ACTA UNIVERSITATIS AGRICULTURAE SUECIAE

VETERINARIA 105

Opsonisation and Neutrophil Phagocytosis in Foals and Adult Horses

Gittan Gröndahl

SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

Opsonisation and Neutrophil Phagocytosis in Foals and Adult Horses

Gittan Gröndahl

Akademisk avhandling som för vinnande av veterinärmedicinsk doktorsexamen kommer att offentligen försvaras i föreläsningssal Ettan, Klinikcentrum, SLU, Uppsala, onsdagen den 23 maj 2001, kl. 9.15.

Abstract

Infectious diseases are the main cause of disease and deaths in foals. The neutrophil phagocyte is a part of the early, non-specific immune response, which is essential in defending the host against microbial invasion. Coating of microbes with opsonic factors such as antibodies and complement from serum is required for optimal phagocytosis.

Flow cytometric methods were developed and used for studies of equine neutrophil phagocytosis and serum opsonisation of *Saccharomyces cerevisiae*, *Escherichia coli* and *Actinobacillus equuli*, and of the expression of complement receptor subunit CD18 on neutrophils. Foals from birth to 2 months of age and adult horses were studied.

Incubation for 15 min was found sufficient for both opsonisation and phagocytosis. Opsonisation of yeast and *A. equuli*, but not of *E. coli*, was observed already at low serum concentrations (<3%).

Complement activation was needed for optimal phagocytosis of all the microbes studied. The classical pathway was required for yeast phagocytosis at low serum concentrations (1.5%) and is the main pathway for C3 deposition with <50% serum. At higher serum concentrations, the alternative pathway is able alone to provide sufficient amounts of C3. The main form of C3 on yeast cells is iC3b and the rest is C3b. The molecular weights of equine C3 fragments were similar to those of their human equivalents.

In newborn foals, colostrum ingestion was required to achieve a serum opsonic capacity for all the microbes studied. Serum from foals up to 3-4 weeks of age showed a lower capacity to opsonise yeast, but a higher capacity to opsonise *E. coli* compared to serum from older foals and horses. No age-related differences were observed in serum opsonisation of *A. equuli*. Very low serum concentrations of IgG or IgGb were associated with decreased phagocytosis of yeast and *E. coli*, but not of *A. equuli*. However, there were large individual variations in the opsonisation of yeast, irrespective of the concentrations of IgG, by sera from newborn foals and plasma from individual adult donors. This may have been due to different levels of complement activation. Plasma transfusion to healthy 7-day old foals resulted in an increased serum capacity for yeast opsonisation at 14 days.

The neutrophil phagocytosis capacity *per se* was similar in foals and adult horses, and the expression of complement receptor CD18 was also similar, or higher in the foals.

These results emphasise the importance of both antibodies and complement factors in serum for an effective defence against microorganisms in young foals. Intravenous administration of plasma to foals seems to be of some benefit, but additional studies are warranted to characterise the factors involved. Important differences were observed between various microbes in the particular requirements for effective phagocytosis.

The involvement of complement in common equine pathological conditions deserves further attention. A better understanding of the equine complement system may lead to improved prophylactic and therapeutic regimens in foals and adult horses.

Key words: neutrophil, phagocytosis, opsonisation, foals, complement, IgG, transfusion, *Escherichia coli*, *Actinobacillus equuli*, colostrum, flow cytometry

Fakultetsopponent:
Professor Max J. Paape
University of Maryland, USA and University of Ghent, Belgium
Research Physiologist, Immunology and Disease Resistance Laboratory,
Animal and Natural Resources Institute, United States Department of Agriculture,
Beltsville, USA

Betygsnämnd:
Docent Per Jonsson, SLU
Docent Olle Korsgren, Uppsala Universitet
Docent Ulf Magnusson, SLU
Docent Karin Persson Waller, SLU
Professor John Pringle, SLU

Distribution:
Swedish University of Agricultural Sciences
Department of Large Animal Clinical Sciences
P.O. Box 7018, S-750 07 Uppsala, Sweden

Uppsala 2001
ISSN 1401-6257
ISBN 91-576-5931-1

Opsonisation and Neutrophil Phagocytosis in Foals and Adult Horses

Gittan Gröndahl
Department of Large Animal Clinical Sciences
Uppsala

Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2001

Acta Universitatis Agriculturae Sueciae
Veterinaria 105

ISSN 1401-6257
ISBN 91-576-5931-1

Tryck: SLU Service/Repro, Uppsala 2001

Det är den som går vilse som finner de nya vägarna
Niels Kjaer

To all the foals

Abstract

Gröndahl, G. 2001. *Opsonisation and neutrophil phagocytosis in foals and adult horses.* Doctoral dissertation.
ISBN 91-576-5931-1

Infectious diseases are the main cause of disease and deaths in foals. The neutrophil phagocyte is a part of the early, non-specific immune response, which is essential in defending the host against microbial invasion. Coating of microbes with opsonic factors such as antibodies and complement from serum is required for optimal phagocytosis.

Flow cytometric methods were developed and used for studies of equine neutrophil phagocytosis and serum opsonisation of *Saccharomyces cerevisiae*, *Escherichia coli* and *Actinobacillus equuli*, and of the expression of complement receptor subunit CD18 on neutrophils. Foals from birth to 2 months of age and adult horses were studied.

Incubation for 15 min was found sufficient for both opsonisation and phagocytosis. Opsonisation of yeast and *A. equuli*, but not of *E. coli*, was observed already at low serum concentrations (<3%).

Complement activation was needed for optimal phagocytosis of all the microbes studied. The classical pathway was required for yeast phagocytosis at low serum concentrations (1.5%) and is the main pathway for C3 deposition with <50% serum. At higher serum concentrations, the alternative pathway is able alone to provide sufficient amounts of C3. The main form of C3 on yeast cells is iC3b and the rest is C3b. The molecular weights of equine C3 fragments were similar to those of their human equivalents.

In newborn foals, colostrum ingestion was required to achieve a serum opsonic capacity for all the microbes studied. Serum from foals up to 3-4 weeks of age showed a lower capacity to opsonise yeast, but a higher capacity to opsonise *E. coli* compared to serum from older foals and horses. No age-related differences were observed in serum opsonisation of *A. equuli*. Very low serum concentrations of IgG or IgGb were associated with decreased phagocytosis of yeast and *E. coli*, but not of *A. equuli*. However, there were large individual variations in the opsonisation of yeast, irrespective of the concentrations of IgG, by sera from newborn foals and plasma from individual adult donors. This may have been due to different levels of complement activation. Plasma transfusion to healthy 7-day old foals resulted in an increased serum capacity for yeast opsonisation at 14 days.

The neutrophil phagocytosis capacity *per se* was similar in foals and adult horses, and the expression of complement receptor CD18 was also similar, or higher in the foals.

These results emphasise the importance of both antibodies and complement factors in serum for an effective defence against microorganisms in young foals. Intravenous administration of plasma to foals seems to be of some benefit, but additional studies are warranted to characterise the factors involved. Important differences were observed between various microbes in the particular requirements for effective phagocytosis.

The involvement of complement in common equine pathological conditions deserves further attention. A better understanding of the equine complement system may lead to improved prophylactic and therapeutic regimens in foals and adult horses.

Key words: neutrophil, phagocytosis, opsonisation, foals, complement, IgG, transfusion, *Escherichia coli*, *Actinobacillus equuli*, colostrum, flow cytometry

Author's address: Department of Large Animal Clinical Sciences, Swedish University of Agricultural Sciences, P.O. Box 7018, S-750 07 Uppsala, Sweden

Svensk resumé

Studier av opsonisering och neutrofilfagocytos hos föl och vuxna hästar

Gittan Gröndahl, leg vet
Doktorsavhandling, SLU 2001

Infektioner är den vanligaste orsaken till sjuklighet och dödsfall hos unga föl. De vita blodkroppar som kallas neutrofila granulocyter (neutrofiler) bekämpar och oskadliggör bakterier och andra främmande partiklar eller skadade celler i kroppen, genom att ta in och avdöda dessa i en process som kallas fagocytos. Denna livsnödvändiga del av immunförsvaret, som brukar benämnas kroppens första försvarslinje mot infektioner, studerades hos föl och vuxna hästar i detta arbete. Fagocytos kan förekomma både i blodet och andra kroppsvätskor, samt i organ i kroppen där det skett en infektion eller inflammation.

För att neutrofilerna ska känna igen de främmande mikroberna krävs att dessa täcks med olika proteiner från serum, såsom antikroppar (t ex IgG) och komplementfaktorer (framför allt C3). Dessa kallas opsoniner och processen heter opsonisering. Neutrofilerna har särskilda igenkänningsreceptorer på cellytan för opsoniner.

I det första delarbetet togs metoder fram för att studera fagocytosförmågan hos hästneutrofiler (I). Neutrofilerna fick fagocytera fluorescerande jästpartiklar. Därefter kunde fagocytosförmågan bestämmas genom att mäta andelen aktiva neutrofiler och fluorescensen i varje cell med hjälp av flödescytometri. Trypanblått användes för att släcka fluorescensen hos icke upptagna jästpartiklar. Det gjorde det möjligt att också särskilja jästpartiklar som enbart fäst till ytan av neutrofilerna från de som var helt upptagna inne i cellen.

Adhesionsfasen var kort och jästcellerna togs snabbt tas upp i cellen. De flesta neutrofiler hade fagocyterat redan efter 15 min inkubering. Både serum och renade antikroppar (IgG) mot jäst fungerade opsoniserande, men serum var mer effektivt än IgG.

Därefter studerades den opsoniserande förmågan hos plasma från olika blodgivarhästar (II). Plasmainfusioner används för behandling av föl och vuxna hästar med svåra infektioner, förlust av plasmaproteiner eller nedsatt immunförsvar, till exempel om fölen inte fått i sig tillräckligt med antikroppar från råmjölken. Plasma används även som förebyggande behandling mot infektioner hos föl på många stuterier. Det har dock inte tidigare funnits så mycket dokumentation om vilka egenskaper som är viktiga för en sådan plasma. Olika blodgivarhästars plasma stimulerade fagocytos av jäst i olika hög grad i våra studier. Detta iakttogs både när neutrofiler från unga föl och vuxna hästar användes. Genom inaktivering av komplementfaktorerna visades att komplementsystemet var viktigare för den opsoniserande förmågan än antikroppar i dessa försök.

I nästa studie undersöktes om det sker en åldersutveckling av fagocytosförmågan hos nyfödda föl (III). Bakgrunden till denna frågeställning var det faktum att föl har en mycket högre infektionskänslighet än vuxna hästar. Nyfödda föl drabbas särskilt ofta och allvarligt av framför allt bakterieinfektioner.

Resultaten visade att förmågan till fagocytos av jäst var låg hos nyfödda föl och sedan utvecklas kraftigt under de första levnadsveckorna, för att uppnå full kapacitet vid 3-4 veckors ålder. Den nedsatta förmågan hos de nyfödda fölen avspeglades både i ett sämre upptag per neutrofil och en lägre andel neutrofiler som var aktiva. Bristen visade sig ligga i serums opsoniserande kapacitet, medan själva neutrofilerna i princip är mogna redan

från födseln. Koncentrationen av IgG i serum gick inte att korrelera med den iakttagna ökningen av opsoniseringsförmågan. Därför kan komplementsystemet vara det avgörande för opsoniseringen.

En grupp av de undersökta friska fölen erhöll plasmabehandling vid en veckas ålder vilket ledde till en ökad opsoniseringsförmåga av jäst, medan de obehandlade fölen inte visade någon uppgång under samma tid.

Neutrofilernas uttryck av s.k. CD18-receptorer undersöktes också. Dessa medverkar i vidhäftning och fagocytos av mikrober täckta med komplementfaktor C3, och en sjuklig brist på CD18 leder till dödliga infektioner hos människor och djur. Fölens neutrofiler visade sig uttrycka fler CD18-receptorer än de vuxna hästarnas celler gör, varför receptoruttrycket alltså inte utgjorde en riskfaktor för ökad infektionskänslighet hos föl.

För att vidare karakterisera den viktiga opsoniseringsprocessen, utformades en metod för att mäta täckningen av antikroppar (IgG) och komplementfaktor C3 från hästserum på ytan av jäst (IV). Specifika antikroppar riktade mot IgG och C3 hos hästar användes i analysen. Dessa märktes med fluorescerande markörer. Mängden IgG och C3 på jästytan kunde på så sätt mätas med en flödescytometri. Effekten av att opsonisera med olika serumkoncentrationer och under olika lång tid studerades. De två vanligaste aktiveringsvägarna (klassisk respektive alternativ) för C3 undersöktes också separat.

Resultaten visade att både C3 och IgG binds från hästserum till jäst, och att C3 är den viktigaste opsoninen av dessa. Om komplementsystemet inaktiverades, band istället mer IgG till jästytan. Opsoniseringen inleddes snabbt (inom 2-4 min) och började redan vid låga serumkoncentrationer (0.75%) vilket är viktigt för ett effektivt immunförsvar.

C3 binds huvudsakligen via den klassiska vägen vid låga serumkoncentrationer och den klassiska vägen var snabbare än den alternativa. Den alternativa vägen kan dock också fungera väl vid höga serumkoncentrationer eller längre inkubationstider.

Western blot-analys visade att C3 på jästen framför allt är i form av iC3b. De olika fragmenten av C3 hos häst var mycket lika C3-fragmenten hos människa vad gäller molekylstorleken.

Opsonisering av sjukdomsframkallande bakterier (V) undersöktes sedan i serum hos unga föl. De två bakterier som är de vanligaste vid blodförgiftning (s.k. fölsjuka) hos nyfödda föl under en veckas ålder, *Escherichia coli* och *Actinobacillus equuli* valdes för dessa studier.

Effektiv fagocytos av *E. coli* visade sig kräva betydligt högre serumkoncentrationer (40%) än fagocytos av jäst och *A. equuli* (<5%). Inaktivering av komplementsystemet i serum ledde till en kraftigt minskad fagocytos, vilket tyder på att C3 har en viktig roll även för fagocytos av bakterier.

Förmågan att opsonisera *E. coli* och *A. equuli* var mycket låg i serum hos föl innan fölen diat råmjölk (colostrum) som ger dem deras första antikroppar. Under de följande veckorna i livet var opsoniseringsförmågan när det gäller *A. equuli* i stort sett lika i fölserum som i serum från moderstona. Opsoniseringsförmågan för *E. coli* var högre hos de studerade fölen före 3 veckors ålder än senare.

Sammanfattningsvis visar studierna att både antikroppar och komplementfaktor C3 i serum är nödvändiga för en effektiv fagocytos av svampar och patogena bakterier, och att intag av råmjölk är mycket viktigt för immunförsvaret hos nyfödda föl. Ytterligare studier av olika bakterietyper bör göras, liksom studier av värdet av plasmatransfusioner till friska och sjuka föl och de ingående plasmakomponenterna.

Ökad kunskap om komplementsystemet hos hästar krävs och kan förhoppningsvis leda till förbättrade behandlingsåtgärder vid många vanliga hästsjukdomar, exempelvis hälta, fång, kolik och allergiska luftvägsproblem.

Contents

Appendix

The thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Johannisson, A., Gröndahl, G., Demmers, S. and Jensen-Waern, M., 1995. Flow-cytometric investigations of the phagocytic capacities of equine granulocytes. Acta Vet. Scand., 36: 553-562.

II. Gröndahl, G., Johannisson, A. and Jensen-Waern, M., 1997. Opsonic effect of equine plasma from different donors. Vet. Microbiol., 56: 227-235.

III. Gröndahl, G., Johannisson, A., Demmers, S. and Jensen Waern, M., 1999. Influence of age and plasma treatment on neutrophil phagocytosis and CD18 expression in foals. Vet. Microbiol., 65: 241-254.

IV. Gröndahl, G., Johannisson, A., Jensen-Waern, M. and Nilsson Ekdahl, K., 2001. Opsonization of yeast cells with equine iC3b, C3b, and IgG. Vet. Immunol. Immunopathol. In press.

V. Gröndahl, G., Sternberg, S., Jensen-Waern, M. and Johannisson, A., 2001. Equine neutrophil phagocytosis of the neonatal pathogens *Actinobacillus equuli* and *Escherichia coli*. Submitted for publication.

Offprints are published by kind permission of the journals concerned.

Abbreviations

The following abbreviations are used in the text:

BALF	Bronchoalveolar lavage fluid
BPIP	Bactericidal/permeability increasing protein
C1, C3, C3a, C3b, C4a, C5, C5a, C9	Complement factors
C3bBb, C4b2a	Alternative and classical C3 convertases
CD4	Cell receptor expressed mainly on helper T cells
CD8	Cell receptor expressed mainly on cytotoxic T cells
CD11b/CD18	Cell receptor for iC3b (=CR3)
CD16, CD32, CD64	Cell receptors for IgG (=FcγRIII, FcγRII, FcγRI)
CD35	Cell receptor for C3b, iC3b and C4b
CFU	Colony forming units
CL	Chemiluminescence
CR1, CR3	Complement receptors (=CD35, CD11b/CD18)
EDTA	Ethylene diamine-tetraacetic acid
EGTA	Ethylene glycoltetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FcγRI-III	Cell receptors for IgG
FCM	Flow cytometry, flow cytometric
FITC	Fluorescein isothiocyanate
FL1, FL2, FL3	Green, orange and red fluorescence, respectively
FSC	Forward scatter
G-CSF	Granulocyte colony stimulating factor
HI	Heat inactivated
HNP	Human neutrophil peptide
HRP	Horseradish peroxidase
iC3b	Cleaved complement factor 3b
IgA, IgG, IgM	Immunoglobulins A, G and M
IgGa, IgGb, IgGc, IgG(T)	Subisotypes of IgG
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
NADP	Nicotinamide-adenine dinucleotide phosphate
OD	Optical density
PMA	Phorbol myristate acetate
PMN, PMNL	Polymorphonuclear neutrophils
PS	Pooled adult horse serum
RIA	Radioimmune assay
RID	Radial immunodiffusion
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SSC	Side scatter
TNF-α	Tumour necrosis factor-alpha

Introduction

Neutrophil phagocytosis is an exciting miracle, a battle of good and evil that is fought in our bodies again and again without us even noticing it. It is like the most intricate of the kids' computer games, but it is reality. Here we have the heroes: the mighty and clever **neutrophils**, that most of time are sitting and biding their time along the highway, on the blood vessel wall. As soon as stress hits the body, the alarm goes off, stress hormones are squirted out and the neutrophils leave their hideaway and start patrolling. If you take a blood sample just before, when the horse (for example) is calm and relaxed, you find a certain number of neutrophils in that sample. Only a minute later, if you have frightened the animal, the neutrophil count in the blood can be doubled, when these cells have joined the circulating pool. The neutrophils are, in my mind, the Kamikaze pilots of the body. They can smell, but they have no nose. They can see, but they have no eyes. They can eat, but they have no mouth. They can grip, but they have no hands. They can crawl, but they have no legs. And they can find the enemy and kill it, by ingesting and capturing the hostile microbe inside themselves and pouring toxic substances such as household bleach on it until they both die. Thus, in this process, called **phagocytosis**, they are killed themselves, and they are pushed out of the system as pus. They do it for their community, your body.

This is my more poetic or dramatic and simplistic way of looking at this fascinating process. How do the neutrophils know what to kill? It would be a catastrophe if they were to run amok and start harming the body's own cells. This happens in some diseases, such as emphysema in smokers. Inadvertent release of chemicals from the neutrophils may damage the surrounding tissue and cause permanent scarring and impaired function, such as in chronic udder inflammation in cows (Paape et al., 2000). The only time when phagocytes are allowed to destroy the body's own cells is when these are injured, infected, changed by cancer or dying, and the killer lymphocytes then act as humane destroyers and the neutrophils (and macrophages, another type of professional phagocyte) will be the garbage men. If there are such injured body cells, these will leak out substances that attract the neutrophils. The neutrophils follow the smell along the blood road and get to the right address, the site of damage. There, they squeeze through the blood vessel wall without any blood leaking out, a process called **diapedesis**. They continue crawling between the cells towards the injury in a process called **chemotaxis**. Dying cells put out "eat-me" flags on their surface that are recognised by phagocytes, which destroy these cells without further ado (Savill and Fadok, 2000).

When strangers such as bacteria intrude the body, the neutrophils do not automatically see them. Our heroes may pass close by and not notice that there is an intruder. They need help, and the help comes in the form of small guard dogs, proteins that are flowing around in the blood along with the blood cells. Some of them are specialised for certain bacteria, and these are the **antibodies** or **immunoglobulins**, for example IgG. They are produced by plasma cells if the lymphocytes have met this particular microorganism before, so that the body is

immunised, perhaps through a vaccination. Other proteins can react the first time they meet a foreigner, and the most important of these is **complement factor 3, C3**. Such proteins will react to features on the surface of the bacteria that they recognise as strange. Antibodies and C3 coat the foreign microbes and suddenly the neutrophil may recognise these, and react by binding to the coated bacteria through its **receptors for IgG and C3**. This coating is called **opsonisation** and the proteins are called **opsonins**. The microbes now become tasty for the neutrophils, and these terms actually stem from the Greek word "opson" (ὄψου), meaning "appetising flavour" or "deliciously cooked food" (Liddell and Scott, 1996). As a side note, there is also a Greek word "opsofagos" (ὀψοφάγος), used for "gourmet, one who eats delicacies, such as fish and other dainties" - obviously bacteria were not on the menu of the ancient Greek.

If anything in this system fails or is lacking, the battle is won by the bacteria and the body will be heavily infected by proliferating intruders, and perhaps die of a generalised infection, septicaemia. This happens more easily in young, newborn animals than in adult animals, and bacterial infection is the main cause of foal diseases. *Escherichia coli* and *Actinobacillus equuli* are the two bacteria that are most frequently found in blood from newborn foals with severe infections (Koterba et al., 1984; Wilson and Madigan, 1989; Raisis et al., 1996), although they are common in the normal flora of adult horses without causing disease. Each of these bacteria has been isolated in about 20% of positive blood cultures from Swedish foals with septicaemia (Båverud, 2001).

My objectives with this work were to learn how neutrophil phagocytosis and opsonisation can be studied in horses and foals, and to use these methods to gain important information on the apparently immature defence system against bacterial infections in young animals.

Neutrophil granulocytes

The different kinds of white blood cells were first described in the 18th century (Lundh, 1992). Those carrying a multi-lobed nucleus and small grains in the cytoplasm were named granulocytes. Of these, some cells were difficult to stain with dyes, and hence, were called neutrophilic granulocytes (neutral to staining), as opposed to eosinophilic and basophilic granulocytes, which take up acid and basic dyes, respectively. The polymorphonuclear neutrophil granulocyte or leucocyte is often abbreviated PMN or PMNL, and it is also called a neutrophil, which is the term mainly used in this thesis.

The function of neutrophils is to capture and destroy foreign material through phagocytosis. In the late 19th century, Elie Metchnikoff described phagocytes as "wandering cells", which he observed around the sharp thorns with which he had pierced transparent starfish larvae (Metchnikoff, 1883). He hypothesised rightly that these cells were important for the defence against the damage inflicted by the thorns.

In a resting adult healthy horse, there are about 2.4-6.0 $*10^9$ neutrophils per litre blood, which is about 50% of the total leucocytes (reference values from

Department of Clinical Chemistry, SLU). Increased numbers are seen in inflammatory and infectious diseases, and in certain tumours. Neutrophils are produced by the bone marrow and released into the blood, and about 12 hours later they move into the tissue (Tizard, 1996). The life span of a neutrophil is only a few days, and it cannot divide. In a human, 100,000,000,000 granulocytes or 1 dl are produced daily (Engström, 1994). The neutrophil is rounded and about 12 μm in diameter (Tizard, 1996). The nucleus of an immature neutrophil is irregular and sausage-like (band-shaped), and in the mature cell it is segmented and dense. The cytoplasm is finely granular and has two major types of enzyme-rich granules, primary and azurophilic, and secondary granules. The enzymes are further described in the next section and in Table 1.

Neutrophil functions

Neutrophils, together with blood monocytes and tissue macrophages, are professional phagocytes. Eosinophil granulocytes and dendritic cells are also able to phagocytose, and various other types of cells may occasionally engulf particles. However, none of these are as effective against microbes as the professional phagocytes. Of these, neutrophils are generally the first to reach the site of infection, but macrophages survive in the tissue for longer periods of time. In the following, aspects of neutrophil phagocytosis will be considered. Specific details on equine neutrophils are not well investigated, and the following outline is thus based mainly on studies in other species.

Neutrophil phagocytosis is continuous, but can be divided schematically into four stages, although these are not necessarily separated in time and space: chemotaxis or the migration of neutrophils, adherence to the target, ingestion, and digestion or killing, as introduced before.

Circulating neutrophils are triggered to leave the blood as a result of increased adhesiveness of the cells in the blood vessel walls. Selectins, proteins expressed on these endothelial cells, bind to selectins and integrins on neutrophils (reviewed by van Eeden et al., 1999) resulting in diapedesis. Tissue injury and bacterial invasion cause production and release of factors that attract neutrophils to the site by migration in the tissue, chemotaxis. Chemotactic factors include complement factors C3a and C5a, fibrinopeptide B, platelet factor 4, leucotriene B4 and bacterial peptides (Stossel, 1974; Tizard, 1996). When the neutrophil encounters a foreign particle, it covers it with a pseudopod and adherence (attachment) or binding between recognition receptors and opsonins or microbial ligands occurs.

The neutrophil receptors for the different opsonins have not been thoroughly studied in horses (Banks and McGuire, 1975). Neutrophils in humans express receptors for the Fc part of the IgG molecule, named FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). However, while CD16 and CD32 are expressed by the vast majority of neutrophils (Payne et al., 1993), CD64 expression is generally low but can be induced by the cytokines interferon-γ and

granulocyte colony-stimulating factor, G-CSF (Eckle et al., 1990; Hoglund et al., 1997).

For complement, two main complement receptors (CR) are described, CR1 (CD35) for C3b and CR3 (CD11b/CD18) for iC3b (Arnaout et al., 1983) (Fällman et al., 1993; Nilsson and Nilsson Ekdahl, 1998). CR3 also mediates binding directly to diverse microbial ligands, including lipopolysaccharide, LPS (Cain et al., 1987; Ehlers, 2000). Expression of CD18, a subunit in CR3, is enhanced in bovine neutrophils by proinflammatory mediators such as tumour necrosis factor-alpha, TNF-α, and a derivative of C5a, C5a(des-Arg) (Rainard et al., 2000). Deficiency of CD18, leading to seriously impaired phagocytosis and fatal infections, has been described in humans (Crowley et al., 1980) (Bowen et al., 1982), cattle (Kehrli et al., 1990) (Worku et al., 1995) and dogs (Trowald-Wigh et al., 1992).

The linking of opsonic antibodies and neutrophil receptors such as FcγRII triggers phagocytosis and also activation of the neutrophil surface enzyme NADPH oxidase, resulting in a 100-fold increase in oxygen consumption. Linking with CR3, however, requires additional signals to induce this cell activation (reviewed by Ehlers, 2000).

	Enzymes	*Reactive oxygen species*	*Reactive nitrogen species*
Oxidative	Myeloperoxidase Superoxide dismutase NADPH oxidase Cytochrome b558	Superoxide anion Hydroxyl radicals Singlet oxygen Hydrogen peroxide Hypochlorous acid Chloramines	Nitric oxide Nitrogen dioxide Nitrous acid Peroxynitrite
	Enzymes	*Antimicrobial peptides*	*Nutritional*
Non-oxidative	Lysozyme Elastase Cathepsin G β-glucoronidase Proteinase 3 Collagenase	Defensins Azurocidin BPIP, Bactericidal permeability increasing protein	Lactoferrin (iron sequestration) Tryptophan degradation

Table 1. Antimicrobial mechanisms of phagocytes. Adapted from Giguère and Prescott, 2000.

Once bound to the neutrophil surface, the microbe is ingested into the cell and becomes enclosed in a phagosome in the cytoplasm. The acidic milieu in the phagosome is antimicrobial. The engulfed particle is destroyed through several mechanisms, which are described in the following and summarised in Table 1.

Respiratory burst – When NADPH oxidase is activated, NADPH is converted to $NADP^+$, with release of electrons. This results in formation of superoxide anions, which generate hydrogen peroxide, H_2O_2, which further can react with intracellular halide ions (Cl^-, Br^-, I^- or SCN^-) to produce hypohalides. These two reactions are catalysed by the enzymes superoxide dismutase and myeloperoxidase. The hypohalides, of which the most potent is hypochlorous acid (HOCl; remember, the active ingredient of laundry bleach and swimming pool disinfectants) kill the microbes by oxidising their proteins (Hampton et al., 1998; Babior, 2000). In addition, production of reactive nitrogen species such as peroxynitrite, another oxidant, has been shown (Hampton et al., 1998; Babior, 2000). A titbit for trivial pursuit is that myeloperoxidase is a cousin of haemoglobin, but it is green instead of red, and is the cause of the greenish colour in pus (Babior, 2000).

Lysosomal enzymes – The granules, or lysosomes, in the cytoplasm fuse with the phagosome and release their enzymes and antimicrobial proteins (Table 1). These digest bacterial walls and kill most microorganisms but the susceptibility of the microbes varies. In addition, lactoferrin can prevent bacterial growth by binding to iron, a necessary nutrient for the bacteria (Giguère and Prescott, 2000).

The azurophilic or primary granules contain myeloperoxidase, which is required for the oxidative burst. Other contents are β-glucoronidase, defensins, serprocidins, lysozyme, and bactericidal permeability increasing protein (BPIP), all of which are antimicrobial proteins with a broad spectrum of activity against bacteria, protozoans, enveloped viruses, and fungi (Sørensen and Borregard, 1999; Newman et al., 2000). The defensins in humans are HNP-1, HNP-2 and HNP-3 (Newman et al., 2000). The serprocidins are cationic glycoproteins such as azurocidin, cathepsin G, elastase and proteinase 3. Cathepsin G can kill microorganisms both by enzymatic and non-enzymatic mechanisms (Newman et al., 2000). BPIP has a strong affinity for LPS in Gram-negative bacteria, but may also inhibit yeasts (Newman et al., 2000) and it has opsonic activity (Iovine et al., 1997). Acyloxylase hydrolase in bovine neutrophils decreases the toxicity of lipid A of endotoxins (Dosogne et al., 1998). Lysozyme, collagenase and lactoferrin are found in secondary or specific granules of neutrophils (Sørensen and Borregard, 1999).

Opsonisation

The enhancing effect of serum on phagocytosis was discovered some time after phagocytosis studies began, and was named opsonisation by Sir Almroth Wright, who introduced typhoid vaccination (Wright and Douglas, 1903). At that time there was controversy as to whether resistance to infection was cellular or humoral. The cellular theory was proposed by Metchnikoff, who was studying phagocytosis in Paris (Metchnikoff, 1883), and the humoralists were led by Ehrlich in Germany (Turk, 1994). Also debated was the question of whether the

opsonic effect was specific or not, and studies were performed to find the origin of opsonins (Simon et al., 1906). For this purpose, different body organs were ground up and used for opsonisation, and it was shown that the opsonins were essentially components of blood.

Almroth Wright was the model for the main character in George Bernard Shaw's play "The Doctor's Dilemma" (1906), where the opsonic theory is presented as follows:

> What it comes to in practice is this. The phagocytes won't eat the microbes unless the microbes are nicely buttered for them. Well, the patient manufactures the butter for himself all right; but my discovery is that the manufacture of that butter, which I call opsonin, goes on in the system by ups and downs – nature always being rhythmical, you know – and that what the inoculation does is to stimulate the ups and downs, as the case may be.
>
> Drugs can only repress symptoms; they cannot eradicate disease. The true remedy for all diseases is Nature's remedy. Nature and Science are at one, Sir Patrick, believe me; though you were taught differently. Nature has provided, in the white corpuscles as you call them, in the phagocytes as we call them – a natural means of devouring and destroying all disease germs. There is at bottom only one genuinely scientific treatment for all disease, and that is to stimulate the phagocytes. Stimulate the phagocytes. Drugs are a delusion. Find the germ of the disease; prepare from it a suitable anti-toxin; inject it three times a day a quarter of an hour before meals; and what is the result? The phagocytes are stimulated; they devour the disease; and the patient recovers – unless, of course, he's too far gone.

The humoral and cell mediated theories could only be reconciled much later, when the roles of T-cell receptors and cytokines were elucidated. The suggested use of therapeutic immunisations was more or less left in abeyance, as it gained a bad reputation for being associated with the death of treated patients. It is also difficult to envisage how an injection of microorganisms in an already heavily infected individual can enhance resistance to the infection (Turk, 1994). Also, the antibacterial drugs were introduced with great success. It was Alexander Fleming in Wright's laboratories in London who first discovered penicillin.

The current standpoint in these issues is that opsonins may be either specific, as in the case of antibodies, or non-specific, such as complement. The main method used to distinguish between these two has been heating of serum, just as in the days of Wright. This destroys complement but leaves the action of immunoglobulins intact (Stossel, 1974). However, we now know a little bit more about these and other proteins that coat foreign particles, such as the serum acute phase proteins mannose-binding lectins, and C-reactive protein, as well as the lung surfactant proteins A and D, all of which have been shown to have an opsonic effect in human phagocytosis (Holmskov, 2000; Song et al., 2000). The importance and sometimes even the existence of these in the horse still deserve further investigation.

Antibodies

Opsonic antibodies or immunoglobulins include IgG, the predominant immunoglobulin type in serum, and IgM, which is produced earlier in the immune response. The reference range in adult horse serum is 10-15 g/L for IgG and 1-2 g/L for IgM (Tizard, 1996). Four well-defined subisotypes of IgG in the horse have described, IgGa, IgGb, IgGc and IgG(T) (Sheoran and Holmes, 1996).

Antibodies are produced by plasma cells as a result of the response of B and helper T cells to specific antigen. Plasma cells are distributed throughout the body, but the greatest numbers are seen in the spleen, lymph nodes and bone marrow. Antibodies are present in many body fluids, but their highest concentrations are normally found in the blood serum. Compared to that in equine serum, a several-times higher concentration of immunoglobulins is found in equine colostrum, the first secretion of the mammary gland after parturition (Kohn et al., 1989; Sheoran et al., 2000).

Complement

The main opsonic component in the complement system is C3. This is but one in a system of about 20 different complement factors, many of which are not yet thoroughly characterised in horses. The principal biological functions of the complement system are cytolysis, opsonisation, activation of inflammation, solubilisation and phagocytic clearance of immune complexes, and promotion of humoral immune responses (Abbas et al., 1997).

Complement activity is also present in primitive life forms (invertebrates), and is considered to be one of the first immunity mechanisms in the evolutionary process, appearing as long as 700 million years ago (Song et al., 2000).

Complement factors are primarily synthesised in the liver, but also by other cells, e.g. monocytes/macrophages (reviewed by Nilsson and Nilsson Ekdahl, 1998). C3 is abundant in horse serum, where concentrations of 1-2 g/L have been found in normal horses (Perryman et al., 1971).

Coating of foreign particles with C3 depends on the cleavage of serum C3 to opsonic C3b by convertases produced by different pathways in a cascade reaction (Figs. 1 and 2). Potent pro-inflammatory split products in this process include the anaphylatoxins C3a and C5a.

The alternative complement pathway is promoted by spontaneously formed C3b, which persists on activating surfaces, where the breakdown of native C3b by factors H and I is restricted (Pangburn, 1983). Subsequent steps result in the formation of the alternative C3 convertase, C3bBb.

In the classical pathway, on the other hand, antibodies bound to the antigen initiate activation of the complement factor C1, which in a series of reactions leads to the formation of the classical C3 convertase C4b2a (Fig. 1).

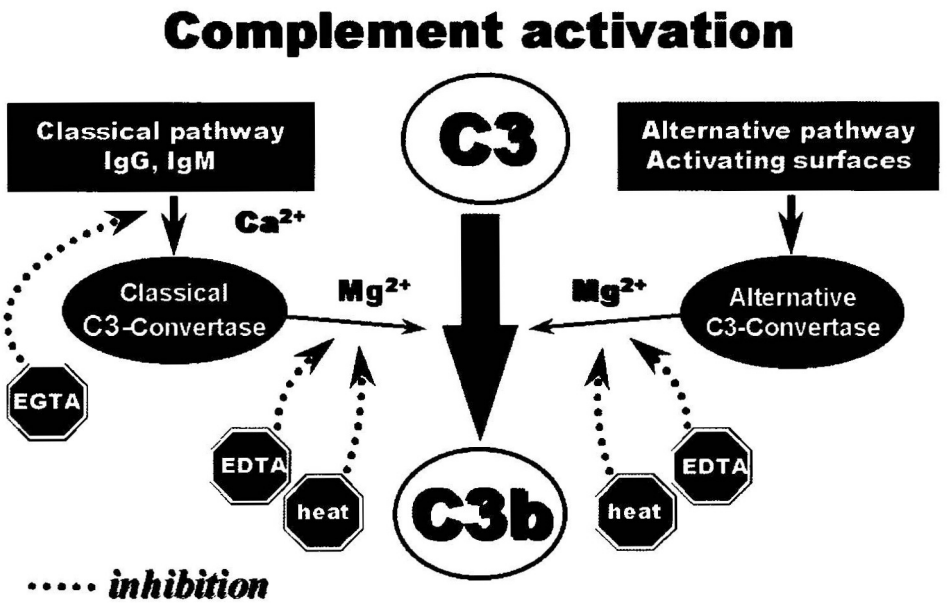

Figure 1. Schematic view of activation of the classical and alternative complement pathways for conversion of C3 to C3b. Possibilities of blocking of the classical pathway with EGTA or of both pathways with EDTA or heat inactivation are indicated.

The classical pathway is dependent on Ca^{2+} (Fig. 1), and thus it can be blocked selectively by addition of the calcium chelator ethylene glycol tetraacetic acid, EGTA (Fig. 1) (Fine et al., 1972; Kozel, 1996). Both the alternative and the classical convertases require magnesium ions, and thus these pathways may be blocked by addition of the chelating agent ethylene diaminetetraacetic acid, EDTA (Fig. 1) (Fine et al., 1972).

C3 convertase can also be formed through the more recently described, but probably ancient, lectin pathway (reviewed by Holmskov, 2000 and Song et al., 2000). In this pathway, collectins such as mannose-binding lectin, an acute-phase serum protein synthesised by the human liver, is bound to sugars on the bacterial surface, and this binding activates specific serine proteases leading to activation of the classical pathway. Other collectins include lung surfactant proteins A and D in humans and conglutinin and collectin-43 in Bovidae (Holmskov, 2000; Song et al., 2000).

Conversion of C3 leads to amplification of particle-bound C3b, which may be further cleaved to iC3b (Fig. 2) by factor I in the presence of a co-factor, e. g. factor H. C3b and iC3b are both opsonic, but bind to different receptors on the phagocytes as earlier mentioned.

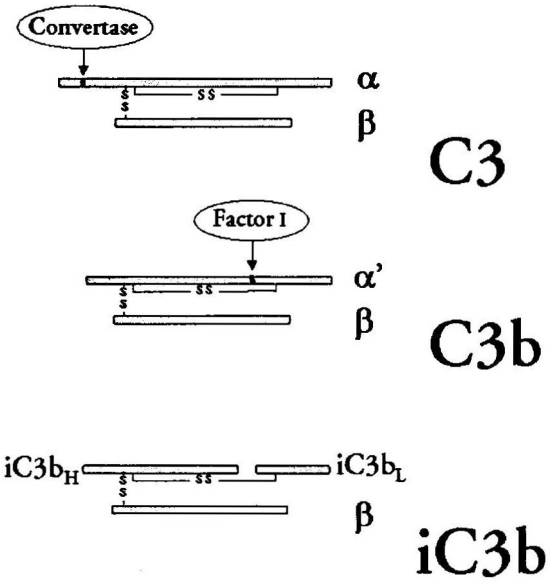

Figure 2. Schematic view of the cleavages of the α chain of C3 by the C3 convertases and factor I, sequentially generating C3b and iC3b. Further cleavage products (C3c and C3dg) are not shown in the figure. The α, α' and β chains are indicated, as well as the heavy and light fragments of iC3b ($iC3b_H$ and $iC3b_L$, respectively). The locations of the interchain disulphide bonds are marked SS. Modified from Kozel, 1996.

Detailed studies of equine C3 and the opsonisation process have been sparse in equine immunology despite the probably important role of complement in infectious, inflammatory and immune-mediated disorders in horses. Purification of equine C3 was first described by Perryman et al. (1971). The molecular masses of its alpha and beta chains have been characterised (Kay et al., 1986; Boschwitz and Timoney, 1993), and has been reported to show electrophoretic polymorphism (Kay et al., 1986). C3 deposition has been studied on the strangles-inducing bacterium *Streptococcus equi* subsp. *equi* (Boschwitz and Timoney, 1994), and on erythrocytes following infection with equine infectious anaemia virus (McGuire et al., 1969).

Studies have more often addressed the haemolytic and conglutinating activities of equine complement (see review in Leid et al., 1985). In haemolytic assays the end-point of complement activation, the cytolytic effect of the membrane attack complex C5b-C9, is evaluated. It has been shown that systems using equine serum and erythrocytes from the rabbit, swine, guinea pig or chicken are functional (Perryman et al., 1971; Barta et al., 1973; Leid et al., 1985; Reis, 1989; Rashmir-Raven et al., 1992). However, sheep red blood cells (commonly used in human and guinea pig complement assays) were lysed poorly by equine complement in early studies (Rice and Boulanger, 1952; Barta et al., 1973; and Grant, 1977 with references), but were useful in later studies (Leid et al., 1985;

Lavoie et al., 1989; Bernoco et al., 1994), which was explained as being due to the use of a higher antibody concentration.

Phagocytosis assays

The basic procedure in a phagocytosis assay is to incubate neutrophils with a target particle to ingest under constant mixing, and then monitor the intake, or the loss of extracellular targets. Some assays use the killing capacity or respiratory burst as a measure of phagocytic activity. Isolated granulocytes, a leucocyte suspension after erythrocytolysis, or whole blood may be used. The targets may be bacteria, fungi or synthetic beads, microbes being more physiological. Most targets need opsonisation for ingestion, and serum or serum factors are therefore added before or during the incubation with the leucocytes. To distinguish between ingested targets and those adherent to the neutrophil, fluorescent particles can be used, and quenchers for extinguishing fluorescence in extracellular particles. Quenching agents described include crystal violet (Hed, 1977), trypan blue (Bjerknes and Bassøe, 1984), tannic acid (Giaimis et al., 1994) and ethidium bromide (Raidal et al., 1998b). Alternatively, dual-colour methods using antibodies have been applied (Sveum et al., 1986), and also antibiotics that selectively lyse extracellular bacteria (Hampton and Winterbourn, 1999).

Phagocytosis has been studied by several methods (reviewed by Hampton and Winterbourn, 1999). The methods aim at elucidating different important moments or steps in phagocytosis, where changes may lead to an increased disposition to infections and disease. Some of the advantages and disadvantages of the methods are presented in the following, and references to equine studies are made where applicable.

Microscopy

Microscopy allows a direct visual assessment of phagocytosis. If fluorescent particles are used, intracellular particles can be identified by quenching techniques. The number of particles per neutrophil can be counted, and the percentage number of neutrophils adhering to or ingesting targets is calculated (Bernoco et al., 1987; Hietala and Ardans, 1987a; Trowald-Wigh and Thorén-Tolling, 1990; Wichtel et al., 1991). This method is valuable for checking the accuracy of other methods. Only a limited number of neutrophils can be examined, however, and the manual analysis is laborious and time-consuming, which is a drawback in experiments with a large number of samples. Small particles such as bacteria are difficult to evaluate because of low resolution. Electron microscopy overcomes this problem, but it is not practical in studies on kinetics or in large materials.

Flow cytometry

Flow cytometry (FCM) is an elegant way to rapidly process a large number of samples, as thousands of cells can be analysed within one minute. The cells pass single-file through a laser beam, and light scatter and fluorescence properties of

each cell are analysed in order to recognise different cell populations. Target particles are fluorescence labelled, and quenching agents may be added to distinguish between extra- and intracellular particles. The percentage number of neutrophils adhering to or ingesting prey is calculated. The loss of extracellular targets due to phagocytosis can be quantified by counting the numbers of these targets in a constant volume. If particles with constant fluorescence, such as beads, are used, the number of ingested particles may be accurately determined by measuring the fluorescence in each neutrophil.

The assay can be combined with concurrent assessment of the oxidative burst, by using non-fluorescent substrates that convert into fluorescent dyes under the influence of reactive oxygen species (Smits et al., 1997; Bassøe et al., 2000). The phagocytic contribution of neutrophils and monocytes may also be estimated simultaneously (Bassøe et al., 2000).

FCM analysis of phagocytosis was the main technique used in the present investigations, and has been used on equine cells by other authors also (Foerster and Wolf, 1990; Raidal et al., 1998b; Flaminio et al., 2000).

Radiolabelled particles

With the use of radiolabelled particles, the radioactive uptake of the neutrophils is measured (Kishore, 1981; Couignoul et al., 1984). The handling of radioactive material before, during and after assays involves a safety issue not encountered in other techniques.

Microbiological assays with viability assessment techniques

In microbiological assays, viable bacteria are phagocytosed. The loss of extracellular bacteria is measured. By plating on agar and counting of colony-forming units after lysis of phagocytosing neutrophils, the loss of viability can also be determined (Yager et al., 1986; Morris et al., 1987; Hampton et al., 1994). Another way of assessing viability is to investigate the ability of bacteria to incorporate [^{3}H]-thymidine into newly synthesised DNA (Martens et al., 1988; Hampton and Winterbourn, 1999), or to use acridine orange, which can differentiate between viable and dead bacteria by a shift in the emission peak from green to orange (Hietala and Ardans, 1987b).

An advantage of these assays is that the target is physiological, and that concurrent determination of the killing capacity is possible. With acridine orange, FCM analysis can be performed.

The tedious work with multiple dilutions, plating and colony counting is a drawback. With both the plating and thymidine uptake techniques, a decreased rate of cell division or bacteriostasis can be misinterpreted as cell death. There is also a risk of loss of viable bacteria due to release of cytotoxic factors from the neutrophil, or by aggregation of bacteria. In assays with longer incubation times, division of extracellular bacteria may instead confuse the results (Hampton and Winterbourn, 1999).

Assays for respiratory burst

The respiratory/oxidative burst or increased consumption of molecular oxygen in the phagocytosing neutrophil has been used as a measure of the phagocytic capacity. The oxidative burst is the main killing mechanism of neutrophils (Table 1). The neutrophil oxidative burst can be stimulated by various soluble and particulate stimuli, including chemoattractants, certain cytokines, phorbol esters, calcium ionophores, lectins, and opsonised as well as unopsonised microorganisms (Dahlgren and Karlsson, 1999). As activation of the oxidative burst may be initiated without neutrophil ingestion of microbes, it may cause difficulties in interpretation, if the assay is intended solely for measuring phagocytic activity. Thus, most of these methods are better defined as measurements of the respiratory burst capacity than of the phagocytic capacity.

Many methods for measuring the respiratory burst capacity are described, commonly using photometry, fluorometry, luminometry or precipitation reactions for detection of cellular release or intracellular production of the generated reactive oxygen species (reviewed by Dahlgren and Karlsson, 1999). Reduction of nitroblue tetrazolium has been used for measuring the oxidative burst in horse neutrophils (Takai et al., 1986). The most frequently used method in reported studies is the chemiluminescence (CL) assay. In this assay, the oxygen species excite activity-amplifying dyes that release energy in the form of light, which is quantified. CL has been used in studies of equine neutrophils (Jacobsen et al., 1982; LeBlanc and Pritchard, 1988; Magnusson and Jonsson, 1991). Relatively recent FCM techniques for measuring the respiratory burst have also been employed with equine blood (Raidal et al., 1998a), even simultaneously with measurements of phagocytic activity (Flaminio et al., 2000).

Assays for opsonisation

The opsonic capacity of serum may differ between individuals or groups, for example between young and adult animals. The concentration of opsonins in serum can be measured by different techniques. However, this may not automatically be equivalent to the functional opsonic capacity of serum for a certain microbe, if other factors have an impact on the opsonisation process, such as characteristics of the microbe (Kozel, 1996) or interactions between serum factors. Neither are such methods suitable for studies of the mechanisms and kinetics of opsonisation. A more accurate way is to measure the concentration of opsonins on the surface of the microbe after incubation with serum. Further, it is essential that the function of this coating is evaluated by testing the opsonised particle in a standardised phagocytosis assay (Väkeväinen, 2001).

Coating of microbes and synthetic surfaces with opsonins has been evaluated with immunological methods, employing labelled antibodies for C3 or IgG in radioimmune assays, RIA (Newman and Mikus, 1985; Cain et al., 1987), enzyme-linked immunosorbent assays, ELISA (Rainard and Boulard, 1992), spectrophotometry (Hed and Stendahl, 1982) or immunohistochemistry (Kozel et al., 1984) (Boschwitz and Timoney, 1994). Radio-iodinated C3 has also been used (Pangburn et al., 1983; Nilsson and Nilsson, 1985; Kozel and Pfrommer,

1986). A more recent addition to the analytical methods is FCM immunoassays for the detection and quantification of opsonins on target surfaces (Hall et al., 1993; Gemmell, 1997; Krediet et al., 1998; Rokita et al., 1998). Some advantages of FCM are that it is faster than histological techniques, it does not involve work with radioactive material, and that information can be obtained for single particles in the solution as opposed to results gained from ELISA, providing an opportunity to observe differences in subpopulations of target cells.

Immune defence mechanisms in foals

The most common cause of morbidity and mortality in foals is septicaemia, which is responsible for more than 40% of the 5% mortality observed in foals up to six months of age (Cohen, 1994). The higher prevalence of serious infections observed in foals compared to adult horses has motivated research aiming at characterising the immunological background of this difference.

Passive transfer of maternal antibodies

One of the first things to be discovered in foal immunology was the importance of colostrum ingestion in foals. In the 1970s McGuire and co-workers found that hypogammaglobulinaemia in foals was associated with infections (McGuire et al., 1975; McGuire et al., 1977). Foals, like other ungulates, are born without antibodies, since the placenta in these animals does not allow transfer of maternal immunoglobulins into the umbilical cord as in humans. Thus, colostrum intake is critical, and the window for successful transfer over the intestine is only a few hours up to 24 hours after birth. The maternal immunoglobulins in foal serum reach a peak after ingestion of colostrum and then gradually decline. Typically, serum IgG reaches a minimum at about 60 days of age before autologous production leads to an increase again (McGuire et al., 1975).

In Australia, determination of "adequate" serum IgG levels are now often required by insurance companies prior to the provision of mortality insurance for foals (Raidal, 2000). However, many foals do well despite low serum IgG (Kohn, 1989). Other factors such as a low density of pathogens in the environment at well-managed farms probably reduce the occurrence of infections in such cases.

The standard treatment for failure of passive transfer in foals is intravenous transfusion of plasma from an adult horse (Clabough, 1988). There is also commercially available plasma from horses hyperimmunised against foal pathogens such as rotavirus, *Rhodococcus equi* (Chaffin et al., 1991; Machangú and Prescott, 1991; Martens et al., 1991), or *E. coli* (Morris et al, 1986; Chaffin and Cohen, 1998), which may be indicated in prophylactic treatments in endemic situations. However, the effect of such treatments have not yielded consistent results.

The main link between low IgG and infections in foals is thought to be decreased opsonisation and phagocytosis of pathogenic bacteria (Giguère and Prescott, 2000). IgG binds directly to surface antigens, enhances phagocytosis via Fc receptors on neutrophils, and also activates the classical complement pathway, indirectly leading to opsonisation with C3. However, the opsonic requirements of foal pathogens other than *R. equi* (Yager et al., 1986; Hietala and Ardans, 1987b; Martens et al., 1987; Yager et al., 1987; Martens et al., 1988), and possible mechanisms by which foal pathogens may evade phagocytosis have not been thoroughly evaluated.

Figure 3. Intravenous treatment with adult horse plasma is often used to foals with a low serum concentration of antibodies (IgG) due to failure of passive transfer from colostrum.

Complement in foals

Effective opsonisation is, as mentioned, also dependent on complement activation. Complement factors are produced already in the equine foetus, but the serum concentration of C3 in newborn foals has been found to be only 30% of the adult standard and to reach normal adult values at 2 weeks (Bernoco et al., 1994). Foals up to 1-4 weeks of age show less serum haemolytic and conglutinating complement activity than adults (Barta and Barta, 1976; Hietala and Ardans, 1987b; Lavoie et al., 1989; Bernoco et al., 1994). This is consistent with findings in calves (Rice and Duhamel, 1957; Renshaw and Everson, 1979), piglets (Rice and L'Ecuyer, 1963), lambs (Rice and Silverstein, 1964) and human infants (Stossel et al., 1973; Ferriani et al., 1990).

Neutrophil phagocytosis in foals

Studies of neutrophil functions in foals have been carried out with different methods (Bernoco et al., 1987; Hietala and Ardans, 1987a; Hietala and Ardans, 1987b; Morris et al., 1987; Yager et al., 1987; LeBlanc and Pritchard, 1988; Martens et al., 1988; Wichtel et al., 1991; Flaminio et al., 2000; Raidal, 2000). The results of these studies are further discussed later on in this thesis. These investigations have provided useful information, but there is still a need for more detailed studies, such as the temporal development of the involved functions, and the relevance of the results from one target microbe on the characteristics of another. These are aspects that were considered in the present work.

Other factors in the innate immunity of foals

Other factors in the innate immunity have been poorly studied in the horse and foals, for example cationic antimicrobial peptides, such as defensins, cathelicidins, protegrins and granulysin. These are abundant in neutrophils, but some are produced by epithelial cells of several organs, and some can be found in glandular secretions in the respiratory, digestive and urogenital tracts (Lehrer and Ganz, 1999; Travis et al, 2001). These peptides have been found to have a broad activity against a range of infectious bacteria and fungi.

Lymphocyte subpopulations in foals

Studies in foals have shown that the peripheral lymphocyte counts at birth are adequate, and increase during the first 3 months of life. The increase is due to contributions of CD4+ and CD8+ T cells as well as B cells (Flaminio et al., 2000). The number of B cells is larger in foals of ages 2-4 months than in adult horses, the larger number coinciding with an increase in serum immunoglobulin concentrations in foals (Flaminio et al., 2000). During the first month of life, foals have fewer cells expressing equine MHC class II, which are considered to be antigen-activated memory cells (Lunn et al., 1993; Flaminio et al., 2000).

Broncho-alveolar leucocytes in foals

Since the respiratory system is so commonly affected in both mild and severe infectious diseases in foals, the local immunology of the lung has attracted considerable interest. The leucocyte pattern in the lung differs between newborn foals and adult horses, and the number of these cells is smaller in broncho-alveolar lavage fluid (BALF) from foals. There is a higher proportion of macrophages and fewer CD4+ and CD8+ T lymphocytes in newborn foals (Zink and Johnson, 1984; Balson et al., 1997; Flaminio et al., 2000). Further, the proportion of B cells in BALF was found to be higher in 1-week-old foals than in mares in one study (Balson et al., 1997), but B cells were almost undetectable in foals up to one month old in another report (Flaminio et al., 2000). The proportion of neutrophils in BALF from foals varied, as in that of adult horses (Zink and Johnson, 1984; Balson et al., 1997).

Aims of the investigations

The general aims of the present series of investigations were to establish methods for studying neutrophil phagocytosis and the opsonic capacity of serum in horses, and to use these methods in more detailed studies of these functions in general and in clinical studies of foals. The main hypothesis tested was that the phagocytic function in foals is impaired compared to that of adult horses.

The specific aims of the studies were:

- to establish flow cytometric methods for analysis of the phagocytic capacity of equine neutrophils, using *Saccharomyces cerevisiae* yeast cells (paper I), or the foal pathogens *Escherichia coli* and *Actinobacillus equuli* (V) as targets

- to investigate the kinetics of attachment to and ingestion of yeast (I), and the kinetics of opsonisation (IV)

- to measure the serum concentrations of IgG (II, III) or the subisotypes IgGa and IgGb (V), and to study the deposition of IgG on yeast cells (IV) in relation to phagocytosis

- to investigate the dependency of opsonisation and phagocytosis on serum complement factor C3 (II, IV, V) and on the classical and alternative complement activation pathways (IV), and to characterise equine C3 fragments on opsonised yeast (IV)

- to assess the potential of plasma from different donors to enhance yeast phagocytosis in neutrophils from foals and adult horses (II)

- to elucidate the capacity for opsonisation and phagocytosis in foals of different ages as compared to adult horses, using yeast (II, III, V), *E. coli* and *A. equuli* (V) as targets, and to determine the expression of the neutrophil surface receptor CD18 in foals compared to adult horses (III)

Material and methods

A brief summary of the materials and methods used and some additional information are given below. For more detailed accounts, see papers I-V.

Animals and experimental design

Horses and foals of the Standardbred trotter breed were used in all the studies, with the addition of other breeds where stated. The horses were clinically healthy at samplings, with the exceptions mentioned in paper III. Sampled mares were barren and not lactating unless otherwise indicated (dams in studies III and V). Pooled serum in all studies was prepared from 10 adult horses (geldings and mares). The batches were not always the same in the different studies. Besides normal management procedures, the experimental design involved only blood sampling from the horses, and administration of plasma intravenously once in six foals in study III. All studies were approved by the Ethical Committee for Animal Experiments, Uppsala, Sweden.

Study I

In study I, neutrophils from 5 adult mares were used for investigations of the kinetics of attachment and ingestion of yeast cells opsonised with pooled serum or anti-yeast IgG in a flow-cytometric assay. The variation of the method was studied with neutrophils from two other horses (one Thoroughbred and one Swedish Warmblood). The dynamics of quenching of fluorescein isothiocyanate (FITC) fluorescence in yeast with trypan blue was assessed.

Study II

The opsonic capacity of plasma from 3 geldings, used as plasma donors for foals at a stud farm, was evaluated in this study. Phagocytosis assays were performed with yeast using neutrophil granulocytes from 7 foals, aged 7-8 days (Study A) and from 7 adult, non-lactating mares (Study B). The serum concentration of IgG in the plasma samples was measured. The effect of heat inactivation of complement on the opsonic capacity was determined in each plasma sample.

Study III

Study III dealt with the influence of age on neutrophil phagocytosis and the expression of CD18, and with the serum opsonic capacity and serum concentrations of IgG.

Foals (n = 13) from two farms, their lactating dams (n = 13) and the sires of the foals (n = 3), were used. Six of the foals (Farm A) were treated at one week of age with plasma from one of 3 adult geldings. The foals were sampled at seven time-points, at the ages of 2, 7, 14, 20, 28, 42 and 56 days. Blood was collected on one occasion only from the adult horses. During the study, four foals in Farm A developed respiratory infections, of which two were treated with antibiotics and two did not require treatment.

Neutrophils from these horses and foals were tested in phagocytosis assays with yeast, opsonised with a) autologous serum (serum from the same individual as the neutrophils), b) pooled serum or c) anti-yeast IgG. In addition, blood from 3 adult geldings was used in studies of phagocytosis with adult neutrophils and foal serum.

Study IV

The opsonic deposition of C3 components and IgG on yeast cells by pooled serum was examined in study IV. The kinetics and effects of different serum concentrations on opsonisation were assessed, as also were the contributions of the classical and alternative complement activation pathways. C3 fragments on yeast were characterised. The deposition of opsonins was related to the phagocytosis results, using neutrophils from 3 adult horses.

Study V

The serum capacity for opsonisation of *E. coli*, *A. equuli* and yeast, and the serum concentrations of IgGa and IgGb were assessed in foals before colostrum ingestion and at 2, 7, 14, 21, 28, 35 and 42 days of age (median n=5, range 3-12 at each sampling). The foals were not the same as in earlier studies. Some Swedish Warmblood foals were included. Sera from lactating mares (n=7) and pooled serum were tested for comparison with foals. Neutrophils from one non-lactating mare were used for phagocytosis with all the sera and microbes.

Phagocytosis assays

Phagocytosis assays were performed with equine neutrophils from EDTA-blood after erythrocytolysis with a commercial lysing reagent (papers I-III) or a buffer containing NH_4Cl (IV-V).

Yeast and bacteria were labelled with FITC. The target particles were opsonised with fresh equine serum (II, III), frozen and thawed equine serum (I-V), frozen and thawed equine plasma (II) or freeze-dried and resuspended rabbit anti-yeast IgG (I, III). Total or selective complement pathway inhibition in serum was achieved by heat inactivation (II, IV, V) or addition of EDTA or EGTA (IV). The serum concentrations and incubation times varied in the methodological studies, but the standard incubation time was 15 min and the standard serum concentrations were 5% for yeast cells and *A. equuli* and 40% for *E. coli*.

The opsonisation was performed and the particles were washed before incubation with neutrophils in studies I-IV. Yeast cells opsonised with pooled serum (PS) or IgG and washed were frozen in aliquots and thawed for each experiment in study III. In study V, the opsonised bacteria and yeast were not washed before neutrophils were added.

For phagocytosis, the ratio of neutrophils to yeast particles was 1:5 in all studies and the volume used was 1 ml, in 5-ml tubes with end-over-end rotation at 37°C for 15 min except in studies of kinetics. In assays with bacteria, optical density instead of bacterial counts was used to achieve the best reproducibility of

bacterial concentrations. Phagocytosis was terminated in all samples by adding EDTA and embedding the samples in ice prior to the FCM analysis.

Assays for opsonisation

The deposition of C3 fragments and IgG on opsonised yeast cells was analysed separately with a FCM immunoassay (IV). For the detection of C3, a sheep anti-horse C3 polyclonal antibody conjugated to a green-fluorescing fluorochrome was used. For deposited IgG, a biotinylated polyclonal rabbit anti-horse IgG antibody was used, followed by streptavidin-R-phycoerythrin. Complement activation was inhibited with EGTA, EDTA or heating of serum as described previously.

CD 18 receptor assays

Monoclonal mouse anti-equine-CD18 antibodies with FITC-fluorescent secondary rabbit anti-mouse antibodies were used to detect expression of CD18 on equine neutrophils with FCM (III). An isotype control antibody was used for detection of unspecific binding, and staining with propidium iodide for exclusion of dead cells.

Flow cytometric analyses

FCM analyses were conducted with a sorting flow cytometer. The light sources and software employed are outlined in the respective papers.

For each cell, forward light scatter (FSC) indicating the size, and side scatter (SSC) indicating the granularity or complexity of the cell, as well as the fluorescence were recorded in all samples and assays. Gates for yeast cells, granulocytes and lymphocytes were set on the basis of light scatter properties.

Green fluorescence (FL1) from FITC was recorded in the phagocytosis assays. Values of 30,000 leucocytes were recorded in the experiments with bacterial assays and of 50,000 events (yeast cells and leucocytes) in tests with yeast. Trypan blue was used for quenching of FITC-yeast not engulfed by phagocytes (Bjerknes and Bassøe, 1984). The percentage of neutrophils attaching to or ingesting prey and the mean fluorescence of the neutrophils were calculated.

Values of 2,000 yeast cells were recorded in the opsonisation assays, with FL1 for C3 detection and FL2, i.e. orange fluorescence of phycoerythrin, for IgG detection.

In CD18 assays, 20,000 leucocytes were investigated for FL1 (CD18 or isotype control detection) and FL3 (red fluorescence for dead cells stained by propidium iodide). The FITC fluorescence of viable granulocytes was evaluated, and the differences between CD18-stained samples and samples stained with an isotype control antibody were calculated for each horse and sampling occasion.

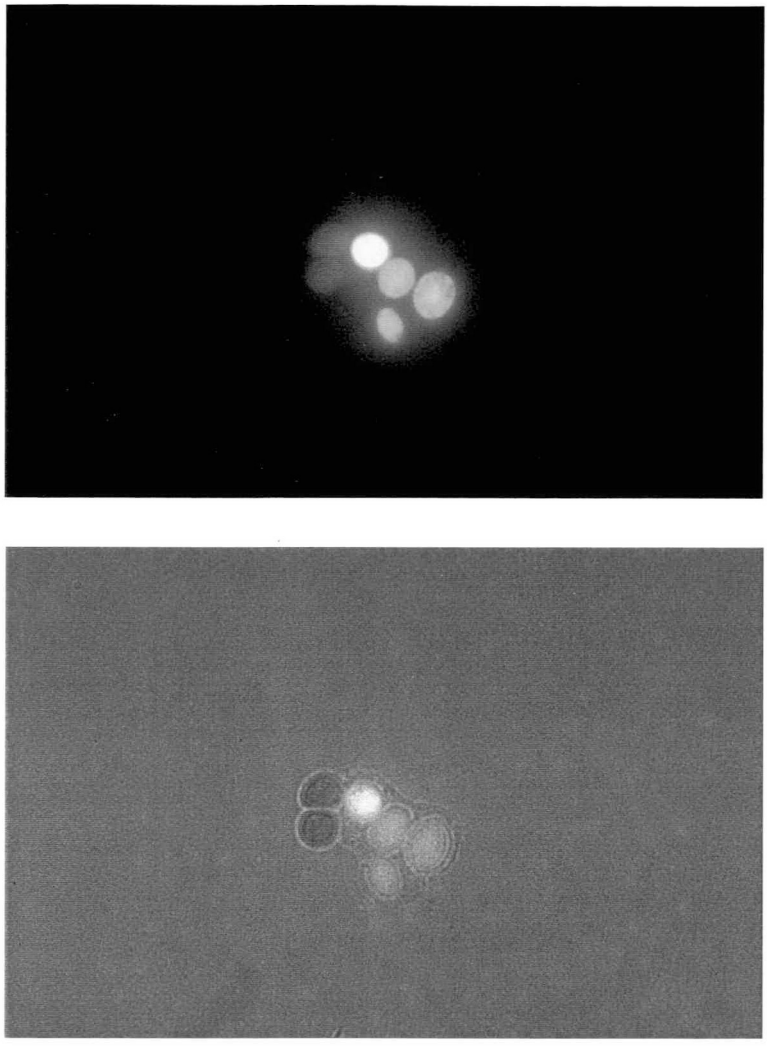

Figure 4. Equine neutrophil after phagocytosis of FITC-fluorescent yeast cells in fluorescence microscope. The upper picture shows the fluorescence of four ingested yeast cells. In the lower picture, application of background light allows visibility of the cell membrane and, to the left, two attached yeast cells which are quenched with trypan blue can be observed.

Quantification of serum IgG and subisotypes of IgG

Radial immunodiffusion (RID) was used for measuring serum concentrations of IgG (II, III) and of the subisotypes IgGa and IgGb (V). Serum protein electrophoresis was used for determination of albumin and globulin fractions in serum (II, III).

Analysis of C3 fragments from serum and particles by SDS-PAGE and Western blot

Covalently bound C3 fragments on yeast cells opsonised with equine pooled serum were released with methylamine in a buffer containing sodium dodecyl sulphate (SDS) and phenylmethylsulphonyl fluoride (a protease inhibitor) (Nilsson and Nilsson, 1985). C3 was also isolated from zymosan-activated human and equine serum by matrix-bound immune precipitation using anti-human C3c antibody.

The released C3 fragments from opsonised yeast cells, and C3 from native equine serum and from zymosan-activated equine and human sera were analysed by SDS-PAGE in 10% polyacrylamide gels under reducing conditions, followed by either Coomassie brilliant blue staining or transfer to nitrocellulose membranes (IV). Human factors C3 and iC3b were used as controls.

C3 fragments on the nitrocellulose membranes were detected in a Western blot analysis, using biotinylated anti-equine C3 followed by streptavidin conjugated to horseradish peroxidase (HRP) and an HRP colour development reagent.

Statistical analyses

The data are expressed as means ± SEM or with ranges. Differences in measured values between time-points within a group were analysed by the Wilcoxon signed rank test. The Mann-Whitney U test was used to analyse differences between groups. The level of significance was set at $p<0.05$, unless otherwise indicated.

Results and discussion

Methodology for phagocytosis assays

Yeast assays

In the initial part of the present investigations, a flow-cytometric method for studying phagocytosis of equine neutrophils was developed. The purpose was to find a means of analysing large materials, facilitating studies of groups or herds of animals. As presented later on (Table 3), studies on the ingestion capacity of equine neutrophils in the literature at that time had mostly been performed with the help of microscopy (Bernoco et al., 1987; Hietala and Ardans, 1987b; Wichtel et al., 1991) or plating techniques (CFU) (Morris et al., 1987; Yager et al., 1987).

Although FCM had proven to be useful and to allow a fast and accurate analysis of the phagocytic capacity of neutrophils in both humans (Bjerknes and Bassøe, 1984; Hed et al., 1987) and animals in studies at this university (Matsson et al., 1985; Saad and Hageltorn, 1985; Thuvander et al., 1987; Thuvander et al., 1992; Jensen-Waern et al., 1994), there was only one report on its use in equine phagocytosis (Foerster and Wolf, 1990).

The assay that we first developed for equine neutrophil phagocytosis has since been used satisfactorily in a number of studies including all those presented in this thesis and others (Jensen-Waern et al., 1999; Sternberg et al., 1999; Demmers et al., 2001). It employs serum- or IgG-opsonised FITC-fluorescent yeast fungi, *Saccharomyces cerevisiae*, as prey. It is a convenient and accurate assay with low variation. The coefficient of variation in study I was 0.13-0.31, and with increased experience in our laboratory, it is now 0.06 (paper V).

In this assay, granulocytes are identified as a distinct cell population when forward and side scatter properties are plotted (Fig. 5a). After ingestion of FITC-yeast cells, neutrophils gain fluorescence (Fig. 5c and 5e) and the increases in size and granularity cause a shift towards the upper right corner in the scattergram (Fig. 5b).

Opsonisation is necessary for equine neutrophil uptake of yeast, as shown by the fact that less than 0.5% of the neutrophils phagocytosed non-opsonised yeast cells (III, IV). When serum is used as an opsonin, the process is completed more rapidly than with IgG, and the proportion of phagocytic neutrophils after 15 min is two to three times higher, both when foal and adult neutrophils are used (I, III). This relation was also found in studies in pigs (Jensen-Waern et al., 1994), but not in dogs (Trowald-Wigh and Thorén-Tolling, 1990), and in humans the opposite tendency has been observed (Hed, 1979). Our results indicate that serum factors other than IgG are important for equine neutrophil phagocytosis of yeast, assumedly mainly complement C3, as suggested in studies on human neutrophils (Hed and Stendahl, 1982).

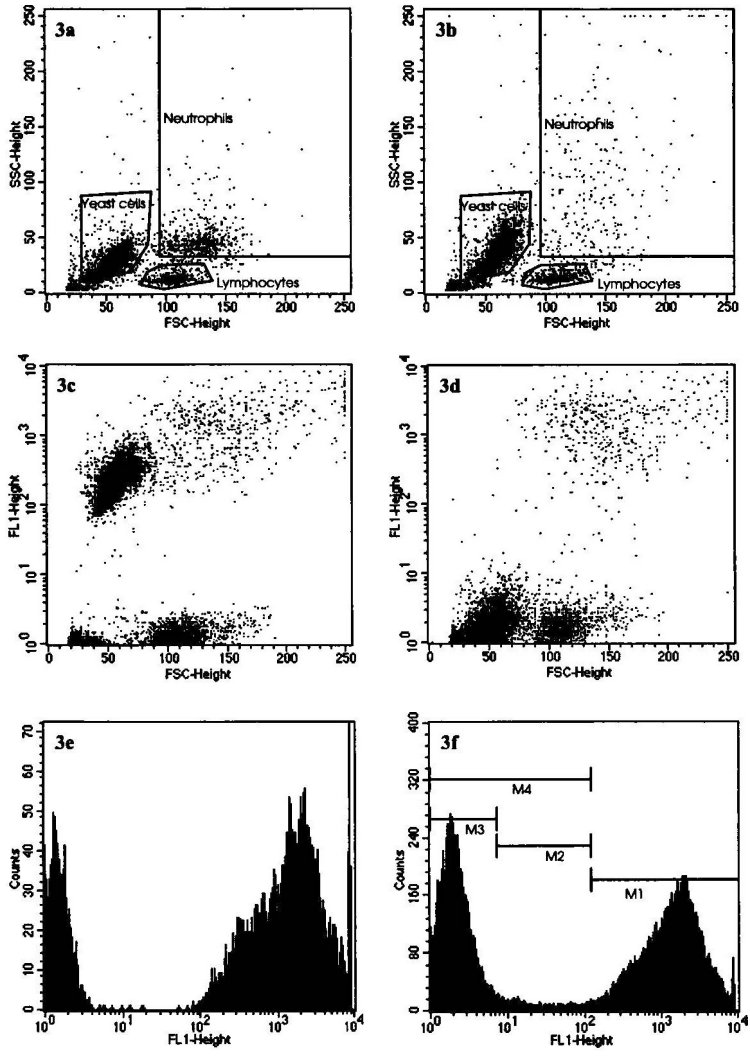

Figure 5. Flow cytometric analysis of equine neutrophil phagocytosis of opsonised FITC-fluorescent yeast cells. *a)* Before phagocytosis. Windows for neutrophils, yeast cells and lymphocytes are indicated in the scattergram. X axis: FSC, forward scatter indicating size; Y axis: SSC, side scatter, indicating granularity. *b)* After 15 min of phagocytosis. Note the increases in size and granularity of the neutrophils, causing a shift towards the upper right corner in the scattergram. *c)* Green fluorescence (FL 1; Y axis) of all cells in panel *b*. *d)* FL1 of all cells in panel *b* after trypan blue quenching. The fluorescence has decreased in extracellular yeast cells but remains in phagocytosing neutrophils. *e)* FL1 of the cells in the neutrophil window in panel *b* depicted in a histogram. Most cells have gained fluorescence by the ingestion of FITC-yeast cells. X axis: FITC fluorescence (arbitrary units); Y axis: number of cells. *f)* Same as panel *e*, but after trypan blue quenching. M1 indicates neutrophils with ingested yeast cells (fluorescent) and M3 are neutrophils without ingested yeast cells. The difference between M3 in this histogram and the equivalent cluster in panel *e* consists of neutrophils with attached yeast cells only. M2 are cells with intermediate fluorescence, and these consist of extracellular yeast aggregates. The percentage number of phagocytosing neutrophils is calculated as M1/(M1+M3).

The quenching agent trypan blue quickly decreased the fluorescence of extracellular yeast (Fig. 5c-d), so that a low level was reached within 4-5 minutes (I). Phagocytosing neutrophils were left fluorescent (Fig. 5f), since this dye does not penetrate living cells, as opposed to crystal violet, which is lysosomotropic (Bjerknes and Bassøe, 1984; Hed et al., 1987). Quenching made it possible to separate neutrophils with ingested particles from those which had only attached or adhered to yeast particles. In kinetic experiments, attachment and ingestion started within minutes of incubation (I). The adherence was rapidly followed by engulfment, a finding similar to results in other phagocytosis systems (Peterson et al., 1977; Kuypers et al., 1989; Martin and Bhakdi, 1992). Thus, in subsequent studies it was decided to analyse quenched samples only. The distinction between attachment and internalisation may not be critical in opsonophagocytosis studies, as also discussed by Lehmann et al. (2000).

Besides the percentage of phagocytic neutrophils, a semi-quantitative measure of the ingestion activity of the individual cells was calculated as the ratio of the mean fluorescence intensity in the phagocytic neutrophils to that in the free target particles, and designated the phagocytosis or ingestion index (II, III, V). The relationship between the number of ingested particles and this index was not linear. This is explained by the dampening effect of the intracellular pH on FITC fluorescence, leading to underestimation of the number of ingested particles per cell (Bassøe et al., 2000). For a more detailed estimation of this number, other fluorescent labels which are not affected by pH can be used, such as lucifer yellow (Sveum et al., 1986).

Trypan blue quenching also eliminated the problem of yeast cell aggregates, which was encountered in some batches of yeast. Aggregates of yeast cells passing the laser in the instrument have light scatter properties similar to those of neutrophils, as a result of which, in combination with their fluorescence, they can be falsely interpreted as phagocytosing cells. Samples were passed through a thin insulin syringe needle to break these clumps. This procedure improved the results but they were not thoroughly satisfactory. A region in the fluorescence histogram was therefore set for the low fluorescence which remained in quenched aggregates of yeast, and which was intermediate to that of phagocytosing and non-phagocytosing neutrophils (Fig. 5f). In this way aggregates could be excluded from the analysis in calculations of proportions of active neutrophils in studies II-V.

Defective yeast opsonisation *in vitro* in humans has been associated with clinical problems such as a general opsonic defect (Turner et al., 1986), frequent infection in children (Soothill and Harvey, 1976) and abnormalities in the alternative pathway of complement, e.g. absence of essential co-factor activity (Turner et al., 1985a; Turner et al., 1985b). The model with yeast was therefore chosen for the subsequent clinical studies of foals and adult horses (III), and of the opsonic potential of plasma from different donors (II). As described below,

opsonisation proved to be a critical factor in these studies, and the opsonisation of yeast with equine serum was further characterised in study IV. However, we were also interested in testing the opsonic capacity of foal serum for pathogens commonly isolated in neonatal infections, which is why the model was adapted and tested with the use of *E. coli* and *A. equuli* in study V.

Bacterial assays

The established phagocytosis assays with *E. coli* and *A. equuli* were accurate and highly reproducible, with coefficients of variation of <0.05. The sensitivity of the method for detecting changes in opsonin concentrations was high, according to serum dilution assays. However, there was a striking difference in the amount of serum needed for phagocytosis of *A. equuli* and *E. coli*. To recruit half of the neutrophils in phagocytosis, less than 5% serum was needed for actinobacilli, as against over 40% serum for *E. coli*. This may be an effect of cellular constituents in the strain of *E. coli* used. This strain was a serum-resistant isolate from a foal with septicaemia. Serum resistance, a virulence factor, has been reported consistently in *E. coli* isolates from septic foals in contrast to isolates from faeces of adult horses (Hirsch et al., 1993). Certain outer membrane proteins, LPS, and the capsule have been considered responsible for the resistance to lysis by complement proteins in serum (reviewed by Hirsch et al., 1993), and the same mechanisms may have accounted for the decreased opsonisation observed.

It is important that all parameters are strictly controlled in this type of assay. As an example, an increased ratio between bacteria and neutrophils augmented both the number of ingesting neutrophils and their mean fluorescence (V). The optical density (OD) of bacterial suspensions was used to keep the concentrations of bacteria consistent (Asbury et al., 1984; Morris et al., 1987; LeBlanc and Pritchard, 1988), and by diluting bacteria, a linear relation with OD was confirmed. Counting of bacteria in a Bürker chamber gave less consistent results in our hands.

It has been reported that the ratios between bacteria and neutrophils can be assessed by counting the numbers of each in a certain volume (time period) in the flow cytometer (Bassøe et al., 2000). However, owing to the large difference in size between bacteria and neutrophils, logarithmic amplification of scatter parameters was required for visualisation of bacteria and neutrophils in the same plot. With this setting, signals from the majority of the neutrophils disappeared when data were transferred from the instrument to the computer system. The reason for this is unclear, but possible causes may involve logarithmic amplifiers, software bugs or incorrect operation of the instrument. When, instead, one cell type at a time was collected with the settings for each, correct results were obtained and ratios could be determined before and after incubation.

In contrast to the expected results, there were more extracellular bacteria per neutrophil after incubation than before (data not presented previously). This was due to a loss of neutrophils, which was also observed after incubation without bacteria, and is interpreted as an adherence of neutrophils to tube walls. There

was no loss of lymphocytes, and there were no traces of cell debris that would have implied lysis of neutrophils. Shaking the sample vigorously just before analysis and flushing the walls of the tube with the help of a pipette only succeeded in detaching some of these neutrophils. Samples with high percentages of phagocytosing neutrophils showed the greatest loss of neutrophils in the fluid phase, indicating that activation may also have led to increased adherence. To my knowledge, this problem has not been pointed out before and deserves to be elucidated, including the effect of the tube material.

Another finding in these experiments was that too high concentrations of bacteria gave false positive (fluorescent) neutrophils and lymphocytes. The reason for this is that at high concentrations or high speeds of flow, extracellular bacteria contaminate the volume analysed per leucocyte in the flow cytometer. To solve this problem, the fluorescence of extracellular bacteria can be quenched by quenching dyes such as trypan blue, or the flow rate can be decreased. However, using a correct ratio is probably a better solution. If these parameters are under control, it is our experience that a rapid and consistent way of measuring the phagocytosis of bacteria by FCM can be achieved.

In our experience, trypan blue was not effective in quenching the FITC-bacteria used in study V (data not presented before). Neither was staining with propidium iodide satisfactory. Both dyes can normally be used for detecting dead cells. Attempts to culture the bacteria on blood agar confirmed that the FITC- and formaldehyde-treated microorganisms were indeed non-viable despite the poor staining by these dyes. The reason for the poor staining remains unclear, but it may be an effect of the fixation method. In this study, formaldehyde fixation was chosen because it was considered as essential to keep the membrane antigens as intact as possible. This stabilisation of membrane proteins might decrease the diffusion of dye into the bacterial cells, as compared to other methods of fixation such as heat or ethanol treatment. With heat treatment of bacteria, quenching with trypan blue appears to work well (Raidal et al., 1998b). Other methods of labelling extracellular fluorescent target particles in phagocytosis have been described, such as the use of the quenching agent tannic acid (Giaimis et al., 1994), use of ethidium bromide (Raidal et al., 1998b), and dual-colour methods employing antibodies (Sveum et al., 1986), but the use of these with formaldehyde-fixed particles remains to be evaluated.

Preparation of blood

Blood was transported to the laboratory immediately after sampling, and the results were not altered by storage for up to 6 hours after sampling in pilot assays. Blood and prepared leucocytes were stored on ice in initial studies, but later they were kept at room temperature with similar results. The morphology of isolated neutrophils was slightly changed after ice cooling, and it was considered more physiological to avoid the large changes in environmental temperatures.

EDTA was used as an anticoagulant, but pilot assays showed that heparin-coated tubes are also suitable. Neutrophils from a suspension of leucocytes after erythrocytolysis with NH_4Cl buffer were used in the phagocytosis assays. Owing

to the capacity of FCM to distinguish between cell populations, it was not necessary to isolate the leucocytes further. Pilot studies showed good repeatability between samples prepared in this way, in accordance with results obtained with 0.2% NaCl for lysis of red blood cells (Paape and Miller, 1988). Hypotonic lysis with distilled water caused larger changes in the membrane phospholipid organisation than did lysis with NH_4Cl medium in one study (Van Oostvedt et al., 1999). Isolation by density sedimentation centrifugation increases neutrophil purity but may result in undesirable effects, such as decreased viability and increased background for CL assays (Hietala and Ardans, 1987b), selection of a cellular sub-population or modulation of receptor functions (Jacobsen et al., 1982; Fearon and Collins, 1983; Hed et al., 1987; Vachiéry et al., 1999), and other changes in the structure of the cell membrane (Van Oostvedt et al., 1999).

Methodology for opsonisation assays

To further study the characteristics of the coating of opsonins on yeast, a FCM immunoassay for opsonic coating both with complement C3 and with IgG was developed (IV). The results were compared with those from a phagocytosis assay, and the bound C3 fragments were released and characterised with SDS-PAGE and Western blotting. When complement opsonisation is of interest, an assay measuring the coating function is more valuable than measuring serum concentrations of the native proteins, or the haemolytic capacity of serum complement. The traditional haemolytic assay, on the other hand, reflects the final result of the full complement cascade reaction and involves many other steps and proteins following the initial activation and deposition.

The immunoassay of opsonic coating worked well, with a low variation. Worthy of note is the difference between the methods used for detection of C3 and IgG. A fluorophore-labelled antibody to C3 for detection in one single step worked well, whereas use of anti-IgG from several sources labelled with various fluorophores did not work in pilot studies, because of the low signals (data not shown). An amplification protocol was necessary. Thus the primary anti-IgG was biotinylated and fluorophore-labelled streptavidin was then applied, which provided sufficient signals. As there is no reason to believe that the affinity for IgG was low in all the tested antibodies, this suggests that the yeast cells are coated with much less IgG than C3. It is also consistent with the finding that inhibition of complement activation leads to a significant increase in IgG coating. The more abundant opsonin on the yeast cells would thus normally consist of complement.

The inhibitory effect of EDTA on complement activation in serum was shown at a concentration of 3.5 mM or higher, but no such effect was seen with <2 mM.

Phagocytosis and opsonisation in foals and adult horses

The methods developed were employed in three clinical studies, aimed at elucidating the capacity for neutrophil phagocytosis in foals of different ages and the impact of plasma and serum from foals and adult horses on this process (II, III, V).

The main finding was that opsonisation was the limiting factor for phagocytosis in foals. The foal neutrophils showed a mature capacity for phagocytosis of yeast when serum or plasma from adult horses or IgG was used as an opsonin (Table 2; II, III). The values obtained were similar to those in studies of adult horse neutrophils, or higher. The expression of CD18, a part of the C3b-receptor, in foal neutrophils was also similar to or higher than that of adult neutrophils (III).

However, serum from newborn foals showed no opsonic capacity in yeast assays before colostrum intake, and thereafter the capacity was low compared to that of adult serum (II, III, V). This ability increased with age ($p<0.05$), showed substantial individual variations, and reached the levels of adult horses after 3-4 weeks (Table 2; III, V).

An increase in serum opsonic capacity followed plasma treatment of foals (III). Individual foals showed a low serum opsonic capacity consistently over a period of time (III, V) and there were significant differences between plasma donors (II), suggesting that individual traits may be more important for the immunological defence against infections than age-related characteristics that are statistically evident in a large population. Low opsonic potentials of foal sera were observed with the use of foal as well as adult neutrophils, reinforcing the conclusion that there is an opsonic deficiency in the sera of some foals.

Precolostral serum did not induce phagocytosis of *E. coli* or *A. equuli* either, although after colostrum intake, this ability was restored in the group of foals studied to the level observed in adult serum or higher (V). The serum opsonic capacity for *E. coli* was better in foals 2-14 days old than at 28 days of age, but when *A. equuli* was used, there were no age-related changes in opsonic capacity in the foals studied.

Inactivation of serum complement resulted in significantly decreased phagocytosis of all microbes studied (II, IV, V).

The results from the present investigations were essentially in line with those from neutrophil phagocytosis studies in foals reported in the literature, which are summarised for comparison in Table 3 and discussed in the following. The investigations described in this thesis contribute to the accumulated knowledge with the presentation of more detailed temporal results. The same foals have been followed closely at many well-defined time-points, with studies of the functions of both neutrophils and sera, including precolostral sera. The results include percentages of phagocytic neutrophils as well as the mean uptake, which provides extended information. The serum opsonic capacity is compared with serum concentrations of IgG, which is the number one parameter used in

clinical practice to identify foals at risk for bacterial infections. Equine neutrophil phagocytosis of *E. coli* and *A. equuli*, the two most common isolates in foal sepsis, has not been reported on before. These studies should therefore provide information of relevance for clinical practice.

Age (days)	Farm A			Farm B			Adult horses	
Opsonisation with autologous serum								
2	51 ± 11	*	(n=6)	52 ± 6	**	(n=6)		
7	23 ± 5	***	(n=6)	66 ± 15		(n=5)		
14	72 ± 8		(n=6)	51 ± 10	**	(n=7)		
21	53 ± 14	*	(n=5)	85 ± 5		(n=7)		
28	63 ± 13		(n=6)	83 ± 6		(n=7)		
42	83 ± 5		(n=6)	80 ± 7		(n=7)		
56	85 ± 3		(n=6)	88 ± 2		(n=7)		
Adult							82 ±3	(n=16)
Opsonisation with pooled adult serum								
2	86 ± 2	††	(n=6)	86 ± 3	††	(n=7)		
7	75 ± 1		(n=3)	84 ± 3	†	(n=7)		
14	85 ± 3	††	(n=6)	81 ± 3		(n=6)		
21	87 ± 2	††	(n=4)	81 ± 3	†	(n=6)		
28	79 ± 5		(n=6)	84 ± 1	††	(n=7)		
42	80 ± 5		(n=6)	82 ± 3	†	(n=7)		
56	76 ± 3		(n=6)	86 ± 1	†††	(n=7)		
Adult							72 ± 2	(n=16)
Opsonisation with IgG								
2	50 ± 6	††	(n=6)	43 ± 5	††	(n=7)		
7	36 ± 10		(n=3)	38 ± 7		(n=7)		
14	44 ± 7	†	(n=6)	32 ± 8		(n=6)		
21	47 ± 7	†	(n=4)	37 ± 5		(n=6)		
28	35 ± 7		(n=6)	37 ± 5		(n=7)		
42	49 ± 3	††	(n=6)	42 ± 6	†	(n=7)		
56	31 ± 4		(n=6)	32 ± 3		(n=7)		
Adult							27 ± 3	(n=16)

* = p<0.05, ** = p<0.01, *** = p<0.001, significantly lower compared to adult horses
† =p<0.05, †† = p<0.01, ††† = p<0.001, significantly higher compared to adult horses

Table 2. Percentage numbers of neutrophils phagocytosing yeast (paper III). Means ± SEM.

The foal neutrophil per se

The results of these investigations confirmed the finding in most other studies using adult serum or immunoglobulins as an opsonin, that there were no differences in phagocytic capacity between foal and adult neutrophils (Couignoul et al., 1984; Bernoco et al., 1987; Martens et al., 1988; Wichtel et al., 1991; Flaminio et al., 2000). These other studies all applied different microorganisms as targets, namely yeast, *Rhodococcus equi*, *Streptococcus equisimilis* and *Staphylococcus aureus*, and various techniques, such as microscopy, bacteria killing assays, and FCM (Table 3), but led to the same conclusion.

However, there are also some divergent results. In the aforementioned report of Martens *et al* (1988), two out of 13 foals showed neutrophils with a clearly lower killing capacity for *R. equi* when sampled in the first week of life. In another study a lower uptake of *S. aureus* was found in neutrophils from 2-week-old foals than from adult horses when the bacteria was opsonised with autologous serum, i.e., from the same donor as the cells (Raidal, 2000). The phagocytosis by foal neutrophils did not improve when adult instead of foal serum was used in that study, implying that the decreased function had a cellular cause. However, in the same study the oxidative burst, stimulated by phorbol myristate acetate (PMA), was similar in adult and foal neutrophils.

Additional studies performed on the foals used in study III showed a lower CL response to PS-opsonised zymosan in the neutrophils from foals up to 2 weeks of age compared to those of older foals and from adult horses (Demmers et al., 2001). This low respiratory burst was observed at the same time as normal ingestion of yeast cells, pointing to the need for different techniques to evaluate the various neutrophil functions and to detect more subtle changes.

In contrast to those results, foal neutrophils have been reported to show better killing of *R. equi* compared to adult neutrophils (Yager et al., 1987). However, the two groups compared were reported in different papers and it is not clear whether they were assayed together.

It is possible that methodological issues account for the divergent results, and the experiments may have to be repeated to evaluate these findings further, including studies of the impact of foal serum on phagocytosis by adult cells.

Table 3 (next page). Summary of studies on neutrophil phagocytosis in foals. Abbreviations used: antistaph. ab = antistapylococcal antibodies; abs-PS = PS absorbed against the target particle; CFU = colony forming units; CL = chemiluminiscence; d = days; fluo = fluorescence; HI = heat inactivated; mo = months; ND = not defined; PMN = polymorphonuclear neutrophil; precol = precolostrum; PS = pooled adult serum; *S. aureus* = *Staphylococcus aureus; S. cerevisisae* = *Saccharomyces cerevisiae; S. equisimilis* = *Streptococcus equisimilis.*

	Morris *et al*, 1987	**Yager *et al*, 1987**	**Hietala+Ardans, 1987**	**Martens *et al*, 1988**	**Couignoul *et al*, 1984**	**Wichtel *et al*, 1991**
Foals, n and breed	n=8; ponies	n=8; ponies+mixed	n=10; mixed	n=10-13; ponies+QH	n=4, ponies	n=6; mixed
Foal ages	3-7 d (once)	2-14 weeks (once)	1-4 months (once)	1-8 d; 29-45 d (twice)	2-4 months (once)	0-7 months (7 periods)
Adult controls	n=8; dams	n=3; mixed breeds	Not used	n=13; dams	n=6; dams + others; mixed	n=28; mixed breeds
Method	CFU	CFU	Microscopy	Thymidium uptake	Radiolabelled bacteria	Microscopy
PMN preparation	Density gradient	Density gradient	Dextran sedimentation	Density gradient	Density gradient	Buffy coat
Target particle	*S. aureus*	*R. equi, S. aureus*	*R. equi*	*R. equi*	*S. aureus*	*S. equisimilis*
Opsonisation	5%; ND serum; direct	5%; PS; direct	Concentration ND; PS, PS HI, abs-PS; before	33%; adult serum; direct	Bovine antistaph. ab; direct	10%; PS; before
Opsonisation, time	(same as phag.)	(same as phag.)	20 min	(same as phag.)	(same as phag.)	30 min
Phagocytosis, time	3 h	15 min	0.75-6 h	0.5-2 h	45 min	30-90 min
Results	Uptake (bact/PMN) and killing similar in foals and mares	Fast and effective killing by foal PMNs, better than adult PMNs	Effective killing after 3 h with PS and PS HI, but not with abs-PS or without opsonisation	Similar mean killing by foal and adult PMNs, but 2/13 foals with lower killing capacity at first time-point.	Ingestion of bacteria similar in foal and adult PMNs.	Bacteria/PMN increased up to 113 d, killing decreased. No difference between foals and adult horses.

	Flaminio *et al*, 2000	**Raidal, 2000**	**Raidal, 2000**	**Bernoco *et al*, 1987**	**Hietala + Ardans, 1987**	**LeBlanc+Pritchard, 1988**
Foals, n and breed	n=12; mixed	n=8; not defined	n=5; not defined	n=6-11; mixed	n=20; mixed	n=9; mixed
Foal ages	0-4 months (7 periods)	0-2 d (5 times, of which 2 precolostral)	14 d (once)	Precol., 4-12 d, 1 mo, 2.5-5 mo (4 times)	0-6 months (biweekly)	0-28 d (4 times, of which 1 precolostral)
Adult controls	n=1; dam	ND; dams?	n=12; ND	n=6-11; dams+stallions	n=20; dams	n=2; mixed
Method	Flow cytometry	Flow cytometry	Flow cytometry	Microscopy	CL	CL
PMN preparation	Density gradient	Erythrocytolysis	Erythrocytolysis	Dextran sedimentation	Dextran sedimentation	Dextran sedimentation
Target particle	*S. aureus*	*S. aureus;* ox burst: PMA	*S. aureus;* ox burst: PMA	*S. cerevisiae* (yeast)	*R. equi*	*S. zooepidemicus*
Opsonisation	10-40%; adult serum; before	14%; foal or dam (?) serum; direct	14%; foal or dam? serum direct	40%; mare, foal or pooled plasma; before	16%; mare or foal sera; before	10%; foal or adult serum; before
Opsonisation, time	30 min	(same as phag.)	(same as phag.)	15 min	20 min	20 min
Phagocytosis, time	15 min	10-30 min	30 min	20 min	ND	2-25 min
Results	Ingestion (mean fluo/PMN) and oxidative burst similar in foals and mare. Low phagocytosis with 10% serum.	No change in % phag PMN in foal sera, or oxidative burst stimulated by PMA. Foal and mare PMNs not compared. Precolostral foal serum induced lower phagocytosis in mares.	% phag PMN similar with foal and adult serum in foal PMNs. Lower in foal PMNs than in adult PMNs with autologous sera. Ox burst stimulated by PMA similar in foal and adult PMNs.	Precolostral sera induced low numbers of yeast associated with foal and mare PMNs. Foal PMNs similar capacity to mare PMNs with PS.	Lower CL with foal than with adult sera in foal and adult PMNs. CL of foal PMNs and foal sera varied with age using *R. equi*, but not when using zymosan. CL dependent on IgG and complement.	CL of foal PMNs and adult sera varied with age. Foal sera stimulated lower CL than adult sera in adult PMNs. Precolostral foal sera induced lower CL in adults.

Table 3. Summary of studies on neutrophil phagocytosis in foals.

The opsonic capacity of foal serum

Reports on the opsonic capacity of serum from foals prior to ingestion of colostrum are sparse (Table 3), despite the widely accepted assumption of the benefit of colostrum for opsonisation and phagocytosis. In concert with our results for yeast, *E. coli* and *A. equuli* (V), precolostral serum induced very low phagocytosis of yeast by both adult and foal neutrophils compared to serum from older foals in the study of Bernoco *et al* (Bernoco et al., 1987). Similarly, low CL produced by adult neutrophils was observed with precolostral serum and *Streptococcus zooepidemicus* (LeBlanc and Pritchard, 1988). Precolostral sera also induced low phagocytosis of *S. aureus* by neutrophils from mares in the study of Raidal (2000). When foal cells were used, however, no difference was noted between the effect of serum from foals before and after colostral intake in that study, an unexpected finding that was not explained.

The obvious characteristic of precolostral foal serum is the absence of IgG antibodies, which probably is the main cause of the low opsonic activity observed by us and others. The influence of IgG on opsonisation is discussed further below.

Besides immunoglobulin transfer, colostrum intake might have transferred cellular activity as well as had an effect on the complement activity in the foals of the present study. These processes may together cause or contribute to the increase in opsonic capacity observed after colostrum ingestion.

The role of colostrum ingestion on the capacity for opsonisation with complement C3 in foals is not clear. Colostrum of women and cows contains a high concentration of C3, which is both opsonic and bactericidal for *E. coli*, but there is also complement-inhibiting activity (Reiter and Brock, 1975; Ogundele, 1999; Tregoat et al., 1999; Ogundele, 2000). Studies in foals and calves showed increasing serum haemolytic or bactericidal complement activity during the first period of life after ingestion of colostrum (Barta et al., 1972; Mueller et al., 1983; Bernoco et al., 1994). However, these changes were observed days later than the increase in serum IgG concentrations, suggesting that they are not due to passive transfer of complement factors. Opsonisation with C3 and its effect on phagocytosis are further discussed later in this thesis.

In addition to humoral factors such as antibodies and complement, colostrum contains functional leucocytes (Johnson et al., 1980; Bhaskaram and Reddy, 1981). These have been showed to enter the circulation and contribute to the cellular and humoral immunity of neonatal piglets (Williams, 1993), calves (Riedel-Caspari, 1993) and lambs (Tuboly et al., 1995). Similar studies have not been performed on horses, and the question whether or not colostral cells may influence the capacity for phagocytosis in foals remains to be elucidated.

In the present studies, the opsonic capacity of serum or plasma from foals even after colostrum ingestion was deficient compared to that of serum from adult horses in yeast assays (Table 2; II, III, V). This finding is in accordance with previous reports (Table 3) (Bernoco et al., 1987; Hietala and Ardans, 1987b; LeBlanc and Pritchard, 1988). As mentioned, we observed deficient opsonisation

of yeast up to 3-4 weeks of age, but not of *E. coli* or *A. equuli* (II, III, V). In the other studies *R. equi* or *S. zooepidemicus* were used as target particles in CL assays, or the number of yeast particles per neutrophil was evaluated by microscopy (Table 3). The influence of the age of the foals on the serum opsonic capacity was not clear from these reports.

In contrast to our results with yeast and *A. equuli*, and to results of studies using other bacteria, we found a better serum opsonic capacity for *E. coli* in foals 2-14 days old than at 28 days of age. The reasons for these age differences are not yet elucidated. If phagocytosis of this serum-resistant strain of *E. coli* is more dependent on opsonisation with IgG than with complement, the higher serum IgG in foals during the first two weeks of life may have provided the better opsonisation observed compared to that in older foals.

The roles of IgG and C3 in opsonisation

The differences in serum opsonic activity observed between foals before and after colostrum ingestion, during the first weeks of life, between foals and adult horses, and between adult plasma donors, warranted further studies on the opsonisation process.

The influence of IgG on opsonisation and phagocytosis

As low serum IgG is implicated as the main predisposing factor for infections in foals (McGuire et al., 1977; Clabough et al., 1991; Robinson et al., 1993), concentrations of IgG (III) or of the two major subisotypes IgGa and IgGb (V) in serum were compared with phagocytosis results. Similar studies on these targets were absent from the literature. Neither IgGa nor IgGb was detected in precolostral foal sera, but both peaked on day 2 (Fig. 6), coincident with a significant increase in opsonic activity. Nevertheless, there was no correlation between the concentrations of these immunoglobulins in serum from the foals and horses studied and the percentages of phagocytic neutrophils either in yeast or bacterial assays.

The mean serum IgG of all foals at the age of 2 days was 14.1 g/L in study III. During the 2-month study period, the mean serum IgG concentrations decreased to 7.2 g/L in the plasma-treated foals from Farm A and to 4.7 g/L in the foals from Farm B. In study V, there was more IgGb than IgGa in serum from foals up to 21 days of age, but because of the earlier start of endogenous production of IgGa, the reverse was found at 42 days (Fig. 6).

When, instead, serum samples were grouped on the basis of thresholds of IgGa or IgGb concentrations, significantly lower phagocytosis was observed when IgGa was below 0.9 g/L or IgGb was below 1.9 g/L in serum. This association was noted for IgGb and phagocytosis of yeast and *E. coli*, and for IgGa and yeast uptake. For *A. equuli*, no such associations were observed. Either IgG has no influence on opsonisation of this bacterium or, which is more likely, IgG was not a limiting factor in the postcolostral sera, which may have contained enough antibodies for phagocytosis under the conditions employed.

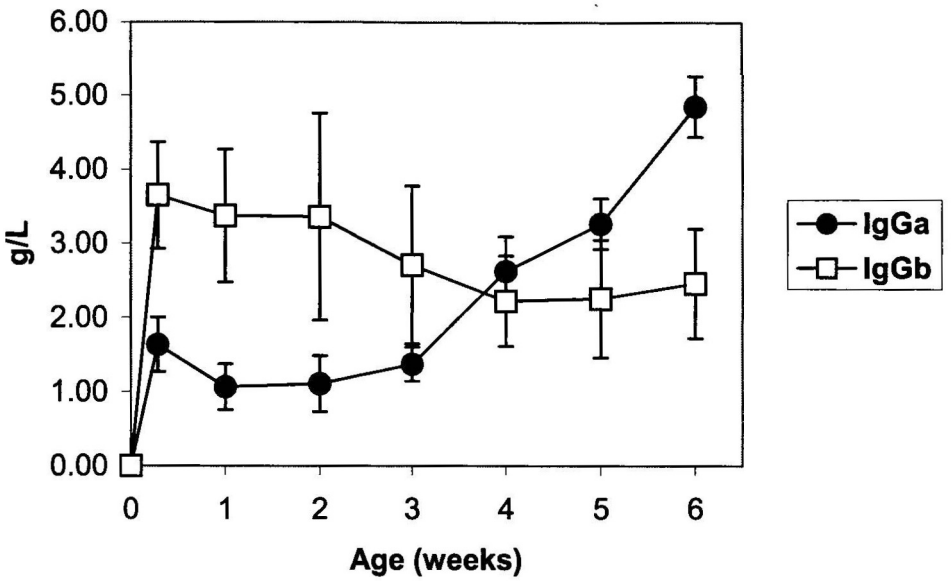

Figure 6. Serum concentrations of IgGa and IgGb in foals from birth to 6 weeks of age (paper V).

The results of the clinical studies indicated that IgG played a role in the opsonisation and phagocytosis of the microbes studied, but that other factors were probably also important. Further investigations are needed to clarify any influence of other immunoglobulins in serum, such as IgA or IgM, on the phagocytosis as well as the specificity of immunoglobulins. Results obtained after heat inactivation of serum and plasma suggested that complement was probably a significant opsonin. To further investigate the characteristics of IgG and C3 opsonisation, the coating of yeast by opsonins from serum was examined in study IV.

Results from the flow-cytometric immunoassay showed that there was indeed IgG on the yeast cells and that the levels were dependent on the concentration of serum used (IV). This finding is in contrast to the results of Hed (Hed and Stendahl, 1982) who considered that serum-opsonised yeast cells were coated with C3b only, as no anti-yeast activity was found by immunofluorescence in the sera used in their assays. In the present studies, IgG was detected at as low a serum concentration as 0.75%, and the fluorescence levels increased 15-fold with 90% serum. Substantial amounts of IgG were already deposited within the first minutes of incubation, but the values continued to increase over a 30-min period when 50% serum was used. Some results indicated that normally there is less IgG than complement C3 on serum-opsonised yeast cells, but quantitative experiments are required to confirm this.

A somewhat unexpected finding was that inhibition of C3 by heating or by addition of EDTA or EGTA enhanced the fluorescence indicating deposition of IgG three- to six-fold (IV). Spatial competition between C3 and IgG may account for this. Thus, more sites for antibody binding may be exposed when complement coating is diminished on the yeast surface. Masking of cell surface antigens by bound C3b and iC3b has previously been described in erythrocytes (Pangburn et al., 1983). *In vivo*, turning to more IgG opsonisation in a situation with only a small amount of complement would presumably be beneficial for maintenance of the phagocytic function.

Attempts to evaluate the impact of IgG opsonisation on neutrophil phagocytosis of yeast in the normal situation are not without technical difficulties. If complement activation in serum is inhibited in order to separate and study the effect of IgG, more IgG is deposited on the yeast cells, as discussed, compared to opsonisation with unaltered serum. This probably leads to an increased Fc-receptor-mediated phagocytosis in these samples. When experiments of this type were performed, the serum IgG was not sufficient for inducing phagocytosis at 1.5% serum, at which concentration unaltered serum induced phagocytosis in about half of the neutrophils (IV). At 6.25% complement-inactivated serum, there was suboptimal phagocytosis, with about half of the activity observed with intact serum. However, at an opsonising serum concentration of 50%, the phagocytic activity remained unchanged by complement inactivation, suggesting that IgG is sufficient for phagocytosis at these high levels.

These results should be interpreted with caution and the proportion or impact of IgG-mediated phagocytosis with unaltered serum can be expected to be lower. With human granulocytes, even though anti-yeast IgG (derived from immunised rabbits) can be a very efficient opsonin *in vitro* (Hed and Stendahl, 1982), it has been shown that normal human serum contains insufficient amounts of anti-yeast IgG for effective opsonisation (Cain et al., 1987). In the present studies (I, III), opsonisation of yeast with only 5% horse serum was at least two to three times more effective for equine neutrophil phagocytosis than using an abundance of rabbit anti-yeast IgG (0.1 g/L). These results, taken together with the results from our studies with complement-inactivated serum, suggest that the conclusion for human serum also holds true for horse serum. Similar studies should be carried out on the various equine pathogenic bacteria to gain additional knowledge.

To study the impact of IgG opsonisation further, experiments could be conducted with the help of serum after depletion of IgG, provided that this process does not harm the effect of other opsonins. For example, absorbing serum against the intended target particle can be used, but in this process complement activation must be controlled with, for example, EDTA, in order to achieve a serum with untampered complement factors. The use of specific antibodies to block the different opsonin receptors is another possible approach.

The influence of C3 on opsonisation and phagocytosis

Complement activation was found to be of critical importance to the equine neutrophil phagocytosis of yeast, *E. coli* and *A. equuli*. When serum complement was inactivated in the standard phagocytosis assays, phagocytosis of these microbes was reduced by more than half (paper V, $p<0.05$). Heat inactivation of plasma from two donors in study II resulted in over 90% reduction of the proportion of neutrophils phagocytosing yeast ($p<0.05$).

Further, in conditions with low concentrations of opsonins, such as with 1.5% serum, complement activation by the classical pathway is required for phagocytosis of yeast cells, as shown in study IV.

The C3 fragments from horse serum on yeast were separated by electrophoresis (SDS-PAGE) and characterised by Western blotting (Fig. 7; paper IV). Most of the C3 on yeast was in the form of iC3b, with a smaller amount of C3b. This is in accordance with the findings with yeast opsonised with human serum (Newman and Mikus, 1985; Cain et al., 1987). There were no signs of the small fragments C3dg or C3c, indicating that a further cleavage of iC3b had not occurred.

C3 opsonisation is a dynamic process, in which bound C3b is further cleaved to iC3b by factors I and H concurrently with the cleavage of more C3 to C3b by C3 convertase (Figs. 1 and 2) (Newman and Mikus, 1985). So, although alternative pathway activators such as yeast provide a protected environment for C3b that favours the binding of factor B over factor H (Pangburn and Müller-Eberhard, 1978; Kazatchkine et al., 1979), C3b may still be converted to iC3b to some degree. At high serum concentrations, when C3 will soon cover most of the activator surface, the environment changes so that it more resembles a non-activator of the alternative pathway (Pangburn et al., 1983). At this point more C3b can be converted to iC3b and this may participate in the deceleration of the initial rapid accumulation of C3 observed in study IV and discussed below.

The molecular weights of purified equine C3 fragments were in the same range as those previously described (Kay et al., 1986; Boschwitz and Timoney, 1993), and were approximately as follows: α-chain 100 kDa, β-chain 70 kDa, the heavy iC3b fragment 68 kDa, and the light iC3b fragment 45 kDa (Figs. 2 and 7). The equine and human C3 fragments were of similar size. The molecular weights should preferably be evaluated from purified samples, as shown by the difference in pattern between native serum (lane 3) and serum that was activated and purified (lane 2) in Figure 7. In the native sample, other proteins such as albumin distort the picture, and are the reason why the heavy iC3b fragment appears as if it is pushed further down in the gel in lane 3.

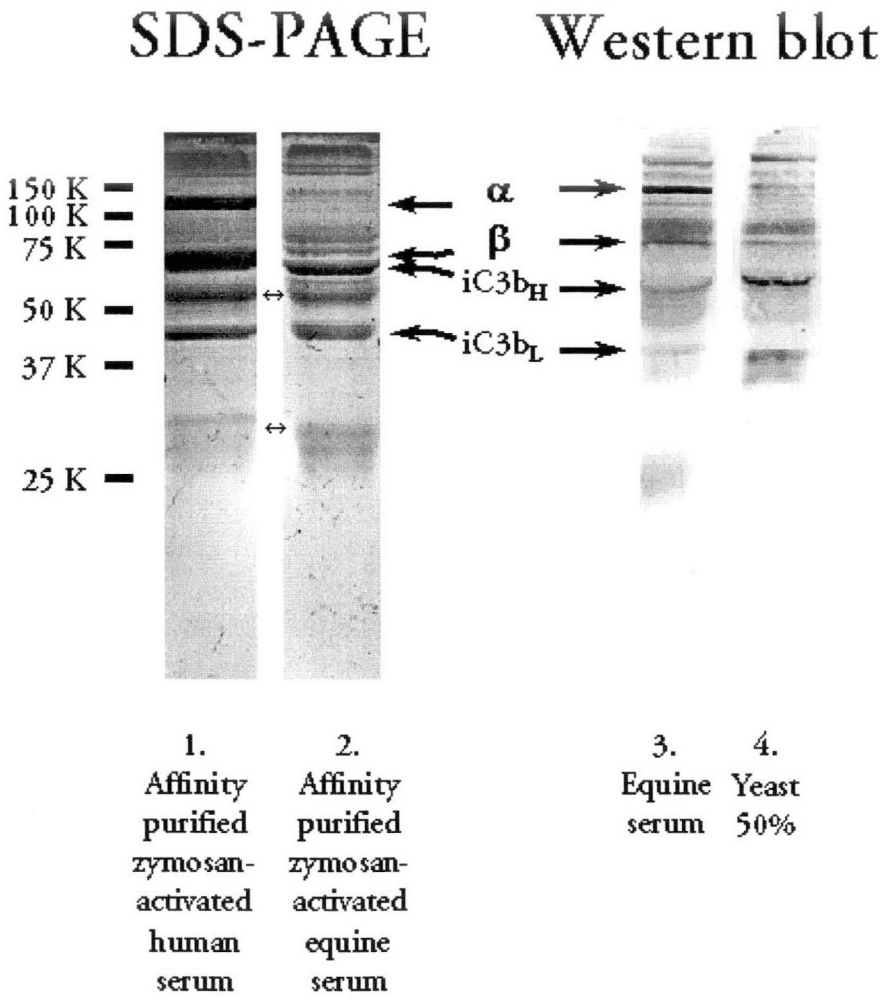

Figure 7. Agarose gel electrophoresis (SDS-PAGE, lanes 1-2) and Western blotting analyses (using anti-equine C3, lanes 3-4) of serum and C3 fragments from yeast (paper IV). Lanes 1 and 2 show affinity-purified C3 fragments from human and equine serum activated with zymosan. Lane 3 shows intact equine serum and lane 4 displays C3 fragments eluted from yeast opsonised with 50% equine serum for 15 min. The locations for equine and human C3 fragments are indicated. The small arrows between lanes 1 and 2 indicate the locations for heavy and light chains of the antibody used for purification of the sample.

C3 was not activated in the frozen and thawed native serum used in the studies, as shown by the integrity of the α-chain in such samples. This demonstrates that frozen equine serum samples can be used accurately in studies of complement functions, a question that has sometimes been raised.

Complement activation and deposition of C3 on yeast cells occurred as a result of activation by both the classic and alternative pathway and was dependent on the serum concentration (Fig. 8a). Activation of the complement cascade was necessary and there was no passive adsorption of C3 to the yeast surface, as shown by the fact that C3 deposition was effectively inhibited both by the addition of EDTA and by heating the serum to 56°C for 30 min.

Selective inhibition of the classical pathway by EGTA was useful in evaluating the potential for each of the two main pathways (Fig. 8). In conditions with a limited supply of opsonic factors, such as when low serum concentrations or short incubations are used, C3 deposition is dependent on classical pathway activity. However, when the serum concentration is high or when the incubation time is long, the alternative pathway alone can provide for maximal C3 deposition (Fig. 8).

In the studies of the kinetics of the alternative pathway, a 15 min delay or lag preceding the rapid C3 accumulation was observed, compared to serum without inhibition, which resulted in high C3 deposition as early as at 2 min, and increasing to a plateau at 15 min (Fig. 8). These results would explain the importance of IgG in a clinical situation, in that IgG significantly potentiates C3 deposition by initiating the classical pathway, resulting in C3 deposition at an earlier stage or at lower serum concentrations. Thus, IgG and C3 work synergistically in opsonisation and facilitation of phagocytosis.

The lag period observed with the alternative pathway can be ascribed to the slow spontaneous formation of metastable C3b and its random attachment to activator particles, followed by amplification by the alternative C3 convertase. Similar kinetics has been reported from studies of human serum and zymosan (Pangburn, 1983), and also from examinations of *Cryptococcus neoformans*, where it was shown that deposition of C3 by the alternative pathway is initially focal and expands over the surface from these sites (Kozel et al., 1991). In contrast, classical pathway activation in that study caused a simultaneous and synchronous initiation of C3 accumulation over the entire cell wall.

The plateau observed in the kinetics of C3 deposition may be interpreted as a result of a steady-state between the rates of three processes: the deposition of C3b, the conversion of C3b to iC3b and the release of C3b and iC3b (Pangburn, 1983; Newman and Mikus, 1985). The analysis of deposited C3 fragments showed that there was a rapid conversion from C3b to iC3b. This may explain the abrupt termination in amplification, as iC3b cannot be used in the formation of C3

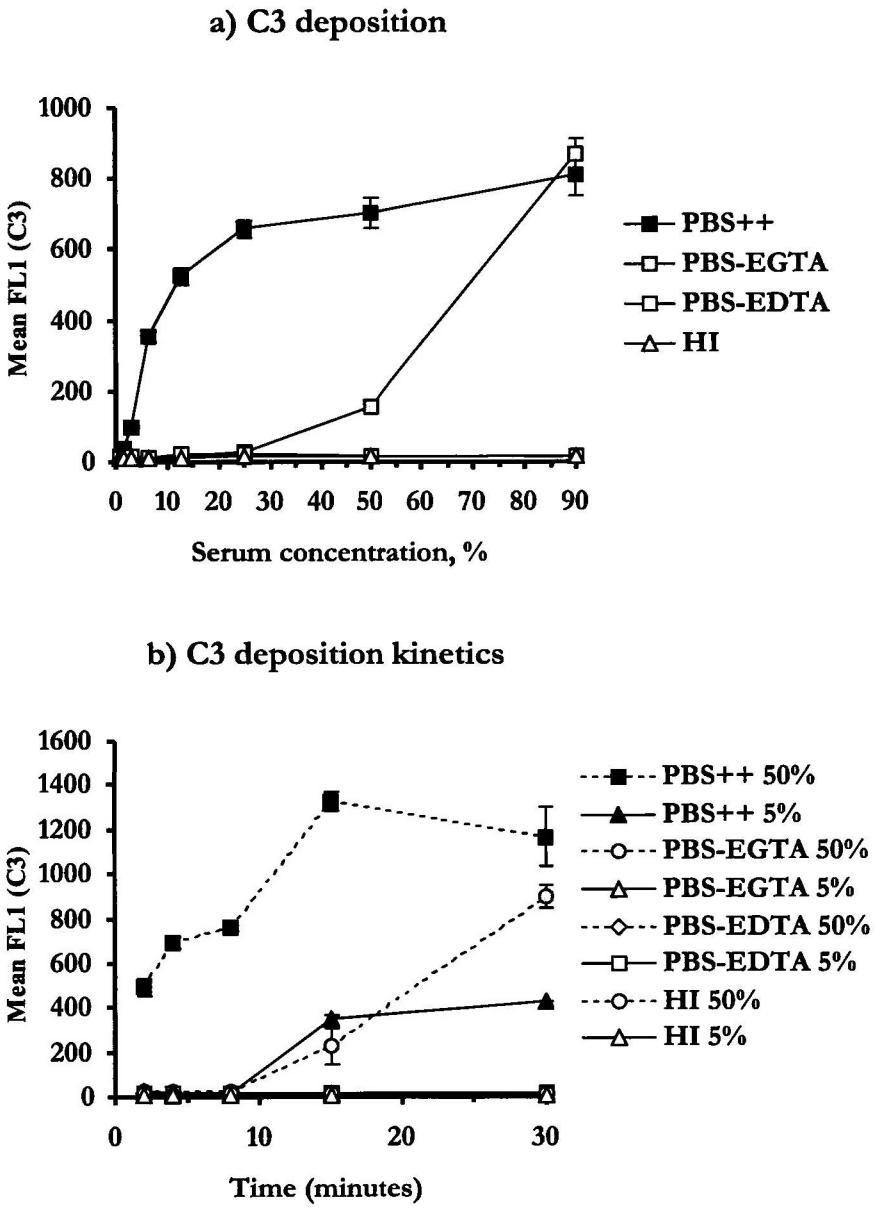

Figure 8. Deposition of C3 on yeast particles. *a)* The effects of the serum concentration and complement inactivation. Samples were opsonised for 15 min. *b)* The kinetics of deposition of C3 with and without complement inactivation. Samples were opsonised with 5 or 50% serum as indicated. The serum was untreated or heat-inactivated (HI). Buffers used were PBS with the addition of Ca^{2+} and Mg^{2+} (PBS++), 10 mM EDTA (PBS-EDTA) for inactivation of complement activation, or 10 mM EGTA (PBS-EGTA) for inactivation of only the classical pathway of complement activation. Error bars represent SEM.

convertase. Consumption of complement factors may also account for the occurrence of the plateau, when the serum:particle ratio is low.

The observed ability for phagocytosis at very low opsonic serum concentrations (1.5%) is important for an efficient host defence, e.g. extravascular phagocytosis at infection sites. The characteristics of complement activation and opsonisation of microorganisms may differ between species and subspecies. Increased knowledge of the requirements for opsonisation of the various equine pathogenic bacteria by C3 is desirable, as some bacterial virulence factors interfere with C3 activation, opsonisation and phagocytosis (Barrio et al., 2000)

General summary and conclusions

- Accurate and reproducible flow cytometric methods were established for equine neutrophil phagocytosis of the yeast fungus *Saccharomyces cerevisiae* and the bacteria *Escherichia coli* and *Actinobacillus equuli*, two important pathogens in foals.

- Opsonisation is required for phagocytosis of these microbes, and phagocytosis is observed already at low serum concentrations (<3%) for yeast and *A. equuli*, but several-fold higher serum concentrations are required for the serum-resistant *E. coli* strain studied. Opsonisation and attachment of yeast cells to neutrophils are fast processes and are rapidly followed by ingestion. The capacity for phagocytosis at very low opsonic concentrations is presumed to be especially important for extravascular phagocytosis at infection sites.

- Passive transfer of immunity by colostrum is required in newborn foals to achieve a serum opsonic capacity for all the microbes studied. Very low serum concentrations of IgG or IgGb in foals after colostral ingestion are associated with decreased phagocytosis of yeast and *E. coli*, but not of *A. equuli*.

- Complement activation is required for optimal phagocytosis of all the microbes studied. Classical pathway activation is necessary to achieve phagocytosis of yeast cells at low concentrations of opsonins (1.5% serum), and it is required for C3 deposition on yeast if <50% serum is used. At higher serum concentrations, the alternative pathway is able alone to provide sufficient amounts of C3. The main form of C3 on yeast cells is iC3b and the rest is C3b. The equine C3 fragments have similar molecular weights as their human equivalents.

- Transfusion of adult plasma to healthy foals at 7 days of age results in an increased capacity for yeast opsonisation and phagocytosis. Plasma from individual horses has different opsonic capacities, regardless of IgG concentrations.

- Serum from foals up to 3-4 weeks of age shows a lower capacity to opsonise yeast, whereas opsonisation of *E. coli* is enhanced compared to serum from older foals and horses. No age-related differences were found in serum opsonisation of *A. equuli*. Newborn foals show a large individual variation in serum opsonic capacity, which partly depend on their abilities for complement activation. The neutrophil phagocytosis capacity *per se* is similar in foals and adult horses, and the expression of complement receptor CD18 is also similar in the foals, or higher.

Suggestions for future studies

The roles of C3 and IgG in opsonisation of yeast and bacteria by serum from newborn foals should be studied further. This may be achieved by quantifying these opsonins on target particles with the flow cytometric immunoassay presented in this thesis.

The concentrations of various complement factors and other serum factors, e.g, collectins, involved in complement activation should also be quantified in foal and adult horse serum. The impact of specific antibodies on phagocytosis needs to be further evaluated, especially in view of the fact that hyperimmunised plasma (with high specific titres to, for example, *R. equi* or *E. coli* antigens) is marketed heavily in some countries. Possible serum factors that are suspected to be deficient can be tested by titration to restore the function of foal sera with low opsonic capacity.

The opsonic requirements for common species of pathogenic bacteria should be further elucidated. There might also be differences between various strains of the same bacteria. The opsonic capacity of foal sera for other foal pathogens is currently being investigated by our group.

The effect of intravenous plasma treatment on opsonisation and phagocytosis in healthy and sick equine neonates needs further attention. The optimal plasma for such treatments has not yet been characterised and the potential of the different constituents is still unclear.

The involvement of complement activation deserves to be studied further in many equine conditions where inflammation is present. These include common problems recognised by every veterinary clinician, as the horse seems to be a rather sensitive animal compared to most other species.

Some diagnoses where studies of this type would be warranted include aseptic and septic synovitis, tendinitis, rhabdomyolysis ("tying-up"), laminitis, pleuropneumonia, small airway disease, uveitis, thrombophlebitis, immune-mediated vasculitis, lymphangitis, colitis and endotoxaemia. These are all common conditions in horses and cause considerable distress, sometimes necessitating euthanasia.

A better understanding of the equine complement system may result in improved prophylactic and therapeutic treatment regimens in foals and adult horses.

References

Abbas, A. K., Lichtman, A. H., and Pober, J. S. (1997). Cellular and molecular immunology, 3 edn (Philadelphia, W B Saunders Company).

Arnaout, M., Todd III, R., Dana, N., Melamed, J., Schlossman, S., and Colten, H. (1983). Inhibition of phagocytosis of complement C3- or Immunoglobulin G-coated particles and of C3bi binding by monoclonal antibodies to a monocyte-granulocyte membrane glycoprotein (Mo1). *J. Clin. Invest.* **72**, 171-179.

Asbury, A. C., Gorman, N. T., and Foster, G. W. (1984). Uterine defense mechanisms in the mare: Serum opsonins affecting phagocytosis of *Streptococcus zooepidemicus* by equine neutrophils. *Theriogenology* **21**, 375-385.

Babior, B. M. (2000). Phagocytes and oxidative stress. *Am. J. Med.* **109**, 33-44.

Balson, G. A., Smith, G. D., and Yager, J. A. (1997). Immunophenotypic analysis of foal bronchoalveolar lavage lymphocytes. *Vet. Microbiol.* **56**, 237-246.

Banks, K., and McGuire, T. (1975). Surface receptors on neutrophils and monocytes from immunodeficient and normal horses. *Immunology* **28**, 581-588.

Barrio, B., Vangroenweghe, F., Dosogne, H., and Burvenich, C. (2000). Decreased neutrophil bactericidal activity during phagocytosis of a slime-producing *Staphylococcus aureus* strain. *Vet. Res.* **31**, 603-609.

Barta, O., and Barta, V. (1976). The equine complement system. Paper presented at: International Conference on equine diseases IV.

Barta, O., Barta, V., and Ingram, D. G. (1972). Postnatal development of bactericidal activity in serum from conventional and colostrum-deprived calves. *Am. J. vet. Res.* **33**, 741-750.

Barta, O., Barta, V., and Williams, E. I. (1973). A method for titrating equine haemolytic complement. *Z. Immun.-Forsch.* **146**, 114-122.

Bassøe, C.-F., Smith, I., Sørnes, S., Halstensen, A., and Lehmann, A. K. (2000). Concurrent measurement of antigen- and antibody-dependent oxidative burst and phagocytosis in monocytes and neutrophils. *Methods* **21**, 203-220.

Bernoco, M. M., Liu, I. K. M., and Willits, N. H. (1994). Hemolytic complement activity and concentrations of its third component during maturation of the immune response in colostrum-deprived foals. *Am. J. vet. Res.* **55**, 928-933.

Bernoco, M. M., Liu, K. M., Wuest-Ehlert, C. J., Miller, M. E., and Bowers, J. (1987). Chemotactic and phagocytic function of peripheral blood polymorphonuclear leococytes in newborn foals. *J. Reprod. Fertil.* **Suppl. 35**, 599-605.

Bhaskaram, P., and Reddy, V. (1981). Bactericidal activity of human milk leukocytes. *Acta Paed. Scand.* **70**, 87-90.

Bjerknes, R., and Bassøe, C. F. (1984). Phagocyte C3-mediated attachment and internalization: Flow cytometric studies using a fluorescence quenching technique. *Blut* **49**, 315-323.

Boschwitz, J. S., and Timoney, J. F. (1993). Purification and characterization of equine complement factor C3. *Vet. Immun. Immunopath.* **38**, 139-153.

Boschwitz, J. S., and Timoney, J. F. (1994). Inhibition of C3 deposition on *Streptococcus equi* subsp. *equi* by M protein: a mechanism for survival in equine blood. *Infect. Immun.*, 3515-3520.

Bowen, T., Ochs, H., Altman, L., Price, T., Van Epps, D., Brautigan, D., Rosin, R., Perkins, W., Babior, B., Klebanoff, S., and Wedgwood, R. (1982). Severe recurrent bacterial infections associated with defective adherence and chemotaxis in two patients with neutrophils deficient in a cell-associated glycoprotein. *J. Ped.* **101**, 932-940.

Båverud, V. (2001). Foal septicaemia- bacterial findings and resistance patterns in Sweden (Course material) (Uppsala).

Cain, J., Newman, S., and Ross, G. (1987). Role of complement receptor type three and serum opsonins in the neutrophil response to yeast. *Complement* **4**, 75-86.

Chaffin, M. K., and Cohen, N. D. (1998). Randomized controlled trial of effects of *Escherichia coli* antiserum on serum immunoglobulin G concentrations and morbidity and mortality rates in foals. *J. Am. vet. med. Ass.* **212**, 1746-1750.

Chaffin, M. K., Martens, R. J., Martens, J. G., and Fiske, R. A. (1991). Therapeutic effects of immune plasma in foals with *Rhodococcus equi* pneumonia. *Equine vet. J.*, 23-29.

Clabough, D. L. (1988). Diseases of the equine neonate. *Eq. Vet. Sci.* **8**, 5-10

Clabough, D. L., Levine, J. F., Grant, G. L., and Conboy, H. S. (1991). Factors associated with failure of passive transfer of colostral antibodies in Standardbred foals. *J. vet. internal Med.* **5**, 335-340.

Cohen, N. D. (1994). Causes of and farm management factors associated with disease and death in foals. *J. Am. vet. med. Ass.* **204**, 1644-1651.

Couignoul, F. L., Bertram, T. A., Roth, J. A., and Cheville, N. F. (1984). Functional and ultrastructural evaluations of neutrophils from foals and lactating and non-lactating mares. *Am. J. vet. Res.* **45**, 898-902.

Crowley, C., Curnutte, J., Rosin, R., André-Schwartz, B., Gallin, J., Klempner, M., Snyderman, R., Southwick, F., Stossel, T., and Babior, B. (1980). An inherited abnormality of neutrophil adhesion: Its genetic transmission and its association with a missing protein. *N. Engl. J. Med.* **302**, 1163-1168.

Dahlgren, C., and Karlsson, A. (1999). Respiratory burst in human neutrophils. *J. Immunol. Methods* **232**, 3-14.

Demmers, S., Johannisson, A., Gröndahl, G., and Jensen-Waern, M. (2001). Neutrophil functions and serum IgG in growing foals. *In manuscript.*

Dosogne, H., Capuco, A. V., Paape, M. J., Roets, E., Burvenich, C., and Fenwick, B. (1998). Reduction of acyloxyacyl hydrolase activity in circulating neutrophils from cows after parturition. *J. Dairy Sci.* **81**, 672-677.

Eckle, I., Reifer, R., Hoferichter, A., Kolb, G., and Havemann, K. (1990). High affinity binding of immunoglobulin G fragments to human polymorphonuclear leukocytes. *Biol. Chem. Hoppe Seyler* **371**, 1107-1111.

Ehlers, M. R. W. (2000). CR3: a general purpose adhesion-recognition receptor essential for innate immunity. *Microbes and Infection* **2**, 289-294.

Engström, C., ed. (1994). Nationalencyklopedin (Höganäs, Bokförlaget Bra Böcker AB).

Fearon, D., and Collins, L. (1983). Increased expression of C3b receptors on polymorphonuclear leukocytes induced by chemotactic factors and by purification procedures. *J. Immun.* **130**, 370-375.

Ferriani, V. P., Barbosa, J. E., and de Carvalho, I. F. (1990). Serum haemolytic classic and alternative pathways of complement in infancy: age-related changes. *Acta Paed. Scand.* **79**, 322-327.

Fine, D., Marney, S., Colley, D., Sergent, J., and Des Prez, R. (1972). C3 shunt activation in human serum chelated with EGTA. *J. Immun.* **109**, 807-809.

Flaminio, M. J. B. F., Rush, B. R., Davis, E. G., Hennessy, K., Schuman, W., and Wilkerson, M. J. (2000). Characterization of peripheral blood and pulmonary leukocyte function in healthy foals. *Vet. Immun. Immunopath.* **73**, 267-285.

Foerster, R. J., and Wolf, G. (1990). Phagocytosis of opsonized fluorescent microspheres by equine polymorphonuclear leukocytes. *J. vet. Med. B* **37**, 481-490.

Fällman, M., Andersson, R., and Andersson, T. (1993). Signaling properties of CR3 (CD11b/CD18) and CR1 (CD35) in relation to phagocytosis of complement-opsonized particles. *J. Immun.* **151**, 330-338.

Gemmell, C. (1997). A flow cytometric immunoassay to quantify adsorption of complement activation products (iC3b, C3d, SC5b-9) on artificial surfaces. *J Biomed. Mater Res.* **37**, 474-480.

Giaimis, J., Lombard, Y., Poindron, P., and Muller, C. D. (1994). Flow cytometry distinction between adherent and phagocytized yeast cells. *Cytometry* **17**, 173-178.

Giguère, S., and Prescott, J. F. (2000). Equine immunity to bacteria. *Vet. Clin. North Am. Equine Pract.* **16**, 29-47.

Grant, C. K. (1977). Complement "specificity" and interchangeability: measurement of hemolytic complement levels and use of the complement-fixation test with sera from common domesticated animals. *Am. J. vet. Res.* **38**, 1611-1617.

Hall, R., Strugnell, T., Wu, X., Devine, D., and Stiver, H. (1993). Characterization of kinetics and target proteins for binding of human complement component C3 to the surface-exposed outer membrane of *Chlamydia trachomatis* serovar 12. *Infect. Immun.* **61**, 1829-1834.

Hampton, M. B., Kettle, A. J., and Winterbourn, C. C. (1998). Inside the neutrophil phagosome: Oxidants, myeloperoxidase, and bacterial killing. *Blood* **92**, 3007-3017.

Hampton, M. B., and Winterbourn, C. C. (1999). Methods for quantifying phagocytosis and bacterial killing by human neutrophils. *J. Immunol. Methods* **232**, 15-22.

Hampton, M. B., Vissers, M. C. M., and Winterbourn, C. C. (1994). A single assay for measuring the rates of phagocytosis and bacterial killing by neutrophils. *J. Leukocyte Biol.* **55**, 147-152.

Hed, J. (1977). The extinction of fluorescence by crystal violet and its use to differentiate between attached and ingested microorganisms in phagocytosis. *FEMS Microbiology Letters* **1**, 357-361.

Hed, J. (1979) Studies on phagocytosis by human polymorphonuclear leukocytes using a new assay which allows distinction between attachment and ingestion, Medical dissertation (PhD), Linköping University, Linköping.

Hed, J., Johansson, S., and Larsson, P. (1987). The use of fluorescence quenching in flow cytofluorometry to measure the attachment and ingestion phases in phagocytosis in peripheral blood without prior cell separation. *J. Immunol. Methods* **101**, 119-125.

Hed, J., and Stendahl, O. (1982). Differences in the ingestion mechanisms of IgG and C3b particles in phagocytosis by neutrophils. *Immunology* **45**, 727-736.

Hietala, S. K., and Ardans, A. A. (1987a). Interaction of *Rhodococcus equi* with phagocytic cells from R. equi-exposed and non-exposed foals. *Vet. Microbiol.* **14**, 307-320.

Hietala, S. K., and Ardans, A. A. (1987b). Neutrophil phagocytic and serum opsonic response of the foal to *Corynebacterium equi*. *Vet. Immun. Immunopath.* **14**, 279-294.

Hirsch, D. C., Kirkham, C., and Wilson, W. D. (1993). Characteristics of Escherichia coli isolated from septic foals. *Vet. Microbiol.* **34**, 123-130.

Hoglund, M., Hakansson, L., and Venge, P. (1997). Effects of in vivo administration of G-CSF on neutrophil functions in healthy volunteers. *Eur. J. Haematol.* **58**, 195-202.

Holmskov, U. L. (2000). Collectins and collectin receptors in innate immunity. *APMIS* **108, Suppl. 100**, 5-59.

Iovine, N. M., Elsbach, P., and Weiss, J. (1997). An opsonic function of the neutrophil bactericidal/permeability-increasing protein depends on both its N- and C-terminal domains. *Proc. Natl. Acad. Sci. USA* **94**, 10973-10978.

Jacobsen, K., Gintz, T., Reed, S. M., Newbry, J., Bayly, W. M., Perryman, L. E., and Leid, R. W. (1982). Isolation of equine neutrophils and analysis of functional characteristics by chemiluminiscence and bactericidal assays. *Am. J. vet. Res.* **43**, 1912-1916.

Jensen-Waern, M., Johannisson, A., Trowald-Wigh, G., and Ederoth, M. (1994). Methods for evaluation of the adhesive and phagocytic capacities of porcine granulocytes. *J. vet. Med. B* **42**, 625-.

Jensen-Waern, M., Lindberg, Å., Johannisson, A., Gröndahl, G., Lindgren, J., and Essén-Gustavsson, B. (1999). The effects of an endurance ride on metabolism and neutrophil function. *Equine vet. J.* **Suppl. 30**, 605-609.

Johnson, D. F., France, G. L., Marmer, D. J., and Steele, R. W. (1980). Bactericidal mechanisms of human breast milk leukocytes. *Infect. Immun.* **28**, 314-318.

Kay, P., Dawkins, R., Bowling, A., and Bernoco, D. (1986). Electrophoretic polymorphism and molecular structure of equine C3. *Anim. Genet.* **17**, 209-215.

Kazatchkine, M., Fearon, D., and Austen, K. (1979). Human alternative complement pathway: membrane-associated sialic acid regulates the competition between B and β1H for cell-bound C3b. *J. Immun.* **122**, 75-81.

Kehrli, M. J., Schmalstieg, F., Anderson, D., van der Maaten, M., Hughes, B., Ackermann, M., Wilhelmsen, C., Brown, G., Stevens, M., and Whetstone, C. (1990). Molecular definition of the bovine granulocytopathy syndrome: Identification of deficiency of the Mac-1 (CD11b/CD18) glycoprotein. *Am. J. vet. Res.* **51**, 1826-1836.

Kishore, R. (1981). Radiolabeled microorganisms: comparison of different radioisotopic labels. *Rev. Infect. Dis.* **3**, 1179-1185.

Kohn, C. W., Knight, D., Hueston, W., Jacobs, R., and Reed, S. M. (1989). Colostral and serum IgG, IgA, and IgM concentrations in Standardbred mares and their foals at parturition. *J. Am. vet. med. Ass.* **195**, 64-68.

Koterba, A., Brewer, B., and Tarplee, F. (1984). Clinical and clinicopathological characteristics of the septicaemic neonatal foal: Review of 38 cases. *Equine vet. J.* **16**, 376-383.

Kozel, T., Highison, B., and Stratton, C. (1984). Localization on encapsulated *Cryptococcus neoformans* of serum components opsonic for phagocytosis by macrophages and neutrophils. *Infect. Immun.* **43**, 574-579.

Kozel, T., and Pfrommer, G. (1986). Activation of the complement system by *Cryptococcus neoformans* leads to binding of iC3b to the yeast. *Infect. Immun.* **52**, 1-5.

Kozel, T., Wilson, M., and Murphy, J. (1991). Early events in initiation of alternative complement pathway activation by the capsule of *Cryptococcus neoformans*. *Infect. Immun.* **59**, 3101-3110.

Kozel, T. R. (1996). Activation of the complement system by pathogenic fungi. *Clinical Microbiology Reviews* **9**, 34-46.

Krediet, T., Beurskens, F., van Dijk, H., Gerards, L., and Fleer, A. (1998). Antibody responses and opsonic activity in sera of preterm neonates with coagulase-negative staphylococcal septicemia and the effect of the administration of fresh frozen plasma. *Pediatr. Res.* **43**, 645-651.

Kuypers, T. W., Eckmann, C. M., Weening, R. S., and Roos, D. (1989). A rapid turbidimetric assay of phagocytosis and serum opsonizing capacity. *J. Immunol. Methods* **124**, 85-94.

Lavoie, J.-P., Spensley, M. S., Smith, B. P., Bowling, A. T., and Morse, S. (1989). Complement activity and selected hematologic variables in newborn foals fed bovine colostrum. *Am. J. vet. Res.* **50**, 1532-1536.

LeBlanc, M. M., and Pritchard, E. L. (1988). Effects of bovine colostrum, foal serum immunoglobulin concentration and intravenous plasma transfusion on chemiluminiscence response of foal neutrophils. *Anim. Genet.* **19**, 435-445.

Lehmann, A. K., Sørnes, S., and Halstensen, A. (2000). Phagocytosis: measurement by flow cytometry. *J. Immunol. Methods* **243**, 229-242.

Lehrer, R. I., and Ganz, T. (1999). Antimicrobial peptides in mammalian and insect host defence. *Current Opinion in Immunology* **11**, 23-27.

Leid, R. W., Coley, S. C., Blanchard, D., and Perryman, L. (1985). Equine alternative pathway activation by unsensitized rabbit red blood cells. *Vet. Immun. Immunopath.* **9**, 71-85.

Liddell, H. G., and Scott, R. (1996). A Greek-English Lexicon (Oxford, Clarendon Press).

Lindahl, G., Sjöbring, U., and Johnsson, E. (2000). Human complement regulators: a major target for pathogenic microorganisms. *Current Opinion in Immunology* **12**, 44-51.

Lundh, B. (1992). Hematologi. In Nationalencyklopedin, K. Marklund, ed. (Höganäs, Bokförlaget Bra Böcker AB), p. 522.

Lunn, D., Holmes, M., and Duffus, P. (1993). Equine T-lymphocyte MHC II expression: variation with age and subset. *Vet. Immun. Immunopath.* **35**, 225-238.

Machangú, R., and Prescott, J. (1991). Role of antibody to extracellular proteins of *Rhodococcus equi* in protection against *R. equi* pneumonia in foals. *Vet. Microbiol.* **26**, 323-333.

Magnusson, U., and Jonsson, K. (1991). A method for the accurate measurement of opsonic activity in uterine secretions of the mare. *Theriogenology* **36**, 737-747.

Martens, J. G., Martens, R. J., and Renshaw, H. W. (1988). *Rhodococcus (Corynebacterium) equi:* Bactericidal capacity of neutrophils from neonatal and adult horses. *Am. J. vet. Res.* **49**, 295-299.

Martens, J. G., Martens, R. J., Renshaw, H. W., and Hietala, S. K. (1987). *Rhodococcus equi*: Equine neutrophil chemiluminescent and bacterial responses to opsonizing antibody. *Vet. Microbiol.* **14**, 277-286.

Martens, R. J., Martens, J. G., and Fiske, R. A. (1991). Failure of passive immunisation by colostrum from immunised mares to protect foals against *Rhodococcus equi* pneumonia. *Equine vet. J.* **Suppl. 12**, 19-22.

Martin, E., and Bhakdi, S. (1992). Flow cytometric assay for quantifying opsonophagocytosis and killing of Staphylococcus aureus by peripheral blood leukocytes. *Journal of Clinical Microbiology* **30**, 2246-2255.

Matsson, P., Fossum, C., and Larsson, B. (1985). Evaluation of flow cytometry and fluorescence microscopy for the estimation of bovine mononuclear phagocytes. *J. Immunol. Methods* **78**.

McGuire, T., Henson, J., and Burger, D. (1969). Complement (C'_3)-coated red blood cells following infection with the virus of equine infectious anemia. *J. Immun.* **103**, 293-299.

McGuire, T. C., Crawford, T. B., Hallowell, A. L., and Macomber, L. E. (1977). Failure of colostral immunoglobulin transfer as an explanation for most infections and deaths of neonatal foals. *JAVMA* **170**, 1302-1304.

McGuire, T. C., Poppie, M. J., and Banks, K. L. (1975). Hypogammaglobulinemia predisposing to infection in foals. *JAVMA* **166**, 71-75.

Metchnikoff, E. (1883). Untersuchungen über die intracelluläre verdauung beim wirbellosen thieren (stavn?). *Arb Zoologischen Inst Univ Wien* **5**, 141.

Morris, D. D., Gaulin, G., Strzeminski, P. J., and Spencer, P. (1987). Assessment of neutrophil migration, phagocytosis and bactericidal capacity in neonatal foals. *Vet. Immun. Immunopath.* **16**, 173-184.

Morris, D. D., Whitlock, R. H., and Corbeil, L. B. (1986). Endotoxemia in horses: Protection provided by antiserum to core lipopolysaccharide. *Am. J. vet. Res.* **47**, 544-550.

Mueller, R., Boothby, J. T., Carroll, E. J., and Panico, L. (1983). Changes of complement values in calves during the first month of life. *Am. J. vet. Res.* **44**, 747-750.

Newman, S. L., Gootee, L., Gabay, J. E., and Selsted, M. E. (2000). Identification of constituents of human neutrophil azurophil granules that mediate fungistasis against *Histoplasma capsulatum. Infect. Immun.* **68**, 5668-5672.

Newman, S. L., and Mikus, L. K. (1985). Deposition of C3b and iC3b onto particulate activators of the human complement system - Quantitation with monoclonal antibodies to human C3. *J. Exp. Med.* **161**, 1414-1431.

Nilsson, B., and Nilsson Ekdahl, K. (1998). Components of the alternative pathway. In The complement system, K. Rother, G. Till, and G. Hänsch, eds. (Berlin, Springer-Verlag), pp. 23-49.

Nilsson, B., and Nilsson, U. R. (1985). An assessment of the extent of antigenic analogy between physiologically bound C3 and C3 denatured by sodium dodecyl sulphate. *Scand. J. Immunol.* **22**, 703-710.

Ogundele, M. O. (1999). Inhibitors of complement activity in human breast-milk: a proposed hypothesis of their physiological significance. *Mediators of Inflammation* **8**, 69-75.

Ogundele, M. O. (2000). Activation and deposition of human breast-milk complement C3 opsonins on serum sensitive Escherichia coli 0111. *J. Reprod. Immun.* **48**, 99-105.

Paape, M. J., and Miller, R. H. (1988). Sources of variation introduced into a phagocytosis assay as a result of the isolation of neutrophils from bovine blood. *Am. J. vet. Res.* **49**, 1210-1213.

Paape, M. J., Shafer-Weaver, K., Capuco, A. V., Van Oostvedt, K., and Burvenich, C. (2000). Immune surveillance of mammary tissue by phagocytic cells. *Adv. Exp. Med. Biol.* **480**, 259-277.

Pangburn, M. (1983). Activation of complement via the alternative pathway. *Fed. Proc.* **42**, 139-143.

Pangburn, M., and Müller-Eberhard, H. (1978). Complement C3 convertase: cell surface restriction of β1H control and generation of restriction on neuraminidase-treated cells. *Proc. Natl. Acad. Sci. USA* **75**, 2416-2420.

Pangburn, M., Schreiber, R., and Müller-Eberhard, H. (1983). C3b deposition during activation of the alternative complement pathway and the effect of deposition on the activating surface. *J. Immun.* **131**, 1930-1935.

Payne, N. R., Frestedt, J., Hunkeler, N., and Gehrz, R. (1993). Cell-surface expression of immunoglobulin G receptors on the polymorphonuclear leukocytes and monocytes of extremely premature infants. *Pediatr. Res.* **33**, 452-457.

Perryman, L., McGuire, T., Banks, K., and Henson, J. (1971). Decreased C3 levels in a chronic virus infection: equine infectious anemia. *J. Immunol.* **106**, 1074-1078.

Peterson, P. K., Verhoef, J., Schmeling, D., and Quie, P. G. (1977). Kinetics of phagocytosis and bacterial killing by human polymorphonuclear leukocytes and monocytes. *J. Infect. Dis.* **136**, 502.

Raidal, S., Bailey, G., and Love, D. (1998a). Flow cytometric determination of oxidative burst activity of equine peripheral blood and bronchoalveolar lavage-derived leucocytes. *Vet. J.* **156**, 117-126.

Raidal, S. L. (2000). Immunity in foals (Barton, Kingston, ACT, Rural Industries Research and Development Corporation).

Raidal, S. L., Bailey, G. D., and Love, D. N. (1998b). The flow cytometric evaluation of phagocytosis by equine peripheral blood neutrophils and pulmonary alveolar macrophages. *Vet. J.* **156**, 107-116.

Rainard, P., and Boulard, C. (1992). Opsonization of *Streptococcus agalactiae* of bovine origin by complement and antibodies against group B polysaccharide. *Infect. Immun.* **60**, 4801-4808.

Rainard, P., Riollet, C., Poutrel, B., and Paape, M. J. (2000). Phagocytosis and killing of Staphylococcus aureus by bovine neutrophils after priming by tumor necrosis factor-alpha and the des-arginine derivative of C5a. *Am. J. vet. Res.* **61**, 951-959.

Raisis, A. L., Hodgson, J. L., and Hodgson, D. R. (1996). Equine neonatal septicaemia: 24 cases. *Aust. vet. J.* **73**, 137-140.

Rashmir-Raven, A., Coyne, C., Fenwick, B., Gaughan, E., Andrews, G., and DeBowes, R. (1992). Inhibition of equine complement activity by polysulphated glycosaminoglycans. *Am. J. vet. Res.* **53**, 87-90.

Reis, K. J. (1989). A hemolytic assay for the measurement of equine complement. *Vet. Immun. Immunopath.* **23**, 129-137.

Reiter, B., and Brock, J. H. (1975). Inhibition of Escherichia coli by bovine colostrum and post-colostral milk. I. Complement-mediated bactericidal activity of antibodies to a serum susceptible strain of E. coli of the serotype O 111. *Immunology* **28**, 71-82.

Renshaw, H., and Everson, D. (1979). Classical and alternate complement pathway activities in paired dairy cow-newborn calf sera. *Comp. Immun. Microbiol. infect. Dis.* **1**, 259-267.

Rice, C., and Duhamel, L. (1957). A comparison of the complement, conglutinin, and natural anti-sheep red cell antibody titres of the serum of new born and older calves. *Can. J. comp. Med.* **21**, 109-116.

Rice, C., and L'Ecuyer, C. (1963). Complement titres of naturally and artificially raised piglets. I. In piglets of different birth weights. *Can. J. comp. Med. vet. Sci.* **27**, 157-161.

Rice, C., and Silverstein, A. (1964). Haemolytic complement activity of sera of foetal and new-born lambs. *Can. J. comp. Med. vet. Sci.* **28**, 34-37.

Rice, C. E., and Boulanger, P. (1952). The interchangeability of the complement components of different animal species. IV. In the hemolysis of rabbit erythrocytes sensitized with sheep antibody. *J. Immunol.* **68**, 197-205.

Riedel-Caspari, G. (1993). The influence of colostral leukocytes on the course of an experimental *Escherichia coli* infection and serum antibodies in neonatal calves. *Vet. Immun. Immunopath.* **35**, 275-288.

Robinson, J. A., Allen, G. K., Green, E. M., Fales, W. H., Loch, W. E., and Wilkerson, C. G. (1993). A prospective study of septicaemia in colostrum-deprived foals. *Equine vet. J.* **25**, 214-218.

Rokita, E., Makristathis, A., Presterl, E., Rotter, M., and Hirschl, A. (1998). *Helicobacter pylori* urease significantly reduces opsonization by human complement. *J. Infect. Dis.* **178**, 1521-1525.

Saad, A. M., and Hageltorn, M. (1985). Flow cytometric characterization of bovine blood neutrophil phagocytosis of fluorescent bacteria and zymosan particles. *Acta vet. Scand.* **26**, 289-307.

Savill, J., and Fadok, V. (2000). Corpse clearance defines the meaning of death. *Nature* **407**, 784-788.

Sheoran, A. S., and Holmes, M. A. (1996). Separation of equine IgG subclasses (IgGa, IgGb and IgG(T)) using their differential binding characteristics for staphylococcal protein A and streptococcal protein G. *Vet. Immun. Immunopath.* **55**, 33-43.

Sheoran, A. S., Timoney, J. F., Holmes, M. A., Karzenski, S. S., and Crisman, M. V. (2000). Immunoglobulin isotypes in sera and nasal mucosal secretions and their neonatal transfer and distribution in horses. *Am. J. vet. Res.* **61**, 1099-1105.

Simon, C., Lamar, R., and Bispham, W. (1906). A contribution to the study of opsonins. *J. Exp. Med.* **8**, 651-680.

Smits, E., Burvenich, C., and Heyneman, R. (1997). Simultaneous flow cytometric measurement of phagocytic and oxidative burst activity of polymorphonuclear leukocytes in whole bovine blood. *Vet. Immun. Immunopath.* **56**, 259-269.

Song, W., Sarrias, M., and Lambris, J. (2000). Complement and innate immunity. *Immunopharmacology* **49**, 187-198.

Soothill, J. F., and Harvey, B. A. M. (1976). Defective opsonization. A common immunity deficiency. *Arch. Dis. Child.* **51**, 91-99.

Sørensen, O., and Borregard, N. (1999). Methods for quantification of human neutrophil proteins, a survey. J. Immunol. Methods 232, 179-190.

Sternberg, S., Johannisson, A., Magnusson, U., and Jensen-Waern, M. (1999). Effects of *Actinobacillus equuli* culture supernatants on equine neutrophil functions and survival. *J. vet. Med. B* **46**, 595-602.

Stossel, T. P. (1974). Phagocytosis (First of three parts). *New Engl J Med* **March 28**, 717-723.

Stossel, T. P., Alper, C. A., and Rosen, F. S. (1973). Opsonic activity in the newborn: Role of properdin. *Pediatrics* **52**, 134-157.

Sveum, R., Chused, T., Frank, M., and Brown, E. (1986). A quantitative fluorescent method for measurement of bacterial adherence and phagocytosis. *J. Immunol. Methods* **90**, 257-264.

Takai, S., Morozumi, Y., Higashiyama, S., and Tsubaki, S. (1986). Nitroblue tetrazolium reduction by neutrophils of newborn foals, adult horses, and a foal infected with *Rhodococcus (Corynebacterium) equi. Jpn. J. Vet. Sci.* **48**, 405-408.

Thuvander, A., Johannisson, A., and Grawé, J. (1992). Flow cytometry in fish immunology. In Techniques in Fish Immunology, J. S. Stolen, T. C. Fletcher, D. P. Anderson, S. L. Kaatari, and A. F. Rowley, eds. (Fair Haven, SOS Publications).

Thuvander, A., Norrgren, L., and Fossum, C. (1987). Phagocytic cells in blood from rainbow trout, Salmo gairdneri (Richardson), characterized by flow cytometry and electron microscopy. *J. Fish Biol.* **31**, 197-208.

Tizard, I. R. (1996). Veterinary immunology: an introduction, 5 edn (Philadelphia, W.B. Saunders Company).

Travis, S. M., Singh, P. K., and Welsh, M. J. (2001). Antimicrobial peptides and proteins in the innate defense of the airway surface. *Current Opinion in Immunology* **13**, 89-95.

Tregoat, V., Montagne, P., Cuilliere, M., Bene, M. C., and Faure, G. (1999). C3/C4 concentration ratio reverses between colostrum and mature milk in human lactation. *J. Clin. Immunol.* **19**, 300-304.

Trowald-Wigh, G., Håkansson, L., Johannisson, A., Norrgren, L., and Hård af Segerstad, C. (1992). Leucocyte adhesion protein deficiency in Irish setter dogs. *Vet. Immun. Immunopath.* **32**, 261-280.

Trowald-Wigh, G., and Thorén-Tolling, K. (1990). Phagocytosis induced by yeast cells in canine granulocytes: A methodological study. *Acta vet. Scand.* **31**, 87-95.

Tuboly, S., Bernath, S., Glavits, R., Kovacs, A., and Megyeri, Z. (1995). Intestinal absorption of colostral lymphocytes in newborn lambs and their role in the development of immune status. *Acta Vet. Hung.* **43**, 105-115.

Turk, J. L. (1994). Almroth Wright - phagocytosis and opsonization. *J. Royal Soc. Med.* **87**, 576-577.

Turner, M., Grant, C., Seymour, N., Harvey, B., and Levinsky, R. (1986). Evaluation of C3b/C3bi opsonization and chemiluminiscence with selected yeasts and bacteria using sera of different opsonic potential. *Immunology* **58**, 111-115.

Turner, M., Seymour, N., Kazatchkine, M., and Mowbray, J. (1985a). Suboptimal C3b/C3bi deposition and defective yeast opsonization. I. Evidence for the abscence of essential co-factor activity. *Clin Exp Immunol* **62**, 427-434.

Turner, M., Seymour, N., Kazatchkine, M., and Mowbray, J. (1985b). Suboptimal C3b/C3bi deposition and defective yeast opsonization. II. Partial purification and preliminary characterization of an opsonic co-factor able to correct sera with the defect. *Clin Exp Immunol* **62**, 435-441.

Vachiéry, N., Totté, P., Balcer, V., Martinez, D., and Bensaid, A. (1999). Effect of isolation techniques, in vitro culture and IFNγ treatment on the constitutive expression of MHC Class I and Class II molecules on goat neutrophils. *Vet. Immun. Immunopath.* **70**, 19-32.

van Eeden, S. F., Klut, M. E., Walker, B. A. M., and Hoggs, J. C. (1999). The use of flow cytometry to measure neutrophil function. *J. Immunol. Methods* **232**, 23-43.

Van Oostvedt, K., Dosogne, H., Burvenich, C., Paape, M. J., Brochez, V., and Van den Eeckhout, E. (1999). Flow cytometric procedure to detect apoptosis of bovine polymorphonuclear leukocytes in blood. *Vet. Immun. Immunopath.* **70**, 125-133.

Väkeväinen, M., Jansen, W., Saeland, E., Jonsdottir, I., Snippe, H., Veriteul, A., and Käyhty, H. (2001). Are the opsonophagocytic activities of antibodies in infant sera measured by different pneumococcal phagocytosis assays comparable? *Clinical and diagnostic laboratory immunology* **8**, 363-369.

Wichtel, M., Amderson, K., Johnson, T., Nathan, U., and Smith, L. (1991). Influence of age on neutrophil function in foals. *Equine vet. J.* **23**, 466-469.

Williams, P. P. (1993). Immunomodulating effects of intestinal absorbed maternal colostral leukocytes by neonatal pigs. *Can. J. Vet. Res.* **57**, 1-8.

Wilson, W. D., and Madigan, J. E. (1989). Comparison of bacteriologic culture of blood and necropsy specimens for determining the cause of foal septicemia: 47 cases (1978-1987). *J. Am. vet. med. Ass.* **195**, 1759-1763.

Worku, M., Paape, M., Di Carlo, A., Kehrli, M., and Marquardt, W. (1995). Complement component C3b and immunoglobulin Fc receptors on neutrophils from calves with leukocyte adhesion deficiency. *Am. J. vet. Res.* **56**, 435-439.

Wright, A. E., and Douglas, S. R. (1903). An experimental investigation of the role of the body fluids in connection with phagocytosis. *Proc. R. Soc. London* **72**, 357.

Yager, J. A., Duder, C. K., Prescott, J. F., and Zink, M. C. (1987). The interaction of *Rhodococcus equi* and foal neutrophils *in vitro*. *Vet. Microbiol.* **14**, 287-294.

Yager, J. A., Foster, S. F., Zink, M. C., Prescott, J. F., and Lumsden, J. H. (1986). *In vitro* bactericidal efficacy of equine polymorphonuclear leukocytes against *Corynebacterium equi*. *Am. J. vet. Res.* **47**, 438-440.

Zink, M. C., and Johnson, J. A. (1984). Cellular constituents of clinically normal foal bronchoalveolar lavage fluid during postnatal maturation. *Am. J. vet. Res.* **45**, 893-897.

Acknowledgements

These studies were carried out at the Department of Large Animal Clinical Sciences and the Department of Pathology, Swedish University of Agricultural Sciences (SLU), Uppsala. The present heads of these departments, Associate Professor Mats Forsberg and Professor Lennart Jönsson, and their predecessors are thanked for this opportunity.

This work was supported by research grants from the Swedish Horse Race Totalization Board (ATG), the Agria Foundation for Research, and Hoechst. The flow cytometer was financed by a grant from the Knut and Alice Wallenberg Foundation. Grants from the Michael Forsgren fund and the Stina Johansson in Fränsta fund made it possible for me to participate in this project initially.

I wish to express my sincere gratitude to my supervisors and all the people who have helped me and those who have been there for me in life during this exciting time.

My special thanks are due to:

Professor Marianne Jensen-Waern, Manne, my supervisor, for leading me through this work with never-failing enthusiasm and support, deep and broad knowledge, cheerful an remarks and personal warmth, spiced with inspiriting anecdotes from all aspects of life and science. I admire you also for your total ignorance of the word impossible and your ability to remember and care about the golden side of life. If I ever become a real scientist, or a supervisor, I want to be like you.

Associate Professor Anders Johannisson, my co-supervisor, for introducing me to the technically intricate flow cytometry (I thought it was a cockpit of a jumbo-jet when I first saw the lab), for all our late working nights in the lab, for all the help, for your fantastically kind, sweet and stoic Jämtlandic mood even when my lab book was full of question marks and drawings of unhappy small faces, and for sharing the joyful moments :) when solutions to our problems were thought out, when the results looked good and many other times, not forgetting when we experienced Australia. Your qualities are also something to strive for.

Professor Emeritus Sune Persson, for allowing me to enter this project once upon a time and for the support I have felt all along.

Johan Carlsten, my supervisor as a student, for encouraging me to go on and try to study this area further after my student paper on foal pneumonia.

Kristina Nilsson Ekdahl and Bo Nilsson, the best complementologists I have ever met, for enthusiastic and knowledgeable guidance into their fascinating world of the small proteins most veterinary researchers regard as "Old Maid" ("Svarte Petter") and haven't touched in the last decades, and for placing their lab and technicians at my disposal at the Department of Clinical Immunology and Transfusion Medicine, University Hospital, Uppsala.

Susy Demmers, my co-author and the most passionate and skilled Foal Mama there is, for all the work with supplying these studies with a myriad of samples from her own horses, and for being a good friend and colleague, and for pleasant travel companionship on trips to Denmark, Canada, England and Italy to learn more about immunology in foals and horses.

Sanna Sternberg, my co-author, for working after hours in borrowed laboratories to prepare the bacteria and for interesting discussions on all topics from horses to other pertinent issues in life.

Foozieh Ghazi, Uppsala University Hospital, for excellent technical assistance with SDS-PAGE and immunoblots.

Marie E, Kristina K, Karin T, Yvette R and Robert J for helping me out in the lab when I was a confused beginner and also later, when I was a sometimes stressed PhD student.

The owners and staff of Erikssund and Västerbo stud farms, for letting their foals and horses be included in the studies and for their help at samplings.

Helena Vallin and Anders Perers, Department of Veterinary Microbiology, Section of Immunology, SLU, for fruitful scientific advice and help with the antibodies.

Dr Pascal Rainard, France, for highly valuable advice through e-mail on analyses of complement and opsonisation.

Dr. Sharon Hietala, University of California, Davis, for help with ELISA analyses.

Librarians of KC and Ultuna libraries, for their rapid service with provision of hundreds of reference articles during these years.

Maud Marsden, for excellent and enjoyable linguistic revision of most manuscripts, including the thesis.

David Tilles and Richard Hopkins, for fast and good help with linguistic revisions.

Pia, Kia, Agnes, Anna, Miia, Johan, Ove, Mats, Åsa and all my other colleagues, former and present PhD-students and staff at KM stordjur for making it so nice to go to work, and for all the kind gossip, laughter and joy that we have shared together at work, parties, conferences and ski trips.

Carin Wrange and family, for letting me share your love for the horses Bocian and Alma, and for warm friendship.

Niklas, Martin, Åsa, Vivi, Eric, Per, Nena, Agne with spouses, my old friends from Falun, for long friendship with many fun memories for almost 20 years now, and for not knowing a lot about my work.

Gunnar & Lena, Maria & Sören, Monica and other good friends from my study years, for lasting friendship

Annika and Stabbe, for being my very best girl friends through party times, travels all around the world, and times of despair. I hope that we will always stay close.

Lennart, for good partnership and memorable adventures during one third of my life.

Peter, Tina, Mika and Rikard, my brothers and sisters, for each being a very special person that I love and feel proud of.

Mamma Anita and Pappa Bigge, for everything, of course, but especially for your love for animals and your ability to look at things from new angles that you have passed on to me, and the creativity, curiosity and critical thinking (some say I have too much of that) that you have always encouraged and which has been a good help in this work.

Erik, my love, my Man on this planet and in the sky, for making me feel that there is no limit... to what one can do... to all the good things in life... or to love

Acta Universitatis Agriculturae Sueciae
presents doctoral theses from the Faculty of Agriculture, Landscape Architecture and Horticulture, the Faculty of Forestry and the Faculty of Veterinary Medicine at the Swedish University of Agricultural Sciences. Each faculty has its own subseries, Agraria, Silvestria and Veterinaria, respectively, with separate numbering.

ISSN 1401-6257
ISBN 91-576-5931-1