# Experimentally induced Swine Dysentery

## with Special Reference to Alterations in Lymphocyte Sub-populations, Cytokines and Amino Acids in Blood

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# ABSTRACT

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Swine dysentery is an enteric disease that is present in most pig-producing countries. It is caused by the spirochete *Brachyspira hyodysenteriae* and the typical manifestation is severe muco-haemorrhagic diarrhoea that may lead to a high mortality in affected herds if it is left untreated.

The aim was to study the immunological and metabolic responses associated with swine dysentery. Pigs were orally inoculated with *Brachyspira hyodysenteriae* strain $B204^R$ after a provocative feeding regime with soy bean meal. Blood was sampled before inoculation and repeatedly during the incubation, dysentery and recovery periods. Counts of leucocyte and lymphocyte sub-populations and levels of the cytokines IL-1β, IL-6, Il-10, TNF-α and IFN-γ, the acute phase protein Serum Amyolid A, glucose, lactate and 18 amino acids were determined.

The experimental infection model was successful and ~65% of the pigs developed dysentery with a deterioration of the body condition and no weight gain. The presence of the pro-inflammatory cytokine IL-1β in serum was associated with the manifestation of dysentery and the production of Serum amyloid A. During the dysentery period the numbers of monocytes, neutrophils and CD8α+ lymphocytes increased in the blood. Glucose and lactate levels showed no alteration during dysentery, but the serum concentrations of gluconeogenic amino acids, mainly glutamine, alanine, tyrosine and serine were decreased during that period. Most parameters were returned to their pre-inoculation levels during the recovery, but the presence of the anti-inflammatory cytokine IL-10 and the production of *B. hyodysenteriae* specific antibodies were associated with the recovery of sick animals. The susceptibility to disease after inoculation appeared to be related to low levels of CD8α+ lymphocytes and high levels of γδ T cells in blood prior to inoculation. In conclusion, this thesis shows that the cellular and humoral immune responses as well as the metabolic response in blood were altered by swine dysentery.

*Keywords:* Pigs, Brachyspira hyodysenteriae, infection, neutrophils, γδ T cells, monocytes, CD8α, metabolism, amino acids, cytokines, antibodies.

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# POPULÄRVETENSKAPLIG SAMMANFATTNING

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Titel: *Experimentally induced swine dysentery with special reference to alterations in lymphocyte sub-populations, cytokines and amino acids in blood.*

Svindysenteri är en vanligt förekommande tarmsjukdom som främst vållar problem i besättningar med växande tamgrisar. Drabbade grisar tappar ofta i tillväxt och dödligheten kan vara hög om de inte behandlas. Den bakomliggande orsaken är en bakteriell infektion i grovtarmen. Bakterien, *Brachyspira hyodysenteriae,* sprids via avföringen från smittade grisar och orsakar en slemmig och blodig diarré. Smittade besättningar kan behandlas med antibiotika, men resistenta stammar av bakterien blir allt vanligare. Vaccination är ett bra behandlingsalternativ men än så länge finns inga effektiva vacciner. Kunskap om grisens immunologiska svar mot infektionen är väsentlig för att kunna utveckla verkningsfulla vacciner.

För att undersöka de immunologiska förändringar som sker vid svindysenteri genomfördes försök där 31 grisar experimentellt infekterades med *B. hyodysenteriae* oralt. Av dessa utvecklade 20 en slemmig diarré varav 15 också hade blod i avföringen. Blodprov togs före infektionen och vid upprepade tillfällen under inkubationstiden, sjukdoms- och återhämtningsfasen. Blodet analyserades bl.a. med avseende på olika populationer av vita blodkroppar, cytokiner, akutfasproteinet Serumamyloid A och antikroppar mot *Brachyspira hyodysenteriae*.

De grisar som före infektionen hade låga halter av CD8α+ lymfocyter och höga halter av så kallade γδ T celler var mer mottagliga för dysenteri. Detta kan vara en del av förklaringen till varför vissa blir sjuka medan andra förblir friska. Grisar som utvecklade symtom hade förhöjda nivåer av monocyter och neutrofiler under sjukdomsperioden. De här vita blodkropparna är mycket viktiga fagocyter som effektivt kan ”äta” upp bakterier. Andelen aktiverade lymfocyter (CD45RA-) steg under dysenteriperioden varav de flesta var CD8α+ lymfocyter, vilket är intressant eftersom det är en grupp vita blodkroppar som främst har ansetts vara involverade i virusinfektioner. Två viktiga inflammationsframkallande cytokiner, IL-1β och TNF–α, detekterades under diarréfasen. Dessa cytokiner produceras framförallt av monocyter och är viktiga för att aktivera olika delar av immunförsvaret, bl.a. akutfasreaktionen. Serumamyloid A är en komponent i denna reaktion och en ökning av proteinet sågs under svindysenteri. När de drabbade grisarna började återhämta sig kunde man se en ökande produktion av cytokinet IL-10 och antikroppar mot *B. hyodysenteriae* i blodet. IL-10 är ett cytokin som är viktigt för att en effektiv produktion av antikroppar ska kunna ske. Dessutom dämpar IL-10 generellt inflammation och immunförsvaret när det inte längre behövs. Under återhämtningsperioden uppvisade de vita blodkropparna i stort sett samma mönster som innan infektionen förutom γδ T cellerna som ökade.

Vid infektioner går generellt energikrävande funktioner som tillväxt ner på sparlåga då det behövs extra energi av immunförsvaret för celldelning, bakteriell bekämpning m.m. Vid diarré kan näringsupptaget från tarmen vara starkt begränsat på grund av nedsatt aptit, förändrad tarmflora och en allt för snabb passage av födan genom tarmen. Då kan ämnesomsättningen rubbas vilket kan leda till en låg blodsockernivå. För att återställa blodsockernivån kan en nedbrytning av grisens energiedepåer inträffa. Cytokinerna IL-1β och TNF–α är viktiga för att initiera den här nedbrytningen och de aminosyror som då frisätts kan användas för proteinproduktion eller, genom att omvandlas till socker i levern, ge energi åt det stora antalet vita blodkroppar som försöker bekämpa infektionen.

För att undersöka de effekter som svindysenteri har på ämnesomsättningen analyserades blodet med avseende på glukos, mjölksyra och olika aminosyror.

Majoriteten av de sjuka grisarna hade normala blodsockernivåer under sjukdomsperioden, vilket tyder på att de kan kompensera för det ökade behovet av energi från immunförsvaret. Dock kunde man se stora koncentrationsförändringar i ett flertal olika aminosyror, framför allt de som kan omvandlas till socker. Aminosyrorna glutamin och alanin, vilka utgör viktiga energikällor för vita blodkroppar, sjönk drastiskt under dysenterin. Aminosyran lysin ökade i slutet av dysenterin och i början av återhämtningsfasen. Tarmen har ett stort behov av lysin och den ökning som sågs i blodet kan orsakas av en lägre användning av lysin från den skadade tarmen.

Sammanfattningsvis restes ett systemiskt immunförsvar med produktion av typiska cytokiner samt en ökning av fagocyterande celler under sjukdommen. Dessutom bildades specifika antikroppar under återhämtningsfasen vilket också kan ha betydelse för att bekämpa infektionen. Dysenterin orsakade också systemiska förändringar i ämnesomsättningen med sänkta blodnivåer av flera viktiga aminosyror, vilket skulle kunna vara relaterat till ett ökat behov av dessa aminosyror.

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## The morphology of porcine white blood cells is unique in the animal kingdom

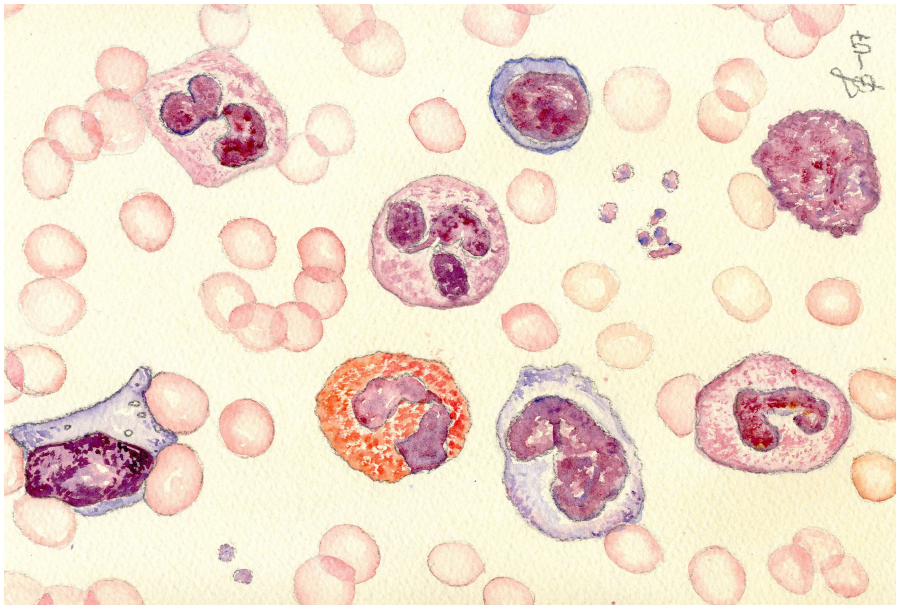

*Figure A: An example of stained white blood cells from a pig viewed by incandescent light. Magnification 100x.*

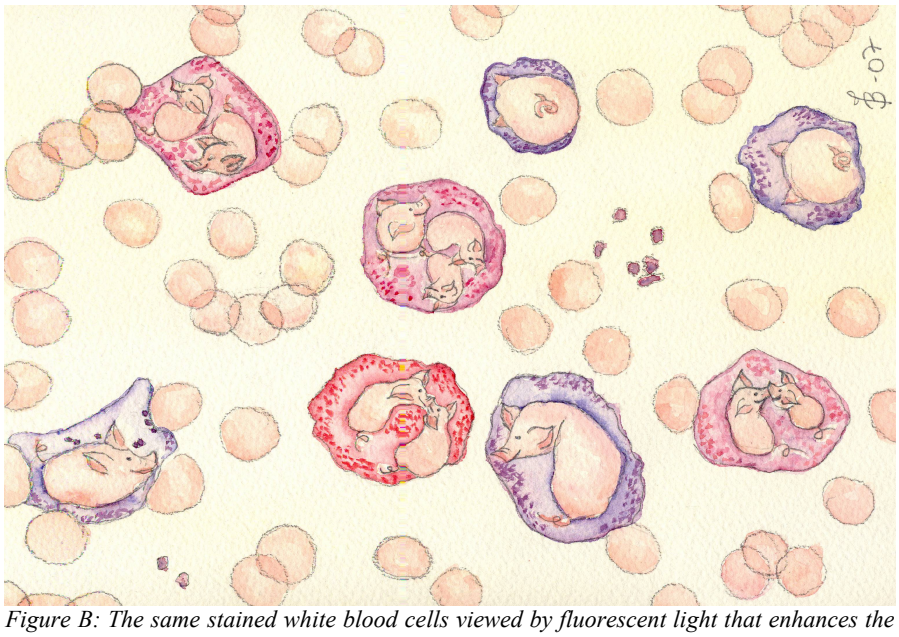

*Figure B: The same stained white blood cells viewed by fluorescent light that enhances the morphology of the nucleus.*

***I would like to express my sincere gratitude to my wonderful mother-in-law, Ingrid Borbély, who has made these paintings for my thesis.***

# CONTENTS

# APPENDIX

## List of original papers

**This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:**

**I.** Jonasson R, Johannisson A, Jacobson M, Fellström C, Jensen-Waern M. Differences in lymphocyte subpopulations and cell counts before and after experimentally induced swine dysentery. *J Med Microbiol*. 2004; 53: 267-72.

**II.** Jonasson R, Andersson M, Råsbäck T, Johannisson A, Jensen-Waern M. Immunological alterations during the clinical and recovery phases of experimental swine dysentery. *J Med Microbiol*. 2006; 55: 845-55.

**III.** Jonasson R, Essen-Gustavsson B, Fossum C, Jensen-Waern M. Blood concentrations of the cytokines IL-1β, IL-6, IL-10, TNF-α and IFN-γ during experimentally induced swine dysentery. Submitted 2007.

**IV.** Jonasson R, Essen-Gustavsson B, Jensen-Waern M. Blood concentrations of amino acids, glucose and lactate during experimental swine dysentery. *Res Vet Sci*. 2007; 82: 323-331.

# ABBREVIATIONS

**The following abbreviations are used in the text:**

| | |
|---|---|
| ADCC | Antibody-dependent cell-mediated cytotoxicity |
| CD | Cluster of differentiation |
| ELISA | Enzyme-linked immunosorbent assay |
| HPLC | High performance liquid chromatography |
| IFN-γ | Interferon-gamma |
| Ig | Immunoglobulin |
| IL | Interleukin |
| LPS | Lipopolysaccharide |
| M-cells | Microfold cells |
| MHC | Major histocompatibility complex |
| NK cells | Natural killer cells |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate buffered saline |
| SAA | Serum amyloid A |
| SDS | Sodium dodecyl sulphate |
| sIgA | Secretory immunoglobulin A |
| TCR | T cell receptor |
| Th cell | T helper cell |
| TNF | Tumour necrosis factor |

# LIST OF LYMPHOCYTE SUB-POPULATIONS

**The following cell populations are synonymously referred to in the text:**

| | |
|---|---|
| CD3+ cells | T cells |
| CD8α+ cells | Double positive T cells, Cytotoxic T cells, NK cells and some γδ T cells |
| CD8α+CD4- cells | Cytotoxic T cells, NK cells and some γδ T cells |
| CD4+CD8α+ cells | Double positive T cells |
| CD8α+CD8β+ cells | Cytotoxic T cells |
| CD4+CD8α– cells | T helper cells |
| CD21+ cells | B cells |
| CD45RA+ cells | Naïve/resting lymphocytes |
| CD45RA- cells | Activated/memory lymphocytes |

# INTRODUCTION

## Swine dysentery

Swine dysentery is a contagious bacterial disease that primarily affects grower and finisher pigs. It was first described in the early 1920s in the USA (Whiting *et al*. 1921). In 1960 swine dysentery was also found to occur in Sweden (Ronéus, 1960) and nowadays it is present in most pig-producing countries. This disease was later established to be caused by the anaerobic oxygen-tolerant gram-negative spirochete *Brachyspira hyodysenteriae* (Taylor & Alexander, 1971), but the commensal intestinal flora is also necessary for the development of dysentery (Whipp *et al.,* 1979). The bacteria are highly motile and colonise the luminal surface and mucosal crypts of the large intestine where it causes excessive mucus production, haemorrhages and tissue necrosis. Two structural elements of *B. hyodysenteriae* have been identified as toxic; hemolysin and lipopolysaccharide (LPS). The hemolysin has been shown to be cytotoxic and is essential for the pathogenicity (Lysons *et al.,* 1991). The LPS is endotoxic and can provoke inflammatory lesions (Greer and Wannemuehler, 1989). The first clinical sign of disease is generally loose grey faeces which usually progresses to the typical manifestation of swine dysentery: muco-haemorrhagic diarrhoea. In addition to diarrhoea, the affected animals show a deterioration of the body condition and decreased daily weight gain. If the infection is left untreated it may lead to severe dehydration, acidosis and hyperkalemia that result in a mortality as high as ~80% in affected herds (Raynaud *et al.,* 1980).

Swine dysentery is mainly controlled by the use of expensive eradication programmes and by treatment with antimicrobial agents, such as tiamulin and tylosin. However, the extensive use of antibiotics in therapy and as growth promoters has resulted in a decrease of the antimicrobial susceptibility worldwide (Molnar, 1996, Lobova *et al.,* 2004). Attempts have been made to produce effective vaccines (Fernie *et al*., 1983, Waters *et al*., 1999a, 1999b), but the developed vaccines have mainly resulted in a reduced severity of lesions instead of inducing specific IgA antibodies (La *et al.,* 2004). An understanding of the host immune responses during dysentery is therefore imperative for the development of prophylactic measures, including vaccination programmes.

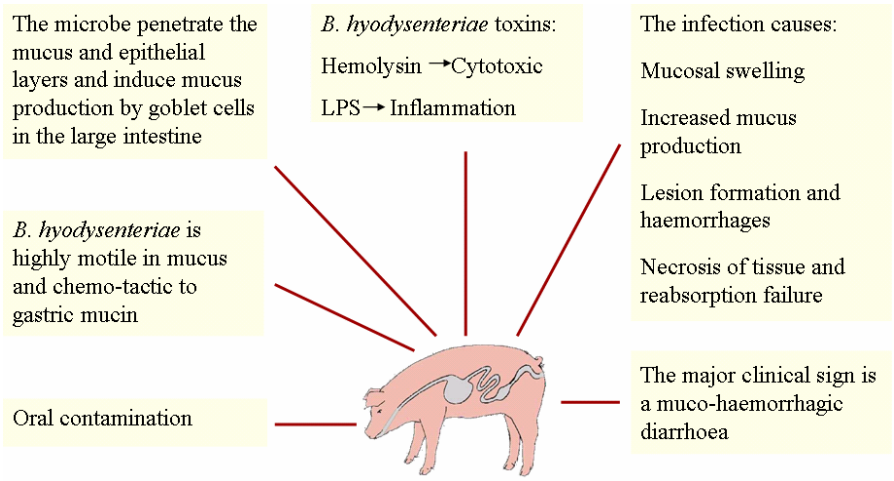


Figure 1. A brief overview of the infection with *B. hyodysenteriae* and its consequences for the pig. More information about the genus *Brachyspira* can be obtained at the homepage of the Swedish Brachyspira Working Group (http://www.brachyspira.se).

## The innate immunity

Bacterial infections that cause diarrhoea are initially controlled by the innate immune system and have usually been defeated before the adaptive immunity has developed (Mims et al., 2001). The reason for this is that many of the diarrhoea-associated diseases are caused by "hit and run" infections. In order to cause an infection a microbe initially needs to gain access to the body. The innate immune response is the first line of defence against the invading microbe. The mucus lining the intestinal tract acts as a protective layer for the underlying epithelial surface and is an example of a physical barrier that blocks adherence and penetration of microbes, while the acidity of the gastric juice is an example of a chemical barrier. If these barriers are broken or bypassed, many other innate defences help to protect the host. Cytokines, acute phase proteins, lysozymes, complement factors, leukotrienes and prostaglandins are some examples of soluble bactericidal or immune stimulating factors released during the innate defences. The production of these mediators is important for enhancing the innate immune responses and in directing the adaptive immune responses against either a cell mediated immune response or a humoral response (Romagnani, 1996).

Cytokines are generally released locally in tissues but are also released systemically in the blood during an infection in pigs (Jesmok *et al.,* 1992, Fossum *et al.* 1998, Zhu *et al.* 2004). A vast number of different cytokines are currently described in the literature and the number is continuously increasing. During the inflammatory process some of these, on the basis of their principal effects, can be

grouped as proinflammatory, i.e. tending to induce inflammation, and some as anti-inflammatory, causing inflammation to decrease. The production of the proinflammatory cytokines interleukin (IL)-1β, tumour necrosis factor (TNF)-α and IL-6 can be induced by bacterial cell wall components, such as lipopolysaccharides (Degre, 1996), and initiate/enhance inflammatory responses. They also often play important roles in the pathogenesis of many infections. IL-1β is one of the key cytokines involved in inflammation. It generally causes fever, induces production of acute phase proteins and induces leucocyte adhesion to inflamed tissues (Dinarello, 2000). Both IL-1β and TNF-α induce anorexia (Dinarello, 1987) and increase protein catabolism in rats and mice (del Rey & Besedovsky, 1987, Flores *et al.,* 1989). TNF-α is also commonly expressed during many infections. The gastrointestinal tract is known to be sensitive to TNF-α and this cytokine has in mice been shown to facilitate lesion development, tissue necrosis and development of vascular thrombi in the gut (Beutler *et al.,* 1985). IL-6 is also a key cytokine and it has been suggested that it may be a bio-marker of bacterial infections in pigs. IL-10 is one of the more important anti-inflammatory cytokine, which may down-regulate the production of proinflammatory cytokines and generally protects against systemic inflammation (for review see Opal & Depalo, 2000). Interferon (IFN)-γ is an important activator of macrophages and the cytotoxic T cell pathway (Murray, 1990). The knowledge of the cytokine response during swine dysentery is poor and most of the available information comes from *in vitro* studies. Considering the complexity of an infectious disease it is of great importance to also study the *in vivo* responses during an infection.

Several cytokines, such as IL-1β, IL-6 and TNF-α, can induce an acute phase response. This response involves about 30 different proteins, some of which are included in the complement cascade. They are mainly produced by hepatocytes and generally help the host defences in restoring and maintaining homeostasis, but their presence may also be connected to malaise and fever. Serum amyloid A (SAA) is a common acute phase protein which has been shown in pigs to be clinically relevant (Heegaard *et al*., 1998). This protein is induced by the presence of LPS, IL-1β, IL-6 and TNF-α (for review see Jensen & Whitehead, 1998). SAA has several important functions during immune responses, such as enhancement of tissue infiltration of monocytes, polymorphonuclear cells and T cells into the inflamed tissues (Badolato et al. 1994, Xu et al. 1995). The complement system is also an important part of the innate defence and consists of an enzyme cascade of at least 20 proteins that act as opsonins, promote chemotaxis and increase vascular permeability. This system is very important for the mediation of inflammatory responses.

The innate immune system also includes cells such as neutrophils, monocytes/macrophages, dendritic cells, eosinophils, basophiles, mast cells and natural killer (NK) cells that can react non-specifically to foreign antigens. Neutrophils and monocytes/macrophages migrate rapidly into the intestinal tract during infections where they phagocyte and kill invading micro-organisms. These cells are also an important link between the innate and the adaptive immune systems and in the case of macrophages and dendritic cells, their antigen processing and presentation via MHC class II is crucial for the adaptive immunity.

## The adaptive immunity

T and B lymphocytes are essential in adaptive immunity through their antigen-specific recognition of an epitope. During infections there are polyclonal responses of T and B cells, but certain clones will have more effective immune responses against the invading microbe than others. Lymphocytes comprise several different sub-populations of cells that are mainly recognised by their different surface receptors/markers. For instance, lymphocytes carrying the CD3 receptor are recognised as T cells. These T cells can be further sub-classified by the presence of CD8β as cytotoxic T cells, of CD4 as helper T cells (Th1 and Th2 cells) and of TCR1 as γδ T cells. CD8α+ cells recognise the MHC class I receptor that is present on all cells and display antigens from intracellular organisms/proteins, while CD4+ cells recognise the MHC class II receptor that is present on antigen presenting cells and mainly display antigens from extracellular organisms. Dendritic cells, macrophages and B cells all act as professional antigen presenting cells that are exceptionally efficient at phagocytosis or endocytosis of antigens. A fragment of this antigen is then bound to a class II MHC molecule that is subsequently displayed on the membrane. The T cell recognises and interacts with the antigen-class II MHC molecule complex on the membrane of the antigen presenting cell. An additional co-stimulatory signal is then produced by the antigen presenting cell, leading to activation of the T cell. If activated by the right MHC class displaying a suitable antigen for the specific T cell receptor, the T cell will become activated and undergo clonal expansion. The effect is dependent on the phenotype of the T cell. The CD8β+ cells can release cytotoxic and antimicrobial factors that kill infected cells. The CD4+ Th1 cells mainly produce cytokines such as IFN-γ and IL-2 that can modulate the cell-mediated immune responses, while the CD4+ Th2 cells mainly produce cytokines such as IL- 4, IL-5 and IL-10 that can induce proliferation of B cells and their maturation into antibody-producing plasma cells. Pigs are unique in that they have significant numbers of extra-thymic T cells that carry both the CD4 and CD8 markers. These are often referred to as

"double positive" T cells and are considered to be mature antigen-experienced memory/effector cells derived from Th cells, which when activated also express CD8 (Pescovitz *et al*., 1994, Zuckermann & Husmann, 1996). CD45RA is expressed on naïve/resting CD4+ and CD8+ T cells, and on B cells, NK cells and dendritic cells.

The T cell repertoire of pigs, and most ruminants, is distinguished by unusually large numbers of circulating γδ T cells. These cells generally only remain for a short time in the circulation and are considered to be important in the early response against infections at epithelial surfaces (Skeen & Ziegler, 1993, Boismenu & Havran, 1994). γδ T cells are important in the immune response of young pigs before other lymphocyte subsets mature (Yang & Parkhouse, 1996). The TCR1 receptor of γδ T cells has broader antigen specificity than the αβ T cell receptor (Constant *et al.*, 1994, Schoel *et al.*, 1994), and the capacity of these cells for antigen recognition and their subsequent activation are not dependent upon MHC class I or class II presentation (Schild *et al.,* 1994, Tanaka *et al.,* 1995).

B cells are generally identified with a CD21 marker. Upon specific antigen recognition and binding of antigen to the membrane-bound antibodies, B cells may differentiate into antibody-producing plasma cells.

## The antibody response

After initial antigen recognition by B cells, small amounts of specific antibodies are often formed locally within a few days. It takes approximately one to two weeks before these antibodies are produced in sufficiently large quantities as to be detectable in serum. A second exposure to the same antigen results in the formation of large amounts within a few days. The antibodies produced can be divided into different immunoglobulin classes. Immunoglobulin (Ig) G is the most common class found in the blood and increases in local tissues during an inflammatory process. IgA is the most common antibody class found on mucosal surfaces and is of great importance for the gastro-intestinal tract. The lymphoid organs and the submucosa of the intestinal tract are the main compartments for the antibody responses during intestinal infections. IgM is confined to the vascular system and through its short life-span the presence of IgM can indicate a recent or a persistent infection. Recognition of and subsequent binding of an antibody by its complementary antigen can result in antimicrobial activities through several actions, such as prevention of adherence of the antigen/microbe to the host cells, activation of the classical complement pathway and activation of macrophages and neutrophils.

## The immunology of the gastrointestinal tract

The gastro-intestinal tract is the largest and one of the most immunologically active regions in the body. The mucus that is produced by goblet cells protects the underlying epithelia by entrapping pathogens to sIgA. The epithelial layer is a mechanical barrier that transport nutrients from the lumen, but it also contains high numbers of CD8α+ cells and γδ T cells that may mediate cytotoxicity against foreign antigens. Beneath the epithelium lays the lamina propria. The porcine lamina propria around crypts harbours high numbers of IgA secreting plasma cells (Brown and Bourne, 1976), while T cells predominate in the villi. Peyer's patches are organised lymphoid structures in the intestine. This structure is covered by specialised epithelial cells, called M-cells. These cells sample antigens in the intestinal lumen and present them to macrophages beneath. The processed antigen is then presented by the macrophage to B and Th cells in the lymphoid structure. Here, an initial recognition of foreign luminal antigens occur, which with the aid of Th cells is followed by B cell activation, proliferation and a switch to produce mainly IgA. The activated B cells enter the bloodstream and then migrate to the lamina propria of infected sites were they differentiate into antibody secreting plasma cells.

## The metabolic response

The immune response that is triggered by an infection increases the demand for energy. In general, fat cannot be utilised efficiently during infections (Wannemacher *et al.,* 1981, Friman & Ilbäck, 1998) and therefore a switch from fatty acids as the preferred energy source to glucose normally occurs in many tissues (Klasing & Johnstone, 1991). Thus the production of glucose through gluconeogenesis will cover a large proportion of the increased energy requirements (Wannemacher *et al.,* 1981). Glucose is an important energy source for most activated immune cells (Meszaros *et al.,* 1991), and during stressful states it mainly derives from gluconeogenic amino acids, from degradation of liver glycogen and from the recycling of lactate into glucose (Wolfe and Burke, 1978). Neutrophils, monocytes and lymphocytes all have a high consumption not only of glucose but also of the amino acid glutamine (Newsholme *et al.,* 1985, Curi *et al.,* 1997). Several amino acids are of major importance during infections, both directly as structural elements of new proteins and as gluconeogenic substrates for glucose production. The amino acid requirement of a pig is mainly supplied by the diet, but is also partly met by the gastrointestinal microflora (Torrallardona *et al.,* 2003). During infections however, amino acids can also be provided through increased protein catabolism of mainly muscle tissue. The degradation of skeletal muscle protein is an important source of

alanine and glutamine during stress. The muscle glutamine pool can be reduced by as much as 50% during stress in humans (Furst *et al.,* 1990). The increased numbers of immune cells during infections may require more energy than the feed can provide, especially in the case of a decreased feed intake, an increased passage of ingesta and an altered gastrointestinal microflora in a diarrhoeic animal.

The interactions between the different branches of the immune system and the metabolic responses during an infection are complex (figure 2) and only partially understood. Further studies are therefore needed to elucidate how they are interrelated.

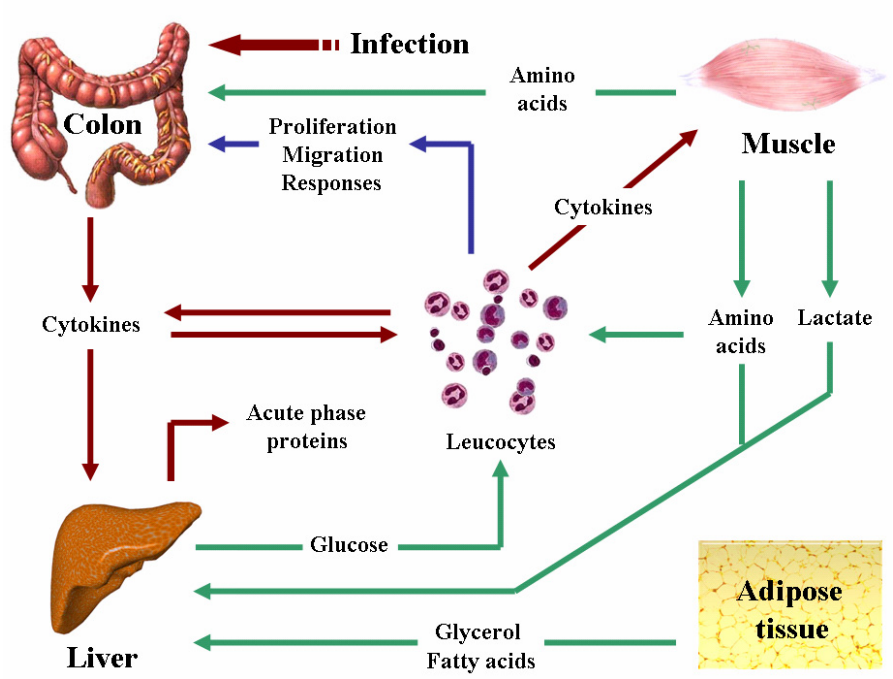


Figure 2. The interplay between leucocytes, inflammatory mediators (cytokines and acute phase proteins) and metabolites (glucose, amino acids and fatty acids) during infections.

# THE AIMS OF THIS THESIS

The principal aim of this work was to experimentally induce swine dysentery and to study immunological and metabolic responses in the blood associated with the disease.

The specific aims were:

- to determine if any changes in leucocyte populations occur during the course of swine dysentery (studies I and II)
- to determine if systemic cytokines can be detected in affected animals during the course of swine dysentery (study III)
- to determine if *B. hyodysenteriae*-specific antibodies are produced during swine dysentery (study II)
- to determine if metabolic alterations occur during the course of swine dysentery (studies IV)

# MATERIALS AND METHODS

## Animals

The Ethical Committee for Animal Experiments, Uppsala, Sweden, approved all experiments. Clinically healthy conventional crossbreed (Yorkshire x Swedish Landrace) pigs of both sexes were obtained from a conventional piglet-producing herd with a well-known health status and known to be free from dysentery. They were kept at the experimental facilities at the Department of Clinical Sciences, SLU, Uppsala, Sweden.

*Experiment 1 (study I)*

Twenty-one pigs with a mean weight of 21 kg (range 17-25 kg) prior to inoculation were used. Throughout the study 12 pigs were group-housed, with three animals per pen, and nine were housed individually.

*Experiment 2 (studies II, III and IV)*

Ten pigs with a mean weight of 23 kg (range 20-29 kg) prior to inoculation were used and housed individually throughout the study.

All animals had free access to water and were fed twice a day with an excessive amount of a finisher diet (Singelveg® SPK, Lantmännen, Stockholm, Sweden). On arrival, faecal samples were analysed for the presence of parasite eggs, *Brachyspira spp., Salmonella spp.* and *Yersinia spp.* and found to be free of these pathogens. They were given at least one week to acclimatise to the new environment and personnel before the experiment started. During this acclimatisation the bedding material consisted of straw and they were handled frequently each day.

## Animal infection model

The infection model, which was used in all studies, included a provocative feeding regime to facilitate onset of infection (Jacobson *et al.,* 2004). Briefly, four days prior to oral inoculation and during the three following days of inoculation every second meal was replaced by a pure soybean meal. In addition, the bedding material was replaced by synthetic fur blankets during the experimental period in order to minimise fibre ingestion from straw, which could have interfered with the infection model. From the first day of inoculation and onwards throughout the studies, all individually housed animals were moved in-between the pens once a day. The inoculum consisted of 30 mL per day (90 mL in total) of a brain-

heart infusion broth containing approximately $10^7$-$10^9$ *B. hyodysenteriae* strain B204$^R$ (ATCC 31212) /mL. The bacteria were propagated as described by Jacobson *et al.* (2004) and prior to inoculation the bacterial growth, motility and purity were evaluated by phase contrast microscopy.

## Clinical examinations

Thorough clinical health examinations and evaluation of faeces, with respect to consistency and presence of blood and mucus, were performed daily on all animals. The animals were weighed once a week throughout the experiment and the daily weight gain was calculated.

In experiment 2, day 1 of the swine dysentery period referred to the first day with haemorrhagic diarrhoea, and day 1 of the recovery period referred to the day when a change from diarrhoea to normal or just slightly loose faeces occurred.

## Blood sampling

All blood samples were obtained by puncture of the jugular vein and collected into vacutainer tubes without additives or with EDTA or heparin. The blood samples that were not intended for staining of leucocytes were centrifuged, after which serum or plasma was collected and stored at -80°C until further analysed.

*Experiment I*

Blood was sampled before the soybean diet and inoculum were given. In pigs that developed dysentery blood samples were also taken at clinical signs, i.e. diarrhoea, and in those that remained healthy samples were obtained at euthanasia (figure 3).

*Experiment II*

Blood samples were collected from all pigs before the soybean diet and inoculum were given (pre-inoculation), and at days 4 and 14 post-inoculation. In pigs that developed dysentery further blood samples were taken once a day during the first 4 days with clinical signs, and on days 1, 3, 7, 11 and 15 of the recovery period. In pigs without any clinical signs of disease blood samples were obtained before inoculation and on days 4 and 14 post-inoculation, and also on days 21, 28 and 35 (figure 3).

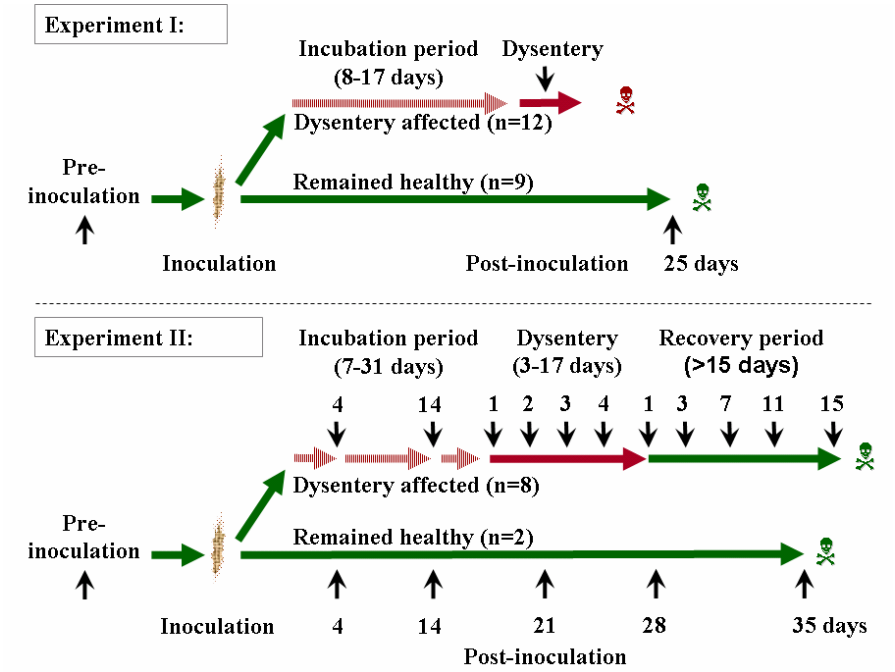


Figure 3. Experimental designs of experiments I and II. Black arrows indicate days when blood was sampled.

## Sampling of faeces

Faecal samples were collected with rectal swabs and examined for shedding of *Brachyspira spp.* as described by Fellström and Gunnarsson (1995).

*Experiment I*

Faecal samples were collected daily from all animals after the inoculation.

*Experiment II*

Samples were collected from all animals once a week throughout the study period. In addition, dysentery-affected animals were sampled once a day during the period with clinical signs of disease.

## Euthanasia

The euthanasia of all pigs was always followed by a thorough pathological examination.

*Experiment I*

All pigs were euthanised by captive bolt and exsanguination. Depending on the severity of the disease, the animals were euthanised after two to six days with clinical signs, which on average meant 17 days (range 11-20) after inoculation. The pigs that remained clinically healthy were euthanised 25 days (range 21-27) after inoculation.

*Experiment II*

The experimental period lasted for a total of 65 days. All pigs were euthanised with an overdose of pentobarbital sodium after sedation. Pigs that recovered from swine dysentery were euthanised 44 days (range 35-56) after inoculation and the pigs that remained clinically healthy were euthanised 35 days after inoculation. One animal had to be killed on the second day of muco-haemorrhagic diarrhoea because of its poor general appearance.

## White blood cell counts (studies I and II)

Total and differential white blood cell counts were analysed with an electronic cell counter (Cell-Dyn 3500, Abbott, Wiesbaden, Germany).

## Staining of peripheral blood lymphocytes (studies I and II)

Immunostaining was performed on EDTA-preserved blood samples within 12 hours after collection. Briefly, erythrocytes were lysed and then centrifuged. The resulting leucocyte pellet was then washed and resuspended in PBS with foetal calf serum. Half a million cells/mL were double-stained with primary porcine monoclonal antibodies (VMRD, Pullman, WA, USA) or with isotype controls (DakoCytomation, Glostrup, Denmark) as summarised in Table 1. After washing the cells, they were incubated with phycoerythrin (PE)-conjugated and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (CALTAG, Burlingame, CA, USA). The cells were washed again, resuspended and finally fixed in PBS with paraformaldehyde pending flow cytometric analyses.

## Flow cytometric analyses (studies I and II)

A total of 30 000 cells per sample were analysed for forward light scatter, orthogonal light scatter and green and orange fluorescence in a BD LSR flow cytometer (BDBiosciences, San José, CA). Lymphocytes, gated for by light scatter parameters, were further evaluated for FITC and/or PE fluorescence. The results were

evaluated by region analysis, except for the CD4/CD8α double staining where quadrant analysis was used. Evaluation was performed with the Cellquest software (BDBiosciences, San José, CA).

Table 1. Combinations of monoclonal primary antibodies to porcine antigens used during the double-staining procedures in studies I and II

| | **Anti-IgG1 FITC conjugate** | | **Anti-IgG2a PE conjugate** | |
|---|---|---|---|---|
| | **IgG1 isotype** | **Clone no.** | **IgG2a isotype** | **Clone no.** |
| Study I | CD3 | 8E6 | CD4 | PT90A |
| | CD8α | PT36B | CD4 | PT90A |
| | CD8α | PT36B | CD8β | PG164A |
| | CD25 | PGBL25A | CD4 | PT90A |
| | γδ (Po-TcR1-N4) | PGBL22A | CD4 | PT90A |
| | CD 21 | BB6-11C9 | CD8β | PG164A |
| Study II | CD8α | PT36B | CD4 | PT90A |
| | CD8α | PT36B | CD8β | PG164A |
| | γδ (Po-TcR1-N4) | PGBL22A | CD45 RA | PGB78A |
| | CD 21 | BB6-11C9 | CD45 RA | PGB78A |

## Serum amyloid A assays (studies II, III and IV)

SAA assays were performed with a commercially available ELISA kit (Tridelta Phase range SAA kit, Tridelta Development Limited, Greystones, Wicklow, Ireland).

## Serum antibody assays (study II)

Sera obtained on the following sampling occasions were analysed: before inoculation, day 2 of clinical signs of dysentery, and days 1, 7, 11 and 15 of the recovery period. In the pigs that remained healthy sera obtained before inoculation, and 14 and 28 days after challenge were used for the analyses. Sera from each pig collected on these separate sampling occasions were pooled, resulting in six pooled samples for the dysenteric pigs and three for the healthy pigs.

In addition, unpooled sera obtained from each of the swine dysentery-affected pigs on recovery days 7, 11 and 15 were analysed separately for specific serum antibodies. An absorption procedure was used in order to remove general antibodies to *Brachyspira spp*., thus leaving only those with the specificity for *B. hyodysenteriae* strain $B204^R$ (ATCC 31212). In brief, bacterial pellets of *B. innocens*, *B. pilosicoli*, *B. intermedia, B. murdochii* and *B. aalborgi* were diluted in a resuspension buffer and sonicated on ice. This mixture was then added to every serum sample and the samples were incubated for 90 min at 37 °C before use.

*Western blot of unabsorbed or absorbed sera*

Whole cell proteins from the type strain *B. hyodysenteriae* ($B78^T$) were separated by standard SDS-PAGE as described by Laemmli (1970). Bacterial samples (~2 µg of protein) were mixed with a sample buffer, heat treated, applied onto an SDS-polyacrylamide gel and together with a pre-stained, broad range, protein size marker (New England Biolabs, Beverly, MA) were subjected to electrophoresis (Mini-protean II gel system, Bio-Rad, Hercules, CA). The separated proteins were transferred onto a PVDF membrane with a Mini Trans-Blot cell (Bio-Rad). The membrane was blocked in TBS with dried non-fat milk powder and then incubated with absorbed or un-absorbed sera from the pigs in experiment II. A peroxidase-conjugated secondary anti-swine IgG antibody (DakoCytomation, Glostrup, Denmark) was used during a conjugate incubation. The membranes were washed and bound antibodies were visualised with a chemiluminescent substrate system (ECL-system and Hyperfilm ECL, Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions

## Cytokine assays (study III)

Serum levels of Il-1β, IL-6, IL-10, TNF-α and IFN-γ were determined with commercially available ELISA kits (Quantikine Porcine Immunoassays, R&D systems Europe Limited, Abingdon, UK), according to the manufacturer's instruction.

## Amino acid assays (study IV)

Free amino acids were measured in sera after precipitation of proteins with trichloroacetic acid followed by centrifugation. The amino acid content was measured fluorometrically after reversed-phase HPLC separation according to the method of Pfeifer *et al.* (1983) with some modifications. Briefly, a C18 column (Resolve™ C18 90Å, Waters Corporation, Massachusetts, USA) was used at a fixed temperature of 45°C. Precolumn derivatization of samples was performed with *o*-phthalaldehyde prior to injection. Separation was achieved with a gradient of mobile phase A (acetate buffer, methanol, tetrahydrofuran (93: 2: 5% v/v)) and mobile phase B (methanol, $ddH_2O$, tetrahydrofuran (65: 30: 5% v/v)).

## Glucose and lactate assays (study IV)

Plasma glucose concentrations were determined with a modified method described by Lowry and Passonneau (1972) and detected fluorometrically (FL 600®, Bio-Tek Instruments, Inc., Vermont, USA). Plasma lactate concentrations were measured with a lactate

analyser (Analox GM7, Analox Instruments Ltd., London, UK), according to the manufacturer's instruction.

## Statistical analyses, calculations and missing data

Data in text are presented as mean ± SE (studies I and II) or as mean ± SD (studies III and IV). The statistical calculations were performed with SigmaStat software (SPSS Science, Chicago, USA) and differences were regarded as statistically significant at $p<0.05$.

*Study I*

An unpaired *t*-test was used to compare differences before inoculation between the group that remained healthy and the group that developed swine dysentery after inoculation. Differences within each group, i.e. between pre- and post-inoculation values, were analysed with a paired *t*-test.

*Studies II, III and IV*

In order to avoid the effect of differences in growth rates during the fattening period, the daily weight gain was divided by the animal's live weight and presented as daily weight gain per kg live weight. In study II the proportion of stained cells was multiplied by the total number of lymphocytes determined from the total and differential counts in order to calculate the absolute numbers of lymphocytes in the various sub-populations.

Analysis of variance (ANOVA, Holm-Sidak method) for repeated measures was used to compare differences between measurement times. Correlation coefficients were calculated with the Pearson Product Moment Correlation. The results at day 14 post-inoculation are means from six pigs, since two animals had already developed dysentery at that time. Of the eight animals with clinical symptoms, one had to be euthanised on the second day of clinical symptoms and is therefore missing at later sampling points. In addition, the results at days 7, 11 and 15 of the recovery period are means from six out of seven animals used in studies II and IV, while there is an additional sample missing at day 7 in study III. The missing samples during recovery are from different pigs on different occasions.

# RESULTS AND DISCUSSION

## PRIOR TO INOCULATION

All animals were clinically healthy and the numbers of leucocytes and the SAA concentrations were within the reference values before the inoculation. However, there were interindividual differences in certain lymphocyte sub-population levels. Interestingly, some of these individual differences appeared to be associated with the susceptibility to dysentery after inoculation.

### Leucocyte and lymphocyte sub-populations (studies I and II)

There were no differences in the total numbers of neutrophils, monocytes or lymphocytes before inoculation between animals that subsequently developed dysentery and those that remained healthy (table 3). However, the pigs that subsequently got swine dysentery in experiment I had lower proportions of CD8α+ cells and higher proportions of γδ T cells before inoculation than those that did not develop swine dysentery (figure 4). This was not possible to verify in study II (experiment II). These individual differences between healthy animals may be related to exposure to certain microbes/antigens prior to the experiment that triggered shifts within lymphocyte populations. There may also be a normal genetic variation among pigs, such as the effect of the inherited MHC (termed SLA in pigs) molecule type on susceptibility to infections (Lunney 1993, 1994), which may explain why some individuals are more susceptible to certain diseases than others. It has earlier been proposed that such a genetic variation may influence the defence against infection in farm animals (Müller and Brem, 1991). In addition, a high glucocorticoid level during stress strongly influences immune functions through its immunosuppressive properties. The restraint of the pigs with snare may have stressed an individual, but this type of short restraint has previously been shown not to influence neither neutrophil nor lymphocyte numbers in blood (Roozen & Magnusson, 1996, Magnusson *et al*., 1998). Furthermore, pigs have been shown to be more resistant to the immunosuppressive effects of glucocorticoids than many other species (Flaming *et al*., 1994).

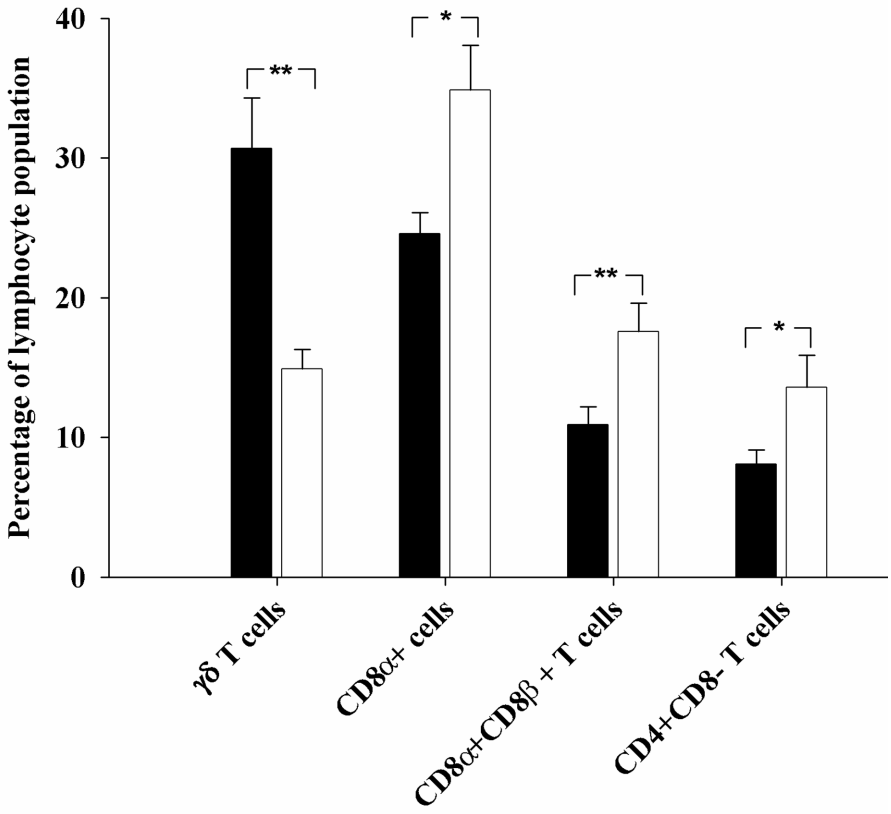


Figure 4. Proportions of γδ T cells, CD8+ cells, CD8+CD8β+ and CD4+CD8- T cells in the blood before the inoculation in study I. ■ denotes pigs that subsequently developed swine dysentery (n=12) and □ pigs that remained healthy (n=9). * $p < 0.05$; ** $p < 0.01$.

Nonetheless, there may be a connection between a low proportion of CD8α+ cells, a high proportion of γδ T cells and a higher susceptibility to dysentery. The presence of γδ T cells in mice is associated with a higher susceptibility to *Salmonella choleraesuis* infections (Emoto *et al.,* 1995), higher morbidity and mortality during *Trypanosoma cruzi* infections (Lima & Minoprio, 1996) and a slow and incomplete recovery from *Pneumocystis carinii* infections (Steele *et al*., 2002). Thus, in the protection against some infections the presence of γδ T cells may not be of advantage for the animal. In an attempt to explain this it may be necessary to look at the regulatory functions of γδ T cells. One of these is their ability to down-regulate the recruitment and function of CD8α+ T cells during infections, which could explain why the pigs with high proportions of γδ T cells had low proportions of CD8α+ cells. The CD8α+ cells have been shown to be a responding cell population during swine dysentery (Waters *et al*., 2000) and are normally present in large numbers in the intestinal epithelia (Fujihashi *et al*., 1990, Guy-Grand

& Vassalli, 1993). The low proportions of these cells prior to inoculation could therefore be a factor that influences the susceptibility to dysentery. In addition, the presence of γδ T cells in mice has been shown to be associated with a lower production of IFN-γ as compared to mice without γδ T cells (Williams *et al.,* 1996, Lima & Minoprio, 1996, Steele *et al*., 2002). CD8α+ cells are major producers of IFN-γ and a down-regulation of CD8α+ cells by γδ T cells (Steele *et al*., 2002) may lead to lower IFN-γ levels. As mentioned, IFN-γ is important for the activation of macrophages (Murray, 1990) and it might be speculated that low proportions of CD8α+ cells, and consequently low levels of IFN-γ, may result in reduced activation of macrophages after inoculation; thus, this may lead to increasing susceptibility to infections that are dependent on macrophage phagocytosis for their resolution.

## THE INCUBATION PERIOD

The incubation periods varied between 8 and 17 days in experiment I and between 7 and 31 days in experiment II (figure 5). The length of this period may be influenced by the monocyte levels.

### Leucocyte and lymphocyte sub-populations (studies I and II)

*Monocytes*

In experiment II two animals had a longer period of incubation (29 and 31 days compared with an average of 15±3 days in the other pigs) than the others. This may have been due to re-inoculation through faecal matter from dysenteric animals, as they were moved between the pens, rather than being an actually longer incubation period. However, the longer period may also have been related to their monocyte counts during that time, since they had higher numbers of monocytes before inoculation than the other animals had (4.0 and $3.3x10^9$/L vs. an average of $1.7\pm0.3x10^9$/L). This relationship was in general seen in both studies I and II, where the pigs with the highest monocyte counts before inoculation had the longest incubation period (study I: correlation coefficient 0.73, p=0.003, n=12; study II: correlation coefficient 0.76, p=0.03, n=8). However, a higher monocytes count prior to inoculation does not appear to influence the development of dysentery, since there was no difference in the pre-inoculation levels of monocytes between animals that developed swine dysentery and those that remained healthy after inoculation (table 3).

*CD8α+ T cells and γδ T cells*

The pigs that developed dysentery in experiment II showed an increase in the total number of circulating lymphocytes 4 days after inoculation (figure 6). These lymphocytes appear be CD8α+ cells (figure 7). Further, irrespective of the development of clinical signs of dysentery, all animals showed a general increase in circulating CD8α+ cells and γδ T cells on days 4 and 14 post-inoculation (figure 7 and 8). The numbers of γδ T cells increase in response to a variety of infections in different species (Hiromatsu *et al*., 1992, Rothwell *et al*., 1995, Koets *et al*., 2002) and have been shown to increase in pigs during immunisation with *B. hyodysenteriae* antigens (Waters *et al*., 2000, Bassaganya-Riera *et al*., 2001). The increase that was seen after inoculation in experiment II may have been a response to the introduction of *B. hyodysenteriae* antigens. As mentioned earlier, γδ T cells have broader antigen specificities (Constant *et al.*, 1994, Schoel *et al.*, 1994) than αβ T cells, and may thus be part of the early defence against *B. hyodysenteriae* after inoculation.

## Cytokines (study III)

There was an increase in IL-1β at day 14 of the incubation period (figure 9). This was associated with the development of dysentery and will be further addressed in that section below. However it is of interest that this increase was noted prior to clinical signs, which could indicate the progression of the incubation period to dysentery.

In addition, an increase in TNF-α was observed 4 days after the inoculation (figure 9). However, this increase was seen in all animals and may reflect a general response to the introduction of novel *B. hyodysenteriae* antigens rather than the development of dysentery.

## Amino acids (study IV)

A decrease in the serum serine concentration was observed 14 days after inoculation through day 7 of the recovery period in the pigs that developed dysentery (figure 12). This was associated with the dysentery and will also be further addressed below. Several other alterations in amino acid levels, such as leucine, isoleucine, aspartic acid and taurine, were noted at 4 and/or 14 days post-inoculation (see table 2 in paper IV), but these were seen to occur in all animals. Whether these alterations were due to the introduction of *B. hyodysenteriae* or to the change in feeding regime during inoculation is unclear at this time.

## THE DYSENTERY PERIOD

### Clinical signs of dysentery

In total, 20 out of 31 pigs (both experiments included) developed clinical signs of swine dysentery after inoculation (table 2). The animal infection model was successful and the morbidity was ~65%. All pigs that displayed clinical signs were shedding *B. hyodysenteriae* continuously during the dysentery period. The manifestation of the muco-haemorrhagic diarrhoea coincided with a deterioration in the general appearance of these animals. Otherwise, none of the 31 pigs displayed any clinical signs of disease other than those that were related to swine dysentery. In addition, the pathological examinations of the 11 animals that remained healthy after inoculation and of the 7 pigs that recovered from dysentery did not reveal any significant pathological findings.

Table 2:

| | Pigs (n) | Incubation time (days) | Diarrhoea type |
|---|---|---|---|
| *Experiment I (study I)* | 21 | | |
| Dysentery | 12 | 13 (range 8-17) | Muco-haemorrhagic : 7 pigs<br>Milder non-haemorrhagic : 5 pigs |
| Healthy | 9 | - | - |
| *Experiment II (studies II – IV)* | 10 | | |
| Dysentery | 8 | 17 (range 7-31) | Muco-haemorrhagic : 8 pigs |
| Healthy | 2 | - | - |

*Experiment I (study I)*

Six of the 12 pigs that developed dysentery were group-housed and the other 6 were of the animals that were housed individually, but moved in-between pens.

*Experiment II (studies II, III and IV)*

Eight pigs developed dysentery (figure 5). They had diarrhoea for an average of seven days (range 3-17 days) and muco-haemorrhagic diarrhoea for an average of four of these days (range 3-6 days). One pig had to be euthanised during the period with muco-haemorrhagic diarrhoea for welfare considerations and the pathological examination in this animal confirmed severe colitis.

The two animals that did not display clinical signs of dysentery had a steady daily weight gain of 25±2 g $kg^{-1}$ throughout experiment II. The animals that developed dysentery also showed a steady daily weight gain prior to clinical signs of disease (24±1 g $kg^{-1}$), but during the muco-haemorrhagic diarrhoea they had no daily weight gain at all. Their weight gain then increased again during the recovery period to 20±0 g $kg^{-1}$.

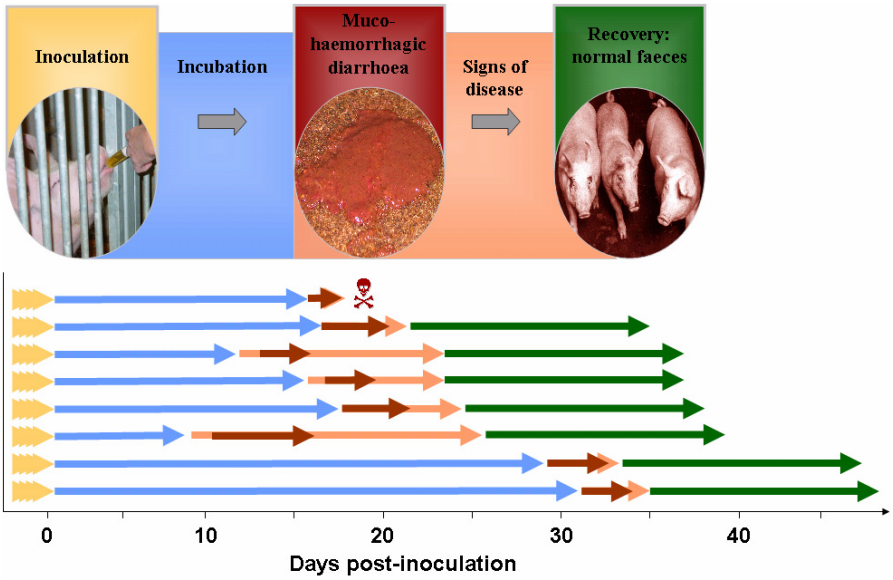

Figure 5. Illustration of the individual incubation (blue), dysentery (diarrhoea: peach, muco-haemorrhagic diarrhoea: red) and recovery (green) periods of the dysenteric animals (n=8) in experiment 2.

## Leucocyte populations (studies I and II)

*Monocytes*

A two-fold increase in the number of monocytes was observed during the period with clinical signs of dysentery both in study I (table 3) and study II (figure 6). This increase was significantly larger ($p<0.001$) in pigs with haemorrhagic diarrhoea (n=7; from $1.5\pm0.2x10^9$/L to $4.7\pm0.6x10^9$/L) than in those with milder non-haemorrhagic diarrhoea (n=5; from $1.5\pm0.4x10^9$/L to $2.5\pm0.7x10^9$/L). Monocytes and neutrophils have previously been shown to increase and to be present at colonic lesions during dysentery (Wilcock & Olander, 1979, Albassam *et al.* 1985, Jacobson *et al.* 2007). Thus, even though their role is still unclear, these cells seem to be important in the pathogenesis of swine dysentery.

*Neutrophils*

In both study I (table 3) and study II (figure 6) there was a two- to three-fold increase in neutrophils during the dysentery period. However, in study I this increase was also observed in the group without clinical signs of disease. The activation of neutrophils during dysentery may be beneficial, but can also be a factor that exaggerates the pathogenesis. This is supported by a study in mice in which the severity of intestinal lesions, oedema and epithelial erosions during

*B. hyodysenteriae*-induced colitis was found to be moderated when the intestinal neutrophils were reduced in number or totally absent (Sacco *et al.,* 2000). The migration of neutrophils to the intestinal tract and their release of active inflammatory and microbicidal mediators, such as prostaglandins, nitrogen oxide, reactive oxygen species and hydrolytic enzymes could be important contributors of the oedema and lesions that are associated with swine dysentery. Nevertheless, neutrophils are an essential part of the immune defence and they are probably necessary for the clearance of the spirochete.

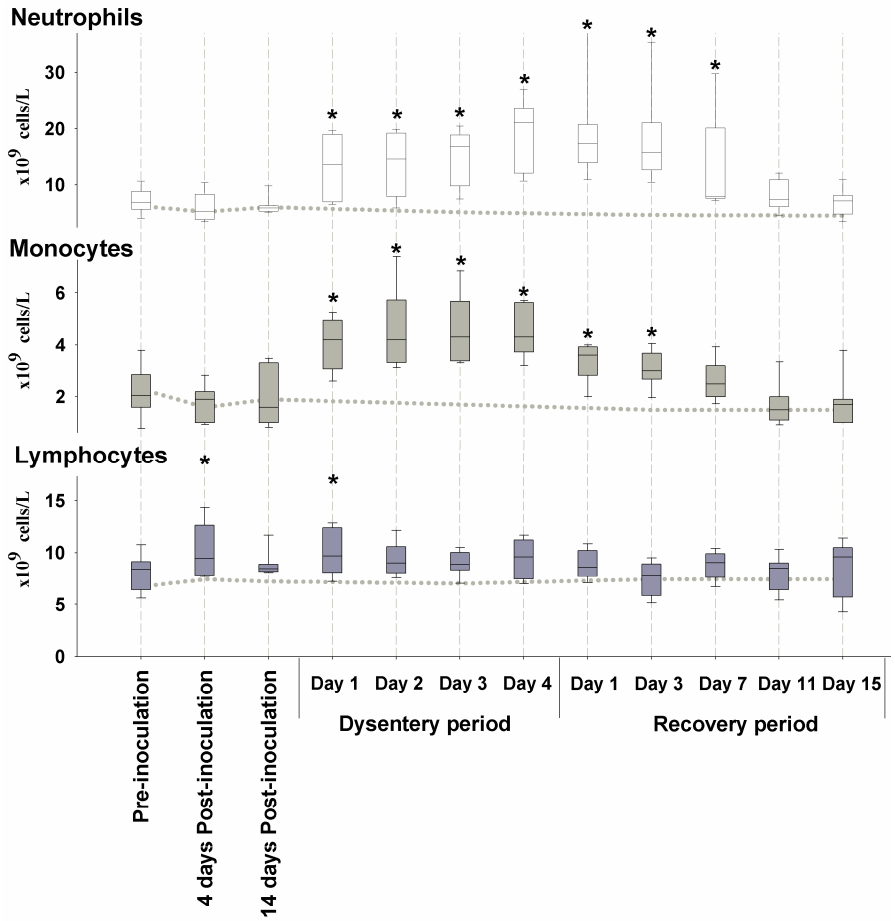


Figure 6. Leucocyte populations in the blood of dysenteric pigs pre-inoculation, 4 and 14 days post-inoculation, during days 1 to 4 with clinical signs of dysentery, and during the recovery period of study II. * denotes significant change from the pre-inoculation value. The boundaries of the boxes indicate the 25th percentile, the median and the 75th percentile. Whiskers indicate the 95th and 5th percentiles. The dotted lines behind each box plot show the mean of the two animals that remained healthy. The sampling points pre-, 4 days post- and 14 days post-inoculation are the same in all animals, but the remaining sampling points in the healthy animals are at 21, 28 and 35 days post-inoculation.

Table 3. Leucocyte and lymphocyte sub-populations in the pigs that developed dysentery and in those that remained healthy (study I).

| | Dysentery group (n=12) | | Healthy group (n=9) | |
|---|---|---|---|---|
| | **Before inoculation** | **Swine dysentery** | **Before inoculation** | **Euthanasia** |
| **Cell counts (x$10^9$/L)** | | | | |
| Leucocytes | 18.5 ± 2.1 | 23.9 ± 1.7 | 19.3 ± 1.5 | 21.2 ± 1.6 |
| Neutrophils | 7.3 ± 1.2 | 11.6 ± 1.2 * | 7.6 ± 0.7 | 10.7 ± 1.5 * |
| Monocytes | 1.5 ± 0.2 | 3.8 ± 0.5 * | 2.1 ± 0.3 | 2.3 ± 0.4 |
| Lymphocytes | 9.1 ± 1.3 | 8.3 ± 1.0 | 9.5 ± 1.5 | 8.1 ± 0.9 |
| **Lymphocyte sub-populations (%)** | | | | |
| T cells (CD3+) | 52.7 ± 3.7 | 61.7 ± 3.9 * | 53.6 ± 4.1 | 60.4 ± 4.0 * |
| γδ T cells | 30.7 ± 3.5 † | 29.0 ± 3.6 | 14.9 ± 1.4 | 20.6 ± 1.3 * |
| CD3+CD4+ T cells | 14.4 ± 1.5 | 17.4 ± 1.4 * | 20.1 ± 2.9 | 22.3 ± 3.2 |
| CD8α+ cells | 24.6 ± 1.5 † | 29.9 ± 2.7 | 34.9 ± 3.1 | 30.5 ± 2.0 |
| CD8α+CD8β+ T cells | 10.9 ± 1.3 † | 11.6 ± 0.7 | 17.6 ± 2.0 | 15.2 ± 1.7 |
| CD4+CD8α- T cells | 8.1 ± 1.0 † | 8.8 ± 0.8 | 13.6 ± 2.3 | 14.4 ± 2.8 |
| CD4+CD8α+ T cells | 5.8 ± 0.9 | 8.9 ± 0.7 * | 9.0 ± 2.0 | 8.7 ± 1.2 |
| B cells (CD21+) | 20.4 ± 1.9 | 20.9 ± 3.0 | 24.4 ± 1.5 | 22.1 ± 1.9 |

† $p<0.05$ denotes a difference before inoculation compared to the healthy group.
* $p<0.05$ denotes the change from before the inoculation within a group.

## Lymphocyte sub-populations (studies I and II)

The total number of circulating lymphocytes remained unchanged in study I but in study II an increase was seen at day 1 of the dysentery period. The total lymphocyte count is seldom reported to increase during swine dysentery. Instead, it is often of greater importance to examine the alterations in different lymphocyte sub-populations and such alterations occurred in both studies. In study I there was a total increase in T cells (CD3+ cells) during dysentery, but this was also seen in the healthy animals. In study II the intension was to triple stain the peripheral blood lymphocytes. Unfortunately, the monoclonal antibody against porcine CD3 was defective and the manufacturer was unable to replace it in time. Therefore it was not possible to analyse this expression directly in study II.

*CD8α+ cells*

In both study I (table 3) and study II (figure 7) cells bearing CD8α were altered during dysentery. An increase in CD8α+ cell populations during dysentery has been reported by Waters *et al*. (2000). Thus, it appears that CD8α+ cell populations are important during swine dysentery and should therefore be further investigated.

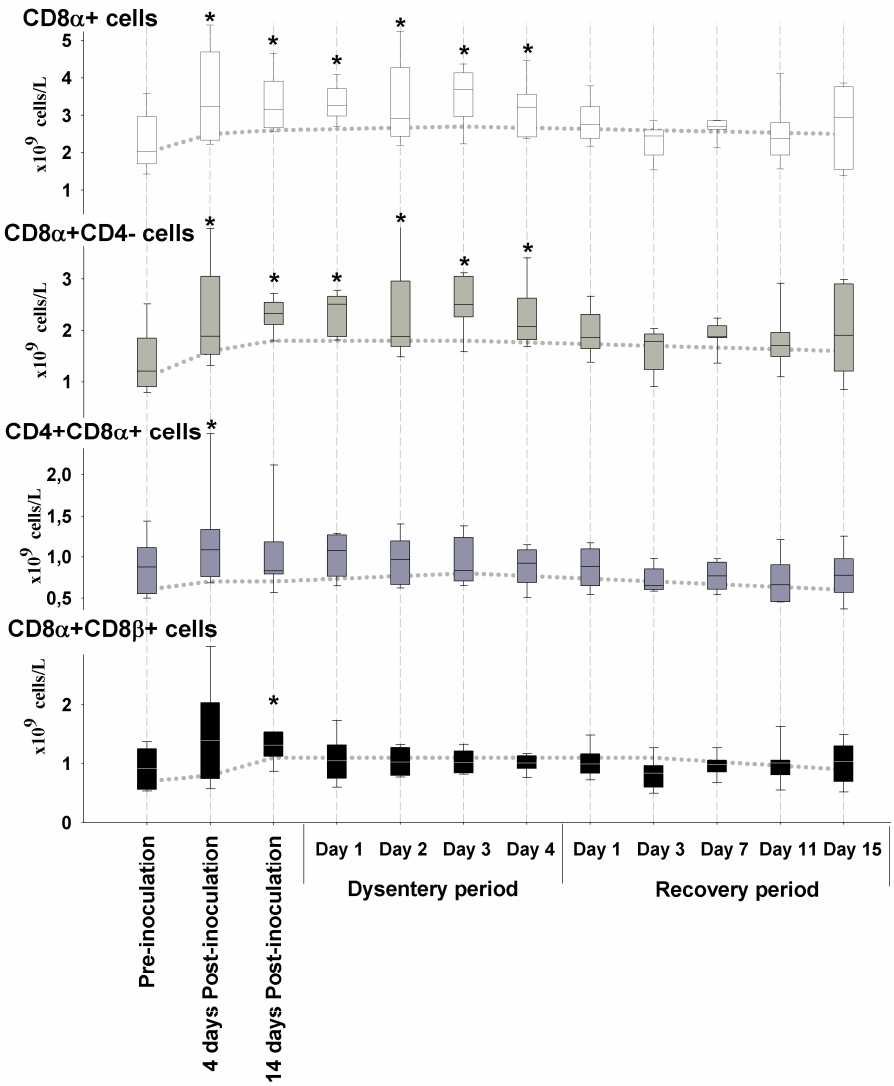


Figure 7. CD8α+ lymphocyte sub-populations in the blood of dysenteric pigs before inoculation, 4 and 14 days post-inoculation, during days 1 to 4 with clinical signs of dysentery, and during the recovery period of study II. * denotes significant change from the pre-inoculation value. The dotted line behind each box plot shows the mean of the two animals that remained healthy. For further information on sampling occasions and box boundaries see figure 6.

In study I there was a tendency of total CD8α+ cells to increase during dysentery (p=0.063). Cytotoxic T cells, NK cells, CD4+CD8α+ T cells and a fraction of γδ T cells all express the αα-homodimer of the CD8 receptor, but the CD8α+ cells that increased during dysentery in study I were mainly CD4+CD8α+ T cells. In study II these cells were seen to increase during the incubation period but not during dysentery. The CD4+CD8α+ T cells have been reported to increase substantially with age in pigs (Yang &

Parkhouse, 1996). However, there was no increase in CD4+CD8α+ T cells in the animals that remained healthy, or during recovery, and it is therefore unlikely that the increase was related to age. In a study by Waters *et al* (2000), no increase in CD4+CD8α+ T cells was observed during swine dysentery, but *B. hyodysenteriae* antigens have been shown to induce a proliferation response by porcine CD4+CD8α+ T cells *in vitro* (Waters *et al*., 1999a). The CD4+CD8α+ T cells that increased during dysentery could be antigen-experienced memory cells (Pescovitz *et al*., 1994, Zuckermann & Husmann, 1996) and through their production of IL-10 they may participate in the enhancement of developing B cell responses (Levy & Brouet, 1994; Ober *et al*., 1998).

In study II there was a significant increase in total CD8α+ cells during the dysentery period. In contrast to study I the majority of these CD8α+ cells were CD4-, thus not CD4+CD8α+ T cells, and also CD8β-, thus not cytotoxic T cells. Vaccination studies with *B. hyodysenteriae* antigens have shown increased peripheral levels of CD8α+ γδ T cells and CD8α+ natural killer cells (Waters *et al.*, 1999a, Bassaganya-Riera *et al.* 2001). Even though some γδ T cells also express CD8α, no increase in γδ T cell levels was seen during dysentery and it is more likely that the CD8α+ cells that increased during dysentery were CD8α+ NK cells. NK cells produce many important cytokines, such as IFN-γ, and may also direct cytotoxic attacks against target cells that undergo various forms of stress during infections. Bacterial LPS has previously been shown to augment murine NK cell activity (Djeu *et al.,* 1979). This augmentation has also been reported to occur with bacterial extracts from *B. hyodysenteriae* (Greer and Wannemuehler, 1989), but it is also possible that the cellular stress induced in the damaged epithelia during dysentery triggers NK cell activity.

*CD45RA cells (study II)*

The total numbers of CD45RA– cells increased during the period with clinical signs of dysentery and then returned to the pre-inoculation level during recovery (figure 8). These were not B cells, since there were no alterations in CD45RA+ B cells, and the increase is probably associated with the increase in CD8α+ cells. CD45 is required for efficient T cell receptor activation and the gene expression is regulated such that naïve T cells are CD45RA+ and when activated they become CD45RA– (Janeway, 1992). The increase in CD45RA- cells during dysentery shows that an increase in the number of activated and proliferating lymphocytes occurs during clinical signs of dysentery.

*γδ T cells*

No general change in the peripheral γδ T cell levels during clinical signs of dysentery was observed either in study I or study II, but the two pigs with the longest episode of diarrhoea in study II (17 and 12 days vs. an average of 5 ±1 days in the other animals) had the lowest levels of γδ T cells during the dysentery and recovery periods. It has been reported that swine dysentery induces a loss of intraepithelial colonic γδ T cells in pigs (Hontecillas *et al.,* 2005). This is most likely caused by the epithelial erosions that occur in the colon during dysentery (Hutto & Wannemuehler, 1999, Hontecillas *et al.,* 2005). The loss of intraepithelial γδ T cells during dysentery may further aggravate the colitis, since the absence of γδ T cells in mice has been shown to cause decreased epithelial proliferation and increased intestinal damage (Komano *et al*., 1995). This might be attributable to the immunoregulatory role of intraepithelial colonic γδ T cells. Whether the loss of local γδ T cells in the intestinal tract influences the numbers of γδ T cells in the blood is not known, but a general decrease was not observed in the present investigations.

*B cells (CD 21+ cells)*

There was no increase in B cells in either study I (table 3) or study II (figure 8). This does not necessarily mean that there was no activation of B cells, in view of the possibility that a local proliferation might have occurred without being apparent in the circulation. The numbers of B cells in the blood in study II were generally lower after inoculation. It has been reported that a gradual decrease of B cells occur in pigs several weeks after weaning (Solano-Aguilar et al. 2001), but it is not known whether this still occur at the same age as the animals used in experiment II. The decrease in study II may have been due to increased migration of B cells from the blood into the gastro-intestinal tract in response to the introduction of *B. hyodysenteriae*. However, in a study in which biopsy specimens were subsequently taken from the colon of dysentery-affected pigs the numbers of colonic B cells showed no tendency to increase at clinical signs of disease (Jacobson *et al*., 2007). In addition, T cells but not B cells from pigs vaccinated with *B. hyodysenteriae* antigens have been reported to proliferate in response to *in vitro* stimulation with the same antigens (Waters *et al*., 1999a). This has raised the question whether the recovery from swine dysentery could primarily be dependent on non-humoral defences through the activities of CD8α+ cells, neutrophils and macrophages.

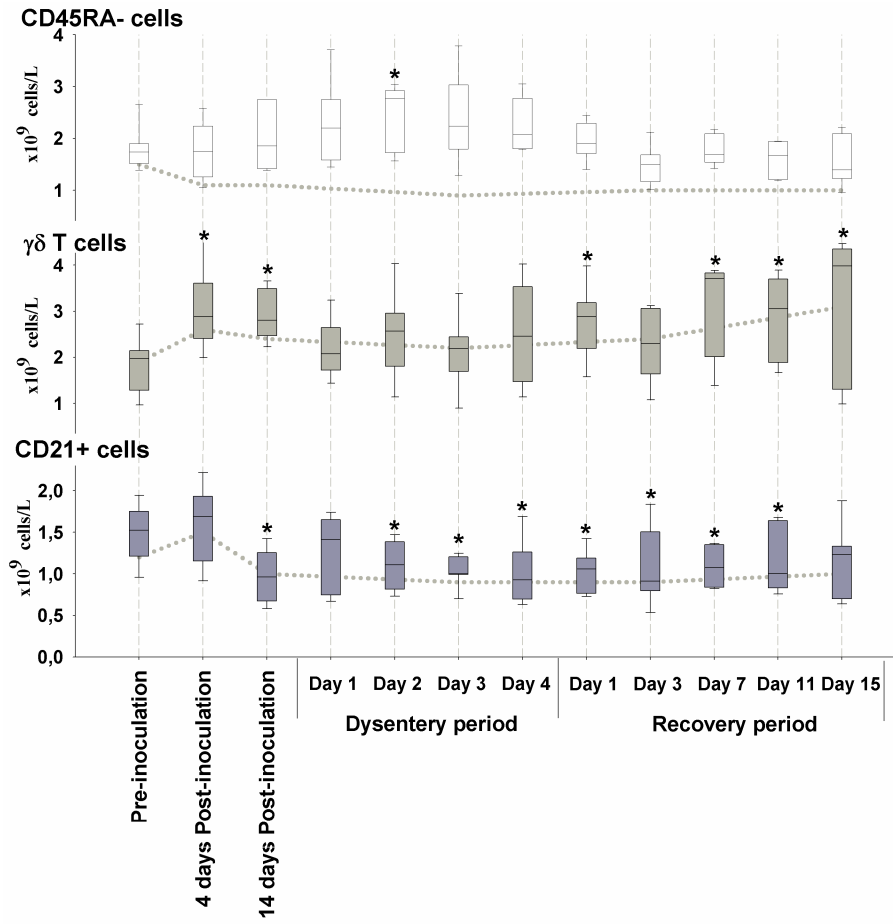


Figure 8. Lymphocyte sub-populations in the blood of dysenteric pigs pre-inoculation, 4 and 14 days post-inoculation, during days 1 to 4 with clinical signs of dysentery, and during the recovery period in study II. * denotes a significant change from the pre-inoculation value. The dotted line behind each box plot shows the mean of the two animals that remained healthy. For further information on sampling occasions and box boundaries see figure 6.

## Cytokines (study III)

*IL-1β*

An increase in the serum concentrations of IL-1β was seen in all dysenteric animals at onset of clinical signs of the disease (figure 9). IL-1β is produced by a variety of porcine cells, such as macrophages and even intestinal epithelial cells (Stadnyk, 2002). It is possible that the increases in monocytes, and perhaps also the damaged epithelial cells of the colon, are significant contributors to the IL-1β produced during dysentery. Bacterial LPS and endotoxins are common inducers of IL-1β (Huether *et al.,* 1993) and LPS and endotoxin extracts from *B. hyodysenteriae* have been shown to induce IL-1β *in*

*vitro* (Greer and Wannemuehler 1989, Sacco *et al.,* 1996). The results of study III indicate that production of IL-1β is also induced in the *in vivo* situation. IL-1β is an endogenous pyrogen and is generally associated with pyrexia. However, swine dysentery does not as a rule appear to induce fever and the only animal with an elevated body temperature during clinical signs of disease was the pig that had to be euthanised because of severe signs of dysentery.

IL-1β can also elicit local responses, such as increased vascular permeability and oedema, and together with TNF-α it can augment the effects of prostaglandins and thereby alter the intestinal epithelial cell ion transport in pigs (Kandil *et al.,* 1994). With this in mind, the effects of IL-1β could play a major role in the development of diarrhoea during swine dysentery. Furthermore, IL-1β is known to cause neutrophilia in rats (Ulich *et al.,* 1987) and to be an activator of human neutrophils (Smith *et al.,* 1986). This activation can induce migration of neutrophils from the blood into the intestinal tract during dysentery. As mentioned earlier, increased numbers of circulating neutrophils were observed during dysentery and the presence of IL-1β in the blood probably enhances their migration to the infected colon. Furthermore, IL-1β has been shown to induce anorexia, weight loss and catabolism in rat skeletal muscle (Ling *et al.,* 1997). IL-1β has also been shown to reduce intestinal absorption and blood levels of alanine in rats (Argilés *et al.,* 1989). Alanine is the major gluconeogenic substrate that is released during catabolic stress and, as will be discussed further below, there was a decrease in plasma alanine during dysentery in the pigs of experiment II. Thus, IL-1β could influence the catabolic processes and may thus contribute to the decrease in alanine during dysentery.

*TNF-α*

An increase in TNF-α was observed during the period with dysentery (figure 9), but it is difficult to say whether this was associated with clinical signs of dysentery, since the two animals that remained healthy displayed a similar increase after inoculation. One explanation of this might be that the apparently healthy animals have harboured a sub-clinical *B. hyodysenteriae* infection, even though no shedding of bacteria was observed. The general increase may also reflect a response to a co-infection with other bacteria that induce serum TNF-α in pigs, such as the common *E. coli* (Jesmok *et al.,* 1992, Zhu *et al.,* 2004). In addition, even though all animals were clinically healthy before the inoculation, low serum levels of TNF-α were noted at that sampling occasion. Nonetheless, similar levels of TNF-α have been reported previously in other apparently clinically healthy pigs (Zhu *et al.,* 2004) and may not be so uncommon.

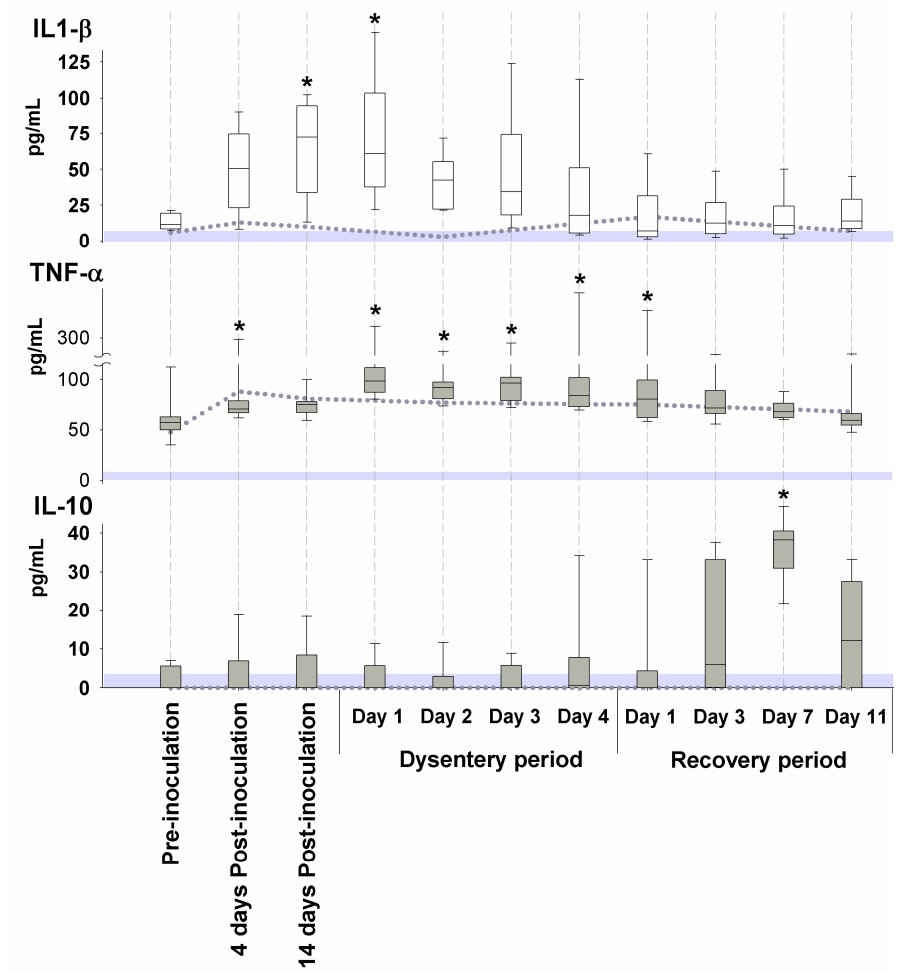


Figure 9. Concentrations of IL-1β, TNF-α and IL-10 in the blood of dysenteric pigs before inoculation, 4 and 14 days post-inoculation, during days 1 to 4 with clinical signs of dysentery, and during the recovery period in study III. The dotted line behind each box plot shows the mean of the two animals that remained healthy and the shaded areas above zero represent the detection limit of the assays. * denotes a significant change from the pre-inoculation value. For further information on sampling occasions and box boundaries see figure 6.

The production of TNF-$\alpha$ is rapidly induced by LPS and other bacterial cell wall products in a variety of porcine cells, but especially in macrophages/monocytes (Baarsch *et al.,* 1991). As mentioned earlier, this cytokine can facilitate lesion development in the intestinal tract (Beutler *et al.,* 1985), and the increase during dysentery may therefore influence the pathogenesis and aggravate the colitis. In addition, TNF-$\alpha$ also induces anorexia, skeletal muscle catabolism (Ling *et al.,* 1997) and may enhance the catabolic effects of IL-1β. Studies with *B. hyodysenteriae* LPS and endotoxin extracts show discrepancies regarding TNF-α induction. When injected

intravenously into pigs these extracts failed to induce TNF-α in serum (Nibbelink *et al.,* 1997). In addition they failed to induce TNF-α in porcine macrophages *in vitro* (Sacco *et al.,* 1996), but the endotoxin has been shown to induce TNF-α in murine cells (Greer and Wannemuehler, 1989). As mentioned earlier it is important to discriminate not only between *in vitro* and *in vivo* situations, but also between studies with bacterial extracts and actual infections, in which a multitude of factors, other than LPS and endotoxins, may cause the release of certain cytokines.

*IL-6*

IL-6 generally displays slow and stable plasma kinetics (for review see Bozza *et al.,* 2005) and has been proposed to be a useful biomarker of bacterial infections in pigs. It has been reported that IL-6 is present in the serum for several days after an *Actinobacillus pleuropneumoniae* infection in pigs (Fossum *et al.,* 1998). However, low concentrations of IL-6 (30-40 pg/mL) were only detected in three of the eight animals afflicted with dysentery in experiment II and similar levels were seen in the two pigs that remained healthy after inoculation. LPS and endotoxin extracts from *B. hyodysenteriae* have been reported to induce IL-6 in serum when injected intravenously in pigs (Nibbelink *et al.,* 1997), but failed to induce IL-6 expression in porcine macrophages *in vitro* (Sacco *et al.,* 1996). In view of these findings and the absence of IL-6 in five of the pigs with diarrhoea, it appears that IL-6 is not a reliable marker of swine dysentery. It is possible that the pigs without IL-6 in serum had circulating levels at an occasion other than that covered in experiment II, but this would nonetheless indicate that IL-6 was not a reliable biomarker of this disease. Furthermore, the low IL-6 levels observed after inoculation in the two pigs that remained healthy could also suggest the presence of a sub-clinical infection. This elevation of serum IL-6 may also have been a reaction to the inoculation itself, as has been shown to occur after endothelial damage in pigs (Johansson *et al.,* 2002). IL-6 is important for immune functions, but it may also play a role in the regulation of substrate metabolism. In humans IL6 is considered to be a potent modulator of the fat metabolism (van Hall *et al.,* 2003, Petersen *et al.,* 2005) and it may also influence the glucose metabolism (Stensberg, 2003). There are also indications that IL-6 may initiate catabolism of rat muscle tissue *in vitro* (Ebisui *et al.,* 1995).

*IFN-γ*

There were no detectable levels of IFN-γ in the serum of any of the animals, which does not exclude the possibility that this cytokine might have been produced locally in the intestinal tract. In a previous study it was found that dysenteric pigs showed up-regulated

expression of IFN-γ in colonic lymph nodes (Hontecillas *et al.* 2002). Considering the increase in monocytes during dysentery and the significance of IFN-γ for monocyte activation (Murray, 1990), participation of IFN-γ in some phase of dysentery seems plausible. In addition, the increase in CD8α+ lymphocytes during dysentery adds to the assumption that IFN-γ is participating, since these cells are major producers of IFN-γ. Monocytes readily produce IL-6 and IL-10, but when activated by IFN-γ they can also produce IL-1β and TNF-α (Lucey *et al*., 1996). The finding that all these cytokines were detected in the serum of most dysenteric pigs in experiment II further points to the importance of monocytes as effectors during dysentery. It has been reported that blood lymphocytes from pigs vaccinated with *B. hyodysenteriae* antigens produced IFN-γ when re-exposed to these antigens *in vitro* (Waters *et al.,* 1999b). This production was not initiated in isolated colonic lymphocytes, demonstrating that there are differences in the localisation of cytokine expression during immune responses. This compartmental difference may also be true for other cytokines than IFN-γ.

### Serum amyloid A (studies II, III and IV)

IL-1β, TNF-α and IL-6 (for review see Jensen & Whitehead, 1998) are all important inducers of hepatic production of the acute phase protein SAA and elevated levels of SAA were seen during the dysentery period in experiment II (see data in papers II to IV). There were large inter-individual variations in the SAA response, but most of the affected pigs had a 30- to 60-fold increase during the first three days of clinical signs, which is in accordance with an earlier report (Jacobson *et al.*, 2004). The three sick animals that had detectable levels of IL-6 were among the four animals with the highest SAA concentrations during disease. IL-6 is a potent inducer of SAA, but it requires IL-1 in order to be able to act as an inducer (Marhaug and Dowton, 1994). This explains why the two pigs that remained clinically healthy had no detectable SAA, in spite of the presence of IL-6. SAA generally provokes infiltration of monocytes and neutrophils into inflamed tissue (Badolato et al. 1994, Xu et al. 1995) and might thereby enhance their migration into the colon during swine dysentery. Furthermore, SAA may influence the metabolism and could have induced some of the alterations in amino acids levels that are described below.

### Glucose, lactate and amino acids (study IV)

The alteration in glucose, lactate and amino acid concentrations were used as indicators of metabolic changes during dysentery. However, in consideration of homeostatic effects we cannot rule out the possibility that apparently unaffected levels could in fact be the

result of an increase in catabolism or utilisation of the substance in question at the same time as an equivalent increase in *de novo* synthesis or release from tissues occur.

Given that the sick animals had no weight gain during dysentery, it is likely that nutrients were diverted from growth processes to yield the energy essential for the basal metabolism and the immune responses. The increased consumption of glucose that takes place in leucocytes and many tissues during immune responses could make an animal hypoglycaemic if the oral intake of feed, intestinal uptake of glucose or the gluconeogenesis is insufficient to compensate for this need. There was a decrease in the total serum concentration of gluconeogenic amino acids during clinical signs of dysentery (figure 10), primarily due to a drop in glutamine, alanine, serine and tyrosine concentrations (figure 11 and 12). This may have been caused by a fall in luminal uptake/synthesis during diarrhoea or by an increased utilisation of these amino acids. As they are gluconeogenic, they could have been converted into glucose.

Bearing in mind the increases in monocytes, neutrophils and lymphocyte sub-populations that were observed during dysentery, much of the available glucose may have been consumed by these cells. There was negative correlation between the alanine level and the blood counts of neutrophils and monocytes (see table 3 in paper IV), which most likely reflects the high utilisation of glucose by these cells. Nevertheless, no significant changes in plasma glucose were seen in the dysentery-affected animals (figure 10) and it appears that the demand for glucose was met with an adequate gluconeogenesis. Conversely, two animals had elevated levels of glucose during dysentery. Such hyperglycaemic levels have been reported during experimental swine dysentery (Somchit *et al.,* 2003). The released glucose together with an increased insulin resistance during an infection may under certain conditions result in hyperglycaemia (for review see Mizock, 1995). In rats, this insulin resistance has been shown to be most prominent in skeletal muscles (Lang *et al.,* 1990).

In addition, there were no significant alterations in plasma lactate during dysentery (figure 10). However, an increased lactate production may be masked by an augmented recycling of lactate into glucose, since lactate is a key gluconeogenic substrate during stress (Wolfe and Burke, 1978). There was a positive correlation between plasma lactate levels and lymphocyte counts in the dysentery-affected animals (see table 3 in paper IV). This correlation could be associated with the high production of lactate from glucose that is seen in lymphocytes (Pithon-Curi *et al.,* 2004).

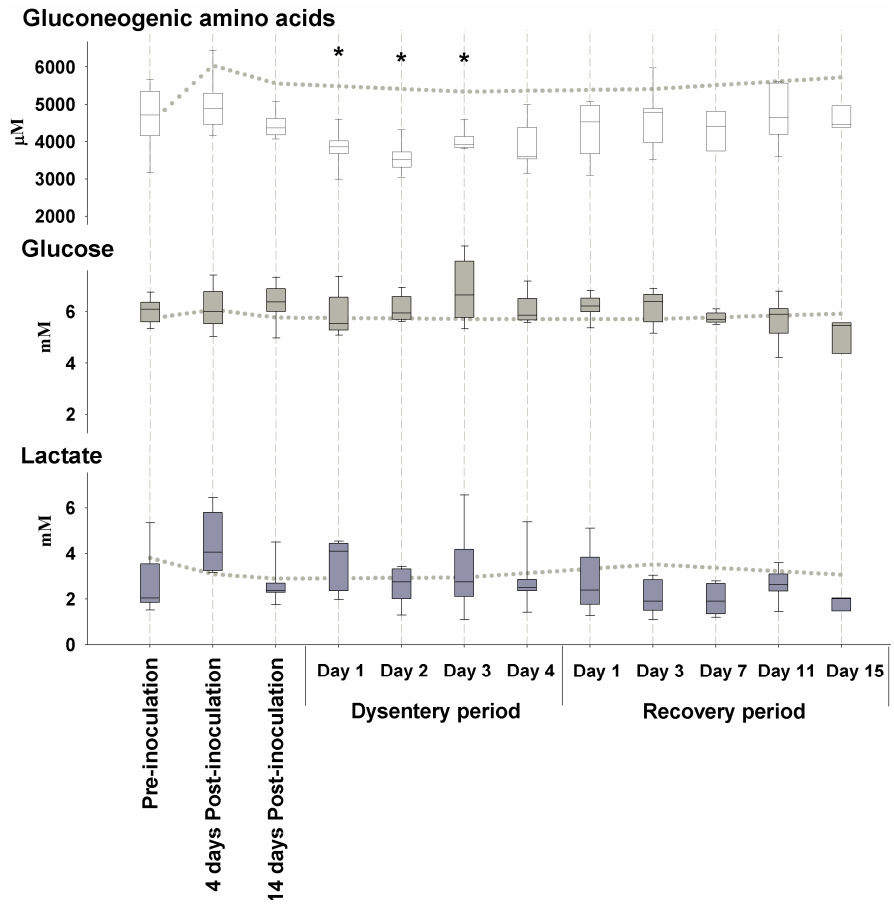


Figure 10. Concentrations of gluconeogenic amino acids, glucose and lactate in the blood of dysenteric pigs before inoculation, 4 and 14 days post-inoculation, during days 1 to 4 with clinical signs of dysentery, and during the recovery period in study IV. * denotes a significant change from the pre-inoculation value. The dotted line behind each box plot shows the mean of the two animals that remained healthy. For further information on sampling occasions and box boundaries see figure 6.

*Glutamine and alanine*

There were substantial drops in the plasma glutamine and alanine concentrations during clinical signs of dysentery (figure 11). A decrease in plasma glutamine and alanine has previously been reported to occur in *E. coli* infected pigs (Yoo *et al.,* 1997). During infections, the liver shows increased gluconeogenesis and increased synthesis of glutathione and acute phase proteins (Austgen *et al.,* 1991). The observed decrease during dysentery could thus derive from an increased hepatic uptake and breakdown of these amino acids into glucose and from synthesis of proteins. In addition, the decrease in glutamine is probably also connected to the high glutamine utilisation by activated monocytes, neutrophils

(Newsholme *et al.,* 1985, Curi *et al.,* 1997) and lymphocytes (Newsholme *et al.,* 1985). Monocytes and neutrophils rely on the metabolism of glucose and glutamine for endocytosis and for the synthesis and release of active compounds (Ogle *et al.,* 1994, Newsholme *et al.,* 1999). This could explain the negative correlation that was found between glutamine levels and the numbers of neutrophils and monocytes (see table 3 in paper IV). Lymphocytes have a high need of glucose and, in particular, glutamine. This amino acid has been shown to be essential for their activation and proliferation processes (for review see Calder & Yaqoob, 1999). However, there was no correlation between glutamine levels and lymphocyte counts, but this may have been due to the absence of major variations in total lymphocyte counts during the dysentery period.

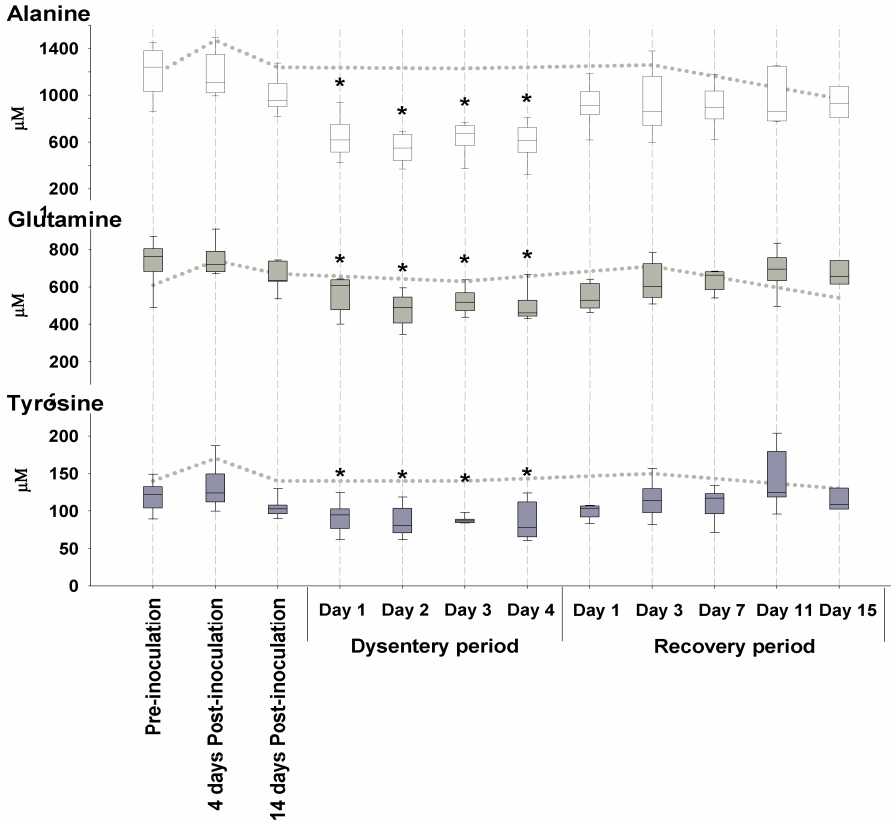


Figure 11. Concentrations of alanine, glutamine and tyrosine in the serum of dysentery-affected pigs before inoculation, 4 and 14 days post-inoculation, during days 1 to 4 with clinical signs of dysentery, and during the recovery period in study IV. * denotes a significant change from the pre-inoculation value. The dotted line behind each box plot shows the mean of the two animals that remained healthy. For further information on sampling occasions and box boundaries see figure 6.

The fall in glutamine levels during dysentery could have adverse effects on the intestinal tract. Glutamine is essential for and utilised in large amounts by the intestinal mucosa and in rats it has been found to have a protective effect against atrophy of the intestinal mucosa (O'Dwyer *et al.,* 1989). Thus it is possible that the mucosal and epithelial erosions that occur during swine dysentery lead to an increased utilisation of glutamine by the damaged mucosa. Consequently, a decreased availability of glutamine could aggravate the atrophy of intestinal cells during dysentery. Furthermore, alanine has an important regulatory influence on a group of inhibitory amino acids that suppress protein catabolism (Pösö & Mortimore, 1984). The decrease in alanine and in the inhibitory amino acids glutamine and tyrosine during the dysentery period may lead to muscle protein degradation during this period.

*Lysine, tyrosine and serine*

The serum lysine concentrations increased at the end of the dysentery period and at the beginning of the recovery period (figure 12). In a study of intestinal lysine metabolism in pigs, van Goudoever *et al.* (2000) found indications that the portal-drained viscera (i.e. intestines, stomach, spleen and pancreas) have a high utilisation of lysine and that it was the mucosa that was responsible for this utilisation. Further, this study also showed that all the utilised lysine was obtained from the blood. The increase in lysine during the late dysentery period in study IV may be due to reduced oxidation and uptake of lysine from the blood by the damaged and eroded mucosa and epithelial layer of the intestines.

The decreased concentrations of tyrosine (figure 11) and serine (figure 12) during dysentery could be related to the production of acute phase proteins, since, at least in humans, SAA contain high amounts of tyrosine (Reeds *et al.,* 1994) and serine is an amino acids that acute phase proteins in general are especially rich in (Grimble *et al.,* 1990). In addition, during inflammation there is an increased production of substances such as glutathione that require sulphur-containing amino acids (Grimble *et al.,* 1992). One of the most important sulphur-containing amino acids is cysteine. In the amino acid analysis in study IV this amino acid could not be quantified, but the carbon skeleton of cysteine is derived from serine. Thus, it could be that the drop in serine is related to an increased demand of cysteine during dysentery.

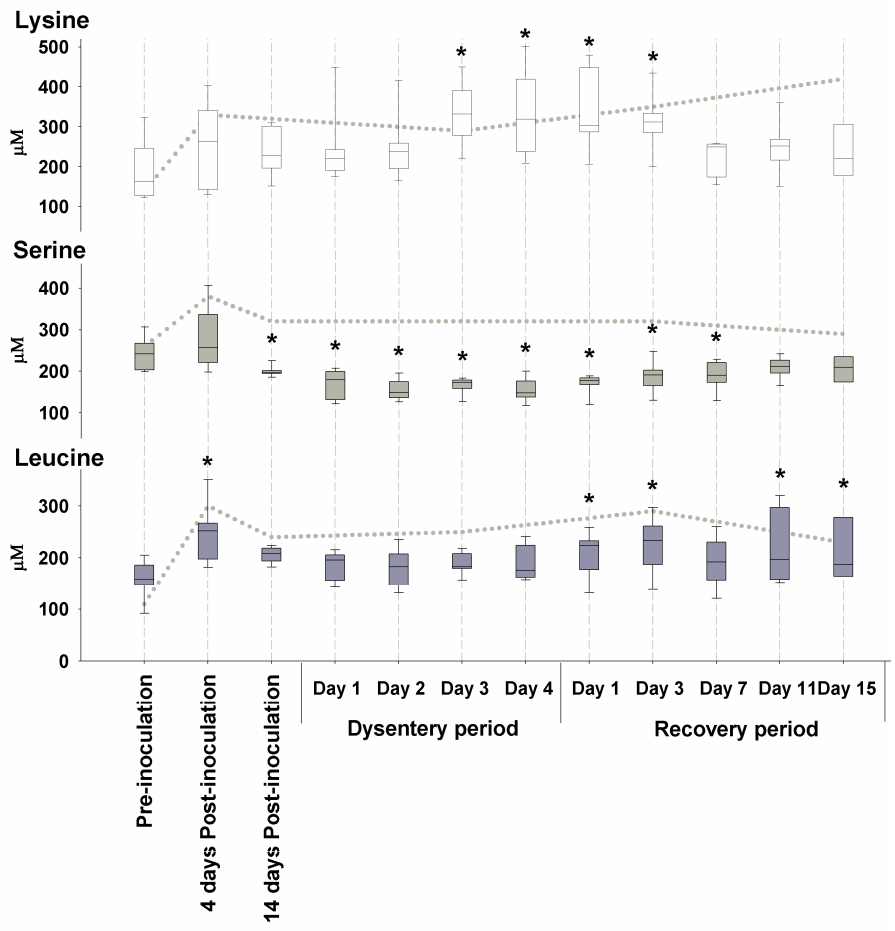


Figure 12. Serum levels of lysine, serine and leucine before inoculation, 4 and 14 days post-inoculation, during days 1 to 4 with clinical signs of dysentery, and during the recovery period in study IV. * denotes a significant change from the pre-inoculation value. The dotted line behind each plot represents the mean of the two animals that remained healthy. For further information on sampling occasions and box boundaries, see figure 6.

## THE RECOVERY PERIOD

All but one of the dysentery-affected pigs in experiment II recovered spontaneously after the period with clinical signs of disease. Five of these pigs stopped shedding *B. hyodysenteriae* after an average of eight days of recovery, while the remaining two animals were still shedding at euthanasia. Several factors, such as SAA, daily weight gain and most cytokines, lymphocyte sub-populations and metabolites returned to their pre-inoculation levels during the recovery period. However, the numbers of γδ T cells increased and the presence of IL-10, *B. hyodysenteriae*-specific antibodies and the elevated concentrations of lysine and leucine may have been associated with the recovery period.

### Lymphocyte sub-populations (study II)

*γδ T cells*

The numbers of γδ T cells increased throughout the recovery period (figure 8). However, they also increased in the animals that remained healthy and a similar increase after inoculation was seen in the healthy group in study I (table 3). Thus, the increase in γδ T cells may not have been associated with recovery from dysentery. As mentioned above, swine dysentery induces a loss of intraepithelial colonic γδ T cells (Hontecillas *et al.,* 2005) and the increase in these cells in the blood during recovery may be due to a proliferation aimed at replenishing the loss of intraepithelial γδ T cells in the colon. In addition, murine intestinal intraepithelial γδ T cells have been shown to stimulate both proliferation and differentiation of epithelial cells *in vitro* (Boismenu & Havran, 1994). Thus, an increase in γδ T cells in the blood and perhaps subsequently in the colon during recovery may promote the regeneration of the damaged intestinal epithelium.

### Cytokines (study III)

*IL-10*

IL-10 increased during the recovery period, and at day 7 of this period all the sampled animals showed an increase (figure 9). The appearance of this anti-inflammatory cytokine during the recovery period coincided with the disappearance of clinical signs of disease and with the appearance of *B. hyodysenteriae*-specific serum antibodies (see below). The latter finding may be due to the stimulatory effect exerted by IL-10 on B cells to increase their antibody production and to induce Ig-class switching and plasma cell differentiation (Rousset *et al.,* 1992, Saeland *et al.,* 1993, Rousset *et al.,* 1995). IL-10 is primarily produced by Th2 cells, monocytes and

B cells (Howard & O'Garra, 1992, Opal *et al.* 1998), but also by intestinal epithelial cells (Stadnyk, 2002), and the increase in IL-10 that was seen during recovery could in part derive from the regenerating epithelial layer in the colon.

## Antibodies (study II)

Despite the absence of an increase in B cell numbers, there was a production of *B. hyodysenteriae* specific serum antibodies during recovery (figure 13), which indicates activation of B cells. As is shown in figure 13a, antibodies were present in sera collected during the recovery period and the absorbed sera in figure 13b show that some of these were specific for *B. hyodysenteriae* antigens. The specific antigen at ~16 kDa has previously been reported to be the outer membrane-associated lipoprotein SmpA of *B. hyodysenteriae* (Thomas *et al.,* 1992, Thomas & Sellwood, 1993), and the weaker band at ~30 kDa is most likely the membrane lipoprotein BmpB (Lee *et al.,* 2000).

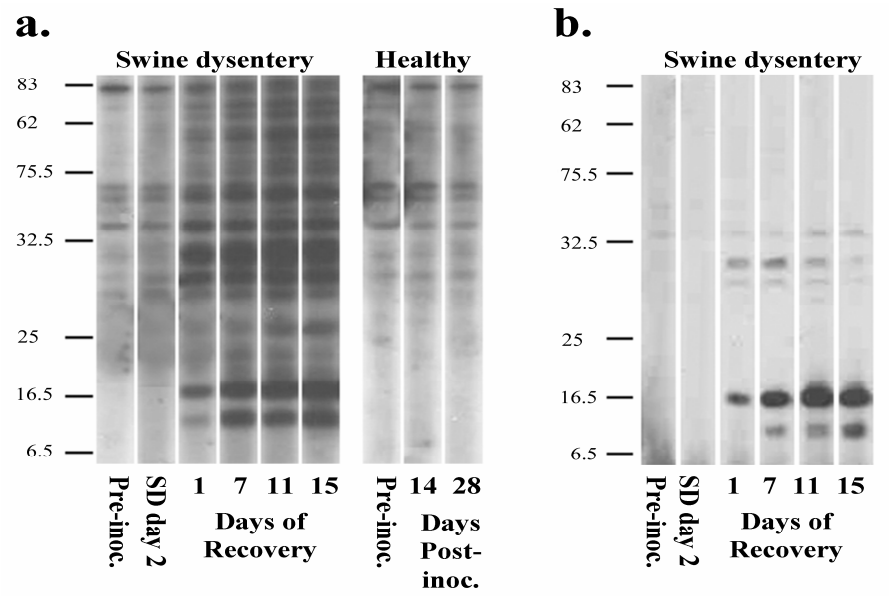


Figure 13. Immunoblot analysis showing the presence of antibodies before and after inoculation with *B. hyodysenteriae* in study II. (a) Serum antibodies bound to whole cell proteins from *B. hyodysenteriae*. (b) B. hyodysenteriae-specific antigens in sera after absorption with *B. intermedia, B. innocens, B. murdochii, B. pilosicoli and B. aalborgi.* Molecular size markers are shown on the left sides (kDa).

Antibodies against SmpA have been shown to inhibit the growth of *B. hyodysenteriae in vitro* (Thomas & Sellwood, 1992). The binding of these antibodies to *B. hyodysenteriae* and subsequent binding to Fc receptors on immune cells such as neutrophils, macrophages and

NK cells may initiate an antibody-dependent cell-mediated cytotoxicity (ADCC) that could be an important part of the recovery. *B. hyodysenteriae* specific antibodies have previously been shown to be present in sera (Joens *et al.,* 1979) and colonic washings after onset of clinical signs of swine dysentery (Joens *et al.,* 1984). In study II the recovery of the affected animals coincided with the appearance of *B. hyodysenteriae*-specific serum antibodies. However, in previous studies the presence of antibodies has not shown relationship to the recovery from dysentery. Nevertheless, pigs that have recovered from swine dysentery are immune to reinfection (Joens *et al.,* 1979, Joens *et al.,* 1983) and this immunity appears to be mediated by antibodies (Joens *et al.,* 1979, Harris and Glock, 1981). In addition, there is evidence of B cell mediated humoral responses, with production of circulating IgG and IgA antibodies and locally mucosa-associated sIgA against *B. hyodysenteriae* (Joens *et al*., 1984, Rees *et al*., 1989). Thus, the relative importance of the antibody-mediated immunity for the recovery from infection with *B. hyodysenteriae* is not known and needs to be further addressed.

## Amino acids (study IV)

*Lysine*

Lysine was increased at the end of the dysentery period and at the beginning of the recovery period, after which it returned to the pre-inoculation levels (figure 12). This might be explained by lower lysine utilisation by the damaged intestines, as discussed above.

*Leucine*

There was also an increase in leucine during the recovery period (figure 12). Given that pigs are unable to synthesise branched-chain amino acids, such as leucine, an increase in the plasma might be explained by enhanced intestinal uptake or a release from primarily skeletal muscle. Leucine has been reported to be an effective activator of skeletal muscle protein synthesis in rats (Jefferson and Kimball, 2001). The increase in leucine may thus be related to muscle protein repletion during the increased weight gain during recovery from dysentery. However, an increase in leucine was also seen in the pigs that remained clinically healthy and may thus be a general effect of growth in fattening pigs rather than a factor associated with dysentery.

## GENERAL SUMMARY

The susceptibility to dysentery may have been related to low levels of CD8α+ lymphocytes and high levels of γδ T cells before inoculation. Also, a longer incubation period was associated with high monocyte counts before disease. Development of disease was related to high numbers of monocytes, neutrophils and CD8α+ lymphocytes and a production of the pro-inflammatory cytokines IL-1β and TNF-α. These cytokines could have initiated the SAA production that was seen during dysentery. There were no general alterations in glucose levels and it appears that the demand for glucose was met with an adequate gluconeogenesis during disease. The concentrations of glutamine, alanine, tyrosine and serine were decreased during dysentery and may have been associated with a high energy utilisation of activated leucocytes. In contrast, lysine increased at the end of dysentery and in the beginning of the recovery period. During the recovery there was a production of the anti-inflammatory cytokine IL-10 and *B. hyodysenteriae*-specific antibodies indicating the presence of an activation of B cells. In addition, γδ T cells increased throughout the recovery period.

## FUTURE STUDIES

- The animals that remained healthy also displayed changes in some lymphocyte sub-populations, cytokine and amino acid concentrations similar to those observed in dysenteric pigs. Whatever the reason, investigations are needed to understand why some pigs are resistant to disease and others develop dysentery.
- Very little is known about the response of the complement system during dysentery. This system constitutes an important innate defence and its role in the protection and recovery from swine dysentery needs to be studied.
- In addition to glucose and amino acids, fat is also an important energy source and therefore it would be of interest to study the lipid metabolism in dysenteric pigs.
- The analysis of cytokine concentrations in pig is mainly limited to consecutive detection of one cytokine per assay. A multiplex flow cytometry assay is therefore under development and will enable simultaneous detection of multiple porcine cytokines.
- The local immune response in the gut needs to be further investigated. Apart from morphological examinations, techniques like microarray will be used to detect the cytokine mRNA-expression in colon biopsies from dysenteric pigs.

# CONCLUSIONS

- Experimentally induced swine dysentery was associated with changes in leucocytes and lymphocyte sub-populations with increases of monocytes, neutrophils and CD8α+ lymphocytes.

- Development of swine dysentery was associated with production of the pro-inflammatory cytokines IL-1β and TNF-α and the anti-inflammatory cytokine IL-10 increased during recovery.

- *B. hyodysenteriae*-specific antibodies were detected during recovery from disease indicating an activation of B lymphocytes.

- Metabolic alterations were found during the course of swine dysentery with decreases in the amino acids glutamine, alanine, tyrosine and serine. This may have been associated with a high energy utilisation of activated leucocytes.

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## *CONSIDERATIONS*

***It is always good to step back and ask yourself what you're doing. It gives you a perspective and the realisation that your work won't change the world. As a helping hand I have therefore added some words of wisdom before each Appendix.***