affect survival (Morris et al. 1990). It is clear that the complement, coagulation and contact activation pathways are pivotal in the aetiopathogenesis of the inflammatory response and disease syndrome observed in equine colic derived endotoxaemia. Studies of C1-inh, a key inhibitor of these pathways, may lead to a greater understanding of this important disease, eventually leading to clinically useful assays and improved intervention therapies.

Preliminary work

Where C1-inh has been measured in horses, along with other coagulation factors, significant positive correlations were found between fibrinogen, C1-inh and plasminogen values (Topper & Prasse 1998). In that study, no comparison was made between C1-inh levels and disease severity or survival. Significant increases in C1-inh levels were not found in the colic group when compared with normal horses (n=11). However, these authors only measured levels of functional C1-inh and it is clear from studies in human septic patients that it is the levels of proteolytically cleaved, *inactive* C1-inh that are related to outcome of disease (Nuijens et al. 1989). Preliminary work carried out by the author identified a commercially available (Sigma) goat polyclonal antibody against human C1-inh that appeared to have excellent cross-reactivity

and, more importantly, selectivity to equine C1-inh. Furthermore, functional C1-inh activity was demonstrated in equine plasma in our laboratory using a later version of the commercial assay employed by Topper and Prasse (1998) (unpublished data).

Aims

This project aims to characterise a specific equine complement component, *complement 1 esterase-inhibitor* (C1-inh), to determine if this protein is a key inhibitor of the complement, coagulation and contact activation pathways in horses. The project aims to determine if pathways are important in the aetiopathogenesis of the inflammatory response and disease syndrome observed in equine colic-derived endotoxaemia.

Specific objectives:

- To develop novel assays to measure C1-inh in the plasma of horses. The diagnostic approaches will be both at protein and gene expression levels.
- To assess whether there are significantly different levels of C1-inh (functional and non-functional) in three groups of horses: non-endotoxaemic control horses, survivors of colic and non-survivors of colic.
- To measure levels of endotoxin in equine plasma samples using a kinetic chromogenic Limulus Amoebocyte Lysate assay. Levels of endotoxin will be correlated to levels of C1-inh, other measures of endotoxaemia (clinical parameters) and survival.
- To evaluate the potential for functional circulating C1-inh, to be considered as a novel therapy for use in horses with endotoxaemia.

Hypothesis

C1-inh protein is important in regulating the early complement, contact and coagulative cascades in colic-associated endotoxaemia, and that the level of functional activity of this protein may be important in determining the outcome (i.e. morbidity and/or mortality) of the disease process.

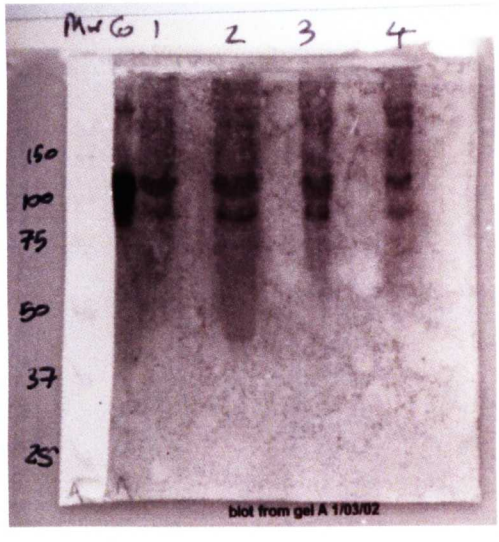
Chapter 1

Assessment of antibody detection of equine C1-inh

Introduction

One of the main aims when the project was conceived was to develop an assay to measure levels of C1-inh (and ideally both C1-inh/substrate complexes and cleaved C1-inh) in equine plasma from clinical colic cases and then to link these levels to clinical outcome and levels of circulating LPS. This facet of the project was based on preliminary work undertaken by JMS in 2002 as part of a Home of Rest for Horses residency project. This study had identified a commercially available goat polyclonal antibody against human C1-inh that showed apparently excellent cross-reactivity and more importantly selectivity to equine C1-inh (see photograph below). The aims of the work in the first part of this chapter were to establish whether the same antibody or others donated to us would show the same cross-reactivity and selectivity to equine C1-inh, in order that a detection assay could be developed for use in the horse in health and disease. All blots shown were reproduced and images of blots are chosen as representative. Unless specified otherwise, all plasma samples (e.g. human, 'non-endotoxaemic' horse and 'endotoxaemic' horse) were from the same source.

Figure 1.1: Western blot from the preliminary work to this project showing apparent cross-reactivity of a commercial polyclonal antibody against human C1-inh to a protein in equine plasma (lanes 1 to 4). This image is representative of the results found in several blots



Mw = molecular weight markers, Co = positive control [purified human C1-inh], lanes 1 → 4 = plasma from four different horses. Blot probed with a goat anti-human C1-inh polyclonal antibody

Objectives

- To develop an immunoassay capable of measuring C1-inh in plasma samples from horses.
- To correlate those levels with severity of clinical disease (especially endotoxaemia) and outcome in terms of morbidity and mortality.

Materials

Abcam Ltd.: Antibody to human Complement protein C3c [AB 8783]

Biorad: Precision Plus protein standards [161-0374]

Insight Biotechnology, PO Box 500, Wembley, Middlesex, HA9 7YN: Polyclonal antibody to kallikrein [BP 572]

Invitrogen Ltd.: SeeBlue pre-stained standard [LC 5925]

Severn Biotech Ltd.: custom peptide generation/ antisera production, Acrylamide/ Bis-acrylamide [20-2100-10]

Sigma Chemical Co. Ltd., Poole, Dorset, UK: Antibody to human C1-inh [C 8159], BCIP/ NBT tablets [B 5655], kallikrein from porcine pancreas [K 3627], Trypsin inhibitor type I-S [T 9003], Protein A Agarose pre-packed column [PAI-1EA], methylamine hydrochloride [M 0505], Sigmamarker [M 3788], rabbit polyclonal anti-goat IgG antibody [A 4062], Complement C1 esterase [C 2412], Complement protein C1q [C 1740], TEMED

Sigma-Genosys Ltd.: custom peptide generation/ antisera production

Methods

Generic methods

Sodium dodecyl sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) standard protocol using the BioRad Miniprotean 3 system

Gels used were denaturing (SDS), discontinuous (resolving and stack gels) and single acrylamide concentration (7.5% or 8.0% unless otherwise stated).

Sample Buffer

30.625 ml distilled water

3.125 ml 1M Tris-HCl pH 6.8

5.0 ml Glycerol

10.0 ml 10% SDS

1.25 ml 0.05% (w/v) bromophenol blue

Running buffer (10x)

15.0 g Tris

72.0 g Glycine

5.0 g SDS

To make 8% SDS-PAGE gels (x4):

Resolving gel:	Stacking gel:
9.3 ml distilled water	2.7 ml distilled water
5.3 ml 30% acrylamide mix	0.67 ml 30% acrylamide mix
5.0 ml 1.5M Tris (pH 8.8)	0.5 ml 1.0M Tris (pH 6.8)
0.2 ml 10% SDS	0.04 ml 10% SDS
0.2 ml 10% ammonium persulphate (APS)	0.04 ml 10% APS
0.012 ml N, N, N', N'-Tetramethylethylenediamine (TEMED)	0.004 ml TEMED

1. Samples were incubated at 37°C for 1 hour with an equal volume of sample buffer (non-reducing). For reducing conditions see details for each experiment. Where DTT was used to reduce samples, these were incubated as above; for β-mercaptoethanol reduction, samples were boiled in reducing sample buffer for 3 minutes.
2. Each gel was electrophoresed at a constant 200V until the dye front reached the bottom of the gel. It was either Coomassie stained or Western Blot transferred

Protein transfer from gel to polyvinyldifluoride (PVDF) paper using the BioRad Miniprotean system

Blotting buffer

3.03 g Tris

14.4 g Glycine

800 ml distilled water

200 ml methanol

Stored on ice/ water

1. PVDF paper was cut to the appropriate size to cover the gel to be transferred (8.5cm x 6.5 cm for a full gel). An appropriate number of filter papers were also cut.
2. The PVDF paper was soaked in methanol for 30 min prior to use.
3. For each 'blotting sandwich' two sponges, and 2 x 6 filter papers were soaked in blotting buffer and then each sandwich was made up as follows; clear support, sponge, 6 x filter papers, PVDF paper, gel, 6 x filter papers, sponge, black support.
4. The blot was then transferred for 1 hour at 150V.

Coomassie staining

0.2% Coomassie stain	Destain
1 g Coomassie blue	1.5 l methanol
150 ml methanol	3.15 l distilled water
50 ml glacial acetic acid	350 ml glacial acetic acid
300 ml distilled water	

1. The gel was placed in a petri dish containing 0.2% Coomassie stain and agitated gently for at least 2 hours.
2. The Coomassie stain was poured away and several changes of destain solution were added to the petri dish containing the gel and the dish was gently agitated.
3. The gel was rehydrated in distilled water for 12 hours before drying.

Western Blotting

1. The PVDF paper was blocked by agitation in 5% skimmed milk in PBS/Tween for 1 hour at room temperature (RT).
2. The blotted paper was probed with the primary antibody/ antisera in PBS/ Tween (unless otherwise stated) for 1 hour at RT by gentle agitation.
3. The blot was probed with the secondary antibody conjugated with alkaline phosphatase (diluted in PBS/ Tween)
4. Appropriate controls were also probed (e.g. secondary antibody only)
5. All blots were washed in PBS/ Tween 3x for 10 min between each antibody step.
6. Finally, alkaline phosphate substrate (5-bromo-4-chloro-3-indolyl phosphate [NCIP] tablets re-constituted to manufacturer's instructions) was added to each blot and the colour developed for 10 minutes.

Enzyme-linked Immunosorbant Assay (ELISA)

Carbonate buffer

0.1M Na_2CO_3 and 0.1M NaH_2CO_3 combined until the pH was 9.6

Glycine buffer

7.5 g 0.1M glycine

100 mg $MgCl_2.6H_2O$

70 mg $ZnCl_2$

Combined and made up to 500 ml and pH 10.4 using distilled water.

Standard protocol

1. Serial dilutions of antigen/ peptide in carbonate buffer were made and 50 µl loaded into each well on microtitre ELISA plate as required
2. The plate was then incubated for 2 hour at 37°C and then overnight at 4°C
3. The plate was then washed 3x with PBS
4. The plate was blocked with 100 µl of 1% BSA/PBS/Tween per well, followed by incubation for 1 hour at 37°C and then 30 min at 4°C
5. The plate was washed 3x with PBS/Tween
6. Serial dilutions of the primary antibody/antisera were made up in PBS/Tween and 50 µl loaded into each well on plate as required
7. The plate was incubated for 1 hour at 37°C and then 30 min at 4°C
8. The plate was washed 3x with PBS/Tween
9. Serial dilutions of the secondary antibody conjugated with alkaline phosphatase were made up in PBS/Tween and 50 µl loaded into each well on plate as required
10. The plate was then washed 3x with PBS/Tween
11. Then 50 µl of $1mgml^{-1}$ of alkaline phosphatase substrate (Sigma 104) in cold (4°C) glycine buffer was added to each well
12. The plate was then incubated at 37°C in the dark and was read intermittently as required at 405 nm on a plate reader to determine the optical density change.

Testing of antisera to human C1-inh for cross reactivity against equine C1-inh

Three different polyclonal antibodies against human C1-inh were tested for cross reactivity against equine C1-inh. Two commercially available polyclonal antibodies against human C1-inh were tested (Sigma Chemical Co. Poole, Dorset & Diasorin Ltd, Wokingham, UK). A third polyclonal antibody (named INCA) was donated by Dr Ineke Bos from Sanquin Research, Holland. The first and third antibodies are designed for use in ELISAs to detect human C1-inh. The Diasorin antisera is designed to be used in a nephelometric (inhibition of immunoprecipitation) assay for detecting C1-inh levels in human plasma.

Objective

- To test the antisera using Western blots for cross reactivity with equine C1-inh.

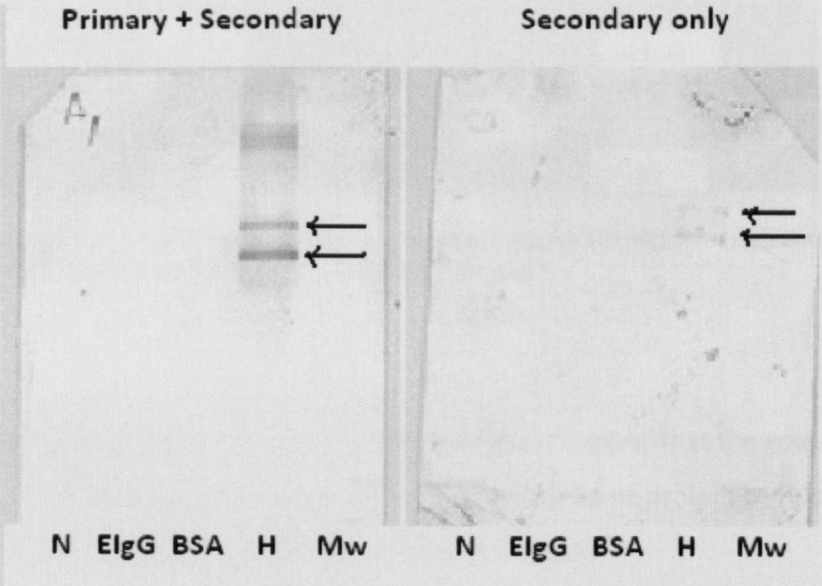
Western blotting

All gels were cast, ran, transferred, blots probed and developed as described above. Important differences in materials and methods are detailed in the results legends.

Results

Diasorin polyclonal antibody

Figure 1.2 Western blots probed by Diasorin polyclonal antibody against human C1-inh



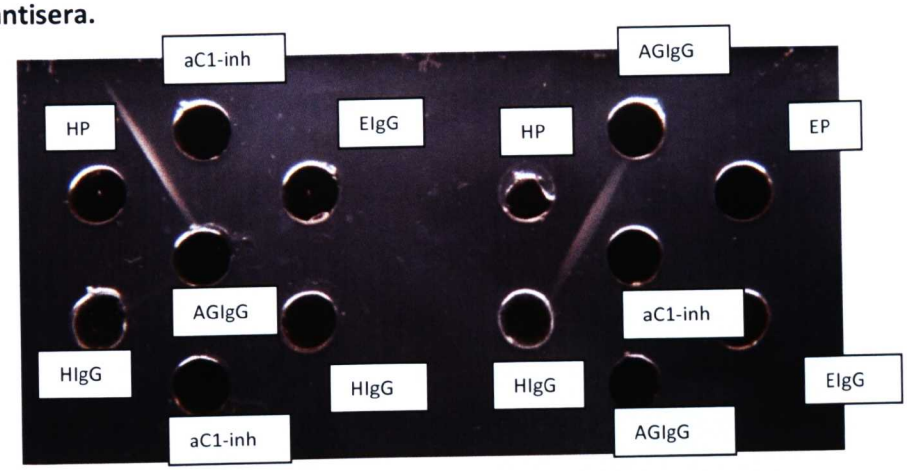
Primary antibody: Diasorin goat antisera against human C1-inh [1:1000] (diluted in PBS/ Tween), Secondary antibody: rabbit anti-goat IgG conjugated with alkaline phosphatase [1:30000] diluted in PBS/ Tween.
N = normal horse plasma, EIgG = equine IgG, BSA = bovine serum albumin, H = human plasma, Mw = molecular weight markers* * as no markers are identifiable, no molecular weights are shown in the picture.
Arrows: point to two similar bands seen on both the blots

Most of the proteins appeared to be transferred when comparing the pre and post-transfer gels. There were two bands in the lane that had the human plasma control as well as some non-specific staining at the higher molecular weights. There was no obvious reaction to anything specific in equine plasma. It also appeared that the secondary antibody was cross-reacting with something in human plasma; this effect was amplified in the presence of the primary antisera.

Double Immuno-diffusion

A 1% agarose gel was made up and allowed to cool and solidify on a glass base. Ten holes were punched into the gel in a defined pattern. 20 µl each of neat human (HP) and equine (EP) plasma, human (HIgG) and equine (EIgG) IgG, neat antisera against human C1-inh (aC1-inh) and anti-goat IgG (diluted to 1:100 in PBS/ Tween) (aGIgG) were added to the wells. The gel was then incubated at 37°C overnight.

Figure 1.3 Double immunodiffusion results showing reactivity between human plasma and anti C1-inh antisera.



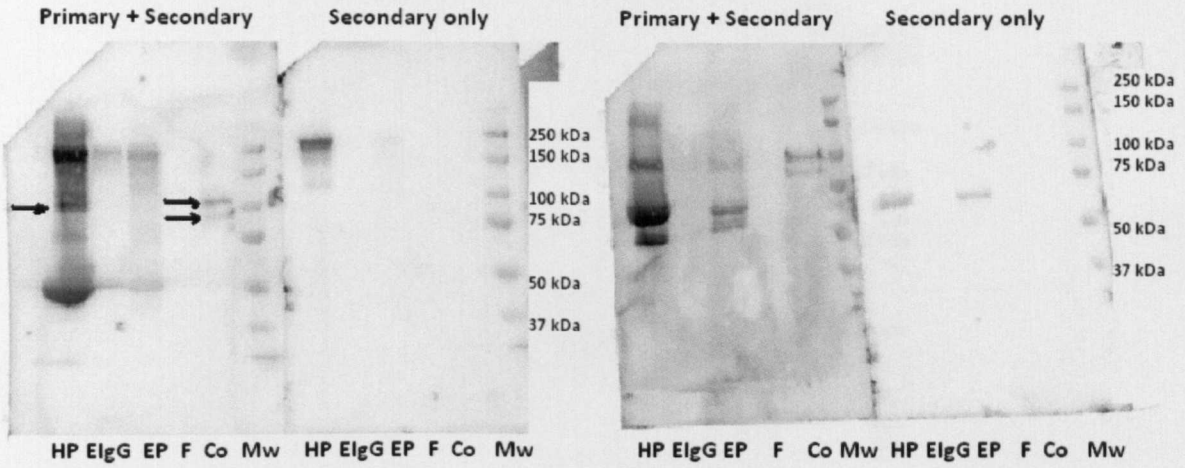
HP = human plasma, EP = equine plasma, HIgG = human IgG, EIgG = equine IgG, aC1-inh = neat antisera against human C1-inh, AGIgG = anti-goat IgG (diluted to 1:100 in PBS/ Tween)

Conclusion

These results, along with the results from the Western Blots suggest that the goat antisera against human C1-inh does not cross react with any specific equine protein components (e.g. equine C1-inh) and thus were of no further use for this project.

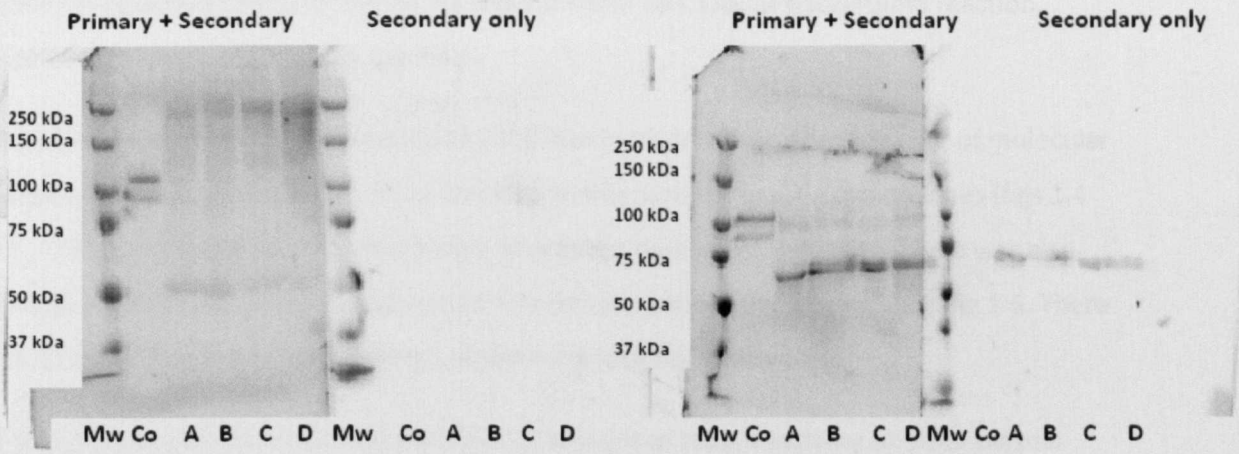
'INCA' polyclonal antibody

Figure 1.4: Western blots of 'INCA' polyclonal anti human C1-inh antibody against various protein solutions. Left: Non-reduced blot Right: Reduced blot



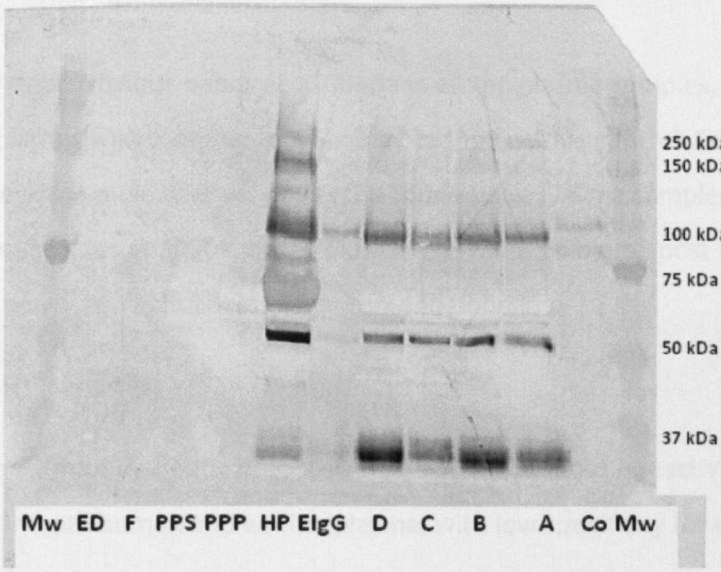
Primary antibody = lapine polyclonal antibody against human C1-inh (INCA) (1:2000), secondary antibody = anti rabbit IgG conjugated with alkaline phosphatase (1:10000)
HP = human plasma, EIgG = equine IgG, EP = equine plasma, F = pooled fractions from jacalin agarose chromatography, Co = purified human C1-inh, Mw = molecular weight marker, arrows = bands corresponding to C1-inh

Figure 1.5: Western blots testing 'INCA' polyclonal anti human C1-inh antibody against plasma from several horses. Left: Non-reduced blot Right: Reduced blot



Primary antibody = lapine polyclonal antibody against human C1-inh (INCA) (1:2000), secondary antibody = anti rabbit IgG conjugated with alkaline phosphatase (1:20000*)* weaker secondary concentration utilised to reduce background staining
Mw = molecular weight marker, Co = purified human C1-inh, A = horse A, B = horse B, C= horse C and D = horse D
Horse A and B = Colic cases: small intestinal lipoma (clinically endotoxaemic)
Horse C = Colic case: large colon displacement (not clinically endotoxaemic)
Horse D = foot penetration (not clinically endotoxaemic)

Figure 1.6: Western blot of reduced samples probed with: primary antibody = INCA (biotinylated) (1:2000), developed with extravadin and alkaline phosphatase substrate



Mw = Molecular weight marker, ED = end dialysis pooled sample from Jacalin Agarose chromatography, F = pooled fractions from Jacalin Agarose chromatography, PPS = post-PEG precipitation supernatant, PPP = post-PEG precipitation precipitate, HP = human plasma, EIgG = equine IgG, D = horse D plasma, C = horse C plasma, B = horse B plasma, A = horse A plasma

Summary of results for INCA

INCA reacted to purified human protein on both reduced and non-reduced blots. INCA appeared to detect C1-inh in human plasma but there was a lot of background reaction, apparently to immunoglobulins (fig 1.4).

There was a very faint band at around the 100 kDa mark and over a broad range of molecular weights between >100 kDa to around 250 kDa in non-reduced equine plasma lanes (figs 1.4 and 1.5). More obvious bands were visible in reduced equine plasma lanes. There was also staining in thick bands between 50 and 65 kDa on the reduced horse samples in fig 1.5. There was no obvious difference in staining between the four horse samples.

As with human plasma, there was a significant amount of cross-reactivity to other plasma constituents in both the reduced and non-reduced blots. There was some reactivity from the INCA antibody for the non-reduced equine IgG lane (fig 1.4).

The secondary antibody appeared to react at 1:10000 with non-reduced human plasma and reduced human and equine plasma (fig 1.4). The molecular weights of the proteins the secondary antibody was reacting with indicate the proteins could be immunoglobulin fractions. The secondary antibody showed little cross reactivity at the 1:20000 concentrations on the non-reduced samples but did show reactivity for some proteins at around 75 kDa in the reduced horse samples (possibly IgG heavy chain fragments) (fig 1.5).

There was no reactivity for the pooled fractions (from the jacalin agarose chromatography: see chromatography chapter) from any antibody.

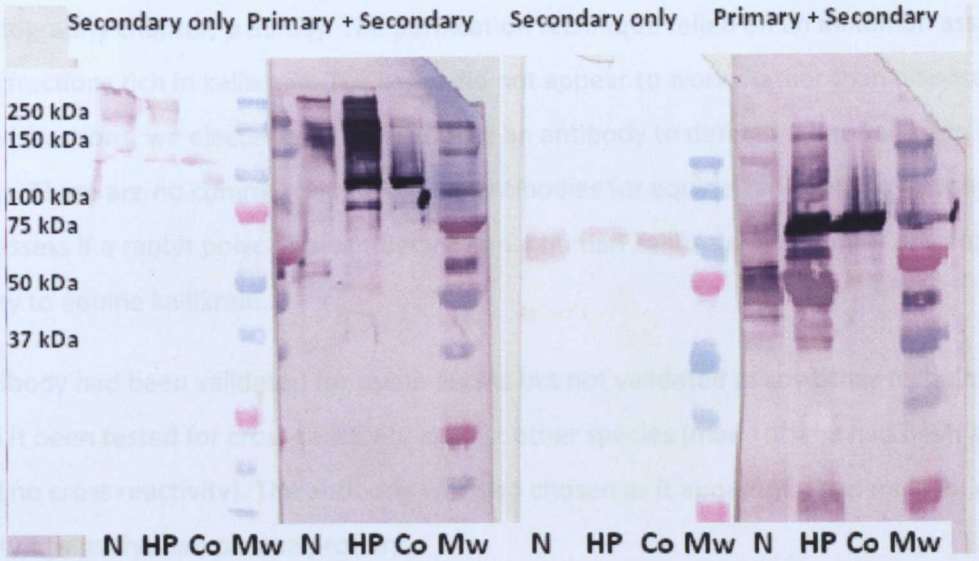
In fig 1.6, there are also obvious bands at ~100kDa in all the plasma samples, with the human plasma displaying bands with a similar morphology to the positive control. Equine IgG had a fainter band at the same molecular weight as the four equine plasma samples. All the plasma samples had obvious bands at 50kDa and ~25kDa in the reduced blots, most likely corresponding to heavy and light chains from immunoglobulins.

Conclusion

The INCA polyclonal antibody clearly demonstrated significant cross – reactivity to immunoglobulins in both human and equine plasma, with low specificity for equine C1-inh

Sigma polyclonal antibody

Figure 1.7: Western blots of non-reduced (left) and reduced (right) samples probed with: primary antibody = caprine polyclonal against human C1-inh [1:1000], secondary antibody = anti-goat IgG conjugated with alkaline phosphatase [1:30000]



Mw = molecular weight marker, Co = purified human C1-inh, HP = human plasma, N = horse plasma

The primary antibody showed high affinity for human C1-inh in non- reduced and reduced blots. On the non-reduced blot the primary antibody appeared to bind to a lot of material between 150 and 250kDa in both plasma samples but especially in the human plasma sample. As the secondary antibody had also shown some cross-reactivity in this region it was hard to discern the relative contribution to the staining in the primary and secondary blot.

There were no obvious distinct bands on the non-reduced blot for any of the equine samples. On the reduced blot the most distinct reaction appeared at between 50 and 75kDa. In this experiment, the secondary antibody seemed to react with constituents in all the sample lanes in both the reduced and non-reduced blots.

Conclusion

These findings indicate that this primary antibody is unlikely to be of any use in developing assays to measure C1-inh in equine plasma or to aid in purification of C1-inh from equine plasma as no bands appeared ~ 105 kDa where equine C1-inh was expected and the primary and secondary antibodies appear to be cross-react with other plasma constituents

Use of a rabbit polyclonal antibody against human kallikrein to attempt to detect equine kallikrein in chromatography fractions

The rationale for purifying equine kallikrein from plasma was so it could be utilised as a substrate in an inhibition assay to detect equine C1-inh in chromatography fractions (see chromatography chapter, p 63-69). The purification technique relied on an inhibition assay to identify fractions rich in kallikrein. The assay did not appear to work. Rather than dispose of the collected fractions, we elected to attempt utilise an antibody to detect equine kallikrein in the fractions. There are no commercially available antibodies for equine kallikrein, so the objective was to assess if a rabbit polyclonal antibody against human kallikrein would have any cross reactivity to equine kallikrein.

The antibody had been validated for use in ELISAs but not validated in any other techniques, nor had it been tested for cross-reactivity against other species (many others had been and showed no cross reactivity). The antibody was also chosen as it apparently had minimal cross reactivity against human plasma proteins.

Objective:

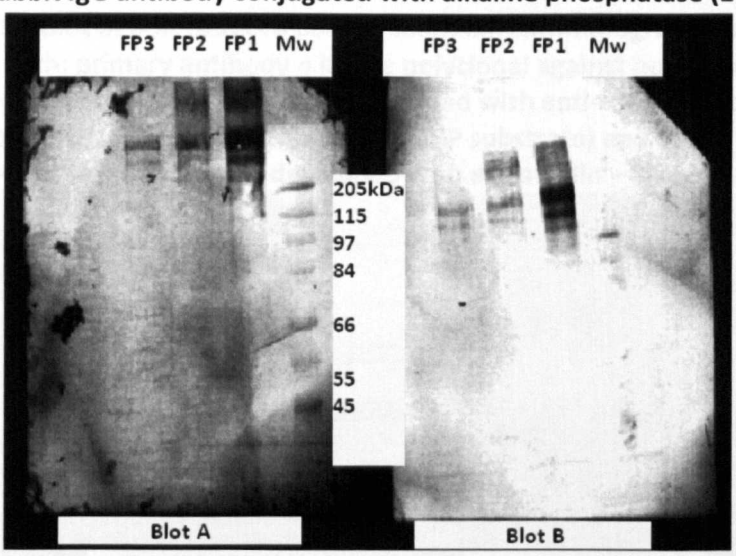
- To determine if an anti-human kallikrein antibody could detect equine kallikrein using Western blotting

Gel running and transfer

As described above. The samples loaded were; A = pooled fractions 19-28 (equal volume to sample buffer), B = pooled fractions 29-37 (equal volume to sample buffer), C = pooled fractions 38-40 (equal volume to sample buffer). The gels were run and then cut in half; one half of each gel underwent Coomassie staining as described above. The other half of each gel was transferred onto PVDF paper as described above. The blots then underwent Western Blotting.

Results

Figure 1.8: Western blot of non-reduced pooled kallikrein chromatography fractions probed with: primary antibody = lapine polyclonal against human kallikrein (Acris)(1:500), secondary antibody = anti-rabbit IgG antibody conjugated with alkaline phosphatase (1:10000).



FP1 = pooled fractions F19-F28, FP2 = pooled fractions F29-F37, FP3 = pooled fractions F38-F40 from kallikrein purification experiment (see chromatography chapter)

There were bands of similar appearance but of varying intensity across all three pooled fractions between 116 and 205 kDa with the main band ~160 kDa. Human plasma kallikrein is ~80 kDa so there was a possibility that the bands at 160 kDa could be dimers or complexes of immunoglobulins and kallikrein as the purification technique is supposed to separate kallikrein along with immunoglobulins.

Thus, we decided to test to see if the bands were caused by immunoglobulins as we had not at this stage carried out a secondary only antibody probe of the blots.

Stripping of antibodies from blots

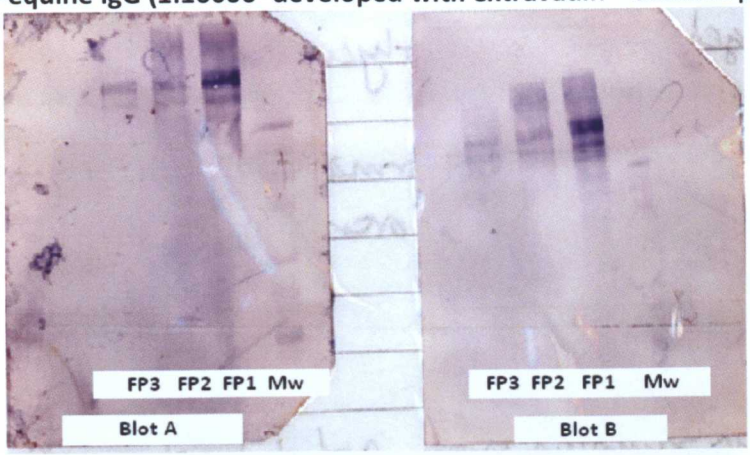
All the antibodies were stripped from both blots using the following technique;

25 ml of 100mM Tris HCl pH 7.0, 0.385 g of DTT, and 5 ml of 20% SDS were mixed and made up to 50 ml with distilled water before adding to the blots which were then gently agitated for 30 min at 65°C and then washed with PBS/Tween for 3x 10 min. The blots were re-blocked with 5% skimmed milk in PBS/ Tween for 1 hour at RT and re-probed with fresh antibodies.

The background stain from the alkaline phosphatase substrate had not been washed away from the blots. Thus, to distinguish any staining from the second probe, blot A was probed with anti-rabbit IgG conjugated with horse radish peroxidise (1:10000- developed with HRP substrate) and blot B was probed with biotinylated anti-equine IgG (1:10000- developed with extravadin – alkaline phosphatase).

Results

Figure 1.9: Western blot of non-reduced pooled kallikrein chromatography fractions after IgG stripping probed with: primary antibody = lapine polyclonal against human kallikrein (Acris)(1:500), secondary antibody = blot A was probed with anti-rabbit IgG conjugated with horse radish peroxidise (1:10000- developed with HRP substrate) and blot B was probed with biotinylated anti-equine IgG (1:10000- developed with extravadin – alkaline phosphatase).



FP1 = pooled fractions F19-F28, FP2 = pooled fractions F29-F37, FP3 = pooled fractions F38-F40 from kallikrein purification experiment (see chromatography chapter).

There was no new staining from the either of the blots.

Conclusion

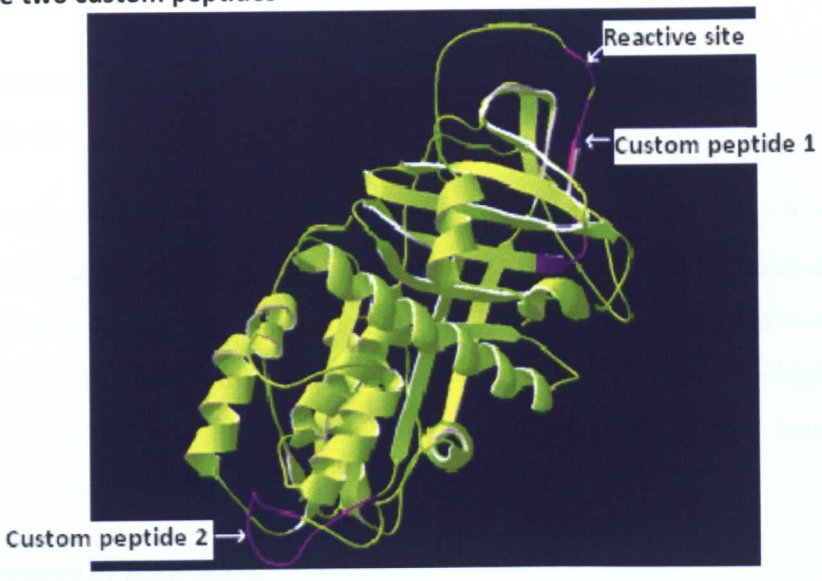
The conclusion was that the original staining had not been due to IgG and therefore the primary antibody had bound to something other than immunoglobulins which we hoped would be kallikrein. We then decided to try to make an affinity column using the primary antibody to see if we could isolate kallikrein from equine plasma (see chromatography chapter, p70).

Testing of antisera raised against custom peptides generated by Sigma-Genosys

The available anti-C1-inh antibodies tested had not shown enough (or any) cross-reactivity and selectivity to be suitable for any immunoassay of purification technique for equine C1-inh. Molecular biological techniques were utilised to determine the nucleotide and amino acid sequence of equine C1-inh with a view to try to model the 3D structure of the protein. Building a model helped to identify differences in the structure between the equine and the human models and to identify areas on the human model that could act as epitopes. Amino acid sequences for the comparable area on the equine model and another area that was on the reactive loop of the equine model were selected to be made into custom peptides (see molecular biology chapter). Those custom peptides were made by a commercial company (Sigma-Genosys Ltd, Haverhill, UK) and inoculated into four rabbits to raise antisera against them.

Sequences: Custom peptide 1 = S1: **LIFHVDQPF** Custom peptide 2 = S2: **LCRMTEDPQDLQV**

Figure 1.10: Picture of 3D model of equine C1-inh generated by Swissmodel depicting the location of the two custom peptides



Custom peptide synthesis and immunisation protocol

The peptides were ordered and made to the following specification by Sigma-Genosys. Detailed descriptions of their peptide generation HPLC protocols are available on their website [http://www.sigma-genosys.eu.com]. The peptides were conjugated with Keyhole Limpet Haemocyanin (KLH) to improve their antigenicity (i.e. the peptides were linked to a larger molecule as, on their own; they may be too small to provoke an antibody response).

Custom peptide 1 = S1: **CLIFHVDQPF**

Theoretical mass:	1218.46	Observed mass:	1216
Amount shipped:	15 mg (lyophilized powder)		
Conjugation:	Keyhole Limpet Haemocyanin		

Custom peptide 2 = S2: **LCRMTEDPQDLQV**

Theoretical mass:	1419.66	Observed mass:	1419
Amount shipped:	15 mg (lyophilized powder)		
Conjugation:	Keyhole Limpet Haemocyanin		

Immunisation protocol for both peptides

The protocols were approved by the IACUC designated member in the institution and is summarised in the appendix at the end of this chapter. Each peptide (S1 and S2) was injected into two rabbits; S1 into B87 and B88, S2 into B89 and B90.

Solubilisation of peptides

The peptides were lyophilised prior to dispatch by the company. Their quoted purity was quite poor (S1 = 59%, S2 = 55%).

Following the manufacturer's instructions, S1 dissolved easily in PBS to make a 5 $mgml^{-1}$ solution (15 mg of peptide powder dissolved in 3 ml PBS), but S2 did not dissolve despite the addition of a further 2 ml of PBS (= 3.75 $mgml^{-1}$). Glacial acetic acid was added to make a final concentration of 0.1% but that still did not dissolve all the powder. Due to the high level of impurities in S2, it was hoped that the undissolved powder represented these impurities and enough of the custom peptide had dissolved.

Testing of antisera at each bleed

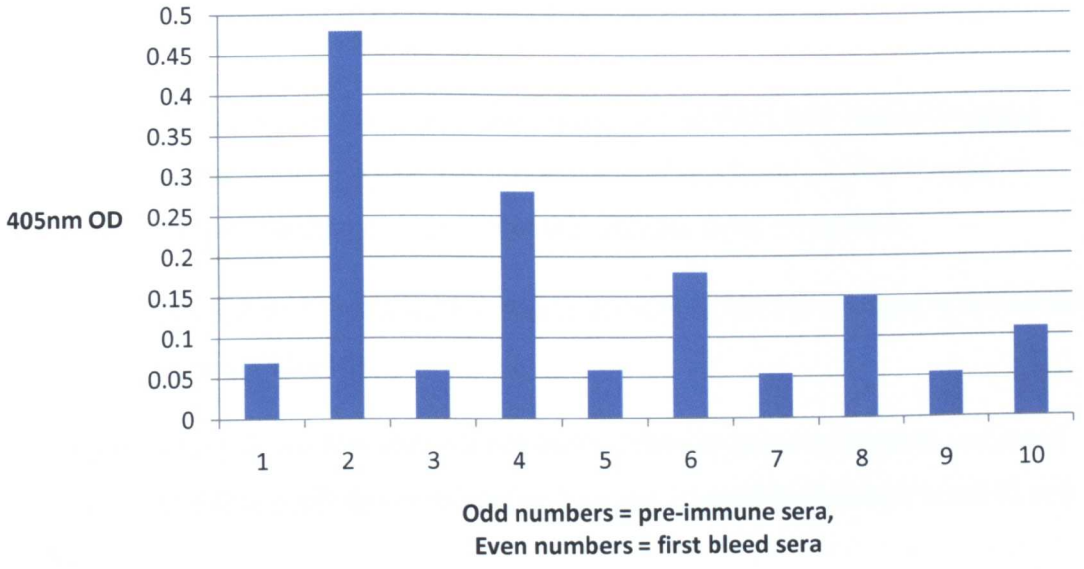
ELISAs

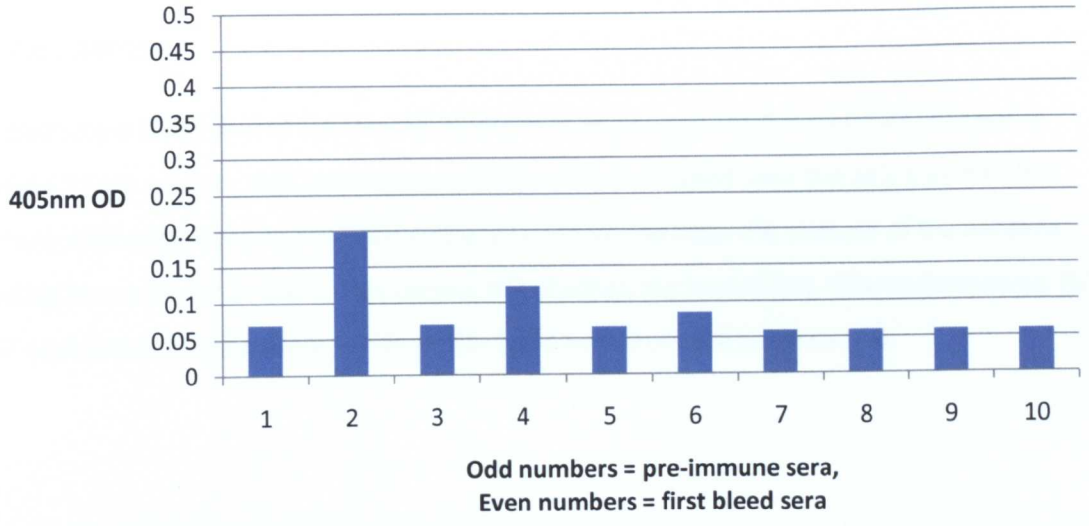
The ELISAs were run under our standard protocol described at the start of the chapter. The details are: The peptide concentrations were achieved by making a 5 μgml^{-1} solution from the stock (5 $mgml^{-1}$ and 3.75 $mgml^{-1}$) and serially diluting from the 5 μgml^{-1} solution. The antisera concentrations were made by making a 1:500 stock solution and diluting from that stock. The

secondary antibody was an anti-rabbit IgG conjugated with alkaline phosphatase and was used at a 1: 10000 dilution. The plates were read at 30, 60, 90, 120 and 150 min

Results

Figure 1.11: Example of ELISA results for first bleed. Top chart = peptide S1 at 1µg ml^{-1}/ B88 at 150min, Bottom chart = peptide S2 at 1µg ml^{-1}/ B90 at 150min.





Antisera dilutions were; series 1,2 = 1/500, series 3,4 = 1/1000, series 5,6 = 1/2500, series 7,8 = 1/5000, series 9,10 = 1/10000. Negative control not included on chart

The antisera from rabbits immunised with S1 (B87 and B88) had a stronger reaction compared with antisera from rabbits immunised with S2 (B89 and B90).

In our laboratory an OD $\Delta \geq 0.5$ is necessary before a sera titre is regarded as being positive. In this context, the antisera result from B88 was only positive at 1:500 and at 1 $\mu g ml^{-1}$ coating antigen, and the antisera result from B90 was not positive at all. The results from the other two rabbits were similar (B87 similar results to B88 and B89 similar results to B90) so are not included to avoid excessive data presentation. Standard interpretation of ELISAs is that a positive result is if the mean OD reading at the end of the analysis is greater than +2 standard deviations of the mean of the control.

The bleed #2 antisera from the two rabbits immunised with S1 (B87, B88) had not changed much in terms of their maximum OD Δ, but the antisera did react over a broader range of peptide concentrations, possibly as a result of more epitopes being recognised.

The bleed # 2 antisera from the two rabbits immunised with S2 had not changed in comparison to the ELISA results from bleed #1.

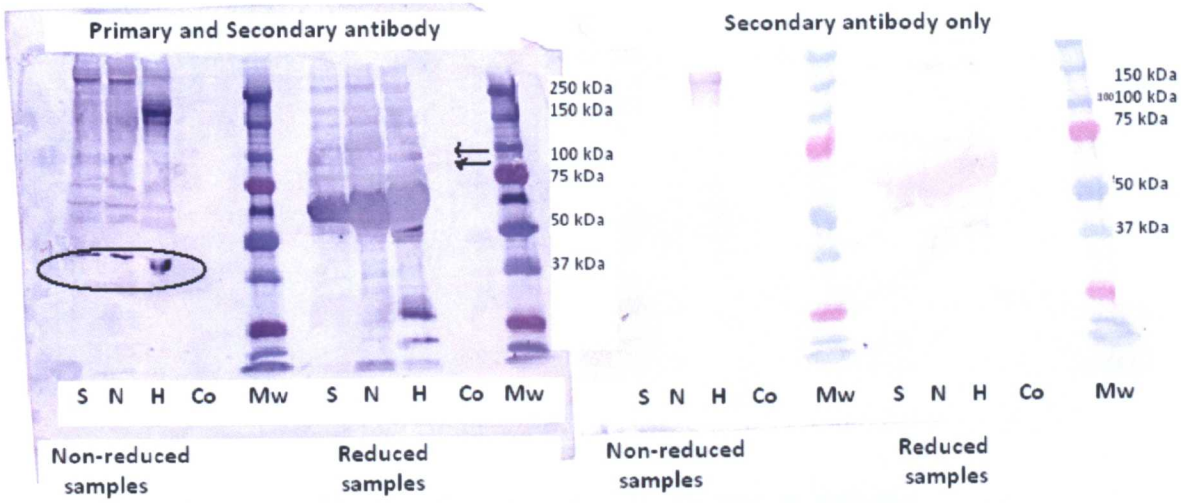
The ELISA results for B88 and B90 were not noticeably different between bleed #2 and bleed #3. The results for B87 and B89 demonstrated an increase in reactivity between bleed #2 and bleed #3.

There was no change in reactivity between the terminal sera and bleed #3 sera.

Western Blotting

Antisera from B88 bleed #1 was tested by Western blotting to see if it could identify entire natural C1-inh protein. N.B. more protein than usual was loaded onto the gels and the blots probed with relatively concentrated antisera (1:50) to maximise the chances of the antisera binding to any C1-inh present. Throughout this chapter, the human (H), non-endotoxaemic (N) and endotoxaemic (S) plasma ran on all the blots were from the same source.

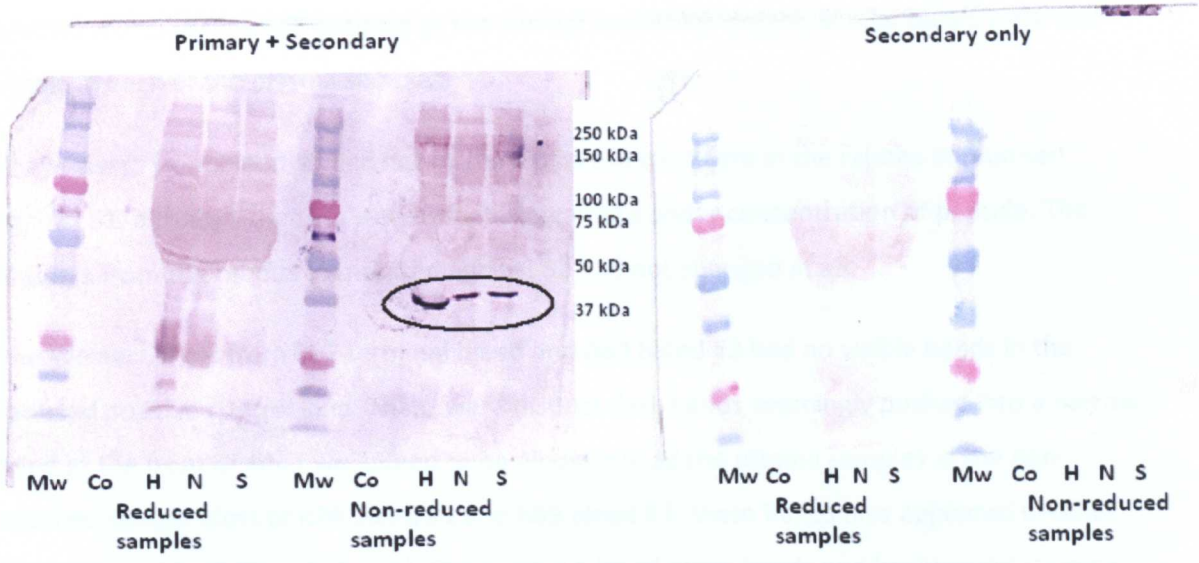
Figure 1.12: Western blot of reduced and non-reduced samples probed with antisera from the first bleed.



Primary antibody= B88 bleed #1 antisera (1:50), secondary antibody = anti-rabbit IgG conjugated with alkaline phosphatase (1:10000). Arrows point to bands in positive control lane, oval highlights strongly staining bands in front of albumin. Mw = molecular weight marker, Co = purified human C1-inh, H = human plasma, N = plasma from a non-endotoxaemic horse, S = plasma from an endotoxaemic horse.

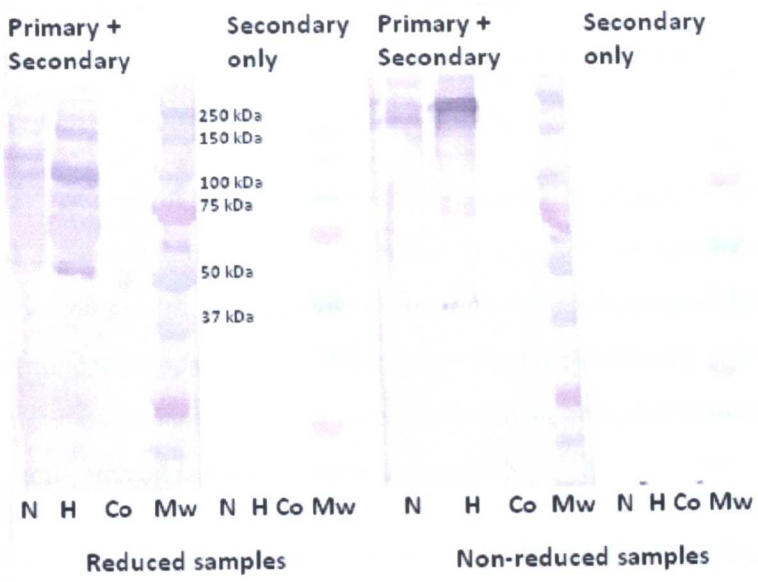
We used Western blotting to test the sera from bleed #3; in particular we were interested in testing B89 as the sera from bleed #3 had appeared to react more strongly to the peptide S1 in comparison to sera from bleed #2.

Figure 1.13: Picture of Western blot of reduced and non-reduced samples from bleed #3;



Primary antibody = B89 bleed #3 antisera (1:100), secondary antibody = anti-rabbit IgG conjugated with alkaline phosphatase (1:10000). Oval highlights strongly staining bands in front of albumin. Mw = molecular weight marker, Co = purified human C1-inh, H = human plasma, N = plasma from a non-endotoxaemic horse, S = plasma from an endotoxaemic horse.

Figure 1.14: Picture of Western blot of reduced and non-reduced samples from the terminal bleed;



Primary antibody = B87 terminal antisera (1:100), secondary antibody = anti-rabbit IgG conjugated with alkaline phosphatase (1:10000). Mw = molecular weight marker, Co = purified human C1-inh, H = human plasma, N = plasma from a non-endotoxaemic horse

Conclusion

After bleed #1, the ELISA results suggested that the rabbits immunised with S1 were beginning to mount an anti-C1-inh antibody response. The rabbits immunised with S2 had not yet responded very well. There were two positive control bands in the reduced Western blot probed with B88 bleed #1 antisera at the correct molecular weight. Similar bands were also visible in each of the plasma samples.

The antisera from bleed #3 had not increased its effective titre in the rabbits immunised against S1, although the sera were effective against a wider concentration of peptide. The antisera from the rabbits immunised against S2 had not changed at all.

The Western blots from B87 terminal bleed and B89 bleed #3 had no visible bands in the reduced positive control lane. There were distinct dark bands seemingly pushed into a narrow band at the front of what we judged to be albumin in all the plasma samples in the non-reduced sample blots of B88 bleed #1 and B89 bleed #3, these bands also appeared in other blots (e.g. at bleed #2: not shown). There were a lot of other bands and background staining which was unsurprising as we had used relatively concentrated antisera.

The secondary antibody seemed to react to reduced albumin in the plasma samples. The sera did not seem to demonstrate obvious cross reactivity for C1-inh and produced many bands on

the blots. Also, there appeared to be too much protein loaded into the wells (especially albumin) which lead to distortion of the way the proteins ran on the gel.

The terminal bleed from B87 had a similar band profile for plasma samples to the Sigma commercial anti human C1-inh under reducing conditions.

Using lower amounts of protein loaded onto the gels reduced the amount of non-specific reactivity from the secondary antibody. The antisera still appear to be binding to high molecular weight proteins (immunoglobulins?) in the non-reduced samples and to a number of proteins in the reduced samples including bands at ~65 kDa (albumin?), and bands at ~25 kDa (light chain immunoglobulins?). Interestingly, at the lower amounts of protein, the bands at ~39 kDa in the non-reduced samples are not apparent.

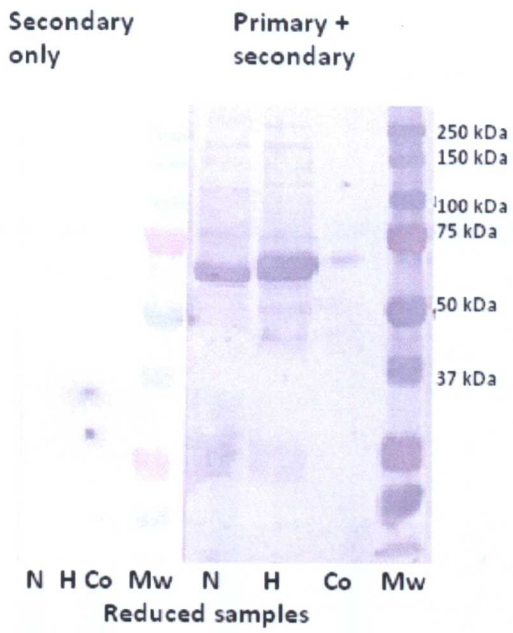
The antisera did not show enough specificity for equine C1-inh in plasma to be utilised for developing an immunoassay or for immunopurification of the protein from plasma

Effect of using 1% BSA/PBS/Tween to dilute antibodies prior to probing blots

The antisera from all the rabbits appeared to bind to a protein of a similar molecular weight to albumin in reduced plasma samples. We attempted to improve specificity of the antisera through the use of 1% BSA/PBS/Tween as the diluent buffer (c.f. PBS/Tween).

Results

Figure 1.15: Western blot of reduced samples testing the antibody diluent buffer with 1% BSA added:



Primary = B89 terminal antisera (1:100), secondary Ab = anti-rabbit IgG conjugated with alkaline phosphatase (1:10000). Mw = molecular weight marker, Co = purified human C1-inh, H = human plasma, N = plasma from a non-endotoxaemic horse.

Conclusion

The use of 1% BSA/PBS/Tween failed to improve specificity. The antisera apparently detected a band ~70 kDa in the Co lane; we are concerned that this could be 'spill over' albumin from the human plasma lane. The secondary antibody does not show much cross reactivity under these conditions.

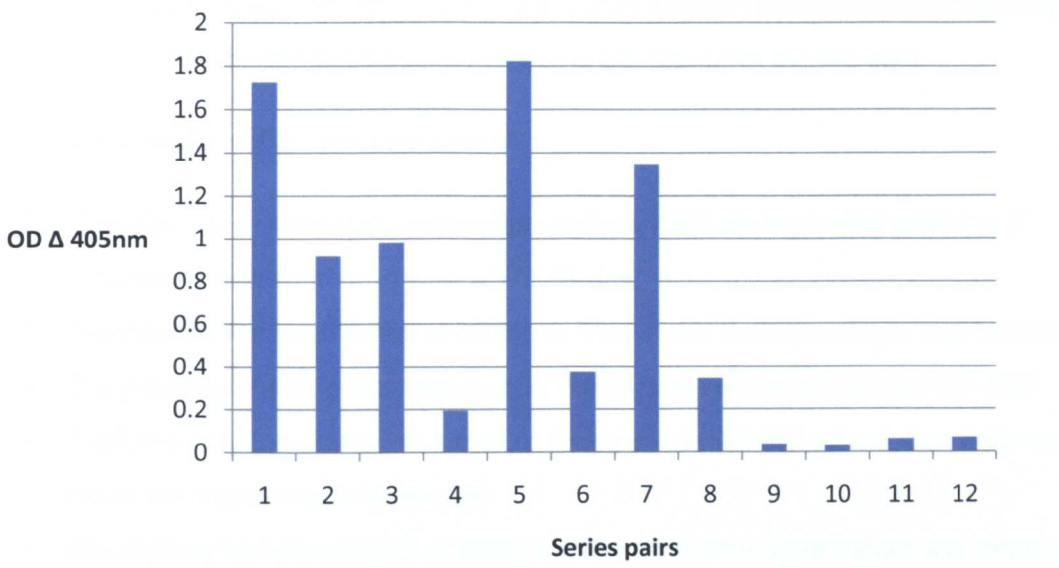
Use of ELISA to investigate cross reactivity of the rabbit antisera to albumin

The ELISA was run under our standard protocol described at the start of the chapter.

The peptides were made to 1 μgml^{-1} using carbonate buffer. Half of the antisera were diluted in PBS/Tween, the other half were diluted in 1% BSA/PBS/Tween. The secondary antibody was an anti-rabbit IgG conjugated with alkaline phosphatase and used at a 1: 10000 dilution.

Results

Figure 1.16: Graph of ELISA results to test the effect of incubating rabbit antisera against custom peptides with BSA



Peptide concentration = 1 μgml^{-1}
Antisera dilution 1:100
Series pairs: odd numbers antisera diluted in PBS/Tween, even numbers diluted in 1% BSA/PBS/Tween
Series 1,2 = B87 bleed #3; 3,4 = B88 bleed #3; 5,6 = B89 bleed #3; 7,8 = b90 bleed #3; 9,10 = negative control PBS/Tween, 11,12 = negative control 1% BSA/PBS/Tween

Conclusions

Incubation with BSA dramatically reduces the reaction of the antisera with their respective C1-inh peptides.

Purification of IgG from antiserum and coupling to a Sepharose 4B cyanide bromide column

The above experiment indicated that at least some of the antibodies present in the antisera from each of the rabbits were reacting with BSA; this supposition was also supported by our experience with the antisera in Western blotting. We attempted to separate BSA reactive antibodies from the antisera in the hope that remaining antibodies would show more selectivity to the peptides and ideally natural C1-inh.

Objective

- To precipitate IgG from the antisera and use an albumin coupled chromatography column to separate out albumin reactive antibodies from the IgG pool

40% salt precipitation of IgG from antiserum

1. Two volumes of saturated ammonium sulphate (SAS) solution were added to 3 volumes of antiserum drop-wise whilst stirring.
2. The mixture was centrifuged at 3000g for 15 min and the supernatant was removed
3. The precipitate was re-dissolved in 1.5x the original volume of the serum in PBS
4. 1 volume of SAS solution was added to 1 volume of the PBS/ IgG solution drop-wise whilst stirring to re-precipitate IgG
5. The mixture was centrifuged at 3000g for 15 min and the supernatant was removed
6. The precipitate was dialysed against 100x volumes of the coupling buffer (see below)
7. Read the OD at 280nm and calculated the $mgml^{-1}$ and total mg (divided OD 280 nm by 1.4)

Results

After dialysis the protein concentration for the IgG from each rabbit's sera was;

B87 = 11.6 $mgml^{-1}$ B88 = 6 $mgml^{-1}$ B89 = 9.8 $mgml^{-1}$ B90 = 5.1 $mgml^{-1}$

Coupling of Albumin to Sepharose 4B cyanide bromide (CNBr) column

1. The required amount of Sepharose 4B CNBr was weighed out [1g of lyophilized powder = ~3.5 ml final medium]
2. The powder was suspended in ice cold 1mM HCl. The medium was allowed to swell and was then washed for 15 min with more 1mM HCl through a sintered glass funnel (porosity G3); using ~200 ml of 1mM HCl per gram of powder.
3. The ligand (albumin) was dissolved in the coupling buffer [0.2M $NaHCO_3$ pH 8.8, containing 0.5M NaCl] to result in 6 ml of coupling buffer per gram of powder (5 mg of albumin per ml of gel was recommended by the manufacturer)
4. The coupling/albumin solution was added to the gel suspended in coupling buffer and mixed by rotating end-over-end for 1 hour at RT to allow the ligand to couple with the gel.
5. Excess ligand was then washed away with > 5 volumes of coupling buffer
6. Any remaining active $-NH_2$ groups on the gel were blocked by transferring the medium into 1M ethanolamine pH 8.0 and letting the gel stand for 2 hours
7. The gel was then washed with > 3 cycles of alternating pH, with > 5 gel volumes of each buffer [0.1M acetic acid/ sodium acetate pH 4.0, containing 0.5M NaCl followed by 0.1M Tris-HCl, pH 8.0 containing 0.5M NaCl]
8. The gel was equilibrated with 50mM sodium phosphate/ 250mM NaCl/ 5mM EDTA, pH 7.4 buffer
9. The serum/ IgG solution (in this case diluted 10x with PBS) was added to column after passing through sham treated column first to remove non-specific binding
10. High protein fractions (determined by 280 nm OD) were collected and pooled
11. Bound IgG was eluted from column using 0.5M acetic acid
12. Fractions collected and pooled that contained acetic acid were dialysed immediately against PBS
13. The column was washed with > 5 gel volumes of equilibration buffer before adding further samples.

Results

The following fractions were obtained and pooled according to peak protein concentrations as determined by 280nm OD absorbance. The initial serum protein concentrations are also included for comparison (serum = supernatant after first salt precipitation; serum A = supernatant after second salt precipitation).

Fractions	Protein concentration [via 280nm absorbance]
B87 serum	1.3 $mgml^{-1}$
B87 serum A	0.06 $mgml^{-1}$
B87 IgG pool	4.8 $mgml^{-1}$
B88 serum	1.6 $mgml^{-1}$
B88 serum A	0.07 $mgml^{-1}$
B88 IgG pool	3.1 $mgml^{-1}$
B88 IgG eluted pool 1	0.05 $mgml^{-1}$
B88 IgG eluted pool 2	0.05 $mgml^{-1}$
B89 serum	3.9 $mgml^{-1}$
B89 serum A	0.04 $mgml^{-1}$
B89 IgG pool	4.3 $mgml^{-1}$
B89 IgG eluted pool 1	0.05 $mgml^{-1}$
B90 serum	3.3 $mgml^{-1}$
B90 IgG	3.3 $mgml^{-1}$

ELISA to test anti-peptide IgG preparations

The ELISA was run under our standard protocol. The details are as follows.

The peptides were made to 1 μgml^{-1} using carbonate buffer. The antisera concentrations were made by making a 1:100 stock solution and serially diluting from that stock. Where the IgG solutions (e.g. serum or IgG pool) were > 1 $mgml^{-1}$ they were diluted in PBS/Tween to make 1 $mgml^{-1}$ solution for addition to the wells. The other IgG solutions were added at the concentration they were at but added in PBS/Tween. The secondary antibody was an anti-rabbit IgG conjugated with alkaline phosphatase and was used at a 1:10000 dilution.

Results

These results were obtained after a much longer incubation time (of the substrate) compared to the other ELISAs in this chapter i.e. 3 hours at 37°C followed by 15 hours at 4°C c.f. 250 min at 37°C.

The reactions of the IgG preparations were all weaker in comparison to the neat sera. Despite having ~20x less protein, the post elution IgG fractions reacted most strongly. These fractions should have contained the antibodies that bound to the column. The fractions that had the best reactions were B88IgGEP1, B88IgGEP2, B89SA and B89IgGEP1; these fractions were tested under Western Blotting using standard protocols described earlier.

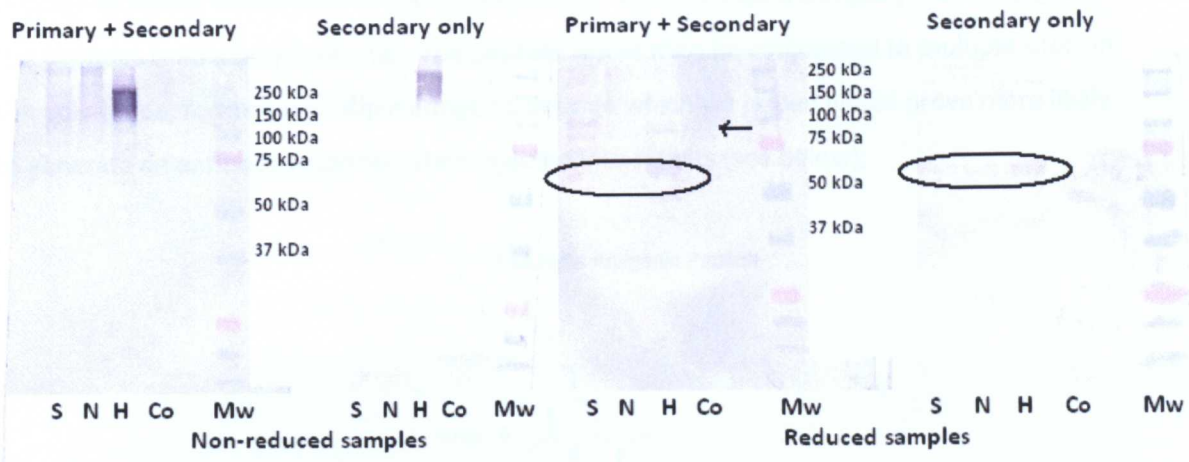
Testing of the pooled fractions B88IgGEP1 and B89IgGEP1 in Western blots

Western blotting

The blots were probed as described above using the following antibody solutions: e.g. B89IgGEP1 (1:200), secondary = anti-rabbit IgG antibody conjugated with alkaline phosphatase (1:10000).

Results

Figure 1.17: Western blots of immunoglobulin eluted sera [B89IgGEP1 shown only]:



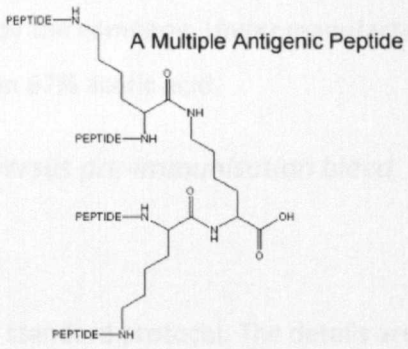
Primary antibody = B89IgGEP1 (1:200), secondary antibody = anti-rabbit IgG conjugated with alkaline phosphatase (1:10000).
Mw = molecular weight marker, Co = purified human C1-inh, H = human plasma, N = plasma from a non-endotoxaemic horse, S = plasma from an endotoxaemic horse.
Arrow: points to faint band ~100 kDa in purified human C1-inh sample lane.
Oval: outlines bands on both blots in plasma samples which are around 100 kDa mark.

Conclusions

Under non-reducing conditions there is very little apparent difference between the IgG pools tested or the secondary antibody. Under reducing conditions the secondary antibody seems to react with albumin. It is hard to discern how much this is responsible for the bands seen on the primary and secondary blot. The primary IgG solution from B89 appeared to be able to detect a faint band in the control lane where one would expect C1-inh to be. There were similar bands in all the plasma samples, as well as others of similar molecular weight. However, at least in the equine plasma samples, at least one of these bands was visible in the secondary antibody-only blot. It was concluded that the IgG purified sera would not be useful in developing an immunoassay for, or immunopurification of equine C1-inh in plasma.

Testing of antisera raised against the custom peptide generated by Severn-Biotech

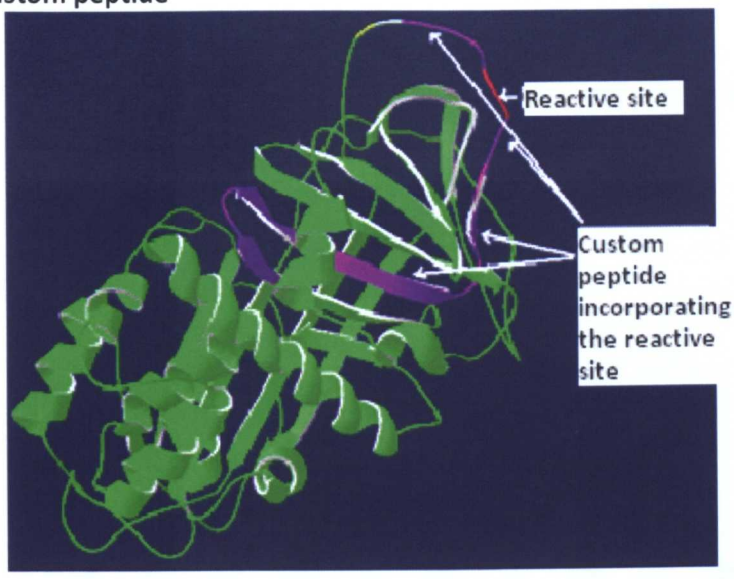
As the antisera raised against the custom peptides generated by Sigma-Genosys had proved unsuitable to assay or purify equine C1-inh, we elected to repeat the process but using a slightly different strategy. This time we used a longer peptide sequence which overlapped with the reactive site and the areas known to be antigenic on the human protein. We hoped that a longer peptide would be more likely to adopt a similar 3D structure to the structure the peptide sequence would form in the whole protein. We also used a company that conjugated the peptides onto a poly-lysine tail. The peptide would then be conjugated to multiple sites on the poly-lysine, forming a multiple antigenic peptide which we hoped would prove more likely to generate an antibody response when injected into rabbits (see below);



Custom peptide sequence:

Custom peptide = LIV1-MAP: **AVSVARNLLIFHVDQPFLEV [underlined = sequence from S1)**

Figure 1.18: Picture of 3D model of equine C1-inh generated by Swissmodel depicting the location of the custom peptide



The multiple antigenic peptide was custom generated by Severn-Biotech (Kidderminster, UK) using semi-preparative and preparative scale C18 reverse phase high performance liquid chromatography (more details: www.SevernBiotech.co.uk)

The residues of the custom peptide formed 54.9% by weight of the final peptide.

The immunisation protocol for LIV1-MAP is summarised in the appendix at the end of this chapter

The peptide was injected into two rabbits; B55 and B56.

Solubilisation of peptides

The peptides were lyophilised by the company. Under manufacturer's instructions, a stock solution of 5mgml^{-1} was made in 67% acetic acid.

Testing of sera from bleeds versus pre-immunisation bleed

ELISAs

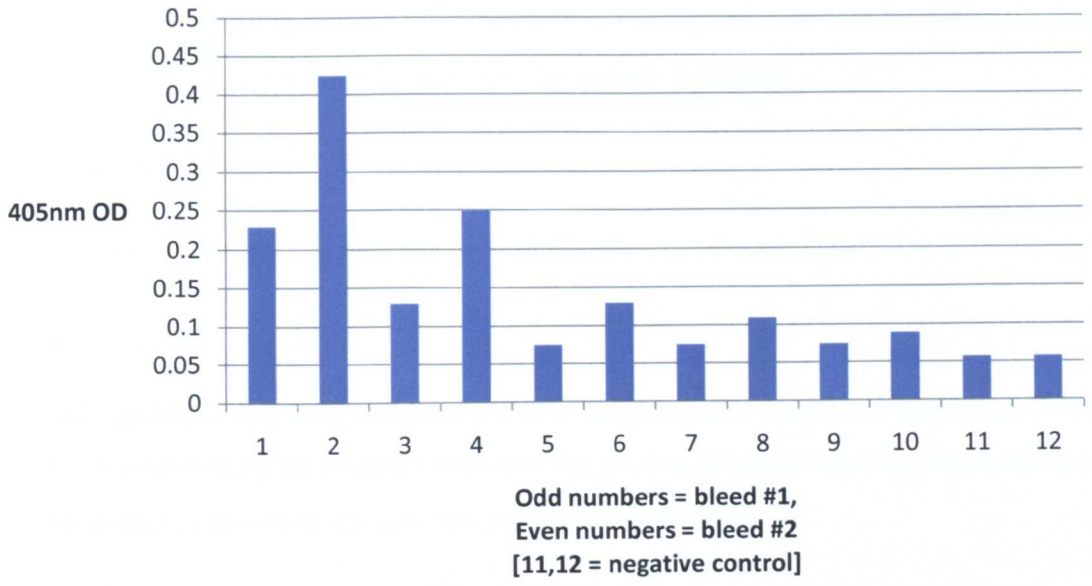
The ELISAs were run under our standard protocol. The details are as follows.

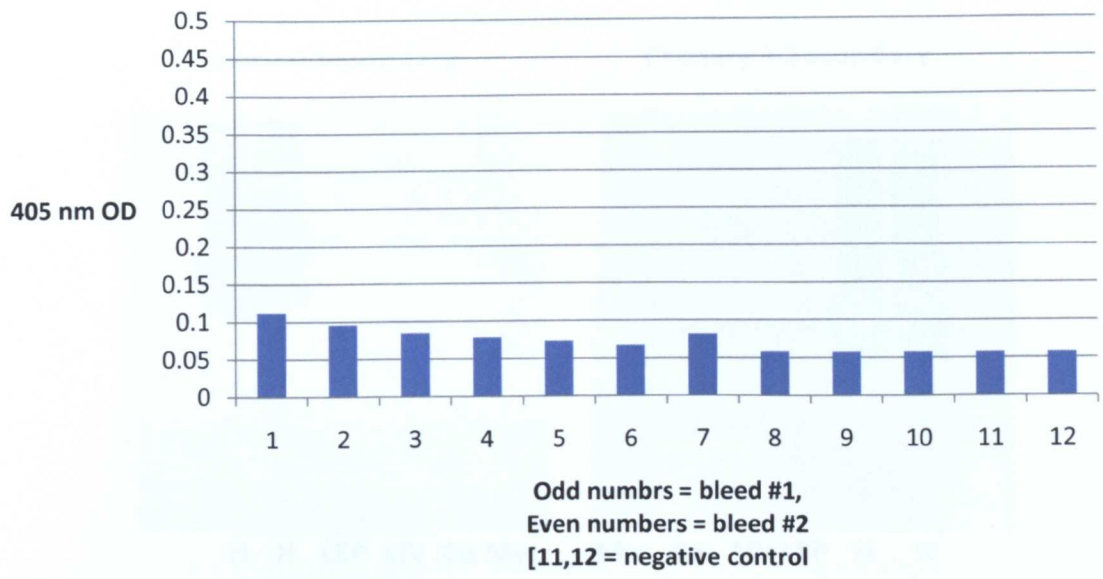
The peptide concentrations were achieved by making a 5 µgml^{-1} solution from the stock in carbonate buffer and serially diluting from the 5 µgml^{-1} solution. The antisera concentrations

were made by making a 1:500 stock solution in PBS/ Tween and diluting from that stock. The secondary antibody was an anti-rabbit IgG conjugated with alkaline phosphatase and used at 1:10000 dilution.

Results

Figure 1.19: Example of ELISA results; bleed #1 versus bleed #2. Top chart = peptide at 2µg ml^{-1}/ B55 at 150min, Bottom chart = peptide at 2µg ml^{-1}/ B56 at 150min.





Antisera dilutions were; series 1,2 = 1/500, series 3,4 = 1/1000, series 5,6 = 1/2500, series 7,8 = 1/5000, series 9,10 = 1/10000, series 11,12 = negative control.

Bleed #2 antisera reacted more strongly than bleed #1, B55 sera reacted more strongly than B56 (see above). The bleed #3 antisera, from both rabbits, reacted more strongly against the peptide in comparison to the antisera from bleed #2. Once again B55 reacted strongest. In the final ELISA, bleed #3 antisera was loaded to compare with the terminal bleed antisera. There was not much difference between the reactivity of the terminal antisera and bleed #3 antisera from both rabbits.

Conclusions

The sera from both rabbits showed some reaction to the peptide with sera from B55 reacting more strongly. Specificity testing by western blotting was indicated.

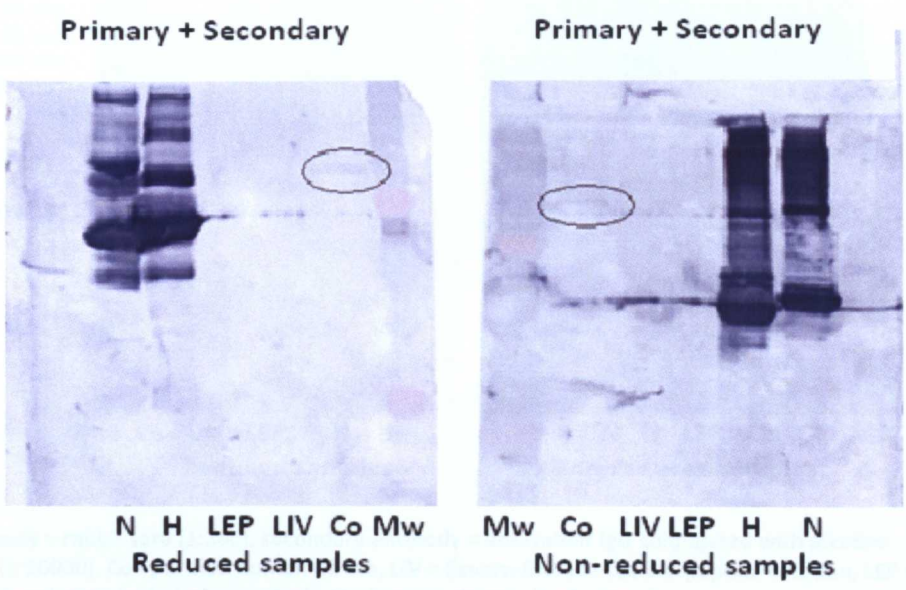
Western blots

The antisera were tested following standard protocols.

Results

Both blots reacted very quickly and the molecular weight markers were not very clear. There was a lot of cross reactivity to many proteins in the plasma samples. However, there did appear to be some weak cross-reactivity with the purified human C1-inh.

Figure 1.20: Western blot probed by B55 terminal bleed sera against non-reduced and reduced samples;



Primary antibody = B55 TB (1:200), secondary antibody = anti-rabbit IgG conjugated with alkaline phosphatase (1:10000). Co= purified human C1-inh, LIV = Severn-Biotech custom peptide solution, LEP = Leptin custom peptide solution (poly-lysine control), H = human plasma, N = horse plasma. N.B no molecular weight markers are identifiable on these blots Eclipses highlight faint bands staining ~100kDa in the positive control lane.

The profiles of the bands on the reduced and non-reduced plasma samples were different between B55 and B56. Neither antisera detected purified human C1-inh. B56 antisera did have bands in both the leptin and LIV1-MAP lanes but these bands were of the same molecular weight to human albumin. B55 had a stronger reaction to what appears to be equine albumin as well as a band between 75 – 100 kDa in reduced human plasma.

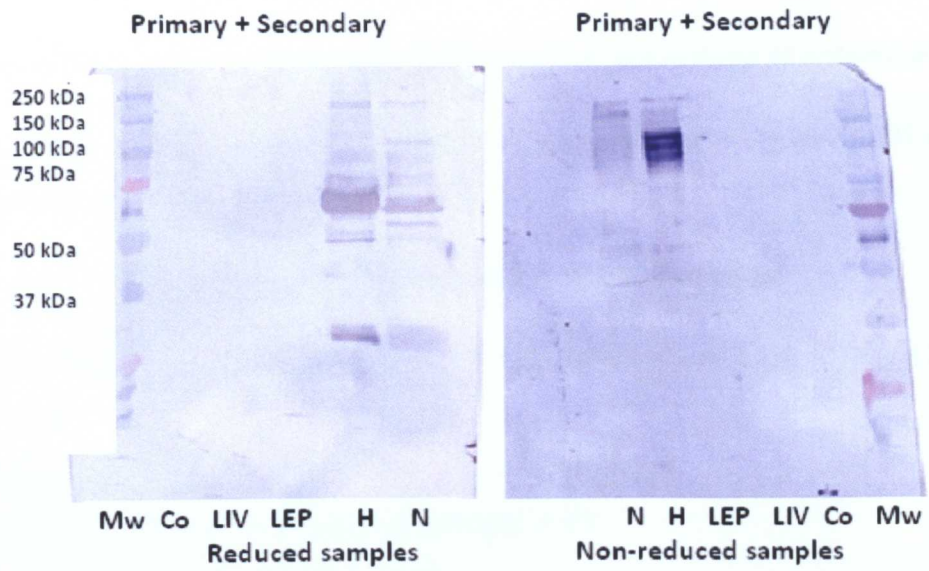
Testing of normal rabbit serum as a primary antisera

Due to the consistent high level of cross-reactivity of all the antisera tested to proteins in the plasma samples we elected to try to establish how much of the reactivity was due to the generated Abs and how much was a result of 'normal' rabbit sera constituents.

To do this we repeated the experiment above but used serum from a normal non-immunised rabbit as the primary antisera.

Results

Figure 1.21: Western blot probed by normal rabbit sera on non-reduced and reduced samples



Primary antibody = rabbit sera (1:200), secondary antibody = anti-rabbit IgG conjugated with alkaline phosphatase (1:10000). Co= purified human C1-inh, LIV = Severn-Biotech custom peptide solution, LEP = Leptin custom peptide solution (poly-lysine control), H = human plasma, N = horse plasma

Conclusions

The sera from the 'normal' rabbit demonstrated a similar band profile to sera from the immunised rabbits on the reduced blot but with less intense staining. The non-reduced blot only showed cross-reactivity at higher molecular weights where one would expect immunoglobulins to be, there was very little staining of bands at molecular weights below 100 kDa. There were only bands in the plasma sample lanes in both blots and no reaction to purified human C1-inh. These results suggest that rabbit sera *per se* has cross-reactivity to human and equine plasma but that the immunised sera tested have heightened cross reactivity and may appear to be reacting to further proteins than normal rabbit sera.

PEG precipitation of immune complexes from antisera

There was a lot of cross-reactivity / non-specificity in the Western blots run on B55. Based on the experiences with the antisera from the previous custom peptides, an experiment was designed to determine if the antisera from B55 and B56 demonstrated any cross-reactivity to albumin by precipitating any immune complexes formed against BSA using PEG and testing the complexes in ELISA and Western blotting for differences to the antisera in their ability to react with the custom generated peptide [LIV1-MAP], purified C1-inh and plasma samples.

PEG precipitation of immune complexes formed by incubating B55 bleed #3 antisera with BSA

Equal volumes of a 1 in 5 dilution of B55 bleed #3 antisera and $2mgml^{-1}$ of BSA in PBS were mixed together and incubated for 1 hour at 37°C.

Principle: free IgG is soluble in 2% PEG but is insoluble when complexed.

1. 20% PEG 6000 in PBS was made up e.g. 6 ml 20% PEG to 3 ml 0.2M EDTA (pH 7.6) and 1 ml PBS
2. 30 µl of 20% PEG was added to 150 µl of each test serum
3. The solutions were mixed and left overnight at 4°C
4. Centrifuged at 2000g at 4°C for 20min
5. The tubes were placed on ice and the supernatant removed
6. Each precipitate was re-suspended with 2 ml of 2% PEG in 0.01M EDTA in PBS
7. Centrifuged at 200g for 20 min at 4°C

8. The supernatants were removed and the precipitates re-dissolved in 150 µl PBS before incubating at 37°C for 1 hour to ensure that the precipitated complexes have re-dissolved.

No visible precipitates were noted during the attempts to form immune complexes.

Results

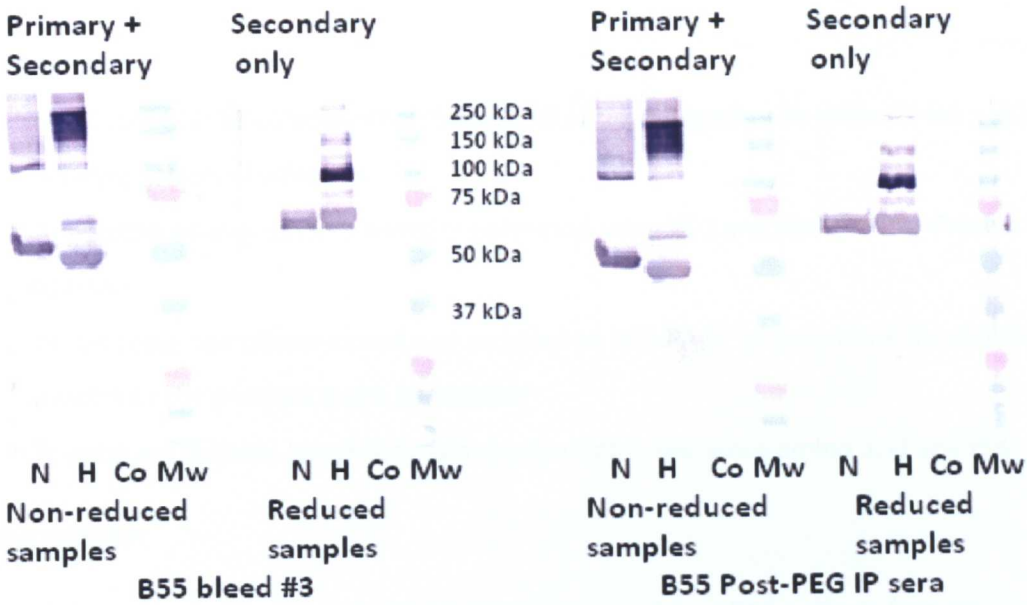
ELISA

There was no difference in reactivity between the bleed #3 antisera and the final supernatant.

Western blotting

There were no obvious differences between the two tested primary antibody solutions;

Figure 1.22: Western blots probed by B55 bleed #3 sera and the same sera after immunoprecipitation on reduced and non-reduced samples



Primary antibody = DILUTED B55 bleed #3 (1:200) [blots on left] or supernatant from final PEG step [blots on right], secondary antibody = anti-rabbit IgG conjugated with alkaline phosphatase (1:10000).
Mw = molecular weight markers, Co = purified human C1-inh control, h = human plasma, N = normal horse plasma

Conclusions

There did not appear to be any cross reactivity of B55 antisera to BSA. No immune complexes were precipitated and so it was not surprising that the supernatant reacted in the same manner to the original antisera when tested by ELISA and Western blotting. There were bands evident at ~100 kDa in all blots.

Precipitation of immune complexes formed by incubating B55 bleed #3 antisera with equine plasma

According to Western blot experiments, B55 bleed #3 antisera appeared to react with various proteins in equine plasma. An experiment was designed to try and determine what those proteins were by incubating the antisera with equine plasma to allow IgG-protein immune complexes to form and then attempt to obtain those complexes and run them as reduced samples on gels to observe the molecular weights of the released proteins. If any proteins were of similar molecular weight to C1-inh, the bands could be cut out from the gel and sent for sequencing to determine the identity of the protein.

After our previous experience with PEG precipitation we elected to utilise two techniques to try to obtain immune complexes, PEG precipitation and a Protein A- Agarose column.

Aims:

- To incubate horse plasma and B55 bleed #3 antisera together to allow C1-inh - IgG immune complex formation
- To precipitate any immune complexes formed using PEG and purify using Protein A - Agarose
- To run these complexes as reduced samples on SDS-PAGE to determine the molecular weights of the proteins in the complexes
- To send any suitable bands for mass spectrometric sequence amino acid analysis

PEG precipitation

Neat equine plasma was incubated with an equal volume of neat B55 antisera (previous experiments using dilute solutions did not produce any precipitates). The solutions were mixed and incubated at 37°C for 1 hour and 4°C for 1 hour, before following the PEG precipitation protocol detailed above.

Note: the first experiment produced a precipitate that would not dissolve in PBS, we repeated the experiment except that rather than dissolving the precipitate in PBS we dissolved it directly into sample buffer.

Protein A on agarose

The equine plasma/ antisera were combined as above except that 1:5 dilutions of each (in PBS) were used as the starting solutions. After incubation the mixture was added to Protein A -

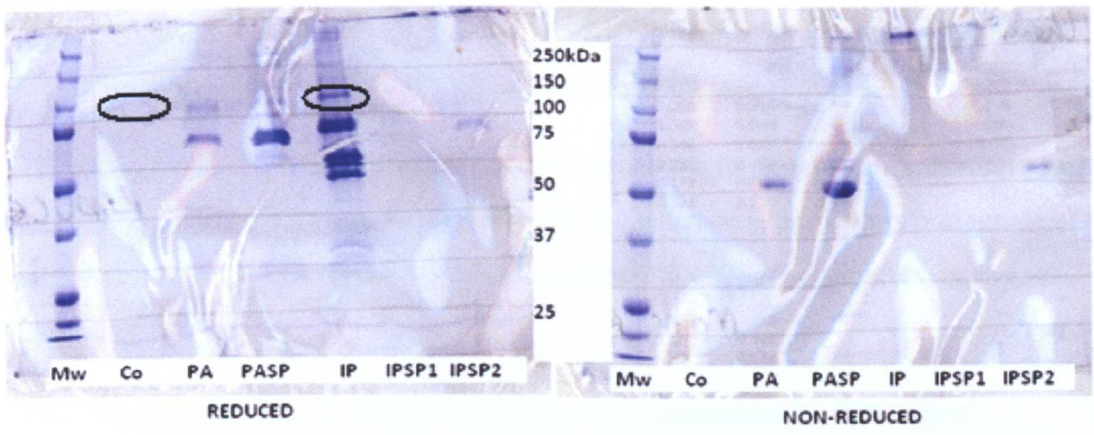
Agarose (10 ml of protein A on agarose per 200 µl of plasma/ antisera solution) and then was mixed for one hour before centrifugation at 200g for 10 min. The supernatant was removed from the precipitate.

Gel running

Two 8.0% SDS-PAGE gels were used with 5 µl of sample per well. The gels were made up as follows; molecular weight markers, purified human C1-inh Protein A on agarose precipitate (20 µl in 180 µl sample buffer), Protein A on agarose supernatant (20 µl of plasma in 180 µl sample buffer), PEG precipitate (added directly to 200 µl sample buffer), PEG precipitate first supernatant (20 µl in 180 µl sample buffer), PEG precipitate second supernatant (20 µl in 180 µl sample buffer).The gels were stained with Coomassie blue and dried.

Results

Figure 1.23: Photograph of SDS-PAGE gels loaded with reduced and non-reduced samples from immunoprecipitate experiment.



Mw = molecular weight markers, Co = purified human C1-inh, PA = Protein A immunoprecipitate, PASP = protein A supernatant, IP = PEG immunoprecipitate, IPSP1 = first PEG supernatant, IPSP2 = second PEG supernatant. Eclipses = bands of interest (see conclusion)

Conclusion

Bands were visible at ~100 kDa in both the Protein A on agarose precipitate and supernatant lanes, but the ~100 kDa band in the PEG immunoprecipitate lane was closest to the molecular weight of the purified human C1-inh in the control lane (see circles in fig 1.23).

Sequencing

The gels were re-run and the bands around 100 kDa from the three lanes described above were cut out and sent to The University of Manchester for mass spectrometric sequence analysis.

Results

The amino acid sequence results for the three bands were returned and each consisted of a mixture of immunoglobulins and fibrinogen.

40% salt precipitation of IgG from rabbit anti peptide serum and coupling of this IgG to a Sepharose 4B cyanide bromide column

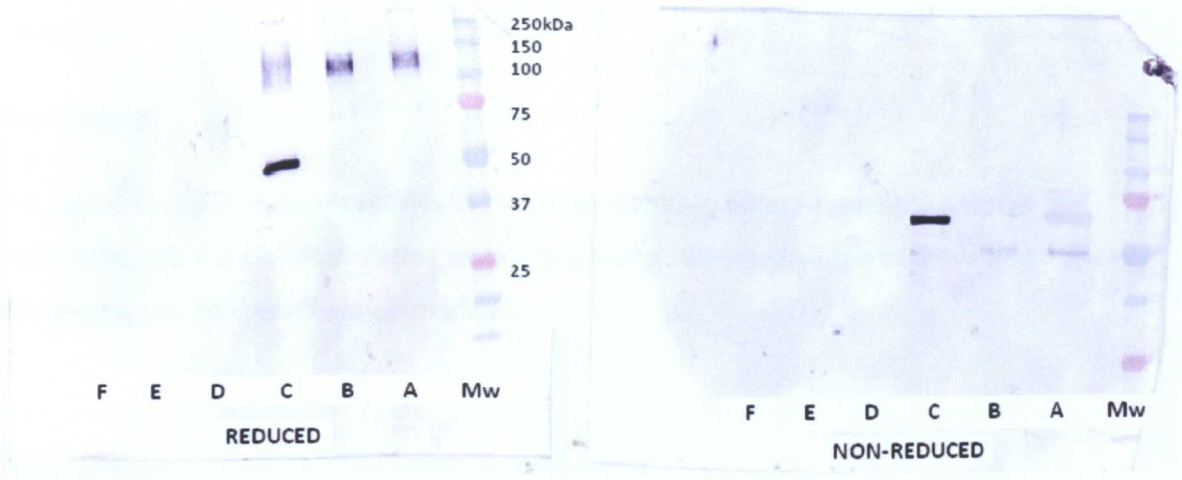
In a further attempt to improve the specificity of the antisera for C1-inh, IgG was precipitated from the antisera and coupled to a sepharose column for possible affinity purification of C1-inh from equine plasma. Coupling of the Sepharose 4B CNBr column and loading/washing and eluting of the columns were carried out as described earlier (see above).

The following fractions were obtained and pooled according to protein concentration peaks (determined by 280nm OD absorbance). The salt precipitation supernatants protein concentrations are also included for comparison (serum B = supernatant after second salt precipitation)

A: B55 serum supernatant = 1.3 $mgml^{-1}$ B: B55 serum supernatant B = 0.06 $mgml^{-1}$
C: B55 IgG column pool 1 = 3.6 $mgml^{-1}$ D: B55 IgG column pool 2 = 0.06 $mgml^{-1}$
E: B55 IgG column pool 3 = 0.25 $mgml^{-1}$ F: B55 IgG column pool 4 = 0.29 $mgml^{-1}$

Western blots were carried out to attempt to identify proteins reacting with B55 TB sera present in each pool.

Figure 1.24: Western blots of reduced and non-reduced immunoprecipitate/ affinity purified samples.



Primary antibody = 1:400 B55 TB sera, secondary antibody = 1:10000 anti rabbit IgG conjugated with alkaline phosphatase. A→ G = pooled samples described above.

The only detectable proteins in either the Coomassie stained gels or the blots under either reducing or non reducing conditions occurred in the two supernatants of the first fraction pool, i.e. the fractions that collected immediately after loading the column and during washing that should contain proteins that were not bound to the IgG on the column. None of the pooled fractions that appeared during the acetic acid elution had detectable protein in them. This may suggest the elution solution had not been able to elute the proteins from the column bound IgG.

Elution of column using 100mM glycine pH 2.4

In order to free any proteins that may be bound to coupled IgG on our Sepharose column, a low pH glycine buffer was used to elute any bound proteins. The column was washed with 5x gel volume of 10mM sodium phosphate pH 6.8, before the elution buffer 100mM glycine pH 2.4 was applied to the column. Each fraction was collected in a tube with 1 in 20 v/v 1M sodium phosphate pH 8.0 to neutralise the low pH glycine buffer.

There were only two fractions where the 280 nm OD increased. We attempted to identify the proteins in the fractions using SDS-PAGE and Western blotting

Results

No proteins were detected at all by either Coomassie staining of the gels or by Western blotting. This was despite loading sufficient protein onto the gels according to the 280 nm OD readings.

Conclusions

The attempts to use custom peptides to immunise rabbits in order to generate antisera that could detect equine C1-inh in plasma were unsuccessful. We were unable to prove that equine C1-inh exists by immuno-chemical methods.

Discussion

Human C1-inh is a unique protein, even amongst the serpin protein superfamily. The physical properties of human C1-inh were covered in the thesis introduction. Briefly, human C1-inh has 478 amino acids and a calculated Mw of 76 kDa (Perkins et al. 1990). However, on SDS-PAGE gels it migrates with an apparent Mw of ~105 kDa; the main reason for this being that human C1-inh is heavily glycosylated (~30% by weight) (Bock et al. 1986). The migration of purified human C1-inh on SDS-PAGE gels is similar under non-reducing and reducing (DTT) conditions (Ziccardi and Cooper 1979). Freshly purified human C1-inh runs as a single band; however, because most purified preparations contain some proteases, purified human C1-inh can appear as two bands (one at ~105 kDa and one at ~95 kDa) especially after a long period in storage or after several freeze-thaw cycles (Harpel and Cooper 1975, Sim and Reboul 1981). The band at ~95 kDa has been termed modified human C1-inh and is proposed to correspond to a major fragment of C1-inh after digestion by proteases such as plasmin or trypsin (Harpel and Cooper 1975, de Agostini et al. 1985). Sim and Reboul (1981) also report the consistent presence of other minor components of C1-inh in purified preparations which run at ~60 kDa and ~30 kDa on non-reducing SDS-PAGE gels; the proportion of these minor components also increases with storage. Sim and Reboul (1981) did not speculate as to the identity of these components; it is probable they are cleaved products of C1-inh and contaminating protease reactions, but whether this is so has not been established.

The preliminary work which formed the basis of the grant application for this project demonstrated that, at that time, a goat polyclonal antibody against human C1-inh (Sigma), apparently strongly and specifically, identified in equine plasma two bands in Western blots

(one at ~105 kDa and one at ~95 kDa) similar to those produced if purified human C1-inh was probed by the same antibody. Further discussion of the premise that equine C1-inh may have a similar molecular weight and amino acid sequence to the human protein can be found in the molecular biology chapter. It was felt that an immunological based assay would provide the greatest chance of developing an assay that would be specific and sensitive. As a result the first series of experiments detailed in this chapter were devoted to identify a suitable antibody to either be used in an assay itself, or at least to help purify equine C1-inh from plasma. More specific antibodies could then be generated against the native equine protein.

The first antibody tested was antisera used in a nephelometric assay in the Royal Liverpool University Hospital Immunology department for detecting levels of human C1-inh in plasma. The antisera did not show any cross reactivity to any proteins in equine plasma, but did cross-react with two bands (~105 kDa and ~95 kDa) in human plasma and showed non-specific cross reactivity at higher molecular weights. The secondary antibody also showed significant cross-reactivity to proteins at similar molecular weights so it was unclear how much of the staining is due to the primary antisera. Alternatively, as a polyclonal reagent, it may not be specific for human C1-inh, but may bind other, unrelated, proteins. When this antisera was tested in the DID experiments there was clear reactivity and precipitation of immune complexes between human plasma and the goat antisera against human C1-inh, as expected. There was no precipitation between the goat antisera and equine plasma, which along with the results from the Western Blots, suggested that the tested goat antisera against human C1-inh did not cross react with any specific equine protein (equine C1-inh) and thus was of no use for this project.

The INCA antibody we tested was designed to be used in ELISAs and had not apparently been tested in Western blots prior to this study. In our experiments INCA appears to be able to detect human C1-inh in Western blots. Furthermore, INCA appeared to react to form faint bands at ~100 kDa in equine plasma in all but one reduced blot and in one non-reduced blot. This reaction was apparently weak under non-reducing conditions and only marginally stronger under reducing conditions. Interestingly, under reducing conditions INCA also detected two bands around 60 kDa in both human and equine plasma. The secondary antibody demonstrated cross-reactivity for a single band at this Mw, which was assumed to be albumin. It is unclear how much of the staining for the higher Mw band in the primary + secondary blots was due to secondary-only cross-reactivity, but it is clear that INCA was reacting with a protein at ~60 kDa. At that stage, the identity of that protein was not speculated upon but it could be one of the minor components consistently reported in purified preparations of C1-inh (Sim and

Reboul 1981). The extravadin experiments were interesting for several reasons. These experiments demonstrated that INCA cross-reacted with purified equine IgG at a molecular weight very close to where we expected equine C1-inh to be. In fact, as there was only one band in the equine plasma samples at that MW, we could not be confident if the staining was due to IgG fractions of C1-inh or other proteins or any combination of the three. Once again, INCA demonstrated strong staining for a protein that ran just in front of albumin in all the plasma samples. There was also strong staining at ~30 kDa in all plasma samples and the purified equine IgG; at that stage we assumed the staining at that Mw was due to cross-reactivity with light chain IgG. Due to the relative lack of specificity of INCA to equine C1-inh in plasma, coupled with the facts that the reaction is apparently weak and may be stronger under reducing conditions, it was concluded that INCA would not be of use either to help purify C1-inh from equine plasma, or in an assay for equine C1-inh.

The tests on the Sigma goat polyclonal antibody against human C1-inh were hugely disappointing. This was the antibody which gave such promising results in 2002. The antibody reacted with human C1-inh (interestingly, by this stage our source of purified human C1-inh was showing the characteristic double banded profile). However, on non-reduced blots there was no obvious cross-reactivity with any proteins in equine plasma around 100 kDa on non-reduced Western blots. On reduced blots the antibody did show a little more cross-reactivity but around the 60 – 75 kDa area and not at 100 kDa. The antibody did appear to cross react with both human and equine albumin as well as proteins of around that Mw. Whether any of these proteins were the minor component described above was not determined. The Sigma antibody not only showed less, if any, specificity for equine C1-inh itself, the secondary antibody appeared to cross react with plasma constituents. These findings mean that the primary antibody was unlikely to be of any use in developing assays to measure C1-inh in equine plasma or to aid in purification of C1-inh from equine plasma. Sigma gave assurances that the antibody was from the same source as in 2002, using the same antigen and from the same goats. If this is the case then the difference in antibody performance may be explained by the possibility that, even though it was the same goat herd, the individuals comprising that herd may have changed resulting in different antibodies being produced. Alternatively, even if the individual goats were the same, it is likely that since 2002, the goats had had repeated immunisations of human C1-inh and the precise nature of their antibody response to the human protein may have been modified sufficiently so that the antibodies no longer recognised epitopes on equine C1-inh. Alternatively, the experiments from 2002 may have been flawed although several Western blots repeatedly produced the same result.

The Western blots appeared to show that the antibody against human kallikrein was cross-reacting with proteins between 116-205 kDa in equine plasma. Human plasma kallikrein is 88 kDa (de Agostini et al. 1985), and we estimated that equine plasma kallikrein would be a similar molecular weight, although no information to confirm this estimation was available. The Western blot results may have been due to the antibody binding equine plasma kallikrein or due to binding with equine immunoglobulins. This series of experiments using the polyclonal kallikrein antibody were carried out in the first 6 months of the project and the experimental design and conclusions made from the experiment are potentially flawed. There was no positive control in the original Western blot to tell if the antibody could detect its target molecule in the Western blotting system. No naive blot was probed with the secondary antibody only, which would have provided valuable information about whether the bands seen were due to the primary or the secondary antibody. The fact that after stripping and re-probing the blots no new staining was observed does not necessarily mean that the original staining was not due to immunoglobulins.

At this stage of the project, all the antibodies tested had not demonstrated sufficient potential for use in either an equine C1-inh assay or in methods to attempt to purify the protein from plasma. Other methods of purifying C1-inh from plasma had been attempted but were unsuccessful (see chromatography chapter, p62). However, the molecular biology experiments had been successful and had elucidated the entire nucleotide and amino acid sequence of equine C1-inh. This allowed the generation of a 3D-model of the protein. The model allowed us to predict likely amino acid sequences that would form epitopes on the whole protein. Custom peptides were generated and rabbit antisera raised against them. The first set of antisera was from Sigma-Genosys. Initially, the results showed promise. In some bleed #1 Western blots there were two bands in the reduced positive control at the correct molecular weight and in a similar pattern against purified C1-inh observed when INCA had been used. Similar bands were also visible in each of the plasma samples. There were distinct dark bands seemingly pushed into a narrow band at the front of what was assumed to be albumin in all the plasma samples in the non-reduced sample blot; this band was just visible in the reduced human plasma lane. We had observed similar bands with INCA but mainly in reduced blots.

The peptide antisera did not increase their effective titre in subsequent bleeds, although the sera were effective against a wider concentration of peptide in the ELISAs suggesting epitope spread (McCluskey et al. 1998). The antisera from the rabbits immunised against S2 had not apparently changed at all. In the Western blot from B87 bleed #3 there were no visible bands

in the reduced positive control lane (c.f. bleed #1) and the strong staining thin bands just below albumin were again apparent in the non-reduced plasma samples. This information can be interpreted in a number of ways. Namely, the lack of staining in the positive control lane suggests that the previous result was either an artefact, or was because the sera tested was from a different rabbit or because the sera was 4x more diluted for this second series of experiments in an attempt to reduce background. The narrow bands may be due to a non-C1-inh specific reaction of the rabbit antisera in general or the 3D epitope that the antibodies are responding to is similar/ identical to one on a protein (or cleaved protein) that has a similar molecular weight to albumin. However, these bands had been detected by more than one antibody (INCA, sera from B87, B88 and B89) against C1-inh, increasing the probability that these bands may represent the 60 kDa minor component of purified C1-inh reported previously (Sim and Reboul 1981). Interestingly, these bands were most apparent in non-reduced blots, in comparison to INCA that had reacted with proteins at a similar molecular weight under reducing conditions.

It was also noticed that the sera consistently appeared to react with albumin on reduced blots. Attempts were made to try to reduce this cross-reactivity by incubating the antisera with 1% BSA, whilst the strength of staining was reduced; the antisera still appeared to react with albumin in the plasma samples. When the ELISAs were repeated using antisera incubated with 1% BSA, the reaction of the antisera with its respective peptide was dramatically reduced. It is difficult to interpret these slightly conflicting results. The ELISA results suggest that some activity of the antisera in the ELISA was removed by incubating the antisera with BSA. The Western blots suggest that if there was any difference in antisera activity after incubation with BSA, then it was to reduce general 'non-specific' cross-reactivity but it did not affect the production of reactive bands where albumin ran on the blots. Together, the results suggest that the antisera seemed to produce a degree of non-specific reactivity in the different systems they were tested in.

In an attempt to remove some of this non-specific cross-reactivity, IgG from each antisera were purified by salt precipitation and run through a BSA coupled Sepharose column. When the products of this experiment were tested in an ELISA or in Western blots, no improvement in specificity was noted.

It appeared in this case that our attempts to use custom generated peptides to raise antisera against epitopes that would also cross-react with the natural whole protein were unsuccessful. Despite some promising results, most of the ELISA results demonstrated that the antisera had

not reacted well against the custom peptides and the antisera demonstrated poor cross-reactivity and low specificity for the native protein.

A final attempt at antisera production that would cross-react with the whole natural equine C1-inh using custom peptides was made. This time a longer stretch of amino acids was used to generate a peptide (LIV1-MAP) and a different approach (poly-lysine) was employed to try to improve the antigenicity of the peptides. This time only one peptide was generated and immunised into two rabbits.

Again initial results were promising; ELISAs showed that B55 reacted more strongly to the peptide compared to B56, although neither demonstrated very strong reactions. The first Western blots were carried out on B55 bleed #2, and were remarkable for the very fast time in which they reacted. There was a lot of cross reactivity to many proteins in the plasma samples. However, there did appear to be some cross-reactivity for the purified human C1-inh. At this stage, a Western blot was repeated using 'normal' rabbit serum as the primary antibody. The 'normal' serum did not react with very much in human or equine plasma in non-reduced blots; it did produce a very similar band profile to those produced by the other rabbit antisera in previous experiments. This finding added to the already cautious interpretation of reduced blots using rabbit sera.

Again attempts to improve antibody specificity of the rabbit antisera (e.g. PEG precipitation of immune complexes formed when BSA was incubated with the sera and B55 IgG was salt precipitated and then coupled to a Sepharose column) were unsuccessful. The experiment incubating equine plasma with B55 antisera, followed by precipitation of the resultant complexes, did result in bands on reduced SDS-PAGE gels in the area where C1-inh would be expected to be. Unfortunately, when those bands were cut out and sequenced (using mass spectrometry) the results suggested that the bands consisted of a mixture of immunoglobulins and fibrinogen. These proteins were present in the cut out bands but that does not necessarily preclude the existence of other proteins, such as C1-inh, being present within those bands. Sequencing by mass spectrometry tends to only reliably sequence the most predominant protein in any given sample (Johnson et al. 2005).

The antisera generated against the custom peptides ultimately proved not to meet our requirements. It was perhaps relevant that none of the three peptides generated large immune responses in any of the rabbits. One reason for this was likely to be that the each of the peptides had relatively low purity (all <60%). The methods used to generate custom peptides

are more reliable if the peptide to be generated contains hydrophilic residues every 5 amino acids (www.sigma-genosys.co.uk). The selected peptide sequences from equine C1-inh were targeted specifically to correspond with areas comparable to the human protein that were known epitopes; this meant that the selected peptides were less than ideal in terms of reliability of antisera production. If further attempts at peptide generation are made it is likely that peptide sequences would be selected that were likely to lie on the periphery of the protein but also would be more likely to be generated with increased purity. It was noteworthy that all the antisera generated against custom peptides showed most promise in Western blots in the early bleeds, and the specificity of the rabbit's antibody response then apparently deteriorated over subsequent bleeds. This response is also likely to be due to the low purity of the peptides against which the rabbits were being immunised and an "epitope-spreading" response to contaminating proteins.

The interpretation of the Western blots was never easy. The main reason was that plasma was being run on the blots in most experiments. Plasma is not the ideal medium to run in Western blots as it contains so many potentially antigenic components. As a result, the anti-rabbit IgG tended to cross react with components in plasma, especially in reduced blots. Also, 'normal' rabbit serum was shown to be highly reactive to human and equine plasma on reduced blots.

Some of the antibodies tested did apparently detect bands in equine plasma where C1-inh might be expected but unfortunately not with enough specificity. One other consistent feature of some of the antibodies (namely INCA and antisera from B87, 88 and 89) was the strong affinity of the antibodies for proteins that ran just in front of albumin in the plasma samples at ~60 kDa as well as for some proteins ~30 kDa in reduced samples. No attempt was made to identify these proteins for two reasons. Firstly, in the case of the ~60 kDa proteins it would be extremely difficult to cut out the bands accurately in an SDS-PAGE gel and even from a blot after staining because each lane loaded with plasma ran slightly differently due to the amount of albumin. Secondly, in both cases the bands were either running in or very near to other proteins which would be in relative abundance (e.g. albumin and light chain Ig respectively), which would likely result in sequencing approaches only identifying the predominant proteins.

One approach that may have helped determine if antibodies were reacting to C1-inh but was not attempted; was the use of competition assays, i.e. incubating the antibodies/ antisera with substrate prior to probing the blots to see if band staining was reduced with increasing concentrations of substrate.

Finally, this chapter has highlighted a potential problem with the use of unvalidated reagents. Putting aside the issue of lack of cross-reaction between antibodies raised against human epitopes to bind to the same molecules in other species, there are other problems which have been raised in this chapter. For example, many of the reagents which are commercially available and used routinely in human diagnostic laboratories may not have been rigorously tested to check on their specificity. In veterinary science, there are relatively few specific antibodies available because they are not commercially viable. Hence, in veterinary laboratories we need to check on potential cross-species reactions. It is the experience in our laboratories that whilst there are a few useful cross- reactions, most searches end in disappointment. Simple referencing to reaction with the human proteins frequently indicates that the reagents used routinely in human medicine simply do not have the stated reactivity, but are almost invariably used without any agency or body attempting adequate quality control, beyond reproducibility. This is an issue which needs addressing by both reagent producers and end users.

Appendix to chapter 1

Immunisation protocol for Sigma-Genosys antisera production:

Day 0: Pre-immune serum collection	5 ml
Day 0: Immunize	200 µg of peptide in Complete Freund's Adjuvant (CFA)
Day 14: Immunize	100 µg of peptide in Incomplete Freund's Adjuvant (IFA)
Day 28: Immunize	100 µg of peptide in IFA
Day 42: Immunize	100 µg of peptide in IFA
Day 49: Test bleed #1	5 ml: ELISA
Day 56: Immunize	100 µg of peptide in IFA
Day 63: Test bleed #2	5 ml
Day 70: Immunize	100 µg of peptide in IFA
Day 77: Test bleed #3	5 ml
Day 84: Terminal bleed, collect sera	

Immunisation protocol for Severn Biotech antisera production

The peptide was injected into two rabbits; B55 and B56.

Day 0: Pre-immune serum collection	10 ml
Day 0: Immunize	200 µg of peptide in Complete Freund's Adjuvant (CFA)
Day 14: Immunize	200 µg of peptide in Incomplete Freund's Adjuvant (IFA)
Day 28: Immunize	200 µg of peptide in IFA
Day 35: Test bleed #1	1-10 ml
Day 42: Immunize	200 µg of peptide in IFA
Day 49: Test bleed #2	2-10 ml
Day 56: Immunize	200 µg of peptide in IFA
Day 63: Test bleed #3	3-10 ml: ELISA
Day 70: Immunize	200 µg of peptide in IFA
Day 77: Terminal bleed, separate sera	

The antisera were shipped with 0.1% sodium azide as preservative

Chapter 2

Use of chromatography techniques to purify equine proteins

Introduction

Protein purification can be a useful in step in the creation of reagents and the characterisation of biological function of target proteins. This strategy was explored for C1-inh.

Human C1-inh is heavily glycosylated (Bock et al. 1986). Glycosylation of proteins is a post-translational event, and may affect the 3D structure of the protein and therefore the epitopes to which antibodies may react. The ultimate aim of the project was to develop a robust assay to detect equine C1-inh in plasma in clinical cases. Such an assay would most likely be an immunological based assay, therefore purified natural equine C1-inh would provide the best antigen to generate a selective antibody capable of detecting the protein in plasma.

There are numerous papers detailing various methods of C1-inh purification, more recent methods using affinity chromatography with monoclonal antibodies against human C1-inh (Alsenz and Loos 1987). As an antibody against equine C1-inh was not available, earlier papers which used other chromatographical techniques to purify proteins were reviewed and techniques were selected that could be replicated in our laboratory (Salvesen et al. 1985).

Attempted purification of C1-inh from equine plasma using fractional precipitation of proteins with polyethylene glycol (PEG), diethylaminoethyl (DEAE) Sepharose ion exchange chromatography, and Cibacron Blue-Sepharose affinity chromatography

Introduction

This series of steps to attempt to purify C1-inh from equine plasma was based mainly on a method published by Salvesen et al. (1985) for the purification of C1-inh from human plasma. One reason this protocol was selected was that we had no assay validated for detecting equine C1-inh in any of the fractions, but Salvesen et al. (1985) reported that the elution of human C1-inh coincided with that of ceruloplasmin which imparts a blue colour onto the fractions. It was hoped that this would also be the case for equine C1-inh, allowing identification of those fractions which were likely to contain the target protein.

Methods

Collection of plasma

Plasma was collected from an anaesthetised horse just prior to the horse being euthanized for a non-infectious chronic performance limiting orthopaedic condition. Blood (1l) was collected via a 10G jugular catheter into plastic blood collection bags containing a citrate based anticoagulant, and was allowed to stand for 20 minutes to allow blood cells and plasma to separate. The plasma was then carefully separated into plastic containers and frozen at -20°C until needed.

PEG fractionation of proteins from plasma

1. Fifty % (w/v) of PEG 6000 was added to 400 ml of equine plasma to make a 12.5% (w/v) final solution
2. The mixture was stirred slowly at room temperature (RT) for 30 min and the precipitate removed by centrifugation @ 5000rpm at 17°C for 40 min
3. The supernatant* (490 ml) was removed and 50% (w/v) of PEG 6000 added slowly to give a final concentration of 30% (w/v) PEG. The mixture was again stirred at RT for 30 min. *It was assumed that because there was an increase in volume of the supernatant

that there was still some PEG in the supernatant; as there was no way of knowing how much PEG was present it was assumed that the starting concentration of PEG in the supernatant was 12.5%

4. The centrifugation step was repeated and the precipitate collected and re-dissolved in 200 ml 0.02M Na_2HPO_4, 0.05M NaCl pH 7.0 buffer and stored at 4°C

DEAE Sepharose chromatography

1. A 2 x 30 cm column of DEAE-Sepharose (Pharmacia) was packed and washed through for 4 hours with the equilibration buffer (EB) [200 ml 0.02M Na_2HPO_4, 0.05M NaCl pH 7.0]
2. The dissolved PEG pellet (see above) contained an estimated 55 $mgml^{-1}$ (280 nm absorbance) of protein which was dialysed against 2 l EB at 4°C in a 12000 Mw semi-permeable membrane for 40 hours (2 changes) to remove excess PEG
3. After dialysis the estimated [protein] was ~42 $mgml^{-1}$ (280 nm absorbance), further EB was added to the solution to result in a final estimated [protein] of 21 $mgml^{-1}$
4. 150 ml of the protein solution was loaded onto the DEAE-Sepharose column at 35 $mlhr^{-1}$
5. The column was then washed with 5 bed volumes of EB
6. The column was then developed with a linear salt gradient (total volume 800 ml) from 0.05M NaCl to 0.2M NaCl in EB 6.5 ml fractions were collected and their 280 nm absorbance measured to detect peak [protein]
7. The column was washed with 100 ml of 0.02M Na_2HPO_4, 0.5M NaCl pH 7.0

Cibacron Blue-Sepharose chromatography

1. A 1.5 x 30 cm column of Cibacron Blue-Sepharose (Pharmacia) was packed and washed through with equilibration buffer (EB) [200 ml 0.05M TRIS HCl, 0.08M NaCl pH 7.8]
2. The fractions from the DEAE Sepharose step that were coloured blue were pooled and made to 200 ml by adding 0.05M TRIS HCl, 0.05M NaCl pH 7.8 buffer
3. The diluted fractions were loaded onto the column at 25 $mlhr^{-1}$, before the column was washed with 5 bed volumes of EB
4. Bound proteins were then eluted stepwise by the addition of 0.1M, 0.15M and 0.2M NaCl in 0.05M TRIS HCl pH 7.8. Four ml fractions were collected and their 280 nm absorbance measured to detect peak protein concentration
5. Peak protein concentration fractions were collected and run on SDS-PAGE gels

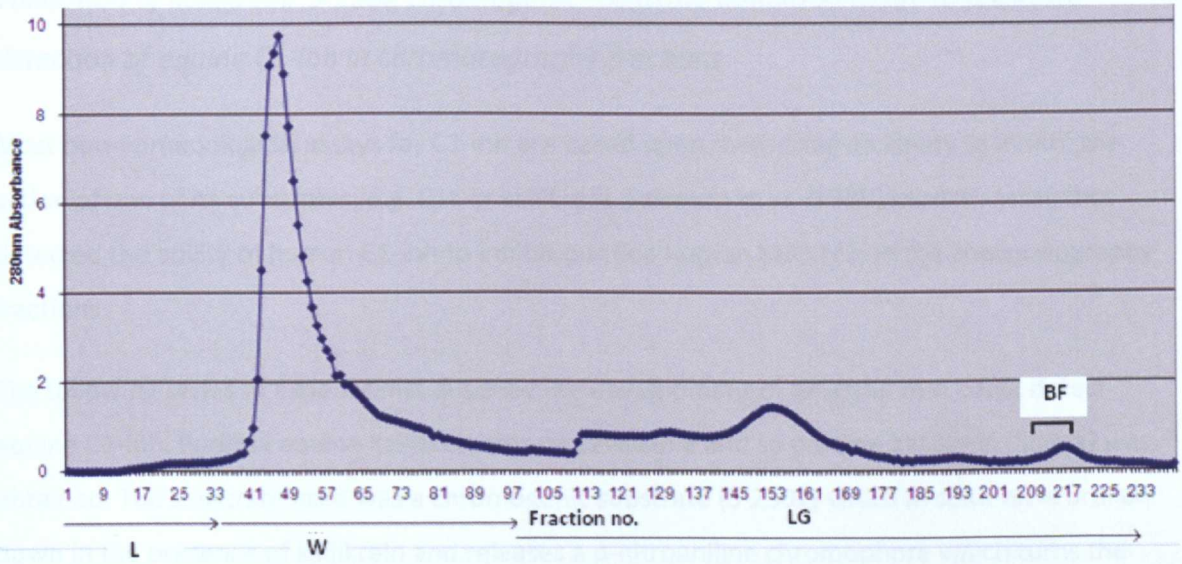
Gel running and staining

Two x 7.5% SDS-PAGE gels were cast with 10 sample wells (as described in the antibody chapter). Each gel was loaded with 5 µl of sample per well.

The gels were run under standard non- reducing running conditions (samples in sample buffer heated at 65°C for 20 min) and stained with Coomassie blue after running, and de-stained as described in the antibody chapter.

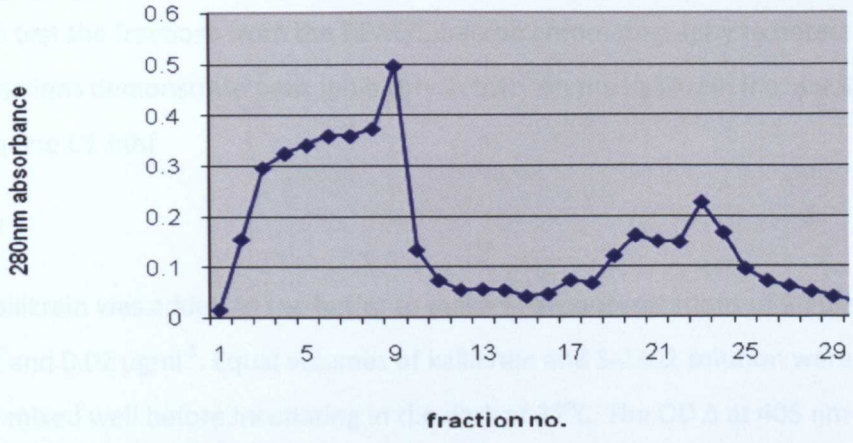
Results

Figure 2.1: Graph showing 280 nm absorbance of fractions from the DEAE Sepharose chromatography



L= loading of sample, W = washing of column, LG = developing column with linear gradient. BF = fractions that eluted blue (F210- 218)

Figure 2.2: Graph showing 280 nm absorbance of fractions from Cibacron Blue Sepharose chromatography



Conclusion

The DEAE sepharose chromatography resulted in elution of some fractions which were coloured blue (presumably by equine ceruloplasmin). The fractions containing the peak protein concentration did show different band patterns when run on SDS-PAGE gels. Unfortunately, the molecular weight markers did not stain very well which made interpretation of the gel difficult. Thus, it was considered that an attempt should be made to detect equine C1-inh using its known biological properties.

Validation of kallikrein/ S-2302 chromogenic substrate inhibition assay to facilitate detection of equine C1-inh in chromatography fractions

Most non-immunological assays for C1-inh are based upon measuring its ability to inhibit the action of one of its substrates, e.g. C1s or kallikrein. Salvesen et al. (1985) used an assay that detected the ability of human C1-inh to inhibit purified human kallikrein in the chromatography fractions.

The following series of experiments describe the development of an assay that could detect equine C1-inh. Purified equine kallikrein was not available and so porcine kallikrein (Sigma) was obtained. The substrate used was a chromogenic substrate (S-2302) which in solution is broken down in the presence of kallikrein and releases a p-nitroaniline chromophore which turns the solution yellow. This colour change can be measured spectrophotometrically at 405nm.

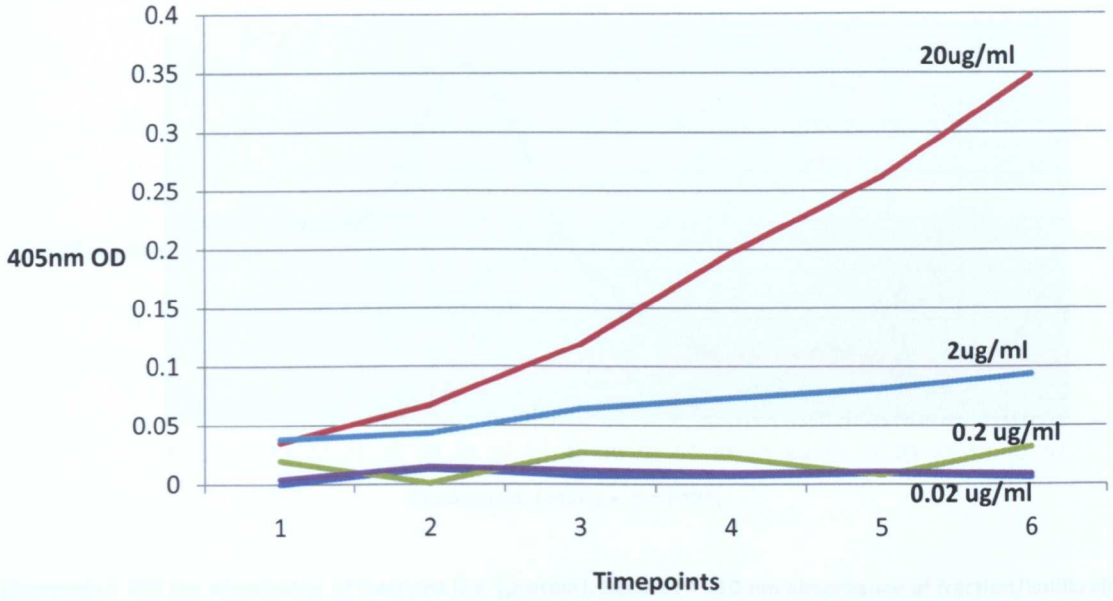
Objectives

- To test if the porcine kallikrein can break down the S-2302 substrate
- To test the fractions from the DEAE/Cibacron chromatography to detect which fractions demonstrate peak inhibitory activity on the kallikrein (i.e. are likely to contain equine C1-inh)

Validation

Porcine kallikrein was added to the buffer to make final concentrations of 20 μgml^{-1}, 2 μgml^{-1}, 0.2 μgml^{-1} and 0.02 μgml^{-1}. Equal volumes of kallikrein and S-2302 solution were added to each other and mixed well before incubating in the dark at 37°C. The OD Δ at 405 nm was recorded at the following time points, 0, 10, 15, 20, 30 and 45 minutes.

Figure 2.3: OD 405 nm readings for the different concentrations of kallikrein incubated with 0.2mM S-2302



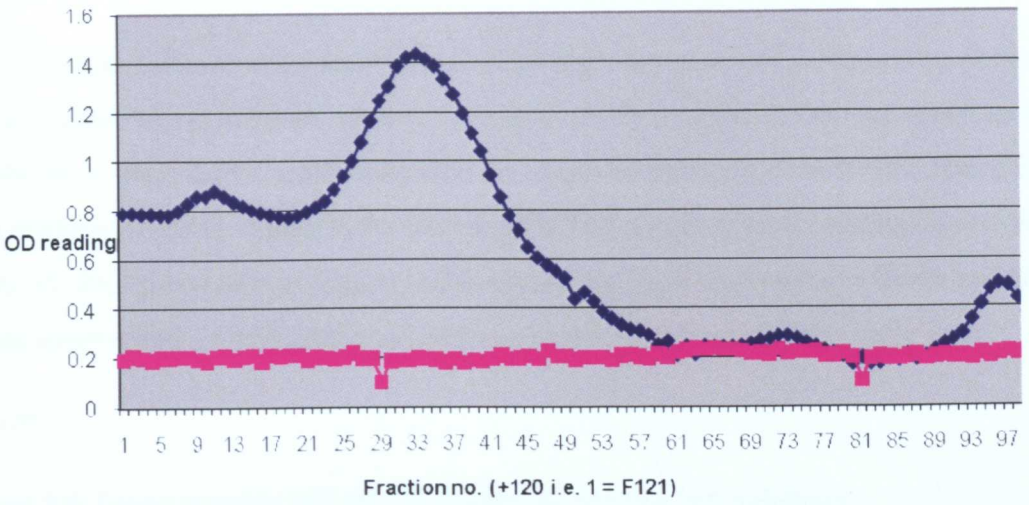
Conclusion

Porcine kallikrein produced a linear increase in colour change over time. The degree of colour change appeared to be proportional to the amount of kallikrein present.

Testing of fractions for inhibition of kallikrein

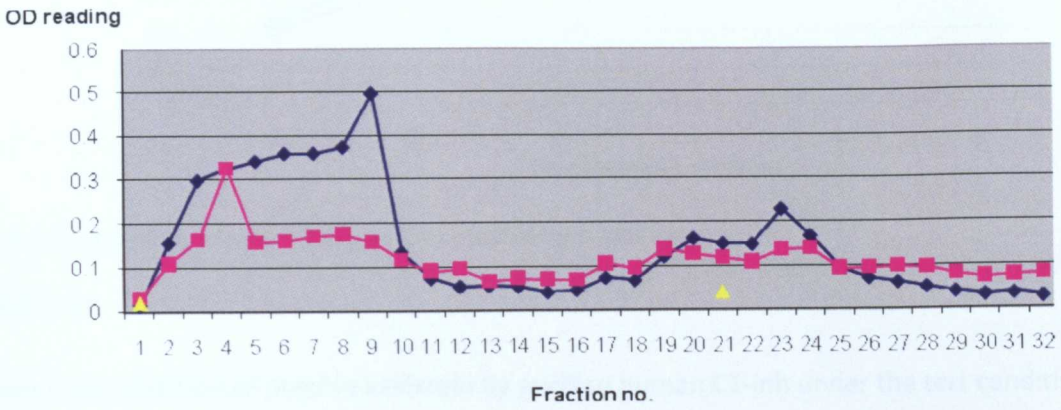
Fractions from the DEAE chromatography and Cibacron blue chromatography were tested for inhibition of porcine kallikrein activity against S-2302 after incubation at 37°C for 30 minutes.

Figure 2.4: Testing DEAE Sepharose fractions for inhibition of kallikrein.



Diamonds = 280 nm absorbance of fractions (i.e. [protein]. Squares = 410 nm absorbance of fraction/kallikrein/ S-2302 after incubation at 37°C for 30 min

Figure 2.5: Testing Cibacron Blue-Sepharose fractions for inhibition of kallikrein.



Diamonds = 280 nm absorbance of fractions (i.e. [protein]. Squares = 410 nm absorbance of fraction/kallikrein/ S-2302 after incubation at 37°C for 30 min. Yellow triangles = blank/ S-2302 readings.

Conclusion

There was no apparent inhibition of porcine kallikrein by the fractions tested. Indeed, the OD changes seemed to correlate with the amount of protein in the fractions.

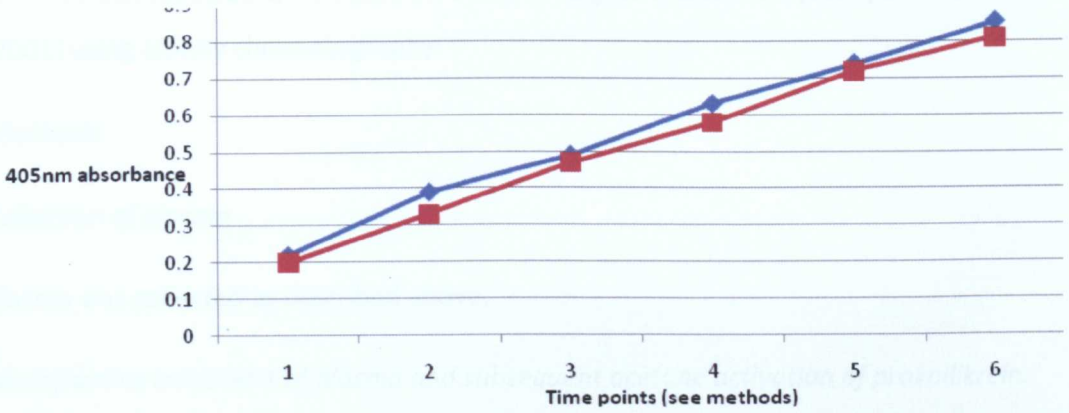
Inhibition of porcine kallikrein by purified human C1-inh

The results of the experiment above led to a review of the assay. It was important to see if porcine kallikrein was inhibited by C1-inh from another species.

C1-inh inhibits kallikrein in an equimolar manner (Salvesen at el 1985), thus excess purified C1-inh was added to the kallikrein solution; 170 µg of purified human C1-inh was added to 2 μgml^{-1} kallikrein in assay buffer, which was added in equal volumes to 0.2mM S-2302. The solution was mixed well and incubated in the dark at 37°C. 405 nm absorbance readings were taken at 5, 10, 15, 20, 25, and 30 min. 2 μgml^{-1} kallikrein in assay buffer without any C1-inh added in equal volumes to 0.2mM S-2302 was used as a control and treated in the same way.

Results

Figure 2.6: Graph showing 405 nm OD change for the two test solutions



Diamonds = C1-inh/ kallikrein/ S-2302; squares = kallikrein/ S-2302 only

Conclusion

There is no inhibition of porcine kallikrein by purified human C1-inh under the test conditions.

Attempted purification of plasma kallikrein from equine plasma using methylamine treatment of plasma, acetone activation of pro-kallikrein, and affinity chromatography using Sepharose 4B cyanide bromide (CNBr) coupled with soy bean trypsin inhibitor (SBTI)

Introduction

It was apparent from the experiment above that kallikrein from one species (porcine) is not inhibited by C1-inh from another species (human). It is likely therefore, that equine C1-inh does not inhibit porcine kallikrein. In order to be able to develop an inhibition assay to detect equine C1-inh in fractions, attempts were made to purify equine plasma kallikrein to use as a substrate for the inhibition assay.

The methods followed were based on those of Nagase and Barrett (1981) and Tada et al. (2001) using affinity chromatography.

Methods

Collection of plasma

Plasma was collected as described above.

Methylamine treatment of plasma and subsequent acetone activation of prokallikrein.

1. 400 ml of plasma was adjusted to pH 8.2 with 2M TRIS/ HCl pH 8.6
2. Solid methylamine HCl was added to the plasma/ TRIS solution to make 0.2M methylamine HCl. The solution was stirred gently for 1 hour at room temperature (RT)
3. Acetone was added to result in 20% v/v; the solution was left for 3 hours at RT
4. The solution was dialysed against 20x vol of dH_2O at 4°C for 16-19 hour
5. The pH of the solution was adjusted to 5.8 with 1M HCl and then left for 30 min before centrifuging at 1500G for 10 min
6. The supernatant was adjusted to pH 7.5-7.8 by the addition of 1M TRIS/ HCl buffer pH 7.8 NaCl was added to 1M and Briij 35 added to 0.1%.

Coupling of SBTI and Sepharose 4B CNBr

1. Excess 0.1M $NaHCO_3$, pH 9 was added to 15 g of dry S4B CNBr to form an estimated 45 cm^3 of gel.

2. The gel was made to react with 100mg of Soya Bean Trypsin Inhibitor (SBTI) in 0.1M $NaHCO_3$, pH 9 by gentle stirring overnight at 20°C
3. The gel was then treated with 50mM Glycine in 0.1M $NaHCO_3$, pH 9 for 2 hours
4. The gel was then washed thoroughly with 0.1M sodium formate, pH 3.0, and then 5mM NaOH over a sintered glass funnel

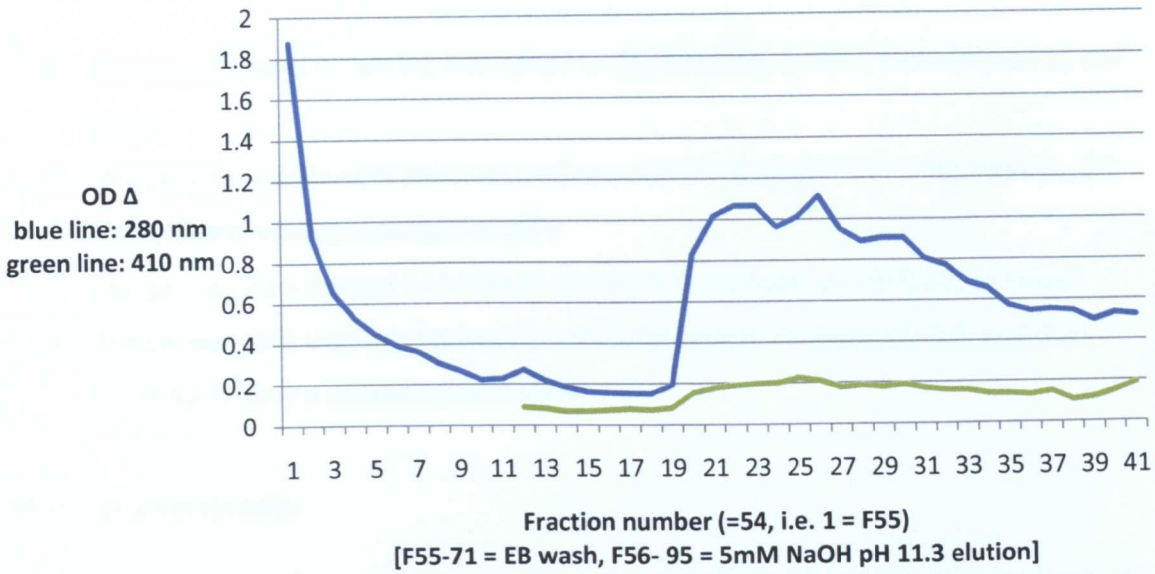
Affinity chromatography

1. The gel was equilibrated with equilibration buffer (EB) = 20mM TRIS HCl/ 1M NaCl, pH 7.8 over a sintered glass funnel and then packed into a 2 x 30 cm column
2. The processed plasma was run through the column @ 50 $mlhr^{-1}$ for 2 hour and then 20 $mlhr^{-1}$ overnight
3. The column was washed with > 4 bed volumes of EB (250 ml)
4. The column was eluted with 5mM NaOH pH 11.2, at a flow rate 50 $mlhr^{-1}$. Five ml fractions were collected in tubes containing 0.5ml of 1M TRIS/ HCl pH 7.8 to neutralise the pH

Testing of fractions for inhibition of kallikrein

An equal volume of each fraction was mixed with an equal volume of 0.2mM S-2302 solution. The absorbance at 405 nm was read after 30 min of incubation at 37°C. The spectrophotometer was zeroed to the control (equal volume of buffer only and S-2302) prior to the first reading.

Figure 2.7: Graph of results of Sepharose 4B CNBr coupled with SBTI fractions.



Blue line = 280 nm absorbance of fractions estimated relative protein concentration in each fraction. Green line = 410 nm absorbance of fractions, i.e. colour change after incubation of fraction/ S-2302.

Conclusion

There was no obvious increase in the breakdown of S-2302 in any of the equine plasma fractions. The breakdown profile appears once again to follow the protein concentration. The increase in breakdown seen after F74 (20 in graph) follows the increase in 280nm absorbance but not by as much as one would expect if the majority of the kallikrein was being eluted at this point (i.e. 5x increase in [protein] is matched by a ~2.5 x increase in S-2302 breakdown).

Attempt to purify proteins in pooled fractions using an anti-human kallikrein polyclonal antibody coupled to Sepharose 4B CNBr

When pooled fractions from the previous experiment were ran on Coomassie stained SDS-PAGE gels and were probed in Western blots with the rabbit polyclonal antibody against human kallikrein (BP572, Acris Antibodies GmBH), bands were observed on the blots between 116-205 kDa which were not apparent on the gels suggesting that the antibody was binding to something in the pooled fractions. Human plasma kallikrein is ~88 kDa (de Agostini et al. 1985) but the Mw for equine kallikrein has not been published.

To attempt to elucidate what proteins were bound by the antibody, the antibody was coupled to a Sepharose 4B CNBr gel in an affinity chromatography column.

Coupling of Antibody BP572 and Sepharose 4B CNBr

1. Excess 0.1M $NaHCO_3$ pH 9.0 was added to dry S4B CNBr to form an estimated 12 cm^3 of gel
2. The gel was reacted with 200 µl of antibody: BP572 (3.0$mgml^{-1}$) in 0.1M $NaHCO_3$, pH 9.0 by gentle stirring overnight at 20°C
3. The gel was then treated with 50mM Glycine in 0.1M $NaHCO_3$, pH 9.0 for 2 hours
4. The gel was then washed thoroughly with 0.1M sodium formate, pH 3.0, and then 5mM NaOH over a sintered glass funnel

Affinity chromatography

1. The gel was equilibrated with equilibration buffer (EB) = 20mM TRIS HCl/ 1M NaCl, pH 7.8 over a sintered glass funnel and then packed into a 10 ml plastic syringe
2. The pooled fractions 19-28 from the Sepharose 4B CNBr/ SBTI chromatography were run through the column at 5 $mlhr^{-1}$
3. The column was washed with > 4 bed volumes of EB (50 ml) and the column was then eluted with 5mM NaOH pH 11.2, at a flow rate of 5 $mlhr^{-1}$
4. 2.5 ml fractions were collected in tubes containing 0.5mL of 1M TRIS/ HCl pH 7.8 to neutralise the pH. The protein concentration was estimated using 280 nm absorbance.

Freeze drying of fractions to increase protein concentration

Fractions 11, 15, 19 and 21 were run on SDS-PAGE gels to determine the molecular weights of the proteins. In order to increase the visibility of any bands under Coomassie staining, the [protein] of each fraction was increased by freeze drying the fractions overnight.

After freeze drying, the dried fractions were re-constituted in 500 µl PBS. Freeze drying had resulted in at least ~5 fold increase in protein concentration

e.g. 280 nm absorbance: F 11 = 1.97 (~1.4 $mgml^{-1}$), F 15 = 0.925 (~0.6 $mgml^{-1}$), F 19 = 2.75 (~1.9 $mgml^{-1}$), F 21 = 1.79 (~1.3 $mgml^{-1}$)

The fractions then were run on SDS-PAGE gels.

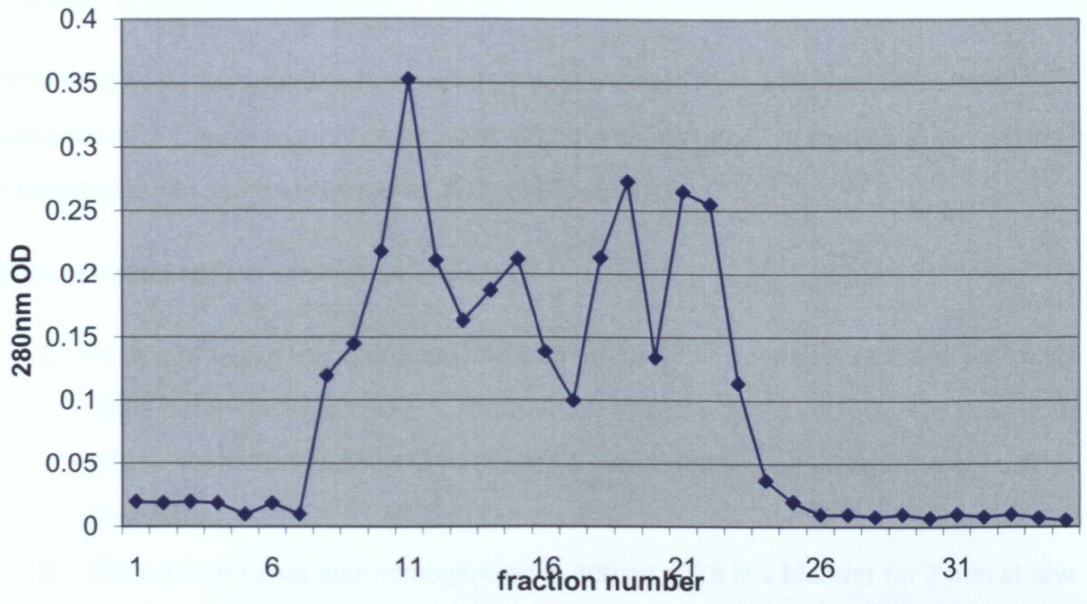
SDS-PAGE

Two x 7.5% SDS-PAGE gels were cast with 10 sample wells (as described in Antibody chapter). Each gel was loaded with 5 µl of sample per well. The gels were run under standard non-reducing running conditions (samples in sample buffer heated @ 65°C for 20 min)

Gels were stained with Coomassie after running, and de-stained as described in the antibody chapter.

Results

Figure 2.8: Graph showing the 280 nm absorbance for each of the fractions from the Antibody BP572 affinity chromatography column.



The readings start from when the pooled fraction solution was loaded onto the column (F6). The elution buffer was applied from fractions 15 -20.

Conclusion

On Western blotting, there are no bands around ~80 kDa (see figure 1.8 in the antibody chapter, p17). The fractions that had peak protein concentration after the elution buffer was applied (i.e. F 19 and F21 which should contain the proteins bound to the antibodies), did show staining for proteins between 116 and 205 kDa but the staining was very diffuse. Hence, the antibody was probably binding to proteins in the fractions non-specifically, or proteins were binding to the column non-specifically and being eluted in the high pH buffer. Due to the diffuse staining and the Mw of the proteins it was concluded that it would be unlikely that kallikrein would be purified from these fractions.

Attempt to purify pro-kallikrein from equine pancreas using DEAE Sepharose and phenyl Sepharose

The attempts to purify equine plasma kallikrein had been apparently unsuccessful. In order to attempt to obtain a supply of equine kallikrein to use in an inhibition to detect equine C1-inh in chromatography fractions, attempts were made to purify pro-kallikrein from equine pancreas.

The method was based on that published by Al-Hamidi et al. (1991) for extraction of bovine pro-kallikrein from pancreatic tissue.

Methods

Collection of pancreatic tissue

Pancreatic tissue was obtained immediately post mortem from a healthy adult horse euthanized for clinico-economical reasons. Small sections (<0.4 cm thickness) were flash frozen in liquid nitrogen and then stored at -80°C until required

Homogenisation of pancreatic tissue

1. Forty g of tissue was defrosted and cleaned of fat and connective tissue with a scalpel blade before being chopped into small pieces and washed with equilibration buffer (EB) = 0.05M TRIS HCl/ 0.1M NaCl pH 7.4 to remove any blood and was then dried with filter paper.
2. The washed tissue was homogenised in 400 ml of EB in a blender for 2 min at low speed and 1 min at high speed
3. The crude homogenate was filtered through sterile muslin and then submitted to 2 centrifugation steps; 7 min at 300g followed by 30 min at 18000g. After each centrifugation the supernatant was filtered through muslin before being used for the next step. The precipitate was discarded.

Batch adsorption

1. The final homogenised solution was added slowly with gentle stirring to 250 ml DEAE Sepharose that had previously being equilibrated in EB.
2. After 15 min of gentle stirring the supernatant was removed from the gel by suction through a glass funnel filled with glass wool connected to a Buchner flask.

3. The adsorbed proteins were removed by washing the gel with 200 ml 0.05M TRIS HCl/ 1.0M NaCl pH 7.4 and removing by suction as above. The gel was then washed with EB
4. The filtrate from step 3 was dialysed overnight in 10L of EB

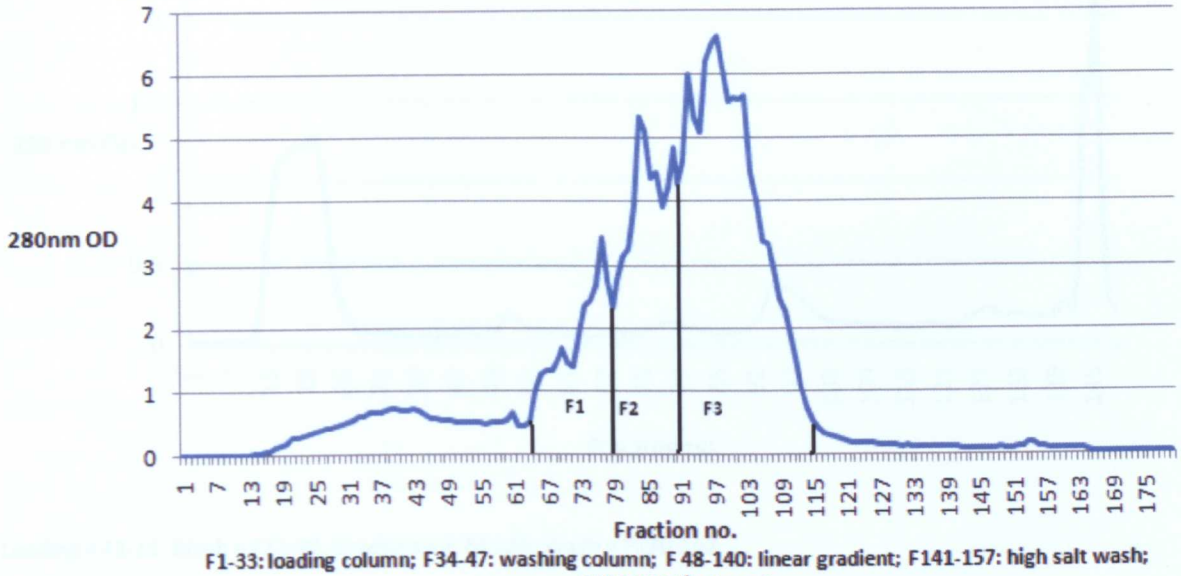
Ion exchange chromatography

1. After dialysis there was 196 ml of solution with an estimated protein concentration of 5 mg ml^{-1}. This solution was applied (at 45 $mlhr^{-1}$) to a 2.5 x 35 cm column of DEAE Sepharose equilibrated with EB.
2. After the protein solution was loaded, the column was washed with 100 ml of EB before the adsorbed proteins were eluted with a linear gradient composed of 0.05M TRIS HCl/ 0.1M NaCl pH 7.4 (EB) and 0.05M TRIS HCl/ 0.7M NaCl pH 7.4, 600 ml gradient total volume
3. Finally, 100 ml of 0.05M TRIS HCl/ 1.0M NaCl pH 7.4 was run through the column before the column was washed with 200 ml of EB
4. Seven ml fractions were collected throughout this process, fractions were pooled (see results below) and frozen at -20°C before the next step

Hydrophobic interaction chromatography

1. A 75cm^3 column of phenyl Sepharose was packed and equilibrated with 0.05M TRIS HCl/ 1M $(NH_4)_2SO_4$ pH 7.0 (EB) overnight at 10 $mlhr^{-1}$
2. The pooled fractions from the previous step were made to contain 0.05M TRIS HCl/ 1M $(NH_4)_2SO_4$ pH 7.0 and applied to the column. The column was washed with 75 ml of EB
3. Adsorbed proteins were eluted with a reverse gradient consisting of 0.05M TRIS HCl/ 1M $(NH_4)_2SO_4$ pH 7.0 to 0.05M TRIS HCl pH 7.0, 400 ml total gradient volume
4. Finally, the column was washed with 0.05M TRIS HCl/ 50% (v/v) ethylene glycol/ 0.02% NaN_3 before storage or before equilibrating the column for the next solution to be loaded
5. Fractions of 7 ml were collected and their protein concentration estimated by 280 nm absorbance
6. Each of the fraction pools from the previous step underwent hydrophobic interaction chromatography (i.e. steps 2 -6 were repeated for each pool)

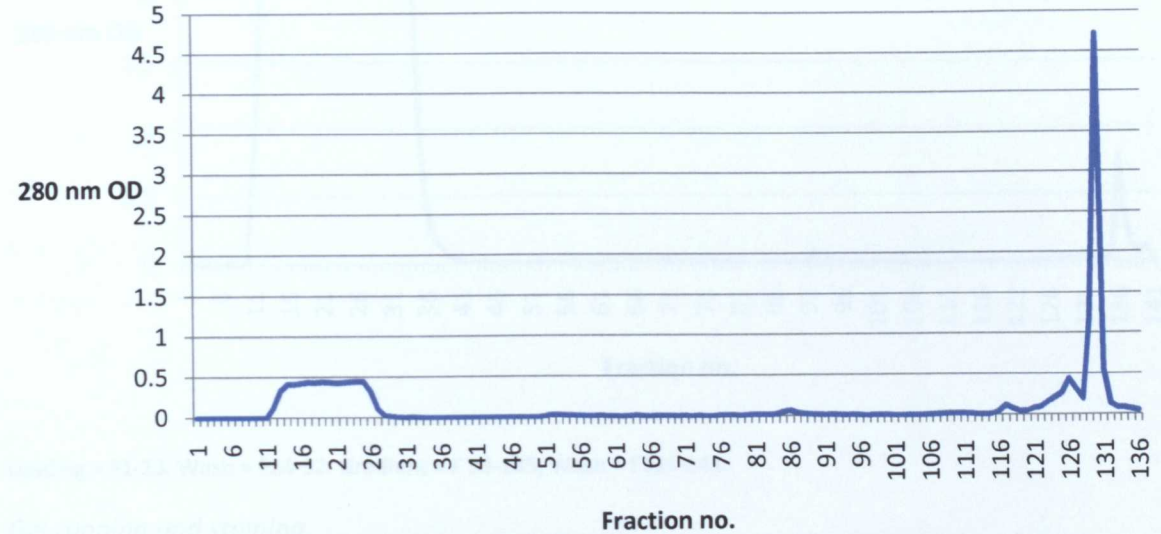
Figure 2.10: Graph showing 280 nm absorbance for fractions from DEAE Sepharose chromatography



F1-33: loading column; F34-47: washing column; F 48-140: linear gradient; F141-157: high salt wash; F158-181: final wash

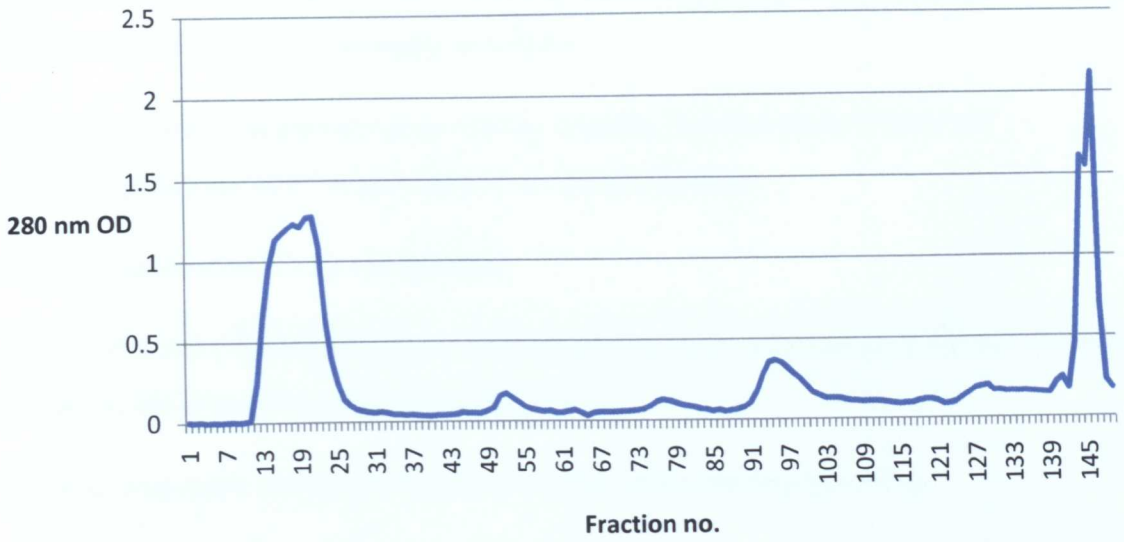
F1 = pooled fractions 64 – 79, F2 = pooled fractions 80 – 91, F3 = pooled fractions 92 – 115

Figure 2.11: Graphs showing 280 nm absorbance from hydrophobic phenyl Sepharose chromatography for pooled fractions F1 –F3
Fraction pool F1:



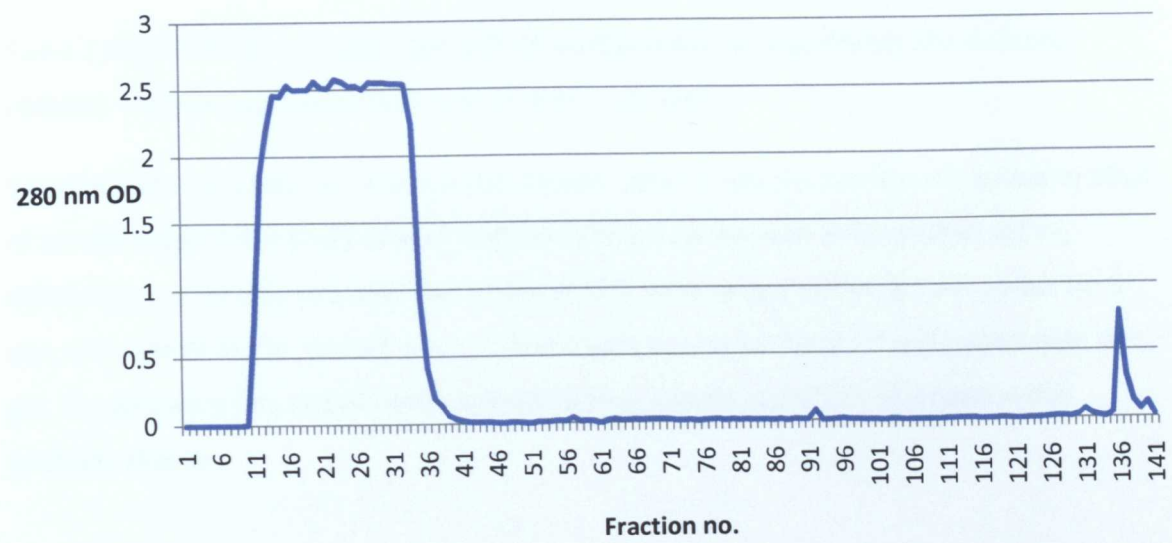
Loading = F1-14. Wash = F15-25. Gradient = F 26-118, Wash = F129-136.

Fraction pool F2:



Loading = F1-11. Wash = F12-19. Gradient = F 20-129, Wash = F130-148

Fraction pool F3:



Loading = F1-23. Wash = F24-32. Gradient = F 33-135, Wash = F136-141.

Gel running and staining

For each of the three runs of the phenyl Sepharose chromatography (i.e. each of the pooled fractions from the DEAE Sepharose chromatography), fractions showing the greatest 280nm absorbance were run on SDS-PAGE gels and stained with Coomassie blue to determine the molecular weights of proteins contained within the fractions.

The peak protein fractions from the phenyl Sepharose chromatography run with fraction pool F2 and fraction pool F3 did not show any bands apart from F52 from fraction pool F2 which had a band of the same molecular weight as albumin.

The gels run with peak protein concentration fractions from the phenyl Sepharose chromatography run with fraction pool F1 are detailed below;

6 fraction pools were run on the two gels;

Pool A = F92-102 (~0.62 $mgml^{-1}$) from DEAE Sepharose chromatography (i.e. sample of pool F3 that went into phenyl Sepharose chromatography)

The remaining pools were from the phenyl Sepharose chromatography step

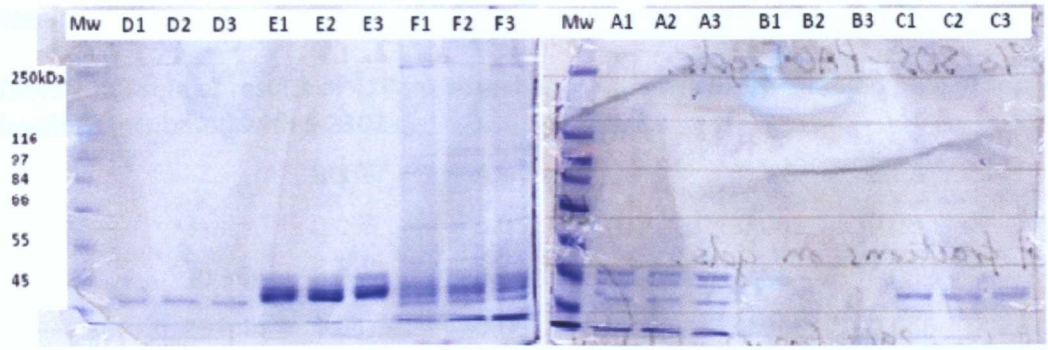
Pool B = F12-27 (~0.77 $mgml^{-1}$) Pool C= F86 (~0.1 $mgml^{-1}$) Pool D= F117-119 (~1.24 $mgml^{-1}$)

Pool E= F124-128 (~0.46 $mgml^{-1}$) Pool F= F129-131 (~3.87 $mgml^{-1}$)

Two x 7.5% SDS-PAGE gels were cast with 10 sample wells (as described in the antibody chapter). Each gel was loaded with 5 µl of sample per well.

Samples were run under non-reducing conditions. 20 µl of sample solution was added to 20 µl of sample buffer three times as each triplicate of each sample was to be treated slightly differently, e.g. A1 was incubated for 1 hour at 37°C with sample buffer, A2 was boiled for 3 min with sample buffer, and A3 was just mixed with sample buffer at RT and loaded onto the gel. The gels were run, stained with Coomassie blue, and de-stained as described in the antibody chapter.

Figure 2.13: Picture of dried gels from DEAE Sepharose chromatography experiment detailed on previous page.



Mw = molecular weight marker, A = fraction pool F92-102 (~0.62 mgml^{-1}) from (i.e. sample of pool F3 that went into phenyl Sepharose chromatography), B = fraction pool F12-27 (~0.77 mgml^{-1}) , C= fraction pool F86 (~0.1 mgml^{-1}), D= fraction pool F117-119 (~1.24 mgml^{-1}), E= fraction pool F124-128 (~0.46 mgml^{-1}) Suffix 1= sample was incubated for 1 hour at 37°C with sample buffer, Suffix 2 = sample was boiled for 3 min with sample buffer, and suffix 3= sample was mixed with sample buffer at RT and loaded onto the gel.

Conclusion

The molecular weight of bovine prokallikrein is ~20 kDa, but in other species it can be as high as 40 kDa (Al-Hamidi et al. 1991). There were discrete bands between 36 and 45 kDa for pools A, C, D and E in the fractions from DEAE –Sepharose separation and phenyl-Sepharose separation. These could have contained equine prokallikrein.

Testing fraction pools from phenyl Sepharose chromatography for inhibition of S-2302

Samples from the fraction pools that showed bands between 36 and 45 kDa in the experiment above (A, C, D and E) underwent freeze drying overnight to increase protein concentration before being reconstituted in 500 µl PBS.

After reconstitution the 280nm absorbance for the pools was;

A = 1.11 =~0.79 mgml^{-1} C = 1.02 = ~0.72 mgml^{-1} D = 3.42 = ~2.44 mgml^{-1} E = 4.09 = ~2.92 mgml^{-1}

In order to approximate the same amount of protein to mix with S-2302, 10 µl of samples A and C was added to 990 µl of assay buffer (see above), 10 µl of sample D was added to 2.9 ml of assay buffer and 10 µl of sample E was added to 3.99 ml of assay buffer.

An equal volume of sample and buffer solution was added to 2mM S-2302, mixed well and incubated in the dark at 37°C, the 410nm absorbance was measured after 3, 10, 25, 40 and 60

min. For control, an equal volume of buffer and S-2302 that had been treated in the same way was also measured for 410nm absorbance.

Results

Table 2.1: Table of results of 410nm absorbance readings for the pooled phenyl Sepharose fractions incubated with S-2302

	Buffer + S	A + S	C + S	D + S	E + S
3 min	0.065	0.062	0.06	0.062	0.06
10 min	0.06	0.061	0.066	0.06	0.059
25 min	0.06	0.061	0.059	0.065	0.066
40 min	0.064	0.065	0.061	0.062	0.065
60 min	0.064	0.065	0.064	0.065	0.064

Conclusion

There was no increase in breakdown of S-2302 by any samples from any of the fraction pools, although the methodology was supposed to purify pro-kallikrein, it was hoped that there would be some residual kallikrein activity (see discussion).

Attempted purification of equine C1-inh from plasma using PEG fractionation of plasma proteins, Jacalin agarose chromatography and hydrophobic phenyl Sepharose chromatography

This final attempt to purify equine C1-inh from plasma was based on a methods published by Pilatte et al. (1989), with a few minor adaptations. The principle behind this approach is that jacalin binds highly glycosylated proteins and as (human) C1-inh is a highly glycosylated plasma protein jacalin may be useful to help separate C1-inh from other plasma proteins.

Method

Plasma collection

Horse blood was collected via a 10G jugular into plastic blood container containing a 20x stock solution of the following anticoagulant/ inhibitor mixture (ρ-nitrophenyl-ρ'-guanidobenzoate

(NPGB), disodium ethylenediaminetetraacetic acid (EDTA) and soy bean trypsin inhibitor (SBTI),to achieve 250 ml blood + final concentration of NPGB 25μM, EDTA 10mM, and SBTI 50μM

The plasma was separated by centrifugation of the whole blood in plastic bottles at 1500g for 10 min.

After this step all procedures were performed at 4°C and polypropylene containers were used.

PEG fractionation

1. The inhibitor treated plasma was brought to a final concentration of 21.4% PEG 3350 (w/v), by adding PEG 3350 powder with constant stirring for 1 hour. The precipitate was removed by centrifugation at 10,000g for 30 min.
2. The supernatant was adjusted to a final concentration of 45% PEG 3350 (w/v), equilibrated for 1 hour and centrifuged to recover precipitated proteins (10,000g for 30 min)
3. The precipitate was kept and the supernatant discarded

Jacalin-agarose chromatography:

1. The 45% PEG precipitate was solubilized and made to 100 ml in equilibration buffer (EB): PBS containing 10mM EDTA and 25μM NPGB, and applied at $30ml hr^{-1}$ to a 20 ml syringe containing 10 ml of Jacalin-agarose equilibrated in the EB.
2. Following application of the sample, the column was washed at the same flow rate with EB + 0.5M NaCl until 280nm absorbance approached zero. The column was then eluted with 0.125M melibiose in the same buffer.
3. Fractions (2.5 ml) were collected. The protein concentrations were estimated using 280nm absorbance. Fractions with peak protein concentration after melibiose elution were pooled and went forward to the next step

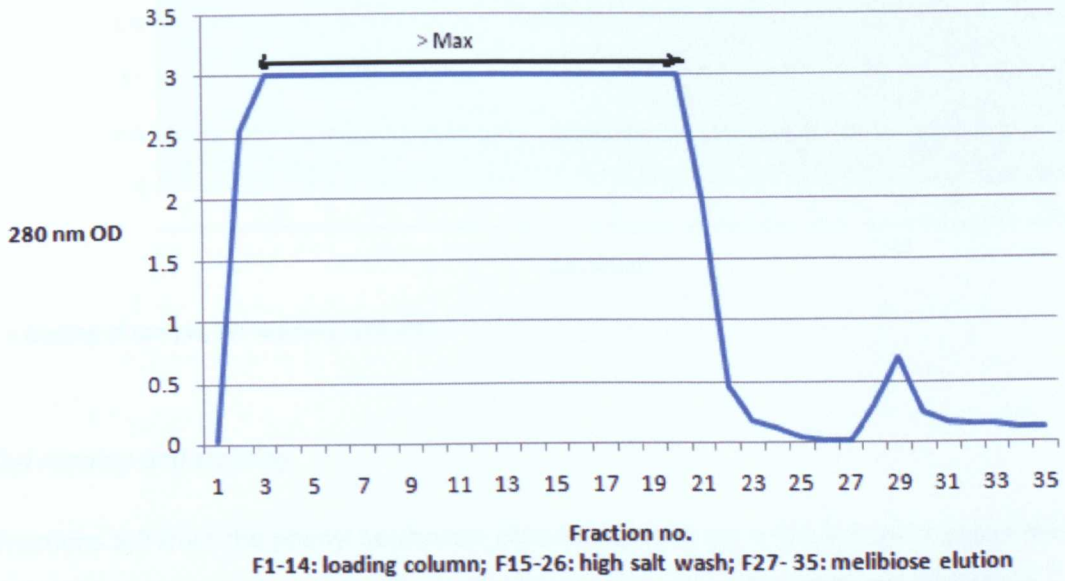
Phenyl sepharose chromatography:

1. The fractions containing the material eluted from the Jacalin-agarose column (after melibiose) were pooled and concentrated under vacuum and made 0.4M $(NH_4)_2SO_4$
2. The pooled fractions were then applied to a column of 7ml phenyl Sepharose equilibrated with PBS/ 0.4M $(NH_4)_2SO_4$. The column was washed with 10 column volumes of the same buffer.

3. Protein concentration was monitored by 280 nm absorbance and the peak corresponding to the material not retained by the column was pooled, concentrated to a final concentration of ~4mgml^{-1} and dialysed with PBS. (C1-inh was supposed to pass through the column and other proteins retained)

Results

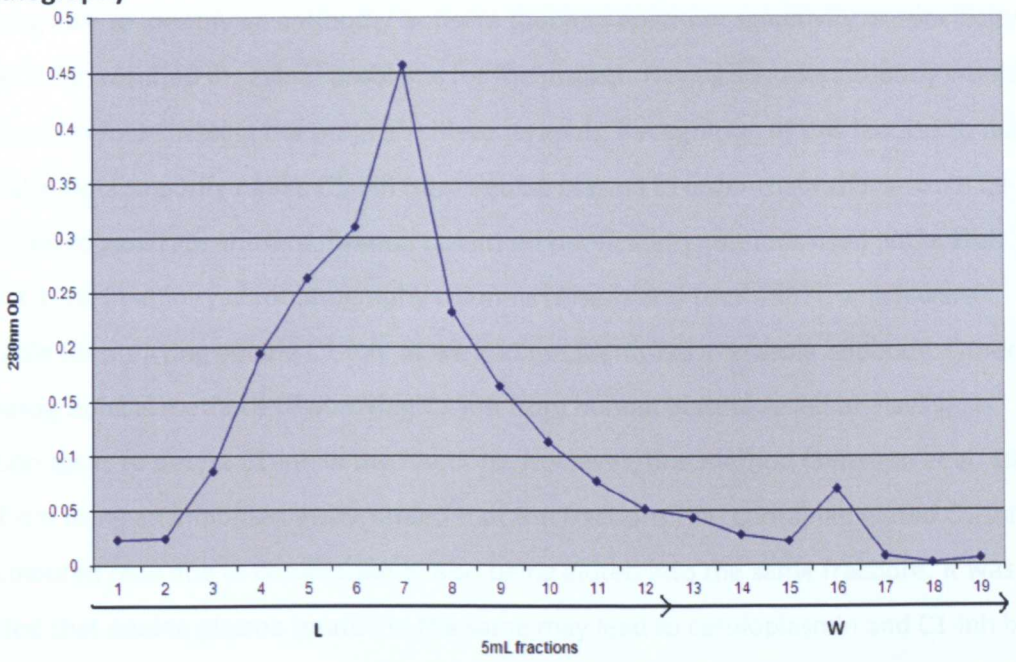
Figure 2.14: Graph showing 280 nm absorbance of fractions from Jacalin agarose chromatography



F1-14: loading column; F15-26: high salt wash; F27- 35: melibiose elution

>Max = > than maximum reading for 280 nm absorbance

Figure 2.15: Graph showing the 280 nm absorbance of fractions from phenyl Sepharose chromatography



L = loading of sample, W= washing with PBS

Gel running and staining

Fractions 5-9 from the phenyl Sepharose chromatography were pooled and dialysed over night in 5 l PBS at 4°C. The pooled fractions were then concentrated by filter centrifugation (Microcon YM-10, YM-100, Millipore UK Ltd.) to give a final 280 nm absorbance of 0.642 (~0.46 $mgml^{-1}$).

Two x 7.5% SDS-PAGE gels were cast with 10 sample wells (as described in the antibody chapter). Each gel was loaded with 5 µl of sample per well. The gels were run under standard non- reducing running conditions (samples in sample buffer heated @ 65°C for 20 min), stained with Coomassie and de-stained as described in the antibody chapter.

Results

No staining was detected ~100kDa, where C1-ihnh was expected to stain.

Western blotting

See antibody chapter (Figure 1.6)

Conclusion

C1-inh was not detected in any of the pooled fractions.

Discussion

Not being able to identify an antibody/ antisera that had apparent selectivity or specificity for equine C1-inh resulted in several problems for the project. Having such an antibody would have been useful in helping the project achieve its goals. Recognition of this fact led to many, varied attempts to purify native C1-inh from equine plasma in order that rabbits could be immunised to generate antisera. Several published purification methods used antibodies against C1-inh in affinity chromatography columns (Alsenz and Loos 1987), which were unsuitable for purifying equine C1-inh, as we had not identified a suitable antibody. Other chromatographical methods of purifying C1-inh from human plasma relied on having an inhibition assay to detect C1-inh in the fractions. However, one method (Salvesen et al. 1985), apart from using an inhibition assay, stated that the fractions that contained eluted C1-inh were coloured blue due to ceruloplasmin also being eluted into the same fractions. It was predicted that equine plasma treated in the same may lead to ceruloplasmin and C1-inh being eluted in the same fractions. On reflection, this was perhaps a rather naive assumption, however, at that stage the results of the PCR experiments were not available and the preliminary project had provided evidence that equine C1-inh was very similar to the human protein. Hence, assuming that the human and equine proteins have similar functions and structure, it was felt that it was worth replicating the method using equine plasma (although there was no information available on equine ceruloplasmin to allow comparison to the human protein). The DEAE sepharose chromatography resulted in elution of fractions which were coloured blue (presumably by equine ceruloplasmin), which went forward into the next chromatography step. It might have been prudent at that stage to run those fractions on SDS-PAGE gels to assess if proteins of a similar Mw to C1-inh were present in the fractions. This was not done for two reasons; firstly, the aim was to purify as much equine C1-inh as possible and so every ml of blue coloured fractions was loaded onto the Cibacron blue column, secondly the fractions still required further purification and if bands were observed at ~100 kDa, there was no way to tell if they were due to C1-inh or other proteins. When the fractions from the Cibacron blue column containing peak protein concentrations were run on SDS-PAGE gels, different band patterns were apparent including several bands at molecular weights that C1-inh might be expected to be detected. It was clear there was a need to develop an assay for detecting the presence of equine C1-inh in the fractions.

Salvesen et al. (1985) measured the concentration of C1-inh in solution by its ability to inhibit the activity of plasma kallikrein that they had purified. Unfortunately, no equine plasma kallikrein was available, so we attempted to develop an assay using porcine kallikrein (Sigma)

and a chromogenic substrate (S-2302). Whilst it was demonstrated that porcine kallikrein reacted with the substrate in an apparently dose dependent manner, there was no obvious inhibition of porcine kallikrein by any of the fractions in the assay. Indeed the OD changes seem to correlate to the amount of protein in the fractions. The reason for this is unclear; it may be that equine plasma kallikrein was present in the fractions, or at least some other proteases that might have activity on the substrate. This latter explanation is perhaps more probable as it would be unlikely that equine kallikrein would be present in all the fractions. It is also possible that something in the fractions was potentiating the action of the porcine kallikrein.

In an example of poor experimental planning, it was only after the total lack of inhibition of porcine kallikrein that whether C1-inh from one species could inhibit kallikrein from another species was tested. Whilst the complement system is relatively well conserved across mammalian species, it has been demonstrated that heterologous components do not necessarily react with one another (Gigli and Austen 1971). This proved to be the case with no inhibition of porcine kallikrein by purified human C1-inh under the test conditions. If human C1-inh did not inhibit porcine kallikrein then it was probable that equine C1-inh would not either and this may have explained why there was no inhibition of porcine kallikrein by any of the tested fractions. As a result, attempts were made to purify equine plasma kallikrein from plasma following the method used by Salvesen et al. (1985) (originally published by Nagase and Barrett (1981)), with some of the modifications to the methylamine treatment and subsequent acetone activation of pro-kallikrein suggested by Tada et al. (2001). Once again, due to the lack of information regarding equine plasma kallikrein, the assumption was that it would be very similar to the human protein and so the purification process would work. S-2302 was used as a substrate to check for kallikrein activity in the fractions. There was no obvious increase in the breakdown of S-2302 in any of the fractions; the breakdown profile once again followed the protein concentration (as measured by 280nm absorbance). The kallikrein should have been eluted with 5mM NaOH pH 11.2-11.4. The increase in breakdown seen after F74 followed the increase in 280 nm absorbance but not by as much as one would expect if the majority of the kallikrein was being eluted at this point (i.e. 5x increase in protein concentration was matched by a ~2.5 x increase in S-2302 breakdown).

The pooled fractions did not show obvious bands around 90 kDa on SDS-PAGE or when probed on Western blots using an antibody against human kallikrein. However, the antibody against human kallikrein did produce bands in the Western blots that were not apparent on the gels which suggested the antibody had an affinity for a protein in some of the fractions. An affinity

column was made coupling the antibody against human kallikrein to attempt to purify the proteins the antibody had apparent affinity for from the fractions. The fractions that were expected to contain the proteins bound to the antibody did show staining for proteins between 116 and 205 kDa but the staining was very diffuse with no clear bands to cut out and attempt protein sequencing.

In a final attempt to obtain a substrate to use in an inhibition assay, a method of purifying bovine pancreatic prokallikrein published by Al-Hamidi et al. (1991) was followed. When the pooled fractions were run on SDS-PAGE gels there were discrete bands between 36 and 45 kDa for pools A, C, D and E. The molecular weight of bovine prokallikrein is ~20 kDa, but in other species it can be as high as 40 kDa (Al-Hamidi et al. 1991). On reflection, it might have been better to cast higher density gels (e.g. 10% acrylamide) as the target protein may have run at the bottom of the gel in the wave front and not be identifiable, also a higher density gel would have resulted in a better spread of proteins at these molecular weights. It is possible that equine prokallikrein is present in some of these bands. There was no increase in breakdown of S-2302 by any samples from any of the fraction pools, although the methodology was supposed to purify pro-kallikrein, at the time it was hoped that some of the prokallikrein would have become activated and react with S-2302. This assumption may be flawed in two respects; firstly, that any prokallikrein would become activated spontaneously at all, or in sufficient amounts to activate S-2302, and secondly, that activated pancreatic prokallikrein would act on S-2302. Al-Hamidi et al. (1991) had to activate their purified prokallikrein by incubating it with trypsin, suggesting spontaneous activation is unlikely. Pancreatic prokallikrein is a trypsin like proteolytic enzyme and so should be able to react with S-2302 but no validation experiments were carried out (i.e. we did not obtain a purified source of pancreatic prokallikrein to validate for activity against S-2302). Notably, Al-Hamidi et al. (1991) utilised the physiological substrate for pancreatic prokallikrein (kininogen) to test for activity. Without further assay validation work it cannot be stated whether or not prokallikrein was present in the fractions. However, there is no published evidence that C1-inh is able to inhibit pancreatic kallikrein and so the value of pursuing this avenue is in doubt.

The last attempt at purifying equine C1-inh from plasma was based on the principle that jacalin binds highly glycosylated proteins and as (human) C1-inh is a highly glycosylated plasma protein, jacalin may be useful to help separate C1-inh from other plasma proteins. Once again, only being able to identify proteins based on them being present in fractions in enough

quantities to be detected on SDS-PAGE gels at the estimated Mw proved to be an insensitive technique and no fractions were detected that contained equine C1-inh.

Unfortunately, our attempts at purifying equine proteins from plasma or tissues were entirely unsuccessful as we were not able to identify the target proteins. This is in no small part due to the fact that we did not possess robust and validated assays for some of the proteins (C1-inh and prokallikrein). In addition, there has been comparatively little research on the proteins that we were attempting to purify and so assumptions had to be made that their structure/ biology would be similar enough to the equine proteins of interest.
There is a commercially available functional assay for human C1-inh (Berichrom GmBH) which has been considered for assay of levels of C1-inh in equine plasma by the author (unpublished data) and (Topper and Prasse 1998), but this assay has not been validated for equine plasma/ C1-inh, and had not been validated for use with chromatography fractions. Furthermore, the assay was too prohibitively expensive (in terms of hardware and consumables) for this project (see also final discussion).

Retrospectively, a more rational approach to the problem of attempting to develop antibodies effective against equine C1-inh would have been to adopt molecular biological approaches, rather than attempting to purify the native protein from plasma utilising chromatographical techniques.

Chapter 3

Molecular biological analysis of equine C1-inh

Introduction

This chapter describes the use of molecular biological techniques to determine the amino acid sequence of equine C1-inh.

There is a lot of information available on the human C1-inh nucleotide and amino acid sequence and their relation to structure and function. In contrast, there is comparatively little, or no, information on the nucleotide and amino acid sequence of the majority of equine proteins, including C1-inh.

Furthermore, the equine genome has not yet been entirely mapped or published making it harder to compare sequences from human or other species to the horse genome (although in the second half of 2007 the equine genome has appeared in the 'pre-species' section on the Ensembl website [www.Ensembl.org/Equus_caballus/index.html). With the limited knowledge available, it was still quite feasible to design systems which gave us the opportunity to detect both DNA and mRNA for specific target equine proteins, such as C1-inh, C3 and C5.

It is a reasonable assumption that equine C1-inh exists. The complement system and its constituent cascade proteins are relatively well conserved across mammals (Gigli and Austen 1971). Preliminary experiments in 2002 demonstrated that a goat polyclonal antibody against human C1-inh cross-reacted with a protein of similar molecular weight in equine plasma. However, at this stage of the project, there was little evidence to support this initial finding. Molecular biological techniques were employed to produce data which would provide information about the structure and function of equine C1-inh.

Objectives

Our aims for this part of the project were to

- Determine if C1-inh is present in the horse
- Determine the nucleotide and amino acid sequence for the protein
- To attempt to relate the generated sequences to structure to enable us understand the apparent lack of cross-reactivity of the antibodies tested
- To use quantitative real-time PCR to assess if there were differences in gene expression for equine C1-inh in different normal and pathological tissues

Materials

Advanced Biotechnologies Ltd.: Superladder low 100 bp ladder [SLL 100]

Applied Biosystems: RNAlater [7021], 6x gel loading dye [AB 0594]

Cogenics Lark Inc.: DNA sequencing

Eurofius Genetic Services Ltd.: Oligonucleotide primers

Falcon cell culture flasks

Starlab: 1-200 µL Micropipette Tips, 100-1000 µL Micropipette Tips

Invitrogen Ltd, Paisley, Scotland, UK: 1 kb plus DNA mass ladder, Duldecco's Modified Eagle Medium, Trizol® Reagent, SuperScript™II reverse transcriptase, foetal calf serum, trypsin-EDTA.

Promega, Southampton, UK: 1 Kb DNA ladder [G 5711], M-MLV Reverse Transcriptase RNase H minus, random primers, oligo$(dT)_{15}$ primer, AMV primer extension buffer, primer extension sodium pyrophosphate T4 polynucleotide kinase 10X buffer, ethidium bromide, 5x Passive lysis buffer.

Qbiogene-ALEXIS Ltd, Bingham, Nottingham, UK: GeneClean Turbo Kit.

Qiagen Ltd, Dorking, Surrey, UK: Rneasy spin kit, Qia Quick PCR purification kit.

Methods

Basic protocols

Preparation of RNA/cDNA from various equine tissues

Collection of tissues

Tissues (heart, kidney, large intestine, liver, lung, skeletal muscle, skin, small intestine and spleen) were obtained under aseptic conditions post mortem (less than 1 hour after death) from horses euthanized with cinchocaine/ quinalbarbitone. The horses were euthanized for economic reasons due to chronic non-septic orthopaedic disease which impaired full athletic function. Small (<0.5 cm) pieces of tissue were placed into RNAlater™ and stored at -20°C. Some pieces of liver were also flash frozen in liquid nitrogen and stored at -80°C.

Processing tissues

Tissues were cut to obtain pieces weighing ~100 mg. These pieces were wrapped in foil and dropped into liquid nitrogen. Dismembrator (Mikro-Dismembrator; Sartorius Stedim Biotech S.A.) chambers and ball were also placed in liquid nitrogen.

After freezing, each piece of tissue was placed into the dismembrator chambers along with the ball. The frozen tissue was then homogenised at 2000 rpm for 2 min and placed into 1 ml Trizol in a 1.5 ml eppendorf tube. The homogenised samples in Trizol were left at room temperature (RT) for 5 minutes before centrifugation at 12,000g for 10 min at 2 - 8°C.

Phase separation

Chloroform (200 µl/1 ml Trizol) was added to the sample suspension before shaking the tube vigorously by hand for 15 sec. The tubes were left at RT for 2 -3 min before centrifugation at 12,000g for 15 min at 2 - 8°C. The colourless aqueous upper phase containing RNA was transferred to a new 1.5 ml eppendorf tube.

Precipitation of RNA

Isopropanol (500 µl/ 1 ml Trizol) was added to the aqueous phase and mixed. The solution was left at -20°C for an hour (or -80°C overnight). The sample solution was then centrifuged at 12,000g for 10 min at 2 - 8°C. The resultant pellet was washed with 100% ethanol (1ml/ 1ml Trizol used) and centrifuged at 7,500g for 5 min at 2 - 8°C. This step was repeated with 75%

ethanol. The supernatant was removed and the pellet air-dried for 5-10 min in a tissue culture hood. The pellet was re-suspended in 50 µl of nuclease free water.

Removal of genomic DNA

RNAsin (1 µl), 5 µl of 10x buffer and 1µl RQ1 RNase-free DNase were added to the RNA in nuclease-free water from the step above. The solution was mixed and then left at 37°C for an hour before being placed on ice prior to the next step.

RNeasy clean up

The volume of treated RNA was adjusted to 100 µl by adding nuclease-free water. 350 µl of RLT buffer (containing 2-mercaptoethanol) was added and mixed thoroughly. 250 µl of 100% ethanol was added. The sample was applied immediately to a RNeasy mini spin column in a collection tube; the column was then centrifuged for 15 sec at >8,000g.

The column was then transferred to a new collection tube and 500 µl of RPE buffer (containing ethanol) was added; the column was then centrifuged for 15 sec at >8,000g. The flow through was discarded, a further 500 µl of RPE buffer (containing ethanol) was placed onto the column and the column was centrifuged for 2 min at >8,000g to dry the column. RNA was eluted from the column by pipetting 50 µl of nuclease-free water directly onto the membrane and centrifuging the column for 1 min at >8,000g.

Preparation of cDNA using SuperscriptII RNase H-reverse Transcriptase

RNA (10 µl), 1 µl of oligo dT (500 µg/ml) [or 200ng random hexamer primers 0.4 µ], 1 µl dNTP mix (10 mM each dATP, dCTP, dTTP, dGTP) and nuclease-free water to 12 µl were assembled in a microcentrifuge tube. The mixture was heated to 65°C for 5 minutes then quickly chilled on ice. Then 4 µl of 5x first strand buffer, 2 µl 0.1M DTT and 1 µl RNasin were added to the tube and the solution mixed gently. When using oligo dT, the tube was heated to 42°C for 10 min; when using random hexamers the tube was heated to 25°C for 2 min. One µl of SuperscriptII was added and the solution mixed gently by pipetting. For random hexamers, the solution was heated again to 25 °C for 2 min. (this step was not necessary if oligo dT was used). The tubes were then incubated at 42°C for 50 min. The reaction was inactivated by heating to 70°C for 15 min. cDNA was now ready for PCR.

Polymerase Chain Reaction

Reactions were carried out in 0.25 ml microcentrifuge tubes which contained 1 µl dNTP solution (10 mM each dATP, dCTP, dTTP, dGTP), 0.13 µl HotStart™ Taq polymerase (50 U/µl), final concentration of 12.5 pmol (0.25µl) of the appropriate sense primer, final concentration of 12.5 pmol of the appropriate anti-sense primer (0.25 µl), 2.5 µl 10x buffer (final concentration 10 mM $MgCl_2$), 2.5 µl Q solution and 2 µl of cDNA prepared from equine tendon fibroblasts grown in cell culture media or from equine liver. A final volume of 25 µl was achieved. The PCR reactions were performed in a Applied Biosystems GeneAmp PCR system 9700 thermocycler. Cycling conditions consisted of a step of denaturation at 94°C for 2 min, then 25 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, elongation at 72°C for 1 min, followed by a final step of elongation at 72°C for 10 min, before cooling to 20°C until the tubes were removed from the thermocycler. In all experiments, negative control tubes were included which substituted water for template; where possible, positive controls were also included.

Agarose gel electrophoresis

PCR products were analysed by agarose gel electrophoresis. Agarose gels were composed of 1% (w/v) agarose in 1x TAE buffer (2.42 g Trizma base, 0.57 ml glacial acetic acid and 1 ml 0.5 M EDTA (pH 8.0) in 1L of dH_2O) containing 5 µl per 100ml (10 mg/ml) ethidium bromide (EtBr). After the gel had polymerised, it was transferred to a gel electrophoresis tank and submerged in 1x TAE buffer. Samples (5 µl of PCR product) were prepared for running in 5x DNA gel loading buffer containing 1 mM EDTA, 10 mM Tris, 30% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 0.1% xylene cyanole FF. After loading samples along with a suitable DNA mass ladder (5 or 10 µl of 1 kb plus DNA mass ladder and/or 5 or 10 µl of low DNA mass ladder: volume loaded depended on size of wells) electrophoresis was performed at constant voltage of 80 V for 40-50 min. The DNA products were then visualised under a UV transilluminator and a Polaroid photograph taken.

PCR product cleaning

The remaining volume of each PCR product was then 'cleaned' using GENECLEAN ™ Turbo (QBio systems). Each PCR product was placed into a 1.5 ml nuclease-free microcentrifuge tube, 5 volumes GENECLEAN ™ Turbo Salt Solution was added and mixed by pipetting.

<600 µl DNA/ Salt Solution was transferred into a GENECLEAN ™ Turbo cartridge placed inside a cap-less collection tube, before centrifuging at >8,000g for 5 sec until all liquid had passed through the filter. 500 µl prepared GENECLEAN ™ Turbo wash solution was added to the cartridge and centrifugation was repeated. The collection tube was emptied and the centrifugation step was repeated for 3 minutes to remove any residual wash solution.

The cartridge was then inserted into a fresh collection tube and 30 µl of GENECLEAN ™ Turbo Elution Solution was added directly onto the filter in the cartridge. The cartridge was allowed to stand for 5 min at RTP, before finally centrifuging at >8,000g for 1 min to transfer the eluted DNA into the collection tube.

PCR product sequencing

The cleaned PCR products were sent to Cogenics Lark Technologies for sequencing. The appropriate sense and anti-sense primers for each product were sent with the products. PCR product sequences were assessed for quality by analyzing the sequence chromatograph outputs (ChromasPro: Technelysium Pty Ltd), and then were compared to each other, the human sequence (NM_000062) and the equine ESTs used to design the primers using the ClustalW (version 1.81) facility, found on the website; www.expasy.ch.

Determination of the nucleotide sequence for equine C1-inhibitor esterase

Primer design

The mRNA sequence for human C1-inh (accession number NM_000062) was compared for homology against an equine Extended Sequence Tag (EST) database (The University of Georgia) and the National Centre for Biotechnology nucleotide database (www.ncbi.nlm.nih.gov/blast) to detect equine mRNA sequences of >85% homology. Accession number CX601951 and the EST sequence (APL07, since assigned: BM780519) showed greatest homology. The ESTs appeared to correlate to two parts of the human sequence, one EST (CX601951) to the trans-serpin region of the protein, the other (BM780519) to the conserved serpin region of the protein. The equine ESTs were then compared for homology against the BLAST(n) search in EST and nucleotide data bases at the website National Centre for Biotechnology database (www.ncbi.nlm.nih.gov/blast) to evaluate homology with any other proteins. Both equine ESTs showed greatest homology to human C1-inh or predicted C1-inh from other species.

The equine EST sequences were then used to design primers. Primers were designed on PrimerXpress (Applied Biosystems). The primer pairs were designed to search for sequences in each region that showed homology to NM_000062 but also DNA sections that could be used to provide sequences for the missing areas of sequence between the two regions. The annealing temperature of the primers was chosen in the range 50-65°C, the percentage of G/C nucleotides was between 40-60% and the length of the gene specific primers was between 15 and 21 nucleotides. Primers were also assessed for potential 'primer-dimer' formation. The following primers were ordered from MWG Biotech (www.mwgbiotech.com);

EqC1InhTS1_for: 5'- GGC ATA GAG CCG CAA - 3'

EqC1InhTS2_for: 5' - GAG CCG CAA ATC ATG - 3'

EqC1InhTS_rev: 5' - CTC CGA GAG GCC TTC ACA A - 3'

The primers above were designed to cross the nucleotide sequence that corresponds to the trans-serpin domain on the human protein.

EqC1Inh_for: 5' - CGT ACC CCA TGA ACT TTG CCT - 3'

EqC1Inh_rev: 5'- CCA GAG CAG GAA GAG GAA GG - 3'

The primers above were designed to generate nucleotide sequence that corresponds to the 3' end of the human protein. The reactive site of human C1-inh is known to be close to the 3' end. Both EqC1InhTS1_for and EqC1InhTS2_for were also designed to be run with EqC1Inh_rev.

In the first experiments the following primer pair combinations were used:

EqC1InhTS1_for /EqC1InhTS_rev: estimated amplicon length 605

EqC1InhTS2_for/EqC1InhTS_rev: estimated amplicon length 598

EqC1Inh_for/ EqC1Inh_rev: estimated amplicon length 800

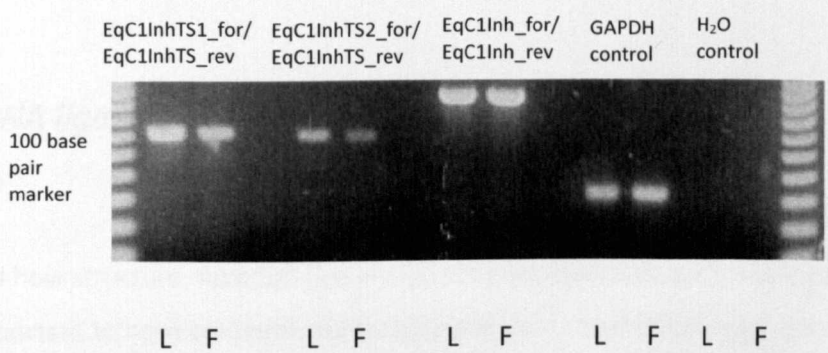
The primer pairs were designed to reveal the nucleotide sequence of the trans-serpin domain and the serpin domain, as a result the produced sequence from the first set of experiments did not overlap.

In the next experiments the following primer pair was used to reveal the nucleotide sequence between the first two obtained sequences; modifications to the cycling conditions for each primer pair are also provided;

EqC1InhTS1_for/ EqC1Inh_rev: elongation at 72°C for 1 min 20 sec. 30 cycles of denaturation.

Results

Figure 3.1: Photograph of PCR products from the primer pairs used in the first experiment



L = Liver cDNA,
F = tendon fibroblast cDNA

2nd experiments;

The products showed 100% homology to the equine ESTs and ~84% homology to NM_000062. The nucleotide sequence determined from the PCR experiments at this stage is shown below. This sequence was blasted (PubMed) to check for homology to other nucleotide sequences. The sequence had homology to C1-inhibitor from man and other species but showed little homology to other proteins.

Equine PCR C1-inh product summary (lower case identifies bases that were assigned after studying chromatographs)

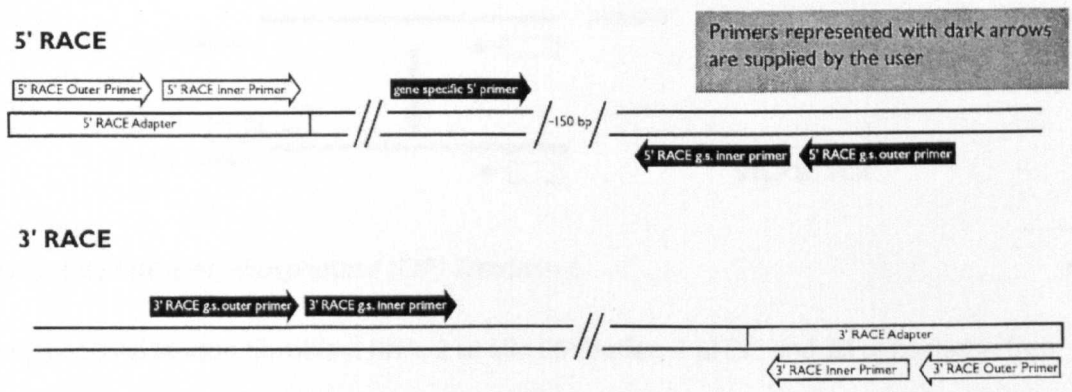
AGACATCTTGGACATCTTGAagggaACTGTGCCCAAgaCAGTATCCATTCAAGACACGGTGAGC
TCTTCCACCTTGCCGGAAACCAAGGAGACCAACACAACAGTTAGTGCCACTTTGGGATCTACCA
CTCAACCAACTACCCAGCCCACTACTGAGTTCGGCTGCCCGGAGCCTGACATCTCCTGCCCTGA
CTTGGGCAATCATTCAGCAGAGATGATGTTGGGGGACGCTTTGACAGATTTCTCCGTGAAGCTG
TACCACGCCTTCTCAGCAGTGAAGAAACCcagGAGCAACATGGCCTTTTCCCCATTCAGCATCG
CCAGCCTCCTCACTCAGGTCCTGCTTGGGGCTGGGGACAGCACCAAGAAAAACCTGGAGAGCGT
CCTCTCGTACCCCATGAACTTTGCCTGTGTCCACCAGACCATGAAGGCCTTCAGGTCTAAAGGC
TTCAGCTCAGCCTCTCAGATCTTCCACAGCCCAGACCTGGCCATAAAGAACGCGTTTGTGAACG
CCTCTCGGAGCCTGTATGGCAGCAGCCCCAGAGTCCTGGGAAATGACAGTCAAGTCAACTTGGA
GCTCATCAACGCCTGGGTGGCAGAGAACACCAACCACAAGATCAGCCGGCTGCTAGACAGCCTG
CCCGCCGATGCTCGCCTTGTCCTCCTCAATGCCGTCTCCCTGAATGCCAAGTGGAAGAAAACAT
TTGATCCGAAAAATACCAGGATGGAGCCCTTTTACGTTAAATCCTCTGTGAAAAAAGTGCTTAT
GATGAGTAGCAAGAAGTACCCTGTGGCCCATTTCACTGACCAGATTCTGAAGGCCAAGGTGGGA
CAACTGCAGCTCTCCCACAACCTTAGCTTGGtGATCCTGGTGCCCCAGGACATGAAACAACATC
TTGAAGACGTGGAGCAGGCTCTCAGCCCCTCTGTCTTcAAGGCCATcCTGAAgAAGCTGGAGAT
GACCAAGATCCAgCCTACTCTCCTGAtGATTCCCCGAATCaAAGTAAAgAGTAGCCaGGAcATG
CTGACAATCATGGAgAAGCTAGAATTcTTTGACTTTaGTTACgACCTcAACCTGtGCAGGAtGA
CTGagGACCcGGAtcTTcAGGTTT

5′ and 3′ RNA ligase mediated rapid amplification of cDNA ends (5′and 3′ RLM-RACE)

To understand how structure, function and antigenicity compare between equine and human C1-inh it is important to have complete nucleotide and amino acid sequences. On human C1-inh, the reactive site against C1/ kallikrein is near to the C-terminus (3') and the N-terminus (5') domain is unlike that of any other known amino acid sequence. In order to obtain the full sequence of equine C1-inh we used **RNA Ligase Mediated Rapid Amplification of cDNA Ends PCR (RLM RACE PCR).**

Briefly, the cDNA was snipped and an adaptor sequence of artificial cDNA added onto either the 5' or 3' end of the cDNA, these adaptor regions had specific primers designed for them which were paired with gene specific primers designed to begin a short way in from the end of the cDNA. Primers were designed to strict specifications e.g. 20-24 bp long, 50%GC content, <3 GC residues in 3' most 5 bases, no G at the 3' terminus. RLM RACE PCR also required production of specially prepared cDNA, the mRNA we used to do this was equine tendon fibroblast mRNA, as it was previously shown to be positive for C1-Inh mRNA.

Figure 3.2: A schematic of the rationale behind the primer design is shown below;



3' RACE primers

3' gene specific outer (for): 5'-CTC TCC TGA TGA TTC CCC GAA T-3'

3' gene specific inner (for): 5'-GTA TCC AGG ACA TGC TGA CAA T-3'

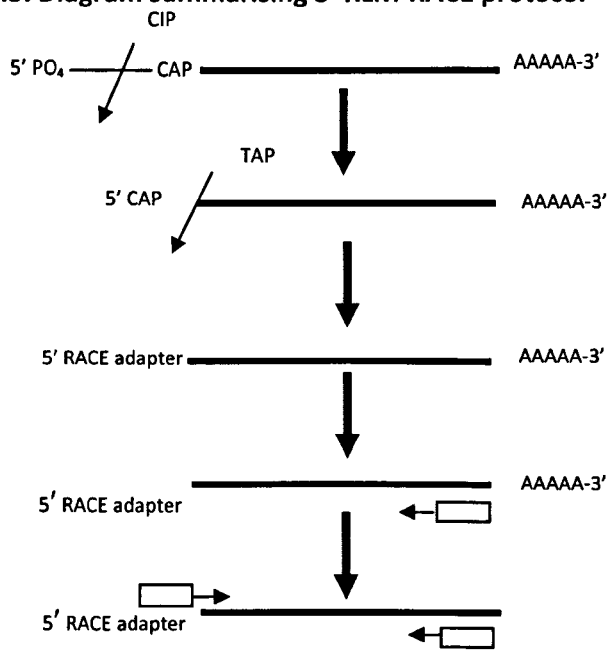
5' RACE primers

5' gene specific (for): 5'-ACA TCT TGA AGG GGA CTG TGC C-3'

5' gene specific outer (rev): 5'-AGG ACG CTC TCC AGG TTT TTC T-3'

5' gene specific inner (rev): 5'-CCA TGT TGC TCC TGG GTT TCT T-3'

Figure 3.3: Diagram summarising 5' RLM-RACE protocol



STEP 1: CIP treatment to remove 5' PO_4 from degraded mRNA, rRNA, tRNA and DNA

STEP 2: TAP treatment to remove cap from full length mRNA

STEP 3: 5' RACE adapter ligation to decapped mRNA

STEP 4: Reverse transcription

STEP 5: PCR

Step 1: Calf Intestine Phosphatase (CIP) Treatment

Ten µg equine tendon fibroblast RNA, 2 µl 10x CIP buffer, 2 µl CIP and 20 µl nuclease-free water were assembled in a microcentrifuge tube, mixed gently and incubated for 1 hour at 37°C. The CIP reaction was terminated with chloroform:phenol extract. Fifteen µl ammonium acetate solution, 115 µl nuclease-free water and 150 µl acid phenol: chloroform were added to the microcentrifuge tube and vortexed thoroughly before centrifuging for 5 min at room temperature at top speed in a microfuge. Chloroform (150 µl) was added and the mixture vortexed before the centrifugation step was repeated. The aqueous phase was transferred to a new microcentrifuge tube and 150 µl isopropanol was added, the solution was mixed and chilled on ice for 10 min. After chilling the solution was centrifuged at top speed for 20 min, the liquid phase was removed and the pellet rinsed with ice cold 70% ethanol. The tube was centrifuged for 5 min at top speed and the ethanol carefully removed, allowing the pellet to air dry. The pellet was re-suspended in 11 µl nuclease-free water with part of the sample being stored and part going forward to step 2.

Step 2: Tobacco Acid Pyrophosphatase (TAP) Treatment

CIP treated RNA (5 µl from step 1), 1 µl 10x TAP buffer, 2 µl TAP and 2 µl nuclease-free water were assembled in a microcentrifuge tube, mixed gently and incubated for 1 hour at 37°C.

Step 3: 5'-RACE adaptor ligation

CIP/ TAP treated RNA (2 µl), 1 µl 5'-RACE adaptor, 1 µl 10x RNA ligase buffer, 2 µl T4 RNA ligase (2.5 U/µl) and 4 µl nuclease-free water were assembled in a microcentrifuge tube, mixed gently and incubated for 1 hour at 37°C.

Step 4: reverse transcription

Ligated RNA (2 µl), 4 µl dNTP mix (10 mM each dATP, dCTP, dTTP, dGTP), 2 µl random decamers, 2 µl 10x RT buffer, 1 µl RNAse inhibitor, 1 µl M-MLV reverse transcriptase and 8 µl nuclease-free water were assembled in a microcentrifuge tube, mixed gently and incubated for 1 hour at 42°C.

Step 5: Nested PCR for 5' RLM-RACE PCR

Outer 5' RLM-RACE PCR

RT reaction product (1µl), 1 µl dNTP solution, 0.13 µl Taq polymerase (50 U/µl), 2.5 µl 10x buffer (final concentration 10 mM $MgCl_2$), 2.5 µl Q solution, 1 µl 5' RACE gene specific outer primer (10 µM), 1 µl 5' RACE outer primer and 17.37 µl nuclease-free water were assembled in a microcentrifuge tube* and then cycled as follows: Cycling conditions consisted of a step of denaturation at 94°C for 3 min, then 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, elongation at 72°C for 30 sec, followed by a final step of elongation at 72°C for 7 min.

Inner 5' RLM-RACE PCR

Nested PCR product (1-2 µl), 1 µl dNTP solution 0.13 µl Taq polymerase (50 U/µl), 2.5 µl 10x buffer (final concentration 10 mM $MgCl_2$), 2.5 µl Q solution, 1 µl 5' RACE gene specific inner primer (10 µM), 1 µl 5' RACE inner primer and 17.37 µl nuclease-free water were assembled in a microcentrifuge tube and then cycled as for the nested PCR step above.

3' RLM-RACE protocol

The 3' RLM-RACE protocol was essentially the same as steps 3 – 5 of the 5' protocol as no removal of phosphate and cap was required; the RACE adaptor was inserted during the RT step.

Step 1: Reverse transcription

RNA (2 µl), 4 µl dNTP mix (10 mM each dATP, dCTP, dTTP, dGTP), 2 µl 3' RACE adaptor, 2 µl 10x RT buffer, 1 µl RNAse inhibitor, 1 µl M-MLV reverse transcriptase and 8 µl nuclease-free water were assembled in a microcentrifuge tube, mixed gently and incubated for 1 hour at 42°C.

Step 2: PCR for 3' RACE

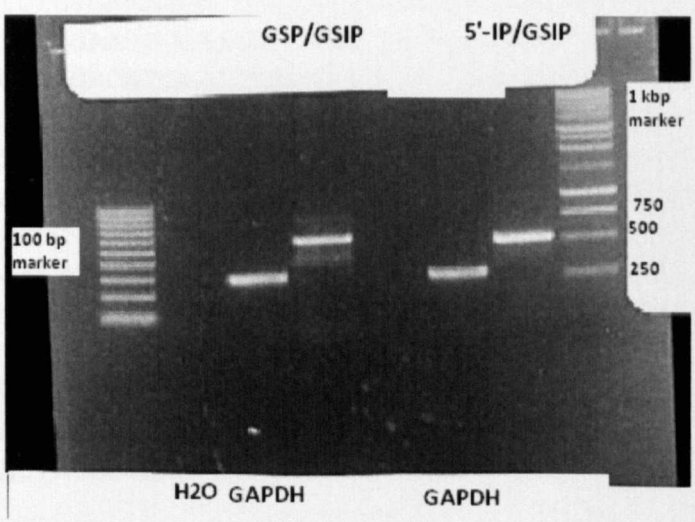
RT reaction product from the 3' end (1 µl), 1 µl dNTP solution, 0.13 µl Taq polymerase (50 U/µl), 2.5 µl 10x buffer (final concentration 10 mM $MgCl_2$), 2.5 µl Q solution, 1 µl 3' RACE gene specific outer primer (10 µM), 1 µl 3' RACE outer primer and 17.37 µl nuclease-free water were assembled in a microcentrifuge tube and then cycled as above;

An inner 3' RACE PCR was not necessary.

The PCR products from all the 5' and 3' RLM-RACE reactions underwent agarose gel electrophoresis, cleaning and sequencing as described above. The resulting sequences were checked and aligned as described above. Various web based tools (ORF finder www.ncbi.nlm.nih.gov/projects/g**orf** and JustBio www.justbio.com), were utilised to translate the above nucleotide sequence into the probable amino acid sequence for equine C1-inh.

Results

Figure 3.4: Photograph of the products from the 5' RLM RACE experiments;



H_2O = water control, GAPDH = positive control, 5-IP/ GSIP = 5' RACE inner primer/ gene specific inner primer PCR product, GSP/ GSIP = gene specific primer/ gene specific inner primer PCR product

The products of the 3' and 5' RACE PCR were cleaned and sent for sequencing. The sequence results were checked by analysing the chromatograms. The results were then blasted against each other, the equine ESTs, human C1-inh and any other protein in the PubMed nucleotide database. The complete nucleotide sequence generated from the PCR and RLM-RACE PCR experiments is shown below. This sequence was then used to determine the most likely amino acid sequence using the ORFfinder tool and was then aligned to human C1-inh using the JustBio hosted alignment tools (www.Justbio.com)

The complete nucleotide mRNA sequence for equine C1-inh (lower case signifies nucleotides allocated on the basis of chromatograph examination):

AAAgGGGTGGGGTGGCAGTCCCCTGCGCCCcGGAGCTCGGAGCTGGCTCCGAGTCTGGCTGACT
TCGCAGGTCCGGACAGACGTCGCCGCCCAGATGGCCTCCAGGCTGACCCCCCTGACCCTCCTGC
TGCTGCTGCTGCTGGCTGGGCATAGAGCCGCCTCAAATCCTGATGATAGCAACCACAGCTTCAC
AGATCCAGAGAGCTTGCAAGGAGAAAGCAAAGGAGACATCTTGGACATCTTGAAGGGAACTGTG
CCCAAGACAGTATCCATTCAAGACACGGTGAGCTCTTCCACCTTGCCGGAAACCAAGGAGACCA
ACACAACAGTTAGTGCCACTTTGGGATCTACCACTCAACCAACTACCCAGCCCACTACTGAGTT
CGGCTGCCCGGAGCCTGACATCTCCTGCCCTGACTTGGGCAATCATTCAGCAGAGATGATGTTG
GGGGACGCTTTGACAGATTTCTCCGTGAAGCTGTACCACGCCTTCTCAGCAGTGAAGAAACCCA
GGAGCAACATGGCCTTTTCCCCATTCAGCATCGCCAGCCTCCTCACTCAGGTCCTGCTTGGGGC
TGGGGACAGCACCAAGAAAAACCTGGAGAGCGTCCTCTCGTACCCCATGAACTTTGCCTGTGTC
CACCAGACCATGAAGGCCTTCAGGTCTAAAGGCTTCAGCTCAGCCTCTCAGATCTTCCACAGCC
CAGACCTGGCCATAAAGAACGCGTTTGTGAACGCCTCTCGGAGCCTGTATGGCAGCAGCCCCAG
AGTCCTGGGAAATGACAGTCAAGTCAACTTGGAGCTCATCAACGCCTGGGTGGCAGAGAACACC
AACCACAAGATCAGCCGGCTGCTAGACAGCCTGCCCGCCGATGCTCGCCTTGTCCTCCTCAATG
CCGTCTCCCTGAATGCCAAGTGGAAGAAAACATTTGATCCGAAAAATACCAGGATGGAGCCCTT
TTACGTTAAATCCTCTGTGAAAAAAGTGCTTATGATGAGTAGCAAGAAGTACCCTGTGGCCCAT
TTCACTGACCAGATTCTGAAGGCCAAGGTGGGACAACTGCAGCTCTCCCACAACCTTAGCTTGG
TGATCCTGGTGCCCCAGGACATGAAACAACATCTTGAAGACGTGGAGCAGGCTCTCAGCCCCTC
TGTCTTCAAGGCCATCCTGAAGAAGCTGGAGATGACCAAGATCCAGCCTACTCTCCTGATGATT
CCCCGAATCAAAGTAAAGAGTAGCCAGGACATGCTGACAATCATGGAGAAGCTAGAATTCTTTG
ACTTTAGTTACGACCTCAACCTGTGCAGGATGACTGAGGACCCGGATCTTCAGGTTTCTGCGAT
GCAGCACCAGATCACGCTGGAGCTGATGGAGTCCGGGGTGGAGGCGGCTGCAGCCACGGCTGTG
TCTGTGGCCCGCAATTTGCTGATCTTCCATGTGGATCAGCCCTTCCTCTTCGTGCTCTGGGACC
AGCAGCACAAGTTCCCTGTCTTCATGGGGCGAGTGTATGACCCCATGGCCTAAGACCTGCAGAG
GACCGGGCAAGGGCAAGCCCTACCCCTCAAACCTCAGCTCTCCACTTGCAGCCCTGCTGCTGCC
TGCCTGGGCTTGCCCCCAGCCACCTTGCACCTTGGGTCCTTTGCCCTCCACCTGAAGGGTTCCC
TCAGGGTCTTGTGAAAGGACCTGCTTTTGTCATCCCTGTGACTCTTTAAATCACTCTCTGCAAC
TTTGTCTGGTACAAGTACACCAGACTCTACAAATAAAACCTGGCAGACCATNAAAAAAAAAAAA
CCTATTGG

Amino acid sequence of equine C1-inh

KGWGGSPLRPGARSWLRVWLTSQVRTDVAAQMASRLTPLTLLLLLLLAGHRAASNPDDSNHSFT
DPESLQGESKGDILDILKGTVPKTVSIQDTVSSSTLPETKETNTTVSATLGSTTQPTTQPTTEF
GCPEPDISCPDLGNHSAEMMLGDALTDFSVKLYHAFSAVKKPRSNMAFSPFSIASLLTQVLLGA
GDSTKKNLESVLSYPMNFACVHQTMKAFRSKGFSSASQIFHSPDLAIKNAFVNASRSLYGSSPR
VLGNDSQVNLELINAWVAENTNHKISRLLDSLPADARLVLLNAVSLNAKWKKTFDPKNTRMEPF
YVKSSVKKVLMMSSKKYPVAHFTDQILKAKVGQLQLSHNLSLVILVPQDMKQHLEDVEQALSPS
VFKAILKKLEMTKIQPTLLMIPRIKVKSSQDMLTIMEKLEFFDFSYDLNLCRMTEDPDLQVSAM
QHQITLELMESGVEAAAATAVSVARNLLIFHVDQPFLFVLWDQQHKFPVFMGRVYDPMA

*DLQRTGQGQALPLKPQLSTCSPAAACLGLPPATLHLGSFALHLKGSLRVL*KDLLLSSL*LFK
SLSATLSGTSTPDSTNKTWQTXKKKKPIX

The amino acid sequence showed relatively good homology to the human protein. Overall amino acid homology was ~68%. The homology for the N-terminal region was poor (see discussion) at ~34%. The homology for the C-terminus (serpin domain) was ~ 80%. (see overall diagram on next page).

Figure 3.5: Amino acid sequence of human C1-inh (top line; also shows structure as determined for human C1-inh) and translated PCR product from horse sequence (bottom line). The colour key is at the foot of the diagram

```
-----SIGNAL PEPTIDE-----[ N-TERMINAL DOMAIN ----------
MASRLTLLTL  LLLLLAGDRA SSNPNATSSS SQDPESLQDR GEGKVATTVI
MASRLTPLTLL LLLLLAGHRA ASNPDDSNHS FTDPESLQ   GESKDGILDI
****** ***  ******* **  ***     *   **** *   ** *     *
-----PROBABLY IMPORTANT FOR METABOLISM----------------
SKMLFVEPIL EVSSLPTTNS TTNSATKITA NTTDEPTTQP TTEPTTQPTI
LK GTV PKT  VSIQDTVSS                        STLPETKETN
 *   * *    **   *  *                         * * *  *
-----AND INTERACTIONS WITH CELLS--------][----←HELIX A
QPTQPTTQLP TDSPTQPTTG SFCPGPVTLC SDLESHSTEA VLGDALVDFS
TTVSATLGST TQPTTQPTTE FGCPEPDISC PDLGNHSAEM MLGDALTDFS
     *     *   *****    ** *   *  **  ** *   ***** ***
          →--←SERPIN DOMAIN→------← HELIX C  →----
LKLYHAFSAM KKVETNMAFS PFSIASLLTQ VLLGAGENTK TNLESILSYP
VKLYHAFSAV KKPRSNMAFS PFSIASLLTQ VLLGAGDSTK KNLESVLSYP
 ********  **   ***** ********** ******  **  **** ****

----←  HELIX D  →-----------------------------------
KDFTCVHQAL KGFTTKGVTS VSQIFHSPDL AIRDTFVNAS RTLYSSSPRV
MNFACVHQTM KAFRSKGFSS ASQIFHSPDL AIKNAFVNAS RSLYGSSPRV
  * ****   * *  **  *  ********* **   ***** * ** *****
--←       HELIX F     →-------------------------------
LSNNSDANLE LINTWVAKNT NNKISRLLDS LPSDTRLVLL NAIYLSAKWK
LGNDSQVNLE LINAWVAENT NHKISRLLDS LPADARLVLL NAVSLNAKWK
* * *  *** *** *** ** * ******** ** * ***** **  * ****
-------------------------------------------------------
TTFDPKKTRM EPFHFKNSVI KVPMMNSKKY PVAHFIDQTL KAKVGQLQLS
KTFDPKNTRM EPFYVKSSVK KVLMMSSKKY PVAHFTDQIL KAKVGQLQLS
 ***** *** ***  * **  ***** **** ***** ** * **********
--------------← HELIX G →----←HELIX H→-------------
HNLSLVILVP QNLKHRLEDM EQALSPSVFK AIMEKLEMSK FQPTLLTLPR
HNLSLVILVP QDMKQHLEDV EQALSPSVFK AILKKLEMTK IQPTLLMIPR
********** *  *  *** ********** *** **** *  *****  **
-------------------------------------------------------
IKVTTSQDML SIMEKLEFFD FSYDLNLCGL TEDPDLQVSA MQHQTVLELT
IKVKSSQDML TIMEKLEFFD FSYDLNLCRM TEDPDLQVSA MQHQITLELM
***  *****  ********* ********   ********** ****  ***
----------------RS*** *  ****----C-TERMINUS----------]
ETGVEAAAAS AISVARTLLV FEVQQPFLFM LWDQQHKFPV FMGRVYDPRA
ESGVEAAAAT AVSVARNLLI FHVDQPFLFV LWDQQHKFPV FMGRVYDPMA
*********  * **** **  * * *****  ********** ******** *
```

IMPORTANT RESIDUES WHERE AMINO ACIDS MATCH E.G. CYSTEINE OR PROBABLE GLYCOSYLATION SITES

PLACE WHERE Bos model started from; the region before is n-terminal and she did not model it

P1-P2 OF REACTIVE SITE LOOP

Underlined are reactive residues where autoantibodies have been identified in humans

See protein modelling section for further information on structure.

Conclusion

Using various PCR techniques we were able to obtain a nucleotide sequence that, when translated using computer based tools, appeared to code for the complete amino acid sequence for equine C1-inh (i.e. it included the signal peptide and the poly-A tail). This sequence aligned well with the serpin domain of human C1-inh; the N-terminal domain of either protein showed less homology to each other and no homology at all to any other protein. The nucleotide sequence and amino acid sequence of equine C1-inh showed greatest homology to human C1-inh and predicted C1-inh from other species (e.g. mouse, cow, chicken). There was less homology for other proteins of the serpin family.

Analysis of various equine tissues for expression of C1-inh mRNA

In humans, C1-inh is thought to be mainly produced by the liver (Johnson et al. 1971, Colten 1972, Morris et al. 1982), with small amounts produced by mononuclear cell types such as monocytes/ macrophages (Randazzo et al. 1985, Reboul et al. 1985) and other cell types such as fibroblasts, platelets and endothelial cells (Ruddy and Colten 1974, Katz and Strunck 1989, Schmaier et al. 1993). Many of the PCR experiments up to this stage of the project were carried out using cDNA made from equine tendon fibroblast RNA as this was good quality mRNA and there was some evidence that fibroblasts could produce C1-inh. Other experiments used cDNA from liver, a known producer of C1-inh in other species. Further tissues were tested for the potential to produce C1-inh by detecting C1-inh mRNA in RNA extracts. Samples were from the following equine tissues; heart, kidney, large intestine, liver, lung, skeletal muscle, skin, small intestine and spleen. All the tissues from this series of experiments came from the same horse.

Aims

- To purify RNA from various equine tissues and prepare cDNA from it
- To use PCR reactions with the primers used in the previous experiments to determine if mRNA for C1-inh is present in those tissues and to see if the nucleotide sequence shows evidence of tissue C1-inh subtypes

We attempted to extract RNA from heart, kidney, large intestine, liver, lung, skeletal muscle, skin, small intestine and spleen. Skin proved to be difficult to homogenise satisfactorily using the dismembrator. No further attempts were made to purify RNA from equine skin.

The rest of the samples were run on an agarose electrophoresis gel to assess RNA quality. RNA from kidney, large intestine, lung, small intestine and spleen was assessed as being of good enough quality to have cDNA made from it.

Tissue survey PCR

Oligo dT cDNA from tissue samples from equine kidney, large intestine, lung, small intestine and spleen was placed into microcentrifuge tubes. Cycling conditions consisted of a step of denaturation at 94°C for 5 min, then 35 cycles of denaturation at 94°C for 30 sec, annealing at 57.8°C for 30 sec, elongation at 72°C for 30 sec, followed by a final step of elongation at 72°C for 7 min, before cooling to 17°C until the tubes were removed from the thermocycler. The following primer pairs were used for each tissue sample:

N-terminal primer pair:

5' gene specific (for): 5'-ACA TCT TGA AGG GGA CTG TGC C-3'

5' gene specific outer (rev): 5'-AGG ACG CTC TCC AGG TTT TTC T-3'

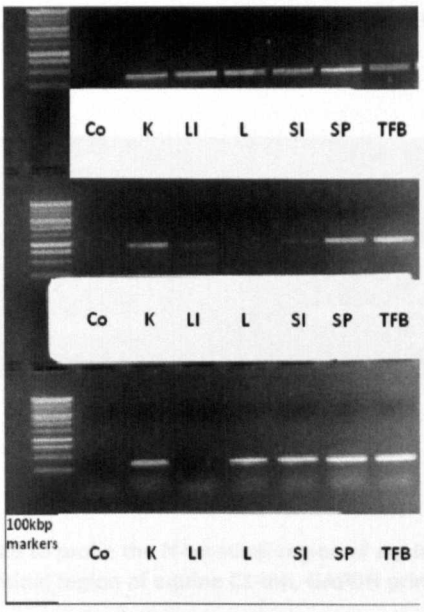
C-terminal primer pair:

EqC1Inh_for: 5' - CGT ACC CCA TGA ACT TTG CCT - 3'

EqC1Inh_rev: 5'- CCA GAG CAG GAA GAG GAA GG - 3'

The PCR products were run on an agarose electrophoresis gel as described above.

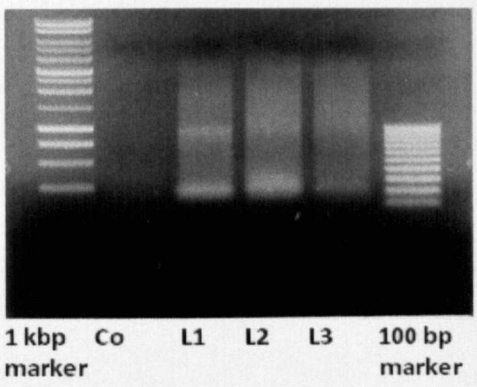
Figure 3.6: Photograph of PCR products from the tissue survey PCR. Top row: N-terminal primer pair, middle row: C-terminal primer pair, bottom row: GAPDH control primer pair.



Co = no cDNA control, K = kidney cDNA, LI = large intestine cDNA, L = liver cDNA, SI = small intestine cDNA, SP = spleen cDNA, TFB = tendon fibroblast cDNA

Further attempts were made to purify quality RNA from the liver samples. These attempts were at first unsuccessful until an adaptation in technique was adopted where Trizol was added to the chambers after homogenisation in order to maximise the amount of homogenised material collected.

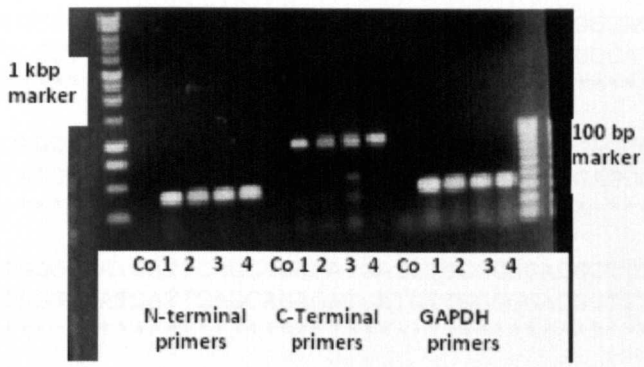
Figure 3.7: Photograph of RNA collected from three liver samples (L1-L3), ran on an ethidium bromide gel.



Co = H_2O control, L1-L3 = RNA samples from 3 equine livers

cDNA was made from the liver RNA using oligo dT and then PCR experiments were run using the protocol above with the exception that the elongation period in the cycling conditions was extended to 45 sec.

Figure 3.8: Photograph of PCR products from equine liver cDNA on an agarose electrophoresis gel.



N-terminal primers = primers designed to probe the N-terminal region of equine C1-inh, C-terminal primers = primers designed to probe the C-terminal region of equine C1-inh, GAPDH primers = primers designed to probe equine GAPDH

Co = H_2O control, 1-4 = RNA samples from 4 equine livers

The PCR products from the various tissues were sent for sequencing. The results were assessed by chromatography before being aligned with each other, the complete equine and human sequence and blasted against other proteins. The sequences where good quality chromatography signals were available showed 100% homology to each other and to the complete C1-inh sequence obtained in earlier experiments.

Figure 3.9: An example of sequence alignment from liver to the complete C1-inh sequence

Top line = PCR results from liver cDNA using C1-inh N-terminal primer pairs aligned to whole equine sequence determined by PCR from tendon fibroblast cDNA (bottom line).

```
  ------------------------------------CANAATTCNTTTCNNA-ACG
  AGACATCTTGGACATCTTGAAGGGAACTGTGCCCAAGACAGTATCCATTCAAGACACG
                                      **  ** * **    * ***

GTGAGCTCTTCCACCTTGCCGGAAACCAAGGAGACCAACACAACAGTTAGTGCCACTTTG
GTGAGCTCTTCCACCTTGCCGGAAACCAAGGAGACCAACACAACAGTTAGTGCCACTTTG
************************************************************

GGATCTACCACTCAACCAACTACCCAGCCCACTACTGAGTTCGGCTGCCCGGAGCCTGAC
GGATCTACCACTCAACCAACTACCCAGCCCACTACTGAGTTCGGCTGCCCGGAGCCTGAC
************************************************************

ATCTCCTGCCCTGACTTGGGCAATCATTCAGCAGAGATGATGTTGGGGGACGCTTTGACA
ATCTCCTGCCCTGACTTGGGCAATCATTCAGCAGAGATGATGTTGGGGGACGCTTTGACA
************************************************************

GATTTCTCCGTGAAGCTGTACCACGCCTTCTCAGCAGTGAAGAAACCCAGGAGCAACATG
GATTTCTCCGTGAAGCTGTACCACGCCTTCTCAGCAGTGAAGAAACCCAGGAGCAACATG
************************************************************

GCCTTTTCCCCATTCAGCATCGCCAGCCTCCTCACTCAGGTCCTGCTTGGGGCTGGGGAC
GCCTTTTCCCCATTCAGCATCGCCAGCCTCCTCACTCAGGTCCTGCTTGGGGCTGGGGAC
************************************************************

AGCACCAAGAAAAACCTGGAGAGCGGTCCTA----------------------------
AGCACCAAGAAAAACCTGGAGAGCGTCCTCTCGTACCCCATGAACTTTGCCTGTGTCCAC
*************************  *
```

Conclusion

That mRNA for C1-inh is expressed by liver, kidney, and large intestine in the horse. Results for lung, small intestine, spleen and tendon fibroblasts indicated that mRNA for C1-inh was also present in these tissues. The sequences obtained from liver, kidney and large intestine showed no evidence of tissue sub-types between each other or the earlier sequence derived from tendon fibroblast or liver from other horses.

Preparation of RNA/ cDNA from equine monocytes

To test whether equine mononuclear cells have mRNA for C1-inh we obtained RNA/ cDNA from equine monocytes using the protocol described below.

Blood collection and Percoll gradient separation

Blood (60 ml) was collected prior to plasma collection from a donor horse into heparinsed syringes before being transferred into 75 cm^3 cell culture flasks (Falcon BDbiosciences) for transport to the laboratory.

All the following steps were undertaken in a cell culture hood unless otherwise indicated. Percoll (85%) was made by adding 90 ml of Percoll solution to 10 ml of sterilised 10x PBS to make Percoll stock. 85 ml of Percoll stock added to 15 ml 1x PBS gave 85% Percoll solution. Equal volumes of heparinised blood and PBS were mixed in tubes. Then equal volumes of the blood/PBS mix were added above the 85% Percoll in new tubes (slowly to minimise mixing). The tubes were centrifuged at 1400g for 30 minutes at 4°C, after which the plasma was decanted and discarded and the interface (containing white cells) from each tube was transferred into a new tube. This was made up to 30 ml by adding PBS before centrifuging at 1400g for 10 minutes at 4°C. The supernatant was discarded and 30 ml fresh PBS was added to the tube, the pellet was mixed thoroughly with the PBS by vortexing, before a further centrifugation at 1400g for 10 minutes at 4°C. The supernatant was discarded and 20 ml of RPMI culture media was added to the tube before vortexing the pellet to mix thoroughly.

The culture media containing the Percoll- harvested and washed cells was then divided equally between two 25cm^3 culture media flasks. The flasks were incubated overnight at 37°C in 5% CO_2. This step was taken to enhance the monocytes' adherence to the plastic flask.

The following morning, all floating cells were washed off using PBS. The process was repeated to ensure only adhered cells remained in the flasks. Then 2 ml Hank's Buffered Saline Solution (HBSS) was added to each flask and gently circulated for 1 min. Trypsin/ EDTA (T/E) (1 ml) was added to each flask and allowed to contact the cells for 20 sec before being removed.

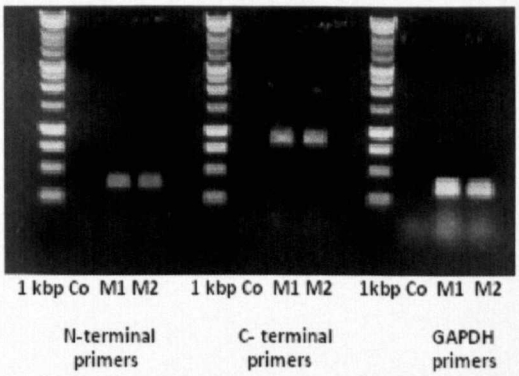
The flasks were then incubated at 37°C for 5 min, before washing off the cells with 1 ml of PBS. The cells were collected into 1.5 ml Eppendorf tubes and counted in a haemocytometer. After which, tubes containing the remainder of the cells were spun at 1400g for 10 min to pellet the

cells, the supernatant was discarded, 1 ml of Trizol was added to each tube and then RNA and cDNA was made from the cells as described above.

PCR reactions were carried out as for the other tissue samples using the same primer pairs, using the same conditions as above for 30 cycles. The PCR products from the two reactions were cleaned and sent for sequencing. The results were assessed by chromatography before being aligned with the equine EST and human sequences and blasted against other proteins. The sequences where good quality chromatography signals were available showed 100% homology to each other and to the complete C1-inh sequence obtained in earlier experiments.

Results

Figure 3.10: Photograph of PCR products from equine monocytes cDNA (M1 and M2) on an agarose electrophoresis gel.



N-terminal primers = primers designed to probe the N-terminal region of equine C1-inh, C-terminal primers = primers designed to probe the C-terminal region of equine C1-inh, GAPDH primers = primers designed to probe equine GAPDH

M1-M2 = monocyte cDNA from 2 monocyte cell cultures

Figure 3.11: N-terminal PCR product aligned to complete equine C1-inh:

```
Top line = C1N-terminal PCR product from equine mononuclear cells
Bottom = complete C1-inh sequence from earlier experiments

 GAGCTCTTCNCCTTGCCGGNAAACCAAGGAGACCAACACAACAGTTAGTGCCACTTTGG
 GAGCTCTTCCACCTTGCCGGAAACCAAGGAGACCAACACAACAGTTAGTGCCACTTTGG
 *********  * *  * * ************************************

GATCTACCACTCAACCAACTACCCAGCCCACTACTGAGTTCGGCTGCCCGGAGCCTGACA
GATCTACCACTCAACCAACTACCCAGCCCACTACTGAGTTCGGCTGCCCGGAGCCTGACA
************************************************************

TCTCCTGCCCTGACTTGGGCAATCATTCAGCAGAGATGATGTTGGGGGACGCTTTGACAG
TCTCCTGCCCTGACTTGGGCAATCATTCAGCAGAGATGATGTTGGGGGACGCTTTGACAG
************************************************************

ATTTCTCCGTGAAGCTGTACCACGCCTTCTCAGCAGTGAAGAAACCCAGGAGCAACATGG
ATTTCTCCGTGAAGCTGTACCACGCCTTCTCAGCAGTGAAGAAACCCAGGAGCAACATGG

************************************************************

CCTTTTCCCCATTCAGCATCGCCAGCCTCCTCACTCAGGTCCTGCTTGGGGCTGGGGACA
CCTTTTCCCCATTCAGCATCGCCAGCCTCCTCACTCAGGTCCTGCTTGGGGCTGGGGACA
************************************************************

GCACCAAGAAAAACCTGGAGAGCGGTCCTA----------------------------
GCACCAAGAAAAACCTGGAGAGCGTCCTCTCGTACCCCATGAACTTTGCCTGTGTCCACC
************************  *
```

Conclusion

mRNA for C1-inh was present in equine monocytes. The nucleotide sequences from the PCR products showed excellent homology to the complete equine C1-inh nucleotide sequence.

Modelling of the protein structure of equine C1-inh

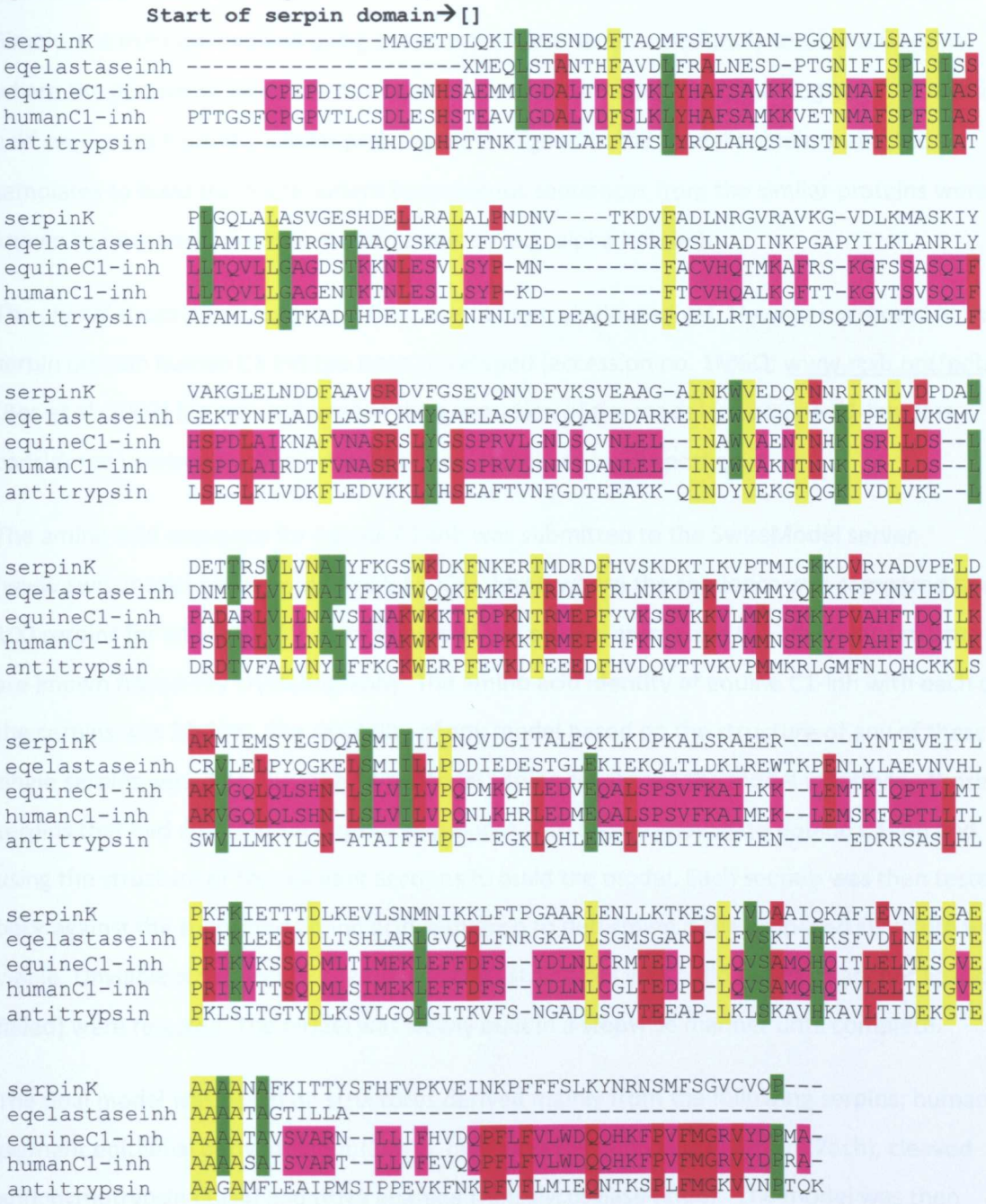
The molecular biological techniques utilised so far in this part of the project were aimed at providing the nucleotide sequence for equine C1-inh and the likely amino acid sequence from the protein. The amino acid alignment of the human and equine sequences (see above) provided useful information. For example, the serpin domain of the equine C1-inh shows good homology with the human protein, particularly around the reactive site loop and where important antigenic residues are found (most autoantibodies against human C1-inh react to epitopes in this region).

To maximise the chances of being able to design custom peptides that would successfully generate antisera that would react against natural equine C1-inh, it was important to model the 3D structure of equine C1-inh, in order to try to identify which parts of the amino acid sequence were likely to be exposed on the periphery of the protein and therefore more likely to act as epitopes for antibodies for subsequent detection systems. The initial studies, using antibodies directed against human C1-inh, had failed to indicate any reactivity to an equivalent equine molecule. The human C1-inh N-terminal amino acid sequence is unlike any other amino acid sequence on the PubMed database. The N-terminal domain of equine C1-inh shows poor homology to the human protein yet when it was blasted on PubMed the only protein it showed homology to was C1-inh. Due to the methodology of protein model generation and because the N-terminal region is unlike any other protein, this part of equine C1-inh could not be modelled. Thus, only the serpin domain of equine C1-inh was modelled.

Amino acid alignment of similar serpins

Using the Clustal W facility the amino acid sequences of the serpin domains of equine C1-inh, human C1-inh, equine elastase inhibitor, antitrypsin and serpin K were aligned to check for homology (see next page).

Figure 3.12: Amino acid alignment of equine C1-inh to human C1-inh three other serpins.



Purple colour = residues that match between equine and human C1-inh, red colour = residues match between equine and human C1-inh and one other serpin, green colour = residues match between equine and human C1-inh and two other serpins, yellow colour = all residues match
serpinK= serpin K
eqelastaseinh = equine elastase inhibitor
antitrypsin = antitrypsin

Model generation

Simply, the method involved using proteins from the same superfamily to C1-inh (serpins) where the precise 3D structure has been elucidated through X-ray crystallography. The amino acid sequences from the similar proteins were aligned to the model protein and acted as templates to build the model where homologous sequences from the similar proteins were known to form various structural components (e.g. alpha helices).

The crystal structure of human C1-inh has not been elucidated yet. However, a model of the serpin domain human C1-inh has been developed (accession no. 1M6Q; www.rcsb.org/pdb/) (Bos et al. 2002) but was not used in the generation of the model for equine C1-inh. It is considered unwise to utilise a model of one protein to build another.

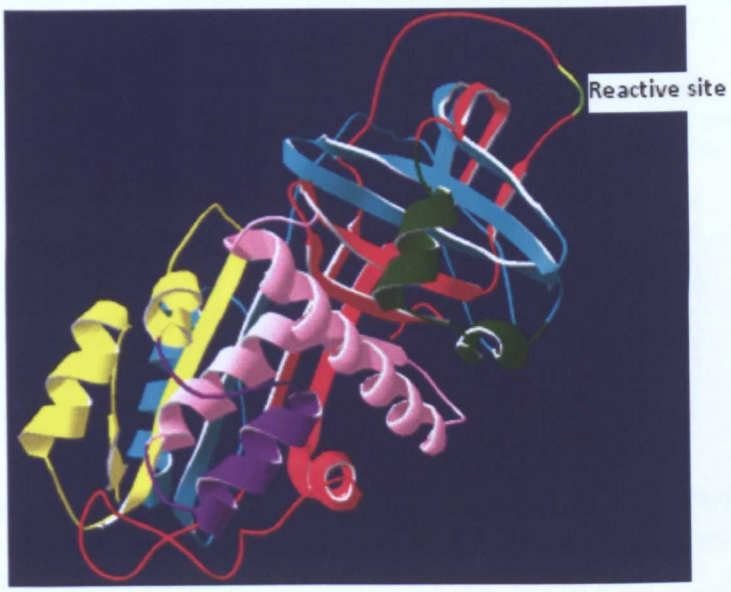
The amino acid sequence for equine C1-inh was submitted to the SwissModel server (www.swissmodel.expasy.org/SWISS-MODEL.html) where the sequence was compared to over 100 serpins for which the 3D co-ordinates of the protein atoms and the secondary structures are known from x-ray crystallography. The amino acid identity of equine C1-inh with each of the serpins was 20-30%. The reliability of any model based on the structure of any of these single serpins would be low. The SwissModel server developed the model identifying those serpins that had different sections of sequences with high homology to equine C1-inh and using the structure of the relevant sections to build the model. Each section was then tested back against the submitted amino acid sequence to determine if the proposed structure was viable. Unviable structures (e.g. amino acid 3D structures which did not fit or anchor ligations failed) were rejected. The model was slowly built in a stepwise manner until complete.

The final model was based on structures derived mainly from the following serpins; human pigment epithelial derived factor (1imV), murine serpina3n (1yxa), trypsin (2ach), cleaved antichymotrypsin (1as4) and tRNA guanine transglycosylase (1Iq8). The model was then compared to each of the serpins above and then to the model for human C1-inh proposed by Bos et al. (2002).

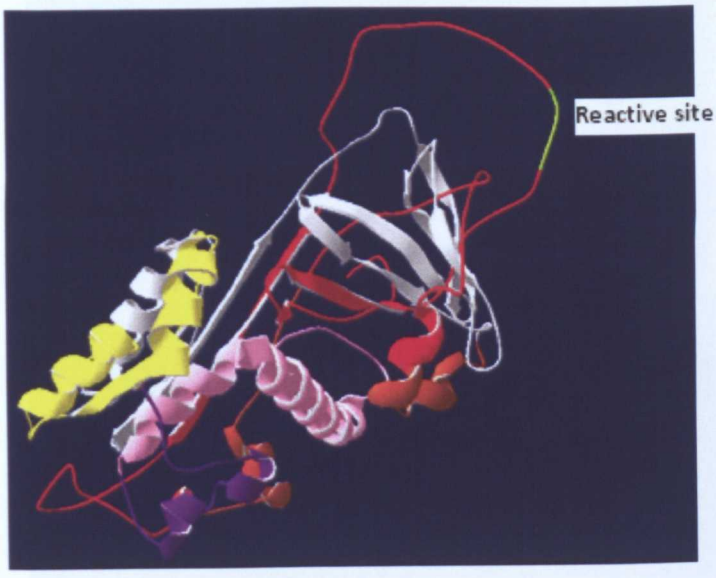
Human C1-inh is very heavily glycosylated (30%) (Bock et al. 1986); in order to estimate likely glycosylation sites on the equine protein we submitted the amino acid sequence to http://www.cbs.dtu.dk/services/NetN Glyc/ to estimate likely N-glycosylation sites and to http://www.cbs.dtu.dk/services/NetO Glyc/ to estimate likely O-glycosylation sites (see appendix for report).

Figure 3.13: 3D model of equine C1-inh (A) and human C1-inh (B) as predicted by Swiss model

A: equine C1-inh



B: human C1-inh



Key:

Helix A [Asn 1 → Gly 50] = Pink, Helix C [Ala 51 → Ala 70] = Dark Purple, Helix D [Cys 71 → Gly 118] = Yellow, Helix F [Asn 119 → Meth 229] = Turquoise, Helix G [Lys 230 -> Lys 250] = Dark Green, Helix H and C-terminus [Lys 251 →..] = Orange, Reactive site [Alanine 331, Aspartine 332, Arginine 333] = Bright Green

Figure 3.14: Human and equine C1-inh models overlaid on each other to demonstrate similarities; *Human: pale grey, Equine: Yellow*

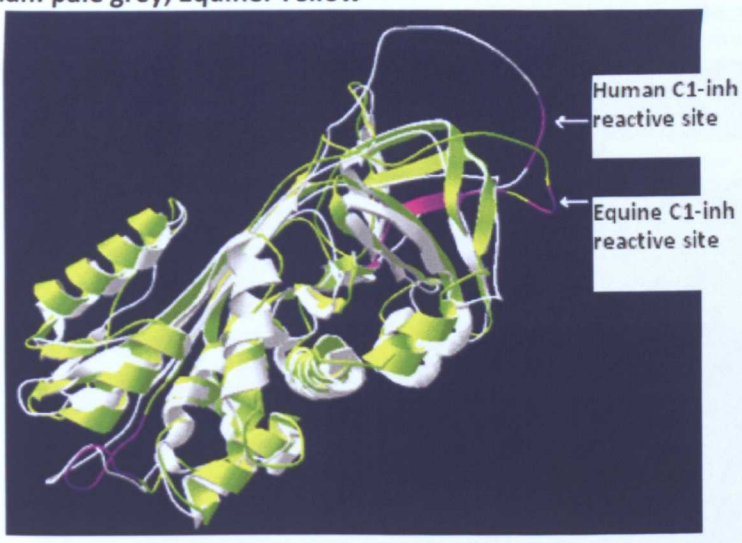
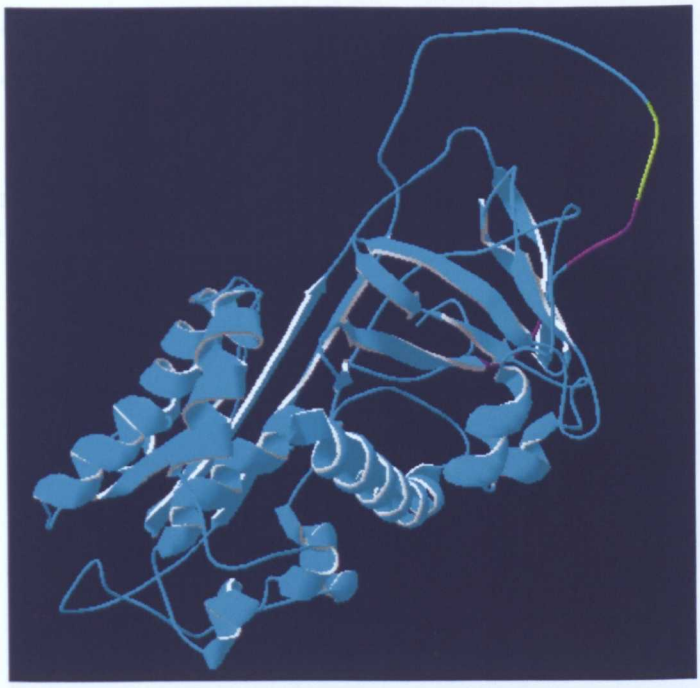
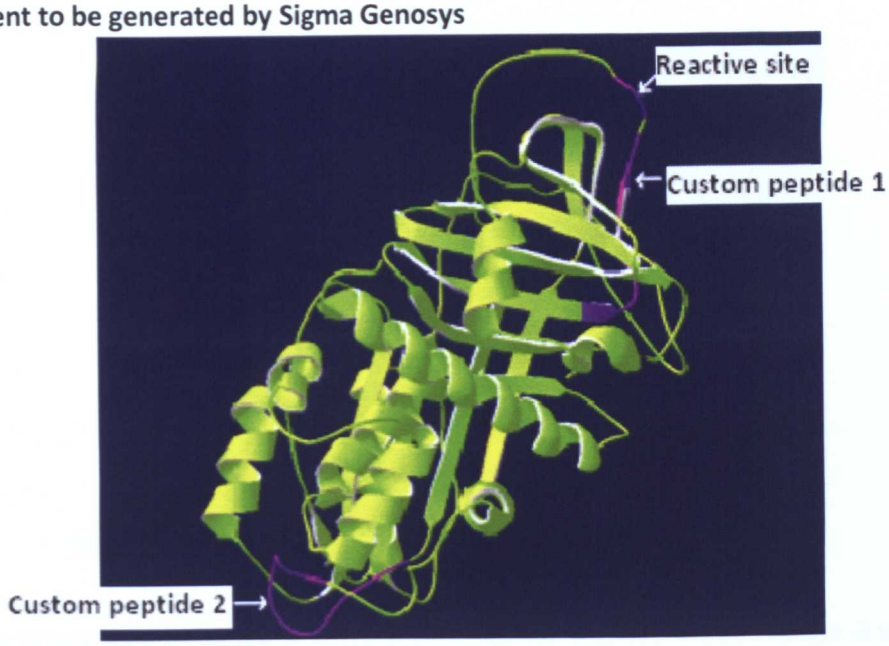


Figure 3.15: Image depicting human C1-inh with the reactive site (highlighted bright green) and two short amino acid sequences near to it which are common epitopes for autoantibodies (highlighted purple)



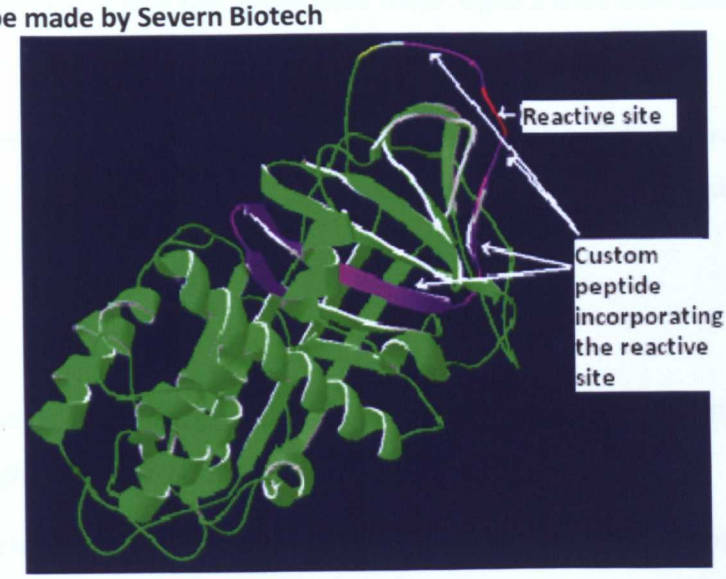
In an attempt to generate antisera that would react with the native equine protein; two custom peptides were designed from the corresponding region on the equine model [Custom peptide 1: Leu 335 → Phe 343]. The amino acid sequence at another site of the protein which the model predicted to be on the periphery of the protein was used for the second custom peptide [Custom peptide 2: Leu 293 → Val 304]. These peptides were generated by Sigma-Genosys and then injected into rabbits in order to generate antisera against the peptides (see antibody chapter).

Figure 3.16: 3D model of equine C1-inh depicting the predicted position of the two custom peptides sent to be generated by Sigma Genosys



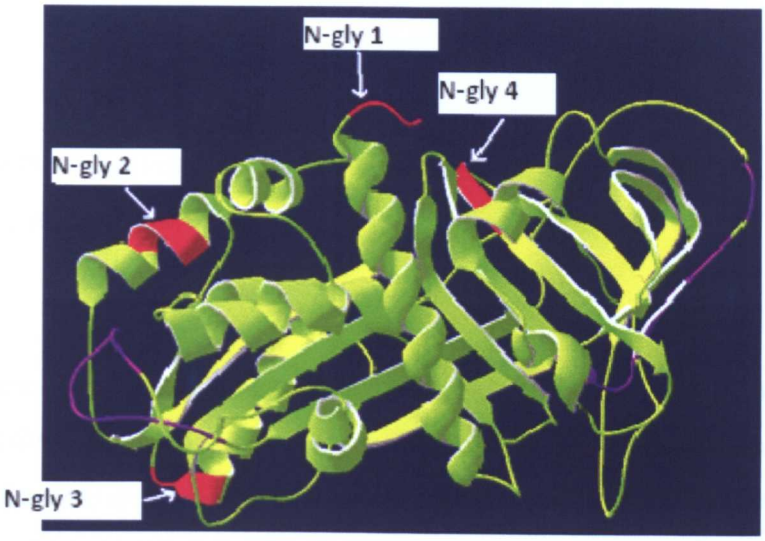
The custom peptide that was made by Severn-Biotech was designed to be longer than the previous peptides and incorporated the reactive site as well as the areas on the model corresponding to where autoantibodies are generated in humans (see next page);

Figure 3.17: 3D model of equine C1-inh depicting the predicted position of the custom peptide sent to be made by Severn Biotech



The results of the amino acid sequence submitted for equine C1-inh for likely glycosylation sites revealed that the O-glycosylation sites were likely to be exclusively in the N-terminal domain (which was not modelled) between residues 50 and 100. The likely N- glycosylation sites on the model are depicted on the next page.

Figure 3.18: Predicted N-glycosylation sites on equine C1-inh (highlighted in red);



N-gly 1 = Asn1 → Ser3, N-gly 2 = Asn104 → Ser106, N-gly 3 = Asn119 → Ser121, N-gly 4 = Asn218 → Ser220

Conclusion

Using Swissmodel, we were able to develop a model for equine C1-inh that closely resembled an independently, manually developed model for human C1-inh. Both models allowed comparison of the reactive sites and identification of likely regions on equine C1-inh that might act as epitopes. The amino acid sequences from these regions were then used to generate custom peptides.

If both models are accurate, the proposed reactive sites are on peripheral loops. The loop in the model for equine C1-inh does not protrude as far as the loop on the model for the human protein.

Determination of the nucleotide sequence for equine complement protein 3 (C3) and complement protein 5 (C5)

Quantitative PCR techniques allow differences in gene expression to be compared between samples in reference to a control gene. The next experiments were carried out to obtain partial nucleotide sequence from equine C3 and C5.

C3 and C5 represent the pro-inflammatory and C1-inh represents the anti-inflammatory side of the complement cascade and the innate immune response. It would be useful to try to establish if there is a difference in the amount of gene expression of C3, C5 and C1-inh in liver

samples depending on whether or not they had come from an endotoxaemic horse, or from mononuclear cells that had or had not been directly exposed to LPS.

Objectives

- To utilise techniques used so far in the project to obtain partial nucleotide sequences for both equine C3 and C5

Primer design

The mRNA sequence for the 3' terminal ends of human C3 (accession number NM_000064) and C5 (NM_001735) were used as described for the human C1-inh sequence to identify equine mRNA sequences of >85% homology from the Extended Sequence Tag (EST) database (The University of Georgia) (APL1_2_H12.g1_A008 and APL1_9_E04.g1_A008 for C3) and the PubMed nucleotide database (www.ncbi.nlm.nih.gov/PubMed/) (accession no. AAWR01024463.1 for C5). The equine EST sequences were then used to design primers. Primers were designed on PrimerXpress. The annealing temperature of the primers was chosen in the range 50-65°C, the percentage of G/C nucleotides was chosen to be between 40-60% and the length of the gene specific primers used for PCR was between 15 and 21 nucleotides. Primers were also assessed for 'primer-dimer' formation.

The following primers were ordered from MWG (GmBh, Germany);

Equine complement protein 3

C3(for): 5'-AGG CTC GGA TGA GGT GCA G-3' C3(rev): 5'-TTC GTC TTG GCA TTC GTC CT-3'

Equine complement protein 5

C5(for): 5'-CTG ATT GTG GGC AAA TGC AG-3' C5(rev): 5'-TCA GCA ACA GCT TCC CCA G-3'

PCR reactions were carried out as described in the basic protocols using the primer pairs above, and oligo dT cDNA from normal horse liver using the same cycling conditions as above except the reactions were repeated for 35 cycles.

Equine complement protein 3:

TAGGCTCGGATGAGGTGCAGGTTGGACAGGAGCGCAGGTTCATCAGCCACATCAAGTGCAGAGA
AGCCCTGAAGCTGGAGAAGGACAAAAAGTACCTCATATGGGGCGTCTCCTCCGACCTGTGGGGA
GAGAAACCCAACATCAGCTACATCATTGGGAAGGACACCTGGGTGGAGCTGTGGCCTGAGGACG
AATGCCAA

Above sequence aligned to the sequence for human C3 (87% homology),][= exon boundaries:

```
                              ] [
EqC3     ------------------------------------------TAGGGCTCGGATGAGG
humanC3 ATGACTTTGACGAGTACATCATGGCCATTGAGCAGACCATCAAGTCAGGCTCGGATGAGG
                                                   *  ************

EqC3     TGCAGGTTGGACAGGAGCGCAGGTTCATCAGCCACATCAAGTGCAGAGAAGCCCTGAAGC
humanC3 TGCAGGTTGGACAGCAGCGCACGTTCATCAGCCCCATCAAGTGCAGAGAAGCCCTGAAGC
         ************** ****** *********** **************************

EqC3     TGGAGAAGGACAAAAAGTACCTCATATGGGGCGTCTCCTCCGACCTGTGGGGAGAGAAAC
humanC3 TGGAGGAGAAGAAACACTACCTCATGTGGGGTCTCTCCTCCGATTTCTGGGGAGAGAAGC
         ***** ** * *** * ******** *****  **********  * ********** *

                    ] [
EqC3     CCAACATCAGCTACATCATTGGGAAGGACACCTGGGTGGAGCTGTGGCCTGAGGA---CG
humanC3 CCAACCTCAGCTACATCATCGGGAAGGACACTTGGGTGGAGCACTGGCCCGAGGAGGACG
         ***** ************* *********** **********  ***** *****   **

EqC3     AATGCCAA--------------------------------------------------
humanC3 AATGCCAAGACGAAGAGAACCAGAAACAATGCCAGGACCTCGGCGCCTTCACCGAGAGCA
         ********
```

Equine complement protein 5:

CTGATTGTGGGCAAATGCAGAGAGAATTGAATCTGACGATCTCGGCAGATACTAGAAAAGAAAG
AGCATGCAAACCAGAGATTGCATATGCTTATAAAGTTAGTATCACATCCATCACTGAAGAAAAT
GCTTTTGTTAAATACACGGCAACCCTCCTGGATGTCTACAAAGCTGGGGAAGCTGTTGCTGAA

Above sequence aligned to the sequence for human C5 (89% homology),][= exon boundaries:

```
EqC5       CTGATTGTGGGCAAATGCAGAGAGAATTGAATCTGACGATCTCGGCAGATACTAGAAAAG
HumanC5    CTGATTGTGGGCAAATGCAGGAAGAATTGGATCTGACAATCTCTGCAGAGACAAGAAAAC
           ********************  ******* ******* ***** ***** ** ******
                                          ][
EqC5       AAAGAGCATGCAAACCAGAGATTGCATATGCTTATAAAGTTAGTATCACATCCATCACTG
humanC5    AAACAGCATGTAAACCAGAGATTGCATATGCTTATAAAGTTAGCATCACATCCATCACTG
           *** ****** ********************************* ***************
                                                                    ][
EqC5       AAGAAAATGCTTTTGTTAAATACACGGCAACCCTCCTGGATGTCTACAAAGCTGGGGAAG
humanC5    TAGAAAATGTTTTTGTCAAGTACAAGGCAACCCTTCTGGATATCTACAAAACTGGGGAAG
            ******** ****** ** **** ********* ****** ******** *********

EqC5       CTGTTGCTGAA-------------------------------------------------
humanC5    CTGTTGCTGAGAAAGACTCTGAGATTACCTTCATTAAAAAGGTAACCTGTACTAACGCTG
           **********
```

Conclusion

Using basic PCR techniques we were able to obtain nucleotide sequences that, when translated using computer based tools, appeared to code for equine complement proteins C3 and C5. The sequences showed 100% homology to the equine ESTs and 87% and 89% homology to the nucleotide sequence for the respective human protein. This sequence was used to design primers for quantitative PCR experiments (see below).

Quantitative real-time PCR analysis of cDNA produced from equine liver and mononuclear cells +/- LPS

Preparation of cDNA

Small samples of liver were collected into RNAlater within an hour after death from 4 horses that had chronic non-septic orthopaedic conditions and six horses who were deemed to be severely clinically endotoxaemic euthanized for clinical reasons. The tissue samples in RNAlater were stored at -20°C until processing to obtain RNA/cDNA.

Blood (60 ml) was collected into heparinsed syringes from four healthy horses (that were to be subsequently used as blood donors) before being transferred into 75 cm^3 cell culture flasks for

transport to the laboratory. The blood samples were processed as described above (preparation of RNA/ cDNA from equine mononuclear cells) in order to obtain two flasks of mononuclear cells per horse.

In this experiment, after the stage when non-adherent cells had been washed off, the media in one flask was changed with fresh RPMI media. The media in the second flask was changed with RPMI media containing 20 μg ml^{-1} *Salmonella typhimurium* LPS (Sigma). The cells in the two flasks were incubated for a further 24 hours in the respective replacement media, before the cells were collected and counted and RNA/ cDNA collected from them as described above.

RNA and oligo dT cDNA was made from the liver and mononuclear cell samples following the protocols described above;

Four 'normal' livers [NL1, NL2, NL3, NL4],

Six 'endotoxaemic' livers [EL1, EL2, EL3, EL4, EL5, EL6] and

Mononuclear cells from 4 horses with some cells from each horse incubated in normal media and some cells exposed to LPS (i.e. 8 cDNA samples from 4 horses [M1-/ M1+, M2-/ M2+, M3-/ M3+, M4-/ M4+]

Primer design and validation

The nucleotide sequences obtained earlier for equine C1-inh (complete), C3 and C5 were checked against the part annotated equine genome pre-species section of the Ensembl website to establish where exon boundaries lay. This was not successful for these sequences. Instead, nucleotide sequences with known exon boundaries for each protein from other species (human, mouse and rat) were compared to determine where common inter-species exon boundaries lay. The horse sequences were then aligned using the Clustal W facility (www.expasy.com/) against the other sequences and probable sites of exon boundaries determined.

Figure 3.19: Example of nucleotide sequence alignment to identify exon boundaries (C1-inh),][= exon boundary;

```
                                                  III                ][   IV
mouse ATGGCCTTTTCCCCATTCAGCATTGCCAGCCTCCTCACACAGGTTCTTCTTGGGGCTGGA 639
                                                  II                 ][  III
rat   ATGGCCTTTTCCCCATTCAGCATTGCTAGCCTCCTCACGCAGGTCCTTCTTGGGGCTGGA 627
                                                  III                ][   IV
human ATGGCCTTTTCCCCATTCAGCATCGCCAGCCTCCTTACCCAGGTCCTGCTCGGGGCTGGG 749

horse ATGGCCTTTTCCCCATTCAGCATCGCCAGCCTCCTCACTCAGGTCCTGCTTGGGGCTGGG 580
      ********************** ** ******** ** ***** ** ** ********
```

The nucleotide sequences surrounding the exon boundaries closest to the C-terminal end of each protein were then used to design primer pairs that either directly crossed exon boundaries or the primer pair product would cross an exon boundary for each protein using the Roche quantitative PCR primer design facility (www. Roche.com).The primer pairs are detailed below and were ordered from MWG. The primers were re-constituted to 100 pmol/ µl using nuclease free water.

C1-inh:

qC1inhfor: 5'- GCT TCA GCT CAG CCT CTC AG – 3' Tm 61.4°C GC content 60%

qC1-inhrev: 5' – CAC AAA CGC GTT CTT GG – 3' Tm 55.3°C GC content 45%

Amplicon length = 62 nt:
gcttcagctcagcctctcagatcttccacagcccagacctggccataaagaacgcgtttg
tg

C3:

qC3for: 5' – CCA CAT CAA GTG CAG AGA AGC – 3' Tm 59.8°C GC content 52.4%

qC3rev: 5' – TCC CAA TGA TGT AGC TGA TGT T – 3' Tm 56.5°C GC content 40.9%

Amplicon length = 113 nt:

ccacatcaagtgcagagaagccctgaagctggagaaggacaaaaagtacctcatatgggg
cgtctcctccgacctgtggggagagaaacccaacatcagctacatcattggga

C5:

qC5for: 5' – GAA AGA GCA TGC AAA CCA GA – 3' Tm 55.3°C GC content 45%

qC5rev: 5' – ACA GCT TCC CCA GCT TTG TA – 3' Tm 57.3°C GC content 50%

Amplicon length = 125 nt:

gaaagagcatgcaaaccagagattgcatatgcttataaagttagtatcacatccatcact
gaagaaaatgcttttgttaaatacacggcaaccctcctggatgtctacaaagctggggaa
gctgt

Real-time PCR by SYBR Green assay protocol

For each primer mix (C1-inh, C3 and C5) a 300nM mix was made by adding 10 µl of the forward and reverse primer to 80 µl of nuclease free water. Mastermix was made up to contain 5 µl SYBR Green, 0.3 µl primer mix and 0.1 µl nuclease free water per well to be used on the 384 well plate. cDNA was diluted to an appropriate concentration (see later).

Loading:

cDNA (4.6 µl) and 5.4 µl Mastermix was loaded into each well by electronic pipette. Adhesive plastic was firmly applied to the plate to ensure no evaporation of well contents whilst in the machine. The plate was wrapped in foil (to protect the plate from dirt) and then centrifuged for 4 min to ensure well contents were mixed and at the bottom of the wells.

The plate was then loaded into the ABI PRISM 7900 sequence detection system and run under standard conditions [10 minutes at 50°C followed by 40 cycles of 95°C for 1 minute and 60°C for 15 seconds, as recommended by the manufacturer (Applied Biosystems)]. Real-time data were analysed by using the Sequence Detection Systems software, version 2.2.1 (Applied Biosystems). The detection threshold was set manually at 0.05 for all assays. Standard curves were generated for each assay (Additional file 1), to confirm that all assays were generated within acceptable limits (efficiency $93\% > x > 107.4\%$) and R^2 values ($R^2 > 0.98$) (with the exception of the genomic contamination assay, in which efficiency was lower, but the detection of any transcript was deemed unacceptable).

Primer validation

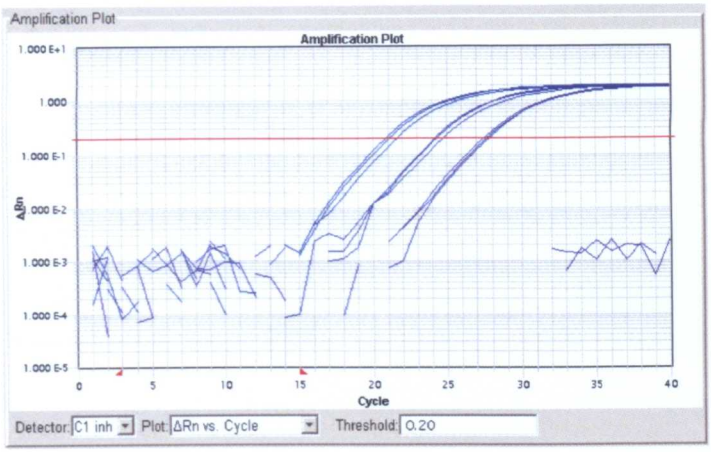
The first plate was used to validate the primer mixes. GAPDH was used as the control primer. The cDNA was made up from equal amounts from each of the normal livers (NL1 → NL4).

Results

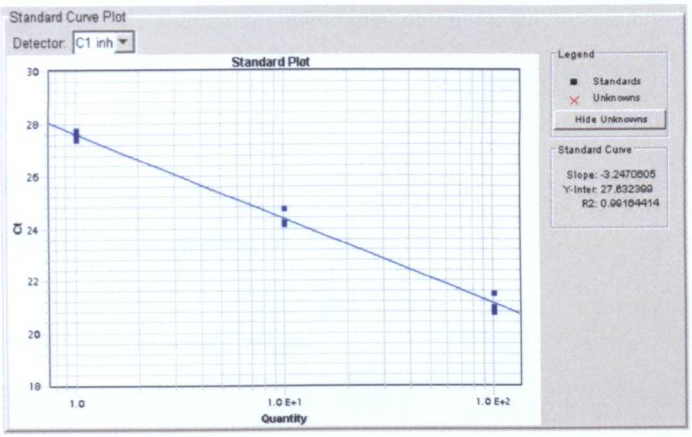
The reactions exhibited some evidence of inhibition due to the cDNA being too concentrated. Accordingly, the standard curves required selection of data points that did not demonstrate inhibited reactions in order to obtain accurate curves. Melting point curves did not demonstrate the presence of primer dimers in any of the reactions. The following standard curves were obtained for the primer mixes.

Figure 3.20: Standard dissociation curves and log dissociation curves for quantitative real-time PCR analysis of C1-inh, C3, and C5 in liver and monocyte cDNA

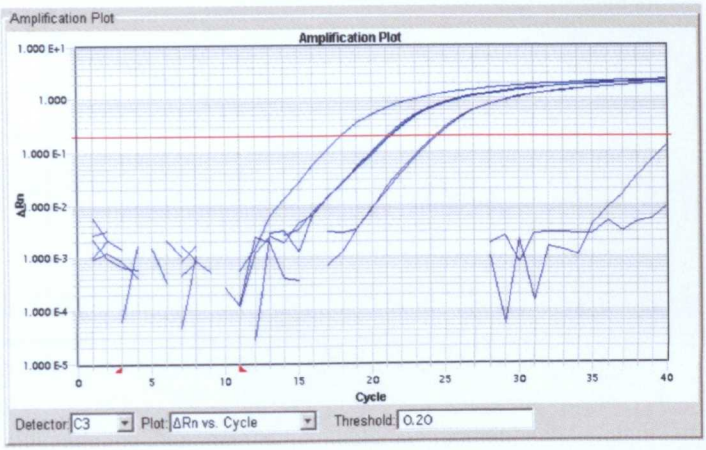
C1-inh standard dissociation curve



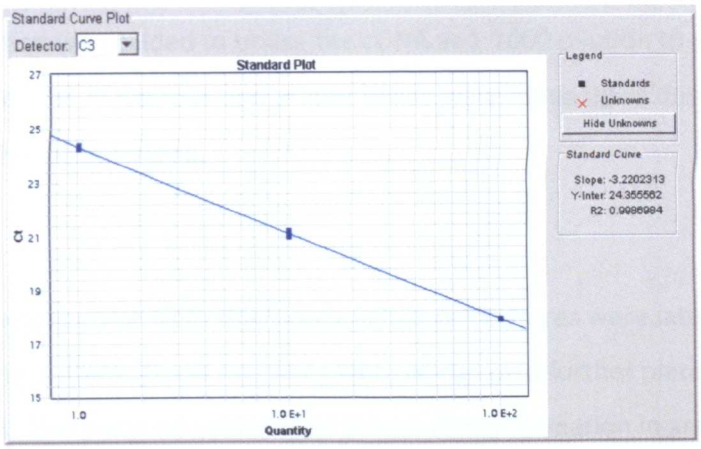
C1-in log dissociation curve



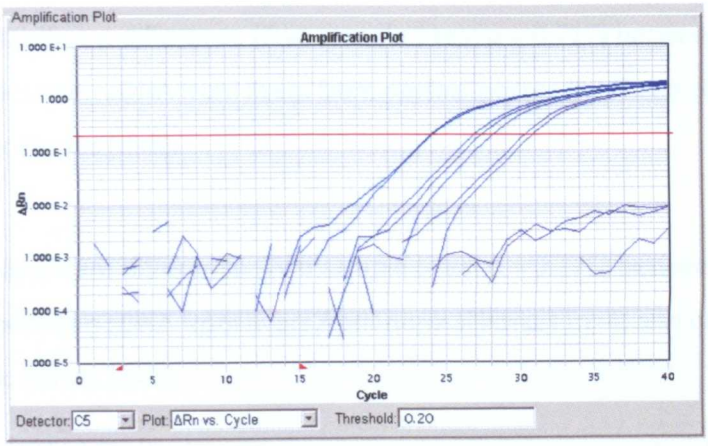
C3 standard dissociation curve



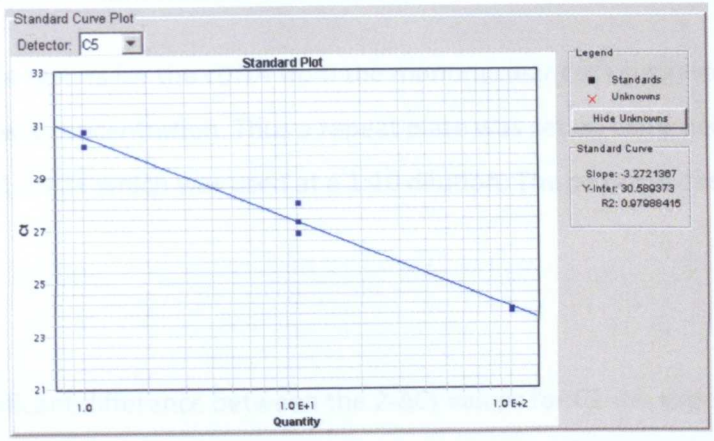
C3 log dissociation curve



C5 standard dissociation curve



C5 log dissociation curve



RT PCR of all cDNA samples

After primer validation we decided to utilise the cDNA at 1:1000 dilution to prevent inhibition of the PCR reactions. The Mastermix and primer mixes were made up as described above and the assay protocol ran as described.

Results

The reactions containing cDNA from the mononuclear cell cultures were late reacting due to too little cDNA being present. These reactions were re-run on a further plate using more concentrated cDNA. There was no evidence of primer dimer formation in any of the reactions.

C1-Inh: There was no significant difference (Two sample t-test p = 0.305) between the 2-ΔCt values for C1-inh expression between cDNA from livers from normal horses (n=4) and cDNA from livers from endotoxaemic horses (n=6).
C3: There was no significant difference (Two sample t-test p = 0.717) between the 2-ΔCt values for C3 expression between cDNA from livers from normal horses (n=4) and cDNA from livers from endotoxaemic horses (n=6).

C5: There was no significant difference (Two sample t-test p = 0.773) between the 2-ΔCt values for C5 expression between cDNA from livers from normal horses (n=4) and cDNA from livers from endotoxaemic horses (n=6).

RT-qPCR of cDNA from mononuclear cell cultures

In the first plate the results for the cDNA from the mononuclear cells suggested that the cDNA was used at too low a concentration. Thus a repeat plate was set up using neat cDNA for all samples except M1-/M1+ which was used at a 1:10 dilution. The protocol described above was followed.

Results

There was no significant difference between the 2-ΔCt values for C1-inh expression between cDNA from mononuclear cells exposed to LPS (n=4) compared to cDNA from cells not exposed to LPS (n= 4) (Paired t-test, p = 0.773), in C3 expression between cDNA from mononuclear cells exposed to LPS (n= 4) compared to cDNA from cells not exposed to LPS (n=4) (Paired t-test, p =

0.715), or in C5 expression between cDNA from mononuclear cells exposed to LPS (n= 4) compared to cDNA from cells not exposed to LPS (n= 4) (Paired t-test, p = 0.510).

Conclusions

There was no significant difference for the 2-ΔCt values (compared to GAPDH) for cDNA from livers from normal horses compared to those from endotoxaemic horses or from cDNA from monocytes exposed to LPS compared to those monocytes not exposed to LPS.

Discussion

Molecular biological techniques were utilised to determine the full nucleotide and amino acid sequence of equine C1-inh and to provide evidence that C1-inh exists in the horse. Three-dimensional modelling of the protein structure revealed differences between the human and equine protein that may explain the lack of cross-reactivity to the equine protein of the tested anti-human C1-inh antibodies.

The first series of experiments produced a 984 base nucleotide sequence. The sequence showed homology only to human C1-inh and appeared to code for around 60% of the protein.

In order to obtain the complete nucleotide sequence; 5' and 3' RLM-RACE PCR of the sequence was undertaken, and proved successful as the resultant 1571 base sequence included the signal peptide and the poly-A tail. This sequence aligned well with the serpin domain of human C1-inh particularly around the reactive site loop and where important residues are found. The homology between the two sequences was best in the serpin domain and towards the C-terminus end of the protein. Nevertheless, there are still important differences, not least in the N-terminal domain and around the reactive site. This is novel information and is also evidence that equine C1-inh exists. Whilst the complement cascade system is relatively well conserved across mammalian species, not surprisingly, antigenic and functional differences do occur (Gigli and Austen 1971); the basis of many of these differences being nucleotide/ amino acid sequence differences. The N-terminal domain of either protein showed less homology to each other and no homology to any other amino acid sequence on the PubMed database. The N-terminal domain of human C1-inh is important for plasma clearance (de Smet et al. 1993) and for interactions with endotoxin (Davis et al. 2007, Zhang et al. 2007) and selectins (Cai and

Davis 2003, Cai et al. 2005). The difference in amino acid sequence and hence structure may affect the function of equine C1-inh and future studies examining the effects of these differences will be informative in understanding the biology of both proteins. Human C1-inh is 478 amino acids and has an estimated Mw of ~78 kDa but runs on SDS-PAGE gels at ~105 kDa due to the degree of glycosylation (Bock et al. 1986, Perkins et al. 1990). The derived amino acid sequence for equine C1-inh is 454 amino acids long and the estimated degree of N and O glycosylation sites is predicted to be different; so it may be reasonable to expect that equine C1-inh may not run exactly as the human protein on SDS-PAGE gels/ Western blots. However, in the preliminary work carried out to this project, a goat polyclonal antibody against human C1-inh detected protein bands in equine plasma run in Western blots at a very similar Mw to purified human C1-inh control.

The next series of experiments were designed to determine what tissues expressed mRNA for equine C1-inh and also to assess whether there was any evidence of tissue sub-types of equine C1-inh. In humans, C1-inh is thought to be mainly produced by the liver (Johnson et al. 1971, Colten 1972, Morris et al. 1982) with small amounts produced by mononuclear cell types such as monocytes/ macrophages (Randazzo et al. 1985, Reboul et al. 1985) and other cell types such as fibroblasts, platelets and endothelial cells (Ruddy and Colten 1974, Katz and Strunck 1989, Schmaier et al. 1993). These experiments demonstrated mRNA in tendon fibroblasts, mononuclear white blood cells, liver, kidney, and large intestine in this horse. Partial results for lung, small intestine, spleen suggested that mRNA for C1-inh was also present in these tissues. Skin proved a very difficult tissue to obtain RNA from and further attempts were abandoned. Some tissues, such as heart and skeletal muscle, did not provide good quality RNA. At the time it was hypothesised that this was due to connective tissues affecting tissue homogenisation. Unfortunately, unlike liver, further attempts at optimising the sample processing and RNA purification protocols were not made for these tissues.

The sequences obtained from liver, kidney and large intestine showed no evidence of tissue sub-types between each other or the earlier sequence derived from tendon fibroblast or liver from other horses. The wide tissue distribution of C1-inh suggests that it has an important role in many tissues. However, these results should be considered with some caution as the results may be due to the PCR amplifying mRNA from various cell types present within the tissue (either through sample contamination or being present in the tissue but not originating from that tissue e.g. white blood cells). Further work (cell type specific) would be required to

determine which types of cells within these tissues were capable of producing C1-inh. Tissue sub-types were not identified for the protein. This may be a true finding or be a result of sample contamination with a cell type (e.g. macrophage), although the same contamination would be unlikely over multiple samples.

The Swissmodel utility was used to generate a 3D model of equine C1-inh based on the derived amino acid sequence that closely resembled an independently manually developed model for the serpin domain of human C1-inh (Bos et al. 2002). Although the overall identity of equine C1-inh was between 20-30% for most of the serpins it was compared to, the Swissmodel facility compares short sequences (~10 amino acids) where the homology may be much higher to individual proteins and builds the model in a stepwise manner from multiple serpins. The N-terminal sequence had to be excluded from the model building process as its sequence is apparently unique and lacks homology with any other protein sequence based on a PubMed blast search. Indeed, when the sequence including the N-terminal was submitted for model development, it was returned as unusable. It was only after the N-terminal sequence was removed and remainder sequence was submitted, that the Swissmodel facility could generate a model. To what, if any, extent the N-terminal structure would affect the model remains as speculation until the crystal structure of this protein is determined, but it is likely that the presence of the N-terminus will affect the overall 3D-structure of the modelled serpin domain and C-terminus and this may be one of the reasons the attempts to use custom peptides to generate antisera with cross-reactivity to the natural protein were unsuccessful.

The model for equine C1-inh demonstrated that the protein is similar to the independently developed model for the human protein (Bos et al. 2002), which provides further evidence that the derived sequence is correct. The protein model allowed us to identify likely epitopes for custom peptide generation. If both models are accurate, the proposed reactive sites for each protein are on peripheral loops. The amino acids around the reactive site form epitopes for auto-antibodies generated against the human protein in patients suffering from acquired hereditary angio-oedema (He et al. 1996). The reactive site loop in the model for equine C1-inh does not protrude as far as the loop on the model for the human protein. Nevertheless, the amino acid sequences used to generate custom peptides were selected from this area as we knew comparable sequences in the human protein were antigenic. The possible reasons for the failure of the custom peptides to generate antisera that could detect the equine protein have been discussed in the antibody chapter.

In comparison to human C1-inh, equine C1-inh has fewer predicted N-glycosylation sites (8 compared to 13 (Bock et al. 1986). The predicted sites for O-glycosylation are all in the N-terminal domain. The function of the significant number of carbohydrate groups in human C1-inh is unknown but some studies suggest they are important in plasma clearance or potentiation by glycosaminoglycans, of the inhibitory capacity of C1-inh with C1s (Bos et al. 2002). Further work will be necessary to determine the degree of glycosylation of equine C1-inh and the role the carbohydrate residues may play in protein function.

The complement cascade has a number of immunological and non-immunological physiological roles (see introduction) and so it is likely that complement proteins and their inhibitors are constitutively expressed. The complement system is also important in the inflammatory response to endotoxaemia, where C1-inh (Liu et al. 2007), C3 and C5 (Hollmann et al. 2008) have been shown to have important roles. There is little evidence as to whether gene expression of these three complement proteins would increase or decrease during endotoxaemia. Intestinal cells have been shown to increase C3 production in response to certain cytokines (interleukin-1 beta and tumour necrosis factor – alpha) (Moon et al. 1997). Conversely, C5 has been shown to modulate systemic tumour necrosis factor-alpha in a mice endotoxaemia model (Barton and Warren 1993). It has been demonstrated that there is an increased level of proteolytic cleavage of C1-inh in sepsis (Nuijens et al. 1989), and that C1-inh can modulate the production of inflammatory mediators in sepsis (Caliezi et al. 2000). There is no information on gene expression of this protein during inflammation, although gene expression of C1-inh has been shown to be reduced in patients with hereditary angioedema (Pappalardo et al. 2004). In this study, RT-qPCR analysis of cDNA from liver from normal and endotoxaemic horses, and from mononuclear cells cultured with or without LPS did not show significant differences in complement gene expression between groups. There was a trend for C1-inh expression to decrease. One may have expected a significant increase or decrease in any of the tested proteins from the complement cascade when an animal is endotoxaemic or when mononuclear cells are exposed to LPS. There are a number of reasons why our experiments did not detect any differences. Firstly, the sample sizes in these experiments were small which decreases the likelihood of finding significant differences. Secondly, the liver samples were all from different horses and individual variations in basal gene expression and/ or gene expression during endotoxaemia may have confounded the results. The mononuclear cells may not have produced a 'normal' response to LPS exposure for a variety of reasons such as inadequate dose/ duration of exposure or because the culture media did not contain lipopolysaccharide binding protein (LBP), which is normally present in plasma and is a vital

component in initiating the mononuclear cell response to LPS through the presentation of LPS to the Toll-like receptor apparatus (Henry and Moore 1988). Cultured equine polymorphonuclear neutrophils did not respond to incubation with LPS unless they were incubated in whole blood and then cultured (Benbarek et al. 1998). However, other workers have demonstrated pro-coagulant responses from similarly cultured equine mononuclear cells exposed to 10 $\mu g ml^{-1}$ *E.coli* LPS in the absence of LBP (Grunig et al. 1991). There may also be differences between the mRNA levels for each protein in the cultured cells. In synovial cells C3 mRNA has been shown to be unstable, becoming degraded in minutes. In the same study the synovial cells had an apparent lag of up to 48 hours before C1-inh mRNA could be detected in culture (Whaley et al. 1992). If any such effects existed in equine mononuclear cells in culture, these may have masked any differences in gene expression between the proteins, especially as RNA was isolated from the cells after incubation with LPS for 24 hours. Finally, the results from this work are expressed as a function of relative gene expression in comparison to a control gene. The control gene (GAPDH) has been widely utilised as a control gene by other workers. In a similar study measuring C1-inh gene expression in mononuclear cells from human blood, GAPDH and CD64 were used as control genes (Pappalardo et al. 2004). The relative expression results did differ between the two control genes although the trends were the same. GAPDH may not actually be an ideal control gene for the equine tissues/ cells tested. Further work should be attempted to identify appropriate control genes for this system. Pappalardo et al. (2004) state that in humans C1-inh gene expression shows high individual variation between both healthy volunteers and hereditary angioedema patients.

Although the results from the RT-qPCR experiments did not provide evidence that there are changes in gene expression for C1-inh in comparison to the control gene, it is possible that these results are correct. The fact that the gene expression/ protein synthesis of C1-inh may not increase under conditions of severe inflammation (such as endotoxaemia) may explain why low levels of functional C1-inh (or high levels of C1-inh breakdown products) are associated with increased mortality levels in people with sepsis (Nuijens et al. 1989), i.e., that there is no capacity to increase production of C1-inh and once the protein supply has been used up the anti-inflammatory effects of this protein are absent. My own feeling is that the fact the expression of the other two genes tested did not apparently change much in either the liver or the mononuclear cell samples suggests that the experiment needs further optimisation and repeating with greater sample numbers in order to improve the likelihood of obtaining valid results. The work outlined in this chapter has established the existence of C1-inh in horses. This work has provided evidence that C1-inh is present in multiple tissues in the horse suggesting a

potential role in the control of local and systemic inflammation. The determination of the nucleic and amino acid sequences has provided further information on the possible structure of equine C1-inh which may explain the apparent lack of cross-reactivity of antibodies against the human protein to the equine protein.

The results from the RT-qPCR experiments did not show differences between C1-inh gene expression in comparison to GAPDH between livers from endotoxaemic horses and non-endotoxaemic horses or between mononuclear cells exposed or not exposed to LPS. However, there were also no differences in the expression of C3 and C5; both important proteins in inflammation and the innate immune response. One might have expected that gene expression of these proteins would have altered (at least in the livers from endotoxaemic horses) in cells that were in a pro-inflammatory state. It is likely that further optimisation/ validation of these RT-qPCR experiments is necessary to better elucidate gene expression of these important proteins in innate immunity and inflammation. For example, experiments to validate genes to act as appropriate positive (e.g. a gene we know to be upregulated after exposure to LPS) and negative controls for the experiment. Therefore, these results do not in any way diminish the likely vital anti-inflammatory role of C1-inh in colic derived endotoxaemia in horses.

Appendix to chapter 3

Glycosylation reports for submitted amino acid sequence for equine C1-inh.

Predicted N-Glyc site sequences are coloured red/ blue in the amino acid sequence below. Table below summarises the predicted sites and likelihood of their presence in the natural protein.

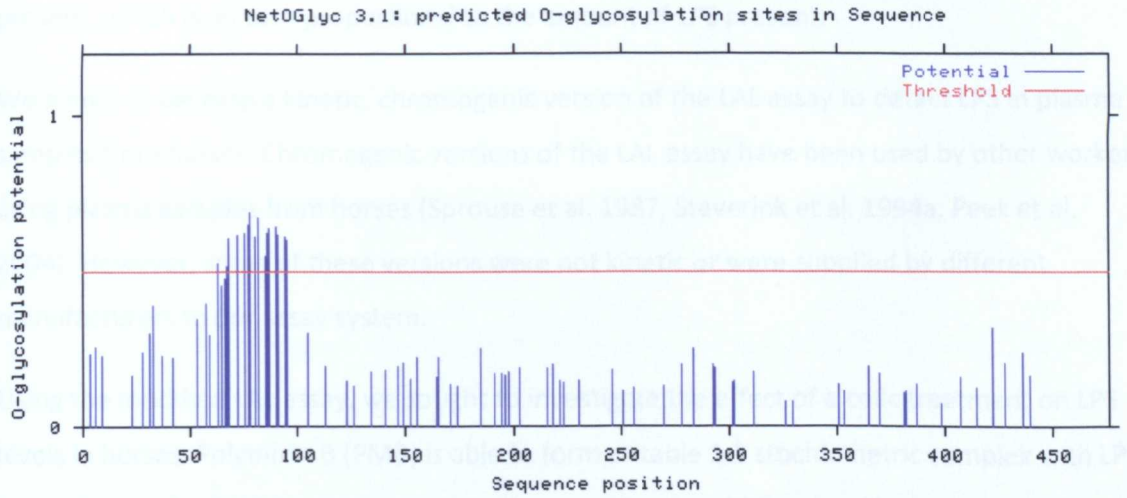
Predicted glycosylation sites from http://www.cbs.dtu.dk/services/NetN Glyc/ and http://www.cbs.dtu.dk/services/NetOGlyc/

```
Name:   Sequence          Length:   476
MASRLTPLTLLLLLLLAGHRAASNPDDSNHSFTDPESLQGESKGDILDILKGTVPKTVSIQDTVSSSTLPE
TKETNTTVS       80
ATLGSTTQPTTQPTTEFGCPEPDISCPDLGNHSAEMMLGDALTDFSVKLYHAFSAVKKPRSNMAFSPFSIA
SLLTQVLLG      160
AGDSTKKNLESVLSYPMNFACVHQTMKAFRSKGFSSASQIFHSPDLAIKNAFVNASRSLYGSSPRVLGNDS
QVNLELINA      240
WVAENTNHKISRLLDSLPADARLVLLNAVSLNAKWKKTFDPKNTRMEPFYVKSSVKKVLMMSSKKYPVAHF
TDQILKAKV      320
GQLQLSHNLSLVILVPQDMKQHLEDVEQALSPSVFKAILKKLEMTKIQPTLLMIPRIKVKSSQDMLTIMEK
LEFFDFSYD      400
LNLCRMTEDPDLQVSAMQHQITLELMESGVEAAAATAVSVARNLLIFHVDQPFLFVLWDQQHKFPVFMGRV
YDPMA
```

(Threshold=0.5)

SeqName	Position	Potential	Jury agreement	N-Glyc result
Sequence	29 NHSF	0.5124	(4/9)	+
Sequence	76 NTTV	0.7717	(9/9)	+++
Sequence	111 NHSA	0.5824	(7/9)	+
Sequence	214 NASR	0.5276	(5/9)	+
Sequence	229 NDSQ	0.6529	(9/9)	++
Sequence	328 NLSL	0.6881	(9/9)	++

Predicted O-Glyc site sequences are represented by straight lines in the graph below. Lines passing the threshold are most likely to be O-Glyc sites in the natural protein. Note: most predicted O-Glyc sites are in the N-terminal region of the protein.



Chapter 4

Determination of levels of endotoxin (LPS) in the plasma of horses using the Limulus Amoebocyte Lysate (LAL) Assay

Introduction

One of the major aims of our project was to measure levels of LPS in the plasma of horses in order to compare them to levels of C1-inh, to relate LPS load to disease (e.g. colic versus non-colic or strangulating lesion versus non strangulating lesion) and clinical outcome, and also to see if plasma LPS measurements related to clinical signs associated with endotoxaemia.

LPS is an essential part of the outer membrane of all Gram negative bacterial cell walls. Structurally, it is made up of three distinct components; lipid A, core oligosaccharide and an O-polysaccharide chain (see main introduction). LPS can come from any of the diverse species of gram negative bacteria within the gut of horses and present in an immeasurable number of fragment sizes. As a result, it is not surprising that, with such a potential for epitope diversity, there is currently no single antibody-based assay available which can detect LPS originating from all bacterial species.

The only assay available which detects all LPS is a bioassay based on an enzyme found in blood cells of the Horseshoe Crab called amoebocyte lysate. Briefly, LPS initiates activation of the enzyme from an inactive proenzyme via a cascade of serine proteases. In the presence of a colourless substrate (S-2423), the active enzyme rapidly catalyzes cleavage of the chromophore, p-nitroaniline (p-NA) which produces a yellow colour that can be measured spectrophotometrically. The colour change is proportional to the amount of active enzyme present, which is, in turn proportional to the amount of LPS present.

We aimed to develop a kinetic, chromogenic version of the LAL assay to detect LPS in plasma samples from horses. Chromogenic versions of the LAL assay have been used by other workers using plasma samples from horses (Sprouse et al. 1987, Steverink et al. 1994a, Peek et al. 2004). However, some of these versions were not kinetic or were supplied by different manufacturers to our assay system.

Using the modified LAL assay, we sought to investigate the effect of a colic treatment on LPS levels in horses. Polymixin B (PMB) is able to form a stable 1:1 stochiometric complex with LPS by binding to the lipid A component (Morrison and Jacobs 1976). It has shown potential as a

therapeutic agent to treat endotoxaemia both *in vivo* (Barton et al. 2004) and *in vitro* (Parviainen et al. 2001). Current experimental models that show PMB to be efficacious in ameliorating the inflammatory response to LPS are limited in that they rely on administration of PMB either prior to or nearly concurrently to LPS administration (Barton et al. 2004), which does not reflect the clinical situation in many cases. PMB began to be used as a treatment for endotoxaemia in horses within our hospital around the time this project began. Thus, we were in a unique situation to determine if PMB administration affected the LAL assay's ability to detect LPS in plasma from clinical cases, and if administration of PMB had an effect on outcome of cases.

Materials

Charles River Laboratories : 100mm Borosilicate Glass Dilution Tubes [TL 300], Control standard endotoxin [E 110], Endochrome K [R 1710 K], LAL Reagent [W 110], 96 Well pyrogen-free Microtitre Plates [TF 1300], Tecan Sunrise reader

Starlab: 1-200 µL Micropipette Tips, 100-1000 µL Micropipette Tips

Helena Biosciences: Biopure Combitips 0.5 ml [0030-069-420], Biopure Combitips 5 ml [0030-069-455]

VWR: Parafilm 10 cm x 38 m

Sartorius: Ministart™ 0.40 µm sterile filter unit

Non-haemolytic *E.coli* 0117 was kindly provided by Mrs Jackie Jones from the Department of Veterinary Pathology, University of Liverpool and had been isolated from a calf diarrhoea sample.

Methods

Basic protocols

Kinetic chromogenic LAL assay protocol

1. Samples were diluted 1:5 in Laboratory Reagent Water (LRW = endotoxin/ pyrogen-free water) and heat treated at 70°C for 15 min, allowed to cool and any required further dilutions were made prior to being loaded onto the plate.

2. Control Standard Endotoxin (CSE) was made up to 1000 $EUml^{-1}$ [1 EU = 100pg =0.1 ng] according to the Certificate of Analysis document supplied by manufacturer. The stock CSE was used to make up a five point standard curve of 50, 5.0, 0.5, 0.05, 0.005 $EUml^{-1}$.
3. 100 µl LRW was added to a pyrogen-free microtitre plate (2 wells), F1, F2 (negative water control). 100 µl of each standard was added in duplicate, at: 0.005, 0.05, 0.5, 5.0, 50.0 $EUml^{-1}$
4. 100µl of each sample was added into wells in quadruplicate
5. 10µl of 5 $EUml^{-1}$ was added to 2 of the 4 equine sample wells to give 0.5 $EUml^{-1}$ positive product controls.
6. The LAL reagent (Endochrome K) was opened and 3.4ml of LRW per vial added. 100µl of LAL reagent was added to every well containing sample or standard
7. The microplate was placed into the plate reader and the assay was read at 405nm at 37°C with repeated readings every 60 seconds. The assay was automatically stopped when the negative (LRW only) controls had reacted to an optical density increase of 0.1.
8. The software (Endosafe™, Charles River Laboratories) compared the reaction time values from each well against the standard curve and, after correcting for the inputted dilution factor, produced mean $EUml^{-1}$ values (the mean of the duplicated wells for each sample).

Collection of blood samples

In all cases, blood samples were obtained from excess blood collected for clinical reasons (e.g. determining packed cell volumes) from horses that had in-dwelling jugular catheters. Owner consent was obtained for all horses on admission to the hospital.

Statistics package

All statistical methods are detailed where appropriate in the text. Statistical analyses were carried out using Minitab 15 (Minitab Inc.), apart from generalised additive multivariable models which were carried out using S-Plus v6.1 (Insightful Corporation).

Sample storage validation for the Limulus Amoebocyte Lysate assay

It was important for any future analysis of C1-inh in plasma samples that sodium citrate was used as an anticoagulant in blood samples from horses. Also, due to the sample collection site and the laboratory being 17 miles apart, a collection system was required that would reduce the potential for contamination through immediate processing and storage of plasma. The company producing the LAL assay (Charles River Laboratories) recommended that 4ml sodium citrate/ ficoll Cell Preparation Tubes (CPT) (Becton Dickinson Vacutainer Cell Preparation Tubes (CPT)) were used and gave assurances that they had experience within their company of using this assay on human plasma collected in these tubes. The advantage of these tubes is that when centrifuged at an appropriate speed, the plasma and the blood cells are separated by a Ficoll gradient and a gel, so that the plasma and cells remain separated during handling and transport/ storage, thus removing the need to process plasma at the time of collection (thereby decreasing collection compliance and increasing potential contamination of the plasma samples).

Due to the laboratory and the sample collection/ storage sites being some distance apart, samples were to be processed and frozen before being analysed in batches. Therefore, a validation assessment was carried out to determine how long plasma samples in sodium citrate CPT gel tubes containing LPS could be stored without deterioration of the LAL assay's ability to detect LPS, i.e., whether there was a significant decline in measurable LPS in plasma over time under the proposed storage system.

Methods

The tubes were tested using the assay protocol (described above) for detectable levels of LPS in them both as 'clean' tubes and after they had been 'spiked' with known amounts of LPS. The "spiking" natural LPS was made by filter sterilising (Sartorius: Ministart™ 0.40 µm sterile filter) a broth of non-haemolytic *E.coli* 0117.

Blood samples from two horses were collected into 4mL CPT vacutainer tubes prior to blood being collected for plasma (the horses were to be euthanased for performance limiting non-septic orthopaedic conditions). 16 CPT tubes of blood were obtained from each horse to provide 8 tubes to be 'spiked' and 8 'unspiked' tubes. 16 CPT tubes were filled with LRW, again to provide 8 'spiked' and 8 'unspiked' tubes. One tube each of 'spiked' and 'unspiked' tubes from horse 1, horse 2 and 'water' were tested on day 1. The remaining tubes were frozen at -

20°C and one set of tubes tested weekly for a further seven weeks to test for deterioration in detectable LPS over the storage period.

Statistics

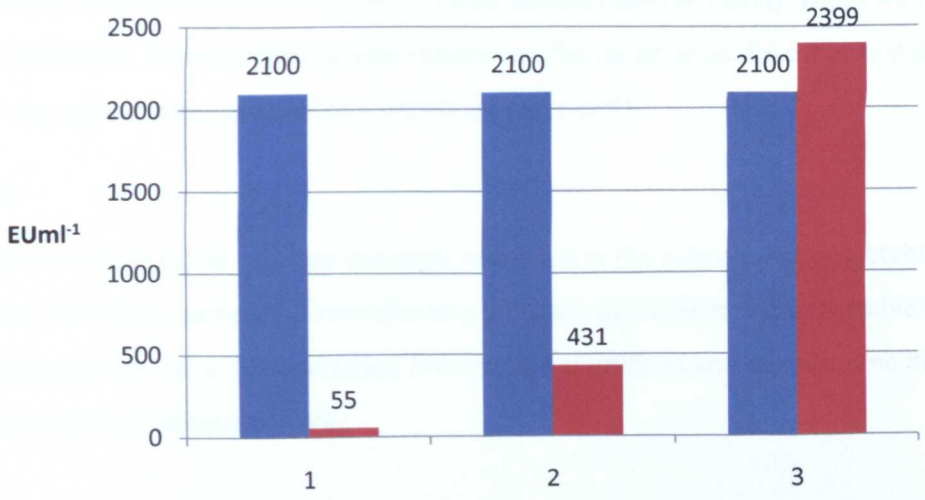
All data was tested for normal distribution using histograms/ box-whisker plots and the Kolmogorov-Smirnov test. Paired t-tests were carried out on the mean $EUml^{-1}$ values over the test period. Significance was taken at $p < 0.05$.

Results

There was ~80% (horse 2) and ~98% (horse 1) reduction in detectable 'spiked' LPS in the plasma after LPS was added to whole blood in the sample tubes.

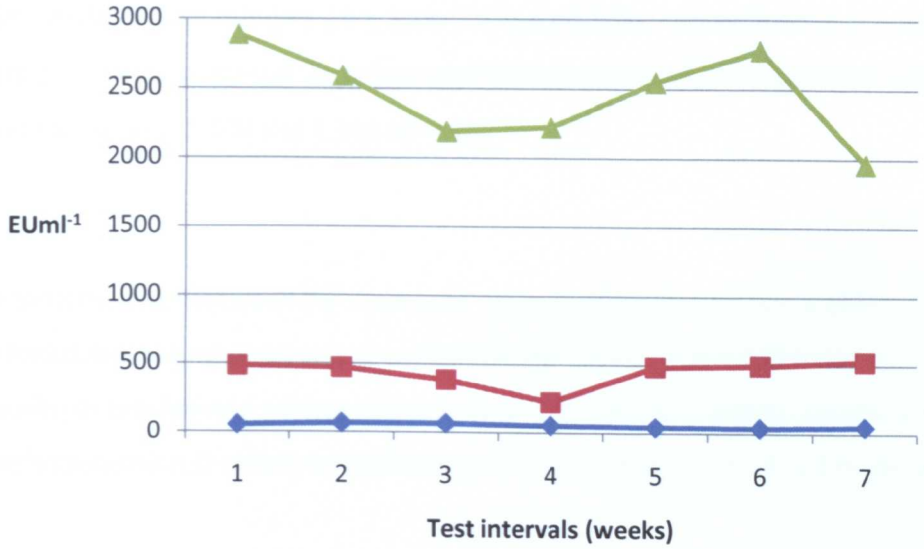
Figure 4.1: Histogram showing the difference between the amount of endotoxin (2100 $EUml^{-1}$) added to the test solutions compared to the amount detectable by the assay after processing.

Blue columns= amount added to solution; red columns = amount detected



Series 1 = Horse 1, Series 2 = Horse 2, Series 3 = Water

Figure 4.2: Graph representing the detectable EUml^{-1} in the three test solutions over a period of several weeks. Diamonds = Horse 1, Squares = Horse 2, Triangles = Water



There was no significant difference between the day one results and the mean of all the other test day results for either horse 1 or horse 2 spiked samples (paired t-test). There was a significant difference between the day one results and the mean of all the other test day results for the spiked water samples ($p = 0.024$) (paired t-test).

Conclusion

Spiking LPS into whole blood resulted in a large reduction in the subsequent detectable LPS from plasma from those samples. There was no significant decrease in the detectable LPS over the storage period for the plasma samples. The storage conditions and storage time did not cause deterioration of detectable LPS.

Correlation of LPS detection with haematological/ blood protein values

The storage validation study demonstrated that there was a large reduction in detectable LPS when natural LPS was spiked into whole equine blood (see discussion). The difference in 'spike recovery' between the samples from the two horses warranted further investigation. To assess whether other components in equine blood were affecting the assay (e.g. plasma proteins, red cells) a further 12 samples from clinical cases (endotoxaemic and non-endotoxaemic) were tested and haematological profiles and plasma protein levels were measured on concurrently collected EDTA samples.

Protocol

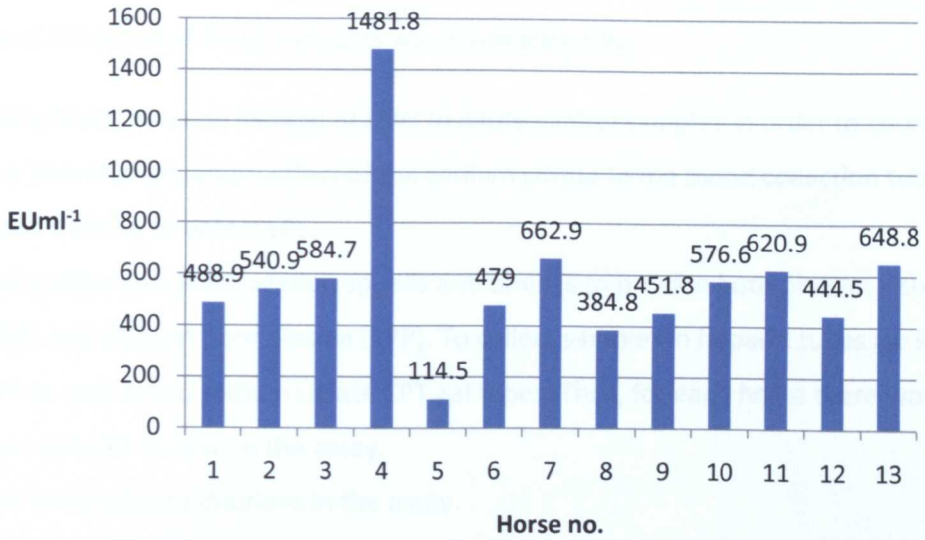
Two sodium citrate CPT gel samples were taken from each case, one assayed at a 1:100 dilution and the other was 'spiked' with filter sterilised non-haemolytic *E.coli* O117 (2100 $EUml^{-1}$) and run in the assay at 1:100 and 1:200 dilution.

Results

There was no correlation between the measured 'spiked' endotoxin values at either dilution to any of the measured haematological (e.g. red/ white cell counts, haemoglobin concentration, platelet counts) or biochemical parameters (e.g. total and fractional protein concentrations). E.g. Pearson's correlation (2 tailed) p= 0.395 for packed cell volume and p= 0.210 for white cell count.

There remained a wide variation in measured spiked values between different horses.

Figure 4.3: Histogram showing the mean measured $EUml^{-1}$ values for the 'spiked' samples from the plasma from thirteen different horses



Two horses apparently had measurable LPS in their 'unspiked' samples. Neither of these horses was deemed to be clinically endotoxaemic.

Tube and centrifugation speed comparison study for optimisation of kinetic chromogenic LAL assay

A wide variation in the detectable 'spiked' LPS between horses was not expected. It appeared that these differences were not correlated to haematological parameters. It was also interesting that although only a small number of samples from horses that were deemed to be clinically endotoxaemic had been tested, the assay had not detected LPS in 'unspiked' samples from those cases. After consultation with the assay manufacturers we identified several possible factors to consider to further optimise the assay to work in clinical equine samples:

1. The sodium citrate in the tubes may be inhibiting the assay.
2. The majority of endotoxin may be bound in the platelets and thus platelet rich plasma may provide better results. The gel in the CPT tubes may also be absorbing endotoxin.
3. The samples we had been using may have been too dilute.
4. A plasma protein called Hagemann Factor may be interfering with the assay.

The potential influence of these variables was investigated by:

1. Using $MgCl_2$ solution instead of LRW to dilute clinical samples in order to counteract any potential inhibitory effect of the sodium citrate in the blood collection tubes on the assay's ability to detect LPS.
2. Using different centrifugation speeds and timings to provide both platelet rich plasma (PRP) and platelet poor plasma (PPP). To collect samples in heparin tubes for PRP and PPP as well as the sodium citrate CPT gel tubes. Thus, for each horse there would be four samples to test on the assay.
3. Use lower plasma dilutions in the assay.
4. Use a specific substrate (S-2423), in order to test for the presence of Hagemann Factor.

The assay protocol was modified and validated in order to accommodate the above changes. Preliminary results showed that 0.25M $MgCl_2$ caused precipitation and could not be used in the assay. 1.56mM calcium chloride was tested and found not to cause precipitation. All samples were diluted with LPS-free 1.56mM $CaCl_2$ (made with LRW).

For the tube/ centrifugation speed comparison test, plasma samples from horses determined clinically to be non-endotoxaemic (normal HR/ PCV and no clinical reason to have endotoxaemia), moderately (HR>50-<70, PCV between 38-45%, clinical disease/ surgical finding that might result in endotoxaemia), and severely endotoxaemic horses (HR > 70 and/ or PCV ≥ 45%, and clinical disease/ surgical findings likely to result in LPS absorption from the gut), were collected into two heparin and two sodium citrate CPT gel vacutainers . One of each vacutainer per horse was then centrifuged at either 150g for 10 minutes or 1700g for 15 minutes to separate the plasma. The tubes were then frozen at -20°C until assayed.

Assay protocol

The assay protocol followed the basic protocol detailed at the start of this chapter with the following exceptions;

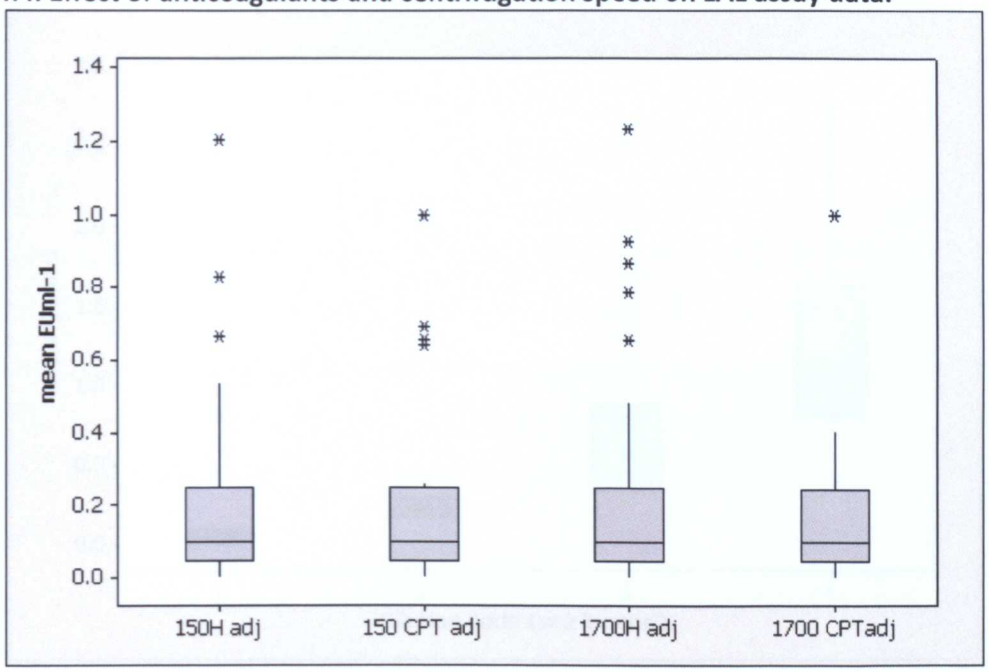
1. The stock CSE was used to make up standard LPS solutions as above; a four point standard curve of 5.0, 0.5, 0.05 and 0.005 $EUml^{-1}$ were added to the plate.

2. 100 µl LRW was added to a pyrogen-free microtitre plate to 2 wells, E1, E2 (negative water control)

Results

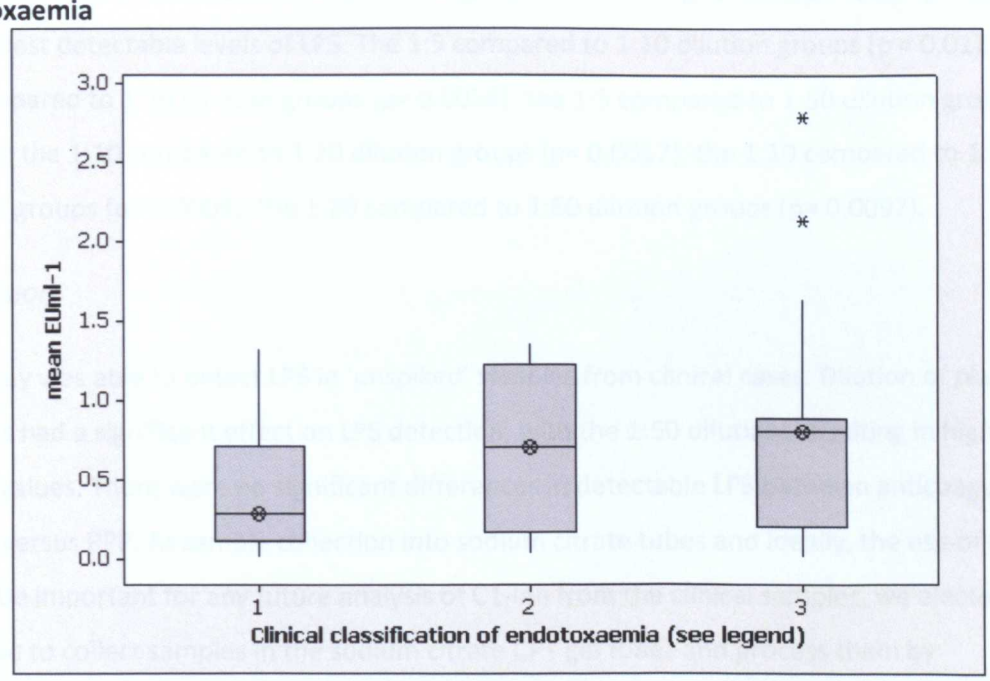
There was no significant difference (two sample t-tests) in mean $EUml^{-1}$ values between the two types of anticoagulant or the two centrifugation speeds. There were no significant differences (Mann-Whitney) between median values for $EUml^{-1}$ for the clinical classifications of endotoxaemia groups.

Figure 4.4: Effect of anticoagulants and centrifugation speed on LAL assay data.



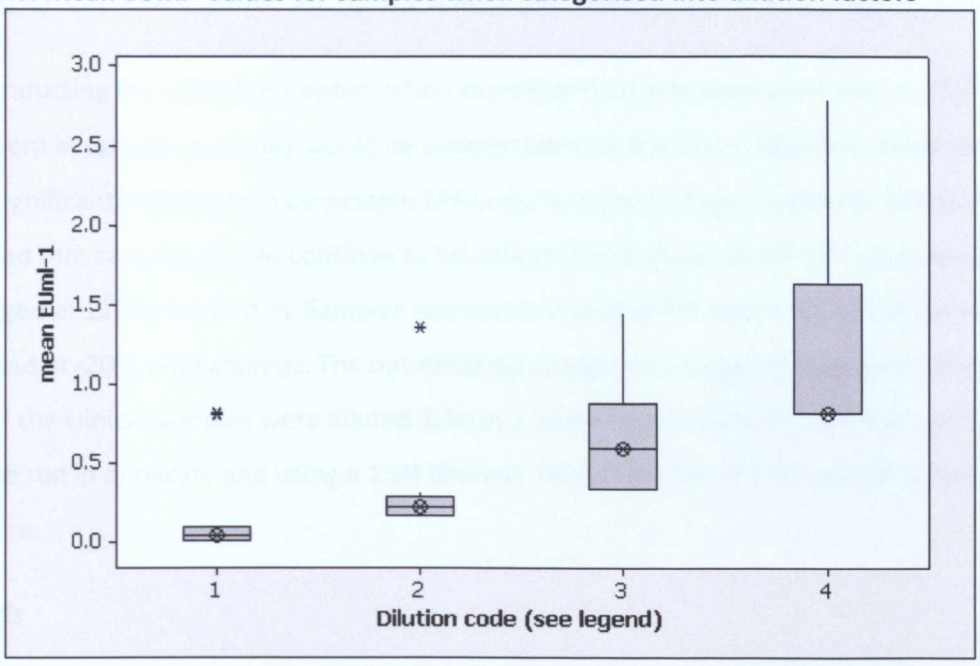
Box plot showing mean EUml^{-1} for the two tested anticoagulants and the two centrifugation settings. 150H adj = Heparin sample spun at 150g for 10 min, 150 CPT adj = Citrate sample spun at 150g for 10 min, 1700H adj = Heparin sample spun at 1700g for 15 min, 1700CPT adj = Citrate sample spun at 17000g for 15 min.

Figure 4.5: Mean EUml^{-1} results categorised according to clinical classification of endotoxaemia



1 = non-endotoxaemic, n= 16, 2 = moderately endotoxaemic, n =8, & 3 = severely endotoxaemic, n =16

Figure 4.6: Mean EUml^{-1} values for samples when categorised into dilution factors



1 = 1:5 dilution, 2 = 1:10 dilution, 3 = 1:20 dilution & 4 = 1:50 dilution

When the results were categorised according to the dilution factor there were significant differences (Mann-Whitney) between all the groups with the higher dilution factor providing the highest detectable levels of LPS. The 1:5 compared to 1:10 dilution groups ($p = 0.01$); the 1:5 compared to 1:20 dilution groups ($p= 0.0038$); the 1:5 compared to 1:50 dilution groups ($p= 0.0001$); the 1:10 compared to 1:20 dilution groups ($p= 0.0017$); the 1:10 compared to 1:50 dilution groups ($p= 0.0009$); the 1:20 compared to 1:50 dilution groups ($p= 0.0097$).

Conclusion

The assay was able to detect LPS in 'unspiked' samples from clinical cases. Dilution of plasma samples had a significant effect on LPS detection, with the 1:50 dilutions resulting in higher EUml^{-1} values. There were no significant differences in detectable LPS between anticoagulants or PRP versus PPP. As sample collection into sodium citrate tubes and ideally, the use of PPP would be important for any future analysis of C1-inh from the clinical samples, we elected to continue to collect samples in the sodium citrate CPT gel tubes and process them by centrifuging at 1700g for 15 min before storage. Samples run on the modified LAL assay would be diluted 1:50 using 1.56mM $CaCl_2$ in LRW.

Testing of clinical equine samples using the optimised LAL assay

After conducting the validation/ optimisation experiments it was concluded that much of the LPS present in samples probably would be undetectable by the assay. However, there would not be significant reductions in detectable LPS under sample storage conditions. It was also concluded that samples should continue to be collected in sodium citrate CPT gel tubes and centrifuged at 1700g for 15 min. Samples were centrifuged at 4°C within 20 min of collection and stored at -20°C until analysis. The optimisation studies also suggested we would get best results if the clinical samples were diluted 1:50 in 1.56mM $CaCl_2$ made in LRW. Each sample would be run in duplicate and using a 1:50 dilution. Four negative controls would be run on each plate.

Methods

The assay was conducted as described above with a four point standard curve (5.0, 0.5, 0.05 and 0.005 $EUml^{-1}$). Each sample was run in duplicate. For the standard curve to be valid, the gradient of the log values of the reaction times when plotted on a graph had to be between -1.00 and -0.98 (i.e. a straight line). A CV % of >20% between each sample result was considered an invalid result and the samples were re-tested on another plate. The samples were coded so that the tester was unaware of which horse, sample or date, the sample was from.

Objective

To obtain plasma samples from horses on admission (and daily for up to three days afterwards) to the Philip Leverhulme Equine Hospital in order to measure LPS levels using the kinetic chromogenic LAL assay. The relationship between levels of LPS and factors such as clinical variables, type of colic, and survival will then be explored. Samples from non-colic cases will be used as a control group.

Statistics

All data were tested for normal distribution using histograms/ box-whisker plots and the Ryan-Joiner test. The $EUml^{-1}$ results were not normally distributed due to the large number of zero values (the limit of detection of the assay was 0.25 $EUml^{-1}$; and any assay result of <0.25 $EUml^{-1}$ value was reported as 0.00 $EUml^{-1}$), accordingly, Kruskal-Wallis (adjusted for ties) tests for two independent variables were carried out on individual sample results with $EUml^{-1}$ being the response/ outcome for comparisons between groups. Cross tabulations with chi – squared

tests were carried out for categorical variables. Significance was taken at $p < 0.05$. Binary logistic regression was carried out with the response of LPS detected in sample recorded as yes or no, at the sample and horse level. All variables with a p value <0.3 were included (and some e.g. PMB administration), were included on an a priori basis) in multivariable models using a stepwise backward elimination model development process, removing factors with the highest p value at each step, until all factors remaining in the model had $p \leq 0.05$.

Results

Descriptive statistics

Two hundred and forty horses provided 375 samples. Some samples were later excluded from the study as being contaminated (see below). The mean age of the study population was 10.8 years (SD = 6.0). Castrated males (122, 50.83%) were the commonest sex, followed by females (108, 45%) with a small number of entire males (10, 4.17%). 63 (26.4%) horses from the study population died or were euthanized whilst in the hospital. 195 (81.6%) of the horses providing samples for the study presented to the hospital with colic, 44 horses did not present as colic (data missing for 1 horse). Of the horses presenting as colic cases, 167 (86.5%) required surgical intervention, 37 (20%- 10 cases data missing) were deemed to be clinically endotoxaemic (HR ≥ 70, PCV ≥ 45% and/or other strong clinical evidence e.g. surgical findings) and 30 colic cases received PMB during their stay (not all of these cases fell into the clinically endotoxaemic category). 56 (28.9%) colic cases died or were euthanized whilst in the hospital.

The assay provided positive results in 38 samples which came from cases which were deemed to be clinically endotoxaemic (true positive). The assay also provided positive results in 38 samples which came from cases which were not deemed to be clinically endotoxaemic (false positive). The assay result was negative in 266 samples which came from cases that were not deemed to be clinically endotoxaemic (true negative). The assay also provided a negative result in 27 samples that came from cases that were deemed clinically endotoxaemic (false negative). Overall, the assay detected LPS in 23.2% (68/294) of samples from colic cases presented at the hospital. At the horse level the assay detected LPS in 26.5% (62/234; after exclusions) of horses presented at the hospital, or in 29% (56/192) of horses presenting with colic.

Table 4.1: LAL assay performance; if clinical signs associated with endotoxaemia are assumed to reflect circulating LPS.
PMB = polymixin B treatment

	True positive [positive assay result/ clinical endotoxaemia criteria met]	Total positive [positive assay result ± clinical endotoxaemia criteria met]	Sensitivity (true positive / total positive x 100)	True negative [negative assay result/ clinical endotoxaemia criteria not met]	Total negative [negative assay result ± clinical endotoxaemia criteria met]	Specificity (true negative / total negative x 100)
All samples	38	65	58.4%	266	304	87.5%
Samples from cases receiving PMB excluded	28	40	78%	241	278	86.7%
Samples from cases receiving PMB	10	25	40%	21	22	95.5%

Univariable analysis

Some horses provided more than one sample and some horses were deemed to be both clinically endotoxaemic and non-endotoxaemic at different times during their stay within the hospital. Accordingly, some analyses were carried out at the sample level. However, as the dataset for all other clinical parameters was only complete for cases at the time of admission then most comparisons have been stratified at the horse level rather than at the sample level. Where this has not been done is made clear in the text. Five sample results were excluded from the analysis as likely contaminated samples (i.e. their $EUml^{-1}$ results were several times higher than the highest value for any clinically endotoxaemic sample).

Equine Colic variables

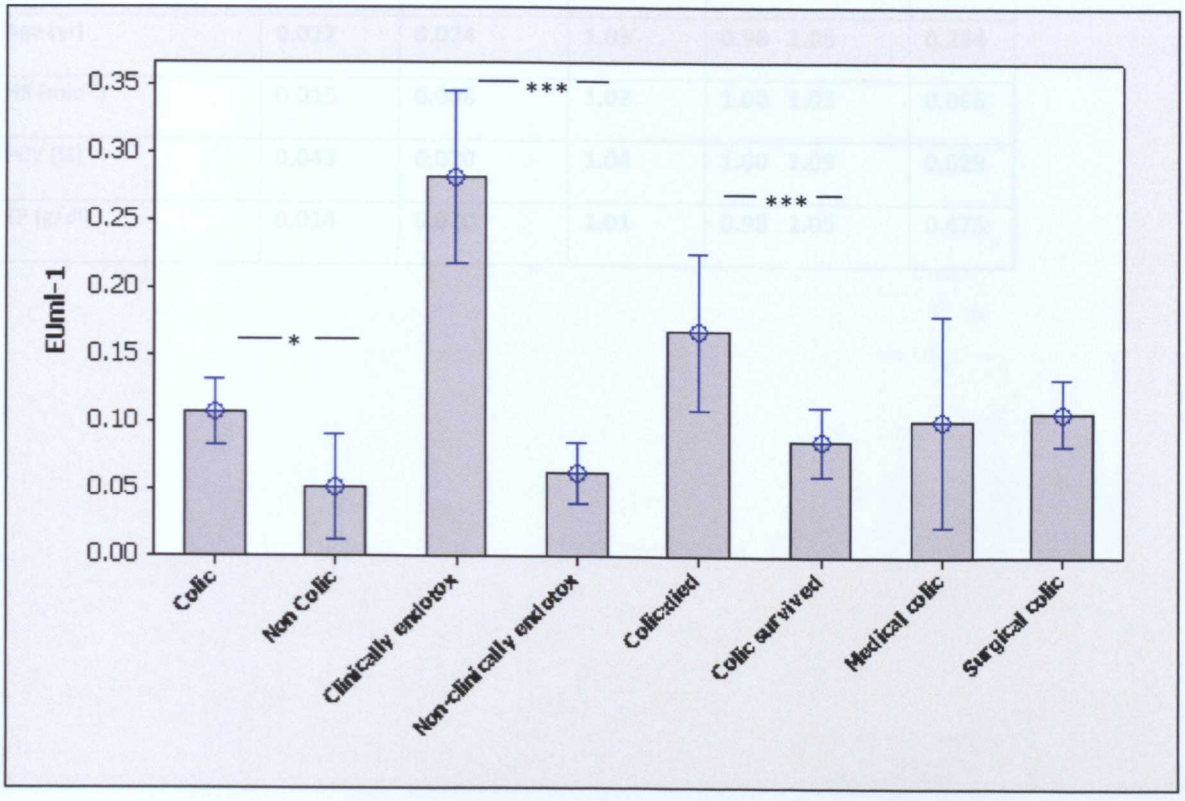
Sample level

Samples from the following cases had significantly higher $EUml^{-1}$ values (Kruskal-Wallis: adjusted for ties); colic cases in comparison to non-colic cases ($p= 0.017$), cases deemed to be clinically endotoxaemic compared to samples from all other clinically non-endotoxaemic cases ($p <0.001$), colic cases deemed to be clinically endotoxaemic in comparison to samples from colic cases not deemed to be clinically endotoxaemic ($p <0.001$), and from colic cases that died whilst in the hospital compared to samples from colic cases that survived the stay in the hospital ($p= 0.001$). $EUml^{-1}$ values were not significantly different between samples from

medical and surgical colic cases (p = 0.722), between samples from large intestinal, small intestinal or other cases (p= 0.233), or between samples from cases with a strangulating lesion or a non-strangulating lesion (p= 0.432).

Figure 4.6: Mean EUml^{-1} values for all assay results from the following samples: Samples (n= 294) from colic cases and samples (n= 75) from non-colic cases; samples (n= 62) from colic cases deemed to be clinically endotoxaemic and samples (n= 227) from colic cases NOT deemed to be clinically endotoxaemic ; samples from colic cases that died whilst in the hospital (n= 67) and samples (n= 223) from colic cases survived; samples from medical colic (n= 39) and samples (n= 255) from surgical colic cases.

Bar = mean EUml^{-1} value; Blue line = 95% confidence interval of the mean, * = P <0.05, *** = P≤0.001



Horse level

As most clinical data was collected on admission, we compared variables collected at this time to whether or not they were likely to result in a positive LPS sample, at the horse level, using both univariable and multivariable logistic regression.

Table 4.2: Univariable logistic regression analysis of continuous variables when compared to detectable LPS yes =1, no = 0. For all horses in the study

Variable [recorded at admission]	Coefficient	SE Coefficient	Odds ratio	95%CI Odds ratio	P-value
Age (yr)	0.027	0.024	1.03	0.98 1.08	0.264
HR (min^{-1})	0.015	0.008	1.02	1.00 1.03	0.066
PCV (%)	0.043	0.020	1.04	1.00 1.09	0.029
TP (g/dl)	0.014	0.020	1.01	0.98 1.05	0.475

Table 4.3: Univariable logistic regression analysis of categorical variables when compared to detectable LPS yes =1, no = 0. For all horses in the study

Variable		LPS >0.25 EUml^{-1} detected? No	LPS >0.25 EUml^{-1} detected? Yes	Odds ratio	95%CI	P-value
Colic	Yes	136	56	1		
	No	36	6	0.40	0.16 1.01	0.037
Condition	Medical	30	7	1		
	Surgical	142	55	1.66	0.69 4.00	0.241
Clinical endotoxaemia?	Yes	15	30	1		
	No	155	32	0.1	0.05 0.21	<0.001
Died in hospital?	Yes	39	22	1		
	No	133	39	0.52	0.28 0.98	0.045
Survivors home in <7d	Yes	65	16	1		
	No	67	23	1.39	0.68 2.88	0.365

Table 4.4: Univariable logistic regression analysis of continuous variables when compared to detectable LPS yes =1, no = 0. For horses presented with colic only.

Variable [recorded at admission]	Coefficient	SE Coefficient	Odds ratio	95%CI Odds ratio	P-value
Age (yr)	0.005	0.025	1.00	0.96 1.06	0.856
HR (min^{-1})	0.016	0.009	1.02	1.00 1.03	0.060
PCV (%)	0.043	0.020	1.04	1.00 1.09	0.032
TP (g/dl)	0.011	0.020	1.01	0.97 1.05	0.574
Total colic time (hr)	0.011	0.008	1.01	1.00 1.03	0.144

Table 4.5: Univariable logistic regression analysis of categorical variables when compared to detectable LPS yes =1, no = 0. For horses presented with colic only.

Variable	LPS >0.25 EUml^{-1} detected? No	LPS >0.25 EUml^{-1} detected? Yes	Odds ratio	95%CI	P-value
Condition					
Medical	20	5	1		
Surgical	116	51	1.76	0.63 4.95	0.285
Clinical endotoxaemia					
Yes	14	30	1		
No	120	26	0.1	0.05 0.22	<0.001
Site of lesion					
Large intestine	26	17	1		
Small intestine	46	22	0.73	0.33 1.62	0.441
Strangulating?					
Yes	61	31	1		
No	71	25	0.69	0.37 1.30	0.252
Died in hospital?					
Yes	34	20	1		
No	102	35	0.58	0.30 1.14	0.119
Survivors home in <7d? Yes	50	14	1		
No	51	21	1.47	0.67 3.21	0.333
PMB administration?					
Yes	20	11	1		
No	115	45	0.71	0.32 1.60	0.417

Summary of univariable analysis

The following groups of horses were more likely to have had LPS detected in samples taken from them (Pearson Chi^2): Horses with any type of colic compared to non-colic cases (p = 0.037), horses with colic that met the criteria for clinical endotoxaemia on admission compared to those that did not (p = <0.001), and horses that died in the hospital compared with those that did not (p = 0.045).

There was no significant difference in outcome for categorised length of time that colic survivors remained in the hospital between horses that had provided positive LPS samples compared to those that did not (p = 0.333). Colic horses that had positive LPS samples were not more likely to die than those that did not (p = 0.119). Whereas, those horses classified as clinically endotoxaemic at some stage of their stay in the hospital were much more likely to die compared to those that were not (p = <0.001) (data not in table above).

Multivariable logistic regression

Binary logistic regression models were built using backward elimination to determine variables which were likely to predict whether a horse was likely to provide a positive LPS sample for all horses in the study and for colic cases only. The model for colic cases is shown below:

Table 4.6: Final Binary Logistic Regression Model after stepwise backward elimination of the likelihood of a horse providing a positive LPS sample [LPS >0.25 $EUml^{-1}$ detected? yes/no] depending on the horse's heart rate on admission (HR on admit), and if the horse did not fit the criteria for clinical endotoxaemia on admission (Clinical endotoxaemia? No) for ALL HORSES PRESENTED TO THE HOSPITAL WITH COLIC. The table shows the mean (SE) values of the coefficients and the odds ratios and their 95% confidence intervals (CI).

	Coefficient	P value	Odds ratio	Lower 95% CI	Upper 95% CI
Constant	2.94106	1.12762			
HR on admit	-0.0274081	0.043	0.97	0.95	1.00
Clinical endotoxaemia? No	-3.06737	0.000	0.05	0.01	0.01

Log-Likelihood = -88.977
Test that all slopes are zero: G = 41.957, DF = 2, P-Value = 0.000
N= 180 cases

The models for ALL COLIC CASES and ALL HORSES were virtually identical due to the number of non-colic cases in the ALL HORSES model that were excluded due to incomplete data.

In summary, the multivariable model shows that when all the variables with a p = <0.3 from univariable binary logistic regression were entered into the multivariable model (so that the influences of each variable on each other is accounted for), the only variables that significantly influenced the model were heart rate and clinical endotoxaemia. Any horse that provided a sample with detectable LPS during its stay was likely to have had an elevated heart rate at the time of admission. A horse that was not deemed to be clinically endotoxaemic was ~20x less likely to provide a positive sample compared to one that was.

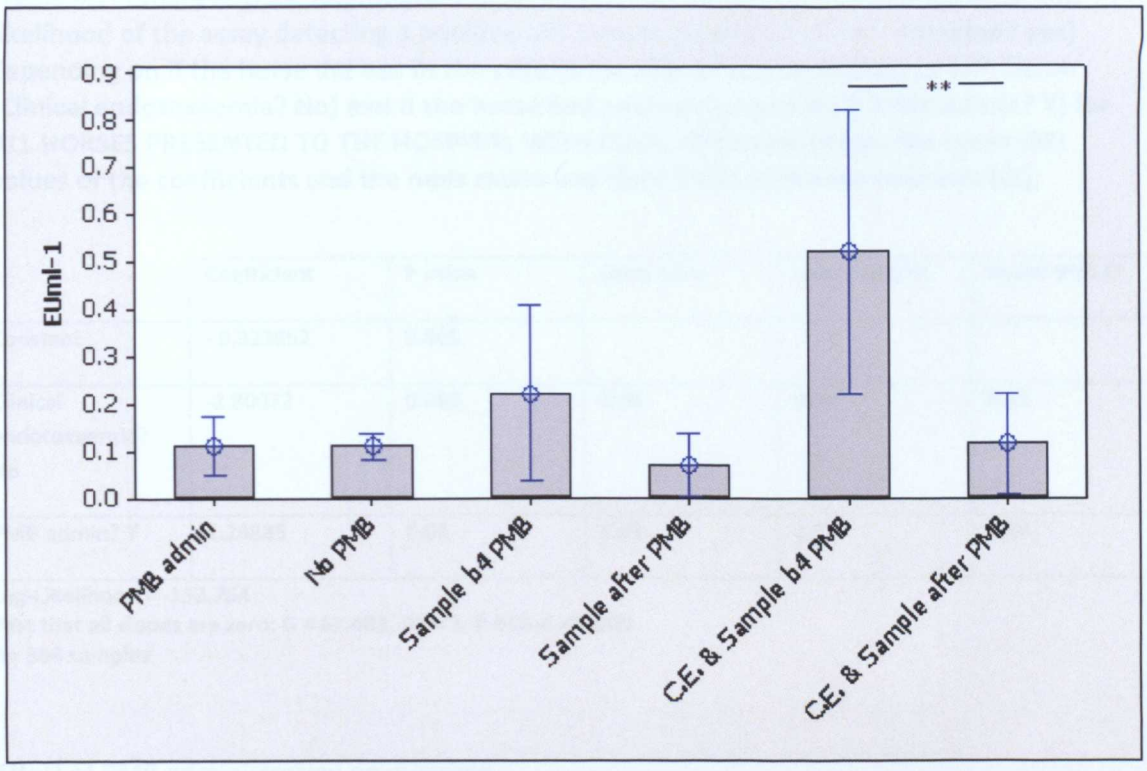
Effect of PMB administration

Sample level

There was no significant difference (Kruskal-Wallis: adjusted for ties) in endotoxin values between samples from colic cases that had received PMB compared to those that had not (p= 0.896), or from those that had received PMB where the samples were known to have definitely been taken before PMB administration compared to those cases where the samples were known to have definitely been taken after PMB administration (p= 0.116).

Not all the samples from colic cases that received PMB were from horses deemed to be clinically endotoxaemic. When the above test was repeated for samples that came from colic cases deemed to be clinically endotoxaemic, samples taken before PMB was administered had significantly higher $EUml^{-1}$ values than those samples taken after PMB (p= 0.004), although the group sizes were small.

Figure 4.7: Mean EUml^{-1} values for all assay sample results from the following samples:
Samples from colic cases receiving polymixin B (PMB admin) (n= 47) and from colic cases that did not receive PMB (no PMB) (n= 243); samples from colic cases receiving PMB where the sample was known to be obtained prior to PMB administration (Sample b4 PMB) (n= 11) and from colic cases receiving PMB where the sample was known to be obtained after to PMB administration (Sample after PMB) (n= 25); samples from clinically endotoxaemic colic cases receiving PMB where the sample was known to be obtained prior to PMB administration (C.E. & Sample b4 PMB) (n= 4) and from clinically endotoxaemic colic cases receiving PMB where the sample was known to be obtained after to PMB administration (C.E. & Sample after PMB) (n= 15).
Bar = mean EUml^{-1} value; Blue line = 95% confidence interval of the mean, ** = P <0.005



Horse level

Horses classed as being clinically endotoxaemic on arrival that received PMB were less likely (Pearson's Chi^2) to have detectable LPS in their samples (LPS only detected in 9/24 samples [37.5%], p = 0.024) compared to those that did not receive PMB (LPS was detected in 29/39 samples [74.4%], p = <0.001).

Also, 'unexpected' negative results (horses that were deemed to be clinically endotoxaemic but the LAL assay did not detect any endotoxin) were obtained more frequently from cases receiving PMB. Forty-seven samples were from colic cases receiving PMB contributed 15 out of 27 false negative results, compared to 243 samples from colic cases that did not receive PMB and contributed 12 out of 27 false negative results (p =<0.001).

The likelihood of a horse providing a positive LPS sample was analysed in a binary logistic regression model, against whether or not the horse was deemed to be clinically endotoxaemic and whether or not it had received PMB. The model suggested that if a horse was not deemed

to fit the criteria for clinical endotoxaemia it was ~ 20x less likely to provide a positive LPS sample in comparison to a horse that was clinically endotoxaemic, and a horse that had not received PMB was ~3.5x more likely to provide a positive LPS sample than one that had received PMB. This was after the influence of the two factors on each other, have been accounted for (see below).

Table 4.7: Binary Logistic Regression Model after stepwise backward elimination of the likelihood of the assay detecting a positive LPS sample [LPS >0.25 EUml^{-1} detected? yes] depending on if the horse did not fit the criteria for clinical endotoxaemia on admission (Clinical endotoxaemia? No) and if the horse had received polymixin B (PMB admin? Y) for ALL HORSES PRESENTED TO THE HOSPITAL WITH COLIC. The table shows the mean (SE) values of the coefficients and the odds ratios and their 95% confidence intervals (CI).

	Coefficient	P value	Odds ratio	Lower 95% CI	Upper 95% CI
Constant	- 0.323852	0.405			
Clinical endotoxaemia? No	-2.80072	0.000	0.06	0.03	0.13
PMB admin? Y	1.24885	0.01	3.49	1.34	9.04

Log-Likelihood = -152.754
Test that all slopes are zero: G = 67.483, DF = 2, P-Value = 0.000
N= 364 samples

Effect of PMB administration on outcome

Although the sample numbers were small in some groups, colic cases that provided a positive LPS sample, as well as having PMB administered, were more likely to die compared to those that provided a positive LPS sample but did not have PMB administered (64% compared to 30%, p = 0.036). Horses with colic that provided a negative LPS sample and had PMB administered were not significantly more likely to die than those that did not receive PMB (40% compared to 23%, p = 0.098). PMB administration appeared to be associated with an increased likelihood of death in colic cases that were deemed to be clinically endotoxaemic (p = 0.007). When the LPS result and PMB administration in colic cases were considered together in a logistic regression analysis, the model showed that horses not receiving PMB were less likely to die (p =0.011, PR =0.36, 95%CI =0.36, 0.79). However, after multivariable analysis of whether a horse died as the outcome for colic cases, the only factor that remained in the model was whether or not the horse was deemed to be clinically endotoxaemic, i.e. horses with colic deemed to be clinically endotoxaemic were five times more likely to die whilst in the hospital (p =<0.001, OR = 0.20, 95%CI= 0.1, 0.42).

Discussion

The chromogenic Limulus amoebocyte lysate assay was first developed based on the agglutination of gelatin by Levin and Bang (1964) and then subsequently developed to detect LPS in human blood in sepsis patients (Tachiyama et al. 1986). Since then it has been modified into other forms (chromogenic) and used for other applications (primarily testing for LPS contamination in pharmaceutical products). Other workers have used chromogenic LAL assays in various guises to measure LPS in the plasma of horses from clinical cases (King and Gerring 1988, Steverink et al. 1994a) and experimental animals (Sprouse et al. 1987, Menzies-Gow et al. 2004, Peek et al. 2004). The majority of these studies have used chromogenic end-point assay systems with one study using a kinetic chromogenic assay manufactured by a different company to that used in this project (Menzies-Gow et al. 2004). Accordingly, it was important that the assay that we intended to use underwent validation and optimisation before being used on clinical samples.

Sample collection/ processing

For any future analysis of equine C1-inh it was important that sodium citrate was used as the anti-coagulant. Heparin potentiates the action of human C1-inh (Bos et al. 2002) and the danger of potentiation for any future functional assay needed to be avoided. EDTA acts to activate complement and so also had to be avoided. The sodium citrate gel CPT tubes used in this study for collection of blood were chosen as they were pyrogen-free and designed to permanently separate plasma and white blood cells from red blood cells/ platelets without danger of subsequent mixing during handling. This was important to the study as the laboratory where samples would be analysed was 17 miles from where the samples would be collected and the tubes would allow sample processing and storage with minimal risk of contamination and maximum chance of collection compliance. As samples were to be analysed in batches, it was important that the tubes were stored at -20°C as the tubes were made of glass and lower temperatures would increase glass brittleness and the risk of breaking.

The blood samples were obtained from excess blood collected for clinical reasons (e.g. determining packed cell volumes) from horses that had in-dwelling jugular catheters. The collection protocol was that the first sample would be obtained as soon after the horse was admitted to the hospital as practicable and then further samples collected every morning thereafter (as long as the horse had a catheter in place and was having blood collected for clinical reasons) for a maximum of three days (i.e. maximum of four samples). Previous studies in clinical colic cases only measured LPS from samples obtained when the horse was admitted

up to a maximum period of 40 hours after surgery (Steverink et al. 1995). The samples were to be collected from both colic and non-colic cases; the non-colic cases acting as controls. The rationale behind this protocol was to allow us to measure LPS and C1-inh in the samples to see if there were any changes over a period of time. However, many cases contributed fewer than four samples with many cases only contributing a single sample. This was partly due to poor compliance with sampling protocols and partly due to the fact that it was not possible to collect four samples from many cases, e.g., intravenous catheters were removed or the horses died.

Storage validation/ anticoagulant/ Platelet-rich-plasma (PRP)-Platelet-poor-plasma (PPP) comparison

The storage validation results demonstrated that the storage conditions did not result in significant loss of detectable LPS but that the amount of detectable LPS was only a small percentage of the amount of LPS 'spiked' into the whole blood. In order to separate the red cells/ platelets from the plasma, the sodium citrate gel CPT tubes require centrifugation at 1500-1700g for 15 min, effectively producing PPP. One study reported that PPP from horses is unsuitable for reliable detection of LPS using the LAL assay by demonstrating a weak but highly significant correlation ($r= 0.53$, $p = 0.001$) between platelet counts and LPS concentrations in plasma when PPP and PRP was tested from 145 paired samples from horses with colic (Steverink et al. 1994b). In contrast to the findings of Steverink et al. (1994b), the study we undertook designed to compare different centrifugation speeds and heparin with sodium citrate, did not find significant differences between PRP/ PPP, or between the anti-coagulant used. Our study was much smaller in terms of number of horses (11); and not all the samples came from colic cases and so would not be expected to provide positive samples which may have reduced the power of the study to detect significant differences. However, 8 comparisons were run for each set of samples (2 centrifugation speeds x 4 dilution factors), making 88 comparisons of PPP/PRP. Peek et al. (2004) also found that PRP proved more reliable than PPP at determining LPS levels from blood 'spiked' with a purified LPS. However, the evidence as to the suitability of PPP/PRP for the LAL assay from human plasma samples is conflicting; Obayashi et al. (1986) report no differences in detectable LPS between PPP/PRP from 'spiked' human whole blood but Tachiyama et al. (1986) report *in vivo* binding of purified LPS with platelets, but that the degree of binding was dependent on the species of bacteria from which the LPS originated, with one *E.coli* species showing no binding at all. The same study did not demonstrate a difference in detectable 'natural' endotoxin from PPP/ PRP from the blood of

surgical patients and suggested natural LPS may not bind to platelets (Tachiyama et al. 1986). It is likely that differences in results between our study and others (Steverink et al. 1994b, Peek et al. 2004) are in part a reflection of different study designs and assay systems/ protocols e.g. we used a purified LPS from a single *E.coli* species in this project and that may not bind to platelets. Further work is needed to determine the best sample type for use of the LAL assay in clinical cases.

The comparison study also did not demonstrate any inhibition of the assay by Hagemann factor (data not shown). The results meant that the samples could continue to be collected in the sodium citrate gel CPT tubes.

LPS was detected in 'unspiked' clinical samples during this study although no significant differences were found in $EUml^{-1}$ values between the non-endotoxaemic, moderately endotoxaemic or severely endotoxaemic groups. This was not surprising as the group sizes were very small resulting in low study power to detect differences.

Levin et al. (1970) first demonstrated that blood contains substances that inhibit the LAL assay. The assay manufacturers recommend diluting all samples to be used in the assay to dilute any inhibitory factors present in the sample solution. The dilution factors were shown to be significantly different with higher dilutions providing the highest $EUml^{-1}$ values. The higher dilutions may have resulted in better performance of the assay as the inhibitory factors will have been diluted more. It is also possible that the assay design itself may be responsible, at least in part, for these findings. The assay software calculates the final $EUml^{-1}$ value by multiplying the 'real' value for the 1:50 dilution by a factor of 50. Any variation/ error in the result will therefore also be multiplied by the same factor, although this would be true of variation/ error that would result in both higher and lower values.

Clinical samples

The assay criteria for testing the clinical samples were designed according to the results of the validation/ optimisation experiments. The assay was conducted with a four point standard (log) curve (5.0, 0.5, 0.05 and 0.005 $EUml^{-1}$). For the standard curve to be valid the R value for the line of best fit of the log values of the reaction times had to be <-0.98 (i.e. nearly a perfect straight line).

A consequence of using 50x dilutions to increase detectable $EUml^{-1}$ values meant that the lower limit of detection of the assay was 0.25 $EUml^{-1}$(0.005 $EUml^{-1}$ x 50). Four negative controls were run on each plate (two wells of LRW and lysate/ chromogenic substrate and two wells of 1.56mM $CaCl_2$ in LRW and lysate/ chromogenic substrate) to account for the fact that the clinical samples were diluted in the $CaCl_2$ solution. Each sample was run in duplicate. A CV of >20% between each sample result was considered an invalid result and the samples were re-tested on another plate (the normal tolerance for CV % in our laboratory for micro plate assays is 15%). The sample processing and storage technique was similar to that employed by Steverink et al. (1995) and relied on collection into pyrogen free blood tubes, rapid chilled centrifugation of samples and storage of plasma at -20°C. Previous methodologies have required acid extraction, heat inactivation, dilution and/ or chelation of cations of samples prior to storage to prevent endotoxin breakdown (Obayashi et al. 1982). In our protocol, heat inactivation and dilution occurred after storage, but the storage validation experiments demonstrated there was no loss of detectable endotoxin during storage.

The LAL protocol we used involved diluting samples to 1:5 v/v before heat treatment at 70°C for 15 min, then further diluting the samples to the final required dilution (1:50 v/v for the clinical samples). This approach was based partly on the manufacturer's instructions and as an attempt to maintain conformity of sample handling during the validation/ optimisation experiments. Menzies-Gow et al. (2004) used 1:25 v/v dilutions followed by heating to 75°C for 30 min; Steverink et al. (1995) used 1:10 dilutions and heated to 75°C for 15 min and King and Gerring (1988) used 1:3 dilutions and heated to 80°C for 20 min. It is not clear what effect, if any, such differences may have had on results. Each group used different sample collection protocols and used different assay manufacturers. The assay conditions utilised in this study resulted from manufacturer's guidance and validation/ optimisation experiments within our laboratory, and this was the case with at least one other group (Menzies-Gow, personal communication). Ideally, for future studies, a standardised assay system would be preferable to allow better comparison of data between studies.

Samples from horses suffering from colic had significantly higher $EUml^{-1}$ values compared to horses that were suffering from other conditions. Colic cases were also more likely to provide samples with detectable levels of LPS within them in comparison to non-colic cases. These results may be expected as the equine gastrointestinal tract contains large numbers of Gram negative bacteria and any disease process which results in increased absorption of Gram negative bacteria/ bacterial cell walls and will likely result in increased LPS in the blood.

Mean $EUml^{-1}$ values for samples from colic cases that were deemed to be clinically endotoxaemic were significantly higher than those from non-endotoxaemic colic cases. Horses deemed to be clinically endotoxaemic were also more likely to provide a positive sample. The same was true for samples from colic cases that died whilst in the hospital in comparison to those that did not. The criteria for a horse to be deemed *clinically endotoxaemic for this part of* the study were the same as those for a horse to be classified as being severely endotoxaemic from the tube comparison study (i.e. HR > 70 and/ or PCV ≥ 45%, and clinical disease/ surgical findings likely to result in LPS absorption from the gut). These criteria are based partly on long term clinical experience of diagnosing endotoxaemia in horses. However, assays of LPS in the blood of these horses are not normally carried out so it is reassuring that there is evidence to support these clinical suppositions. It should be pointed out that not all horses that fulfilled the clinical criteria for endotoxaemia had detectable levels of LPS (see below). The fact that colic cases that died were more likely to provide samples that had higher $EUml^{-1}$ values compared to those that survived being in hospital may reflect that levels of LPS per se, may have some role to play in the outcome of colic cases. Steverink et al. (1995) did not find any difference between levels of LPS between survivors and non-survivors of surgical colic cases in plasma samples taken when the cases were first presented. It was only when the authors compared results from intra-operative samples that non-survivors were found to have significantly higher LPS levels in plasma. The differences in results between the two studies may be explained by differences in sample collection. In our study, we obtained samples from all types of colic cases, and not just surgical cases. Also any case could contribute any number up to a maximum of four samples taken daily for four days; meaning that in surgical cases where more than two samples were obtained; the majority of samples would be post-operative samples. Further work is needed to elucidate what, if any, relation to the presence of different levels of LPS in the blood have on the outcome of clinical colic cases.

King and Gerring (1988) found a direct correlation between detectable LPS levels in the plasma of colic horses with the severity of the causal lesion; no attempt was made in our analyses to grade 'severity' due to the limitations of data recording and the lack of agreed indicators for severity of lesion for all causes of colic. There was no difference in mean $EUml^{-1}$ values between samples from colic cases when compared according to anatomical location of causal lesion (large intestinal, small intestinal or other locations), whether or not the lesion was a strangulating lesion or non-strangulating lesion or whether the colic required medical or surgical intervention, nor were any horses that fell into these categories more likely to provide a positive LPS sample. These findings regarding anatomical location and causal lesion are

supported by Steverink et al. (1995), who also did not find differences in LPS levels between these groups. These findings may be explained by a number of factors. Firstly, within each anatomical division there are lesions which may be expected to cause endotoxaemia and those which would not, it is probable that the results from each type of lesion confound the other. Secondly, when comparing strangulating and non-strangulating lesions, although one might assume that strangulating lesions are more likely to result in endotoxaemia, there are non-strangulating lesions which can also result in endotoxin absorption from the equine gastrointestinal tract (e.g. colitis, and anterior enteritis). It is likely that the chosen sub-categories of anatomical location and causal lesion require further refinement to be able to distinguish which ones are more likely to result in endotoxaemia. The broad intervention category (medical or surgical) is also unlikely to predict likelihood of endotoxaemia as many causes of surgical colic do not result in endotoxaemia and some notable causes of colic which do not require surgical intervention can cause severe endotoxaemia.

When variables which had shown a significant relationship to detectable LPS in samples or to whether horses survived or not were evaluated in multivariable logistic regression models, most variables were eliminated. Only heart rate on admission and whether the horse fitted the study criteria for clinical endotoxaemia remained in the model for whether or not a horse was likely to provide a positive LPS sample. The only variable that significantly influenced the probability of death whilst hospitalised was clinical endotoxaemia.

Multivariable analysis is a useful tool as it allows evaluation of multiple variables and accounts for interactions and confounding that these variables may cause. In terms of the models produced in this analysis, it is not surprising that so many factors which were significant at the univariable level were eliminated from the model; for example, heart rate and PCV are related to cardiovascular status and to each other and so are likely to confound each other in multivariable analysis and only the variable exerting the strongest influence on the fit of the model will remain. When one considers the study definition of clinical endotoxaemia (HR ≥70, PCV ≥ 45%, plus clinical diagnosis likely to result in endotoxaemia), it is clear that this 'variable' is strongly influenced by cardiovascular parameters and may itself be responsible for the elimination of other related variables from the model. The reasons other factors were likely to have dropped out of the multivariable models (e.g. medical versus surgical cases) have been discussed above.

During the study PMB was administered to 32 colic cases that also had samples taken for LPS measurement. PMB is an aminoglycoside antibiotic agent which forms a stable 1:1 complex

with LPS, preventing the lipid A portion of LPS binding to CD14/MD-2 and initiating an inflammatory response (see introduction for mechanism of LPS induced inflammatory response). PMB has been shown to be effective at binding LPS in an ex vivo horse model (Parviainen et al. 2001) and prevented clinical signs of endotoxaemia when administered prior to an LPS infusion in an experimental in vivo equine model (Barton et al. 2004). Despite having potentially serious deleterious side effects (Mackay et al. 1999), the use of PMB to 'treat' endotoxaemia in horses has increased despite a lack of information regarding its efficacy in clinical cases. As far as we are aware, ours is the first study where LPS has been measured before or /and during PMB administration to clinical cases.

There was no significant difference between $EUml^{-1}$ values for samples from all colic cases that had received PMB compared to all those that had not and between samples taken before PMB was administered and those taken after. However, not all colic cases that received PMB fulfilled the study criteria for being clinically endotoxaemic (see above). When the data from clinically endotoxaemic cases was analysed to compare samples from cases that had received PMB but the sample was known to have been taken before PMB administration, to those where the sample was known to have been taken after PMB administration, the samples obtained prior to PMB administration had significantly higher $EUml^{-1}$ values suggesting that PMB was reducing the measurable levels of LPS. However, this result should be interpreted with some caution as the group sizes were very small (4 horses/ 4 samples for those that had had the sample taken before PMB, and 8 horses/ 15 samples in the group where the samples had been taken after PMB administration).

The administration of PMB appeared to have an effect on the LAL assay results, in particular samples that had come from cases receiving PMB were relatively overrepresented in the 'false negative' results. This provides further evidence in clinical cases that PMB affects the assay's ability to detect LPS. Perhaps the most convincing evidence for the effect of PMB administration on the assay was when PMB administration and clinical endotoxaemia were considered together in a logistic regression model for the likelihood of the assay detecting LPS in the plasma samples from colic cases, it was shown that horses not receiving PMB were ~3.5 times more likely to provide a positive LPS sample even when the influence of clinical endotoxaemia is accounted for.

When samples from colic cases that had received PMB were compared to those that had not, with death as the outcome, administration of PMB appeared to be associated with an

increased likelihood of death. However, when this analysis was repeated with only samples from those colic cases that were classified as clinically endotoxaemic then there was no difference between the groups. Also, when whether a horse had provided a positive LPS sample and whether or not it had received PMB were considered together into a multivariable logistic regression model, the model showed that horses receiving PMB were more likely to die (p =0.011, PR =0.36, 95%CI =0.36, 0.79).These results suggest two things; firstly, that PMB is more likely to be given to colic cases that would be classified as clinically endotoxaemic and these cases are more likely to die (c.f. non clinically endotoxaemic cases p =<0.001), and secondly, that when analysed in this way, PMB does not appear to significantly reduce the likelihood of death in clinically endotoxaemic colic cases. This finding is supported at the multivariable analysis level; in the multiple logistic regression model built to identify risk factors for whether a horse died in the hospital or not, the only variable that remained in the model after multivariable analysis was whether or not the horse was deemed to be clinically endotoxaemic. There was also no effect on death as an outcome when PMB administration was analysed in generalised additive models in clinically endotoxaemic horses. However small group sizes (n=18 for endotoxaemic cases receiving PMB versus n= 26 for endotoxaemic cases not receiving PMB) mean the power of these analyses to detect differences is weak. To detect a 25% improvement in survival due to PMB administration, power analysis suggests a group size of 198 horses in each group would be required to have 80% power to detect the difference with 95% confidence.

The results relating to PMB administration provide evidence at this stage that PMB appears to affect the LAL assay's ability to detect LPS in samples from clinical cases, but there is no evidence that PMB administration has a beneficial effect on clinical outcome. Whether PMB is binding LPS or directly affecting the assay cannot be deduced from this data.
The study criteria for clinical endotoxaemia have been discussed already, but in the context of the number of false 'results' it is possible that some cases will have been misclassified according to those criteria i.e. classed as clinically endotoxaemic whereas in fact they were not and vice versa. However, the criteria used are similar to those used by clinicians within our hospital to make judgements on whether a horse presenting with colic is endotoxaemic and are based partly on models developed in our hospital to estimate (long term) postoperative survival in surgical colic cases (Proudman et al. 2002a).

The total number of 'false negative' sample results was 27. The potential influence of PMB administration on 15 of these sample results has already been discussed. The other 12 samples

came from 11 horses where the clinical criteria of clinical endotoxaemia were met when the samples were obtained. In addition, each of the cases had causal lesions of colic that would be expected to result in endotoxaemia (e.g. > 360° large colon torsions, strangulated small intestine, colitis, peritonitis etc.). It is possible that some of these cases were not actually endotoxaemic when the samples were taken and the elevated heart rates/PCVs were due to other causes, although this is unlikely. Experimental horses differ in the magnitude of the cardiovascular dysfunction observed in response to a given dose (30 $ngkg^{-1}$) of purified LPS (Moore et al. 2007). Other work in experimental horses has shown that after the same (30 $ngkg^{-1}$) dose of purified LPS administered over 30 min, the peak plasma concentration of detectable LPS was ~12 $pgml^{-1}$ and these plasma levels of LPS produced increases in heart rate in the study horses (Menzies-Gow et al. 2004). The lowest limit of detection of our assay was 0.25 $EUml^{-1}$ (or 25 $pgml^{-1}$), a consequence of using 1:50 dilutions (see above). There is little information on the pharmacokinetics of LPS in the blood of horses in experimental animals and no information in clinical cases. In rabbits it has been shown that after IV injection of LPS that there is a rapid (<30 min) clearance phase with cellular uptake of LPS by the reticulo-endothelial system followed by a slower reduction (half-life = 12 hours) of detectable LPS through binding of LPS to plasma lipoproteins (Mathison & Ulevitch 1979). It may be that the 'false negative' samples came from horses that were more sensitive to the effects of relatively low plasma levels of LPS that fell below the limits of detection of our assay, or that the initial 'dose' of LPS that had produced the inflammatory response had passed and the LPS levels had fallen below the limit of detection for our assay.

There were 38 'false positive' sample results from 32 horses. Only 8 of these samples came from non-colic cases. None of these samples were excluded as the levels of detected LPS did not suggest obvious contamination, although contamination of samples or the assay is a possible explanation for some of the false positive results. One of the 'false positive' sample results did prove to be the highest level of LPS detected (hyperlipaemia/ hepatic encephalopathy case = 1.14 $EUml^{-1}$ (114 $pgml^{-1}$ or ngl^{-1}) and this sample was re-tested with the same result. There were several samples where the colic case had cardiovascular parameters which only just fell outside the arbitrary levels set for the study criteria of clinical endotoxaemia. It is likely that horses may have had detectable levels of LPS by our assay and have clinical endotoxaemia yet not have cardiovascular parameters that would fit with the study definition of clinical endotoxaemia. Whether this is due to some form of resistance of an individual horse to the cardiovascular dysfunction of endotoxaemia, so-called endotoxin tolerance (Fan and Cook 2004), or whether the horses had had drugs administered that

ameliorate the cardiovascular dysfunction is not clear. Certainly, there was no difference between groups for recorded drug administration for colic cases (prior to admission) that were in the positive, false positive and false negative groups (other than PMB administration), although the quality of data recording in terms of drugs administered prior to hospital referral was poor (data not shown). The 7 non-colic false positive samples came from 6 horses. It was interesting to note that of those 6 horses, 4 had undergone surgery and the 'false positive' samples occurred in the post-operative period. The other two horses both had serious systemic illness (hyperlipaemia/ hepatic encephalopathy and severe ventral oedema).There are reports of transient endotoxaemia in horses after strenuous exercise and stress (Donovan et al. 2007). It may be that the results for these samples were not false at all and many of these cases had endotoxaemia.

Another explanation for the 'false positive' and 'false negative' results is that the assay is a biological assay and as such is susceptible to producing variable results. The impact of such variability was minimised by safeguards in protocol such as analysing samples in duplicate and rejecting those with >20% of co-variance of results between samples (see above).

Five results from the clinical samples were excluded from analysis as they were thought to have been contaminated because their $EUml^{-1}$ values were orders of magnitude higher than any others e.g. (4 – 19 $EUml^{-1}$ or 400-1900 ngl^{-1}). Contamination may have occurred at blood collection, sample processing or during the assay. If occasional samples returned such high values they were repeated on another plate and in most cases the high results were not repeated, suggesting that contamination of some wells was occurring during running the assay, most likely as a result of splashes from when the wells nearby were being 'spiked'. However, in the case of the five excluded samples the high values were repeated more than once suggesting contamination had occurred at some other stage.

In comparison to other studies that used the LAL assay to detect LPS levels in clinical colic cases, we detected LPS in 23.2% of samples from colic cases. Two studies found a comparable level in their study population (King and Gerring 1988, Fessler et al. 1989), whilst Steverink et al. (1995) detected LPS in 41% of samples taken from horses on admission for colic but this had dropped to 23.5% when samples were taken during anaesthesia. Steverink et al. (1995) had a detection limit for their assay of 5 ngl^{-1} which was five times more sensitive than our assay which may explain the higher prevalence of positive samples from their study; indeed, in a different report from the same study the authors suggest that their decision limit for clinical endotoxaemia of 5 ngl^{-1} was too low and should be higher (Steverink et al. 1994a). King and

Gerring (1998) had a detection limit of 100 ngl^{-1}. The samples that were positive for LPS in the population studies by Steverink et al. (1995) had mean LPS levels of 35 ngl^{-1}; range 7-197 ngl^{-1}. In our assay, the results were expressed in $EUml^{-1}$. The assay manufacturers state that in their units 1 EU =100pg =0.1 ng. The positive samples from our study population of colic cases had a mean LPS level of 0.464 $EUml^{-1}$ (46.4 ngl^{-1}); range 0.255 $EUml^{-1}$ (25.5 ngl^{-1}) - 0.930 $EUml^{-1}$ (93.0 ngl^{-1}) SD±0.17 $EUml^{-1}$ (17 ngl^{-1}). It appears that the kinetic chromogenic assay used in our study on our study population produced similar results to those from other studies.

The sensitivity and specificity calculations presented in the results are limited in that they rely on the study criteria of clinical endotoxaemia to determine true and false positive and negative results. A more relevant interpretation is that the assay is apparently able to detect LPS in clinical cases under specific sample collection/ storage/ assay conditions, and that measured levels of LPS are related to clinical parameters and mortality in colic cases. The reasons why some cases classified as clinically endotoxaemic did not have detectable levels of LPS warrant further investigation. The effect of PMB administration on the assay warrants further investigation as do its effects in clinical cases.

It is too early to tell if the LAL assay will prove to be a useful diagnostic/prognostic tool for clinical colic cases. The assay did demonstrate that colic cases were more likely to have detectable LPS in their plasma than non-colics, and those that were more likely to die had higher LPS levels than those that were not. However, the multivariable models suggests that whether a horse provided a positive LPS sample is not a better indicator of mortality in colic cases compared to whether a horse fitted the study criteria of clinical endotoxaemia. It is also worth noting the potential influence that PMB administration has had on how the LAL assay has been assessed. As already discussed, if no cases had received PMB it is likely that the number of false negative cases would be reduced. It is unclear whether the assay results would have remained in the multivariable model to predict mortality in colic cases. This study provided evidence that PMB administration can affect the LAL assay's ability to detect LPS in clinical samples, but there was no evidence of a beneficial effect in the outcome of cases receiving PMB.

The LAL assay has demonstrated real potential as a research tool to help understand the mechanisms and aetiopathogenesis of the equine response to endotoxaemia.

Concluding comments

This project aimed to characterise a specific equine complement component, *complement 1 esterase-inhibitor* (C1-inh) because it is considered a key inhibitor of the complement, coagulation and contact activation pathways which are pivotal in the aetiopathogenesis of the inflammatory response and disease syndrome observed in equine colic derived endotoxaemia. The project also aimed to measure levels of endotoxin in the plasma of clinical cases presented to the hospital and correlates these levels to morbidity and mortality outcomes.

Molecular biological techniques were employed to produce data which would provide structural and functional information on equine C1-inh. This work produced novel and important information. It provided evidence that C1-inh exists in the horse, and is produced in several cell types. The full nucleotide sequence for the protein was determined and the amino acid sequence derived. This information was used to generate a 3D model of the protein which was compared to a model of the human protein. From the 3D model of equine C1-inh protein, likely sequences for use as immunogens were identified. Custom conjugated peptides were made that were then inoculated into rabbits to raise polyclonal antisera for use to detect native equine C1-inh in equine samples. These antisera were tested by ELISA and Western blotting but ultimately did not demonstrate enough cross-reactivity with the native protein. Other methods of producing recombinant immunogens e.g. molecular expression systems are available and will be explored in future work (Wagner et al. 2005). Quantitative real-time PCR was used to assess if there were differences in gene expression for equine C1-inh and pro-inflammatory complement components C3 and C5 in different normal and pathological tissues but the experiments did not show significant differences in complement gene expression between groups. Possible reasons for this were discussed in the relevant chapter. The success of molecular biological methods highlights advantages over chromatographic methods for gaining information about proteins that have yet to be purified/ isolated. With hindsight, the relative success of the molecular biological components of this project suggests that the project may have advanced further overall if these molecular biological techniques had been employed at the start of the project.

Another successful component of the project was the development of a kinetic, chromogenic version of the LAL assay to detect LPS in plasma samples from clinical cases and correlate those levels to other measures of endotoxaemia (clinical parameters) and survival. Three hundred and seventy-five plasma samples from 247 horses were tested. This is the first time LPS has been measured in plasma from so many clinical cases. LPS was detected in 29% of horses

presenting with colic, which is a similar proportion to that reported in other studies. Horses that had detectable LPS were significantly more likely to die whilst in the hospital. In multivariable models to assess likelihood of mortality for colic cases, the presence of LPS or the level of LPS detected were not significantly associated with outcome. However, those horses which met study criteria for clinical endotoxaemia, were significant in multivariable models to predict likelihood of death. The study definition of clinical endotoxaemia was HR ≥70, PCV ≥ 45%, plus clinical diagnosis likely to result in endotoxaemia; it is clear that this 'variable' is strongly influenced by cardiovascular parameters and may itself be responsible for the elimination of other related variables from the model. The results demonstrate that there are many confounding and interlinked parameters that may influence survival from endotoxaemia and provide evidence to support clinical 'anecdotal' belief that elevated heart rates and PCV reflect the degree of endotoxaemia in horses suffering from colic. Further work is needed understand the relationship between LPS levels, cardiovascular parameters and mortality in horses. Work also needs to be undertaken to understand what physiological mechanisms are involved in removing/ neutralising the toxic effect of LPS from the blood. This part of the present study also sought to investigate the effect of a colic treatment (polymixin B) on LPS levels in horses. Whilst there was evidence that polymixin B treatment resulted in a decrease in detectable LPS in samples, there was no evidence that polymixin B treatment decreased mortality in colic cases.

The preliminary work for this project demonstrated that, at that time, a goat polyclonal antibody against human C1-inh, apparently easily and specifically, identified two bands in Western blots similar to those produced if purified human C1-inh was probed by the same antibody, in the plasma of several different horses, that had been run on non-reducing SDS-PAGE gels. These results could not be repeated in the main project, despite using the same antibody from the same commercial source. The reasons for this were discussed in the relevant chapter. Further antibodies against human proteins (C1-inh and kallikrein) were also tested for cross-reactivity to equivalent equine proteins; but little evidence for cross-reactivity was found. The antisera raised against the custom peptides generated from the sequences chosen after building a 3D model of equine C1-inh was also tested but no useful cross-reactivity could be demonstrated. The interpretation of the Western blots was not straightforward, due in part to the fact that plasma was being run on the blots in most experiments. Plasma is not the ideal medium to run in Western blots as it contains so many potentially antigenic components. As a result, the secondary antibodies (especially against rabbit IgG) tended to cross react with

components in plasma, especially in reduced blots. Also, 'normal' rabbit serum was shown to be highly reactive to human and equine plasma on reduced blots.

Chromatographic techniques were employed to attempt to purify equine C1-inh (and kallikrein). Unfortunately, our attempts at purifying equine proteins from plasma or tissues were entirely unsuccessful as we were not able to identify the target proteins. This was in no small part due to the fact that we did not possess robust and validated assays for some of the proteins (C1-inh and prokallikrein). In addition, there has been comparatively very little research on the proteins that we were attempting to purify and so assumptions had to be made that their structure/ biology would be similar enough to the equine proteins of interest.

This project also highlights problems associated with the use of unvalidated reagents. For example, many of the reagents which are commercially available and used routinely in human diagnostic laboratories may not have been rigorously tested to check on their specificity. In veterinary science, there are relatively few specific antibodies available because they are not commercially viable. Hence, in veterinary laboratories it is necessary to check on potential cross-species reactions which may be useful. It is the experience in our laboratories that whilst there are a few useful cross reactions, most such searches end in disappointment. Simple referencing to reaction with the human proteins frequently indicates that the reagents used routinely in human medicine simply do not have the stated reactivity, but are almost invariably used without any agency or body attempting adequate quality control, beyond reproducibility. This is an issue which needs addressing by both reagent producers and end users.

It is hoped that work begun in this thesis will be continued and future work associated with the LAL assay has already been already mentioned. It is intended that the qt-PCR experiments will be refined in order to investigate differences in gene expression of C1-inh, C3 and C5 in different tissues in the horse, in healthy tissues and tissues exposed to LPS. The differences between human C1-inh and equine C1-inh are also worthy of further investigation from both an antigenic and functional perspective and attempts will be made to produce recombinant equine C1-inh. The production of an assay to detect native equine C1-inh from the plasma from clinical cases remains the objective of these studies as our hypothesis that levels of C1-inh may help us to measure the inflammatory response of the horse to circulating LPS has not been rejected. This route is preferable to attempting to validate a commercially available functional assay for human C1-inh (Berichrom GmBH) for use in horses as this assay is prohibitively expensive (in terms of hardware and consumables) and such work would be unlikely to provide as much information on comparative differences between human and equine C1-inh.

I intend to utilise the immunological toolbox facility (www.immunologicaltoolbox.com) to attempt to produce recombinant equine C1-inh or produce polyclonal antibodies against equine C1-inh.

This project has contributed to this area of study in several ways;

- It has established that several different antibodies against human C1-inh show no cross-reactivity to equine C1-inh
- It has proved that equine C1-inh exists
- It has elucidated the full nucleotide and amino acid sequence of equine C1-inh. It has provided insights as to important structural, and therefore potentially functional, differences between the human and equine protein.
- This study has measured LPS in the plasma of many more clinical cases than any other study and has been able to correlate those measures to clinical parameters and outcome. Furthermore, this study has provided some useful preliminary evidence on the effects of polymixin B treatment in endotoxaemic clinical cases on LPS detection and clinical outcome which will be used to inform further studies into this treatment.

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