VITAMIN A, GRANULOPOIESIS, AND SUSCEPTIBILITY TO
MASTITIS IN PERIPARTURIENT DAIRY COWS

by

VALÉRIE VAN MERRIS

Dissertation submitted in fulfillment of the requirements for
the degree of Doctor (Ph.D.) in Veterinary Sciences

Promoters
Prof. Dr. E. Meyer
Prof. Dr. C. Burvenich

Ghent University, Faculty of Veterinary Medicine
Dept. Physiology, Biochemistry, and Biometrics
Milk Secretion and Mastitis Centre (MMRC)
Salisburylaan 133, B - 9820 Merelbeke
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Le bonheur de la vie c’est de savoir qu’il y a toujours des cimes à atteindre et des sommets à conquérir.

B. Chamoux
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<tr>
<td>CFU-G</td>
<td>colony-forming unit-granulocyte</td>
</tr>
<tr>
<td>CFU-GEMM</td>
<td>colony-forming unit-granulocyte, erythroid, monocyte, megakaryocyte</td>
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<td>CFU-GM</td>
<td>colony-forming unit-granulocyte, monocyte</td>
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<td>CFU-M</td>
<td>colony-forming unit-monocyte</td>
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<tr>
<td>CSF</td>
<td>colony-stimulating factor(s)</td>
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<tr>
<td>c.v.</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>DAD</td>
<td>diode array detector</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle Medium</td>
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<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>FSC</td>
<td>forward scatter</td>
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<tr>
<td>G-CSF</td>
<td>granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks’ Balanced Salt Solution</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s modified Dulbecco’s Minimal Essential Medium</td>
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<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>NAP</td>
<td>neutrophil alkaline phosphatase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>p.i.</td>
<td>post-infusion</td>
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<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
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<td>PMN</td>
<td>polymorphonuclear leukocytes</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>RAR</td>
<td>retinoic acid receptor</td>
</tr>
<tr>
<td>RBP</td>
<td>retinol-binding protein</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
</tr>
<tr>
<td>SAA</td>
<td>serum amyloid A</td>
</tr>
<tr>
<td>s.e.</td>
<td>standard error</td>
</tr>
<tr>
<td>SSC</td>
<td>side scatter</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor α</td>
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<td>% F</td>
<td>percent fluorescence</td>
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PREFACE
This doctoral thesis consists of several papers of Valérie Van Merris with respect to the immunosuppression in periparturient dairy cows. The relationship between vitamin A, granulopoiesis, and susceptibility to mastitis is studied during the periparturient period.

The general introduction gives an overview of the structure and function of the bone marrow, and the lineage commitment during hematopoiesis. In addition, chemical and biological data on vitamin A, with special emphasis on the immunity, are provided.

The first part deals with the description of an in vitro culture system for bovine bone marrow cells, and a separation technique for fractionation of bone marrow cells into maturation-related fractions. A simple quantification method was developed for assessing the blood vitamin A profile in bovine serum using liquid-liquid extraction and subsequent HPLC-DAD analysis.

The second part covers the experimental studies performed on bone marrow and vitamin A. In a first chapter, the maturation sequence of bovine bone marrow cells is assessed during normal granulopoiesis. Blood vitamin A profile, including retinol and retinoic acid isomers, during the periparturient period and subsequent experimentally induced Escherichia coli mastitis is revealed in a second chapter. Finally, the involvement of steroids in the alteration of bovine granulopoiesis, and the possible role of retinoids were assessed in vitro in a third chapter.

In the general discussion, results from the current study are fitted in the knowledge obtained from decades of research on the immunosuppression in periparturient dairy cows.

This research work tries to contribute to the better understanding of the immunosuppression in dairy cows at the bone marrow level, and aims at inspiring researchers to unravel this intriguing topic.

Valérie Van Merris.
Merelbeke, December 2003.
GENERAL INTRODUCTION
INTRODUCTION

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1. PERIPARTURIENT PERIOD IN DAIRY COWS

1.1. PHYSIOLOGICAL CHANGES AT PARTURITION

Transition from gestation to lactation in dairy cows involves considerable hormonal and metabolic adaptation, characterised by negative energy, calcium, and protein balance as well as profound changes in circulating steroid hormone, mineral and antioxidant vitamin profile.

Parturition and the onset of lactation impose significant physiological challenges to the homeostatic mechanisms in the dairy cow. Calving is typically associated with a decrease in plasma progesterone concentration and a decrease in plasma estrogens the week before calving, and a sudden increase in cortisol the day before calving (Smith et al., 1973). These hormonal changes play an important role in expulsion of the calf and removal of the placenta. Sex steroids are also responsible for mammogenesis, and need to decline for lactogenesis and galactopoiesis. During early lactation, the amount of energy required for maintenance of body tissues and milk production exceeds largely the amount of energy the cow can obtain from dietary sources. As a result, cows suffer from a negative energy balance and blood ketone levels are elevated.

It is known that around parturition and during early lactation, dairy cows are more susceptible to mammary infection with common environmental pathogens such as Escherichia coli. The incidence of clinical E. coli mastitis is highest between parturition and peak lactation (Erskine et al., 1988; Burvenich et al., 1994). It is well recognised that E. coli mastitis cases occurring during established lactation induce moderate clinical signs, subside very rapidly and are self-curing (Hill et al., 1979). During early lactation, however, a severe systemic response sometimes leading to shock and death, is more prominent. Bacterial, cow and environmental factors are interdependent and influence mastitis susceptibility. It is also indisputable that the severity of E. coli mastitis is mainly determined by cow factors rather than by E. coli pathogenicity (Burvenich et al., 2003). During E. coli mastitis, the host defence status is a cardinal factor determining the pathogenicity and the outcome of mastitis.
1.2. LEUKOCYTE DYSFUNCTION

Among the immune cells affected at parturition, polymorphonuclear leukocytes (PMN) have been of particular interest due to their primary role in innate immune defence against mastitis (Guidry et al., 1976; Burvenich et al., 1994). To fulfil their defensive role, mature PMN perform a cascade of functions to effect successful clearance of pathogens invading the mammary gland. Circulating PMN need to sense the focus of infection, slow down and adhere to the endothelium of capillaries and venules adjacent to the inflammatory locus, migrate through the vessel wall and the interstitium to the infectious site. Once in the udder, they engage in the seeking out and ingestion of bacteria using energy-required and receptor-mediated phagocytosis. Phagocytosis stimulates PMN to undergo massive respiratory burst, producing multiple reactive oxygen species which, combined with myeloperoxidase, initiate the oxidative killing of ingested bacteria. During this process, PMN also need to produce factors to ensure their survival, recruit additional inflammatory cells, inactivate their own toxic products and induce their death pathway to prevent damage to the mammary gland tissue.

*In vitro* determination of leukocytic parameters in blood samples around parturition have shown that the number, functionality and maturity of PMN are altered. A dramatically reduced random migration, diapedesis and respiratory burst activity of PMN are observed the first week after parturition (Kehrli et al., 1989; Cai et al., 1994; Mehrzad et al., 2002). The phagocytic capacity of PMN increases before parturition, being highest on the day of calving, and decreases after parturition (Cai et al., 1994; Detilleux et al., 1995; Dosogne et al., 1999). The oxidative burst activity of PMN decreases after parturition, and recovers to normal values at 4 weeks postpartum (Dosogne et al., 1999; Hoeben et al., 2000; Mehrzad et al., 2001). Interestingly, the total number of circulating PMN rapidly increases around calving. This elevation is largely due to an influx from the bone marrow as immature cells, namely metamyelocytes and band cells, appear in circulation (Moreira da Silva et al., 1998; Mehrzad et al., 2002). High numbers of circulating PMN would seem beneficial for mammary defence against mastitis, given that a large pool of blood cells would be available for recruitment. To be effective, however, these cells must be able to migrate to the infected gland, ingest and kill bacteria efficiently.
It is becoming increasingly apparent that PMN alter their phenotype in response to parturition. The reduced functionality of circulating PMN invites to believe that a decreased immunocompetence occurs during the periparturient period. Because such changes have been observed over the world, in dairy cows with a wide range of milk production (Dosogne et al., 1999), the question arises whether these alterations are to be classified as physiological or pathological. Furthermore, it is also questionable if under these circumstances the inflammatory reaction, characterised by activation of the bone marrow, is also depressed. At the moment no reference is made of any threshold value that could explain the switch from the physiological state (regulation) to the pathological state (disregulation). If such a threshold does exist, researchers are invited to search for the relationship between the leukocytic changes observed after parturition and the susceptibility to mastitis in early lactation. Experimental models with *E. coli* mastitis have demonstrated a clear relationship between pre-infection PMN functions and the severity of subsequent coliform mastitis. The number of circulating PMN, the capacity to produce reactive oxygen species, and the chemotactic response of PMN prior to infection were negatively correlated to milk production, mainly in uninfected quarters, and to colony counts of *E. coli* in milk of infected quarters, and hence the severity of systemic disease (Heyneman et al., 1990; Lohuis et al., 1990; Kremer et al., 1993; Van Werven et al., 1997). This inverse relation indicates that indeed the cows’ resistance is compromised at parturition and during early lactation.

During the periparturient period, circulating PMN are exposed to a rapidly changing steroid environment, with pronounced fluctuations in blood concentrations of cortisol, progesterone and estradiol. Several studies have described relationships between fluctuating steroid concentrations and altered functionality of PMN. Hoedemaker et al. (1992) provided evidence for the influence of cortisol, estrone, 17-β-estradiol and progesterone on bovine PMN function (either stimulatory or inhibitory). In contradiction with Moreira da Silva et al. (1997), Winters et al. (2003) found no significant changes in PMN oxidative burst activity at physiological or pharmacological levels of estrogens. However, Roth et al. (1983) reported that the combination of low estradiol with high progesterone levels is associated with a reduced oxidative metabolism and enhanced random migration of PMN. Recently, Madsen et al. (2002) demonstrated that genes
regulating basic functions of bovine PMN may be repressed at parturition, possibly due to influences of steroid hormones. Hoeben et al. (1999) investigated the effect of hormones and metabolites, of which the concentrations change substantially around parturition, on hematopoiesis. It was demonstrated that the β-hydroxybutyric acid and acetoacetic acid induced significant inhibition of the proliferation of bovine progenitor cells. Bovine pregnancy-associated glycoprotein also reduced the proliferative activity of bovine bone marrow cells. The inhibitory effects of β-hydroxybutyric acid, acetoacetic acid, and pregnancy-associated glycoprotein may be involved in the changes in the number, differentiation and maturity of circulating PMN observed during the periparturient period (Hoeben et al., 1999), as the circulating pool is largely depending on the proliferative capacity of the bone marrow.

1.3. HYPORETINEMIA

Parturition imposes metabolic stress on the cow, causing relative deficiencies of nutritional factors that are necessary for maintenance of the immune system. The peripartum period represents a period of significant decrease in energy, protein and trace element concentrations in blood that may influence the outcome of disease (Goff and Horst, 1997). Blood selenium and vitamin E concentrations decrease during the immediate postpartum period. Deficiencies in either of these essential nutrients have been related to increased incidence and severity of mastitis (Hogan et al., 1993). Significant changes also occur in the blood vitamin A concentrations (Johnston and Chew, 1984; Goff and Stabel, 1990). Goff and Stabel (1990) reported that plasma retinol declined from 625 ng/ml two weeks prepartum to 206 ng/ml the day after parturition. The precipitous decline was attributed to the loss of retinol in mammary secretions (Kimura et al., 1999). Colostrum contains approximately 4300 ng retinol / ml (Johnston and Chew, 1984). A cow producing 10 litres colostrum thus removes 43 mg of retinol from the plasma pool.

Lower vitamin A during the postpartum period have been associated with higher milk somatic cell counts during subsequent lactation (Johnston and Chew, 1984), indicating increased recruitment of PMN to the mammary gland. Dairy cows suffering from clinical mastitis in early lactation had lower plasma vitamin A concentrations during the period immediately after calving (Chew et al., 1982).
Several studies in dairy cows have suggested that vitamin A and its precursor β-carotene could modulate PMN function during the periparturient period (Daniel et al., 1991; Michal et al., 1994) and may even reduce both the incidence and the severity of mammary infections and subsequent inflammation (Chew et al., 1982; Johnston and Chew, 1984; Oldham et al., 1991).
2. HEMATOPOIESIS

2.1. ONTOGENY OF HEMATOPOIETIC TISSUES

During development, hematopoiesis occurs sequentially and in distinct anatomical localisations (Moore, 1975) (Fig. 1). Until recently it was thought that hematopoiesis originated solely from the yolk sac, and that hematopoietic stem cells colonised sequentially the fetal liver, thymus, and bone marrow. Non-yolk-sac regions e.g. the aorta-gonadal-mesonephros region, however, may also contain pluripotent stem cells during embryogenesis (Medvinski and Dzierzak, 1996).

![Figure 1. Human expansion and recession of hematopoietic activity in extramedullary and medullary sites (adapted from Erslev and Gabuzda, 1979).](image)

In cattle, hematopoietic stem cells colonise the fetal liver at 4 weeks of gestation (Rüsse, 1991), whereafter hematopoietic cells also seed to the spleen. The liver remains the dominant hematopoietic organ throughout fetal life. From the fetal liver, stem cells seed the bone marrow where hematopoietic cavities develop around the fourth month of gestation (Rüsse, 1991). In contrast to the fetal liver where hematopoiesis is predominantly erythroid, hematopoiesis occurring in the fetal marrow is mainly myeloid. At birth, the
bone marrow cavities are the only sites of significant hematopoietic activity and are completely engorged with hematopoietic cells. Liver and spleen are inactive but retain their hematopoietic potential throughout cows’ life. A significant number of fat cells have appeared in the diaphysis of long bones ("yellow" marrow) throughout life. These cells replace hematopoietic elements and expand until hematopoietic “red” marrow is found only in vertebrae, ribs, skull, pelvis and proximal epiphysis of femora and humeri.

2.2. BONE MARROW MICRO-ENVIRONMENT

The bone marrow micro-environment is a complex organ in which stromal cells are responsible for providing most factors required for the orderly development of the hematopoietic stem cells. Stromal cells include osteoblasts, fibroblasts, adipocytes, myocytes, endothelial cells, dendritic cells and macrophages (Gronthos and Simmons, 1996). In mammals, hematopoiesis takes place in the extravascular spaces between marrow sinuses (Fig. 2). The sinus wall is composed of a luminal layer of endothelial cells and an abluminal coat of adventitial reticular cells, with a thin interrupted basement lamina between both cell layers (Abboud and Lichtman, 2000). The endothelial cells are broad flat cells that completely cover the surface of the sinus. They form the major barrier and control the system for molecules entering and leaving the hematopoietic spaces. The adventitial surface of the vascular sinus is composed of reticular cells, which form an incomplete lining. The reticular cells synthesise fibers that extend into the hematopoietic compartments and form a meshwork on which stem cells rest. The small marrow sinuses drain into a central sinus that enters the general circulation through an efferent vein. A small afferent artery supplies the hematopoietic tissue. The bone marrow has an extensive nerve supply, but lacks lymph vessels (Aufderheide, 1981).

Hematopoietic cells of different stages of maturation and lineage commitment can be found in distinct areas throughout the bone marrow space, between the vascular sinuses (Fig. 2). The apparent association of lineage-specific progenitors in islets, so-called “niches”, suggests that the differentiation of cells may depend on specific progenitor-stroma interactions (Schofield, 1978). Such niches would consist of specialised stromal cells, producing hematopoietic supportive cytokines that are conductive for the commitment of progenitor cells.
Figure 2. Schematic view of the bovine bone marrow micro-environment. The blood - bone marrow barrier consists of endothelial cells and adventitial cells, separated by a discontinuous basement membrane. Hematopoietic cells develop in distinct compartments, grouping into lineage-specific islets.
2.3. LINEAGE COMMITMENT

After cell division, the two daughter cells of a hematopoietic stem cell have to decide their fate. They can self-renew (remain a hematopoietic stem cell), differentiate and become a more committed progenitor (Fig. 3), or undergo apoptosis (Domen and Weissman, 1999). A fourth fate, which is open under certain conditions (e.g. during fetal life), is to migrate out of the bone marrow and seed other organs. The balance between these cell-fate decisions determines the size of the hematopoietic stem cell compartment.

The earliest stem cells are pluripotent and capable of differentiation to either lymphopoietic or myelopoietic cells. Later unipotent progenitor cells respond to lineage-specific cytokines and mature into precursor cells that may undergo four or five mitotic divisions before terminating in functional blood or immune cells (Fig. 3). The process of development from pluripotent stem cells to mature cells with specific functions thus involves the progressive loss of developmental potential to other lineages. This stepwise developmental process has been considered linear in the sense that once a cell has made a choice, it cannot revert (Kondo et al., 2003). The earliest lineage-potential decision that a hematopoietic stem cell must make is whether to become a lymphoid or a myeloid cell type (Fig. 3), and once it does, this decision is permanent.

The hematopoietic stem cell has the appearance of a small lymphocyte. It is neither synthetically nor mitotically very active, and offers little in the way of unique structural features, thus is not structurally identifiable. The colony-forming unit in culture assays describe a cell that transforms a colony in vitro in a semi-solid medium (such as methylcellulose) in the presence of appropriate growth factors. Therefore colony-forming units identify multipotent (CFU-GEMM), oligopotent (CFU-GM) of committed (CFU-G, CFU-E, BFU-E) progenitors (Fig. 3).
Figure 3. Overview of hematopoietic development: from stem cell to differentiated cell. CFU-S, colony-forming unit-spleen; CFU-GEMM, colony-forming unit-granulocyte, erythrocyte, monocyte and megakaryocyte; CFU-B colony-forming unit B-cells; CFU-T, colony-forming unit T-cells; BFU-E, burst-forming unit-erythrocytes; CFU-E, colony-forming unit-erythrocytes; CFU-GM, colony-forming unit-granulocyte and monocyte; CFU-Meg, colony-forming unit-megakaryocytes; CFU-G, colony-forming unit-granulocytes; CFU-M, colony-forming unit-monocytes; CFU-Eo, colony-forming unit-eosinophils.
3. LIFE SPAN OF PMN: FROM MATURATION TO SENESCENCE

3.1. PROLIFERATION, DIFFERENTIATION AND MATURATION OF GRANULOCYTES

Granulocytes originate from the pluripotent hematopoietic stem cell, first becoming committed to the colony-forming unit-granulocyte, erythrocyte, monocyte and megakaryocyte (CFU-GEMM), thus further differentiating to the colony-forming unit-granulocyte and monocyte (CFU-GM) and the colony-forming unit-eosinophils (CFU-Eo) (Fig. 3). Finally, an unidirectional progression of proliferation, differentiation and maturation from the colony-forming unit-granulocytes (CFU-G) through myeloblasts, promyelocytes, myelocytes, metamyelocytes, and band cells results in the formation of segmented granulocytes (Fig. 4). Precursor cells undergo striking morphological, biochemical and functional changes during granulocytic maturation. These changes are associated with significant changes in cell size and nuclear shape, and with the development of stage-specific proteins.

Covering the multi-step process of granulopoiesis, the hematopoietic compartment in the bone marrow can be divided in 1) the mitotic or proliferation pool, consisting of myeloblasts, promyelocytes, and myelocytes; 2) the non-mitotic or maturation pool with metamyelocytes and band cells; and 3) the storage or reserve pool, primarily comprised of mature granulocytes and some band cells (Jain, 1993; Moreira da Silva et al., 1994). The myeloblast has a high nuclear/cytoplasmic ratio and contains one or more nucleoli. Its dark to blue cytoplasm contains no granules. The promyelocyte, the largest cell of the granulocytic series, can be recognised by its off-center and round nucleus, and a large number of azurophilic granules. The myelocyte is characterised by its indented nucleus and mixed population of granules. This is the first appearance of the specific granules, allowing preliminary characterisation as neutrophilic, eosinophilic or basophilic myelocyte. The metamyelocyte can easily be identified by a kidney-shaped nucleus, and a high number of granules. The nucleus of the band cell is narrow with parallel sides. Irregularity of the nuclear membrane or indentations are features of the mature granulocytes.

Limited studies have been performed on the kinetics of the granulopoiesis in the bovine species. In normal calves the mean time for production of labelled PMN is 5.77 days (Valli et al., 1971). The mean time of arrival of PMN in the bloodstream is 7.01 days.
The half-life of PMN in circulation is 8.9 hours (Carlson and Kaneko, 1975). The lactating adult cow has approximately $2 \times 10^{11}$ mature PMN in the bone marrow and circulating storage pools (Paape et al., 1979). The proliferative pool of the myelocytic series in the bone marrow ranges from 2.8 to 5.3% in cows, and the maturative pool is between 9.4 and 11.6% (Schalm and Lasmanis, 1976; Jain, 1993). The reserve of mature bone marrow PMN is estimated at 12% of all bone marrow leukocytes. In addition to the reserve pool in the bone marrow, there is also a large storage pool containing mature PMN in the bloodstream (Paape et al., 1979). This marginated pool lines venules and capillaries and exists as a buffer to meet the initial demand.

3.2. Regulation of the Granulopoiesis

The process of granulopoiesis is strictly controlled by regulatory growth factors, comprising cytokines and colony-stimulating factors (CSF), to ensure the maintenance of a steady state (Fig. 4). The hematopoietic growth factors have pleiotropic effects on proliferation, differentiation and functional activation of precursor cells (Metcalf, 1989). They interact at various levels of the differentiation cascade, from unipotent stem cell to mature PMN. Each growth factor has distinct lineages of bone marrow cells upon which it acts, although there is some overlap in lineage activity and synergy between factors. The biologic effects of the growth factors are mediated through specific receptors on the surfaces of target progenitor cells. The major cellular sources of the growth factors are monocytes, macrophages, T lymphocytes, and fibroblasts. Growth factors can be classified according to the level at which they act in the granulopoiesis. Late-acting lineage specific factors act on maturing cells. Granulocyte colony-stimulating factor (G-CSF) regulates for example the proliferation and maturation of granulocytic precursors, but also acts with other factors to support the proliferation of primitive stem cells. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) are examples of intermediate-acting non-specific factors that support the proliferation of multipotent progenitors. IL-6, IL-11, G-CSF, and stem cell factor SCF act synergistically with IL-3 to induce dormant hematopoietic stem cells to enter the cell cycle (Metcalf, 1989; Ogawa, 1993).
Figure 4. Action of growth factors, metabolites, hormones, and cytokines on the proliferation, differentiation, and function of PMN.

SCF, stemcell factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; BHBA, β-hydroxybutyric acid; AcAc, acetoacetic acid; bPAG, bovine pregnancy-associated glycoprotein; IL, interleukin; RA, retinoic acid; Adr, adrenaline; GCS, glucocorticosteroid.
3.3. EGRESS AND MOBILISATION FROM THE BONE MARROW

Once hematopoietic progenitors have matured, they are released from the bone marrow in the peripheral circulation. It has been postulated that cells leave the bone marrow more-or-less by a “pipeline” mechanism, the older cells being released first (Maloney and Patt, 1968). The exact mechanism(s) underlying the process of egress is not well understood. These that have been suggested include the anatomical localisation of the hematopoietic cells close to vascular spaces, and the development of transient holes in the venous sinuses to allow egress of marrow cells (Fig. 2). Also the developmental changes in the nucleus and the cytoplasm of the maturing cells that increase their motility, deformability, and chemotactic ability could attribute to the release of terminally differentiated PMN from the hematopoietic compartment into the efferent vascular space (Lichtman, 1970). Van Eeden et al. (1997) assigned a possible role to L-selectin in the release of mature granulocytes from the bone marrow in human, as L-selectin is highly expressed on mature cells in the postmitotic (maturation) pool in the bone marrow (Terstappen et al., 1990). With active bone marrow release an increase in the number of PMN expressing high levels of L-selectin in the circulation is observed. However, levels of L-selectin on circulating PMN never reach levels expressed in the bone marrow, suggesting that L-selectin is shed as PMN enter the circulation (Van Eeden et al., 1997). Recently, Monfardini et al. (2003) confirmed these data for the bovine species.

A number of releasing factors have been implicated in the initiation of marrow egress. Those for granulocytes include G-CSF, GM-CSF, glucocorticoid hormones, steroids, and endotoxines. Under steady-state conditions the majority of more primitive hematopoietic cells are retained in the bone marrow micro-environment. Physiological stressors such as parturition and infection (e.g. mastitis) can increase the number of circulating immature granulocytes in dairy cows (Heyneman et al., 1990; Moreira da Silva et al., 1998; Mehrzad et al., 2002).

3.4. AGEING OF PMN

Life span of PMN can be divided into three different phases: intramedullary (in the bone marrow), circulatory and tissue phase. In the tissue, aged PMN undergo spontaneous apoptosis in the absence of pro-inflammatory agents prior to their removal by macrophages
(Savill et al., 1989), thus preventing the release of their cytotoxic content. Apoptosis of terminally differentiated cells usually occurs after the cells have played their functional roles in immune defence. This type of apoptosis might be the physiological apoptosis of ageing and full life-spanned mature PMN (Whyte et al., 1999).

In cows, PMN apoptosis is influenced by stage of lactation. In the study of Van Oostveldt et al. (2001) blood was obtained from early and mid-lactating cows and subsequently incubated for 4 hours to induce apoptosis. PMN isolated from the blood of early lactating cows exhibited a higher rate of apoptosis than PMN derived from mid-lactating cows. Interestingly, PMN isolated from early lactating cows also display impaired functions (Kehrli et al., 1989). Further, onset of apoptosis has been reported to downregulate phagocytic and respiratory burst activity in bovine PMN (Van Oostveldt et al., 2002). The findings concerning phagocytosis in aged PMN from humans are reasonably consistent. Studies measuring ingestion of opsonised E. coli bacteria have shown a significant reduction in phagocytic ability of ageing PMN. Reduced phagocytosis was based predominantly on a reduced number of bacteria ingested per cell, rather than the number of PMN with phagocytic ability (Butcher et al., 2000). Microbicidal activity of ageing human PMN has been examined by several groups and although data are often contradictory, a majority support a decline in ROS production (reviewed by Lord et al., 2001).

Lipschitz et al. (1987) suggested that the age-related decline in PMN function of mice is a result of a membrane defect. A recent study in rat peritoneal PMN demonstrated an increase in neutrophil membrane fluidity with age, due to a decrease in cholesterol/phospholipid ratio and rise in poly-unsaturated fatty acids (Alvarez et al., 2001).

Another possible pathway is the diminished capacity of PMN to escape from apoptosis following cell challenge with pro-inflammatory mediators. PMN are short-lived cells, and in order to increase their bactericidal potential, their life span is extended at the site of infection by bacterial components, complement and pro-inflammatory cytokines (Lee et al., 1993). Human aged PMN demonstrated an impairment of cell survival following activation with GM-CSF, G-CSF, endotoxin or IL-2 (Fülöp et al., 1997). This
may implicate that older PMN respond less well to infectious stimuli upon recruitment to
the site of infection and also die more rapidly (Butcher et al., 2000).

It may be postulated that increased susceptibility to bovine apoptosis during the
period immediately following parturition contributes to impaired PMN function and
increased severity of coliform mastitis observed during this period (Burvenich et al.,
1994). Thus, although PMN are produced in sufficient numbers in the bone marrow and
are probably recruited to infected mammary gland efficiently, their bactericidal function
may be compromised by their accelerated entry into apoptosis at the site of infection.
4. VITAMIN A

4.1. STRUCTURAL CHARACTERISTICS OF VITAMIN A

The term vitamin A is used for all compounds that exhibit the biological activity of retinol (Norum and Blomhoff, 1992), whereas the term retinoids comprises the natural occurring vitamin A derivatives as well as a large number of synthetic analogues (Sporn and Roberts, 1985). Vitamin A is chemically described as “pale yellow crystalline solid” (Olson, 1996). Retinol, the alcohol form of vitamin A, is an unsaturated monohydric alcohol with the empirical formula C\textsubscript{20}H\textsubscript{30}O (molecular weight = 286.48). The structure of retinol consists of a cyclohexane ring (β-ionone) linked to a poly-unsaturated chain terminating in an alcoholic group (Fig. 5). Modifications in the end-group result in the formation of vitamin A derivatives, retinyl esters, retinaldehyde and retinoic acid. The natural retinoids present some difficulty in manipulation because of the conjugated double-bound system that makes them sensitive to light, oxygen, heat, and therefore susceptible to isomerisation, oxidation, and other degenerative processes.

4.2. OUTLINE OF VITAMIN A METABOLISM

Vitamin A is an essential nutrient for mammals. The main dietary sources of vitamin A are provitamin carotenoids (mainly β-carotene) from vegetables, and preformed retinyl esters from animal sources. Retinyl esters are hydrolysed by a pancreatic esterase in the intestinal lumen, and the free alcohol is absorbed in the small intestine (Fig. 6). Retinol is re-esterified in the mucosa, and incorporated into chylomicrons (Nayak \textit{et al.}, 2001). Carotene is absorbed into enterocytes by passive diffusion, and converted enzymatically to retinol. The conversion of β-carotene to retinol involves two reactions: central cleavage of β-carotene into retinal by β-carotene-15,15’-dioxygenase, and reduction of retinal to retinol (Fig. 5) (Glover, 1960). This reaction yields two molecules of retinal from β-carotene. Cattle also absorb substantial amounts of β-carotene without prior conversion to retinol, and these pigments are responsible for the yellow colour of the blood plasma (Hornbuckle and Tennant, 1997).
Figure 5. Structure of naturally occurring retinoids, and metabolic flow from retinyl ester through retinol and retinal into retinoic acid.
After secretion in the lymphatic system (Fig. 6), nascent chylomicrons undergo lipolysis to give rise to chylomicron remnants (Goodman and Blaner, 1984). Although chylomicron remnants are mainly cleared by the liver, extrahepatic uptake may be important in the delivery of fatty acids, sterols, and retinol in tissues with intensive cell proliferation such as the bone marrow (Hussain et al., 1989). Once chylomicron remnants are taken up by liver parenchymal cells, a rapid hydrolysis to retinol takes place. When vitamin A status is insufficient, i.e. when extra-hepatic organs urgently need vitamin A, the newly formed retinol is bound to retinol-binding protein (RBP), a specific retinol carrier, and secreted in the blood (Blomhoff et al., 1991). When vitamin A status is satisfactory, the newly formed retinol is transferred in the form of retinyl esters to a specific cell type, the hepatic stellate cells (Fig. 6). These cells can store large quantities of vitamin A in characteristic lipid droplets. The lipid droplets are subsequently mobilised to maintain a homeostatic concentration of retinol, and to meet the requirements of the organism. It is worth mentioning that enterocytes and hepatocytes handle retinol in a different way: enterocytes rapidly esterify the absorbed retinol with long-chain fatty acids and secrete the retinyl esters packed with triacylglycerol in the core of chylomicrons; hepatocytes do not incorporate retinyl esters together with triacylglycerol into very-low-density lipoproteins (Ross and Zilversmit, 1977). This difference may be crucial for the regulation of the storage of vitamin A and the delivery to tissues. Remarkably, the vitamin A spectrum is very broad, ranging from deficiency to toxicity (Russell, 2000).

In circulation, vitamin A mainly exists in two forms, including lipoprotein-associated retinyl esters, and retinol bound to RBP and mostly complexed with transthyretin. The RBP-bound retinol is presumably taken up by target cells via a mechanism thought to involve a membranous receptor specific for RBP. After it is internalised, retinol is oxidised to retinal and converted into retinoic acid (Fig. 5).
Figure 6. Schematic representation of vitamin A metabolism: absorption from the small intestine, transport, storage in the liver, mobilisation and formation of retinoic acid in target cell (based on Blomhoff et al., 1991 and Norum and Blomhoff, 1992).

ROH, retinol; RAL, retinal; RA, retinoic acid; RE, retinyl ester; CM, chylomicron; CMR, chylomicron remnant; RBP, retinol-binding protein; TTR, transthyretin; TG, triacylglycerol
4.3. **MECHANISM OF ACTION OF VITAMIN A**

Retinol has no biological activity in itself, but rather serves as a substrate for the biosynthesis of bio-active retinoids. These fall into two categories: 1) 11-cis-retinal, that is covalently bound to the protein opsin to form rhodopsin in the eye, and 2) retinoic acid isomers regulating gene expression.

Although retinoic acid circulates in the blood bound to albumin, most retinoic acid is produced in the target cells (Fidge et al., 1968). Vitamin A-dependent tissue cells including the eye, epithelia, and bone marrow seem to have the metabolic machinery needed to oxidise retinol to retinoic acid. The biochemical process by which retinoic acid is enzymatically formed within tissues has not been unequivocally established. The currently prevailing hypothesis is the following (Blaner and Olson, 1994; Duester, 2000) (Fig. 5): the first and rate-limiting step involves the reversible dehydrogenation of retinol into retinal, and retinal is then irreversibly oxidised into all-trans-retinoic acid by NADP⁺-dependent retinal dehydrogenase(s). Alternative metabolic routes leading to all-trans-retinoic acid may exist but are not fully elucidated (Napoli, 1999). The different retinoic acid isomers are enzymatically interconverted in a reversible way in vivo (Kojima et al., 1994).

The retinoid signal is transduced by two families of nuclear receptors, as first described by Petcovich et al. (1987): the retinoic acid receptor (RAR) family and the retinoid X receptor (RXR) family, each comprising subtypes (α, β, γ), with various isoforms of each subtype (Mangelsdorf et al., 1994). These receptors belong to the superfamily of nuclear hormone receptors and act as ligand-activated transcription factors. The natural ligands for RAR are all-trans-retinoic acid and its stereoisomers 9-cis- and 13-cis-retinoic acid, whereas RXR is solely activated 9-cis-retinoic acid. Most of the effects of retinoic acids are mediated by RAR/RXR heterodimers (Fig. 7). The ligand-receptor complexes act as inducible transcription regulators of several genes by binding to specific retinoic acid response elements, with activation or silencing of target genes as a result (Marill et al., 2003).
Figure 7. Intracellular retinoid signalling. All-trans-retinoic acid (trans RA) binds to nuclear retinoic acid receptor (RAR) and 9-cis-retinoic acid (9-cis RA) to retinoid X receptor (RXR), respectively. Subsequently, the RAR/RXR heterodimer binding to specific retinoic acid response elements (RARE) results in the regulation of gene transcription.

4.4. ROLE OF VITAMIN A IN HEMATOPOIESIS AND PMN FUNCTION

Vitamin A got his first reputation as the “anti-infective” vitamin in 1928 (Green and Mellanby, 1928). Since, the importance of vitamin A in immune function and protection against infections is well-established (Semba, 1999; Stephensen, 2001; Field et al., 2002). Today, it is known that vitamin A can influence host defence mechanisms indirectly through its role in epithelial cell differentiation and host barrier function, or directly through its essential functions in immune processes, including hematopoiesis, apoptosis, and leukocyte function (Semba, 1998).

It has been known for some time that vitamin A and especially retinoic acid can modulate normal human hematopoiesis (Collins, 2002). The implication of retinoic acid in the differentiation of CFU-GM has been further stressed by the abundant presence of retinoic acid receptors in granulocytic precursors (Chomienne et al., 1990). Retinoic acid has the capacity to drive pluripotent hematopoietic progenitor cells into the granulocytic
lineage (Tsai and Collins, 1993). Kastner et al. (2001) unravelled the in vivo mechanisms of RARα as a key mediator for the effects of retinoids on granulopoiesis in rats. The receptor can bi-directionally modulate granulopoiesis, as a differentiation factor when liganded to retinoic acid or as an inhibitor in the absence of the ligand. Both retinoid excess and deficiency seem to affect granulopoiesis, suggesting that a balance between liganded and unliganded receptor molecules exists in vivo under normal conditions (Kastner et al., 2001). The active sequestration of retinol in the bone marrow of vitamin A-deficient rats further stresses the importance of vitamin A in the differentiation of myeloid cells to mature immune cells (Twining et al., 1996).

In hematopoietic cells, apoptosis seems to be an essential component of terminal differentiation, in order to maintain homeostasis in the number of cells (Domen, 2001). Programmed cell death seems to be tightly controlled by retinoids. Retinoic acid regulates the rate of cellular differentiation and apoptosis, depending on the retinoic acid receptor subtype. Mehta et al. (1996) demonstrated that activation of RARs induces the genes linked to cellular differentiation, while activation of RXRs induces genes linked to apoptosis.

Although a reduction in circulating PMN numbers does not appear to be a feature of vitamin A deficiency, PMN display an impaired function that could result in initial exposure to a greater number of pathogenic organisms. Functional studies showed defects in chemotaxis, adhesion, phagocytosis, and ability to generate active oxidant molecules in neutrophils from vitamin A-deficient rats, compared to the control (Twining et al., 1997).

4.5. ACUTE-PHASE RELATED HYPORETINEMIA

Serum retinol levels are maintained within a fairly narrow range despite large fluctuations in dietary vitamin A intake and tissue stores. Only under extreme conditions, such as deficiency or intoxication, the homeostatically regulated retinol levels become affected. Serum retinol concentrations decrease transiently during the acute-phase response of infection or inflammation in human (Beisel, 1998; Schweigert, 2001; Stephensen, 2001) and in rats (Rosales et al., 1996; Rosales and Ross, 1998). During the recovery phase, retinol concentration characteristically returns to normal, sometimes with a small overshoot, within a time course of 1 - 2 weeks (Tabone et al., 1992).
While the mechanisms involved in the changes caused by the acute-phase response on the so-called acute-phase proteins in serum (i.e. serum amyloid A, haptoglobin, albumin, ceruloplasmin) are well characterised, limited information regarding the quantitative or qualitative effects of the acute-phase response on serum levels of retinol is available. Three main, and not mutually exclusive, mechanisms have been proposed to explain the lowering of serum retinol during the acute-phase of infection (Filteau, 1999; Schweigert, 2001; Stephensen, 2001): 1) decreased mobilisation and transport of retinol from the liver, 2) excretion of retinol (and eventually RBP-bound retinol) in the urine, and 3) increased metabolic requirements. The most intensively discussed possibility is the increased consumption of circulating antioxidants during neutralisation of free radicals released by activated PMN (Sies and Stahl, 1995). Retinoids possess potent activities that scavenge singlet oxygen and peroxyl radicals, thus preventing oxidative cell destruction. 4) Recently, a fourth mechanism was postulated. Serum retinol can be specifically taken up by certain tissues that require vitamin A, perhaps those involved in immune response or in tissue repair.

All mechanisms are generally accepted to be mediated through inflammatory cytokines, as they exhibit plausible time courses with respect to changes in retinol distribution (Tabone et al., 1992). The functional importance of the inflammation-induced decrease in serum retinol concentration is not, at present, clear.


General Introduction


Periparturient dairy cows undergo a plethora of physiological changes, including nutritional, hormonal and immunological changes. During this time period cows experience an increased susceptibility to intramammary infections, caused by environmental pathogens. A relationship between the animals’ physiological pre-infection status, especially at the level of the first line defence, and the severity of experimentally induced \textit{E. coli} mastitis has been shown (Heyneman \textit{et al.}, 1990; Lohuis \textit{et al.}, 1990; Kremer \textit{et al.}, 1993; Van Werven \textit{et al.}, 1997) (Fig. 8, \(\textcircled{1}\)). It has previously been hypothesised by Burvenich \textit{et al.} (1994) that an immunosuppression at the bone marrow level could compromise the cows’ immunity around parturition. Although the functional incompetence of less mature PMN appearing in circulation has been postulated (Guidry \textit{et al.}, 1976), the stepwise functional development during granulopoiesis has not been determined (Fig. 8, \(\textcircled{2}\)).

Hormonal and metabolic changes around parturition are unequivocally involved in the reduced functionality of circulating PMN (Goff and Horst, 1997) (Fig. 8, \(\textcircled{3}\)). Furthermore, ketone bodies can alter the proliferative capacity of progenitor cells at the level of the bone marrow (Hoeben \textit{et al.}, 1999). Steroids of which the concentrations change abruptly around parturition might also affect bovine granulopoiesis, thereby reducing the proliferation of progenitor cells, the acquisition of immune functions, and the number of circulating PMN (Fig. 8, \(\textcircled{4}\)).

Retinol is known to decrease spectacularly immediately after parturition (Johnston and Chew, 1984), and several studies have suggested that vitamin A and \(\beta\)-carotene could modulate PMN function during the periparturient period (Daniel \textit{et al.}, 1991) (Fig. 8, \(\textcircled{5}\)). In humans, hyporetinemia is clearly related to the acute-phase response (Schweigert, 2001). Vitamin A concentrations might be affected during acute \textit{E. coli} mastitis in dairy cows (Fig. 8, \(\textcircled{6}\)). Furthermore, vitamin A may be of importance in the regulation of bovine granulopoiesis (Fig. 8, \(\textcircled{7}\)).
Figure 8. Scheme representing the hypothesis. White numbers on black background are known features; black numbers on white background are objectives of the doctoral thesis.

1. Relationship between pre-infection status and severity of coliform mastitis.
2. Are immature cells as functional as their mature counterparts?
3. Steroid hormones alter the functionality of circulating PMN.
4. Do steroid hormones affect the granulopoiesis?
5. Vitamin A modulates PMN function during the periparturient period.
6. Are vitamin A concentrations affected during coliform mastitis?
7. Is vitamin A involved in the regulation of the granulopoiesis?
**REFERENCES**


OBJECTIVES OF THE STUDY

The general objective of this doctoral thesis was to investigate the immunosuppression in periparturient dairy cows at the bone marrow level. The relationship between vitamin A, granulopoiesis and susceptibility to mastitis was studied during the periparturient period. Based on the scheme (Fig. 8) representing the hypothesis of the current study, a number of objectives could be distinguished:

- To determine the functional maturation sequence of progenitor cells during bovine granulopoiesis (Fig. 8, ①).
- To assess the effect of steroids (progesterone, 17-β-estradiol, and hydrocortisone) on the proliferative activity of bovine bone marrow cells (Fig. 8, ②), with respect to the leukocytic changes observed in circulation after parturition.
- To determine the blood vitamin A profile (including retinol and three retinoic acid isomers) during the periparturient period, and during subsequent experimentally induced *E. coli* mastitis in early lactation (Fig. 8, ③).
- To gain insight in the regulation of bovine granulopoiesis by retinoids (Fig. 8, ④).

To attain these purposes, adequate methods were developed and evaluated. A bone marrow cell culture assay optimised for myeloid colony formation was described. A separation technique for fractionation of bone marrow into maturation-related suspensions was developed. An extraction procedure and detection system using HPLC-DAD was set-up to enable simultaneous quantification of naturally occurring retinoids in serum.
PART 1

DEVELOPMENT & EVALUATION OF METHODS
CHAPTER 1
Culture of bovine bone marrow progenitor cells \textit{in vitro}

CHAPTER 2
Separation of bovine bone marrow into maturation-related fractions

CHAPTER 3
Quantification of endogenous retinoids in bovine serum
Adapted from


Chapter 1

ABSTRACT

Bovine bone marrow samples were aspirated aseptically from the sternal cavity of healthy cows. Mononuclear cells were separated by gradient centrifugation (1.077 g/ml specific density), and aspirated from the light density interface. Progenitor cells were subsequently cultured using an in vitro assay optimised for bovine myeloid colony formation. The semi-solid system consisted of 0.9% high-viscosity methylcellulose, 30% of non heat-inactivated fetal bovine serum, 1% of bovine serum albumin, 3% of lymphocyte-conditioned medium, 10 mM 2-mercapto-ethanol, and IMDM supplemented with 200 IU/ml of penicillin, 200 µg/ml of streptomycin and 0.5 µg/ml of amphotericin B. A volume of 1 ml soft methylcellulose-gel containing $1 \times 10^5$ progenitor cells was plated out per 35-mm culturing dish. Culture assays were incubated for 7 days at 37°C in 95% relative humidity and under 5% CO₂ in air. The use of bovine lymphocyte-conditioned medium, produced by stimulation of blood lymphocytes with 4 µg/ml concanavalin A and harvested after 5 days of incubation, resulted in an acceptable myeloid clonogenic output. The availability of an in vitro clonogenic assay for bovine progenitor cells provides a powerful impetus to the investigation of the myelopoiesis.
INTRODUCTION

During the periparturient period, dairy cows show an increased susceptibility to infectious and metabolic diseases (Paape et al., 1995). Circulating polymorphonuclear leukocytes (PMN) play a pivotal role in the defence against the infectious diseases, and in the pathogenesis of acute coliform mastitis. The increased susceptibility to infectious diseases is probably due to changes in the number, function, and maturity of circulating PMN after parturition (Burvenich et al., 1994). Since changes in the hematopoiesis may underlie alterations in functionality and maturity of circulating PMN, culture of bovine bone marrow cells in vitro could provide insight in the changes observed in the circulation during the periparturient period.

One of the problems inherent to the use of in vitro culture assays of hematopoietic cells is the variability in colony growth (plating efficiency) that can be encountered with changes in each component of the medium. Standardisation of the culture medium is not the only condition to maximise the reproducibility of bovine hematopoietic colony formation. Culturing conditions, including temperature, humidity, and CO₂ levels can affect colony growth and morphology, as does the incubation time allowed before the colonies are scored.

MATERIALS AND METHODS

BONE MARROW ASPIRATION

Bone marrow samples were taken from clinically healthy cows at the slaughterhouse of the Ghent University (Melle, Belgium). Before splitting the carcass, 2 ml marrow was aspirated from the third or fourth sternebrae (Van Merris et al., 2001b) by means of a Janus bone marrow needle (Bignell Surgical Instruments Ltd; Arundel, England). The aspiration site was determined by counting of the ribs, starting from the last one. The precise localisation coincided with the intersection of the median line of the sternum and the imaginary line joining the olecranos. Under gentle rotation and with controlled pressure, the needle was pushed into the sternebrae under a right angle till a
subtle ‘plop’ was felt, indicating that the cortex had been pierced and the needle had entered the sternal marrow cavity. The stylet was removed and a 10-ml syringe was fitted onto the cannula. About 2 ml of marrow fluid was aspirated. Samples were transferred in sterile tubes containing 5 ml of Iscove’s modified Dulbecco’s Minimal Essential Medium with 584 mg/l of L-glutamine (IMDM; Gibco BRL, Life Technologies Inc., Gaithersburg, MD, USA) supplemented with 10% of non heat-inactivated bovine serum (FBS; Gibco BRL). Lithium heparin (100 U/ml; Leo Pharmaceutical Product, Zaventem, Belgium) was added as an anticoagulant. Samples were kept at 4°C until isolation.

ISOLATION OF MONONUCLEAR BONE MARROW CELLS

Bone marrow cells were gently mixed before further processing. Progenitor cells were isolated by centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden) with specific density of 1.077 g/ml. Following centrifugation at 400 × g for 20 min at 20°C (Van Merris et al., 2001a), mononuclear bone marrow cells were obtained from the light density interface, and subsequently washed twice (400 × g, 10 min) in IMDM. Finally the pellet was resuspended to obtain a concentration of 1×10^7 cells ml^-1 in IMDM, supplemented with 200 IU/ml of penicillin (Gibco BRL), 200 µg/ml of streptomycin (Gibco BRL) and 0.5 µg/ml of amphotericin B (Gibco BRL).

BOVINE LYMPHOCYTE-CONDITIONED MEDIUM

Peripheral blood (25 ml) was aseptically collected from the external jugular vein of healthy lactating Holstein cows in 30 ml of Alsever solution (Gibco BRL) and further diluted with 27 ml of Dulbecco’s modified Eagle medium (DMEM; Gibco BRL). This cell suspension was centrifuged on Ficoll-Paque (1.077 g/ml) at 1000 × g for 30 min at room temperature. The peripheral lymphocytes collected from the interface were washed twice in Alsever solution (400 × g, 10 min, 20°C), followed by two washings with DMEM (400 × g, 10 min, 20°C). The isolated cells were counted using an electronic cell counter (Technicon H1, Bayer Diagnostics, Tarrytown, New York, USA) and further diluted to a concentration of 1×10^6 cells ml^-1 in DMEM supplemented with 20% of FBS (Myocline Super Plus USA; Gibco BRL). Lymphocytes were stimulated with 4 µg/ml of concanavalin A (Sigma Chemical Co.). After 5 days of incubation in a high-humidity
atmosphere containing 5% CO2 in air, supernatants was harvested (2000 × g, 10 min, 4°C), subsequently aliquotted and frozen (-20°C) until use (Van Merris et al., 2001a).

**CULTURE OF BOVINE BONE MARROW CELLS**

Bovine mononuclear bone marrow cells were cultured using an *in vitro* assay optimised for myeloid colony formation by Van Merris et al. (2001a). The semi-solid system consisted of 0.9% high-viscosity methylcellulose (Methocel®, Fluka Chemie, Buchs, Switzerland), 30% of non heat-inactivated FBS, 1% of deionised bovine serum albumin (BSA fraction V 7.5%; Sigma Chemical Co., St. Louis, MO, USA), 3% of lymphocyte-conditioned medium, 10 mM 2-mercaptoethanol (Sigma Chemical Co.), and IMDM supplemented with 200 IU/ml of penicillin, 200 µg/ml of streptomycin and 0.5 µg/ml of amphotericin B. A volume of 1 ml soft methylcellulose-gel containing 1×10^5 progenitor cells was plated out per 35-mm culturing dish (StemCell Technologies, Vancouver, Canada). All cultures were carried out in duplicate. Sterile water was added in between the wells in order to avoid drying out of the methylcellulose-medium. Culture assays were incubated for 7 days at 37°C in 95% relative humidity and under 5% CO2 in air (Binder GmBH, Tuttlinger, Germany).

**MICROSCOPICAL IDENTIFICATION OF COLONIES**

Cellular growth and colony formation were evaluated using an inversion microscope (PL Fluotar, Leitz, Wetzlar, Germany) at a magnification of ×100. Scoring was performed by a single person, at a magnification of ×250, by means of gridded scoring dishes (StemCell Technologies).

**RESULTS AND DISCUSSION**

A prerequisite for investigations on bovine bone marrow is a simple and reliable technique to obtain active marrow. Aspiration of red marrow from the sternal cavity of cows yielded active bone marrow with viable cells. Blood contamination of the marrow sample was inevitable, yet minimised by limiting the aspirated volume to 2 ml of marrow.
liquid. Excessive suction caused damage of blood vessels and sinuses in the marrow, resulting in dilution of bone marrow with blood (Wilde, 1961).

After 7 days of incubation of mononuclear bone marrow cells, the following myeloid colony types were clearly discernible, based on their typical morphological characteristics. Colony-forming unit-granulocyte, monocyte (CFU-GM) were mixed granulocytic and monocytic colonies containing > 50 cells with a typical dense centre and a very widespread growth pattern away from the dense centre of the colony. Colony-forming unit-granulocyte (CFU-G) consisted of clusters of > 40 spherical granulated cells with a very dense circular structure. Colony-forming unit-monocyte (CFU-M) were clusters of > 20 larger brownish cells that were less densely organised compared with CFU-G. Colony-forming unit-granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM), although rare, consisted of small numbers of granulocytes and macrophages around the periphery multiple lineages of a spherical mass of hemoglobinised erythroid colonies. Bovine colonies started to disintegrate between 7 and 14 days (Fritsch and Nelson, 1990).

Addition of bovine lymphocyte-conditioned medium, harvested from concanavalin A - stimulated blood lymphocytes, resulted in an acceptable clonogenic output of myeloid colonies. Ito and Kodama (1996) demonstrated that bovine lymphocytes stimulated with concanavalin A expressed mRNA for interleukin-2, interleukin-6, interferon-gamma, and granulocyte-macrophage colony-stimulating factor. Because heat-inactivation of FBS (heating at 56°C during 30 min) may destroy cytokines and growth factors, which are naturally present in serum, we preferred to use non heat-inactivated FBS.

The availability of reproducible and quantitative in vitro clonogenic assays for bovine progenitor cells provides a powerful impetus to the investigation of the myelopoiesis. The culturing method is also a useful tool for assessing the effect of a variety of hormones and metabolites on bovine myelopoiesis (Hoeben et al., 1999).
REFERENCES


CHAPTER 2

SEPARATION OF BOVINE BONE MARROW INTO MATURATION-RELATED FRACTIONS

Adapted from

ABSTRACT

A prerequisite for studies on bovine myeloid cells in relation to maturity is a reliable separation method, in order to obtain enriched and partially purified cell fractions of different maturation stages. Since current techniques for bovine bone marrow cell isolation fall short of this requirement, a technique for fractionating bovine bone marrow using a three-layer discontinuous gradient column was developed. Three maturation-related myeloid cell fractions were obtained at specific densities. Early immature myeloid cells, i.e. myeloblasts and promyelocytes, were found at a specific density of 1.060 g/ml. Late immature myeloid cells, i.e. myelocytes and metamyelocytes, were retrieved at 1.080 g/ml. Bands and segmented cells, representing the mature fraction, accumulated in the high density pellet (> 1.080 g/ml). Myeloid cell populations were identified in each fraction by flow cytometry based on their forward and side scatter pattern. Confirmation was provided by light microscopy of flow cytometrically sorted myeloid populations, using morphological characteristics. The developed method provides a unique tool for studying maturation-dependent functions in bovine bone marrow.
**INTRODUCTION**

Bone marrow is a complex matrix that is neither routinely examined in veterinary medicine, nor commonly used for research purposes. Interest has already been focused on the morphological and, consequently, functional immaturity of circulating polymorphonuclear leukocytes (PMN) in relation to the susceptibility to mastitis (Guidry and Paape, 1976; Silva et al., 1989). The increased susceptibility to and severity of coliform mastitis observed during the periparturient period has been related to a low number of PMN in circulation as well as to impaired PMN functions (Heyneman et al., 1990; Moreira da Silva et al., 1998; Dosogne et al., 1999). The initial neutropenia is rapidly counteracted by a mobilisation of PMN into circulation from the marginated pool. This mature reserve pool exists as a buffer to meet the initial demand for PMN. Depletion of the marginal pool triggers cell release from the bone marrow. Finally, immature cells are released into circulation, resulting in a left-shift (Jain et al., 1978). The question rises whether this left-shift compromises the cows’ resistance by supplying more immature cells that are less functional (Guidry and Paape, 1976).

To study this hypothesis, there is a need to assess functional properties, including biochemical events and surface markers, in relation to cellular maturity. A prerequisite to study the bovine granulopoiesis is a reliable separation resulting in cell fractions of different maturation stages. Since current techniques of bovine bone marrow cell isolation fall short for this requirement, a fractionation method using a three-layer discontinuous gradient column was developed.

**MATERIALS AND METHODS**

**BONE MARROW ASPIRATION**

Bovine bone marrow samples were collected from the sternum of adult cows at the slaughterhouse of the Ghent University (Melle, Belgium). Before cleavage of the carcass, 2 ml marrow was aspirated from the third or fourth sternebrae (Van Merris et al., 2001b) by means of a Janus needle (Bignell Surgical Instruments Ltd; Arundel, England). Bone
marrow samples were transferred on ice into sterile tubes containing Iscove’s modified Dulbecco’s Minimal Essential Medium with 584 mg/l of L-glutamine (IMDM; Gibco BRL, Life Technologies Inc., Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and 100 U/ml lithium heparin (Leo Pharmaceutical Product, Zaventem, Belgium).

**PERCOLL GRADIENT PREPARATION**

Percoll media of different densities were prepared according to the supplier’s instructions. Briefly, the required volume of Percoll® (colloidal silica coated with polyvinylpyrrolidone; Sigma Chemical Co., Missouri, USA) was diluted in sterile water and 1/10th of the final volume of 10× phosphate-buffered saline (PBS ×10; Gibco BRL). The formula for calculating the amount of Percoll stock required for making a working solution is

\[
V_p = V \frac{(d - 0.1 \times d_s - 0.9)}{(d_p - 1)}
\]

where
- \( V_p \) = volume (ml) of Percoll stock required
- \( V \) = volume (ml) of the final working solution
- \( d \) = desired density (g/ml) of final working solution
- \( d_s \) = density of 10× PBS
- \( d_p \) = density of Percoll stock

As Percoll stock has a density of 1.130 g/ml and 10× PBS has a density of 1.058 g/ml, the formula can be rewritten to

\[
V_p = V \frac{(d - 1.0058)}{0.130}
\]

The pH of the Percoll media was adjusted to 7.4 before use. Discontinuous three-layer gradient columns were built in 50 ml Falcon tubes (Becton Dickinson, NJ, USA). Each tube was first filled with nine ml of the heaviest Percoll solution with a density of 1.080 g/ml (57% Percoll). Nine ml of media with densities of 1.060 g/ml (42% Percoll) and 1.030 g/ml (19% Percoll) respectively, were carefully layered on top of each other using a peristaltic pump (Varioperpex; LKB-produkter, Sweden). The tubes were kept at
4°C until use. The density gradient columns were calibrated using density marker beads (Sigma Chemical Co.).

**ISOLATION OF BOVINE BONE MARROW**

Bone marrow cells were gently mixed before dilution and further processing. After counting (Coulter Counter Z2; Coulter Corporation, FL, USA), nine ml of marrow cell suspension (IMDM + 10% FBS) containing approximately 50×10⁶ nucleated cells was gently layered on top of the gradient column. Tubes were centrifuged (1000 × g, 20 min, 4°C), resulting in the separation of bovine bone marrow into three fractions, labelled as fraction I, II and III (Fig. 1).

![Diagram](Image)

**Figure 1.** Schematic drawing of the gradient column used for the separation of myeloid cells from bovine bone marrow. The cell suspension is placed upon a discontinuous three-layer gradient column with densities 1.030, 1.060 and 1.080 g/ml. Following centrifugation, three fractions can be recovered: fraction I containing early immature myeloid cells, fraction II containing late immature myeloid cells, and fraction III containing mature myeloid cells.

Fractions I and II were collected by aspiration (3 ml) of the interfaces between light and intermediate Percoll media, and intermediate and heavy media, respectively. Cell fractions were washed twice in 10-ml ice-cold PBS (300 × g, 10 minutes, 4°C) in order to
remove remaining Percoll. Fraction III, the high-density pellet, was obtained by removal of the gradient column. Erythrocytes contaminating the pellet were removed by hypotonic lysis (Hoeben et al., 1997).

The recovery of the density centrifugation, expressed as a percentage, was calculated as the sum of cells in the three different fractions obtained after washing, divided by the initial cell load of the gradient. The variability of the separation method was tested by performing four parallel isolations from one bone marrow pool.

**IDENTIFICATION OF MYELOID POPULATION**

The myeloid region within each fraction was initially defined by flow cytometry (FACScan; Becton Dickinson Immunocytometry Systems, Sunnyvale, CA, USA) using a bovine granulocyte marker. Isolated cells of each fraction were incubated with a saturating amount of a mouse anti-bovine G1 (granulocyte) monoclonal antibody (CH138A; VMRD Inc., Pullman, USA), followed by incubation with a secondary goat anti-mouse IgG (Fab specific) - fluorescein isothiocyanate (FITC) antibody (Sigma Chemical Co.). Fluorescence microscopy (Axiocam2; Zeiss, Germany) was used to confirm the flow cytometric data. Therefore, cells were fixed in 1% paraformaldehyde, incubated with a nuclear dye (Dapi; Molecular Probes Europe BV, Leiden, The Netherlands), and washed twice in PBS prior to cytocentrifugation (Shandon, Runcorn, United Kingdom).

In a second step, cytograms of the three fractions were evaluated on their typical forward scatter (FSC, proportional to the cell size) and side scatter (SSC, measure of cellular granularity) characteristics. To confirm the optimised positioning of the myeloid gate in a FSC versus SSC cytogram, cells of each fraction were sorted on a FACS Vantage cell sorter (Becton Dickinson Immunocytometry Systems).

The distribution of myeloid precursors in the three fractions was assessed by differential counting of 100 myeloid cells. Morphological identification of bovine bone marrow cells was carried out according to Wilde (1964), at a magnification of ×630. Cell suspensions were adjusted to a concentration of 1×10^5 ml^{-1} in PBS with 10% FBS and subsequently cytocentrifuged onto FBS-coated glass slides. The slides were air dried at room temperature, fixed in methanol and stained with azur-eosine-methylene blue (Sangodiff® G, Merck Diagnostica, Darmstadt, Germany).
Viability of the myeloid cells obtained after density centrifugation was measured in the flow cytometer by propidium iodide exclusion (Hoeben et al., 1997).

RESULTS

Following centrifugation of bovine bone marrow through an optimised three-layer gradient column, four density barriers were created (Fig. 1). The first layer, i.e. between the bone marrow cell suspension and the lightest Percoll medium (1.030 g/ml), mainly consisted of lipids and cellular debris. This interface was omitted, whereas three remaining cellular bands were selectively retrieved: fraction I, the interface cell fraction between light (1.030 g/ml) and intermediate (1.060 g/ml) Percoll media; fraction II, the interface cell fraction between intermediate and heavy (1.080 g/ml) Percoll media; and fraction III, the high-density pellet (> 1.080 g/ml). The recovery of the density gradient centrifugation ranged between 25.8% and 36.3%, with a variability of 3.50% (n = 4).

After labelling with the bovine granulocyte marker (G1 mAb - CH138A), green fluorescence was detected with the flow cytometer in a well-defined region for each fraction. Fluorescence microscopy confirmed that the FITC-positive cells in each fraction belonged to the myeloid lineage. In a second step, it was possible to further define the myeloid population of each fraction in a scatter cytogram plotting FSC versus SSC (Fig. 2). The mean percentage of cells gated within each cellular fraction was 27 ± 4.9% in fraction I, 56 ± 2.9% in fraction II and 89 ± 1.8% in fraction III (n = 8). Gated cells were sorted, and light microscopy of the resulting cell suspensions revealed the composition for each fraction (Fig. 2). Fraction I contained the early immature myeloid cells, primarily myeloblasts and promyelocytes; fraction II contained the late immature myeloid cells, primarily myelocytes and metamyelocytes, and fraction III contained the most mature myeloid cells, primarily band cells and segmented granulocytes. A minimal overlap between the three myeloid maturation-related stages was observed (Table 1).

Viability of the myeloid cells was 87 ± 1.6% in fraction I, 96 ± 0.80% in fraction II and 98 ± 0.3% in fraction III (n = 8).
Figure 2. Flow cytometric analysis of the three fractions (fraction I, II, and III) obtained after density gradient centrifugation of bovine bone marrow cells: forward scatter (FSC) versus side scatter (SSC) dot plot, and light microscopy of the gated cells obtained after sorting of the corresponding fractions (original magnification ×630).
Table 1. The distribution of gated myeloid precursors in the three fractions of density gradient separated bovine bone marrow cells. The numbers refer to the mean percentage (± standard error of the mean) of the different maturation stages in each fraction, based on a differential count of 100 myeloid precursors from representative gradient separations of eight cows.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Early immature (%)</th>
<th>Late immature (%)</th>
<th>Mature (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>82 ± 1.9</td>
<td>12 ± 1.9</td>
<td>7.0 ± 2.8</td>
</tr>
<tr>
<td>II</td>
<td>5.0 ± 1.3</td>
<td>86 ± 3.4</td>
<td>9.0 ± 2.9</td>
</tr>
<tr>
<td>III</td>
<td>0 ± 0.4</td>
<td>6.0 ± 2.1</td>
<td>94 ± 2.2</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Bone marrow contains an infinite number of cells, belonging to specific lineages in different stages of development. Therefore, labelling with a selective myeloid marker, or physical separation to purify and enrich myeloid subpopulations are the only valid alternatives to allow unequivocal differentiation. In contrast to human research, specific anti-bovine antibodies against cell-surface antigens restricted to hematopoietic progenitor cells are not commercially available. The study of bovine hematopoiesis is strongly hampered by this deficit. Since monoclonal antibody production is expensive and laborious, screening of well-characterised antibodies for serological cross-reactions is a useful strategy. However, the anti-human CD34 detecting progenitor cell populations in human bone marrow does not cross-react with bovine cells (Sopp and Howard, 1997). Therefore, a physical separation technique is required to isolate myeloid precursors from bovine bone marrow into subpopulations according to their maturity.

Previously, isolation of bovine progenitor cells has only been performed on Ficoll-Paque, a density medium made of synthetic polymer of sucrose with a specific gravity of 1.077 g/ml (Fritsch et al., 1991; Van Merris et al., 2001a). The resulting interface cell population was characterised by a considerable degree of heterogeneity of the progenitor cells. As this one-step density separation technique is limited in efficiency, the use of a complex panel of monoclonal antibodies against leukocyte cell surface differentiation
Chapter 2

antigens has been proposed to further enrich bovine progenitor cells prior to in vitro culture (Fritsch et al., 1991; Muiya et al., 1993). However, from a practical point of view, this option is too expensive and can not be carried out on a routine basis for assessing additional antigen expression and functional properties.

Percoll has successfully been used as a gradient forming medium with adapted densities for isolation of eosinophils and neutrophils from bovine blood (Riding and Willadsen, 1981; Chambers et al., 1983). Moreover, separation of human bone marrow cells has previously been performed on Percoll (Olofsson et al., 1980; Cowland and Borregaard, 1999). Density gradient centrifugation was introduced by Brakke in 1951, isolating potato yellow-dwarf virus on a sucrose solution. The density gradient type used in this study is classified as isopycnic gradient centrifugation, and was first described by Anderson (1955). The gradient column encompasses the entire density range of the cells, and sedimentation must be continued until the cells reach positions at which the density of the surrounding medium is equal to their own (Pretlow and Pretlow, 1989). Thus, low-density cells appear near the top of the column, and the higher density cells closer to the bottom. With this technique, the separation is solely governed by the physical properties of cells, i.e. diameter and density. In a centrifugal field the sedimentation of cells is described by the most general form of the Svedberg equation

\[ \nu = \frac{dx}{dt} = \frac{\phi (\rho_c - \rho_m)}{f} \omega^2 x \]  

where  
\( \nu \) = radial velocity of a sedimenting cell  
\( \phi \) = volume of the cell  
\( \rho_c \) = density of the cell  
\( \rho_m \) = density of the medium  
\( f \) = frictional coefficient  
\( \omega \) = angular velocity (speed of centrifugation)  
\( x \) = radial distance from the axis of rotation
According to Stokes’ law, the frictional coefficient \( f \) of a spherical particle (e.g. a cell) is given by \( f = 6 \pi \eta r \) 
\[ \ldots \quad (2) \]
in which \( \eta \) = viscosity of the medium
\( r \) = radius of the cell

The volume of the cell can be calculated as
\[ \phi = \frac{4}{3} \pi r^3 \] 
\[ \ldots \quad (3) \]

Using equations (2) and (3), the Svedberg equation (1) may be rewritten to:
\[ \nu = \frac{\phi (\rho_c - \rho_m)}{6 \pi \eta r} \quad \omega^2 x = \frac{2 r^2 (\rho_c - \rho_m)}{9 \eta} \quad \omega^2 x \quad \ldots \quad (4) \]

From a superficial examination of this equation (4), it is already apparent that the term that represents the size of the cell is squared and therefore relatively important. Diameter has the greatest influence on the sedimentation of the cell until the cell approaches its density in the gradient and \( \rho_c - \rho_m \) becomes small (de Duve et al., 1959).

In the present study the gradient conditions for fractionation of bovine bone marrow were optimised, yielding three highly enriched and partially purified maturation-related myeloid fractions. The optimisation was performed starting from densities routinely used for blood cell isolation in cows (Heyneman et al., 1990). Early immature myeloid cells were found in greatest number at a density of 1.060 g/ml, whereas late immature myeloid cells were selectively retrieved at 1.080 g/ml. Band and segmented cells accumulated in the high-density pellet (> 1.080 g/ml). A minimal overlap between the three myeloid maturation-related stages was observed. This contamination within a fraction with cells of a different maturation stage can be attributed to the heterogeneity in the density of the different precursors (Cowland and Borregaard, 1999). The overlap should be taken into consideration if very pure maturation stages are required for subsequent experiments.

The densities of the three bovine myeloid maturation-related stages observed in the present study are in the range of the densities of most mammalian cells, i.e. between 1.055 and 1.110 g/ml. Furthermore, the larger hematopoietic cells exhibit lower densities than the corresponding mature types of cells (Pretlow and Pretlow, 1989). Our findings are in
agreement with Fritsch et al. (1991) stating that 98% of bovine progenitor cells have a relative density less than 1.077 g/ml. Chambers et al. (1983) isolated mature bovine blood PMN at a specific density of 1.092 g/ml. The density distribution profile of bovine myeloid bone marrow cells revealed some similarities to the distribution of human cells (Olofsson et al., 1980; Cowland and Borregaard, 1999). The progressive density pattern of immature to mature cellular differentiation occurs in bovine as in feline species (Stiff et al., 1986). Although bovine and feline myeloblasts and promyelocytes, and band and segmented cells are respectively found at the same density, feline myelocytes and metamyelocytes are less dense (1.060 - 1.068 g/ml) than the late immature myeloid cells from cows, accumulating at 1.080 g/ml.

The myeloid region in the three different fractions of bovine bone marrow was successfully determined by flow cytometry using a granulocyte marker (G1 mAb - CH138A). This monoclonal antibody was found to react with antigens expressed on granulocytes isolated from blood of ruminants (Davis et al., 1987). To our knowledge, the present study describes at first the use of the G1 granulocyte marker in bovine bone marrow.

Viability of the myeloid cells after fractionation was > 95% in fraction II and fraction III. The slightly lower viability of myeloid cells from fraction I could be attributed to the presence of apoptotic cells, becoming less dense when loosing their DNA (Shetty et al., 2000). Bone marrow cells belonging to all stages of maturation, ranging from immature blasts to terminally differentiated forms, have been found to be apoptotic. The importance of apoptosis in the bone marrow is maintaining stable populations and regulating hematopoietic stem cell numbers (Domen, 2001).
CONCLUSION

This study describes an unequivocal and reproducible separation method for fractionation of bovine bone marrow into three maturation-related myeloid fractions. The physical separation was performed by means of an optimised three-density layer discontinuous gradient column. Pretlow and Pretlow (1989) have enumerated the relative advantages of Percoll: a non-toxic medium, iso-osmotic throughout the gradient and adjustable to physiological ionic strength and pH. Furthermore, the ease of handling and the reproducibility of separation profiles were appreciated.

Fractionation of bovine bone marrow cells on an optimised Percoll gradient column resulted in three purified and enriched maturation-related fractions containing viable myeloid cells. The myeloid population in the resulting fractions can readily be identified by flow cytometry, based on FSC/SSC cytograms, without laborious and expensive labelling. This method allows further studies on antigen expression and functional development during bovine granulopoiesis.
REFERENCES


Separation of bone marrow cells


CHAPTER 3

QUANTIFICATION OF ENDOGENOUS RETINOIDS IN BOVINE SERUM

Adapted from

ABSTRACT

A high-performance liquid chromatographic method with diode-array detection (HPLC-DAD) was developed for the simultaneous quantification of endogenous retinoids in bovine serum. The described technique was initially based on an extraction procedure and reversed phase HPLC method designed for human serum, optimised and validated for bovine serum. Following denaturation of 2 ml samples, a one-step liquid-liquid extraction was performed. Analytes were separated on a Symmetry C\textsubscript{18} column in a total run time of 60 minutes. The retinoids were monitored at an optimal wavelength of 350 nm as chosen from the retinoid spectra. Retinal was added as internal standard because of its intermediate polarity. Peak-height and peak-area ratios were linear over a concentration range from 1 to 10 ng/ml for all-trans-, 9-cis-, 13-cis-retinoic acid, 100 to 600 ng/ml for retinol and 10 to 100 ng/ml for retinyl palmitate. Coefficients of variation were acceptable for both within-day and day-to-day assays. To allow the simultaneous analysis of the endogenous retinoids with a wide polarity range, recoveries were compromised. Nevertheless they systematically exceeded 60%. The proposed method is simple, and enables fast and accurate routine quantification of acid, alcoholic and esteric forms of retinoids.
INTRODUCTION

Retinoids in the diet are taken up as provitamin carotenoids, that are exclusively synthesised by photosynthetic micro-organisms and by plants, or as preformed retinyl esters from animal sources. The skeleton of natural retinoids is made up of a non-aromatic six-carbon ring structure with a polyprenoid side chain which is terminated with a carbon-oxygen functional group. Following uptake, the cleavage of carotenoids to retinal and reduction to retinol are the first steps of a complex metabolic process. The storage forms of retinol in the liver and adipose tissue are the lipophilic retinyl esters, mainly consisting of retinyl palmitate. Other important metabolites of retinol are the oxidative intermediate retinal, and the further oxidation product retinoic acid, the carboxylic acid form of retinol (Norum and Blomhoff, 1992). Retinoic acid is biologically active at nanomolar concentrations in many tissues. Isomerisation of retinoic acid results in an array of active acid metabolites such as all-trans-, 9-cis-, 13-cis-retinoic acid.

Many HPLC methods for the quantification of retinoids in human serum or plasma have been reported (De Leenheer and Nelis, 1990; Wyss, 1995). However, most of these methods cover only a limited polarity range of retinoids. Protocols suitable for the analysis of polar retinoic acid metabolites cannot simultaneously detect the apolar retinyl esters (Meyer et al., 1994; Lanvers et al., 1996; Teerlink et al., 1997; Miyagi et al., 2001), whereas methods that succeed to separate the less polar retinol from the retinyl esters, fail to separate the more polar retinoic acid metabolites (Hartmann et al., 2001). Papers that describe the simultaneous assay of a wide polarity-range in retinoids, without having to compromise on aspects such as analysis time or recovery are scarce (Barua and Olson, 1998; Barua, 2001).

To the best of our knowledge, no simultaneous quantification of endogenous retinoids in bovine serum or plasma has been described. The few research groups that studied endogenous retinoid levels in bovine species used separate HPLC methods to detect either retinol (Johnston and Chew, 1984) or retinoic acid isomers (Horst et al., 1995). Our aim was to develop a simple method for the simultaneous determination of important natural retinoids in bovine serum. Although they share the same molecular skeleton, retinoids vary considerably in their physical and chemical properties. The
polarity and thereby the solubility of various retinoids range from very soluble to insoluble in polar solvents like water and vice versa in apolar solvents like hexane (Gundersen and Blomhoff, 2001). Therefore, the development of a simultaneous determination method for retinoids always is an analytical challenge.

**MATERIALS AND METHODS**

**SOLVENTS, REAGENTS, AND WORKING CONDITIONS**

The extraction and mobile phase solvents acetonitrile, acetic acid, dichloromethane, \( n \)-hexane, methanol, 2-propanol, and water were HPLC-grade (LiChrosolv® quality). Ammonium acetate was of pro analysis quality. All reagents were obtained from Merck (Darmstadt, Germany).

As a precaution to avoid photoisomerisation, all standard and sample manipulations were carried out in amberised tubes and under dim yellow light.

**SERUM SAMPLES**

Blood samples were aseptically taken by venipuncture in the Vena Jugularis Externa of clinically healthy non-lactating cows. Plain glass tubes (BD Vacutainer Systems, UK) were protected from light, and allowed to clot for 30 min at 37°C. Serum was obtained following centrifugation (1000 \( \times \) g, 20 min, 4°C), transferred into amberised glass vials and stored at -20°C until analysis.

**STANDARD SOLUTIONS**

The standards all-trans-retinol, all-trans-retinal and retinyl palmitate were purchased from Sigma Chemical Co. (St.-Louis, MO, USA). All-trans-, 9-cis-, and 13-cis-retinoic acid, all-trans-acitretin, isopropyl-acitretin and retinyl acetate were generously provided by Hoffmann-La Roche (Basel, Switzerland). Each retinoid standard was dissolved in methanol to produce stock solutions of 100 ng/µl. Working solutions of 0.1, 1, and 10 ng/µl were prepared by serial dilutions of stock solutions with methanol. Following flushing with nitrogen, stock and working solutions were stored at -20°C. Spiked serum
samples for validation were prepared using a serum pool from 10 clinically healthy non-lactating cows.

**EXTRACTION PROCEDURE**

Extraction of retinoids from bovine serum involved protein precipitation, followed by a one-step liquid-liquid extraction. The final protocol was optimised for bovine serum based on the extraction of retinoids described for human plasma by Meyer *et al.* (1994). In a first step, protein denaturation of 2 ml samples was performed with 2.1 ml of acetonitrile following acidification with 100 µl of 2 N acetic acid. After 2 min of vortex mixing, 1.0 ml of water and 7.5 ml of an extraction mixture consisting of *n*-hexane and 2-propanol (6.5:1.5, v/v) were added. The sample was extracted by vortex mixing for 2 min. After centrifugation (1000 × g, 10 min, 4°C) the organic layer was removed and evaporated in a solvent evaporator at 15°C (Speedvac SVC200; Savant, Holbrook, United States) connected to a refrigerated vapor trap RVT400 (Savant). The residue was dissolved in 200 µl of mobile phase solvent A by vortex mixing. Following centrifugation (300 × g, 3 min, 4°C), the supernatant was transferred into an autosampler vial and a volume of 150 µl was automatically injected in the HPLC-system.

**CHROMATOGRAPHIC SET-UP, MOBILE PHASE AND GRADIENT PROGRAM**

The HPLC system consisted of an intelligent gradient pump L-6200A, an autosampler AS-2000A, a diode array detector (DAD) type L-4500, and an Interface D-6000 all from Merck/Hitachi (Darmstadt, Germany). The 3.0 mm i.d. × 150 mm analytical column was filled with an dimethyloctadecylsilyl bonded amorphous silica stationary phase (Symmetry C18, 5 µm particle size; Waters Inc., Massachusetts, USA).

The mobile phase and gradient conditions were based on the method of Barua and Olson (1998), and further optimised for serum of the bovine species. The solvent system consisted of methanol-water (3:1, v/v) containing 10 mM ammonium acetate and 0.025% acetic acid [solvent A] and of a methanol-dichloromethane (4:1, v/v) mixture [solvent B]. A linear gradient from 100% solvent A to 100% solvent B was applied over a period of 30 min, followed by isocratic elution with solvent B for an additional 15 min (Table 1). Between each run, the analytical column was equilibrated with 100% solvent A for 15 min.
The total run time covered 60 min. The flow rate was set at 0.4 ml min\(^{-1}\) and the detection wavelength between 320 nm and 380 nm (from 18 to 40 minutes). Monitoring wavelength of the DAD was set at 350 nm.

**Table 1. Composition of the mobile phase**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>16.0</td>
<td>47</td>
<td>53</td>
</tr>
<tr>
<td>30.0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>45.0</td>
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</tr>
<tr>
<td>45.1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>60.0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

**METHOD VALIDATION**

Method validation was performed using the following parameters: variability in retention time, linearity range and calibration curves, selectivity and specificity, precision, limit of quantification (signal/noise = 5/1), and recovery.

Linearity was calculated by linear least-squares regression analysis plotting peak-height or peak-area analyte to internal standard ratios against the concentration of each analyte. Serum was spiked with 0, 100, 150, 300, 450, and 600 ng/ml for retinol; 0, 1, 2.5, 5, 7.5, and 10 ng/ml for the retinoic acid isomers; 0, 10, 25, 50, 75, and 100 ng/ml for retinyl palmitate, and 50 ng/ml for the internal standard. Additional analyses of linearity were performed on 10 consecutive days spiking serum from the same pool with 0, 1, 5 and 10 ng/ml for the three retinoic acids; 0, 100, 300 and 600 ng/ml for retinol; 0, 10, 50, and 100 ng/ml for retinyl palmitate and 50 ng/ml for the internal standard.

To test for reproducibility (precision), a pool of bovine serum was divided into three pools and spiked with each of the retinoid standards at three different concentration levels (low, medium, high). The added concentrations for the low, medium and high concentration level were 1, 5 and 10 ng/ml for the retinoic acid isomers; 100, 300 and 600 ng/ml for retinol; and 10, 50 and 100 ng/ml for retinyl palmitate, respectively. The amount of internal standard added was kept constant for all samples. For the within-day
Quantification of endogenous retinoids

reproducibility ten samples of each concentration level were analysed on one day, while the day-to-day reproducibility was tested for each of the three concentration levels on ten different days covering a two-month period.

The recovery of each retinoid was determined at three concentration levels (low, medium, and high) and for at least 7 samples. The signal of serum extracts to which increasing amounts of each retinoid were added, was compared to the signal of the corresponding standards injected directly into the HPLC system. Endogenous retinoid levels from blank serum were subtracted.

RESULTS AND DISCUSSION

1. OPTIMISATION OF THE EXTRACTION PROTOCOL

1.1. SERUM VERSUS PLASMA

Because problems with unidentified interferences typical for bovine plasma were initially observed, serum was the preferred matrix for further experiments. The choice of serum over plasma was also encouraged by Aebischer et al. (1999). These authors stated that although no statistical differences existed for vitamins and carotenoids, plasma levels for all analytes were slightly, but systematically lower than the corresponding serum levels.

1.2. SAMPLE VOLUME AND pH

In analogy to its human counterpart, naturally occurring retinoic acid isomers are present at low nanomolar concentrations in bovine serum (Horst et al., 1995). In order to determine trace amounts of analytes in complex matrices, a major enrichment is required. However, liquid-liquid extraction of retinoic acid from biological fluids such as serum is hampered by the fact that retinoic acid, in contrast to most other retinoids that are water-insoluble, is rather water-soluble under physiological pH conditions (Szuts and Harosi, 1991). Retinoic acid will therefore not be extracted optimally when water-immiscible solvents such as \( n \)-hexane are used without adaptation of the pH. The extraction efficiency of acid retinoids can indeed be significantly improved by prior acidification of the water-
phase. Acetic acid or an acetate buffer, and hydrochloric acid are the most commonly described acidifying agents in literature (Meyer et al., 1994; Teerlink et al., 1997; Barua and Olson, 1998; Miyagi et al., 2001). In our protocol a pH of 3 in the aqueous phase was obtained by acidification with acetic acid. A second possibility to improve the detection of trace amounts of analytes, is the use of large sample volumes. Because an increase in sample volume would be inevitably accompanied by an increase in interferences of the extract, a maximum sample volume of 2 ml was used in our final protocol.

1.3. Denaturation Conditions

Due to their lipophilic character, retinoids exhibit strong protein-binding to specific or aspecific serum protein carriers like retinol-binding protein (retinol carrier) and albumin (retinoic acid carrier), respectively. Bound analytes were released by protein denaturation with acetonitrile in combination with acetic acid. Acetonitrile was preferred over other organic solvents such as the most commonly used alcohols (ethanol and methanol), because it resulted in better precipitation of the serum matrix. The release of retinyl esters from lipoproteins was also improved. This observation was supported by literature describing the insolubility of most lipid classes in acetonitrile, the precipitation of lipids and their adherence to the glass (Dueker et al., 1993). Teerlink et al. (1997) reported the critical influence of the acetonitrile content for optimal denaturation, and the beneficial effect of simultaneous acidification. In analogy to these authors, an optimum percentage of 50% acetonitrile was withheld for our protocol.

1.4. Liquid-Liquid Extraction

The classical way of extracting retinoids is by addition of a water-immiscible solvent after vigorous shaking, centrifugation and removal of the organic phase (Wyss, 1995). A mixture of n-hexane and 2-propanol (6.5:1.5, v/v) was preferred for an optimal extraction of the retinoids in bovine serum. This is in analogy with the extraction as performed by Rissler et al. (1991) and Meyer et al. (1994).
1.5. **CHOICE OF AN ADEQUATE INTERNAL STANDARD**

We first evaluated the possibility of a validation without the use of an internal standard. Although exceptional, this option has indeed been previously described by Teerlink et al. (1997) for the simultaneous analysis of multiple retinoids in human serum. However, preliminary validation results showed that the use of an internal standard was required to compensate for losses and other sources of variation. An ideal internal standard should be absent from the original sample, chemically and physically similar to the analyte (i.e. be itself a retinoid), be well resolved from all other chromatographic peaks, and should possess similar characteristics for extraction, chromatography and detection as the analytes (Furr et al., 1994).

Four retinoids were evaluated as potential internal standard: the synthetic acid all-trans-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid (i.e. all-trans-acitretin or Ro 10-1670), its corresponding isopropylester (isopropyl-acitretin or Ro 11-0249), retinyl acetate and finally all-trans-retinal (the aldehyde analogue of retinol). In theory, the latter compound was not suited as an internal standard because it can be formed endogenously by metabolism of retinol. Nevertheless, the fact that we still evaluated it can be motivated as follows: the three other candidates were either susceptible to interference with an unidentified serum compound (Ro 10-1670) or did not compensate equally well for the diverse polarity range of analytes (Ro 11-0249), or co-eluted with retinyl palmitate (retinyl acetate). Most importantly, in none of the chromatograms from blank bovine serum sample extracts a signal with the same retention time or spectrum as retinal was detected. Retinal occurs in significant quantities only in ocular tissue (Furr et al., 1994) and is therefore virtually undetectable outside the eye (Napoli, 1999). All criteria for an ideal internal standard were therefore met by retinal. Moreover, no artefactual oxidation of retinal to retinoic acid during sample preparation was observed upon addition of increasing amounts of retinal to a blank serum sample. The only remaining drawback on the use of retinal, was that it did not compensate equally well for retinyl palmitate as for the other analytes.
2. OPTIMISATION OF CHROMATOGRAPHIC CONDITIONS

2.1. MOBILE PHASE, pH, AND GRADIENT PROGRAM

As mentioned above, the extraction of acid retinoids was significantly improved by acidification of the aqueous phase. The change in pH shifted the carboxylic acid functional group to its non-ionised form. In analogy, acidification of the mobile phase was performed during the subsequent chromatographic step. It allowed homogenous interaction of all non-ionised retinoic acid molecules with the reversed phase stationary phase. Only a very small percentage of acid was added because an acid pH of the mobile phase degraded the acid-sensitive silica phase. However this minimal amount of acetic acid in combination with a low molarity of ammonium acetate avoided tailing of the acid retinoids and masked the presence of residual silanol functions (Barua and Olson, 1998).

The multi-step gradient program was optimised to allow optimal resolution of all analytes in an acceptable run time of about 30 min. An additional 15 min with 100% of solvent B was necessary to elute potentially interfering lipophilic compounds from the column, while the last 15 min with 100% solvent A were required to re-equilibrate the system.

2.2. SELECTION OF MONITORING WAVELENGTH

Because of their conjugated polyene chains, retinoids absorb light maximally at high-ultraviolet wavelengths ranging from 325 to 370 nm. This wavelength range is specific for retinoids (Wyss, 1995), as few other natural compounds absorb light appreciably within this range (Dueker et al., 1993). The choice of a DAD provided the possibility of both quantitative detection and qualitative identification, comparing the spectra of chromatographic peaks to those of reference retinoid standard spectra. The detection of the different retinoid analogues required a compromise for the monitoring wavelength. From the individual standard retinoid spectra it was determined that a maximal sensitivity of the trace amounts of retinoic acids was obtained at 350 nm. In comparison, the same wavelength was used by Meyer et al. (1994) and Miyagi et al. (2001), while Teerlink et al. (1997) and Barua and Olson (1998) detected retinoids in human plasma at 340 nm.
A representative chromatogram obtained from a bovine serum extract using the optimised chromatographic conditions is shown in Figure 1.

Figure 1. Representative chromatogram of bovine serum spiked with retinoids (I.S., internal standard).

3. METHOD VALIDATION

3.1. SELECTIVITY AND SPECIFICITY

No interfering peaks with a similar retention time but a different ultraviolet spectrum as the analytes were observed in the chromatograms of the extracted serum samples. This indicates that the extraction and the chromatographic conditions ensured persistently clean chromatograms. The use of an extra 15 min run time in the gradient program successfully prevented the occurrence of ghost peaks.
3.2. RETENTION TIME

The variability in retention time was found to be minimal both within-day and between-day, with coefficients of variation for all retinoids lower than 1 and 2%, respectively (Table 2).

Table 2. Reproducibility of retention times determined on a given day (n = 10).

<table>
<thead>
<tr>
<th>Retinoid</th>
<th>mean (s.d.)</th>
<th>c.v.</th>
</tr>
</thead>
<tbody>
<tr>
<td>13-cis-retinoic acid</td>
<td>21.6 (0.21)</td>
<td>0.95</td>
</tr>
<tr>
<td>9-cis-retinoic acid</td>
<td>22.7 (0.21)</td>
<td>0.90</td>
</tr>
<tr>
<td>all-trans-retinoic acid</td>
<td>23.3 (0.20)</td>
<td>0.86</td>
</tr>
<tr>
<td>retinol</td>
<td>24.7 (0.15)</td>
<td>0.59</td>
</tr>
<tr>
<td>internal standard</td>
<td>25.7 (0.16)</td>
<td>0.60</td>
</tr>
<tr>
<td>retinyl palmitate</td>
<td>30.1 (0.14)</td>
<td>0.46</td>
</tr>
</tbody>
</table>

3.3. LINEARITY RANGE AND CALIBRATION CURVES

In all cases, with the exception of retinyl palmitate, an acceptable correlation coefficient was obtained (Table 3). These r-values on average exceeded 0.992 for retinol, 0.999 for all-trans-, 0.994 for 9-cis-, and 0.999 for 13-cis-retinoic acid. An r-value of 0.961 was obtained for retinyl palmitate.

3.4. LIMIT OF QUANTIFICATION

Following limits of quantification were obtained: 86 ng/ml for retinol, 1 ng/ml for all-trans-, 3 ng/ml for 9-cis-, 1 ng/ml for 13-cis-retinoic acid, and 17 ng/ml for retinyl palmitate, respectively (Table 3). These data indicate that the analysis method allows the quantification of endogenous retinoid levels. The reference values found in the literature for the bovine range from 1 to 5 ng/ml for retinoic acid and from 150 to 600 ng/ml for retinol (Goff and Stabel, 1990; Horst et al., 1995). No comparable values are available for retinyl palmitate in bovine serum.
Table 3. The r-value (as a measure for linearity) with the number of analyses (n) performed, the limit of quantification (LOQ) in ng/ml, and the recovery expressed as a percentage, for the different retinoids.

<table>
<thead>
<tr>
<th>Retinoid</th>
<th>Linearity r-value (n)</th>
<th>LOQ (ng/ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>retinol</td>
<td>&gt; 0.992 (9)</td>
<td>86</td>
<td>57</td>
</tr>
<tr>
<td>all-trans-retinoic acid</td>
<td>&gt; 0.999 (11)</td>
<td>1</td>
<td>64</td>
</tr>
<tr>
<td>9-cis-retinoic acid</td>
<td>&gt; 0.994 (6)</td>
<td>3</td>
<td>73</td>
</tr>
<tr>
<td>13-cis-retinoic acid</td>
<td>&gt; 0.999 (10)</td>
<td>1</td>
<td>75</td>
</tr>
<tr>
<td>retinyl palmitate</td>
<td>&gt; 0.961 (9)</td>
<td>17</td>
<td>61</td>
</tr>
</tbody>
</table>

3.5. Recovery

Recovery was on average 57% for retinol; 64%, 73% and 75% for all-trans-, 9-cis-, 13-cis-retinoic acid respectively, and 61% for retinyl palmitate (Table 3). Other authors report higher values but did not cover the wide polarity range of retinoids reported here, with the exception of Barua and Olson (1998). This current procedure, set-up for the simultaneous quantification of retinoids, is a compromise but yields in acceptable recovery values for each analyte.

3.6. Precision

The coefficients of variation (c.v. values), as determined from the height ratios, were satisfactory for all retinoids, even for the retinoic acid isomers which were present at trace amount levels only (Table 4). In general, the c.v. values were lower than 10% for the within-day, and lower than 15% for the day-to-day measurements. An exception was the c.v. value at low concentration level of retinyl palmitate, which exceeded 15% both within-day and day-to-day.
Table 4. Within-day (A) and day-to-day (B) reproducibility, expressed as coefficients of variation values expressed as a percentage, n = 10 for all retinoids, except for retinyl palmitate n = 8).

<table>
<thead>
<tr>
<th>Retinoid</th>
<th>low</th>
<th>medium</th>
<th>high</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>retinol</td>
<td>6.28</td>
<td>5.72</td>
<td>5.19</td>
</tr>
<tr>
<td>all-trans-retinoic acid</td>
<td>11.05</td>
<td>7.87</td>
<td>6.08</td>
</tr>
<tr>
<td>9-cis-retinoic acid</td>
<td>4.49</td>
<td>8.23</td>
<td>5.84</td>
</tr>
<tr>
<td>13-cis-retinoic acid</td>
<td>8.33</td>
<td>7.98</td>
<td>4.69</td>
</tr>
<tr>
<td>retinyl palmitate</td>
<td>20.73</td>
<td>17.08</td>
<td>15.45</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Retinoid</th>
<th>low</th>
<th>medium</th>
<th>high</th>
</tr>
</thead>
<tbody>
<tr>
<td>(B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>retinol</td>
<td>13.53</td>
<td>14.22</td>
<td>10.77</td>
</tr>
<tr>
<td>all-trans-retinoic acid</td>
<td>11.56</td>
<td>14.03</td>
<td>10.03</td>
</tr>
<tr>
<td>9-cis-retinoic acid</td>
<td>13.19</td>
<td>16.39</td>
<td>11.10</td>
</tr>
<tr>
<td>13-cis-retinoic acid</td>
<td>10.37</td>
<td>15.82</td>
<td>9.41</td>
</tr>
</tbody>
</table>
CONCLUSION

Our aim was to develop a simple identification and quantification method with low detection limits for the single-run analysis of the principal endogenous retinoids, i.e. retinol, three retinoic acid metabolites (all-trans-, 9-cis-, 13-cis-retinoic acid), and its predominant retinyl ester metabolite (retinyl palmitate) in bovine serum. The HPLC-DAD procedure described above provides a new useful tool for routine monitoring of changes in endogenous retinoid levels. A few more efficient methods have been previously described for the analysis of individual retinoids or a single class of retinoids in bovine serum (retinoic acid by Horst et al., 1995; retinol and β-carotene by Johnston and Chew, 1984). Nevertheless, this is the first report on the fast and accurate screening of a wide polarity-range of endogenous retinoids in bovine serum.
REFERENCES


PART 2

EXPERIMENTAL STUDIES
CHAPTER 4
Functional maturation during bovine granulopoiesis

CHAPTER 5
Vitamin A profile during the peripartum period and experimentally induced *Escherichia coli* mastitis

CHAPTER 6
Differential effects of steroids and retinoids on bovine myelopoiesis *in vitro*
CHAPTER 4

FUNCTIONAL MATURATION DURING BOVINE GRANULOPOIESIS

Adapted from


Granulocytic precursor cells undergo morphological changes in the nucleus and the cytoplasm during the process of granulopoiesis, taking place in the bone marrow. These changes are associated with the development of stage-specific proteins necessary for the highly specialised roles of polymorphonuclear leukocytes in phagocytosis, bacterial killing, and in mediating the inflammatory process. The objective of the current study was to screen the various events that occur upon functional development of granulocytic bone marrow cells in the bovine species.

Cells were obtained from the bone marrow of clinically healthy cows and separated into different stages of maturation using density gradient centrifugation. Three cellular fractions were obtained that were enriched for either early immature, late immature or mature granulocytic cells. Functions and receptor expressions assessed in the three maturation stages were: Fc-IgG2 receptor, CD62L (L-selectin), and CD11b expression; chemotaxis, phagocytosis of *Escherichia coli*, respiratory burst activity, and cellular myeloperoxidase activity. Immature cells expressed already Fc-IgG2 receptor, CD62L, and CD11b on their cytoplasmic membrane. A chemotactic response towards zymosan-activated serum was detected as early as the (meta)myelocytic stage. Phagocytic ability was acquired in the myelocytic stage, but only the more mature forms were readily capable of phagocytosis. Promyelocytes, myelocytes and metamyelocytes showed no respiratory burst activity. Only band and segmented cells produced reactive oxygen species. Myeloperoxidase was present at all stages of maturity. Thus, each of the maturation stages was characterised by a selective expression of one or more functions and receptors. Therefore, sequential biochemical maturation is postulated during bovine granulopoiesis.
INTRODUCTION

It is well known that the polymorphonuclear leukocytes (PMN) constitute the most important non-specific defence mechanism when pathogens invade the bovine udder (Burvenich et al., 1994). In infected glands, the percentage of neutrophils in milk approaches 100%. The increased susceptibility to mastitis in high-yielding cows during early lactation is associated with decreased neutrophil function. Kehrli et al. (1989) hypothesised that an immunosuppression may occur during the periparturient period and therefore predisposes the dairy cow to new infections. Several of the altered neutrophil functions were found to be related to the severity of experimentally induced Escherichia coli mastitis during early lactation (Heyneman et al., 1990; Lohuis et al., 1990; Kremer et al., 1993; Van Werven et al., 1997). The number of PMN prior to infection was also decreased.

The number of circulating PMN is the result of influx and efflux of cells. The influx of cells is related to the release from the bone marrow. The efflux depends on the migration of the cells towards the marginated pool and towards tissues. Neutrophils do not recirculate. There is a unidirectional movement from the bone marrow into the blood, and from circulation towards the tissues. A well-controlled equilibrium exists between the circulating and the marginated pool in the blood (Fig. 1). Upon demand, a physiological mechanism appears for the rapid release of mature granulocytes into the circulation and accelerates the maturation of cells in the bone marrow (Jain, 1993; Moreira da Silva et al., 1994). When maturation is impaired, e.g. due to the increased proliferation rate, a higher number of immature cells will appear in circulation. This phenomenon occurs in periods of stress, such as parturition and mastitis.

Myeloid precursors undergo striking morphological and functional changes during the process of granulocytic maturation. These changes are associated with significant changes in cell size and nuclear shape, and with the development of stage-specific proteins necessary for phagocytosis, and bacterial killing, and in mediating the inflammatory response (Berliner, 1998). In the human, various biochemical and functional properties of PMN are acquired in a well-controlled stepwise manner during granulopoiesis (Glasser and Fiederlein, 1987). The sequence for the functional differentiation of the human neutrophil
has been proposed to be the following: Fc receptors – immune phagocytosis – complement receptors – oxygen-independent microbial killing – oxygen-dependent microbial killing – chemotaxis. If this phenomenon would also occur in cows, the appearance of myelocytes, metamyelocytes and band cells in circulation during infection could be involved in the impaired defence during parturition “stress”. Guidry and Paape (1976) also postulated that the left-shift observed during mastitis could compromise the cows’ resistance by supplying more cells that are morphologically immature and functionally inadequate.

**Figure 1. Lifecycle of bovine PMN, according to the data of Jain (1993).** An unidirectional progression of proliferation, differentiation and maturation from the stem cell through myeloblast (MB), promyelocyte (PM), myelocyte (M), metamyelocyte (MM), band cell (band) and segmented cell results in the formation of mature PMN. Cells are released into circulation, and transported to the tissues where they exert their function in the non-specific defence.
The objective of the present study was to investigate the maturation sequence of the functional properties of bovine granulocytes during proliferation and maturation in the bone marrow. Therefore, functions and receptors playing a crucial role in udder defence and found to be deficient during periparturient *E. coli* mastitis were studied. The Fc-IgG2 receptor, L-selectin and CD11b expression, the ability of chemotaxis, the occurrence of phagocytosis, the respiratory burst activity, and the cellular myeloperoxidase activity were studied in three fractions of granulocytes with different maturation characteristics.

**MATERIALS AND METHODS**

**BONE MARROW SAMPLES**

Bone marrow samples were collected by sternal aspiration in clinically healthy adult cattle at the slaughterhouse of Ghent University, as described previously (Van Merris *et al.*, 2001b). Briefly, samples were taken within 15 min after killing. Before cleavage of the carcass, approximately 2 ml marrow was aspirated from the third or fourth sternebrae. The first drop of each aspirate was used for a control smear. Bone marrow samples were transferred into sterile tubes containing 5 ml Iscove’s modified Dulbecco’s Medium (IMDM; Gibco BRL, Life Technologies Inc., USA), supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and 100 U/ml lithium heparin (Leo Pharmaceutical Product, Zaventem, Belgium). Samples were kept on ice until analysis.

**DENSITY GRADIENT SEPARATION**

Bone marrow cells were fractionated into different stages of maturation using a gradient column, optimised for the bovine species by Van Merris *et al.* (2001a). Briefly, the marrow suspension was layered on top of a discontinuous three-layer gradient column with increasing densities. Following centrifugation, three cellular bands were selectively retrieved: the early immature (fraction I), the late immature (fraction II), and the mature fraction (fraction III). In each fraction, the viability of the cells was determined through the propidium-iodide (PI; Sigma Chemical Co., Missouri, USA) exclusion test (Hoeben *et al.*, 1997).
FC- IgG2 RECEPTOR EXPRESSION

Fc-IgG2 receptor expression was assessed by flow cytometry through direct labelling with fluorescein isothiocyanate (FITC) conjugated sheep anti-bovine IgG2 antibody (Serotec, Oxford, UK), as described by Worku et al. (1994). Each maturation fraction containing $1 \times 10^6$ cells in 100 µl staining buffer [staining buffer is phosphate buffered saline (PBS) supplemented with 1% FBS and 0.09% (w/v) sodium azide] was incubated with a saturating amount of the antibody for 30 min at 4°C in the dark. Cells were washed twice with 300 µl staining buffer, pelleted by centrifugation ($200 \times g$, 10 min, 4°C) and resuspended in 400 µl PBS. Subsequently, the fluorescence was measured by flow cytometry.

CD62L (L-SELECTIN) EXPRESSION

L-selectin expression was assessed by flow cytometry through an indirect labelling technique (Monfardini et al., 1999). Briefly, each maturation fraction was adjusted to $10 \times 10^6$ cells ml$^{-1}$ and a 100 µl pellet was incubated for 30 min at 37°C with 50 µl of anti-bovine L-selectin mAb 11G10 (Wang et al., 1997). After centrifugation ($200 \times g$, 10 min, 4°C) and washing with PBS, pellets were incubated in the dark with a F(ab')$_2$ goat anti-mouse IgG conjugated to FITC (Sigma Chemical Co.) for 30 min at 4°C. Cells were washed twice with PBS, fixed in 500 µl of 1% paraformaldehyde in PBS and kept in the dark at 4°C until analysis.

CD11b EXPRESSION

CD11b expression was assessed by flow cytometry through an indirect labelling technique. Each maturation fraction was adjusted to $1 \times 10^6$ cells in 100 µl staining buffer. Cells were incubated with a saturating amount of mouse anti-bovine CD11b (Probio Ltd., Kent, England) for 30 min at room temperature. Cells were washed twice with 300 µl staining buffer and pelleted by centrifugation ($200 \times g$, 10 min, 4°C). Pellets were incubated in the dark with a F(ab')$_2$ goat anti-mouse IgG conjugated to FITC (Sigma Chemical Co.) for 30 min at 4°C. Cells were washed twice with staining buffer, and resuspended in 400 µl PBS. This staining technique was previously used on circulating bovine PMN and resulted in the labeling of 95 to 99% of the cells (Dosogne et al., 1997).
For the determination of the total CD11b content, the primary bovine antibody was added both before and after the fixation and permeabilisation procedure (Lundahl et al., 1991), carried out with a Cytofix/Cytoperm fixation kit (PharMingen, San Diego, USA). In this way, both cytoplasmic membrane expression and intracellular CD11b content were measured.

CHEMOTAXIS ASSAY

A bicameral model was built to assess the capability of bone marrow cells to react to chemotactic stimuli. Therefore, inserts (12 mm diameter, 3 μm pore size; Millipore PCF, Millipore, Bedford, Mass.) were coated with collagen using a 0.1% solution of calf skin collagen type I (Sigma Chemical Co.) (Monfardini et al., 2000). Zymosan activated serum was prepared by incubating FBS with 1 mg/ml of zymosan (Sigma Chemical Co.) for 30 min at 37°C. The mixture was cooled, subsequently centrifuged (2000 × g, 10 min, 4°C), and the supernatant was stored at -20°C. Prior to use, the serum was diluted 1:5 with Hanks’ Balanced Salt Solution (HBSS w/o Calcium and Magnesium; Gibco BRL) and filtered through a 0.22 μm filter.

To perform the assay, cells were resuspended to 10×10^6 cells ml^-1, and 200 μl aliquots of each maturation stage were placed in the upper chambers. For the treated group 400 μl zymosan activated serum was added into the lower chambers, while for the control group 400 μl HBSS was used. Loaded bicameral systems were then incubated for 90 min at 37°C in 95% air and 5% CO₂. The migrated cells were counted using a Burker chamber at a magnification of ×400. Results were expressed in percentage of cells migrated through the barrier. Each assay was performed in duplicate.

PHAGOCYTOSIS OF ESCHERICHIA COLI

To test phagocytic capacity, the method as described by Hoeben et al. (1997) was used. In this method, phagocytosis is tested without discrimination between attachment and ingestion of E. coli bacteria. Following isolation, cell suspensions were analysed by flow cytometry in a forward versus side scatter (FSC/SSC) dotplot to assess the percentage of granulocytic cells within each fraction (Van Merris et al., 2001a). The three fractions were adjusted to 1×10^6 granulocytic cells in 100 μl HBSS. In a chronological rank order, PI-
labelled *E. coli* bacteria were added to the samples at a ratio of 1:25 and incubated with 5% autologous plasma in a shaking bath at 37°C. Phagocytosis was stopped after 20 min by addition of 0.1 mM N-ethylmaleimide (Sigma Chemical Co.) in the same chronological order. Samples were further diluted with 400 µl PBS, stored on ice and immediately analysed by flow cytometry. Using this method, both non-immunological (i.e. mediated by complement, hydrophobic properties, or lectins) and immunological (i.e. antibody-dependent) phagocytosis was detected.

**Respiratory burst activity**

The respiratory burst activity was measured as described by Smits *et al.* (1997), using dihydrorhodamine as a substrate. Following isolation, cell suspensions were analysed by flow cytometry in a FSC/SSC dotplot to assess the percentage of granulocytic cells within each fraction (Van Merris *et al.*, 2001a). Subsequently, the three fractions were adjusted to 1×10^6 granulocytic cells in 100 µl HBSS and incubated with 5% autologous plasma and 10 µM dihydrorhodamine 123 (Molecular Probes, Leiden, The Netherlands) in a shaking bath at 37°C for 5 min. Samples were further diluted with 400 µl PBS and the spontaneous reactive oxygen species (ROS) production was measured by flow cytometry. The stimulated respiratory burst activity was determined by adding 10 µl 0.1 µM phorbol 12-myristate 13-acetate (PMA; Sigma Chemicals Co.) during 15 min, following the first incubation.

**Cellular myeloperoxidase activity**

Myeloperoxidase (EC 1.11.1.7) positive granules were microscopically analysed in each maturation fraction by staining the slides with benzidine dihydrochloride (Sigma Chemical Co.) (Metcalf *et al.*, 1986).

**Flow cytometric analysis**

Fluorescence was measured using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, California) equipped with a 488 nm argon-ion laser. Percent fluorescence (% F) and mean fluorescence intensity (MFI) of selectively gated granulocytic cells in each maturation fraction (Van Merris *et al.*, 2001a) were used to
 quantify the receptor expression and the functional activity of isolated bone marrow cells. Percent fluorescence is defined as the percentage of granulocytic cells with a fluorescence higher than autofluorescence, attributed to respectively receptor expression (Fc-IgG₂ receptor, CD62L, and CD11b), engulfment of bacteria (phagocytosis) or ROS production (respiratory burst). MFI was used as an index for the average number of receptors expressed, bacteria ingested, or ROS produced per cell. For CD11b and L-selectin expression, the MFI was corrected by subtraction of background fluorescence obtained using an isotype-matched antibody control.

DIFFERENTIAL COUNTING AND MICROSCOPIC ANALYSIS

The distribution of granulocytic precursors in the three maturation-related fractions was assessed by differential counting of 100 cells in each fraction. Morphological identification of bovine bone marrow cells was carried out according to Wilde (1964). Cell suspensions were adjusted to a concentration of 1×10⁵ ml⁻¹ in PBS with 10% FBS. For light microscopy, 200 µl aliquots were centrifuged onto FBS-coated slides in a cytocentrifuge (Shandon, Runcorn, UK). The slides were air dried at room temperature, fixed in methanol and stained with Hemacolor® (Merck Diagnostica, Darmstadt, Germany).

Fluorescence microscopy (Axiocam2; Zeiss, Germany) was used as a qualitative control for all properties measured with flow cytometry. Cell suspensions were fixed in 1% paraformaldehyde, incubated in the dark with a nuclear dye (DAPI; Molecular Probes) for 20 min and washed twice with PBS prior to cytocentrifugation (700× g, 5 min).

STATISTICAL ANALYSIS

The data of IgG₂, CD11b, phagocytosis and respiratory burst activity were computed using statistical software (Statistix 4.1® NH Analytical Software, USA). The normality of the data was tested using the Wilk-Shapiro/rankit plot. The significance of differences in receptor expression or functional activity of the bone marrow cells in the three maturation stages (fraction I, II, III) was determined by a LSD (t) pairwise comparisons of means. Data were expressed as means ± standard error of the mean.

The different maturation stages were compared for L-selectin expression (MFI and percentage) by a mixed model with cow as random effect. The three stages were compared
pairwise by Tukey’s multiple comparisons method with an overall error rate of 5%. The effect of the maturation stage and the activation with zymosan on the percentage of migrated cells was assessed in a mixed model with cow as random effect and maturation stage, zymosan-activation and the interaction between these two factors as fixed effects. Maturation stages and zymosan-activation were again pairwise compared by Tukey’s multiple comparisons method with an overall error rate of 5%.

**RESULTS**

**GRANULOCYTIC ISOLATION AND VIABILITY**

Density gradient centrifugation of bovine bone marrow suspension yielded three enriched cellular fractions: fraction I (early immature fraction) contained mainly myeloblasts and promyelocytes, fraction II (late immature fraction) consisted of myelocytes and metamyelocytes, and fraction III (mature fraction) contained mainly band and segmented cells. Although fractions were not entirely pure, they proved to be useful to study the development of some PMN functions. The mean viability of cells was found to be 86.4 ± 2.28% in fraction I, 95.3 ± 1.33% in fraction II and 98.1 ± 0.78% in fraction III.

**FC-IGG2 RECEPTOR EXPRESSION**

Fc-IgG2 receptor was first detected in myeloblasts and promyelocytes (22.2 ± 2.58% in fraction I). The percent fluorescence increased strongly upon maturation, with 49.0 ± 4.06% fluorescence in the late immature fraction (fraction II, P < 0.001). Fc-IgG2 receptor reached a maximal expression at the band and segmented stages (73.3 ± 4.24% in fraction III, P < 0.001). Moreover, MFI increased significantly (P < 0.001) with each subsequent maturation stage. Results were obtained on eight cows and are summarised in Table 1.
Table 1. Percent Fc-IgG₂ positive cells (% F) and their mean expression (MFI) in fractions I, II, and III. Data are expressed as mean ± standard error of the mean (n = 8).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>IgG₂ receptor expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% F</td>
</tr>
<tr>
<td>I</td>
<td>22.2 ± 2.58</td>
</tr>
<tr>
<td>II</td>
<td>49.0 ± 4.06</td>
</tr>
<tr>
<td>III</td>
<td>73.3 ± 4.24</td>
</tr>
</tbody>
</table>

\[a, b \text{ P < 0.001}\]

CD62L (L-SELECTIN) EXPRESSION

L-selectin was expressed on granulocytic cells of all maturation stages. The percent fluorescence increased from the early immature cells (1.98 ± 0.61% F in fraction I) to the late immature cells (4.86 ± 0.52% F in fraction II, P < 0.01). In the mature fraction, the number of cells expressing L-selectin was a 10-fold higher (51.3 ± 0.60% F in fraction III, P < 0.0001) as compared to the late immature fraction. The MFI values found in the early and late immature fraction were significantly lower than in the mature fraction (P < 0.0001). Results were obtained on nine cows and are summarised in Table 2.

Table 2. Percent L-selectin positive cells (% F) and their mean expression (MFI) in fractions I, II, and III. Data are expressed as mean ± standard error of the mean (n = 9).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>L-selectin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% F</td>
</tr>
<tr>
<td>I</td>
<td>1.98 ± 0.61</td>
</tr>
<tr>
<td>II</td>
<td>4.86 ± 0.60</td>
</tr>
<tr>
<td>III</td>
<td>51.3 ± 0.60</td>
</tr>
</tbody>
</table>

\[a, b \text{ P < 0.01}\]

\[b, c, e, f \text{ P < 0.0001}\]

\[d \text{ non significant}\]
CD11b EXPRESSION

CD11b was expressed on the cytoplasmic membrane of the granulocytic cells at all maturation stages (Fig. 2). The complement receptor was first detected on promyelocytes (3.71 ± 0.49% F in fraction I). A small increase was observed in the percent CD11b positive cells until the metamyelocytic stage (13.4 ± 2.58% F in fraction II, P < 0.001). The number of cells expressing CD11b was found to be highest in the mature cells (76.6 ± 3.07% F in fraction III). Band and segmented cells showed a six-fold increase in percent fluorescence in comparison with the late immature cells (P < 0.001). In contrast, a similar MFI value was observed for the three maturation fractions (Table 3).

![Figure 2. CD11b expression on the cytoplasmic membrane of bovine bone marrow cells through fluorescence microscopy (original magnification at ×630).](image)

Table 3. Percent CD11b positive cells (% F) and their mean expression (MFI) on the cytoplasmic membrane, respectively the total content in fractions I, II, and III. Data are expressed as mean ± standard error of the mean (n = 9).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Cytoplasm membrane expression</th>
<th>Total content CD11b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% F</td>
<td>MFI</td>
</tr>
<tr>
<td>I</td>
<td>3.71 ± 0.49 a</td>
<td>63.0 ± 4.41 f</td>
</tr>
<tr>
<td>II</td>
<td>13.4 ± 2.58 a,b</td>
<td>61.7 ± 5.89 f</td>
</tr>
<tr>
<td>III</td>
<td>76.6 ± 3.07 a,c</td>
<td>58.2 ± 1.31 f</td>
</tr>
</tbody>
</table>

\[ ^{a, b, c, d} P < 0.001 \]

\[ ^{e} P < 0.05 \]

\[ ^{f} \text{non significant} \]
Following permeabilisation, the percent fluorescence in the early immature cells (fraction I) did not differ from the cytoplasmic membrane expression. However, an increase of 80% fluorescence was observed in the late immature fraction (69.6 ± 5.41% F in fraction II, P < 0.001). Also in the mature fraction, more cells stained positively upon permeabilisation (increase of 12%, P < 0.001). MFI in fractions I, II and III increased with 60% (P < 0.001) compared to the value prior to permeabilisation. All results were obtained on nine cows and are summarised in Table 3 and Figure 3.

Figure 3. CD11b expression on bovine bone marrow cells: cytoplasmic membrane expression (□ % F, --- MFI) and total CD11b content (■ % F, ─── MFI) in fractions I, II, and III. Data are expressed as mean and error bars represent standard error of the mean (n = 9).

**CHEMOTAXIS**

Bone marrow cells responded with migration through the collagen-coated inserts following stimulation with zymosan activated serum. More than a three-fold increase (P < 0.0001) in chemotactic response was found for the myelocytes and metamyelocytes (25 ± 3.9% in fraction II) as compared to the myeloblasts and promyelocytes (7.7 ± 4% in
fraction I). Band and segmented cells (fraction III) exhibited almost the same response as the late immature cells to the chemotactic stimulus. In the control group, both the early immature and mature fraction showed low values in random migration through the barrier. In contrast, the late immature cells exhibited a significantly higher migratory response (P < 0.05). Comparing the control group to the zymosan activated serum, it became clear that stimulation with zymosan positively affected the chemotactic response of all bone marrow cells (P < 0.0001 for fractions I and III, and P < 0.01 for fraction II). Results were obtained on twelve cows and are summarised in Figure 4 and Table 4.

Figure 4. Bovine bone marrow cells undergoing chemotaxis following stimulation with zymosan activated serum. Data are expressed as mean and error bars represent standard error of the mean (n = 12) and represent the percentage of migration through the bicameral model (■ zymosan activated serum, □ control).
Table 4. Percent migration of bovine bone marrow cells and through the bicameral model with and without zymosan activated serum (ZAS). Data are expressed as mean ± standard error of the mean (n = 12).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>ZAS</th>
<th>control</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>7.78 ± 4.08</td>
<td>2.31 ± 4.22</td>
</tr>
<tr>
<td>II</td>
<td>25.5 ± 3.98</td>
<td>14.0 ± 4.10</td>
</tr>
<tr>
<td>III</td>
<td>23.1 ± 3.90</td>
<td>3.40 ± 3.95</td>
</tr>
</tbody>
</table>

**PHAGOCYTOSIS OF ESCHERICHIA COLI**

Early immature cells did not attach nor ingest *E. coli*. The small percent fluorescence (1.37 ± 0.28% F in fraction I) was due to the presence of more mature cells, as demonstrated with the fluorescence microscope. Some late immature cells showed a minimal phagocytic ability (3.90 ± 0.43% F in fraction II, P < 0.01). Band and segmented cells were the most active phagocytes showing substantial phagocytic capacity. Nevertheless, only 46.2 ± 3.65% of the mature population was phagocytising *in vitro* (P < 0.001). Results were obtained on 11 cows and are summarised in Table 5.

Table 5. Percent phagocytic cells (% F) and their mean activity (MFI) in fractions I, II, and III. Data are expressed as mean ± standard error of the mean (n = 11).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Phagocytosis</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.37 ± 0.28</td>
<td>20.3 ± 2.47</td>
</tr>
<tr>
<td>II</td>
<td>3.90 ± 0.43</td>
<td>24.6 ± 2.12</td>
</tr>
<tr>
<td>III</td>
<td>46.2 ± 3.65</td>
<td>40.4 ± 1.91</td>
</tr>
</tbody>
</table>

*a* P < 0.01  
*b, c* P < 0.001
Respiratory burst and cellular myeloperoxidase

Early immature cells did not show respiratory burst activity. The small percent fluorescence (2.35 ± 0.39% F in fraction I) was attributed to more mature cells contaminating this fraction. Within the late immature fraction (fraction II) 9.64 ± 1.38% of the cells produced ROS (P < 0.001). In the mature fraction a significantly higher percent fluorescence could be detected (36.6 ± 2.69% F in fraction III, P < 0.001). The MFI was not significantly different between fractions I, II and III. Following PMA stimulation, no differences in percent fluorescence could be detected in early and late immature fractions compared to the basal ROS production. However, the MFI increased three-fold in the late immature fraction following stimulation (fraction II, P < 0.001). On average 57.1 ± 2.70% fluorescence was detected in band and segmented cells (fraction III). The MFI showed a ten-fold increase in comparison to the spontaneous burst levels (P < 0.001). Results were obtained on 11 cows and are summarised in Figure 5 and Table 6.

Figure 5. Respiratory burst activity in bovine bone marrow cells: spontaneous (□ % F, --- MFI) and PMA-induced respiratory burst activity (■ % F, ─── MFI) in fractions I, II, and III. Data are expressed as means ± standard error of the mean (n = 11).
Myeloperoxidase positive granules were observed as discrete blue granules in the cytoplasm of granulocytic cells at all maturation stages, starting with the promyelocyte.

Table 6. Percent cells showing respiratory burst activity (% F) and their mean activity (MFI), with and without PMA stimulation in fractions I, II, and III. Data are expressed as mean ± standard error of the mean (n = 11).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Spontaneous respiratory burst</th>
<th>PMA-induced respiratory burst</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% F</td>
<td>MFI</td>
</tr>
<tr>
<td>I</td>
<td>2.35 ± 0.39 a</td>
<td>12.3 ± 1.92 g</td>
</tr>
<tr>
<td>II</td>
<td>9.64 ± 1.38 a</td>
<td>13.6 ± 2.02 g,d</td>
</tr>
<tr>
<td>III</td>
<td>36.6 ± 2.69 a,b</td>
<td>14.4 ± 2.14 g,e</td>
</tr>
</tbody>
</table>

a, b, c, d, e, f P < 0.001

g non significant

**DISCUSSION**

Bovine PMN play a key role in the first line defence against udder infection, and an impaired functional response has been proposed to contribute to the pathogenesis of coliform mastitis (Burvenich et al., 1994). The importance of cellular maturity in the defence against intramammary infections is supported by the correlation between the severity of mastitis and the total number of circulating neutrophils immediately before infection (Heyneman et al., 1990; Kremer et al., 1993; Vandeputte-Van Messom et al., 1993; Dosogne et al., 1997). Cows with a low number of circulating neutrophils become more severely diseased during subsequent induced *E. coli* mastitis.

The knowledge about the acquisition of functional properties during bovine granulopoiesis is based on studies in human bone marrow (Glasser and Fiederlein, 1987). There is only little information about the functional differentiation of bovine cells. A more in depth investigation of bovine bone marrow cells is therefore essential for the determination of the immune status in cattle, because species variation in neutrophil function exists (Styrt, 1989).
Chapter 4

The inflammatory reaction is initiated by extravasation of PMN (diapedesis). This is followed by a directed movement towards the site of infection (chemotaxis), where phagocytosis and killing of the pathogen will take place. Extravasation of activated PMN occurs after L-selectin adhesion of the cells to the endothelial surface, and is further mediated by specific membrane adhesion molecules, e.g. the β2-integrin CD11/CD18. The entire process of phagocytosis entails recognition and adhesion, followed by internalisation of the pathogen that will be degraded through oxygen-dependent and -independent mechanisms. In order for phagocytosis to proceed, PMN must recognise the bacteria. Immunological recognition is mainly accomplished by IgG2 antibodies which recognise the bacteria through F(ab)-regions and bind to the leukocytes via Fc-receptors on their cytoplasmic membrane. Activation of complement also promotes phagocytosis. Therefore, CD11b functions as a receptor for complement factor C3bi. Once complement and immunoglobulins bind to receptors on the PMN surface, the cell becomes activated and generates ROS. The respiratory burst system is very effective for eliminating Gram-negative bacteria such as *E. coli* (Burvenich *et al.*, 2003). Moreira da Silva *et al.* (1998) observed a minimal respiratory burst of circulating PMN three days after calving. This time period coincides with the maximum number of immature neutrophils found in circulation. The question arises whether the fall in the oxidative burst is the direct consequence of the appearance of immature cells in circulation.

In the present study the Fc-IgG2 receptor was detected in bovine bone marrow as early as the myeloblasts and promyelocytes, and the expression increased with each stage of maturation. No comparative data for the bovine species are described in literature. Only one paper describes that the Fc-IgG2 receptor was present on bovine neonatal blood PMN and that its expression was reduced in comparison to circulating PMN isolated from adult cattle (Zwahlen *et al.*, 1992). On human granulocytic precursors, Fc receptors were also found to be expressed early in differentiation, more specifically at the promyelocytic stage (Glasser and Fiederlein, 1987). L-selectin expression progressively increased throughout the granulocytic differentiation pathway, starting at the early immature stage and with an emphasis at the level of band and segmented cells. Data on human bone marrow cells are in agreement with our findings, reporting a low L-selectin expression during the proliferative phase of the granulopoiesis and an up-regulation starting at the
metamyelocytic stage (Lund-Johansen and Terstappen, 1993; Van Eeden et al., 1995). A possible role is assigned to L-selectin during active marrow release in human, as L-selectin is shed from PMN when they transit from the hematopoietic compartment to the circulating pool (Van Eeden et al., 1997). Furthermore, we demonstrated that CD11b surface antigen was expressed in all maturation stages of bovine bone marrow cells. The CD11b expression on the cytoplasmic membrane increased significantly as precursor cells underwent granulocytic differentiation. However, in a comparable study on the characterisation of bovine progenitors, CD11b was detected only on maturing cells, mostly myelocytes, metamyelocytes and segmented granulocytes (Fritsch et al., 1991). We propose that this discrepancy in results could be due to the difference in isolation procedure and/or the monoclonal antibody used. CD11b has been shown to be present in increasing amounts on cell surface of human bone marrow cells starting from the myelocytic stage (Arnaout, 1990). Rosmarin et al. (1989) observed CD11b expression at low levels already in human myeloblasts. Additionally, we determined the total CD11b content in bovine bone marrow cells. A significant intracellular CD11b pool was detected in bovine myelocytes, metamyelocytes, band and segmented cells. Diez-Fraile et al. (2003) recently demonstrated that circulating bovine PMN can increase their cytoplasmic membrane expression of CD11b upon stimulation through mobilisation of preformed CD11b, stored in specific granules.

In the current study, chemotactic ability was assessed using a bicameral model and zymosan activated serum to mimic C5a production following activation of cells during infection. Bovine bone marrow cells responded to zymosan activated serum since the (meta)myelocytic stage with a considerable migration through the porous inserts. The presence of a C5a receptor in early immature bone marrow cells, as demonstrated in this study, is not enough to sustain locomotion and thus chemotaxis. Following zymosan stimulation cells should develop a polar shape and microfilaments distributed in protrusions. Klut et al. (1997) showed a reduced polarity and F-actin redistribution in bone marrow cells isolated from rabbits. As cell polarity is a morphological feature necessary for locomotion, it is understandable that immature cells have a lower capacity to respond with active migration to chemotactic stimuli. Evaluation of random migration of bovine bone marrow cells revealed a strong random migration in myelocytes and metamyelocytes.
The late immature cell fraction corresponds to the transitional phase from the mitotic (proliferation) to the non-mitotic (maturation) pool in the hematopoietic compartment. Although no comparative data are available, the hypothesis is expressed that migration could be a fundamental step in cell fate decision through the relocation of daughter cells to distinct marrow niches (Kondo et al., 2003).

The present results confirm that immature bovine cells do not exhibit a phagocytic ability. Progenitor cells started to attach and ingest *E. coli* bacteria at the myelocytic stage. A significant phagocytic capacity was detected in band and segmented cells. Moreover, only 50% of these apparently mature granulocytes exhibited phagocytosis. This remarkable observation indicates that specific cellular characteristics required for optimal phagocytosis are acquired in conjunction with their release from the bone marrow (Altman and Stossel, 1974). Although CD11b is already expressed early in maturation, its presence does not guarantee complement-mediated phagocytosis by immature bovine bone marrow cells. According to Lichtman and Weed (1972), immature cells may be less capable of forming pseudopods following phagocytic activation. Our findings are in general agreement with the observations of Silva et al. (1989), who studied immune phagocytosis in bone marrow cells of cows. However, Guidry and Paape (1976) inducing a left-shift in bovine following intramammary *E. coli* endotoxin infusion, observed that the immature peripheral granulocytes are equally phagocytic as their mature counterparts. Decreased phagocytosis as compared to circulating PMN has also been demonstrated in human immature as well as morphologically mature bone marrow cells (Altman and Stossel, 1974; Glasser and Fiederlein, 1987).

Assessment of the respiratory burst activity demonstrated that immature cells isolated from bovine bone marrow were not capable to produce ROS, neither spontaneously, nor after stimulation. Following PMA-stimulation, myelocytes and metamylocytes showed a significant respiratory burst activity, whereas they did not exhibit any spontaneous ROS production. Band and segmented cells on the contrary had a spontaneous respiratory burst activity and PMA induced a significant increase in ROS production. Two enzymes play a keyrole in the ROS production: myeloperoxidase and the membrane-bound NADPH-oxidase. Myeloperoxidase is found exclusively in azurophilic granules and is an abundant early myeloid protein in human cells (Rosmarin et al., 1989).
Also in the present study on bovine bone marrow cells, myeloperoxidase positive granules were found at all maturation stages, starting with the promyelocyte. Based on our results, we suggest that only from the metamyelocytic stage, bovine granulocytic cells dispose of a functionally oxidative burst. Comparative data for the bovine species are scarce. In the study conducted by Silva et al. (1989), the nitroblue tetrazolium reduction, an alternative parameter for ROS production, was only observed in segmented granulocytes from the bovine bone marrow. Also in human bone marrow, only band and segmented cells showed a substantial oxygen-dependent microbial killing using nitroblue tetrazolium reduction (Glasser and Fiederlein, 1987).

**CONCLUSION**

In conclusion, the maturation sequence of the functional properties acquired during bovine granulopoiesis was studied. According to our results, the following sequence of events throughout the maturation of bovine bone marrow cells is proposed: myeloperoxidase activity $\rightarrow$ Fc-IgG$_2$ receptor expression $\rightarrow$ CD62L (L-selectin) expression $\rightarrow$ CD11b expression $\rightarrow$ chemotaxis $\rightarrow$ phagocytosis $\rightarrow$ respiratory burst activity. Compared to the functional differentiation of human neutrophils, no major differences are noticed, with exception of phagocytosis. In human bone marrow, even a rare blast cell had the capacity to phagocytise (Glasser and Fiederlein, 1987), whereas bovine precursor cells started to be phagocytic only at the myelocytic stage.

Our findings support the hypothesis that the release of immature cells from the bone marrow into circulation at parturition or during intramammary infection, may contribute to the decrease in a number of important PMN functions. Moreover, a suppression of the granulopoiesis at the level of the bone marrow during the periparturient period was hypothesised by Burvenich et al. (1994). Because the number of circulating neutrophils largely depends on the proliferative activity of the bone marrow on the one hand, and because the number of circulating neutrophils and their maturity after parturition is of major importance for the defence against mastitis on the other hand (Moreira da Silva et al., 1998), further investigations are strongly recommended.
REFERENCES


CHAPTER 5

VITAMIN A PROFILE DURING THE PERIPARTUM PERIOD AND EXPERIMENTALLY INDUCED E. coli MASTITIS

Adapted from

ABSTRACT

Blood vitamin A profile, including concentrations of retinol and its active metabolite retinoic acid were assessed during the peripartum period and during experimentally induced *Escherichia coli* mastitis in heifers. Serum retinol decreased in all animals in the immediate postpartum period, and normalised within one week after parturition. No significant changes were detected in the concentrations of retinoic acid isomers during puerperium. Following intramammary *E. coli* infusion, all cows showed moderate symptoms of systemic disease besides the local signs of inflammation. The presence of a systemic acute-phase reaction was documented by fever, increase in serum amyloid A and decrease in serum albumin. Retinol concentration in serum also decreased spectacularly during coliform mastitis, and the decline was clearly related to the timing of the acute-phase response. Moreover, a significant increase of all-trans-retinoic acid mirrored by a lowering of 13-cis-retinoic acid was detected during the same time period. The 9-cis isomer of retinoic acid was present in all samples, but remained below the limit of quantification.

Results confirmed the decrease in serum retinol during the peripartum period of dairy cows. The study established that profound changes in vitamin A metabolism occur during the acute-phase reaction of coliform mastitis in heifers. The bovine infection model reproduced the acute-phase related hyporetinemia, as previously observed in human and rats. Furthermore, all-trans-retinoic acid was found to be the most abundant circulating acid isomer during mastitis, providing an indication for a possible key role of all-trans-retinoic acid in the modulation of the immune response.
INTRODUCTION

The importance of vitamin A in immune function and protection against infections is well-established (Semba, 1999; Stephensen, 2001). Recent experiments using various animal models and cell lines suggest that retinoids modulate a variety of processes, including hematopoiesis, apoptosis, cytokine and immunoglobulin production, and leukocyte function (Semba, 1998). Retinol represents the most abundant retinoid in blood, while retinyl esters represent the most abundant storage form in the liver. The major established pathway of enzymatic retinoid activation involves hydrolysis of retinyl ester, reversible oxidation of retinol into retinal, and irreversible oxidation of retinal to retinoic acid (Blaner and Olson, 1994). The immunological effects of vitamin A appear to be mediated primarily through its acid derivatives, which include all-trans-retinoic acid and 9-cis-retinoic acid (Petcovich et al., 1987; Pfahl and Chytil, 1996).

Serum retinol levels are maintained within a fairly narrow range despite large fluctuations in dietary vitamin A intake and tissue stores. Only under extreme conditions, such as deficiency or intoxication, the homeostatically regulated retinol levels become affected. In humans, however, serum retinol concentrations were shown to decrease transiently during the acute-phase response to infection (Beisel, 1998; Mitra et al., 1998; Stephensen and Gildengorin, 2000). This phenomenon can complicate the interpretation of serum retinol as an indicator of vitamin A status, and the concomitant assessment of acute-phase proteins therefore seems inevitable (Stephensen and Gildengorin, 2000). Up till now, little attention was drawn towards the possible involvement of retinoic acid, the active metabolite of vitamin A, during infection and inflammation.

A variety of animal models to study inflammatory diseases have been described in literature, most of them involving rodents. However, controlled infection and disease with living bacteria is rather difficult in these small animal species (Vandeputte-Van Messom et al., 1995). In recent years cow models were developed to study the systemic inflammatory response induced by mammary tissue infection (Burvenich et al., 1988). The success of these models is widespread because of their reproducibility, high self-curing rate and the facility to obtain samples allowing molecular, immunocytochemical and pharmacological studies. Moreover, the severity of the systemic signs following intramammary infection of
cows with *Escherichia coli* can be standardised depending on the stage of lactation (Hirvonen *et al.*, 1999) and parity (Mehrzad *et al.*, 2002). A moderate level of systemic signs after intramammary *E. coli* infection can be obtained during mid-lactation whereas a severe systemic response, sometimes leading to shock and death, is more prominent during early lactation. Older cows also show a large variation in severity during early lactation, whereas in heifers a moderate level of systemic signs is observed (Mehrzad *et al.*, 2002). Severity classification can be obtained easily and accurately through measurement of the milk production in uninfected glands (Heyneman *et al.*, 1990; Vandeputte-Van Messom *et al.*, 1993), and is related to the increase in serum amyloid A (Hirvonen *et al.*, 1999) and intensity of bacterial growth in the infected glands (Kremer *et al.*, 1993).

This study was designed to define the blood vitamin A profile during the peripartum period and during experimentally induced *E. coli* mastitis in heifers. Based on the hypothesis of a compensatory change in retinoid metabolism during inflammation, we especially focused on the retinoic acid isomers, the biologically active forms of vitamin A. For this purpose, a well-defined bovine intramammary infection model with measurable but moderate level of systemic signs was used. Under these well-defined conditions, inference from the results is expected because the inflammatory response is a well-conserved process among vertebrates (Klasing *et al.*, 1987).

**MATERIALS AND METHODS**

**EXPERIMENTAL DESIGN**

**ANIMALS AND HOUSING**

Eight clinically healthy Holstein-Friesian cows being in their first pregnancy were selected. All heifers were in their 7th month of pregnancy on arrival at the dairy farm. The heifers were on a system of zero grazing from arrival until the end of the experimental study. The course of calving was normal, no inflammatory processes were detected during puerperium. Animals were fed twice daily with a ration consisting of corn silage, apple pulp, hay and water ad libitum. Concentrates (Sandilac; Dumoulin Voeders Sanders,
Moorslede, Belgium) were distributed according to milk production. Machine milking was performed daily at 0800 a.m. and 1800 p.m. using a quarter milking device (Packo & Fullwood, Zedelgem, Belgium). The study was approved by the Ethical Committee of the Faculty of Veterinary Medicine (Merelbeke, Ghent University).

**INOCULATION PROCEDURE**

Heifers were in their second to fourth week of lactation when challenge was performed. Before the intramammary *E. coli* challenge, animals were controlled to be free of major mastitis pathogens through two consecutive negative bacteriological examinations with a milk somatic cell count below 200 000 cells/ml on quarter level. A stock of *Escherichia coli* strain P4:032 (Bramley, 1976) was maintained in lyophilisation medium at -20°C. For experimental use, bacteria were subcultured in brain-heart infusion broth (CM225; Oxoid, Nepean, ON, USA) at 37°C during three consecutive days. The bacterial suspension was washed three times and finally resuspended in pyrogen-free phosphate-buffered saline. On day 0, after morning milking, six cows were inoculated in the left front and rear quarters with a suspension containing $1 \times 10^6$ colony-forming units of *E. coli* P4:O32 in a total volume of 10 ml pyrogen-free saline solution per quarter. Following disinfection of the teat ends with 70% ethanol containing 0.5% chloorhexidine, the bacterial suspension was inoculated into the teat cistern by means of a sterile teat cannula. After inoculation, the left quarters were massaged for 30 seconds to distribute the bacterial suspension in the mammary gland.

**SAMPLING PROCEDURE**

Blood samples were drawn aseptically from the external jugular vein by venipuncture, either in evacuated tubes containing 143 I.U. heparin, or in plain tubes wrapped in foil (BD Vacutainer Systems, Plymouth, UK). Serum was obtained from clotted blood following incubation for 2 h at 37°C and subsequent centrifugation ($1000 \times g$, 30 min, 4°C). Serum samples were frozen at -20°C until analysis.
PARTUS STUDY

Daily samples were collected for metabolic profile assay from each cow beginning two days before the expected calving date and continuing for one week into lactation. Concentrations of retinoids, serum amyloid A (SAA) and tumor necrosis factor α (TNF-α) were determined in serum.

MASTITIS STUDY

Blood and milk samples were collected once daily on day -7, -4, -1, 0, +1, +2, +3, +6, +9, +13 relative to challenge. On the day of challenge (d 0), blood and milk samples were collected at 6, 12, and 18 hours post-infusion (p.i.). Whole blood was used for determination of the total blood leukocyte count. Retinoid, SAA and albumin concentrations were quantified in serum. Somatic cell count and colony-forming units of E. coli were assayed in milk.

EXPERIMENTAL PROCEDURES

Serum retinoid concentrations. As a precaution to avoid photoisomerisation, all sample manipulations were carried out in amberised tubes under dim yellow light. Retinoid analogues were analysed simultaneously by HPLC-DAD, using the liquid-liquid extraction and mobile phase conditions as described by Van Merris et al. (2002). Briefly, following acidification of the serum, proteins were denatured with acetonitrile. Retinol, all-trans-, 9-cis- and 13-cis-retinoic acid were extracted using a mixture of n-hexane and 2-propanol (6.5:1.5 v/v). The organic layer was evaporated, the residue dissolved, and retinoids were eluted on a Symmetry C18 column (Waters Inc., Massachusetts, USA). Serum retinoid concentrations were calculated from the chromatographic peak area ratios of each retinoid to the internal standard.

Serum amyloid A and albumin. SAA was determined by a commercial sandwich type ELISA kit (Phase SAA kit; Tridelta Development Ltd, Ireland) according to the manufacturer's instructions. Serum albumin was quantified using radial immunodiffusion plates (Bethyl, Montgomery, USA), based on the Fahey and McKelvey (1965) technique. All samples were run in duplicate.
TNF-\(\alpha\). Concentrations of TNF-\(\alpha\) in blood serum were measured by radioimmunoassay (Blum et al., 2000).

Total blood leukocyte count. The number of circulating leukocytes was counted in an electronic particle counter (Coulter Counter Z2; Coulter Electronics Ltd., Luton, England).

Quarter milk production. Daily quarter milk production, the yield of the evening and subsequent morning milking, was measured daily using a quarter milking device (Packo & Fullwood). The loss in milk production in the uninfected contralateral quarters on day 2 p.i. has been used as a criterion to classify cows as moderate or severe responders to experimental \textit{E. coli} mastitis after parturition (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993).

Milk somatic cell count. Milk somatic cell count was determined using a fluoro-opto electronic method (Fossomatic 400 cell counter; Foss Electric, Hillerod, Denmark), which is based on the binding of ethidium bromide to DNA.

Colony-forming units in the inoculated quarters. The population level of \textit{E. coli} in the infused quarters was determined by ten-fold dilutions of the milk sample in phosphate-buffered saline. Ten \(\mu\)l of the dilutions was plated out in duplicate on Columbia Sheep Blood agar (Biokar Diagnostics, Beauvois, France) and incubated at 37\(^{\circ}\)C for 24 h. The obtained colony count was converted to colony-forming units ml\(^{-1}\), based on the dilution factor.

STATISTICAL ANALYSIS

PARTUS STUDY

A mixed model was fitted to the retinoid, SAA and TNF-\(\alpha\) concentrations, respectively, with day around parturition as fixed categorical effect and cow as random effect. The relationship between retinoid and SAA or TNF-\(\alpha\) concentrations was investigated by a mixed model with SAA or TNF-\(\alpha\) concentration as a continuous variable parameter and cow as random effect. The association between retinoid and SAA or TNF-\(\alpha\) concentration was expressed in terms of the slope (and its standard error, s.e.), which
corresponded to the change in retinoid concentration for a unit increase in SAA or TNF-α, respectively.

**MASTITIS STUDY**

The sampling period was grouped into three time periods: the pre-infection period comprising d -7, -4, -1, 0; the acute-phase period comprising d 0 at 6, 12 and 18 h p.i., and d +1; the convalescence period comprising d +2, +3, +6, +9, +13. A mixed model was fitted to the retinoid concentrations and protein concentrations, respectively, with period (3 levels: pre-infection, acute-phase and convalescence) as fixed effect and cow as random effect to account for the repeated measures structure. Pairwise comparisons between the three periods were performed, adjusting the overall 95% confidence level by Tukey’s multiple comparisons technique. The relationship between retinoid and protein concentrations was investigated by a mixed model with protein concentration as a continuous fixed effect and again cow as random effect. The association between the retinoid and protein concentration was expressed in terms of the slope (and its s.e.), which corresponded to the change in retinoid concentration for a unit increase in protein concentration. A significant relationship corresponded to a slope being significantly different from 0. Finally, the effect of retinoid concentrations in the acute-phase period on the reduction in milk production (related to mastitis severity) was studied by a fixed effect model.

**RESULTS**

1. **PARTUS STUDY**

1.1. **RETINOID CONCENTRATIONS DURING THE PERIPARTUM PERIOD**

Throughout the partus study period, retinol and two retinoic acid isomers (all-trans- and 13-cis-retinoic acid) were detected in all samples at a sufficient level for quantification. The 9-cis isomer was present in all samples, but remained below the limit of quantification (i.e. 3 ng/ml; Van Merris et al., 2002). In all animals serum retinol
concentrations changed significantly ($P < 0.0001$) with time over parturition. Retinol values reached their minimal value the first or second day after calving, started to re-establish the third day postpartum and normalised within one week (Fig. 1). No significant fluctuations were observed in the concentrations of all-trans- and 13-cis-retinoic acid during the peripartum period.

Figure 1. Serum retinol (ROH), serum amyloid A (SAA) and TNF-α concentrations during the peripartum period. Data are means ± standard error of eight cows.

1.2. SAA AND TNF-α CONCENTRATIONS DURING THE PERIPARTUM PERIOD

Parturition induced a significant increase in SAA concentration ($P < 0.0001$). Maximal values were detected in the immediate postpartum period, and returned to baseline values within one week after parturition. Fluctuations were observed in the concentrations of TNF-α during the peripartum period, although no significant differences were detected ($P = 0.29$) (Fig. 1).
1.3. Relation between retinoid and SAA or TNF-α concentrations

A significant linear relationship existed between SAA and serum retinol concentrations with each unit increase in SAA concentration (1 mg/l) leading to a decrease of 1.41 ng/ml retinol (slope = -1.41, s.e. = 0.123, P < 0.0001). There was no significant association between serum retinol and TNF-α concentrations during the peripartum period.

2. Mastitis Study

2.1. Characteristics of heifers with acute E. coli mastitis

Following intramammary E. coli infusion, all six heifers became ill, suffering from acute coliform mastitis. Clinical signs of inflammation, i.e. rubor, tumor, calor, dolor and functio laesa, were present at the infection site. Furthermore, animals showed moderate signs of systemic disease, and all cured spontaneously (Fig. 2).

The number of circulating leukocytes decreased during the acute-phase reaction. This initial leukopenia (6, 12, 18 h p.i.) was counteracted by a leukocytosis lasting until 6 days p.i., where-after total blood leukocyte counts returned to pre-infection values. Milk somatic cell count increased spectacularly at 6 h p.i., remaining high during the convalescence period. The number of colony-forming units of E. coli in the infected quarters was elevated at 6 h p.i., where-after the number of colony-forming units gradually decreased. Rectal temperature peaked at 6 h p.i. (40.07 ± 0.13°C), was still elevated at 12 h p.i., and normalised at 18 h p.i. The mean SAA concentration in the acute-phase period was a ten-fold higher than during the pre-infection period. The maximal concentration of SAA was reached at day 1 p.i. (P < 0.0001). During the convalescence period, mean SAA decreased, but was still elevated compared to the pre-infection value (P < 0.0001). Mean albumin concentration decreased significantly during the acute-phase period, with the lowest concentration to be found at 12 h p.i. (P < 0.0001). Concentration of albumin normalised during convalescence (Table 1).
Figure 2. Characteristics of heifers with acute *E. coli* mastitis: blood leukocyte count (BLC), rectal temperature (RT), colony forming units (cfu) of *E. coli*, somatic cell count (SCC), serum amyloid A (SAA), and albumin (ALB). Data are means ± standard error of six cows.

2.2. RETINOID CONCENTRATIONS DURING *E. COLI* MASTITIS

Throughout the mastitis study period, retinol and two retinoic acid isomers (*all-trans*- and *13-cis*-retinoic acid) were detected in all samples at a sufficient level for quantification (Fig. 3, Table 1). The *9-cis* isomer was present in all samples, but remained below the limit of quantification. Serum retinol concentrations started to decrease at 6 h p.i., reaching the nadir at day 1 p.i. (*P* < 0.0001). During the convalescence period retinol concentrations increased significantly, but remained below pre-infection levels (*P* < 0.01).
In contrast, concentrations of all-trans-retinoic acid increased significantly during the acute-phase reaction, starting at 6 h p.i., and reaching maximal values at 12 h p.i. (P < 0.0001). During the convalescence period all-trans-retinoic acid levels decreased significantly compared to the acute-phase period and returned to pre-infection values. Remarkably, the concentrations of 13-cis-retinoic acid mirrored those of the all-trans analogue. Concentrations of 13-cis-retinoic acid started to decrease at 6 h p.i., reaching a dip at 12 h p.i. (P < 0.0001). During the convalescence period 13-cis-retinoic acid increased, but did not reach the pre-infection values (P < 0.001).
Table 1. Serum amyloid A (SAA), albumin, retinol (ROH), all-trans-retinoic acid (atRA) and 13-cis-retinoic acid (13cisRA) during three time periods of acute coliform mastitis in heifers. Results are means (standard error) of six animals.

<table>
<thead>
<tr>
<th></th>
<th>Period</th>
<th>Concentration</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAA (mg/l)</td>
<td>pre-infection</td>
<td>13.73 (8.15)</td>
<td>&lt; 0.0001&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>acute-phase</td>
<td>118.39 (8.03)</td>
<td>&lt; 0.0001&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>convalescence</td>
<td>86.01 (8.20)</td>
<td>&lt; 0.0001&lt;sup&gt;3&lt;/sup&gt;</td>
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<tr>
<td>Albumin (mg/ml)</td>
<td>pre-infection</td>
<td>30.08 (1.20)</td>
<td>&lt; 0.0001&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>acute-phase</td>
<td>25.58 (1.20)</td>
<td>&lt; 0.0001&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>convalescence</td>
<td>31.03 (1.20)</td>
<td>0.0122&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>ROH (ng/ml)</td>
<td>pre-infection</td>
<td>588.20 (33.62)</td>
<td>&lt; 0.0001&lt;sup&gt;1&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>acute-phase</td>
<td>436.92 (33.62)</td>
<td>&lt; 0.0001&lt;sup&gt;2&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>convalescence</td>
<td>544.41 (33.29)</td>
<td>0.0023&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>atRA (ng/ml)</td>
<td>pre-infection</td>
<td>3.08 (0.33)</td>
<td>&lt; 0.0001&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>acute-phase</td>
<td>4.26 (0.33)</td>
<td>&lt; 0.0001&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
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<td></td>
<td>convalescence</td>
<td>3.19 (0.33)</td>
<td>0.1186&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>13cisRA (ng/ml)</td>
<td>pre-infection</td>
<td>3.72 (0.27)</td>
<td>&lt; 0.0001&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
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<td>acute-phase</td>
<td>2.70 (0.27)</td>
<td>&lt; 0.0001&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>convalescence</td>
<td>3.47 (0.27)</td>
<td>0.0003&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup> pre-infection period compared to acute-phase period
<sup>2</sup> acute-phase period compared to convalescence period
<sup>3</sup> convalescence period compared to pre-infection period

2.3. RELATION BETWEEN RETINOID CONCENTRATIONS AND ACUTE-PHASE PROTEINS

A significant linear relationship existed between SAA and serum retinol concentrations with each unit increase in SAA concentration (1 mg/l) leading to a decrease of 1.12 ng/ml retinol (slope = -1.12, s.e. = 0.087, P < 0.0001). The increase in SAA was concomitant with the decrease in retinol, both reaching their maximal, respectively minimal concentration at day 1 p.i. Albumin concentration was negatively related with all-trans-retinoic acid (slope = -0.142, s.e. = 0.012, P < 0.0001), whereas albumin was positively related with the 13-cis analogue (slope = 0.099, s.e. = 0.011, P < 0.0001). The changes in albumin and both retinoic acid isomers were also concomitant, albumin and all-
trans-retinoic acid reaching their maximal value at 12h p.i., and 13-cis-retinoic acid showing the minimal concentration at the same timepoint.

2.4. RELATIONSHIP BETWEEN RETINOID CONCENTRATIONS AND SEVERITY OF COLIFORM MASTITIS

Based on the criterion of Heyneman et al. (1990) and Vandeputte-Van Messom et al. (1993), all six cows were classified as ‘moderate’ responders. The reduction in milk production in the non-infected quarters on day 2 p.i. was related to the concentration of 13-cis-retinoic acid during the acute-phase reaction, with a decrease of 0.053 ng/ml 13-cis-retinoic acid for each percentage reduction in milk production (slope = -0.053, s.e. = 0.018, P = 0.0412).

DISCUSSION

Serum concentrations of vitamin A derivatives were determined during the peripartum period (2 days before until 7 days after parturition) and during experimentally induced E. coli mastitis (from 7 days before challenge to 13 days p.i.). In addition to the classical retinol determination, the chromatographic set-up (Van Merris et al., 2002) used in this study allowed the simultaneous quantification of three retinoic acid isomers in bovine serum.

We were able to confirm the important decline in serum retinol concentrations in the immediate postpartum period, as previously described in dairy cows by Johnston and Chew (1984) and Goff and Stabel (1990). Colostrum production accounts for the major portion of the decline in retinol after parturition, as demonstrated in a recent mastectomy study (Goff et al., 2002). While one ml of milk has about 1800 ng retinol, colostrum contains approximately 4300 ng retinol/ml (Johnston and Chew, 1984). We detected all-trans- (on average 2.57 ng/ml) and slightly higher 13-cis-retinoic acid (on average 3.45 ng/ml) concentrations in bovine serum during the periparturient period. In contrast, the biologically important isomer 9-cis-retinoic acid remained below the limit of quantification throughout the whole study period. Blaner and Olson (1994) reported that 9-cis-retinoic acid is not always detected in human serum, and that the isomer is found at much lower
concentrations than all-trans- and 13-cis-retinoic acid. In analogy with Goff et al. (2002), concentrations of the acid metabolites all-trans- and 13-cis-retinoic acid were not affected by parturition in heifers. Horst et al. (1995) described an important increase in 9,13-di-cis-retinoic acid during the periparturient period, but quantification of this isomer was not built in our chromatographic set-up. Although large variations were observed in the concentrations of serum TNF-α, no uniform pattern with regard to parturition was detected. This is in accordance with data of Koets et al. (1998), but in contradiction with the in vitro study of Sordillo et al. (1995). The acute-phase protein SAA increased during the immediate postpartum period. It has been suggested that the synthesis and release of this protein is a consequence of normal uterine involution, endometrial degeneration and tissue remodelling (Koets et al., 1998).

Experimentally induced coliform mastitis in dairy cows during early lactation caused sepsis, a systemic inflammatory response to an active infectious process (Bone et al., 1992). All heifers suffered from general illness, with clinical signs of quarter inflammation and moderate systemic symptoms. The presence of an acute-phase response was documented by fever, initial leukopenia followed by leukocytosis, increase in SAA and decrease of albumin in circulation. Interestingly, the acute-phase response was accompanied by a significant decrease in serum retinol during experimentally induced mastitis. This phenomenon is in accordance with the well-described acute-phase related hyporetinemia in human (Beisel, 1998; Mitra et al., 1998; Rosales et al., 2000; Stephensen and Gildengorin, 2000) and rats (Rosales et al., 1996; Rosales and Ross, 1998). Moreover, a significant increase in all-trans-retinoic acid and a decrease in 13-cis-retinoic acid were detected at the same time-point as the minimal concentration of serum albumin.

The mechanism underlying the acute-phase related decrease in retinol is not yet fully elucidated, but a number of possibilities have been proposed (Schweigert, 2001; Stephensen, 2001): 1) decreased mobilisation and transport of retinol from the liver, 2) excretion of retinol in the urine, and 3) increased metabolic requirements. Assuming that the observed changes in retinol serum concentrations reflect an increased requirement, it is tempting to speculate on increased utilisation of retinoids during infection. The most intensively discussed possibility is the consumption of circulating antioxidants during neutralisation of free radicals caused by activation of neutrophils (Sies and Stahl, 1995).
The transient changes in retinoic acid isomers (the metabolically active forms of vitamin A) observed during acute coliform mastitis are remarkable and have not been previously described in literature. During the acute-phase reaction, the increase of all-trans-retinoic acid is largely mirrored by a lowering of 13-cis-retinoic acid. Retinoic acid signals are mediated by specific nuclear receptors, the retinoic acid receptor (RAR) and the retinoid X receptor (RXR), which are part of a complex signalling network, allowing for receptor-receptor and receptor-DNA interaction (Petcovich et al., 1987). Because 13-cis-retinoic acid does not display a strong binding affinity for these retinoid receptors, it is believed that 13-cis-retinoic acid can act by serving as a precursor for the more transcriptionally active all-trans-retinoic acid (Blaner, 2001). Steric isomerisation of 13-cis- to all-trans-retinoic acid, catalysed by an isomerase, may thus be of importance to nuclear retinoid receptor-mediated biological activities. The different retinoic acid isomers have been shown to be enzymatically interconverted in a reversible way in vivo (Kojima et al., 1994).

Alternatively, the increase in all-trans-retinoic acid during the acute-phase reaction can partially be attributed to substantial increases in the oxidation of retinol to all-trans-retinoic acid. A mechanism for this hypothesis can be provided by the knowledge of the metabolic steps catalysed by retinoid dehydrogenases during conversion of retinol to retinoic acid. The first and rate-limiting step involves the reversible conversion of retinol into retinal by alcohol dehydrogenases; retinal is then irreversibly oxidised into all-trans-retinoic acid by NADP+-dependent aldehyde dehydrogenase(s) (Duester, 2000). Regulation of retinoic acid biosynthesis has not been fully elucidated, although prostaglandins may be modulatory at the site of conversion (Napoli, 1993). Nonetheless, our data strongly indicate a transient bio-activation mechanism (Chen and Juchao, 1998) possibly involving significant modifications in the activity of these dehydrogenases and putative isomerase during the acute-phase reaction. Some enzymes from the dehydrogenase families specialised in retinoid metabolism are known to be genetically highly conserved (Duester, 2000). Extrapolation of the present data, obtained during acute coliform mastitis in heifers, to other animal or human models seems therefore justified, although it cannot be excluded that species-specific enzymes might also have contributed to our findings.
While the reduction in serum retinol may reflect increases in general consumptive or oxidative processes, there is no clear evidence to suggest that the reduction has any relationship with the outcome of the animal (Schweigert, 2001), whereas such an association was found for serum concentrations of 13-cis-retinoic acid. The changes in serum retinoid levels may either have an important causal relationship with the outcome, or may simply represent secondary phenomena. The former suggestion would provide a strong argument for the supplementation of vitamin A in dairy cows during the peripartum period as it may reduce the incidence and severity of mammary infections (Chew et al., 1982; Johnston and Chew, 1984; Oldham et al., 1991).

The transient and self-correcting nature of the changes in vitamin A concentrations during coliform mastitis in heifers argues against a true deficiency state, and indicates that acute infection influences the retinoid metabolism in far more complex ways than the simple depletion of vitamin A stores. In humans, it has previously been suggested that the serum retinol concentration that is reached during the acute-phase of infection is due primarily to transient changes in vitamin A metabolism unrelated to total liver stores (Mitra et al., 1998). Furthermore, proportionately similar reductions in serum retinol in response to endotoxin were found in vitamin A-deficient and vitamin A-adequate rats (Rosales et al., 1996; Rosales and Ross, 1998), indicating that the effect of inflammation on retinol concentration does not depend on vitamin A status.
CONCLUSION

In summary, this study confirms the hyporetinemia observed in dairy cows during the immediate postpartum period. Furthermore, it describes at first the blood vitamin A profile during the acute-phase reaction of infection, using a *Escherichia coli* induced mastitis in heifers during early lactation. The cow model used in the study apparently reproduced the marked but transient reduction in serum retinol, as observed during infection in human (Mitra *et al*., 1998; Rosales *et al*., 2000) and rats. Furthermore, all-trans-retinoic acid was shown to be the most abundant retinoic acid isomer during the acute-phase reaction of mastitis in dairy cows, providing evidence of a shift in retinoid metabolism towards the active metabolite. Although interactions between retinoids and important immune response factors such as immune cells, adhesion molecules, and cytokines have been postulated (Semba, 1998), the functional importance of the infection-induced changes in serum retinoid levels awaits further clarification. The present data show the involvement of all-trans-retinoic acid in the acute-phase related hyporetinemia and form a strong indication for the key role of all-trans-retinoic acid in modulating the immune response during infection.
REFERENCES


CHAPTER 6

DIFFERENTIAL EFFECTS OF STEROIDS AND RETINOIDS
ON BOVINE MYELOPOIESIS IN VITRO

Adapted from
ABSTRACT

Pregnancy and parturition impose important physiological changes at the level of the steroid hormones, including progesterone, estrogens and corticosteroids. The alterations in number, maturity and function of polymorphonuclear leukocytes observed in dairy cows at parturition suggest a common causative relationship. The study was designed to investigate the in vitro effect of progesterone, 17-β-estradiol, and hydrocortisone on the proliferation of bovine progenitor cells. A methylcellulose-based medium with lymphocyte-conditioned medium was used, and colonies were scored after 7 days of incubation. At low concentrations, 17-β-estradiol induced inhibitory effects on the proliferation of granulocytic progenitor cells. Hydrocortisone reduced growth of granulocytic and monocytic colonies, whereas myelopoiesis was not altered by progesterone. Furthermore, we studied the effect of retinoids on colony formation of bovine bone marrow cells. All-trans- and 9-cis-retinoic acid exerted stimulatory effects on the growth of granulocytic colonies, and inhibited the proliferation of the monocytic lineage. Addition of the 13-cis isomer also resulted in increased numbers of granulocyte colony-forming units. At supra-physiological concentrations retinoic acid could thus modulate bovine myelopoiesis.

This study indicates that steroids that are produced during pregnancy and that change abruptly around parturition may be responsible for physiological alterations in the bovine hematopoietic profiles observed in circulation during late pregnancy and around parturition. These effects might contribute to the increased susceptibility of highly productive dairy cattle to Escherichia coli mastitis during the periparturient period. We furthermore hypothesise that an important role can be attributed to retinoic acid in the regulation of bovine myelopoiesis. This modulation of myelopoiesis in favour of the granulocytic lineage during the acute-phase reaction may be an adaptive mechanism designed to increase the capacity of the first-line defence in response to intramammary infections.
INTRODUCTION

There is a significant increase in the susceptibility to infectious diseases in dairy cows shortly after parturition. The incidence of clinical mastitis caused by environmental pathogens is highest during the immediate postpartum period (Erskine et al., 1988). During the periparturient period, changes in number, differentiation, maturity and function of circulating polymorphonuclear leukocytes (PMN) have been observed (Cai et al., 1994; Dosogne et al., 1999; Mehrzad et al., 2002). Several of the altered PMN functions as well as the circulating PMN number were found to be related to the severity of experimentally induced *Escherichia coli* mastitis during early lactation (Heyneman et al., 1990; Van Werven et al., 1997). Efficient functioning of PMN is necessary during the early phase of infection in order to clear the mammary gland from invading pathogens.

Pregnancy and parturition impose important physiological changes at the level of the steroid hormones that are produced by the placenta and the adrenal gland (Smith et al., 1973; Convey, 1974). Progesterone is the dominant hormone throughout pregnancy. The concentration of progesterone falls precipitously to nearly undetectable levels the day before parturition. Total plasma estrogen concentrations are relatively low in early lactation, increase ten-fold in midgestation and then remain steadily elevated. One week before parturition, estrogens further increase, rising to a peak value on the day of parturition. Plasma cortisol concentration starts rising three days before calving, and peaks the day after parturition.

Because the hormonal changes are concomitant with the observed changes in function, number, differentiation and maturity of circulating PMN, steroids might be involved in the underlying mechanisms of the above mentioned leukocytic changes. Research has mainly been focused on circulating PMN, as they form the first line defence against infectious diseases. Hoedemaker et al. (1992) provided evidence for the influence of cortisol, estrone, 17-β-estradiol and progesterone on bovine PMN function (either stimulatory or inhibitory). In contradiction with Moreira da Silva et al. (1997), Winters et al. (2003) found no significant changes in PMN oxidative burst activity at physiological or pharmacological levels of estrogens. However, Roth et al. (1983) reported that the combination of low estradiol with high progesterone levels is associated with a reduced...
oxidative metabolism and enhanced random migration of PMN. Recently, Hoeben et al. (1999) provided a first indication of the immunosuppressive effect of some metabolites and hormones whose concentrations change dramatically during the periparturient period of dairy cows. Acetoacetic acid, β-hydroxybutyric acid, and bovine pregnancy-associated glycoprotein were found to have inhibitory effects on the colony formation of bovine bone marrow cells.

Besides hormonal fluctuations, important changes in blood vitamin A concentrations take place during the periparturient period of dairy cows. We have recently observed a shift in retinoid metabolism during experimentally induced E. coli mastitis during the immediate postpartum period, suggesting a key-role of the biologically active metabolite all-trans-retinoic acid (Van Merris et al., 2003).

The aim of the present study was to investigate the in vitro effect of steroids whose concentrations change abruptly around parturition, on the proliferation of bovine progenitor cells. Furthermore, we studied the involvement of retinoids on the myelopoiesis because vitamin A, and especially retinoic acid, are known to modulate the immune response (Stephensen, 2001) and normal hematopoiesis in human (Collins, 2002).

**MATERIALS AND METHODS**

**ISOLATION OF MONONUCLEAR BONE MARROW CELLS**

Bovine bone marrow samples were collected from the sternum of adult cows at the slaughterhouse of the Ghent University (Melle, Belgium). Before cleavage of the carcass, 2 ml marrow was aspirated from the third or fourth sternabrae (Van Merris et al., 2001b) by means of a Janus needle (Bignell Surgical Instruments Ltd; Arundel, England). Bone marrow samples were transferred on ice into sterile tubes containing Iscove’s modified Dulbecco’s Minimal Essential Medium with 584 mg/l of L-glutamine (IMDM; Gibco BRL, Life Technologies Inc., Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and 100 U/ml lithium heparin (Leo Pharmaceutical Product, Zaventem, Belgium).
Bone marrow cells were gently mixed before further processing. Mononuclear bone marrow cells were isolated by gradient centrifugation (400 \times g, 20 min, room temperature) of the bone marrow suspension on Ficoll-Paque (Amersham Bioscience, Uppsala, Sweden) with a specific density of 1.077 g/ml (Van Merris et al., 2001a). Light density mononuclear cells were harvested from the interface and the cell suspension was washed twice (400 \times g, 10 min, room temperature) and finally resuspended in IMDM.

**CULTURE OF BOVINE BONE MARROW CELLS**

Bone marrow mononuclear cells were cultured using the *in vitro* culture assay optimised for the bovine by Van Merris et al. (2001a). The semi-solid culture system for myeloid colonies consisted of 0.9% high-viscosity methylcellulose (Methocel®, Fluka Chemie, Buchs, Switzerland), 30% of non heat-inactivated FBS, 1% of deionised bovine serum albumin (BSA fraction V 7.5%; Sigma Chemical Co., St. Louis, USA), 3% lymphocyte conditioned medium, 10 mM 2-mercapto-ethanol (Sigma Chemical Co.), and IMDM supplemented with penicillin-streptomycin and amphotericin-B (Sigma Chemical Co.). A volume of 1-ml methylcellulose-gel containing 1\times10^5 cells was plated out per 35-mm culturing dish (StemCell Technologies, Vancouver, Canada). All cultures were carried out in duplicate. Colonies were scored after 7 days of incubation.

**EXPERIMENTAL PROCEDURE**

Progesterone (4-pregnene-3,20-dione), 17-β-estradiol (1,3,5(10)-estratriene-3,17-b-diol), hydrocortisone (17-hydrocorticoosterone 21-acetate), all-trans-, 9-cis-, and 13-cis-retinoic acid were purchased from Sigma Chemicals Co. Appropriate stock solutions were prepared in ethanol, and extemporaneously further diluted in IMDM. Final ethanol concentration in culture medium was 0.005%. Before the start of the experiment, the solvent effect of ethanol on the *in vitro* colony formation was investigated. No apparent effect was observed in terms of colony number or colony type when increasing the ethanol concentration up to a final concentration of 0.005%. As retinoids degrade upon exposure to light, all experiments were carried out in subdued light. Previously described culturing medium was supplemented randomly with five final concentrations of progesterone (5, 50, 100, 250, 500 ng/ml), 17-β-estradiol (0.01, 0.1, 1, 5, 10 ng/ml), hydrocortisone (5, 10, 25,
50, 100 ng/ml), all-trans-, 9-cis- and 13-cis-retinoic acid (1, 5, 10, 50, 100 ng/ml). Methylcellulose-based cultures without addition of steroid or retinoids were used as a control.

**COLONY SCORING**

Colony formation was evaluated by a single person using gridded scoring dishes (StemCell Technologies). Colony types were clearly discernible, based on their typical morphological characteristics. Colony-forming unit-granulocyte, monocyte (CFU-GM) were mixed granulocytic and monocytic colonies containing >50 cells with a typical dense centre and a very widespread growth pattern away from the dense centre of the colony. Colony-forming unit-granulocyte (CFU-G) consisted of clusters of >40 spherical granulated cells with a very dense circular structure. Colony-forming unit-monocyte (CFU-M) were clusters of >20 larger brownish cells that were less densely organised compared with CFU-G. Total myeloid colony formation (CFU-total) was the sum of CFU-GM, CFU-G and CFU-M. The mixed myeloid and erythroid colony type CFU-GEMM did not grow under experimental conditions reported here.

Colony formation in each assay was expressed as cloning efficiency, which was the number of colonies (CFU) per 100 cells in culture. Because $1 \times 10^5$ mononuclear bone marrow cells were plated out per well, cloning efficiency of the culture equaled the number of colonies counted in a well, divided by 1000. Comparisons of the treated cultures with untreated cultures (control) was expressed as cloning efficiency index.

Cloning efficiency index was $\frac{\text{cloning efficiency 'treated'}}{\text{cloning efficiency 'control'}} \times 100$.

**STATISTICAL ANALYSIS**

The effects of steroids (progesterone, 17-β-estradiol, hydrocortisone) and retinoids (all-trans-, 9-cis-, 13-cis-retinoic acid) on CFU-G, CFU-M and CFU-total were evaluated in a mixed model with cow as random effect and concentration as a categorical fixed effect using the mixed procedure of SAS®, version 8. Concentrations were compared pairwise by Tukey’s multiple comparisons technique with a global error rate of 5%.
RESULTS

CLONING EFFICIENCY OF CONTROL CULTURES

The cloning efficiency of the cultures incubated without any steroid or retinoid was the number of CFU per 100 untreated cells. This cloning efficiency was further used to express the effect of steroid and retinoid treatment, respectively, as compared to the control culture, in terms of cloning efficiency index. Cloning efficiency index was the cloning efficiency of the treated cells divided by the cloning efficiency of the untreated cells multiplied by 100.

The mean cloning efficiency of the controls was $0.128 \pm 0.016$ for CFU-total, $0.105 \pm 0.011$ for CFU-G, $0.022 \pm 0.006$ for CFU-M ($n = 6$). The number of CFU-GM was too low to draw any conclusion. The cloning efficiency index of the highest concentration of each steroid and retinoid tested on CFU-total, CFU-G and CFU-M is provided in Table 1.

Table 1. Effect of the highest concentration of progesterone, 17-β-estradiol, hydrocortisone, and all-trans- (atRA), 9-cis- (9cisRA) and 13-cis-retinoic acid (13cisRA) tested on total myeloid colony-formation (CFU-total), colony-forming unit-granulocytes (CFU-G), colony-forming unit-monocytes (CFU-M). Data are means ± standard error of the mean of bone marrow mononuclear cell populations of 6 cows, and are expressed as cloning efficiency index.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CFU-total</th>
<th>CFU-G</th>
<th>CFU-M</th>
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<tr>
<td>500 ng/ml progesterone</td>
<td>102.05 (7.00)</td>
<td>100.62 (11.40)</td>
<td>110.14 (20.01)</td>
</tr>
<tr>
<td>10 ng/ml estradiol</td>
<td>64.81 (9.26)</td>
<td>58.76 (11.20)</td>
<td>93.32 (21.72)</td>
</tr>
<tr>
<td>100 ng/ml hydrocortisone</td>
<td>68.45 (6.07)</td>
<td>70.70 (6.59)</td>
<td>57.99 (5.42)</td>
</tr>
<tr>
<td>100 ng/ml atRA</td>
<td>123.45 (6.44)</td>
<td>134.89 (5.93)</td>
<td>70.79 (11.97)</td>
</tr>
<tr>
<td>100 ng/ml 9cisRA</td>
<td>130.62 (6.75)</td>
<td>143.46 (6.91)</td>
<td>72.98 (9.98)</td>
</tr>
<tr>
<td>100 ng/ml 13cisRA</td>
<td>119.31 (5.56)</td>
<td>127.43 (7.09)</td>
<td>83.16 (21.05)</td>
</tr>
</tbody>
</table>
EFFECT OF STEROIDS IN CULTURE

Growth of CFU-G and CFU-M were not altered by progesterone in the culturing medium (Fig. 1A).

17-β-estradiol inhibited the growth of bovine mononuclear bone marrow cells in vitro. The number of myeloid colonies (CFU-total) was reduced at concentrations of 1, 5 and 10 ng/ml (P < 0.0001) as compared to the control. The inhibitory action of 17-β-estradiol was mainly due to the inhibition of CFU-G. A decrease (P < 0.01) in number of CFU-G was detected at a concentration of 0.1 ng/ml. With higher concentrations, the number of CFU-G further decreased (P < 0.0001). The number of CFU-M was not significantly affected by 17-β-estradiol (Fig. 1B).

Hydrocortisone showed inhibitory effects on growth of CFU-total, CFU-G and CFU-M. Total myeloid colony formation (CFU-total) was inhibited at concentrations ranging from 10 to 100 ng/ml hydrocortisone (P < 0.0001) as compared to the control. The growth of CFU-G was significantly affected by hydrocortisone at a concentration as low as 10 ng/ml (P < 0.001), and the growth was further inhibited at concentrations of 25, 50 and 100 ng/ml (P < 0.0001). The inhibitory effect of hydrocortisone on CFU-M paralleled effects on CFU-G (Fig. 1C). The number of CFU-M decreased in presence of 10 ng/ml hydrocortisone (P < 0.01), and at higher concentrations (P < 0.0001).

EFFECT OF RETINOIDS IN CULTURE

All-trans-retinoic acid had a stimulatory effect on CFU-total. The number of CFU-total increased in presence of 10 ng/ml all-trans-retinoic acid, with a maximal increase at 100 ng/ml (P < 0.0001). Differential scoring of the myeloid colonies revealed that this increase resulted from a stimulation of CFU-G (P < 0.0001). All-trans-retinoic acid exerted an inhibitory action on CFU-M. The number of CFU-M was reduced at 10 ng/ml (P < 0.01), and further decreased at higher concentrations (P < 0.001) (Fig. 2A).

The effects of 9-cis-retinoic acid on bovine myelopoiesis were identical to those described for all-trans-retinoic acid. The number of myeloid colonies (CFU-total) was increased in the presence of the 9-cis isomer, starting at a concentration of 10 ng/ml (P < 0.0001). The stimulatory effect of 9-cis-retinoic acid on CFU-G was more pronounced than
the effect induced by all-trans-retinoic acid. Growth of CFU-M was inhibited at concentrations of 10 (P < 0.01), 50 and 100 ng/ml (P < 0.001) (Fig. 2B).

In presence of 13-cis-retinoic acid the number of CFU-total increased only at a concentration of 100 ng/ml (P < 0.0001), lower concentrations having no effect. The stimulatory action of 13-cis-retinoic acid on CFU-total was due to its positive effect on CFU-G at the highest concentration (P < 0.0001). The number of CFU-M was not altered by incubation with 13-cis-retinoic acid (Fig. 2C).

Figure 1. Effect of different concentrations of progesterone (A), 17-β-estradiol (B) and hydrocortisone (C) on total myeloid colony-formation (CFU-total), colony-forming unit-granulocyte (CFU-G), colony-forming unit-monocyte (CFU-M). Data are means ± standard error of the mean of bone marrow mononuclear cell populations of 6 cows, and are expressed as cloning efficiency index. Concentrations that do not have a letter in common are significantly different from each other (Tukey-Kramer pairwise comparisons with global error rate of 5%).
Figure 2. Effect of different concentrations of all-trans- (atRA; A), 9-cis- (9cisRA; B), and 13-cis-retinoic acid (13cisRA; C) on total myeloid colony-formation (CFU-total), colony-forming unit-granulocytes (CFU-G), colony-forming unit-monocytes (CFU-M). Data are means ± standard error of the mean of bone marrow mononuclear cell populations of 6 cows, and are expressed as cloning efficiency index. Concentrations that do not have a letter in common are significantly different from each other (Tukey-Kramer pairwise comparisons with global error rate of 5%).
DISCUSSION

Steroid receptors belong to the nuclear receptor family, which includes two major groups based on their ligand binding and the DNA binding domain: 1) steroid hormone receptors including receptors for estrogen, progesterone, androgens, glucocorticosteroids, and 2) thyroid/retinoid/vitamin D receptors activated by thyroid hormone, retinoic acids, and vitamin D3, respectively (Kumar and Thompson, 1999). Ligands of these receptors mediate pleiotropic cellular processes involved in metabolism, immunity, cellular proliferation and differentiation.

This study investigated the effect of steroids, of which the concentrations change abruptly at parturition, on the proliferation of myeloid bone marrow cells in vitro. Furthermore, the involvement of three retinoic acid isomers, the biologically active forms of vitamin A, in bovine myelopoiesis was assessed. In the current study, 17-β-estradiol and hydrocortisone induced inhibitory effects on the proliferation of bovine myeloid bone marrow cells (CFU-total) at concentrations observed at parturition. Very low 17-β-estradiol concentration (0.1 ng/ml) decreased the in vitro growth of granulocytic colonies (CFU-G). Plasma concentration of estrogens ranges from 0.02 ng/ml in early gestation to 0.3 ng/ml during mid- and late pregnancy, and peaks between 4 and 8 ng/ml at calving (Chew et al., 1977). Hydrocortisone inhibited the growth of myeloid progenitors (CFU-G and CFU-M) at a concentration of 10 ng/ml. Physiological concentrations in dairy cows vary between 4 to 10 ng/ml cortisol, and rise to 30 ng/ml shortly after parturition (Smith et al., 1973). No stimulatory nor inhibitory effects were observed on bovine myelopoiesis in the presence of (supra)physiological concentrations of progesterone.

Comparative data on the influence of steroids on bovine bone marrow cells were so far only provided by Hoeben et al. (1999), who reported similar inhibitory effects of hydrocortisone on bovine myelopoiesis. Intravenous injection of glucocorticosteroids in cows induces the release of immature myeloid cells from the bone marrow in the bloodstream thus leading to increased numbers of circulating PMN, but this leukocytosis is short-lasting and rapidly followed by leukopenia (Paape et al., 1973). Based on the current results, we can hypothesise that this might be due to the inhibitory effect of cortisol on the bone marrow progenitor cells. Hydrocortisone, which has the same activity as cortisol,
strongly inhibited the myelopoiesis \textit{in vitro} at physiological concentrations. The effects of hydrocortisone we observed on bovine bone marrow were similar to those described in mice (Metcalf, 1969) and humans (Bagby \textit{et al}., 1980). Treatment of mice with glucocorticosteroids decreased plasma colony-stimulating factor activity and decreased numbers of CFU-GM (Metcalf, 1969). However, Barr \textit{et al}. (1983) reported that hydrocortisone exhibited a dose-dependent effect on CFU-GM proliferation \textit{in vitro}, i.e. enhancement at low concentrations and inhibition at high concentrations. In contrast, more recent studies demonstrated an increase in the number of granulocytes not only in circulation, but also in the bone marrow, indicating that myelopoiesis is enhanced by \textit{in vivo} administration of hydrocortisone (Maruyama \textit{et al}., 1999; Laakko and Fraker, 2002).

The role of estrogens on hematopoietic progenitor cells is well studied. Early results obtained in mice indicated an inhibitory effect of physiological doses of estrogen on several hematopoietic lineages, including granulopoiesis and thrombocytopoiesis (Fried \textit{et al}., 1974). It was initially thought that the \textit{in vivo} suppression of hematopoiesis was secondary to replacement of the bone marrow cavity by new bone because estrogens induce osteosclerosis (Morse \textit{et al}., 1974). However, Perry \textit{et al}. (2000) showed that the effects of estrogens on murine hematopoiesis precede those on bone formation, thus providing evidence for a primary action of the hormone on the hematopoietic marrow. Estrogens were shown to exert their effect indirectly by acting on stromal cells of bone marrow expressing the estrogen receptor-\(\alpha\) (Smithson \textit{et al}., 1995). Non-hematopoietic elements (e.g. epithelial cells, macrophages, dendritic cells) are important in the regulation of hematopoiesis through release of cytokines, and it is possible that the estrogen receptor-\(\alpha\) may regulate the production and/or secretion of cytokines required for hematopoietic cell development. Recently, Thurmond \textit{et al}. (2000) suggested that the responsiveness of progenitor cells to estrogens in mice was mediated by the estrogen receptor-\(\alpha\) on hematopoietic cells. Although the precise mechanism stays unravelled, effects of estrogens through estrogen receptor-\(\alpha\) on hematopoietic cells seem to induce a blockade of the transition from less mature to more mature progenitor cells.

Our results on the effect of progesterone on bovine myelopoiesis are in agreement with those described by others. Progesterone did not affect progenitor growth in both rats (Benayahu \textit{et al}., 2000) and humans (Barr \textit{et al}., 1983). Only one study reported
progesterone to inhibit the proliferation of human CFU-GM cultured in methylcellulose (Smith et al., 1986).

We could demonstrate that all-trans- and 9-cis-retinoic acid exerted the same effects on total myeloid colony growth (stimulatory), CFU-G (stimulatory), and CFU-M (inhibitory), respectively. The 9-cis analogue induced thereby a more pronounced enhancement of CFU-G than did all-trans-retinoic acid. Addition of 13-cis-retinoic acid to the culture medium also resulted in an increased number of CFU-G. The 13-cis isomer, however, was only effective at a ten-fold higher concentration than its stereo-isomers. Our data on the differential effect of all-trans-retinoic acid in semi-solid cultures of bovine myelopoiesis are in agreement with human studies (Gratas et al., 1993; Van Bockstaele et al., 1993; Douer et al., 2000). The enhancement of granulocytic lineage mediated by all-trans-retinoic acid is associated with decreased production of monocytic, erythroid and mixed granulocytic/monocytic colonies (reviewed by Collins, 2002).

Serum concentrations of all-trans- and 13-cis-retinoic acid remain stable around parturition in dairy cows (Goff et al., 2002), but are affected by the acute-phase reaction during E. coli mastitis (Van Merris et al., 2003). During acute coliform mastitis, an increase of all-trans-retinoic acid is mirrored by decreased 13-cis-retinoic acid concentrations, suggesting that a transient bio-activation occurs in the retinoid metabolism. Significant modifications in the activity of retinoid dehydrogenases and isomerases may facilitate the production of all-trans-retinoic acid during infection. It is known that all-trans- and 9-cis-retinoic acid are transcriptionally more active than 13-cis-retinoic acid (Blaner, 2001). The effectiveness of all-trans-retinoic acid on bovine granulopoiesis at lower concentrations than 13-cis-retinoic acid has previously been reported by Van Bockstaele et al. (1993) for human bone marrow cultures. Retinoic acid signals are transduced by two specific nuclear receptors, the retinoic acid receptor (RAR) and the retinoid X receptor (RXR) (Petcoyvich et al., 1987), each comprising subtypes (α, β, γ), with various isoforms of each subtype. The natural ligands for the RAR are all-trans-, 9-cis-, and 13-cis-retinoic acid, whereas RXR is solely activated by 9-cis-retinoic acid. The abundant presence of retinoic acid receptors in human granulocytic precursor cells has further stressed the implication of retinoic acid in the differentiation of mature PMN (Chomienne et al., 1990). Mehta et al. (1996) demonstrated that retinoic acid regulates the
rate of cellular differentiation and apoptosis in the bone marrow, depending on the retinoic acid receptor subtype. Activation of RAR induces the genes linked with cellular differentiation, while activation of RXRs induces genes linked with apoptosis. Kastner et al. (2001) recently unravelled the in vivo mechanisms of RARα as a key mediator for the effects of retinoids on granulopoiesis in rats. Apparently, the receptor can bi-directionally modulate granulopoiesis, as a differentiation factor when liganded to retinoic acid, or as an inhibitor in the absence of the ligand.

**CONCLUSION**

From the current study we suggest that 17-β-estradiol and hydrocortisone may be responsible for physiological alterations in the bovine hematopoietic profiles observed in circulation after parturition. 17-β-estradiol inhibited the growth of myeloid colonies, and decreased the number of CFU-G. The myeloid pathway was also strongly inhibited by hydrocortisone. Hydrocortisone decreased both the numbers of CFU-G and CFU-M. Although progesterone alone had no effect in this culture assay, it is known that progesterone can enhance the inhibitory effects of estrogen colony formation in mice (Medina and Kincade, 1994). The inhibitory effects of 17-β-estradiol and hydrocortisone on bovine progenitor cells might contribute to the increased susceptibility of highly productive dairy cattle to *Escherichia coli* mastitis shortly after parturition.

We hypothesise that an important role can be attributed to retinoic acid in the regulation of bovine myelopoiesis. In a previous study we have shown a shift in retinoid metabolism to occur during acute *E. coli* mastitis (Van Merris et al., 2003). During the acute-phase reaction the metabolism preferentially results in the production of all-trans-retinoic acid. In the current study, all-trans- and 9-cis-retinoic acid have been shown to exert a potent stimulatory effect on CFU-G and an inhibitory action on CFU-M. This modulation of the myelopoiesis in favour of the granulocytic lineage during the acute-phase reaction may be an adaptive mechanism designed to increase the capacity of the first-line defence in response to intramammary infections.
REFERENCES


GENERAL DISCUSSION
Periparturient dairy cows undergo a plethora of physiological changes, including metabolic, hormonal and immunological changes. During this time period cows experience an increased susceptibility to intramammary infections, most likely as a result of a decreased immunocompetence (Oliver and Sordillo, 1988; Cai et al., 1994). The proportion of all cases of clinical coliform mastitis that develop during the first 2, 4, and 8 weeks postpartum increases from 25% over 45% up to 60% (Erskine et al., 1988). Besides mastitis, other infectious diseases occur most frequently during the first two weeks after parturition. This coincides also with a higher incidence of metabolic disturbances (Goff and Horst, 1997).

Among the immune cells affected during the peripartum period, the polymorphonuclear leukocyte (PMN) has been of particular interest due to its important role in innate immune defence against *E. coli* mastitis (Burvenich et al., 1994). Low numbers of circulating PMN and their decreased immune function during the periparturient period have been extensively studied (Kehrli et al., 1989; Cai et al., 1994). It has however not yet been elucidated at what level these alterations occur, including the possibility that parturition affects cells already at the bone marrow level.

Experimental models with *E. coli* mastitis have demonstrated a clear relationship between pre-infection PMN functions and the severity of subsequent coliform mastitis. The number of circulating PMN, the capacity to produce reactive oxygen species, and the chemotactic response of PMN prior to infection were negatively correlated to milk production, mainly in uninfected glands, and to colony counts of *E. coli* in milk of infected quarters, and hence the severity of systemic disease (Heyneman et al., 1990; Lohuis et al., 1990; Kremer et al., 1993; Van Werven et al., 1997). Today, it is known that severity of bovine *E. coli* mastitis is mainly determined by cow factors rather than by the pathogenicity of the invading pathogen (Burvenich et al., 2003), the animals’ physiological pre-infection status thereby playing a considerable role.
Schalm and Lasmanis (1976) reported that during mastitis the myeloid/erythroid ratio in bovine bone marrow increased above 1.0, reflecting an intensified myelopoiesis, probably to ensure replenishment of the PMN reserve and to return to homeostasis. However, the ability of progenitors to proliferate, as expressed in an increased myeloid/erythroid ratio, does not necessarily guarantee that the resulting PMN are competent (Butcher et al., 2000). Rapid recruitment induced by parturition-stress or during infection increases the rate of PMN production, shortening the maturation time and leading to the release of immature cells in circulation. As a consequence, the appearance of metamyelocytes and band cells in circulation after parturition (Moreira da Silva et al., 1998; Mehrzad et al., 2002) may be responsible for the decrease in immune functions in the periparturient period. Furthermore, during acute coliform mastitis the increase in the number of circulating immature PMN is even more pronounced than after calving, and younger cells (i.e. myelocytes, metamyelocytes and band cells) are observed (Heyneman and Burvenich, 1992; Mehrzad, 2002). Guidry et al., (1976) suggested already that the cows’ resistance may be compromised under conditions of stress (parturition, infection) by providing more cells that are biochemically and functionally less mature.

The functional maturation sequence during bovine granulopoiesis was established and proposed to be the following: myeloperoxidase activity → Fc-IgG2 receptor expression → CD11b expression → CD62L (L-selectin) expression → chemotaxis → phagocytosis → respiratory burst activity (Fig. 1) (Van Merris et al., 2002). Compared to the functional differentiation of human PMN, determined by Glasser and Fiederlein (1987), no major differences were noticed. The acquisition of various biochemical and functional properties in bovine PMN was also found to take place at different stages of maturation in the bone marrow. Therefore, we can postulate the existence of sequential functional maturation during bovine granulopoiesis. The efficiency of PMN against invasion of the mammary gland in general and against *E. coli* in particular, seems to be highly dependent on the rate of diapedesis into the infection site (Hill et al., 1979), and on the ability of these PMN to generate reactive oxygen species (Heyneman et al., 1990; Mehrzad, 2002). Although bovine bone marrow cells express surface CD11b and CD62L relatively early in the development, immature cells are not capable to rapidly migrate to the infected mammary gland, ingest and kill efficiently the invading bacteria (Van Merris et al., 2002). The
limited chemotactic and phagocytic ability in immature cells could be due to membrane rigidity with poor cellular deformability, in association with a large nucleus (Lichtman and Weed, 1972). The incapacity to produce reactive oxygen species can be attributed to an undeveloped membrane-bound NADPH-oxidase, as myeloperoxidase is already present in the rare azurophilic granules at the promyelocytic stage. Our findings support the hypothesis postulated by Guidry et al. (1976), namely that the presence of myelocytes, metamyelocytes and band cells in circulation may compromise the immune response in dairy cows because immature cells are not as functional as their mature counterparts.

Figure 1. Proposed stepwise acquisition of various functional properties in bovine bone marrow cells during granulopoiesis (adapted from Van Merris et al., 2002).

Alkaline phosphatase (E.C. 3.1.3.1) is present in substantial amounts in the cytoplasm of PMN. In humans cells, neutrophil alkaline phosphatase (NAP) levels fluctuate in a variety of physiological (pregnancy, stress) and pathological conditions (infections, myeloproliferative disorders) (Okun and Tanaka, 1978). Although the function
of the enzyme is still unclear, it has been suggested that elevated NAP levels in circulating PMN might reflect a hastened release of bone marrow cells in the bloodstream (Keleman, 1973). Conflicting data on NAP as a marker of cell maturity have been published, but most of the studies indicate that NAP activity is directly related to the age of bone marrow cells, being highest in youngest cells (Williams, 1975; Sato et al., 1985b; Tsuruta et al., 1996). Concomitantly with the left-shift, a significant increase in NAP levels was observed during experimental *E. coli* mastitis in dairy cows (Heyneman and Burvenich, 1992). Van Werven et al. (1998) demonstrated that this increase was rather the result of increased enzyme activity per neutrophil than an increase of the number of neutrophils exhibiting NAP activity. The human NAP-inducing factor was shown to be identical to granulocyte-colony stimulating factor (G-CSF) (Sato et al., 1985a), the growth factor thus being responsible for initiating the synthesis of the enzyme in progenitor cells. Therefore, the increased NAP levels after onset of mastitis were believed to be induced by G-CSF, reflecting an enhanced proliferation of bovine bone marrow granulocytes and an increased release of immature cell forms (Heyneman and Burvenich, 1992).

The underlying mechanism that controls the release of cells from the bone marrow into the circulating pool is not fully elucidated. Van Eeden et al. (1997) assigned a possible role to L-selectin in the release of PMN from the bone marrow in human. With active bone marrow release there was an increase in the number of PMN expressing high levels of CD62L in the circulation. However, levels of CD62L on circulating PMN never reached levels expressed in the bone marrow suggesting that CD62L was shed as PMN enter the circulation (Van Eeden et al., 1995). Recently, we observed higher CD62L expression on mature PMN in the bone marrow of cows than on their circulating counterpart (Monfardini et al., 2003). Elevated levels of CD62L on marrow cells might keep them attached longer over time to the endothelium of the bone marrow sinusoids, and stimuli for release may activate the proteolytic cleavage of CD62L (Van Eeden et al., 1997). Furthermore, it was demonstrated by Monfardini et al. (1999) that bovine circulating PMN also shed L-selectin from their surface during acute coliform mastitis. Because the appearance of immature cells in circulation during mastitis is concomitant with the CD62L shedding (Diez-Fraile et al., 2003), it is possible to hypothesise a similar releasing mechanism in cows. Furthermore, several investigations on bovine circulating PMN around parturition have
reported decreased levels of CD62L (Monfardini et al., 2002), correlating well with the periparturient rise in blood cortisol (Weber et al., 2001). Low CD62L expression inhibits vascular margination and may result in depressed PMN recruitment to inflammatory sites (Tedder et al., 1995). Weber et al. (2001) showed that the glucocorticoid-induced CD62L downregulation, e.g. induced by the cortisol peak at parturition, occurred at a gene-expression level on bovine circulating PMN. *In vivo* dexamethasone treatment of rabbits reduced surface CD62L expression on bone marrow granulocytes (Nakagawa et al., 1999). If glucocorticosteroids inhibit L-selectin gene expression on bovine progenitor cells at the bone marrow level at calving or during acute coliform matitis, young cells may be prematurely released into circulation (Weber et al., 2001).

The appearance of immature cells in the bloodstream may not only have disastrous consequences for the immunocompetence of dairy cows, or must not be the root cause of mastitis susceptibility. These youthful cells may provide periparturient dairy cows with an enhanced first line defence mechanism when intramammary infection occurs (Burton and Erskine, 2003). Recently, bovine neutrophil gene expression was screened around parturition using cDNA microarray analysis (Madsen et al., 2002, 2003). On the one hand, genes with decreased expression clustered into groups encoding proteins responsible for mediating programmed cell death and increased metabolism, and preparing cells for inflammatory activation. On the other hand, genes with increased expression encoded proteins that inhibit apoptosis, enhance DNA repair, and decrease protein synthesis. Apparently, parturition induced a pro-survival gene expression pattern in bovine blood neutrophils. From these results, Madsen et al. (2003) concluded that blood neutrophils change from a scenario of high and effective metabolism with short life span before parturition to low metabolism with prolonged survival at and shortly after parturition. Gene analysis data are consistent with the knowledge that blood PMN shift from a population of short-lived but functionally mature cells to a more immature but longer-lived population as parturition approaches (Moreira da Silva et al., 1998; Hoeben et al., 2000b; Mehrzad et al., 2001, 2002).

The process of programming cells for survival during the periparturient period can be attributed to several mechanisms, the most obvious being the early release of cells from the bone marrow. The rapid increase in circulating PMN is accompanied by an increase in
immature cell forms (Moreira da Silva et al., 1998), indicating an influx of cells from the bone marrow and not from the marginated pool of mature PMN (Guidry et al., 1976). It has been documented that cows do not possess a large reserve in the bone marrow (Paape et al., 1979). When the need for PMN exceeds the ability of the bone marrow to supply, immature forms (including band cells, metamyelocytes and myelocytes) are released into the circulating pool.

Changed phenotypes of blood PMN in response to the periparturient microenvironment may also be responsible for periparturient dysfunction. At parturition, circulating PMN are exposed to a rapidly changing steroid environment, with pronounced fluctuations in concentrations of progesterone, estradiol and cortisol (Smith et al., 1973). In an attempt to explain altered PMN function at parturition, investigators have speculated that fluctuating steroid hormone levels are involved in disease susceptibility because PMN dysfunctions are concomitant with changing hormone profiles (Goff and Horst, 1997). Cortisol (Hoeben et al., 1998), estradiol (Moreira da Silva et al., 1997) and bovine pregnancy-associated glycoprotein (Dosogne et al., 1999) have been documented to inhibit the respiratory burst activity of circulating PMN in dairy cows.

Altered maturation of cells at the bone marrow level may also account for a hastened release of not fully matured cells. Beside their effects on the functionality of circulating PMN, hormones and metabolites of which the concentrations fluctuate around parturition, can affect the proliferative activity of bovine bone marrow cells. First evidence was provided by Hoeben et al. (1999), showing inhibitory effects of β-hydroxybutyric acid, acetoacetic acid, and pregnancy-associated glycoprotein on the myeloid colony formation of bovine progenitor cells in vitro. We could demonstrate that 17-β-estradiol and hydrocortisone may be responsible for physiological alterations in hematopoietic profiles observed in circulation during the periparturient period (Van Merris et al., 2003b). 17-β-estradiol altered the growth of myeloid colonies, and at low concentration already inhibited the in vitro growth of granulocytic colonies. The myeloid pathway was also strongly inhibited by hydrocortisone. Hydrocortisone decreased both the numbers of granulocytic and monocytic colonies at concentrations observed around parturition. Although progesterone induced no effect of itself in the culture assay, it is known that progesterone
can enhance the inhibitory effects of estrogen on colony formation in mice (Medina and Kincade, 1994).

On the one hand, it may be possible that periparturient dairy cows with pronounced left-shift may benefit from the increased number of young PMN in the bloodstream. During the early events of mastitis, milk PMN and epithelial cells produce TNF-α, IL-1, IL-6, IL-8, IFN-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Shuster et al., 1996; Riollet et al., 2000). Locally produced inflammatory cytokines reach the circulation shortly after infection (Shuster et al., 1996; Hoeben et al., 2000a), and may co-ordinate immune activation of circulating youthful cells (Sordillo et al., 1992). Cows that undergo such preparation may be armed with a well-established defence mechanism against mastitis. We may entitle this beneficial process in the aspecific immune defence system of periparturient dairy cows the “PMN preparation pathway”. On the other hand, cows that develop clinical mastitis may be animals that do not mobilise large numbers of bone marrow cells in circulation or properly re-program blood cells for survival around parturition (Burton and Erskine, 2003). The latter might use an overtaking manoeuvre in an attempt to overcome the PMN deficit by the time the mammary gland is already invaded by E. coli bacteria. During the acute-phase response of infection, cows have increased serum concentrations of all-trans-retinoic acid (Van Merris et al., 2003a). Our results indicate that retinoid metabolism preferentially results in the production of all-trans-retinoic acid, suggesting a transient bio-activation mechanism to occur during infection. Retinoic acid isomers, and especially all-trans- and 9-cis-retinoic acid, have been shown to exert a potent stimulatory effect on bovine granulopoiesis and to inhibit monocytic colony formation in vitro (Van Merris et al., 2003b). Modulation of the myelopoiesis in favour of the granulocytic lineage during the acute-phase reaction might be an adaptive mechanism designed to increase the capacity of the first-line defence in response to intramammary infections.

The hypothetical PMN preparation pathway in periparturient dairy cows may, at least partly, explain the remarkable finding of an increased survival of milk PMN during coliform mastitis (Mehrzad, 2002). Viability of PMN isolated from milk increased substantially during E. coli mastitis in early lactation, viability being higher in infected than in non-infected quarters. The modulation of bovine PMN survival in mammary
secretions may be attributed to the genetic anti-apoptotic conversion of circulating PMN induced by parturition, as proposed by Madsen et al. (2002, 2003). On the other hand, the increased milk PMN viability could be related to the intrinsic pathophysiology of *E. coli* mastitis, especially to the kinetics of PMN diapedesis through the blood-milk barrier. During acute coliform mastitis rapid migration of circulating PMN into the mammary gland occurs, resulting in increased milk somatic cell counts (Paape et al., 1996). Following recruitment into the inflamed mammary gland, youthful cells might further mature in presence of locally produced cytokines (Shuster et al., 1996), extending their life span and thereby boosting milk PMN viability. The contribution of the blood-milk barrier (Van Oostveldt et al., 2002) and mammary gland injury (Sladek and Rysanek, 2001) in the modulation of PMN apoptosis have been determined recently. Using an in *vitro* model of the bovine blood-milk barrier, Van Oostveldt et al. (2002) depicted a rapid apoptotic response in bovine PMN migrating through a collagen coated membrane (representative for the extracellular matrix) when compared to PMN migrating through a monolayer of either endothelial or epithelial cells (representative for ductular mammary epithelium).

Another matter may be the anti-apoptotic effect of pro-inflammatory cytokines and/or bacterial products, mediators present in the inflamed mammary gland. Endotoxic lipopolysaccharide, C5a, GM-CSF, TNF-α, IFN-γ, IL-1 and IL-6 retarded the programmed death of human PMN (Colotta et al., 1992; Lee et al., 1993). Delayed apoptosis was associated with prolongation of the functional life span of the population. Cytokine- or endotoxin-induced surviving PMN retained the capacity to produce oxygen metabolites (Colotta et al., 1992). In dairy cows, Mehrzad (2002) demonstrated that milk PMN viability and respiratory burst activity were inextricably interrelated, both cellular characteristics increasing concomitantly during the early phase of acute *E. coli* mastitis.
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From the current studies, we can conclude that the cows’ first line defence mechanism is compromised during the periparturient period by the release of not fully matured cells from the bone marrow. Determination of the stepwise biochemical and functional development during bovine granulopoiesis revealed that immature cells are not as functional and effective in immune defence as their mature counterparts. Steroid hormones, of which the concentrations change abruptly at calving, may partly account for the hastened release of less mature cells during the periparturient period, because steroids inhibit the proliferation and subsequent maturation of granulocytic progenitors in the bone marrow.

Fitting the results from this thesis in the knowledge obtained from decades of research on the immunocompetence of periparturient dairy cows, we formulate the hypothesis of a “PMN preparation pathway”. On the one hand, periparturient cows with pronounced left-shift might benefit from the increased number of immature cells in circulation (Fig. 2, pathway A). The youthful cells may further mature in the bloodstream in presence of inflammatory cytokines, and can form a population of longer-lived PMN ready for recruitment. Subsequent activation of these prepared PMN finally results in a well-established defence mechanism against mastitis. On the other hand, cows that do not mobilise large numbers of bone marrow cells at parturition or that do not properly re-program PMN for survival will easily develop clinical mastitis. By the time the mammary gland is invaded by bacteria, these cows will try to overcome the PMN deficit inducing a transient bio-activation of the retinoid metabolism (Fig 2, pathway B). All-trans-retinoic acid will be preferentially formed during the acute-phase of infection, and may stimulate the granulopoiesis in order to meet the increased demand for functional PMN.
The newly formulated hypothesis of a PMN preparation pathway in periparturient dairy cows forms a strong basis for further research. The precise mechanism for the modulation of the PMN population around parturition needs to be further unravelled. A better understanding of the orchestration of the granulopoiesis, the release from the bone marrow and PMN recruitment is a prerequisite. One should thereby also focus on the molecules that control PMN responsiveness in the early phase of intramammary infection.
Figure 2. Relationship between vitamin A, granulopoiesis, and susceptibility to mastitis in periparturient dairy cows.

A) PMN preparation pathway (PPP) modulates the functionally immature cells into a longer-lived youthful PMN population. These prepared PMN form a well-established first line defence against mastitis in early lactation.

B) Periparturient cows with impaired function, number and maturity of PMN (no PPP) will develop clinical mastitis. In an attempt to overcome the PMN deficit, a transient bio-activation of the retinoid metabolism can occur during the acute-phase of infection. All-trans-retinoic acid will be preferentially formed, and may stimulate the granulopoiesis.
Summary
Periparturient dairy cows undergo a plethora of physiological changes, including metabolic, hormonal and immunological changes. During this time period cows experience an increased susceptibility to intramammary infections, caused by environmental pathogens. Among the immune cells affected during the peripartum period, the polymorphonuclear leukocyte has been of particular interest due to its key role in innate immune defence against *Escherichia coli* mastitis. The number, functionality and maturity of polymorphonuclear leukocytes are altered around parturition, resulting in a decreased immunocompetence that may compromise the cows’ resistance.

The general objective of this study was to investigate the immunosuppression at the bone marrow level during the periparturient period. Therefore, the stepwise functional maturation sequence during bovine granulopoiesis was determined. Because the leukocytic changes observed in circulation around parturition may reflect the proliferative activity of the bone marrow, we aimed to assess the effect of steroids, of which the concentrations change abruptly at calving, on the proliferation and differentiation of bovine progenitor cells. Furthermore, the blood vitamin A profile was studied during the periparturient period and during coliform mastitis. The importance of vitamin A in immune function and protection against infections is well-established in humans. Retinoids can modulate a variety of processes, including hematopoiesis, cytokine production, and leukocyte function. The possible involvement of retinoids in bovine myelopoiesis was also studied.

In the first part, the development and evaluation of methods used in the current study are described. A methylcellulose-based culture assay for bovine bone marrow cells was described (*Chapter 1*). Standardisation of the growth medium and incubation conditions resulted in a reproducible and quantitative clonogenic assay for bovine myeloid progenitor cells.

A prerequisite for studying the stepwise functional maturation sequence of bovine bone marrow cells was a reliable separation method, yielding enriched and purified cell fractions of different maturation stages. A three-layer discontinuous gradient column with optimised densities was developed (*Chapter 2*). Following centrifugation of the marrow suspension on the gradient column, three cellular fractions could be recovered: an early
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immature fraction with primarily myeloblasts and promyelocytes, a late immature fraction consisting merely of myelocytes and metamyelocytes, and a mature fraction containing bands cells and segmented granulocytes. This gradient centrifugation method resulted in viable maturation-related fractions allowing further studies on antigen expression and functional development during bovine granulopoiesis.

In order to study the blood vitamin A profile, an extraction procedure and HPLC method were set-up for the simultaneous quantification of endogenous retinoids in bovine serum (Chapter 3). The final procedure enabled fast and accurate quantification of alcoholic, acid and esteric forms of vitamin A in a single-run analysis.

The second part of the thesis covers the experimental studies performed in order to attain the proposed objectives. In Chapter 4 the various events that occur upon functional development of immune cells in the bone marrow were investigated. Each maturation stage was characterised by a selective expression of one or more receptors and functions. Therefore, sequential biochemical and functional maturation was postulated during bovine granulopoiesis, as follows: myeloperoxidase activity $\rightarrow$ Fc-IgG$_2$ receptor expression $\rightarrow$ CD11b expression $\rightarrow$ CD62L expression $\rightarrow$ chemotaxis $\rightarrow$ phagocytosis $\rightarrow$ respiratory burst activity.

Changes in serum retinoid levels were studied during the periparturient period and also during experimentally induced E. coli mastitis (Chapter 5). Serum retinol concentrations decreased spectacularly in the immediate postpartum period, and normalised within one week after parturition. Following intramammary E. coli infusion, all cows showed moderate symptoms of systemic disease besides the local signs of inflammation. The decline in serum retinol was clearly related to the timing of the acute-phase response of infection. Moreover, a significant increase of all-trans-retinoic acid mirrored by a lowering of 13-cis-retinoic acid was detected during the same time period. The retinoid metabolism resulted mostly in the production of all-trans-retinoic acid, the biologically active metabolite, suggesting a transient bio-activation mechanism to occur during infection. These results provided an indication for a possible key role of all-trans-retinoic acid in the modulation of the immune response.
In the last chapter the differential effects of steroids and retinoids on the proliferative activity of bovine bone marrow cells were assessed in vitro (Chapter 6). Alterations in the bone marrow due to fluctuating steroid concentrations may underlie the changes in number, function and maturity of circulating polymorphonuclear leukocytes observed during the periparturient period. Hydrocortisone and 17-β-estradiol inhibited the proliferation of granulocytic and monocytic progenitor cells, whereas progesterone did not alter the growth of bovine bone marrow cells. These results indicated that the changing steroid environment at calving might be responsible for physiological alterations in the bovine hematopoietic profiles in circulation. Retinoic acid isomers were shown to exert a potent stimulatory effect on the granulocytic colony formation and an inhibitory action on monocytic colony growth. The modulation of the myelopoiesis in favour of the granulocytic lineage by retinoic acid may be an adaptive mechanism designed to increase the capacity of the first-line defence in response to infection.

It may be postulated that the cows’ resistance is compromised during the periparturient period by the release of not fully matured cells from the bone marrow. However, it is still unclear whether immature cells in circulation really alter the immunocompetence and increase the susceptibility to coliform mastitis in early lactation. On the one hand, periparturient cows with pronounced left-shift might benefit from the presence of less mature cells. The youthful cells may further mature in the bloodstream in presence of cytokines. Subsequent activation of these prepared polymorphonuclear leukocytes could finally result in a well-established defence mechanism against mastitis. We may entitle this beneficial process in the aspecific immune defence system the “polymorphonuclear leukocyte preparation pathway”. On the other hand, cows that do not mobilise large numbers of bone marrow cells at parturition will easily develop clinical mastitis. By the time the mammary gland is invaded by bacteria, these cows will try to overcome the deficit in functional polymorphonuclear leukocytes by forming mostly all-trans-retinoic acid, in an attempt to stimulate the granulopoiesis and meet the increased demand.
Summary

The newly formulated hypothesis of a polymorphonuclear leukocyte preparation pathway in periparturient dairy cows forms a strong basis for further research. The precise mechanism for the modulation of the polymorphonuclear leukocyte population around parturition needs to be further unravelled. A better understanding of the orchestration of the granulopoiesis, the release from the bone marrow and cellular recruitment is a prerequisite. One should thereby also focus on the molecules that control the responsiveness of polymorphonuclear leukocytes in the early phase of intramammary infection.
SAMENVATTING
Melkkoeien zijn rond de partus onderhevig aan verscheidene fysiologische veranderingen, en dit zowel op metabool, hormonaal als immunologisch vlak. Tijdens deze kritische periode kan ernstige mastitis, veroorzaakt door omgevingskiemen, optreden. De polymorfonucleaire leukocyt speelt een belangrijke rol in de aspecifieke afweer tegen *Escherichia coli* mastitis. Het functieverlies, de daling in aantal en de gewijzigde maturiteit van circulerende polymorfonucleaire leukocyten tijdens de peripartum periode resulteren in een verminderde afweercapaciteit.

De algemene doelstelling van dit doctoraal proefschrift was de immunosuppressie op het niveau van het beenmerg te bestuderen tijdens de periode rond de kalving. Daarom werd de functionele maturatiesequentie tijdens de granulopoëse bepaald. De leukocytaire veranderingen die in de bloedbaan optreden tijdens de peripartum periode weerspiegelen grotendeels de hematopoëtische activiteit van het beenmerg. De invloed van steroidhormonen, waarvan de concentraties drastisch wijzigen bij kalving, op de proliferatie van progenitorcellen werd bestudeerd. Verder werd ook het vitamine A profiel rond de partus en tijdens *E. coli* mastitis vastgelegd. Het belang van vitamine A in afweer en bescherming tegen infectie is bij de mens wel bekend. Retinoiden hebben een modulerende werking op de hematopoëse, cytokine productie, en functie van polymorfonucleaire leukocyten. De rol van vitamine A in de regulatie van de granulopoëse bij het rund werd eveneens bestudeerd.

Het *eerste luik* van het proefschrift beschrijft de ontwikkeling en validatie van de methoden die in dit onderzoek werden aangewend. Een celcultuur model voor bovine progenitoren werd beschreven (*Hoofdstuk 1*). Standardisatie van het groeimedium en van de incubatieparameters resulteerde in een reproduceerbaar en kwantitatief celcultuur model voor bovine myeloïde progenitoren.

Een reproduceerbare scheidingstechniek waarbij beenmergprogenitoren geïsoleerd worden in functie van hun rijpingsstadium was een absolute vereiste om de granulopoëse te bestuderen. Hiervoor werd een discontinue drielagige gradiënt kolom met geoptimaliseerde densiteiten ontwikkeld (*Hoofdstuk 2*). Centrifugatie van de beenmerg celsuspensie bovenop deze gradiënt kolom resulteerde in drie fracties: vroeg immature
Samenvatting

fractie met hoofdzakelijk myeloblasten en promyelocyten, een laat immature fractie bestaande uit myelocyten en metamyelocyten, en een mature fractie met bandcellen en gesegmenteerde granulocyten. De door gradiënt centrifugatie bekomen celfracties lieten verdere studies naar antigenexpressie en functie-ontwikkeling tijdens de granulopoëse toe.

Voor de simultaanbepaling van endogene retinoiden in bovien serum werd een extractiemethode en een HPLC techniek op punt gesteld (Hoofdstuk 3). Na vloeistof-vloeistof extractie konden in één run van 60 minuten retinol, drie retineenzuur isomeren en retinylpalmitaat ondubbelzinnig gescheiden en gekwantificeerd worden.

Het tweede luik van de thesis beschrijft de experimentele studies die uitgevoerd werden om tot de vooropgestelde doelstellingen te komen. In Hoofdstuk 4 werd de ontwikkeling van biochemische en functionele eigenschappen van beenmergcellen tijdens de granulopoëse bestudeerd. De stapsgewijze maturatie werd als volgt voorgesteld: myeloperoxidase activiteit → Fc-IgG receptor expressie → CD11b expressie → CD62L expressie → chemotaxie → fagocytose → oxidatieve burst activiteit. De expressie van een bepaald patroon van functionele eigenschappen bleek aldus een goede maat te zijn voor de graad van differentiatie en maturiteit van de cellen. Het vitamine A profiel in de periode rond de kalving en tijdens coliforme mastitis in vroege lactatie werd bepaald in Hoofdstuk 5. Serum retinol concentraties daalden spectacular onmiddellijk na de kalving, en normaliseerden binnen de eerste week postpartum. Na intramammarie E. coli infusie vertoonden alle koeien lokale symptomen van inflammatie, alsook systemische ziekte tekenen. De daling in serum retinol was duidelijk gerelateerd met de acute-fase reactie van de infectie. Bovendien trad op hetzelfde ogenblik een significante stijging in all-trans-retineenzuur op, en een daling in 13-cis-retineenzuur. Tijdens infectie resulteerde het retinoid metabolisme preferentieel in de productie van de actieve metaboliet all-trans-retineenzuur. Dit transient bio-activatie mechanisme zou kunnen duiden op een mogelijke sleutelrol voor all-trans-retineenzuur in de modulatie van de immuunrespons.

In het laatste hoofdstuk werden de invloeden van steroiden en retinoiden op de proliferatie-activiteit van beenmergcellen in vitro bestudeerd (Hoofdstuk 6). Hydrocortisone en 17-β-estradiol remden de proliferatie van de granulocytaire en monocytaire progenitor cellen. Progesteron had geen effect op de kolonievorming door
Samenvatting

Wijzigingen in de granulopoëse ten gevolge van fluctuerende steroidconcentraties kunnen aan de basis liggen van het functieverlies, de daling in aantal en de gewijzigde maturiteit van de polymorfonucleaire leukocyten zoals die opgemerkt worden in de bloedbaan kort na de partus. Retineenzuur isomeren stimuleerden de granulocytaire kolonievorming, en inhibeerden de proliferatie van de monocytairereeks. De door retineenzuur geïnduceerde modulatie van de myelopoëse in het voordeel van de granulocytaire cellijn suggereert dat een adaptatiemechanisme kan ontstaan tijdens infectie om aldus de eerstelijns afweercapaciteit te laten toenemen.

Enerzijds zou de afweercapaciteit van de melkkoeien gecompromiteerd kunnen zijn door de aanvoer van onrijpe cellen uit het beenmerg tijdens de peripartum periode. Door de versnelde vrijstelling uit het beenmerg komt de functionele maturatie van de beenmergcellen in het gedrang. Anderzijds zouden de immature cellen in aanwezigheid van cytokines verder kunnen uitrijpen in de bloedbaan. Koeien met een groot aantal jonge cellen in circulatie zouden aldus over een beter uitgebouwde eerstelijns defensie kunnen beschikken. Indien rond de partus te weinig cellen uit het beenmerg vrijgesteld worden, stijgt het risico op klinische mastitis. Wanneer de melkklier geïnfecteerd is, kan een inhaalmanoeuver op gang gezet worden om het tekort aan polymorfonucleaire leukocyten op te vangen. Er zal meer all-trans-retineenzuur aangemaakt worden, om aldus de granulopoëse te stimuleren.

Het exacte mechanisme dat aan de basis ligt van de modulatie van de populatie polymorfonucleaire leukocyten tijdens de peripartum periode is nog onbekend. Daarbij moet het samenspel tussen de granulopoëse, de vrijstelling van cellen uit het beenmerg en de mobilisatie van polymorfonucleaire leukocyten tijdens infectie verder bestudeerd worden.
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Valérie.
CURRICULUM VITAE

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PERSONALIA

Name: Van Merris
First names: Valérie, Monique, Yves, C.
Date of birth: 04.02.1975
Place of birth: Aalst
Nationality: Belgian
Civil status: married

ACADEMIC EDUCATION

1999 - 2003  Ph.D. training in Veterinary Sciences, Ghent University

1993 - 1999  Veterinarian, Ghent University
             graduated on 02.07.2003 with great distinction
             Thesis
             Title: Presence of pathogenic Staphylococci on bodysurfaces of the horse
             Promotor: Dr. L. Devriese

1987 - 1993  High School (ASO), Dames van Maria, Aalst
             Latin-Mathematics

PROFESSIONAL ACTIVITIES

01.01.2002 - 31.12.2003
Ph.D. student at the Ghent University, Faculty of Veterinary Medicine, Department of
Physiology, Biochemistry and Biometrics
IWT-bursary (SB/993161/VanMerris)
Curriculum Vitae

01.01.2000 - 31.12.2001
Ph.D. student at the Ghent University, Faculty of Veterinary Medicine, Department of Physiology, Biochemistry and Biometrics
IWT-bursary (SB/991161/VanMerris)

Scientific worker at the Ghent University, Faculty of Veterinary Medicine, Department of Physiology, Biochemistry and Biometrics

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