The cytokines of bovine mammary gland: prospects for diagnosis and therapy

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Abstract

The lack of efficacy of conventional strategies for the maintenance of healthy udders in domestic cattle has prompted studies on the use of cytokines for this purpose. The adjuvant use of recombinant bovine cytokines, such as IL-2, IFN-γ and TNF-α, in normal mammary gland, mobilizes innate and acquired immunity. However, stimulated immunity does not prevent or eradicate infection, particularly in the case of Staphylococcus aureus mastitis. Cytokines do, however, improve the bactericidal efficiency of certain antibiotics. The subtle and sensitive changes in the cytokine network of normal and mastitic bovine mammary gland may encourage the use of cytokines in the diagnosis and prognosis of udder health. Numerous studies support this hypothesis, and detection and monitoring of cytokines could become an important alternative management for udder health. The use of cytokines in the immunotherapy, diagnosis and prognosis of mastitis will grow with knowledge of the cytokine network in bovine mammary glands and the development of efficient cytokine diagnostic techniques.

Keywords: Cytokines; Mastitis; Interleukins; Bovine; Diagnosis; Therapy; IL-8

1. Introduction

Cytokines are small proteins (less than 50 kDa) that act as intercellular communication signals in hematopoiesis, stress, inflammation, immunity and tissue repair (Belardelli and Ferrantini, 2002; Rouveix, 1997; Wood and Rothel, 1997). The identification of a large number of cytokines and disclosure of their pathophysiological mechanisms has opened innovative frontiers in diagnosis and therapy. Therapeutic strategies have been introduced to manipulate the clinical pathological role of cytokines in human diseases by: (1) blocking cytokine production, (2) stimulating the inhibitory pathways, (3) removing cytokines from circulation, (4) inhibiting cytokine-binding to receptors and (5) inhibiting signaling mechanisms (Gheezzi, 1997).

The bovine mammary gland, an organ of great importance for productive dairy cattle, has attracted considerable research and numerous cytokines have been detected in normal udders; however, their biological activity remains largely undisclosed (Alluwaimi and Cullor, 2002; Leutenegger et al., 2000). Research into the pathobiological role of cytokines in coliform and Staphylococcus aureus mastitis has been undertaken (Sordillo et al., 1997), and recombinant cytokines have been used in an attempt to raise the resistance or potentiate the immune system of bovine mammary gland infected with Escherichia coli or S. aureus (Daley et al., 1991b; Erskine et al., 1998; Kehrli et al., 1991a,b; Nickerson et al., 1989; Pighetti and Sordillo, 1996; Quiroga et al., 1993a,b; Sanchez et al., 1994). The prospective role of cytokines as a tool in dairy health control remains unevaluated as an alternative to the conventional methods of diagnosis and treatment. The purpose of this review is to evaluate cytokines as therapeutic, prognostic and diagnostic tools in udder health.

1.1. Interleukin-1

The cloned bovine cDNA of interleukin-1α (IL-1α) and interleukin-1β (IL-1β) encodes precursor proteins of...
268 and 266 amino acids (aa) with molecular weight (MW) of 30,820 and 30,760, respectively. The mature IL-1\(\alpha\) and IL-1\(\beta\) proteins are 150 and 153 aa with MW of 17,210 and 17,732, respectively. Comparison of the bovine IL-1\(\alpha\) amino acid sequence with human, murine and rabbit indicated a high level of homology at 73%, 62% and 71%, respectively. The homology of bovine IL-1\(\beta\) is 62% and 59% with human and murine IL-1\(\beta\) (Maliszewski et al., 1988a) (Table 1). Interleukin-1\(\alpha\) and 1\(\beta\) have been detected in normal bovine milk cells using the reverse transcriptase-polymerase chain reaction (RT-PCR) (Ito and Kodama, 1996; Okada et al., 1997). Interleukin-1 is crucial to the inflammatory process in the mammary gland infused with endotoxin or with natural or experimental coliform mastitis and bovine epithelial cells in vitro (Okada et al., 1999; Persson Waller, 1997; Persson Waller et al., 2003; Rollet et al., 2000b; Shuster et al., 1996, 1997; Shuster and Kehrli, 1995).

In E. coli infection, a sharp elevation of the IL-1 level is associated with the influx of neutrophils (Rollet et al., 2000b; Shuster and Kehrli, 1995; Shuster et al., 1995, 1997) and it has been postulated that IL-1 is indirectly involved in chemo-attraction of neutrophils in E. coli infection (Shuster et al., 1997). Comparing the responses of monocytes and milk macrophages to lipopolysaccharide (LPS) stimulation, Politis et al. (1991) demonstrated a more profound release of IL-1 by monocytes than by macrophages. Limited secretion of IL-1 in milk may affect its efficiency in lymphocyte activation (Politis et al., 1991). The contribution of the IL-1 response in S. aureus mastitis is negligible or transient, which indicates its minor role in this type of mastitis (Rollet et al., 2000b,c, 2001).

The adjuvant activity of IL-1 has been examined in normal bovine mammary gland at the early stage of the dry period (Nickerson et al., 1993). Intra-mammary infusion of normal gland with IL-1 leads to recruitment of an overwhelming number of somatic cells dominated by neutrophils and elicits a significant increase in rectal temperature. Nevertheless, enhancement of the involution process in quarters infused with IL-1 is less prominent (Nickerson et al., 1993). Although IL-1 attracts a considerable number of neutrophils to the infused udders, the overall immune modulation by IL-1 is minor (Nickerson et al., 1993).

In coliform mastitis, the immunotherapeutic properties of IL-1 are masked by the domination of the lethal proinflammatory nature of the IL-1. Nevertheless, the central role of IL-1 in coliform mastitis could be used as a prognostic criterion. Many studies have shown that IL-1 and other pro-inflammatory cytokines are incriminated in the mediation of signs of acute septic shock (Ohtsuka et al., 2001; Shuster and Kehrli, 1995; Shuster et al., 1993). Hence, monitoring the IL-1 level could be useful in defining the stage of coliform mastitis and the efficacy of the therapeutic intervention.

Interleukin-1 could serve as a suitable treatment adjuvant in S. aureus mastitis (Daley et al., 1991b, 1993; Sanchez et al., 1994). Infusion with IL-1\(\beta\) of mammary gland chronically infected with S. aureus enhances the neutrophil influx and up-regulates inducible oxygen radical formation but has no effect on phagocyte efficiency. Treatment of infected mammary gland with IL-1\(\beta\) increases the intracellular potency of certain antibiotics but not to such an extent as to prevent relapse in the infection (Daley et al., 1991b, 1993.).

Studies on the role of IL-1 receptor antagonist (IL-ra) in the modification of the IL-1 pathogenicity in coliform mastitis reveal interesting therapeutic and diagnostic findings (Persson Waller, 1997; Shuster and Kehrli, 1995; Yamanaka et al., 2000). Interleukin-1ra abolishes the IL-1 bioactivity in endotoxin mastitis but has a trivial effect on the mastitis episode and mammary inflammation (Shuster and Kehrli, 1995). The effectiveness of IL-1ra on the bioactivity of IL-1 is most effective if IL-1ra is administered before the IL-1/IL-1ra receptor interaction (Persson Waller, 1997). Interleukin-1ra fails to prevent endotoxin-induced neutrophil accumulation causes significant inhibition when administered simultaneously with the recombinant IL-1 (Persson Waller, 1997). It has been postulated, therefore, that either IL-1 has a marginal role in endotoxin-induced mastitis, or efficient IL-1ra therapy depends on its presence before IL-1 accumulation (Shuster and Kehrli, 1995).

Development of a bovine IL-1ra enzyme linked immunosorbent assay (ELISA) could prove to be a useful tool in the diagnosis of gram negative mastitis and monitoring the effectiveness of treatment (Yamanaka et al., 2000). The newly introduced ELISA has proven practical in the quantification of bovine IL-1ra in sera.

### Table 1

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>IL-1(\alpha)</th>
<th>IL-1(\beta)</th>
<th>IL-2</th>
<th>IL-6</th>
<th>IL-8</th>
<th>IL-12p35</th>
<th>IL-12p40</th>
<th>IFN-(\gamma)</th>
<th>TNF-(\alpha)</th>
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<td>Human (%)</td>
<td>73</td>
<td>62</td>
<td>69</td>
<td>53</td>
<td>76</td>
<td>82</td>
<td>84</td>
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<td>70</td>
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<td>42</td>
<td>67</td>
<td>58</td>
<td>47</td>
<td>74</td>
<td>74</td>
<td>55</td>
</tr>
<tr>
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<td>17,732</td>
<td>15,452</td>
<td>23,429</td>
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<td>30,000</td>
<td>40,000</td>
<td>16,858</td>
<td>19,100</td>
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</tr>
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<td>CDNA</td>
<td>2.1 kb</td>
<td>1.8 kb</td>
<td>791 bp</td>
<td>32 bp</td>
<td>240 bp</td>
<td>666 bp</td>
<td>984 bp</td>
<td>101 kb</td>
<td>156 bp</td>
<td>783 bp</td>
</tr>
</tbody>
</table>

and whey of mastitic and healthy cows (Yamanaka et al., 2000).

1.2. Interleukin-2

A cDNA clone of bovine interleukin-2 (IL-2) is 791 bp in length and codes for a bovine IL-2 precursor polypeptide of 158 aa with an estimated MW of 17,884 (Reeves et al., 1986). Mature bovine IL-2 comprises 135 amino acids and has a predicted MW of 15,452 Da. Bovine IL-2 has 69% homology with human and 50% with murine IL-2 (Baker, 1987; Cerretti et al., 1986a) (Table 1).

The bioactivity of bovine IL-2 compares with that in humans and mice. Interleukin-2 plays a central role in the immunoregulation of adaptive immune response. It stimulates the T cells to express cytokines and drives the clonal expansion and differentiation of activated T and B cells (Smith, 1988).

Bovine IL-2 has been detected in normal and mastitic cells of mammary gland (Alluwaimi, 2000; Alluwaimi and Cullor, 2002; Ito and Kodama, 1996; Sordillo et al., 1991c; Taylor et al., 1997). Although the exact role of IL-2 in bovine udder has not been clearly explored. Alluwaimi and Cullor (2002) detected an increase in the level of IL-2 transcriptional activity in bovine udder at the late stage of lactation, although its significance could not be delineated. On the other hand, Sordillo et al. (1991c) noted a marked decrease in IL-2 level pre-partum compared with post-partum. Diminished immune cell function and increased risk of mastitis pre-partum could be attributed partly to the low activity of IL-2 (Sordillo et al., 1991c). Although IL-2 was detected in S. aureus and coliform mastitis, its exact pathobiological role remained unclear (Alluwaimi et al., 2003a; Hogan et al., 1993; Riollet et al., 2000b,c; 2001; Yokomizo et al., 1995). However, the transcriptional activity of IL-2 in bovine mammary gland infected with S. aureus decreases at 24 h post-infection (pi). The significant decrease in the IL-2 transcription could support its importance in orchestrating the T cell immune responses in bovine mammary gland (Alluwaimi et al., 2003a).

The use of IL-2 as an adjuvant has been addressed extensively (Hogan et al., 1995; Hughes, 1991; Nickerson et al., 1992, 1993; Pighetti and Sordillo, 1995; Quiroga et al., 1993b; Sordillo et al., 1991a; Wedlock et al., 2000). Pighetti and Sordillo (1995) compared the immunopotentiating efficacy of IL-2 and incomplete Freund adjuvant (IFA) in mounting specific immune responses in bovine mammary gland using keyhole limpet hemocyanin as antigen. Mammary gland infused with recombinant bovine IL-2 (rBoIL-2) expresses a higher antibody titre and antigen-specific lymphocyte proliferation than mammary gland infused with IFA. Hughes (1991) recorded similar results when IL-2-infused mammary glands were sensitized with bovine herpes virus-1 antigen.

The in vitro study of the bactericidal activity of IL-2 treated lymphocytes showed enhanced expression of the major histocompatibility complex-II (MHC-II) (Sordillo et al., 1991b). Moreover, neutrophils from IL-2-treated quarters show active phagocytosis against S. aureus, in vitro (Wedlock et al., 2000).

The efficacy of rBoIL-2 in acceleration of the involution process has been addressed (Hogan et al., 1995; Nickerson et al., 1992, 1993). Intramammary infusion of IL-2 elicits a considerable increase in somatic cell count (SCC), which is dominated by macrophages and plasma cells producing IgG1, IgG2, IgA and IgM. The cytokine initiates considerable histological changes, which are dominated by an increase in connective tissue stroma and a decrease in alveolar epithelial and luminal areas (Nickerson et al., 1992, 1993). Theoretically, IL-2 appears ideal for accelerating the involution process, but Hogan et al. (1995) revealed information contrary to previous claims. They showed that IL-2 fails to provide any crucial benefit as an adjunct in dry cows.

The therapeutic role of IL-2 in treatment of S. aureus mastitis has been investigated (Daley et al., 1991b, 1992; Derosa and Sordillo, 1997; Nickerson et al., 1989; Quiroga et al., 1993a; Reddy et al., 1992) with unanimous results. The infusion of IL-2 in the infected quarters results in a significant immunopotentiating which express by overwhelming recruits of somatic cells, lymphocytes, neutrophils, macrophages and eosinophils, up-regulates the MHC class-II expression and gives a high antibody titre in milk and serum (Nickerson et al., 1989; Quiroga et al., 1993a,b; Reddy et al., 1992). Nevertheless, apparent potent immunomodulation in the IL-2 infused normal and/or mastitic mammary glands has not been resulted in prevention or cure of the infection. The setback in treatment of S. aureus mastitis is closely related to the diagnosis of the infection in its early stages. Looking at SCC within S. aureus-infected quarters, it is not until 24 h or more pi that cells reach levels that are considered highly suspicious by the standards of the National Mastitis Center (Alluwaimi et al., 2001; Daley et al., 1991b). At this stage of the infection, S. aureus has already inflicted irreversible damage on the immunological activity of the recruited and resident cells of the mammary gland (Alluwaimi et al., 2003a; Daley et al., 1991a). Concurrently, IL-2 transcriptional activity of cells from infected quarters is significantly decreased; CD4+ cells are in a stage of anergy and the phagocytic capability of the resident neutrophils is severely paralyzed (Alluwaimi et al., 2003a; Daley et al., 1991a).

Intramammary infusion of the infected quarters with rBoIL-2 cures only 38% of quarters, by clearing the bacteria and restoring the superoxide activity of phagocytic cells. This restoration is transient and reversible unless the source of the pathogen or cytokine is maintained continuously (Daley et al., 1991a, 1992).
It could be postulated, therefore, that the large influx of neutrophils and their immunopotentiating in infected udders infused with IL-2 is most likely due to the introduction of the cytokine at a stage before the *S. aureus* can inflict its irreversible damage. Hence, the use of IL-2 immunotherapy alone for treatment *S. aureus* mastitis or with adjunctive antibiotics could have a successful outcome if the cytokines are introduced when neutrophils are still intact. Such a prerequisite is highly unlikely in the absence of a diagnostic tool sufficiently sensitive to detect infection in its early stage. Nevertheless, IL-2 will continue to be a promising alternative prophylactic against *S. aureus* mastitis.

Exploitation of TaqMan® real-time polymerase chain reaction (PCR) in the study of the transcriptional activity of IL-2 mRNA in *S. aureus*-infected mammary gland reveals a promising tool for diagnosing *S. aureus* infection (Alluwaimi et al., 2001, 2003a). A continuous decrease in the mRNA transcription of IL-2 could be used in the diagnosis and/or treatment of *S. aureus* mastitis. Nevertheless, IL-2 mRNA transcriptional level at different stages of lactation and the dry period in normal udder needs to be elucidated as a vital reference for the differential diagnosis of *S. aureus* mastitis.

### 1.3. Interleukin-6

Bovine interleukin-6 (IL-6) cDNA encodes a full-length IL-6 protein of 208 aa and it is 65%, 53% and 42% homologous with porcine, human and murine, respectively (Droogmans et al., 1992) (Table 1). Interleukin-6 activity in coliform mastitis (Nakajima et al., 1997). In mammary gland infected with *E. coli*, *S. aureus* (Riollet et al., 1992) (Table 1). Interleukin-6 is a well-known neutrophil chemotactic cytokine that is produced by stimulated monocytes, T lymphocytes, macrophages, endothelial cells and a number of tumor cell lines (Matsushima and Oppenheim, 1989). The biological role of IL-8 in attracting neutrophils to infected bovine mammary gland was revealed by blocking the neutrophil chemotactic activity with anti-IL-8 antibodies in mastitic mammary secretions (Barber and Yang, 1998).

The IL-8 mRNA transcriptional level has been studied in healthy bovine mammary gland at mid- and late lactation (Alluwaimi et al., 2003b). Despite the expression of IL-8 mRNA at both these stages of lactation, no significant variation in its transcriptional level was observed (Alluwaimi et al., 2003b).

The expression of IL-8 mRNA has been confirmed in coliform and *S. aureus* mastitis (Alluwaimi et al., 2001; Riollet et al., 2000b,c; Shuster et al., 1997). Interleukin-8 is elevated early in experimental *E. coli* mastitis. Riollet et al. (2000b) detected IL-8 in milk as early as 14–16 h pi and Persson Waller et al. (2003) detected it at 4 h pi, whereas in another study it was detected at 24 h pi (Shuster et al., 1997). In *S. aureus* mastitis, measurement of IL-8 indicated no significant changes at all pi time points (Alluwaimi et al., 2001).

The importance of IL-8 as a major neutrophil chemoattractant prompted considerable interest in revealing its...
major cellular source (Barber et al., 1999; Boudjellab et al., 1998; Persson Waller et al., 2003). Mammary epithelial cell lines stimulated with LPS, IL-1β, *S. aureus* and/or *E. coli* copiously produce IL-8 (Barber et al., 1999; Boudjellab et al., 1998). The main source of IL-8 in the mammary gland has been investigated further by measuring its level in milk and in lymph from efferent and afferent lymphatics of the supra-mammary lymph node (Persson Waller et al., 2003). The level of IL-8 was higher in milk than in lymph, indicating that mammary epithelium rather than sub-epithelial tissue is the major source of IL-8 (Persson Waller et al., 2003).

In a different approach, Boulanger et al. (2003) revealed an indispensable role for the nuclear factor κB (NF-κB) in expression of IL-8. Genes encoding inflammatory proteins possess κB sites in their promoter, as an important transcriptional regulatory factor in up-regulating genes encoding IL-8 and granulocyte–monocyte-colony stimulating factor (GM-CSF). Somatic cells in milk from mastitis-affected cows exhibited intense NF-κB activity with a positive correlation with the elevated level of IL-8 and GM-CSF. Accordingly, expression and induction of IL-8 in mastitis appears to be under the tight control of NF-κB factors (Boulanger et al., 2003).

Recent findings have revealed a key role for IL-8 in *E. coli* mastitis (Lee et al., 2003; Wang et al., 2002). CD14, a 53–55-kDa glycosylphosphatidylinositol-anchored protein expressed on the membrane of monocytes/macrophages that facilitates LPS clearance and prevents LPS-induced septic shock (Lee et al., 2003), induces a pivotal signal in activation of mammary epithelial cells to express IL-8 (Wang et al., 2002). A constitutive level of the soluble form of CD14 (sCD14) occurs in serum, urine and milk (Lee et al., 2003). The normal level of sCD14 in bovine milk correlates with the level of SCC. Normal sCD14 level in milk ranges from 11.39 ± 0.29 to 6.5 ± 0.30 μg/ml in the presence of high and low SCC, respectively. Shedding of membrane CD14 (mCD14) from monocytes and neutrophils is the main source of sCD14. Interleukin-1 and IL-8 are the main factors in facilitating the release of mCD14 from neutrophils and macrophages (Lee et al., 2003).

Soluble CD14 enhances the LPS detoxification by binding to LPS-binding protein (LBP), an acute phase protein in serum, which increases the avidity of sCD14 to acquire LPS and accelerate its transport to high-density lipoprotein (Lee et al., 2003). The previously examined data could lead to formalization of a model in which IL-8 and sCD14 play key roles in modulation of *E. coli* or LPS-induced mastitis. The mechanisms of the envisaged model could be hypothesized as follows: a minute quantity of sCD14 in milk interacts with a small LPS concentration in the early stages of mastitis. The sCD14–LPS complex sensitizes mammary epithelial cells to up-regulate IL-8 and the production of other inflammatory cytokines. Elevated IL-8 draws neutrophils to the mammary gland and upon their activation they participate in increasing the pool of sCD14 by shedding their mCD14. Consequently, sCD14–LBP complex detoxifies free LPS either by transporting LPS to high-density lipoprotein and/or by amplified production of pro-inflammatory cytokines that augment sCD14 production. In addition to its role in the stimulation of IL-8, CD14 also plays a key role in ameliorating septic shock, probably by modulating production of TNF-α (Lee et al., 2003). It could be anticipated that either sCD14–LPS or/and sCD14–LBP represent one of the signals that NF-κB-expressed proteins deal with to initiate the IL-8 transcription.

The above model provides a wide window of opportunity in diagnosis and treatment of mastitis. Prevention of coliform mastitis at risk periods or its treatment could be carried out through the use of IL-8 to increase the sCD14 pool in milk. Hence, IL-8 could act as an adjuvant through the activation of the anti-inflammatory process by sCD14 in the mammary gland. Further studies on the efficacy of the direct use of IL-8 or the use of recombinant sCD14 are essential to verify the therapeutic value of this approach. The level of sCD14 could be a key factor in determining the prognosis for udder health at risk periods, particularly immediately pre-partum and post-partum. The ELISA that has been developed to measure sCD14 could provide a sensitive and reliable tool. In general, diagnostic approaches based on measurement of IL-8 or sCD14 could provide invaluable information about the health of the udder during coliform mastitis and could be used as a tool for forecasting the effectiveness of conventional therapy.

### 1.5. Interleukin-12

Bovine interleukin-12 (IL-12) is a heterodimeric cytokine made of p40 and p35 subunits, of 666 and 984 bp and their MW is 40 and 30 kDa, respectively, connected to form p70 homodimer. Bovine IL-12 p40 subunit amino acid sequence demonstrates 84.4% and 67.6% homology with human and murine sequences, respectively, while, p35 subunit amino acid sequence exhibits 82.2% similarity with human but only 85.6% with murine p35 subunit (Table 1) (Takehara et al., 2000; Zarlenca et al., 1995). Interleukin-12 is an important molecule that regulates cellular dichotomy (T helper-1 and T helper-2) lymphocytes in human and murine cells by enhancing the differentiation of T helper-1 cells, and is an important mediator that links the innate with the specific immunity (Trinchieri, 1995). Interleukin-12 p40 mRNA transcription has been monitored in normal bovine mammary gland at mid- and late lactation (Al-luwaimi and Cullor, 2002) and it is expressed at a significant transcriptional level in late lactation compared with mid-lactation. Significant elevation of IL-12p40 at late lactation could be attributed to its vital role in...
enhancing the immune responses in the mammary gland, particularly activation of natural killer cells through the augmentation of IFN-γ synthesis and regulation of antibody class switching (Trinchieri, 1995).

Interleukin-12 mRNA has also been detected by RT-PCR in mastitic mammary gland (Riollet et al., 2000c, 2001; Taylor et al., 1997). In mammary gland experimentally infected with S. aureus mastitis, IL-12p40 mRNA transcription is significantly elevated by 24 h pi (Alluwaimi et al., 2003a). Significant expression of IL-12p40 in S. aureus infection emphasizes further the central role of this cytokine in the adaptive immunity of bovine mammary gland. The pivotal role of IL-12 in linking innate with specific immunity, and its prominent function in the polarization of CD4 T cells towards type-1 T cells phenotype, mirror its crucial immunoregulatory activity in bovine mammary gland in health and mastitis (Alluwaimi et al., 2003a). However, Riollet et al. (2001) detected no obvious evidence on the polarization of the immune response in glands with S. aureus infection. Nevertheless, this finding does not minimize the key role of IL-12 in the mammary gland immunity. Further studies are awaited to disclose the role of IL-12 in orchestrating the immune response of bovine mammary gland.

The pathophysiological activity of IL-12 in bovine mammary gland suggests a role for this cytokine as an adjuvant and in immunotherapy of mastitis. Use of IL-12 as the adjuvant in immunopotentiation of certain cattle diseases has been reported (Tuo et al., 2000). Application of IL-12 against the tick-borne disease Anaplasma marginale gave promising results. The cytokine-augmented IFN-γ production that led to the enhancement of antibody production, particularly IgG1 and IgG2 subclasses, and phagocytosis by activation of macrophages.

Although application of the IL-12 in bovine mastitis has not been recorded, the potential of IL-12 adjuvant activity that was reported in other bovine diseases should encourage its application in bovine mastitis. For instance, recent findings on the possible immunological role of IL-12 could make it a promising tool for immunotherapy and diagnosis of S. aureus mastitis (Alluwaimi et al., 2003a). Elevation of IL-12 in the infected quarters precedes the significant rise in somatic cells. Monitoring the IL-12 mRNA transcriptional activity could lead to a more convenient and sensitive method for early detection of preclinical S. aureus mastitis (Alluwaimi et al., 2003a). Interleukin-12 alone or with other cytokine markers that play a central role in S. aureus mastitis, such as tumor necrosis factor-α (TNF-α) or IL-2, show promise for immunoprophylaxis and/or diagnosis of S. aureus mastitis. Nevertheless, a diagnostic method like ELISA seems necessary for monitoring the IL-12p40 in health and mastitis. Cloning of bovine IL-12p40 and development of monoclonal antibodies will pave the way for the development of a reliable ELISA test.

1.6. Interferon-γ

Bovine interferon-γ (IFN-γ) is a protein composed of 143 aa with a predicted MW of 16,858 kDa. It has 63% homology with human and 47% with murine IFN-γ (Table 1) (Cerretti et al., 1986b). Bovine IFN-γ has been detected in normal mammary gland by means of RT-PCR (Alluwaimi, 2000; Ito and Kodama, 1996). Although there is no significant elevation of the IFN-γ mRNA transcription, at late lactation transcription is significantly correlated with IL-12 expression in the normal mammary gland (Alluwaimi and Cullor, 2002). The IFN-γ mRNA is also detectable in cells from mastitic glands (Taylor et al., 1997), but Persson Waller et al. (2003) claimed that the concentration of IFN-γ was below the level of detection by ELISA.

Staphylococcal enterotoxins stimulate copious synthesis of IFN-γ and other cytokines. It has been postulated that CD8+ and CD4+ T cells release a substantial amount of IFN-γ due to the super-antigen activity of these enterotoxins (Yokomizo et al., 1995). However, in S. aureus experimental mastitis, IFN-γ transcriptional activity does not change significantly (Alluwaimi et al., 2003a).

Knowledge of the IFN-γ biological activity in bovine mammary gland is mainly derived from the adjuvant activity of recombinant bovine IFN-γ (rBoIFN-γ) in normal and infected mammary glands. Infusion of normal mammary glands with rBoIFN-γ has a great impact on mobilizing specific immune responses, mainly activation of T cells and IL-2 production. In addition, the magnitude of expansion of clonal and antigen specific memory cells is greatly enhanced (Pighetti and Sordillo, 1996).

In addition to the other inflammatory cytokines, IFN-γ is an important mediator in the activation of recruited neutrophils and enhancement of their phagocytosis (Riollet et al., 2000a; Wedlock et al., 2000). In vitro, neutrophils stimulated with rBoIFN-γ express enhanced killing of S. aureus, whereas in vivo activation reveals no significant effect on the neutrophils’ bactéricidal activity (Wedlock et al., 2000). Furthermore, experimentally induced S. aureus mastitis impairs the transcriptional activity of IFN-γ (Alluwaimi et al., 2003a). In coliform mastitis, on the other hand, IFN-γ levels are increased significantly during the course of infection and IFN-γ is found in the whey and serum of infected cattle (Hisada et al., 2001).

Extensive studies have been conducted on the immunoprophylactic properties of IFN-γ in normal bovine mammary gland (Pighetti and Sordillo, 1996; Sordillo and Babiuk, 1991a). Infused quarters express wide andconsiderable modulation of immune responses.
Recombinant BoIFN-γ stimulates and supports lymphocyte activation and clonal expansion. Activated lymphocytes express the high-affinity IL-2 receptor and adhesion molecules, such as lymphocyte function-associated antigen-1 (LFA-1). Recombinant BoIFN-γ also shows significant modulation of lymphocyte trafficking which immensely improves the secondary immune response. Hence, the role of rBoIFN-γ in modulation and protraction of inflammatory responses is evident. Expression and regulation of adhesion molecules or leukocyte and endothelial cells reflect the importance of IFN-γ in leukocyte movement. Finally, the cytokines enhance the specific immune responses in dairy cows by increasing the lacteal and serum antibody titers (Pighetti and Sordillo, 1996). Recombinant BoIFN-γ also significantly enhances the suppressed neutrophils retrieved from mammary gland at the pre-parturient period (Sordillo and Babiuk, 1991a). This period is associated with evident suppression of immune responses in the mammary gland. One of several factors responsible is the elevation of glucocorticoid, an important milk stimulator that has prominent immunosuppressive effects (Guidry et al., 1976). Use of BoIFN-γ to treat stressed neutrophils, which suffer from impaired bacterial phagocytosis, bactericidal activity and chemiluminescence, restores and enhances their bacterial phagocytosis and results in higher chemiluminescent activity (Sordillo and Babiuk, 1991a).

The immunotherapy of IFN-γ in coliform and S. aureus mastitis reveals a picture similar to that encountered with IL-2 treatment (Quiroga et al., 1993a,b; Sordillo and Babiuk, 1991b). Recombinant BoIFN-γ treatment of quarters experimentally infected with E. coli decreases the severity of the infection by restricting bacterial growth and reducing the inflammatory responses by inhibition of endogenous inflammatory mediators, particularly tumor necrosis factor-α (TNF-α), in the infected quarters (Moore, 1996; Sordillo and Babiuk, 1991b; Sordillo and Peel, 1992). The results of treatment of S. aureus-infected quarters with rBoIFN-γ were less encouraging (Quiroga et al., 1993a,b), as the course of disease in infected quarters did not change. Although the rBoIFN-γ treatment enhanced the MHC class-II expression and provoked antibody production, the established infection could not be overcome by the enhanced immunity. The outcome of IFN-γ immunotherapy seems to be affected by the setbacks that were discussed under IL-2 immunotherapy.

1.7. Tumor necrosis factor-α

The bovine DNA fragment of 7.2 kb which comprises bovine lymphotoxin (TNF-β) and TNF-α, has been isolated. The 156-bp cDNA of the above fragment encodes a TNF-α protein of 233 aa with predicted MW of 19,100 (Table 1) (Cludts et al., 1993). Tumor necrosis factor-α has been detected and monitored in normal and infected bovine mammary gland (Alluwaimi and Cullor, 2002, 2003a; Hagiwara et al., 2000; Hisaeda et al., 2001; Hoeben et al., 2000; Ito and Kodama, 1996; Riollet et al., 2001). In normal mammary gland, transcriptional activity of TNF-α is significantly elevated at late lactation compared with mid-lactation (Alluwaimi and Cullor, 2002). Numerous studies have detected an elevated level of TNF-α at all of the lactation, involution and periparturient periods, except a short period before the parturition when it drops below a detectable level. Elevated level of TNF-α could display its indispensable role in maintaining and regulating the immunological function of cells and factors involved in the physiological changes of the mammary gland (Rewinski and Yang, 1994; Sordillo et al., 1997, 1999c).

In natural coliform mastitis, experimental E. coli infection and LPS-infused mammary gland, TNF-α is significantly increased in milk and serum (Hisaeda et al., 2001; Hoeben et al., 2000; Nakajima et al., 1997; Ohtsuka et al., 2001; Perkins et al., 2002; Persson Waller et al., 2003; Shuster and Kehrli, 1995; Shuster et al., 1995, 1996; Slepodzinski et al., 2002). In serum, the level of TNF-α is only extensively elevated in severe clinical cases of coliform mastitis (Hoeben et al., 2000; Nakajima et al., 1997). Persson Waller et al. (2003) demonstrated an increase of TNF-α in afferent lymph 2 h and in milk 4 h post-endotoxin infusion. TNF-α plays an important role in coliform mastitis by inducing plasma haptoglobin, and recruiting and activating neutrophils and elevating intra-mammary and systemic nitrite and nitrate (Blum et al., 2000). In contrast to the sustained and continuous release of TNF-α in coliform mastitis, TNF-α transcriptional activity has only a short episodic elevation in the S. aureus-infected glands at 24 h pi, followed by sharp reduction at 32 h pi. S. aureus enterotoxins stimulate T cells to release TNF-α and/or cytokines, such as IL-2 and IFN-γ (Yokomizo et al., 1995). Tumor necrosis factor-α could be involved in the chemotactic activity for neutrophils, but the immunosuppressive nature of S. aureus infection could influence the synthesis and production of TNF-α (Alluwaimi et al., 2003a).

Infusion of normal mammary gland with recombinant bovine TNF-α (rBoTNF-α) results in an increase in the influx of neutrophils and a decrease in the concentration of certain milk proteins such as α-lactalbumin and β-lactoglobulin. Significant infiltration of IgG1 and IgG2 and serum albumin was also observed (Watanabi et al., 2000). These results greatly support the hypothesis that the influx of blood cells is mainly due to the weakening of the milk–blood barrier. In a further study on the pathophysiological effect of rBoTNF-α, Kushiibiki et al. (2003) observed similar inflammatory changes as those observed during coliform mastitis.
Subcutaneous injection of cows with rBoTNF-α lowers the concentration of tri-iodothyronine and insulin-like growth factor-1. On the other hand, nitrite plus nitrate concentration in plasma and milk are increased (Kushibiki et al., 2003).

The use of bovine TNF-α as an adjuvant or in the immunotherapy of mastitis has not been addressed in detail. For instance, in vitro, TNF-α fails to significantly enhance intracellular killing of *S. aureus* by mammary gland-derived neutrophils and macrophages, although it enhances the bactericidal activity of certain antibiotics (Sanchez et al., 1994). Infusion of normal mammary gland with TNF-α demonstrates a substantial reversible suppression of the lactogenic function of the glands and weakens the milk–blood barrier with transient effect on the systemic inflammatory responses. The observed changes resemble those associated with mastitis (Watanabi et al., 2000).

Tumor necrosis factor-α could be a useful candidate to monitor the severity of coliform mastitis and generate a prognosis, but its elevated level in both healthy and mastitic glands prompts a cautious approach. As well as IL-12, TNF-α could play a vital role in the detection of *S. aureus* infection, but the efficiency and feasibility of this approach will be influenced by the development of accurate and inexpensive measurement techniques.

### 1.8. Granulocyte–monocyte-colony stimulating factor

A bovine GM-CSF cDNA library has been isolated from the BT2 bovine T cell line. The cDNA of 783 bp encodes a protein of 143 aa with a predicted MW of 16,160 (Table 1). Bovine GM-CSF has a high degree of sequence homology with murine and human GM-CSF at both the nucleotide and amino acids levels (Maliszewski et al., 1988b). The predicted protein is 70% homologous with human and 55% homologous with murine GM-CSF. Bovine GM-CSF exerts high species specificity with very limited proliferation effect of recombinant bovine GM-CSF (rBoGM-CSF) on human or murine bone marrow cells (Leong et al., 1989). Cai et al. (1994) failed to stimulate normal and impaired neutrophils from cows with mastitis by using human recombinant GM-CSF.

Bovine GM-CSF mRNA has been detected in bovine alveolar macrophages and peripheral blood mononuclear cells by RT-PCR (Ito and Kodama, 1996). Bovine GM-CSF was monitored in normal bovine mammary gland at mid- and late lactation with a significant elevation in its transcriptional activity in late lactation and positive correlation with TNF-α and IFN-γ expression (Alluwaimi and Cullor, 2002). High mRNA transcription of GM-CSF and synergism of GM-CSF with TNF-α and IFN-γ could account for the basic immunological needs of the mammary gland, such as recruitment and potentiation of different somatic cells at the late stage of lactation.

The role of GM-CSF in *S. aureus* mastitis was negligible (Alluwaimi et al., 2003a). In coliform mastitis; GM-CSF has not been addressed extensively. However, in the udder of ewes infected with *E. coli*, GM-CSF was hardly detectable (Persson Waller and Colditz, 1999; Persson Waller et al., 1997). In neutrophils from cows with chronic mastitis, GM-CSF expression is correlated with the increased activity of NF-κB (Boulanger et al., 2003). It has been postulated that GM-CSF expression is under the control of NF-κB.

Milk neutrophils pulsed with rBoGM-CSF have enhanced bactericidal and chemotactic activities and they increase superoxide anion production (Sordillo et al., 1992). Infusion of GM-CSF into quarters experimentally infected with *S. aureus* showed that this cytokine activates the formation of oxygen radicals in the resident neutrophils but has no effect on the influx of neutrophils into the mammary gland (Daley et al., 1993). Other studies, however, showed that GM-CSF increases the number of neutrophils and enhances their bactericidal activity (Kehrli et al., 1991a,b). Although GM-CSF potentiates milk neutrophils in vitro and exerts a significant enhancement of resident neutrophils against *S. aureus* infection, its effect on the final outcome and eradication of infection remains limited.

The role that GM-CSF could play in udder health and the diagnosis and prognosis of mastitis is not promising due to the minor participation of this cytokine in the udder immune system.

### 1.9. Assessment and evaluation

#### 1.9.1. Cytokines and bovine mastitis immunotherapy

The study of the bovine cytokine network has been made possible through the production of a wide range of bovine recombinant cytokines and these have been used as a new avenue in mastitis therapy and prophylaxis. Emerging strains of bacteria that swiftly develop antibiotic resistance and the highly complex evasion mechanisms of pathogens incriminated in bovine mastitis have prompted study of the immunotherapeutic efficacy of recombinant cytokines in the treatment of bovine mastitis.

There are few reports of cytokine immunotherapy of coliform mastitis. The dominant role of the pro-inflammatory cytokines in driving the pathogenicity and outcome of the infection has biased the investigation of cytokine’s immunoregulatory intervention rather than an immunotherapeutic regimen. The immunoregulatory approaches are primarily based on down-regulating the pro-inflammatory cytokines through use of receptor antagonists or anti-inflammatory medications. In essence, in coliform mastitis, mammary gland immune responses are exhaustively stimulated and manipulated.
in a way to enhance the infection. Hence, the ground for effective strategies that abort the pathogen-initiated inflammatory process is wide open.

In *S. aureus* mastitis, mammary gland immune responses are evidently suppressed or markedly downregulated. Although cytokine immunotherapy, particularly with IL-2 and IFN-γ, showed promise of prophylactic activity in normal mammary gland, the resistance to *S. aureus* infection is not enhanced. The virulence factors of *S. aureus*, in particular numerous cell wall proteins and exotoxins, are potent and lethal to the extent that exogenous cytokine immunostimulation only lessens the severity of the infection but does not completely clear it. Trials of the efficacy of cytokines in the prevention and/or treatment of *S. aureus* mastitis have not produced encouraging results. The failure of cytokine immunotherapy of *S. aureus* mastitis can be attributed primarily to the disease-induced changes in the cytokine profile that cannot be restored by infusion of cytokines (Babiuk, L.A., personal communication). An effective therapeutic approach to *S. aureus* mastitis may require extensive study to illustrate the nature of changes in cytokine network inflicted by the infection. In addition, introduction of cytokines at high-risk periods, such as immediately pre-partum, may impede the course of gestation (Erskine, R.J., personal communication). Nevertheless, cytokine immunotherapy is promising as a prophylactic activity in normal mammary gland, the stimulated immunity failed to show any protective effect in experimental or natural mastitis.

Although the studies on the adjuvant activity of cytokines, especially IL-2 and IFN-γ, have generated promising results on enhancing the immunity of normal glands, the stimulated immunity failed to show any protective effect in experimental or natural mastitis.

### 1.9.2. Cytokine prospects in the diagnosis of bovine mastitis

In contrast to the poor outcome of cytokine immunotherapy for bovine mastitis, cytokines could provide a swift, reliable and highly sensitive means of diagnosis. Cytokines are the signals that dictate immune responses in normal and mastitic udders. Hence, subtle changes in the cytokine network of mammary gland in health and disease could help in detecting early infection and in monitoring the effectiveness of the treatment. However, there are major obstacles to be overcome. The cytokine network in bovine mammary gland has not yet been fully explored and delineation of the network at different stages of lactation and non-lactation is essential. This task will not be possible until better assays are available. Techniques based on real-time PCR or microarrays could be reliable but are not user-friendly for daily control of udder health. The practical application of cytokines as a diagnostic means in bovine mastitis requires automation of the procedures for detecting and monitoring cytokines.

### References


