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Mammary Gland Health and Disease

Immune cells and bioactive substances in function of susceptibility and spreading of infections in human and animals

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ABSTRACT BOOK

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MYCOBACTERIUM PARATUBERCULOSIS AND THE BOVINE IMMUNE SYSTEM: A FUNCTIONAL GENOMICS APPROACH

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Key words Johne's disease, *Mycobacterium paratuberculosis*, functional genomics

Abstract Johne's disease is a chronic infectious disease of ruminants caused by *M. paratuberculosis*, a facultative intracellular bacterium residing predominately within host macrophages. Our research group is focused on understanding the relationship between *M. paratuberculosis* and the host bovine immune system (1). Toward this goal, we have utilized gene expression profiling to highlight effects of *M. paratuberculosis* infection on peripheral blood mononuclear cells (PBMCs) from diseased and control cattle (2, 3). These studies have utilized cDNA microarray resources developed specifically for research on bovine immunobiology (4, 5). Effects of *M. paratuberculosis* on gene expression in a cultured bovine macrophage cell line have also been examined (6). As expected, exposure of PBMCs from infected cows (n=3) to *M. paratuberculosis* resulted in numerous immune cell gene expression changes compared to nil stimulation. Genes that were significantly activated (>1.5X, $P<0.1$) include those encoding IL-12(p35), MMP 3, TIMP 2 and TIMP 4, IL-10 and SLAM. Surprisingly however, expression of many genes was significantly suppressed (>1.5X, $P<0.05$) following overnight exposure to *M. paratuberculosis*, including those encoding IKK-β, IL-1, and TRANK. The outcome of PBMC exposure to *M. paratuberculosis* was found to be highly dependent upon stage of disease (2). A subset of genes showing differential regulation between infected and control cows has been deduced. This list includes several cytokines, matrix metalloproteinases, and cell signalling molecules associated with Toll-like receptor activation and apoptosis/proliferation. In cultured macrophage cells phagocytosis of *M. paratuberculosis* induces both pro- and anti-apoptotic signals. Enhanced expression of numerous signalling molecules, such as Act-2 and CCR-9 is also observed. Based on these data, we suggest that 1) macrophages that have ingested *M. paratuberculosis* are attempting to signal and recruit additional immune cells. 2) *M. paratuberculosis* infected macrophages are attempting to prevent persistent infection through apoptosis. 3) The interaction of *M. paratuberculosis* with macrophages may lead to the prevention of apoptosis, perhaps using the TRAF signalling system.

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MICROBIOLOGICAL AND IMMUNOLOGICAL STRATEGIES FOR TREATMENT OF INFLAMMATORY BOWEL DISEASE

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Abstract

Chronic inflammatory bowel disease (IBD) such as Crohn's disease or ulcerative colitis, affects around 2 in every 1000 individuals in western countries and its incidence, particularly amongst children, is increasing. The symptoms of IBD are extremely unpleasant and impact all aspects of quality of life. They include diarrhea, abdominal pain, rectal bleeding, fever, nausea, weight loss, lethargy and loss of appetite. If left untreated, IBD can even lead to death. Conventional treatment of IBD involves powerful immunosuppressive chemotherapies and often surgical intervention during active, severe disease. Long term anti-inflammatory medication is required where inflammation is less severe or following surgery as a prevention against relapse of the disease. Administered orally or by injection, only a fraction of these drugs reaches the intended target site, the inflamed intestinal lining. This is not only an inefficient way to deliver drugs, but, more important, means that patients are often subject to a spectrum of unpleasant side effects. Interleukin-10 (IL-10) is a cytokine that acts to suppress inflammation. Mice that cannot make IL-10 spontaneously develop IBD. Therefore, IL-10 was considered a good candidate therapeutic in the treatment of IBD. When however administered by injection, the high levels of IL-10 that are distributed throughout the body can lead to a number of side effects and so IL-10 is no better in this respect, than conventional synthetic immunosupressive therapies. *Lactococcus lactis* can be genetically engineered in such way that it secretes biologically active cytokines(1, 2). For this we have made use of both the *lac* controlled T7 promotor system(3) and the constitutive P1 promotor(4), in combination with the usp45 secretion leader(5). Much to our surprise, *L. lactis* growth did not suffer from the constitutive secretion of these cytokines. This led to the idea of using live recombinant *L. lactis* strains for therapeutic purposes. When applied to the mucosa of the nose, the engineered *L. lactis* strains can actively deliver such cytokines. Indeed, following intranasal inoculation of TTFC expresser *L. lactis* that either secrete IL-2 or IL-6, a marked increase of the immune response against TTFC was observed(6). By use of this principle - active in situ delivery of a therapeutic agent via de novo synthesis by genetically engineered bacteria - we developed a new therapeutic approach for IBD(7). Two animal models were used. Intragastric administration of *L. lactis* engineered to secrete murine IL-10 produced a 50% reduction in colitis induced in mice by periodic addition of dextran sulfate sodium, as well as prevented the onset of colitis in IL-$10^{-/-}$ mice. The use of the engineered *L. lactis* gets around the problem of delivering IL-10. This can now be achieved by active in situ synthesis in the intestine, which protects the therapeutic from breakdown along the way through the stomach and small bowel and also avoids its systemic distribution. By this strategy, doses of 10000-fold less IL-10 effectively cure IBD in mouse models as compared to doses adminstered by injection. This approach may provide a novel method for cost-effective and long-term management of IBD in humans. By extrapolation this new technology may lead to the development of a whole spectrum of therapies *via* intestinal delivery, not necessarily restricted to the treatment of chronic bowel inflammation.

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ASSESSMENT OF DIAGNOSTIC TOOLS IN YOUNG CALVES EXPERIMENTALLY INFECTED WITH *M. AVIUM* SUBSPECIES *PARATUBERCULOSIS* (ATCC 19698)

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Key words Paratuberculosis, Experimental Infection, Diagnosis, Map antigens

Abstract
In order to assess the performances of paratuberculosis diagnostic tests, 5 young calves (1-2 weeks old) were experimentally infected by the oral route. The animals were infected with 10 mg (108 CFU) of M. *avium* subspecies *paratuberculosis* (Map, ATCC 19698) per day during 10 days. Blood and faecal samples were taken every week for the first 6 months and every two weeks up to now (15 months). The Cell Mediated Immunity (CMI) was assessed by: i) the Interferon gamma (IFN gamma) assay after *in vitro* stimulation of blood samples with avian and bovine PPD's; ii) the lymphoproliferative responses after in vitro stimulation of blood samples with the same antigens. Humoral responses were detected by an absorbed ELISA. Detection of Map in faeces was done by classical mycobacteriology (HEYM + Mycobactin).
Our results can be summarized as follows: i) the animals showed fluctuating CMI responses in both IFN gamma and lymphoproliferation assays from 4-5 weeks until 10-12 weeks post-infection. It took more than 12 weeks post-infection before some animals were consistently classified Map positive by the IFN gamma; ii) no detectable humoral responses could be evidenced, so far; iii) we were unable to culture Map from faecal samples of these animals, although the ATCC strain was shown to be virulent in mice and all tests and reagents passed the quality control and iv) no paratuberculosis clinical signs could be seen, until now.
All together these results emphasises the difficulties of an early and specific detection of Map infected animals although a combination of available tests was used. More, our results suggest that the absence of positive serology as well as the absence of fecal excretion of Map, could jeopardise "paratuberculosis-free" certification programs based on those techniques [1].
Mycobacterial culture filtrates (CF) are a rich source of secreted and surface exposed protein antigens and they have been reported to contain diagnostic and protective antigens for tuberculosis [2,3]. Map CF proteins were separated according to their molecular weight in 30 fractions and these fractions were tested for their capacity to elicit positive lymphoproliferative and IFN-gamma responses in PBMC cultures from naturally and experimentally infected cattle. Several fractions in the 25-40 kD m.w. induced strong T cell responses. Highest IFN-gamma responses were found to fractions containing the Map homologues of the Ag85 complex. Identification of Map specific antigens in these immunoreactive fractions is in progress

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DETECTION OF MYCOBACTERIUM PARATUBERCULOSIS IN RAW AND PASTEURISED MILK

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Key words *Mycobacterium paratuberculosis*, heat resistance, detection, milk

Abstract *Mycobacterium paratuberculosis* (MAP) has been well known for decades as the causative agent of Johne's disease of cattle and other ruminants. The microorganism is also suspected of playing a role in human Crohn's disease. Milk can be contaminated by faecal contamination or direct shedding, also by asymptomatic animals. Interest in detection of MAP in milk exists as diagnostic tool for paratuberculosis on farms and as a tool to investigate the transmission to man. Different studies indicate that MAP would be frequently present in cows's milk at the farm and in industrial collection tanks. The numbers of MAP cells that are encountered have not been defined due to the lack of a suitable detection method. Detection of MAP in milk by culturing takes up to 12 months. Moreover, these cultures suffer from overgrowth by the competitive microflora and (sub)lethal damage to MAP due to the preceeding decontamination, leading to a decrease in detection efficiency. Although PCR is able to selectively detect the specific IS900 sequence in the MAP genome, it does not distinguish between viable and death cells and therefore, can not be used for the evaluation of survival of MAP to different heat processes. Messenger RNA methods are actually studied for this purpose. Solid phase cytometry (SPC) would allow the enumeration of MAP without growing step (1). The bacteria are recuperated from the milk by an extended sample treatment and concentrated on a membrane filter. All viable bacteria are fluorescently labelled and automatically detected by the ChemScan apparatus (Chemunex). Because most milk and dairy products are heat treated before consumption, the effectiveness of milk pasteurisation for eliminating viable MAP is crucial. Recent research has demonstrated survival of MAP under pasteurisation conditions. This fact cannot simply be explained by the thermal death kinetic data obtained in artificial contamination experiments. Two hypotheses for the survival of MAP to industrial pasteurisation are proposed. It is generally known that MAP cells have the tendency to clump, each clump being counted by the culturing method as 1 cell. The number of cells in naturally encountered clumps is, however, not known. Survival of MAP could be explained on the basis of the kinetic data with clumps of $>10^4$ individual cells (3). On the other hand, MAP could be present in naturally contaminated milk in a heat resistant metabolic status. This could be a heat resistant morphological form or a heat resistant metabolic form induced by certain stress conditions (2).

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MILK LIPIDS WITH ANTI-CANCER POTENTIAL

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Key words milk, lipid, cancer

Abstract
Diet is thought to account for about one-third of all cancer deaths in Western populations. Diet interacts with individual's genetic background resulting in development of cancer, but it can also contain components that may help to prevent cancer. Presence of anti-cancer components in plant-based foods is well recognized. However, there is little appreciation that animal products, in particular cow's milk, contain a number of components with anti-cancer action. Some reviewers suggest that the greatest body of evidence for milk components as anti-cancer agents have developed for whey. However, there is also ample evidence showing that components of milk fat can modify the processes involved with development of cancer. Since 1994 Peter Parodi [1] has written a number of reviews on the anti-cancer compounds of cow's milk often focusing on milk fat.
Milk fat consists of tri- and diacylglycerols (95 and 2%, respectively) and several other minor components including a complex mixture of phospholipids, cholesterol and free fatty acids [2]. The number of fatty acids that have been identified in milk is well over 400. Several fatty acids, including butyric acid, 13-methyltetradecanoic acid (iso-15:0) and conjugated linoleic acid (CLA), have been demonstrated to possess anti-cancer effects in animal models. Buttermilk and whey lipids are rich sources of milk fat globular membrane and associated lipids with anti-tumour effects include sphingomyelin and other sphingolipids. Parodi [1] cites a few studies in which milk fat or butter was compared isocalorically with vegetable fats in animal models of carcinogenesis. These studies clearly demonstrate that milk fat based diets produce fewer tumours than vegetable oil based diets. Of course, these studies cannot distinguish between lower levels of tumour promoting linoleic acid in milk fat vs. specific anticarcinogenic components. CLA content of milk fat can be increased up to ten fold by manipulating the diet fed to the lactating ruminant. When CLA enriched butter was fed to rats from weaning to 55 d age they produced 50% less tumours than the controls after a single dose of methylnitrosourea [3].

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A NOVEL GROWTH PROMOTING ACTIVITY IN MILK

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Key words growth factor, milk, cancer

Abstract

It has long been known that milk contains growth-promoting activity for cells that is additional to its nutritional content.

Epidermal Growth Factor (EGF) has been identified in human, rat, swine and goat milk, and in the rat it has been shown to be significant for the normal development of the pups. EGF has not been found in bovine milk, although Insulin-like Growth Factor (IGF), and Bovine Colostrum Growth Factor (BCGF) which is structurally related to Platelet-derived Growth Factor (PDGF), have been identified. Nevertheless, bovine milk does contain considerable growth-promoting activity for a fibroblastic cell line, Rama 27, which is not significantly stimulated by IGF or PDGF[1].

This novel growth promoting activity has been purified to homogeneity, as judged by amino acid sequencing and mass spectrometry, and identified as the C-terminal fragments of bovine S2-casein, although no growth factor activity is observed from the intact protein. Approximately 1,500,000-fold-purification is achieved by a sequence of acid precipitation, ammonium sulphate fractionation, cation-exchange chromatography, hydrophobic interaction chromatography, reversed phase HPLC and gel filtration. The novel growth factor has been shown to be remarkably potent in stimulating the growth *in vitro* of rat mammary fibroblast cells (Rama 27 cell line). It also stimulates growth in human keratinocytes.. The identity of the activity was confirmed by synthesis of the peptides and by the use of a specific antibody raised against the synthetic peptides in chickens.

The nature of this growth factor may have significance in relation to the process of carcinogenesis.

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LACTOFERRIN STRUCTURE, FUNCTIONS AND APPLICATIONS

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Key words cancer, infection, inflammation, milk proteins.

Abstract
Lactoferrin is a multifunctional 80 kDa glycoprotein of the transferrin family that is present in most of the exocrine secretions, mainly in milk, but is also a major component of the secondary granules of polymorphonuclear neutrophils (PMNs). During inflammation, lactoferrin is released by PMNs in the injured tissues and its concentration in blood rises up to 20 mg/ml.
One of the major properties of lactoferrin is iron binding. However, in contrast to the well-known serum transferrin, an homologous molecule which transports iron from the storage pools to the cells, lactoferrin has to be considered rather as an iron chelator than as an iron transporter. This property, as well as several specific physico-chemical features, account for the variety of biological functions of the molecule. Indeed, lactoferrin was found to act in the innate host defence against infection and severe inflammation. Furthermore, a regulation of the growth of cancer cells by lactoferrin has been described. Some of the molecular mechanisms of action of lactoferrin, but not all, are known. Many of these functions are related to its ability to interact with soluble and membrane target molecules. A particular region of lactoferrin, i.e. the N-terminal sequence of the protein, was found to be responsible for these interactions.
The aim of this talk is to review the overall structure–functions relationships of lactoferrin in host defence against micro-organisms, inflammation and cancer. The current applications of lactoferrin either isolated from milk or produced by recombinant techniques are listed.

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CERAMIDE RICH LIPID RAFTS AND ACID SPHINGOMYELINASE ACTIVITY ARE REQUIRED FOR SPONTANEOUS NEUTROPHIL APOPTOSIS

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Abstract

Neutrophils are short lived, terminally differentiated blood cells, which play a vital role in inflammatory responses. Resolution of inflammation involves removal of effete neutrophils by apoptosis. We have been studying the mechanisms of cytokine mediated inhibition of neutrophil spontaneous apoptosis as wee as the initiation of the cell death programme. Neutrophil spontaneous apoptosis has been proposed to involve the loss of mitochondrial potential leading to activation of caspase 3. Whilst we have confirmed that caspase 3 is activated and mitochondrial membrane potential is lost, we have found that caspase 8 was activated early in neutrophil spontaneous apoptosis and that the Fas (CD95/Apo-1) Death Inducing Signalling Complex (DISC) was formed in apoptotic neutrophils, but did not contain Fas ligand. The generation of ceramide rich lipid rafts has been shown recently to drive Fas DISC formation and caspase 8 activation following Fas ligation in T and B cells. Generation of ceramide at the cell membrane also occurred early in neutrophil apoptosis and neutrophil apoptosis was inhibited by the acid sphingomyelinase inhibitor SR33557. In addition, neutrophils from ASM deficient (ASM knockout) mice showed significantly reduced levels of apoptosis after overnight culture compared to wild type mice. Analysis of detergent insoluble (lipid raft) membrane proteins revealed that Fas, FADD and caspase 8 were present in lipid rafts in apoptotic neutrophils and the lipid raft disruptors Nystatin and Filipin inhibited neutrophil apoptosis and DISC formation. We propose that spontaneous neutrophil apoptosis is initiated by association of DISC components in ceramide rich lipid rafts generated following the activation of ASM at the cell membrane.

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NEUTROPHIL DEATH: CASPASE-DEPENDENT AND CASPASE-INDEPENDENT ROUTES OF APOPTOSIS

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Abstract

Neutrophils (PMN) are the first line of defense against invading pathogens. The production rate of the human body is enormous to compensate the short half-life of these cells. Apart from being potential killers, PMN are a potential threat for the surrounding tissues by causing collateral damage during insufficient or inappropriate clearance. An important way for the PMN to self-eliminate without causing injury is through the process of programmed cell death or spontaneous apoptosis. Apoptosis occurs via the activation of several caspases and regulating members of the so-called Bcl-2 family, which are involved in different steps of the process of cell death. Once undergoing apoptosis, the neutrophils remnants are recognized and engulfed by macrophages and some tissue cells. PMN can be rescued from spontaneous cell death by growth factors (e.g. G-CSF, GM-CSF) and inflammatory triggers (e.g. IFN-γ, IL-1, IL-15, low-dose TNF-α), to sustain their life span when needed. Tumor necrosis factor α (TNF-α) is a cytokine with multiple roles in the immune system, including the induction of cellular functions in neutrophils. TNF-α at high dose can also induce apoptotic signals. Whereas inhibition of caspases usually increases cell survival, we found that inhibition of caspases by the general caspase inhibitor zVAD-fmk did *not* prevent TNF-α-induced PMN death. TNF-α alone caused neutrophil death with characteristic apoptotic features (typical morphological changes, DNA laddering, plasma membrane flip-flop (i.e. exposure of phosphatidylserine (PS)), Bax clustering and translocation to the mitochondria with degradation of mitochondria), which coincided with activation of caspase-8 and caspase-3. However, when caspases were completely inhibited, TNF-α induced PMN to die. This type of cell death lacked the typical nuclear features of apoptosis (i.e. no DNA laddering and aberrant hyperlobulated nuclei without typical chromatin condensation) and demonstrated no Bax redistribution, yet showing mitochondria clustering and PS exposure at the plasma membrane. Several experiments subsequently indicated that the caspase-independent death was mediated by reactive oxygen species (ROS) derived from intact mitochondria. These mechanisms that regulate the programmed cell death in neutrophils and their meaning in neutrophil turnover will be discussed. In sum, there are several ways for a neutrophil to undergo some forms of programmed cell death. TNF-α can at least induce two signals resulting in: a "classical" caspase-dependent, and an alternative "non-classical" pathway of cell death when caspases are blocked.

PROTEOMIC INVESTIGATION OF BOVINE MILK

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Key words proteomics, milk, mastitis, acute phase

Abstract

Proteomics is the large-scale study of the total protein content of a biological sample. The core technology of proteomics is two-dimensional electrophoresis (2-DE), which can separate complex protein mixtures into discrete homogenous protein spots, according to two independent properties: isoelectric point, (pI), and molecular radius, (m_r). Protein spots can then be identified by mass spectroscopy and database searching. It is known that during bovine mastitis, when the udder is infected and inflamed, the composition of milk changes, in both quality and quantity. It has been shown recently that the acute phase proteins, serum amyloid A and haptoglobin are present in milk from animals with naturally-occurring clinical mastitis.

The aim of the study was to utilise the analytical potential of proteomic methods to identify and further characterise the changes in the milk proteins during mastitis. A protocol was developed to establish a consistent procedure for the preparation of milk samples from mastitic and normal cows for analysis. Initial studies showed that casein, being the most abundant protein of milk, had a distorting effect on the 2-DE so that removal of this protein during sample preparation was essential.
Prior to 2-DE, and after removal of lipids from the milk by refrigerated centrifugation, caseins were removed by optimised ammonium sulphate fractionation. The samples were then dialysed overnight to remove salts, which interfere with the 2-DE. In the first dimension, the protein mixture was separated by isoelectric focusing in an immobilised pH gradient of either pH 3-10 or pH 4-7, on a polyacrylamide gel strip. Further separation in the second dimension was performed by SDS-PAGE, to yield discrete homogeneous protein spots after staining with Coomassie blue.

Milk samples were taken from commercial dairy farms from both mastitic, and normal animals, prepared according to the optimised protocol and subjected to 2-DE. The patterns of protein expression of mastitic milk and normal milk showed major differences. The relevant protein spots were excised from the gel, subjected to trypsin digestion and identified by MALDI-TOF mass spectrometry based on the predicted molecular weight of trypsin digest fragments. Initial studies, based on visual inspection of the 2DE gels, have shown that that expression of albumin and serotransferrin in mastitic milk were upregulated; while □-lactalbumin and □-lactoglobulin, the major whey proteins in milk, were reduced. More sensitive and specific spot detection methods such as silver staining and western blotting will be employed in the future to identify alterations to the minor protein components of bovine milk during mastitis.

ACUTE PHASE INFLAMMATORY REACTION IN THE BOVINE MAMMARY GLAND

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Key words mammary gland, cow, inflammation, LPS-challenge

Abstract

Immunological and inflammatory factors such as cytokines, lipid mediators and bacteriostatic enzymes are elevated during mastitis in dairy cows. A study was conducted to determine changes of mRNA expression of various of these factors in the mammary tissue of cows during 12 h after induction of mastitis via intramammary administration of lipopolysaccharide (LPS). Five healthy lactating cows were injected in one quarter with 100 μg E. *coli*-LPS (O26:B6) and the contralateral quarter with saline (9 g/l) serving as control. mRNA expression in mammary biopsy samples of the various inflammatory factors and milk proteins at 0, 3, 6, 9 and 12 h after LPS administration was quantified by real-time RT-PCR. Blood samples were taken following the same time course and rectal temperature was measured at 1-h intervals. Temperature increased until 5 h ($P<0.05$) after LPS administration and decreased to pretreatment levels within 24 h after LPS-challenge. Blood leukocyte number decreased ($P<0.05$) from 0 to 3 h from $7.7\pm1.1 \times 10^9$/l to $5.7\pm1.0 \times 10^9$/l and thereafter recovered to pretreatment levels until 12 h after LPS-challenge. In LPS-challenged quarters tumor necrosis factor α and cyclooxygenase-2 mRNA expression increased to highest values ($P<0.05$) at 3 h after LPS-challenge. Lactoferrin, lysozyme, inducible nitric oxide synthase mRNA expression increased ($P<0.05$) and peaked at 6 h after challenge, while platelet-activating factor acethylhydrolase mRNA increased only numerically. mRNA expression of the investigated factors did not change in control quarters. mRNA expression of insulin-like growth factor-1, 5-lipoxygenase and of αS1-casein (CN), αS2-CN, β-CN and β-lactoglobulin did not change significantly, whereas mRNA expression of α-lactalbumin decreased ($P<0.05$) in LPS-treated and control quarters and that of κ-CN only in the LPS-treated quarters. In conclusion, mRNA expression of most inflammatory factors changed within hours, whereas that of most milk proteins remained unchanged.

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STATE OF THE ART ON THE MOLECULAR MECHANISMS OF NF-□B-MEDIATED GENE INDUCTION

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Abstract

Many inflammatory gene promoters contain κB-responsive elements, which are absolutely required for their induction by inflammatory stimuli, such as TNF. Upon treatment of mouse fibroblast cells with TNF, the transcription factor NF-□B is released from its cytoplasmic complex, migrates to the nucleus and binds onto the □□ sites of various cellular promoters. By mutation analysis of the IL6 promoter, we have shown before that NF-□B is the only transcription factor, which is responsive to TNF and, hence, absolutely necessary for its transcriptional activation by TNF.

Upon inhibition of the the ERK and p38 MAP kinases, which are also activated by TNF, induction of the IL6 gene is abrogated, but not the cytoplasmic activation of the transcription factor NF-□B, nor its DNA-binding capacity. From this we have concluded that phosphorylation of NF-□B by MAP kinases may represent an additional, but necessary signal to fine-tune inflammatory gene expression, and thus to codetermine the transactivation capacity of the induced transcription factor.

The NF-□B subunits, p50 and p65, are, however, not a direct substrate for TNF-induced phosphorylation by MAP kinases; in contrast, the p65 subunit can be specifically phosphorylated *in vitro* and *in vivo* by 'mitogen- and stress-stimulated kinase' 1 (MSK1), which is a nuclear kinase, activated by and located downstream of both the ERK and p38 MAP kinases. MSK1 phosphorylates NF-□B p65 at Ser position 276, which is a crucial position for NF-□B transcriptional activity in fibroblast cells. Upon TNF stimulation, MSK1 associates with NF-□B p65, as follows from co-immunoprecipitation experiments and ChIP analysis; however, this TNF-induced association is abrogated, when the cells are treated with inhibitors of the ERK and p38 MAP kinases together, or by inhibition of MSK1. Finally, in MEF cells from $MSK^{-/-}$ animals, in which the various MAP kinases pathways as well as the cytoplasmic activation of NF-□B have remained unaffected, the TNF-induced transactivation potential of NF-□B is completely gone and inflammatory gene expression is severely decreased.

Therefore, we conclude that in mouse fibroblast cells inflammatory gene expression is effected by a dual signalling pathway; i.e. cytoplasmic activation of the transcription factor complex NF-□B and concomitant phosphorylation of the transactivating subunit p65 by the nuclear kinase MSK1 at position Ser 276. Since MSK1 has already been described to also phosphorylate histon 3 tails, this is to our knowledge the first report indicating a nuclear kinase that phosphorylates the driving transcription factor as well as its neighbouring chromatin environment.

NUCLEAR FACTOR-κB ACTIVITY IN HUMAN AND ANIMAL MODELS OF INFLAMMATION ; THE CASE OF ASTHMA

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Key words asthma, inflammation, NF-κB

Abstract The Nuclear Factor-κB (NF-κB) is a key transcription factor in the regulation of immune and inflammatory processes. Its activity is correlated with different pathological inflammatory conditions such as rheumatoid arthritis, inflammatory bowel disease and asthma [1]. Asthma is a chronic inflammatory disease of the airways, in which many inflammatory cells are involved and a large number of inflammatory genes are overexpressed. Most of these genes depend on NF-κB activity for their expression, suggesting that NF-κB could play a key role in the pathophysiology of asthma [2]. In a first study, Hart and colleagues showed that NF-κB activity was increased in the airways of stable asthmatic patients compared to healthy subjects [3]. Similarly, in heaves-affected horses, an animal model of asthma, NF-κB activity drastically increases in the airways during allergen-induced crisis [4]. In this model, NF-κB activity was strongly correlated to the degree of lung dysfunction and was shown to persist for several weeks after allergen eviction, due to autoregulatory feedback loops [4, 5]. At last, mice deficient in the p50 or c-rel subunits of NF-κB fail to develop allergic airway inflammation [6, 7]. Recent data obtained in our laboratory revealed that the specific inhibition of NF-κB in the airways leads to a significant reduction in pulmonary dysfunction and inflammation following allergen challenge in a mouse model of asthma (Bureau et al., unpublished results). Taken together, these results shed light on the potential role of NF-κB in the pathogenesis of inflammatory disorders, especially asthma, and suggest that specific inhibition of NF-κB activity may be of therapeutic value in the treatment of these diseases.

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26.11.02

CLONING AND EXPRESSION IN PICHIA PASTORIS YEAST OF FULL LENGTH PORCINE LACTOFERRIN cDNA

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Abstract

Lactoferrin is a polyfunctional glycoprotein that has a variety of biological activities, including nonimmune disease protection, immunoregulation, anti-inflammation, probiotic support of intestinal microflora and growth promotion. Lactoferrin in milk is likely to be of particular importance to neonatal mammals that are born in an immature state of development as piglets. Lactoferrin, present in sow milk, could support piglets growth in early life. We report the production of recombinant porcine lactoferrin (rpLF) in methylotrophic yeast *Pichia pastoris*. This heterologous expression system offers the benefits of *Escherichia coli* combined with the advantages of expression in a eukariotic system. The full lenght cDNA of pLF was obtained by reverse reaction from the total RNA of sow's mammary gland. The cDNA was cloned in the expression vector of *Pichia pastoris*, pPIC9, downstream the α-factor, a signal peptide of *Saccharomyces cerevisiae*, that drives the production of the recombinant protein in the growth medium. This chimeric construct is cloned under the control of alcohol oxidase 1 (AOX1) promoter, that allows the expression of lactoferrin in the presence of methanol as inductor. Using this system, pLF is expressed and secreted into the growth medium. The protein was purified from the yeast broth by ultrafiltration; Western blotting analysis showed a band of 80 KDa, indicating a *Pichia pastoris*-porcine similar pattern of glycosilation. The production of rpLF by an heterologous system as *Pichia pastoris* can provide an effective tool to support the piglets health during weaning period.

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PMN PROTEOLYTIC ACTIVITIES AND CASEINOLYSIS IN MILK.

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Key words Mastitis, milk, PMN, proteolysis.

Abstract

Somatic cell count is fairy well correlated with milk proteolysis (1). Polymorphonuclear neutrophils (PMN) represent the major cell type during the inflammatory process (2). PMN granules contain several proteases involved in the degradation of the extracellular matrix, amongst them cathepsins, collagenase and elastase (3). The aim of this work was to quantify proteolytic activities of milk PMN during the inflammatory process and to asses their global capacity of caseinolysis. PMN proteolytic activities was quantified by flow cytometry and their caseinolytic capacity was investigated during a kinetic after the intramammary infusion to 6 dairy cows of 10 □g of LPS of *E. coli*. The activities of cathepsins C and G, collagenase IV (MMP-9) and elastase were significantly higher than before infusion. PMN extracted from milk during the kinetic degrade caseins at both acidic and neutral pH. The role of PMN in endogenous caseinolysis was also clearly demonstrated.

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26.11.02

CHANGES IN LYMPHOCYTE DISTRIBUTION DURING EXPERIMENTALLY INDUCED MASTITIS

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Key words $CD4^+/CD8^+$, lymphocyte, mastitis.

Abstract

The cytotoxic/supressor T-lymphocytes are the predominant subsets of T-cells in tissue of the mammary gland and bovine milk. In contrast, T-helper lymphocytes are the predominant subset in the circulation [1,3]. The second important lymphocyte population consists of B cells [2]. Currently, the specific leukocyte populations mediating the defense mechanism of the udder against coliform infection are not well defined. This study aimed at investigating if changes in lymphocyte distribution occur during experimentally induced mastitis, using two different inoculum doses. Secondly, the purpose was to evaluate which lymphocyte subset could play an important role in the defense against *E. coli* mastitis. Therefore, two groups of Holstein-Friesian heifers were intramammary inoculated with 10^4 or 10^6 cfu, in the front and rear left quarters. Mononuclear cells were isolated and purified by gradient centrifugation from blood and milk samples. CD4, CD8 and CD21, which are specific markers for T-helper-, cytotoxic/supressor T -, and B- lymphocytes, respectively, were stained with bovine monoclonal antibodies and FITC labelled secondary antibodies. Samples were analysed by flow cytometry, and results were expressed as the ratio of $CD4^+/CD8^+$ and percentage (%) of $CD21^+$ lymphocytes. The $CD4^+/CD8^+$ ratio and % $CD21^+$ blood lymphocytes was fairly stable during the early acute phase response i.e. from 0 to 18h post infection (p.i.), in both inoculum doses. From 24h onwards the $CD4^+/CD8^+$ ratio increased similarly for both inoculum doses indicating the presence of higher proportions of T-helper lymphocytes in blood, while the amount of CD21+ cells slightly decreased. In milk, the high inoculum dose triggered a strong and rapid decrease of the $CD4^+/CD8^+$ ratio and elevated $CD21^+$ cell proportions during the first 18h p.i. Thereafter, this ratio increased above prechallenge values while the % of $CD21^+$ cells decreased progressively. Although the ratio of $CD4^+/CD8^+$ of milk lymphocytes displays a similar trend during infection for both groups, it was consistently lower in animals inoculated with the high inoculum dose. The % of $CD21^+$ lymphocyte subsets in milk followed similar kinetics for both inoculum doses during the whole experimental period, the % of $CD21^+$ cells was higher in animals inoculated with 10^6 cfu. The transient decrease of the $CD4^+/CD8^+$ ratio and elevated % of $CD21^+$ cells in milk indicate that T cytotoxic lymphocytes are the first cells to be recruited into the mammary gland, suggesting that *E. coli* infection leads to a cytotoxic or suppressive immune response. Furthermore, high dose inoculation triggers a stronger increase of $CD8^+$ cells in the mammary gland. This implies an important involvement of the $CD8^+$ subset in a faster and more pronounced immune response against coliform mastitis.

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THE EXTRACELLULAR CALCIUM-SENSING RECEPTOR IS LINKED TO A SURVIVAL PATHWAY IN AVIAN GRANULOSA CELLS

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Abstract

Our previous results showed a dual role for calcium in cell death and survival in an in vitto apoptosis model consisting of serum-free cultured granulosa explants (Mussche et al., 2000). A temporary $(Ca^{2+})_e$ load induced apoptosis by increasing $(Ca^{2+})_i$ while a continuous load of $(Ca^{2+})_e$ inhibited apoptosis in a dose-dependent manner. This effect was mimicked by incubation with Mg^{2+} and Gd^{3+}, two activators of the calcium-sensing receptor (CaR). This led us to investigate the possible presence of the CaR in the granulosa explant model system. We detected a 120 kDa CaR by Western Blot using a mouse monoclonal antibody (ADD antibody, NPS Pharmaceuticals Inc.). The CaR was localized by immunocytochemistry in granulosa cells of all follicles belonging to the follicle hierarchy, i.e. those follicles predestined to ovulate. The CaR was still expressed 48h after serum-free culture. The CaR was absent in the granulosa cells of previtellogenic follicles (50 µm –1.5 mm) and in the multi-layered granulosa layer of small vitellogenic follicles (1.5 – 6 mm). These latter represent the pool of follicles from which many are lost through the process of follicular atresia. From inhibitory experiments to elucidate the possible implicated signaling pathway we can conclude sofar that there is no involvement of the ERK and p38 MAPK. The PI3Kinase pathway is a candidate signaling pathway in the CaR-mediated survival. As far as we know this is the first report describing the presence of the Ca^{2+}-sensing receptor in granulosa cells. A correlation between activation of the calcium-sensing receptor and prevention of apoptosis was reported by Lin et al. (1998) for serum-free cultured rat 1A-Myc fibroblasts.

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26.11.02

USE OF SOLID PHASE CYTOMETRY FOR THE RAPID DETECTION OF *MYCOBACTERIUM PARATUBERCULOSIS* IN MILK

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Key words solid phase cytometry, *Mycobacterium paratuberculosis*, milk, rapid detection

Abstract

Mycobacterium paratuberculosis (MAP), the etiological agent of Johne's disease in ruminants, can enter milk destined for human consumption by faecal contamination or direct shedding. As this microorganism appears to survive pasteurisation and a role in human Crohn's disease is suspected, some milk products may become potential health hazards [1]. At present, no method for the rapid detection of viable MAP in milk exists, which seriously hampers the scientific research on this microorganism. Due to its extremely slow growth, a conventional culture can take months, whereas PCR is unable to distinguish between viable and dead cells [2]. We have used solid phase cytometry (SPC) to detect MAP as single cells, i.e. without the need for a growth step, in artificially contaminated pasteurised milk. The SPC procedure consists of a membrane filtration, the labelling of bacteria on the membrane filter with an Ar laser excitable fluorescent dye, the automated counting in 3 min by the ChemScan apparatus (Chemunex, Ivry-sur-Seine, France) and a visual confirmation by means of an epifluorescence microscope [3]. To render a complex matrix like milk filterable and to eliminate the background flora, an extensive sample pretreatment had to be developed. This pretreatment includes a chemical milk destruction, several centrifugation steps, decontamination with 0.75% cetyl pyridinium chloride, an enzymatic treatment, a prefiltration step and a purification by means of hydrophobic C_8 polymeric beads. The MAP cells isolated from the milk are finally labelled fluorescently on the membrane filter using the non-specific viability substrate ChemChrome V6. The combined protocol (milk pretreatment/SPC) allows the detection of 20 MAP cells in 50 ml of milk in less than one working day. However, with a non-specific labelling the selectivity of the method depends on sample pretreatment only. Therefore, on-going research focuses on a selective labelling procedure. Combination of the latter with the sample pretreatment would allow the detection of MAP in naturally contaminated milk.

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ADHESION MOLECULE EXPRESSION ON BLOOD AND MILK POLYMORPHONUCLEAR LEUKOCYTES DURING COLIFORM MASTITIS

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Key words L-selectin, Mac-1, polymorphonuclear leukocyte, mastitis

Abstract

During *Escherichia coli* mastitis, L-selectin (CD62L) is generally shed, whereas Mac-1 (CD11b) expression increases at the surface of the polymorphonuclear leukocytes (PMN) in circulation [1-3]. However, adhesion molecule expression on PMN present at the inflammatory focus during coliform mastitis has not been previously examined. In the present study we addressed the question whether differences can be detected in the expression of adhesion molecules on circulating *vs.* milk PMN. Therefore, mastitis was induced in six early lactating heifers by intramammary administration with 10^4 cfu of *E. coli* in the front and rear left quarters. CD62L and CD11b were immunolabeled at the PMN surface, cell viability was assessed by propidium-iodide staining, and samples were analyzed using flow cytometry. Preinfection blood PMN expressed higher CD62L and lower CD11b levels at the cell membrane as compared to milk resident PMN. However, the percentage of both $CD62L^+$ and $CD11b^+$ PMN was considerably lower for milk PMN. After challenge, from 12 h onwards, CD62L was typically shed, while CD11b density increased on blood PMN. Interestingly, adhesion molecule expression on milk resident PMN followed similar kinetics as for circulating PMN. In addition, the amount of $CD62L^+$ and $CD11b^+$ milk PMN sharply increased to similar levels as detected in blood. The differential expression of adhesion molecules on PMN before infection reflects a greater activation state of PMN in milk, in contrast to the blood compartment. The inverse regulation of CD62L and CD11b observed after coliform mastitis indicates that also milk PMN may also undergo important changes in receptor density. The elevated proportion of $CD62L^+$ and $CD11b^+$ in milk PMN after challenge may be related to the increased PMN viability observed at that time.

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INFLUENCE OF SEX STEROIDS ON *IN VITRO* CHEMOTAXIS OF BOVINE BLOOD POLYMORPHONUCLEAR LEUKOCYTES

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Key words polymorphonuclear leukocytes (PMN), 17-□-estradiol, progesterone, chemotaxis

Abstract

Introduction and objective. Susceptibility of cows to *E. coli* mastitis is primarily a problem during early lactation. Since the role of PMN is paramount in the elimination of bacteria, it can be hypothesized that the decreased resistance to infection could be mediated by alterations in PMN function [1]. Factors contributing to the changes in PMN function have not been fully elucidated, although it has been suggested that major shifts in plasma concentrations of steroids may play a role [2]. Therefore we investigated whether exogenous 17-□-estradiol and progesterone have an influence on the chemotactic ability of isolated blood PMN at the end of pregnancy. During this period it is known that endogenous progesterone levels are high.
Materials and methods. Blood was collected by venipuncture from eight clinically healthy cows in their last 4 weeks of gestation. PMN were isolated through hypotonic lysis and subsequently incubated with different concentrations of 17-□-estradiol and progesterone. After incubation, PMN were added on collagen-coated inserts and stimulated to migrate across the membrane by Zymosan activated serum. The number of migrated PMN was counted with Coulter counter and microscopically (Bürker chamber).
Results and discussion. A significant linear decrease of the chemotactic ability of PMN, isolated from cows at the end of gestation and incubated with 17-□-estradiol and progesterone, was detected ($P<0.05$), due to a reduction of the chemotactic ability at all concentrations compared to the control. It is remarkable that the chemotactic ability of PMN can still be influenced by 17-□-estradiol and progesterone even when these PMN were exposed to high endogenous progesterone levels for a long time. To the authors' knowledge a similar study has not been described before. Most authors report that 17-□-estradiol reduces the chemotactic ability of PMN [3,4] but none of these studies have been done with prolonged exposure of PMN to high endogenous progesterone levels. The results presented here suggest that plasma levels of progesterone and 17-□-estradiol play a critical role in the regulation of the chemotactic ability of PMN although it is not yet proven whether this influence also occurs *in vivo*.

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INDUCTION OF ACUTE E. COLI MASTITIS IN DAIRY HEIFERS: EFFECT ON SURVIVAL OF BLOOD AND MILK NEUTROPHILS

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Key words viability, polymorphonuclear leukocyte, E. coli, mastitis

Abstract

Polymorphonuclear leukocytes (PMN) are crucial in both preventing and protecting the udder from pathogens [1; 2]. The effects of intramammary *Escherichia coli* (E.coli; P4:032 strain) inoculation on survival of blood and milk PMN was investigated. Six healthy early lactation primiparous dairy cows (30 ± 4 days post-calving; Holstein-Friesian) were used. The E. coli-infected and non-infected quarter milk samples (200 ml) were aseptically collected for colony forming unit (cfu)/ml, somatic cell count (SCC), PMN isolation and viability assay immediately before and at 6, 12, 18-24 and 48-72 h after E. coli infection. Immediately after milk sampling, blood (80 ml, from the external jugular vein into heparinised evacuated tubes) was sampled for isolation and viability assay [3]. Clinical signs of acute mastitis were observed in all cows. The viability of blood PMN did not change significantly throughout the experiment. Pre-infection viability of PMN isolated from milk was 63±2%. The viability of E.coli-infected quarters' PMN at post-infection hour (PIH) 6, 12, 18-24 and 48-72 increased to 86, 93, 89 and 76%, respectively ($P<0.001$); this was coincided with increased % milk PMN. Pre-infection values were obtained at 72 h after infection. The viability of non-infected quarters' PMN at PIH 6 increased to 77% ($P>0.001$) and remained slightly higher at PIH 12, 18-24, regaining the pre-infection values at 48 h after infection. From PIH 6 to 24, SCC increased continually in E.coli-infected quarters, coinciding with declining cfu/ml.In primiparous dairy cows PMN reacted against E.coli infection efficiently by boosting their survival in milk. The increased milk PMN viability predominantly resulted from the speed of PMN diapedesis into the mammary gland. This can inversely affect bacterial growth in the mammary gland. The underlying mechanism of increase milk PMN viability remains to be investigated.

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TUNEL- AND CASPASE-3 POSITIVE CELLS IN THE CYCLIC CANINE ENDOMETRIUM

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Key words apoptosis, endometrium, dog, estrous cycle

Abstract

Endometrial apoptosis during the estrous cycle has been studied in several species (1, 2, 3) and is related to the ovarian steroid hormone levels (4, 5, 6). In the dog, no data are available on apoptosis in the endometrium and its possible relation to steroid hormone levels. The aim of the present study is to verify the presence of apoptosis in the canine endometrium and to assess its possible relation with serum progesterone levels, as this might form a basis for further reproductive and pathological research in this species. Apoptotic cell death was assessed in the endometrium of 58 female dogs in different stages of the estrous cycle by using the terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick-end-labeling (TUNEL) assay and by immunohistochemical detection of caspase-3 activity on formalin-fixed, paraffin-embedded sections. For both techniques the apoptotic index (AI) was determined in the surface epithelium, the stroma, the crypts and the basal glands by counting the percentage stained cells on a total of 500 cells of each category. In the surface epithelium and in the stroma TUNEL- and caspase-3 positive cells were rare ($AI < 1$) throughout the estrous cycle, but caspase-3 detection showed for both endometrial cell groups a higher AI ($P < 0.05$) in anestrus compared to early metestrus. In the crypts and in the basal glands, the AI significantly increased in late metestrus and anestrus. However, in the crypts this increase was only significant when caspase-3 detection was used, whereas in the basal glands both the TUNEL and caspase-3 reactions showed significant differences. It can be concluded that TUNEL- and caspase-3 positive cells are present in the different canine endometrial cells during the estrous cycle, but caspase-3 detection showed more significant differences than TUNEL assay. Furthermore, no high AI suggestive for endometrial desquamation could be observed in the canine endometrial surface epithelium, in contrast to other species such as man (2), the rabbit (5), the rat (1) and the hamster (4). Finally, no correlation was found between the apoptotic index in the canine endometrium and the serum progesterone levels.

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DIFFERENTIAL LEUKOCYTE COUNT IN FRACTION-COLLECTED BOVINE MILK

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Key words bovine – milk fraction – differential leukocyte count

Abstract

Introduction – Mastitis is an important health problem in animal husbandry. Nowadays, the control of mastitis is mainly monitored by counting the number of cells in milk (somatic cell count, SCC). A better knowledge of the immunological significance of these cells, especially related to low and high SCC, is required [1]. Recently, two flow cytometric differential leukocyte count methods have been developed [2,3]. Using one of these techniques [2], the variation in differential leukocyte count of bovine milk fractions was studied during milking.
Material and methods – Six Holstein cows in mid-lactation, free of udder infection, were used. During milking, five different fractions were collected [4]: (I) foremilk, (II) cisternal milk, (III) main milk, (IV) strippings and (V) residual milk. SCC was analysed using a fluoro-opto-electronic method. Milk fat content was quantified through mid-infrared spectrophotometry. Milk cells were isolated and differential leukocyte count was carried out using the fluorescent probe SYTO®13 [2], subsequently percentages of PMN, macrophages, lymphocytes and cells with apoptotic features were calculated. Statistical analysis was performed using ANOVA.
Results – SCC did not differ between fraction I, II and III. However, the earliest fractions were significantly different from strippings (IV) and residual milk (V)(P<0.001). Fat content was significantly different between fractions I-II, III, IV and V (P<0.001), with the highest content in residual milk (V). The percentage of lymphocytes and macrophages was significantly lower (P<0.001) in residual milk (V) as compared to the other fractions. The percentage of cells with apoptotic features was highest in fraction V (P<0.001). The percentage of PMN was significantly higher (P<0.01) in fraction I-II.
Conclusion – To our knowledge differential leukocyte count has not been performed previously in different fractions during milking. However, differentiation in foremilk has already been carried out. The cell pattern of foremilk in our results is in accordance with other flow cytometric data for differential leukocyte count [3,5]. The high percentage of cells with apoptotic features in residual milk is remarkable, and seems somehow contradictory with the high viability observed in the same fraction [4]. However, due to the high fat content in this fraction, phagocytic cells can take up many fat globules, triggering the oxidative burst. An increased oxidative burst activity enhances programmed cell death [6].

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VITAMIN A PROFILE DURING *E. COLI* MASTITIS IN HEIFERS

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Key words *E. coli* mastitis, acute-phase reaction (APR), serum amyloid A (SAA), vitamin A

Abstract
Introduction. High yielding milking cows are most susceptible to environmental mastitis in the early stage of lactation [1]. *E. coli* mastitis triggers a strong APR, wherein SAA is related to the severity [2]. It has been described that vitamin A guarantees the normal function of epithelia, which might improve defences of the mammary gland against infection [3]. Furthermore, an association between the somatic cell count and the concentrations of blood vitamin A was observed [4].
Objectives. To investigate the effect of *E. coli* mastitis during early lactation on blood vitamin A profile, and to test the hypothesis of an acute-phase related hyporetinemia.
Material and Methods. Six clinically healthy Holstein-Friesian heifers were infected intramammarily with *E. coli*. The APR was monitored by the determination of SAA (Tridelta's Elisa kit). Vitamin A analogues (all-*trans*-retinol, all-*trans*, and 13-*cis*-retinoic acid) were simultaneously identified and subsequently quantified in serum by HPLC-DAD [5].
Results. The presence of a systemic APR was documented by a significant and transient increase in SAA values from 18 h until 3 days post-infection ($P<0.001$). During the same time period, retinol concentrations decreased to 60% of pre-infection values ($P<0.001$). An inverse and linear correlation (Pearson's r equals – 0.91) was detected between retinol and SAA values. Similarly, 13-*cis*-retinoic acid showed a significant decrease ($P<0.001$), with lowest concentrations between 6 and 24 h post-infection. In contrast, serum levels of all-*trans*-retinoic acid increased strongly ($P<0.001$). In the mean time, the ratio all-*trans* / 13-*cis*-retinoic acid rose above one, and the sum of both isomers peaked at 12 h post-infection.
Conclusion. The present study reveals the profile of vitamin A during *E. coli* mastitis, characterised by a transient acute-phase related hyporetinemia. This phenomenon has recently been described during inflammation in humans (reviewed in [6]). In addition, remarkable changes in the concentrations of all-*trans*- and 13-*cis*-retinoic acid were detected. This may be partly due to a shift from the inactive 13-*cis*-retinoic acid to the active all-*trans* isomer.

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