ABSTRACT

The objectives were to establish the origin of 2 acute phase proteins in milk during subclinical bovine mastitis and to characterize the relationship between those proteins in milk and blood. Haptoglobin (Hp) and mammary-associated serum amyloid A (M-SAA3) appear in milk during mastitis, whereas Hp and serum amyloid A increase in serum during mastitis. The concentrations of these proteins were determined in an experimental model using a field strain of \textit{Staphylococcus aureus} to induce subclinical mastitis in dairy cows. The expression of mRNA coding for these proteins was assessed and the presence of M-SAA3 in mammary tissues was determined using immunocytochemistry.

Increases of M-SAA3 and Hp in milk occurred within 12 h of \textit{Staphylococcus aureus} infusion, with peak concentrations occurring 3 d after infusion of the bacteria. The increase of acute phase proteins in milk (15 h) preceded the increase in serum concentrations of both proteins (24 h). Expression of mRNA for M-SAA3 and Hp increased in both mammary and hepatic tissues 48 h after infusion of the mammary glands. In mammary tissue, the increase of M-SAA3 mRNA was greater than the increase in Hp mRNA expression, whereas in hepatic tissue, the increase in M-SAA3 mRNA was less than that for Hp mRNA. Immunocytochemistry demonstrated that M-SAA3 protein was present within secretory epithelial cells at significantly higher levels in infected mammary glands than in control tissues. These proteins, which have host defense and antibacterial activities, may play a significant role in the early response to invasion of mammary tissues by pathogenic bacteria.

Key words: acute phase protein, mammary gland, mastitis, \textit{Staphylococcus aureus}

INTRODUCTION

The acute phase response (APR) is central to the action of the innate immune system in its response to trauma, inflammation, and infection. The APR is stimulated by proinflammatory cytokines such as IL-1, IL-6, and tumor necrosis factor-\(\alpha\) (TNF\(\alpha\)) released from activated leukocytes at the site of tissue damage (Moshage 1997; Jensen and Whitehead, 1998). These cytokines orchestrate the primary response to infectious diseases and inflammation and stimulate a wide range of systemic reactions. In the liver, these cytokines cause a profound effect with about 7% of all genes being up- or downregulated (Yoo and Desiderio, 2003). Among the hepatic metabolic adaptation to the APR is the production and secretion of acute phase proteins (APP) into the plasma, which play a variety of roles in the restoration of homeostasis (Moshage, 1997; Gabay and Kushner, 1999). There are considerable differences among the APP in different species; thus, in humans, dogs, and pigs, C-reactive protein is a major APP, increasing 100 to 1000 fold in plasma concentration during the APR (Eckersall, 2000). In contrast, C-reactive protein is a constitutive plasma protein in cattle, little affected by the APR. Haptoglobin (Hp) is a major APP in cattle, with the serum concentration increasing up to 1,000 fold in plasma concentration during the APR (Eckersall, 2000). Immunocytochemistry demonstrated that M-SAA3 protein was present within secretory epithelial cells at significantly higher levels in infected mammary glands than in control tissues. These proteins, which have host defense and antibacterial activities, may play a significant role in the early response to invasion of mammary tissues by pathogenic bacteria.
cattle. Mastitis, a prevalent condition of lactating dairy cattle, is caused by bacterial infection of the mammary glands by a variety of gram-positive and gram-negative bacteria (Cullor and Tyler, 1996). Mastitis has been shown previously to induce an increase in the plasma concentrations of Hp, SAA, and α1 acid glycoprotein (Conner et al., 1986; Eckersall et al., 2001b).

In recent investigations, we discovered that Hp and SAA were secreted in bovine milk during clinical mastitis (Eckersall et al., 2001b). It has also been shown that experimentally induced mastitis can stimulate expression of these proteins in milk (Eckersall et al., 2001b; Gronlund et al., 2003). Others have shown that the form of SAA present in milk during mastitis is the bovine equivalent of the human SAA3 isoform, which has been called mammary-associated SAA3 (M-SAA3) and that this isoform is present in bovine colostrum (McDonald et al., 2001).

Two questions remain unanswered following these observations. Where is the site of production of APP that are secreted in mastitic milk, and why are APP secreted in milk when the mammary gland is infected with bacteria? There are 2 likely sites of production of Hp and M-SAA3 secreted in milk. The APP could be synthesized in the liver and exported to the mammary gland during infection by leakage from the systemic circulation or there could be de novo synthesis of the APP within mammary gland tissues. Extrahepatic synthesis of APP has been observed previously. Extrahepatic production of M-SAA3 and Hp has been reported in intestine, lung, and adipose tissue in humans and laboratory animals (Friedrichs et al., 1995; Yang et al., 1995, 2000; Urieli-Shoval et al., 1998, 2000; Vreugdenhil et al., 1999; Ogawa et al., 2000; Fukushima et al., 2002), and it is therefore possible that synthesis of APP may also take place in mammary tissue. Recent data from investigations of the expression of mRNA for M-SAA3 and Hp suggest that mammary tissue can be a source of the APP in bovine milk (Molenaar et al., 2002; His et al., 2004).

The secretion of APP in milk during IMI is presumably related to their roles in innate immunity in resisting the invasion and establishment of pathogens. Haptoglobin acts as a scavenger molecule for free hemoglobin, but also has antioxidant activities (Lim et al., 2000) and this may be an important function in milk. Serum amyloid A also has scavenging roles in the circulation, being involved in lipid transport, and has recently been identified as possessing direct antibacterial activity (Hari-Dass et al., 2005). Furthermore, a potential role for M-SAA3 in milk is suggested by its ability to stimulate the secretion of mucin from intestinal epithelial cells and in this process, act as an indirect antibacterial agent in the neonate (Larson et al., 2003a,b), especially as a component of colostrum.

This investigation was designed to determine whether APP, Hp, and M-SAA3 in mastitic milk are derived from hepatic synthesis or whether the mammary gland itself produces these proteins during the course of IMI.

MATERIALS AND METHODS

Experimental Model of Staphylococcus aureus-Induced Mastitis

An experimental model, designed to mimic subclinical bovine mastitis, was established using a low-virulence strain of Staphylococcus aureus originally isolated from a case of subclinical mastitis in Holstein-Friesian dairy cows as described previously (Knight et al., 2000). Intramammary infusion was carried out twice; for the first study period at d 0 (D0), and for a second study period 27 or 28 d later, which was designated d 0 for the second infusion (D0I-2). The study was performed under a Home Office Project Licence and with the approval of the Ethics Committee of the Hannah Research Institute.

All cows selected for the experiment had been free of clinical mastitis throughout the current and previous lactation and had individual quarter SCC below 0.1 × 10^6 cells/mL at monthly recordings during the current lactation. Before infusion, full bacteriological examination was carried out on milk samples from all 4 quarters of infused and control cows at several times, and shown to be negative for S. aureus and other major mastitis-causing pathogens. Small numbers of colonies of minor pathogens were occasionally isolated from some quarters. On D0, 2 quarters of the S. aureus-infused group of cows (n = 10) were infused with 5 × 10^4 cfu of S. aureus in 10 mL of Ringers buffered salt solution with both the right fore (RF) and right hind (RH) quarters being infused. For the second study period, the left hind (LH) and RH quarters were infused on D0I-2 with the same bacterium. Following the second infusion period, each quarter in the infected cows had received different levels of treatment; thus, the RH quarter had been infused twice with bacteria and was referred to as the “double-infused quarter;” the RF quarter had been inoculated only once at the original D0 and was referred to as the “single, early-infused quarter.” The LH quarter had been inoculated only once at the second infusion (D0I-2) and was known as the “single late-infused quarter,” and the left fore (LF) quarter had no exposure to bacterial inoculum at either time and therefore acted as a within-cow control. Eight healthy dairy cows from the same herd,
and shown to be \textit{S. aureus}-negative on bacteriological culture of milk from all 4 quarters, were used as between-cow controls, and were infused with 10 mL of sterile Ringers buffered salt solution in the same pattern as the infected animals.

Milk and serum samples were taken before infusion and twice daily for analysis of APP: Hp and MSAA3 were determined in milk, whereas Hp and SAA were determined in serum. Milk samples on d 1, 7, and 21 and at d 2\textsubscript{1/2} in the second infusion were submitted for bacteriological examination at the Veterinary Diagnostic Laboratory, University of Glasgow. At the end of the second study period, 48 h after the second infusion, cows were euthanized. Tissue samples from liver and mammary tissues were taken, snap frozen, and stored at $-80^\circ C$ for mRNA analyses using real-time reverse transcription PCR (RT-PCR). Tissue samples were also taken and paraffin-embedded for immunocytochemistry.

**Analysis of Milk and Blood**

An aliquot of the milk sample was transferred to a 25-mL container and a milk preservation metabolism inhibitor (potassium dichromate and mercury chloride) in tablet form (Lactabs Mark II, Thompson & Capper, Ltd., Runcorn, UK) was added, as recommended for analysis of SCC. The preserved sample was dispatched on the day of collection to the analytical laboratory of the Scottish Agricultural Colleges (Auchincruive, Ayr, UK) where the SCC was determined by Fossomatic methodology. Further aliquots of milk, as well as the serum samples, were stored at $-20^\circ C$, and analyzed as described below.

The concentration of the APP in serum or milk were assayed for Hp using the hemoglobin-binding assay method described by Eckersall et al. (1999), and the concentration of SAA was determined (Eckersall et al., 2001) using a commercial ELISA kit (Tridelta Development plc, Dublin, Ireland) according to the manufacturer’s instructions. The concentrations of Hp and M-SAA3 in milk were also determined by ELISA. The same ELISA was used to measure SAA in serum and M-SAA3 in milk, because the antibody in the Tridelta kit is known to react with both isoforms (McDonald et al., 2001); therefore, the method did not distinguish between SAA and M-SAA3. In spite of this deficiency, the analyte measured in milk will be referred to as M-SAA3 to maintain clarity. A sensitive ELISA system (Gronlund et al., 2003), rather than the hemoglobin-binding assay, was used to measure Hp in milk.

It was obtained from Tridelta, and was used according to the manufacturer’s instructions.

**Immunocytochemistry of the Mammary Gland**

Immunocytochemical analysis for M-SAA3 was performed on mammary tissue from the double-infused quarter and the within-cow control quarter from each of the \textit{S. aureus}-infused cows and from the double, saline-infused quarter of each of the control cows. For each quarter, analysis was performed separately on tissue taken from each of 3 regions, namely secretory epithelium, gland cistern, and teat canal. Sections were dewaxed and rehydrated in Histoclear (National Diagnostics, Atlanta, GA) for 2 × 5 min, placed in 100% (vol/vol) ethanol for 10 min, 90% (vol/vol) ethanol for 3 min, and 70% (vol/vol) ethanol for 3 min, and washed in Tris-buffered saline plus 0.05% (vol/vol) Tween (TBS) for 2 × 5 min. Endogenous peroxidases were blocked by incubating in 0.6% (vol/vol) hydrogen peroxide (Sigma Chemical Co., Dorset, UK) in methanol for 5 min at room temperature followed by rinsing in TBS for 2 × 5 min. Nonspecific binding was reduced by incubation with normal rabbit serum (1:10 dilution with TBS) for 20 min, followed by washing in TBS (2 × 5 min).

To stain mammary sections for M-SAA3, sections were incubated with a biotin-labeled rat monoclonal antibody to SAA (Tridelta) known to cross react with this mammary isoform (McDonald et al., 2001) diluted 1:50 in TBS containing 5% (vol/vol) fetal bovine serum for 1 h at room temperature. After rinsing (2 × 5 min) in TBS, sections were incubated for 45 min in an avidin-biotin complex (ABC) solution (Dako, Cambridgeshire, UK) according to the manufacturer’s instructions, followed by further washes. The sections were incubated with a solution of 3,3′-diaminobenzidine prepared according to the manufacturer’s instructions (Sigma), for around 1 min until staining could be seen under a microscope; the sections were then washed in distilled water. Nuclei were counterstained with Mayer’s hematoxylin (Dako) and blued with Scott’s tap water (Sigma).

Semiquantitative scoring of staining was performed by visual analysis using a 3-point scoring system where 0 = no staining, 1 = light staining, and 2 = heavy staining. Analysis was done separately on 5 subcellular locations within each section; namely the lumen of the alveoli or cistern, the apical membrane of the secretory or ductular cells, the basal membrane of these same cells, the cellular cytoplasm, and the extracellular matrix.
Table 1. The nucleic acid sequences of primers and probes for bovine acute phase proteins and proinflammatory cytokines

<table>
<thead>
<tr>
<th>Protein</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hp</td>
<td>ccaagagctgccacagat</td>
<td>g tgaggagcaggtcgcatcgct</td>
<td>acctatggggagcaacgtcc</td>
</tr>
<tr>
<td>M-SAA3</td>
<td>gcaagcgcaagcaggtga</td>
<td>gctgagccagctagcctgctc</td>
<td>cttcctcgccgatctatttctgc</td>
</tr>
<tr>
<td>IL-6</td>
<td>gggcctccagtagttgaga</td>
<td>gtcgctcagttgacaggtt</td>
<td>ttctgggccatctcttcagtgt</td>
</tr>
<tr>
<td>TFN-α</td>
<td>cgcgctgtagaatctcag</td>
<td>ggtctgcctacagcttcagtc</td>
<td>aagccctgtaggttctctgta</td>
</tr>
<tr>
<td>β-actin</td>
<td>gccttagagcttcgcctgc</td>
<td>gccttagagcttcgcctgc</td>
<td>gccttagagcttcgcctgc</td>
</tr>
</tbody>
</table>

1Oligonucleotide primers were designed using Primer Express software.
2Hp = Haptoglobin; M-SAA-3 = mammary-associated serum amyloid A3 (M-SAA3); TNF-α = tumor necrosis factor-α.

Quantitative PCR for mRNA of APP and Cytokines

Isolation of RNA was optimized for mammary (epithelial) tissue and liver tissue using guanidium thiocyanate-based methodology as previously described (Eckersall et al., 2001a). Reverse transcription of total RNA using MuLV reverse transcription was carried out according to O'Shaughnessy and Murphy (1993). Preparation of cDNA was carried out in batches containing only a single tissue type (i.e., liver or mammary gland) to minimize variation in cDNA between comparable tissue samples.

Quantitative real-time Taqman PCR was performed (Dolan et al., 2004) on cDNA for Hp, SAA, IL-6, TNF-α, β-actin (internal housekeeping gene), and an external standard, luciferase (Promega, Southhampton, UK) using a GeneAmp 5700 sequence detection system (PE Applied Biosystems, Warrington, UK). An external standard was included to control for the efficiency of RNA extraction, RNA degradation, and the reverse transcription step. Luciferase mRNA (5 ng per sample) was added to each tissue sample at the start of the RNA extraction procedure. The PCR reaction contained GeneAmp 10× PCR buffer (20 mM Tris-HCl, pH 9; 100 mM KCl; 0.1 mM EDTA; 1.0 mM dithiothreitol; 0.5% Tween 20; 50% glycerol), 2.5 mM MgCl₂, 200 μM of each dNTP, 0.625 U of AmpliTaq Gold polymerase (PE Applied Biosystems), molecular biology grade H₂O (BDH, Dorset, UK), 300 nM each of forward and reverse primers, and 200 nM of probe. Thermal cycling conditions were 2 min at 50°C, followed by 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C. For each quantification assay, samples were tested in duplicate. Taqman probes contained 6-carboxy-fluorescein (FAM) as a 5′-reporter and 6-carboxy-tetramethylrhodamine (TAMRA) as 3′-quencher. Oligonucleotide primers, designed using Primer Express software (PE Applied), are listed in Table 1 and were synthesized by MWG biotech (Ebersberg, Germany). All primer/probe sets had >80% efficiency level allowing multiple primer comparisons at the same time. For relative quantification of APP mRNA, the comparative Ct (cycle threshold) method was used (User Bulletin no. 2, PE Biosystems), which involves normalization of the number of target gene copies to the reference genes (i.e., β-actin and luciferase).

Statistical Analyses

Statistical analysis of the results in the first study period was carried out to compare between control and infused groups. Initial analyses of all raw data used procedures to determine optimum Box and Cox transformation of the data (Minitab, Release 13, Minitab Limited, Coventry, UK). Data for SAA and M-SAA3 underwent a natural logarithmic transformation with the reciprocal identified as the most appropriate transformation for milk Hp concentrations. Somatic cell count and serum Hp measurements underwent no further transformation before formal analyses.

The GLM procedure was fitted to data optimized by Box and Cox transformation using Minitab, Release 13. In the first analysis, results from milk samples taken from individual mammary glands in study period 1 were treated separately. The variates analyzed in the first study period were 1) SCC from control or infused quarters, 2) SAA, 3) Hp, 4) M-SAA3 from control or infused quarters, and 5) Hp from control or infused quarters.

The fixed effects considered were S. aureus (infused or control; 1 df), day (18 df), cow (9 df for infected and 7 df for control), and the interaction between S. aureus and day. The only random effects in the model were cows and cows were nested within treatment group.

At the end of the second study period (i.e., 48 h after Do1-2), the differences between noninfused, control quarters, and infused quarters (double infused, single early-infused, single late-infused) in the levels of SCC and milk concentrations of Hp and M-SAA3 were determined and then analyzed by a Wilcoxon signed ranks test. Differences between serum analytes in infused and control cows and between the expression of mRNA of Hp and SAA/M-SAA3 in tissues were com-
pared using the Mann-Whitney test for nonparametric distributions (Minitab, Release 13).

Immunocytochemical data for M-SAA3 distribution were also analyzed using GLM with S. aureus (double infused, control, or saline; 2 df); subcellular location (5 df), site within quarter (2 df), and the interaction of 1 isolated sample that reached 0.77 × 10^6 cells/mL, and no S. aureus was isolated from any of those milk samples. Because those 2 cows had not developed mastitis, their results were not included in the statistical analyses.

The SCC in milk from the quarters of the 8 control cows were low with a median of 0.036 × 10^6 cells/mL (n = 240) but with isolated and occasional samples having SCC reaching 0.3 × 10^6 cells/mL. On d 24 there was a rise in SCC in quarters of 3 of the control cows to 5.2 to 9.9 × 10^6 cells/mL.

Following infusion, the SCC in milk from infused quarters increased rapidly, from 0.03 ± 0.01 × 10^6 cells/mL (mean ± SEM) preinfusion to reach 9.20 ± 2.40 × 10^6 cells/mL within 36 h after infusion (Figure 1A). In the infused quarter, SCC remained elevated until d 24 when the mean was 5.0 ± 6.9 × 10^6 cells/mL in the milk from the noninfused quarter, the mean SCC remained at pretreatment levels (0.06 ± 0.01 × 10^6 cells/mL) apart from a slight rise to 1.24 ± 0.04 × 10^6 cells per/mL at 36 h posttreatment; the SCC in the quarters of noninfused cows was similarly low in all samples. The GLM analysis showed that there were significant (P < 0.001) differences in the RH quarter between S. aureus-infused and control groups. In addition, day was significant (P < 0.001) in terms of SCC in the infused quarter with the counts rising significantly in the group having quarters infused with S. aureus. In addition, for the RH quarter, the model identified that there was a significant effect of cow (P < 0.001) as well as a significant S. aureus × day interaction on SCC concentration in the infused quarter. In contrast, in the control quarter, only day (P < 0.001) and cow (P < 0.001) were significant.

**APP in Milk and Serum.** In milk from the quarters of cows infused with S. aureus, increases in Hp and M-SAA3 occurred rapidly. The mean concentration of

### Results

**SCC and Bacteriology.** Before the infusion, in milk samples taken on d −28, −21, −14, −7, and 0 from all quarters of all cows, the mean (± SEM) SCC was 0.03 ± 0.002 × 10^6 cells/mL, with a median of 0.02 × 10^6 cells/mL, and a range of 0.002 to 0.36 × 10^6 cell/mL, although only a few isolated samples had results at the top of this range and these were equally distributed among the infused and control quarters.

Clinical signs of mastitis (swelling, redness, pain in the udder, or clots in milk) were not observed in any of the infused cows or quarters. Subclinical mastitis was defined as an elevation in SCC from preinfusion to postinfusion, in the absence of clinical signs. Increased SCC in the infused quarters to at least 2 × 10^6 cells/mL indicated that subclinical mastitis was induced in at least 1 sample collected in the first 4 d postinfusion, and S. aureus was isolated from 6 of those 8 quarters on the day following infusion (Table 2). In contrast, the mean SCC in the within-cow control quarter did not rise above 0.10 × 10^6 cells/mL, and S. aureus was not isolated from any of those samples.

Two of the 10 cows in which the RH quarter was infused with S. aureus did not develop mastitis with their SCC being <0.5 × 10^6 cells/mL, with the exception of 1 isolated sample that reached 0.77 × 10^6 cells/mL, and no S. aureus was isolated from any of those milk samples. Because those 2 cows had not developed mastitis, their results were not included in the statistical analyses.

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### Results

**First Study Period**

### SCC and Bacteriology.

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### Table 2. Bacteriology and selected SCC

<table>
<thead>
<tr>
<th>Day relative to infusion</th>
<th>Infused group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. aureus isolated</td>
<td>SCC &gt;2.0 × 10^6 cells/mL</td>
</tr>
<tr>
<td>S. aureus-infused quarters</td>
<td>−7</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>6/10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1/10</td>
</tr>
<tr>
<td>Control quarters</td>
<td>−7</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0/10</td>
</tr>
</tbody>
</table>

1 The proportions of cows with a quarter in which bacteria (Staphylococcus aureus) were identified and the proportions of cows with SCC elevated above 2.0 × 10^6 cells/mL after the first infusion of either S. aureus or saline (control quarters).

2 ND = Not determined.
Figure 1. The response (mean ± SEM) of A) SCC; B) mammary-associated serum amyloid A3 (M-SAA3); C) haptoglobin in milk following infusion of *Staphylococcus aureus* during the first study period. In A and B, the lines show the response of the quarters infused with bacteria (∇), the within-animal control quarters (○), and quarters in the control animals (●). In C, the responses in the within-animal control quarters and quarters in the control animals were below the limit of detection and the line shows the response in the quarters infused with bacteria (∇).

M-SAA3 increased (Figure 1B) from a pretreatment level of <0.3 μg/mL to a mean concentration of 5.6 ± 12 μg/mL at the first sampling time posttreatment (15 h), and to a peak of 20.9 ± 11.9 μg/mL by d 2. Thereafter, there was some fluctuation, notably with a secondary peak of M-SAA3 at 17 d of 12.3 ± 27 μg/mL. In the control quarter in the same animals, the mean M-SAA3 concentration was <0.9 μg/mL except for peaks of 1.2 ± 2.7 and 1.9 ± 3.8 μg/mL at d 1 and 7, respectively. The mean M-SAA3 concentration in the milk samples from control cows was <0.75 μg/mL at all sampling times. The GLM analysis showed that there were significant (P < 0.001) effects in the infused quarter of day (P < 0.001) and cow. In addition, there was a significant *S. aureus* × day interaction (P < 0.001) in the infused quarter for M-SAA3 with the concentration rising in the infected quarters. In the control quarters, there were only significant effects on the M-SAA3 concentration with day (P < 0.01) and cow (P < 0.001).

The mean Hp concentration in milk samples from quarters infused with *S. aureus* (Figure 1C) was below 0.05 μg/mL, which was the limit of detection of the assay, before *S. aureus* infusion, but the mean Hp concentration rose to 7.2 ± 4.3 μg/mL within 12 h of treatment, and reached a peak at 36 h of 22.9 ± 10.1 μg/mL. There was subsequent fluctuation in the mean Hp concentration with peaks at d 5 of 25.9 ± 25 μg/mL and at d 21 of 25.3 ± 15 μg/mL. In the control quarter in the same cows, the Hp concentration was <0.05 μg/mL in the majority of samples assayed, but detectable levels of Hp were found in occasional samples. The Hp concentration in the milk samples from control cows was <0.05 μg/mL in virtually all samples assayed. The GLM analysis showed that there were significant differences in the infused quarter between *S. aureus*-infused and control groups (P < 0.005) and also a significant effect of day (P < 0.001) for milk Hp with the concentration rising significantly in the infused quarters. In addition, the model identified that there was a significant *S. aureus* × day interaction (P < 0.001) as well as a significant effect of cow (P < 0.001) on milk Hp concentration in the RH quarters. In contrast in the control quarters, only a significant effect of *S. aureus* (P = 0.015) was identified.

In serum from cows in the infused group with quarters infused with *S. aureus*, the mean concentration of SAA increased following infusion from a mean of 1.3 ± 0.4 μg/mL to a peak of 115 ± 37 μg/mL 2.5 d after infection (Figure 2A). The first detectable rise in SAA was 24 h after infection (mean SAA concentration, 76.1 ± 47 μg/mL). The mean concentration of SAA in the cows in the control group, in which quarters were infused with sterile saline, was below 6 μg/mL apart from 2 peaks of increased concentration with 1 before
The response (mean ± SEM) of A) SAA; and B) haptoglobin (Hp) in serum following infusion of *Staphylococcus aureus* during the first study period. The lines show the response in the infected animal group (•/H17034) and the control animal group (○/H17033).

The mean SAA concentrations for these points were 34.3 ± 26 and 46.7 ± 44 µg/mL, respectively, and were due to elevated SAA in single samples from different animals. The GLM analysis for SAA identified a significant difference between *S. aureus* and control groups (*P* = 0.02), as well as a significant effect of day (*P* < 0.001) with the SAA concentration rising significantly in the *S. aureus*-infused animals. In addition, the model identified that there was a significant cow effect (*P* < 0.001) as well as a significant *S. aureus* × day interaction (*P* < 0.001) on SAA concentration.

The mean serum Hp concentration in cows with mammary glands infused with *S. aureus* showed a rise from <0.01 mg/mL on the day of treatment to reach a peak of 0.54 ± 0.12 mg/mL by d 2.5 (Figure 2B). The first detectable increase was found after 24 h when a mean Hp concentration was 0.08 × 0.03 mg/mL. The *S. aureus*-infused group showed elevated mean Hp concentration before infusion on d −7 and −14 but these were due to spikes of increased serum Hp in different individual animals and were not associated with increases in SCC, Hp, or M-SAA in milk from the quarters that were subsequently infused with *S. aureus*.

The serum Hp concentration in the control cows was generally below 0.01 mg/mL with minor peaks at d −14 (0.13 ± 0.12 mg/mL) and d 10 (0.09 ± 0.08 mg/mL). The GLM analysis showed that there were significant differences between *S. aureus*-infused and control groups (*P* < 0.005) as well as a significant effect of day (*P* < 0.001) for serum Hp with the concentration rising significantly in the infected animals. In addition, the model identified that there was a significant cow effect (*P* < 0.001) and *S. aureus* × day interaction on serum Hp concentration between infused and control groups.

**Second Study Period**

**SCC.** The second infusion of quarters with *S. aureus* led to increases in the mean SCC in all quarters that were infused with bacteria. Results are given for the mean (± SEM) of the milk samples taken 48 h after infusion just before euthanasia (Table 3) because this is most relevant to the subsequent immunochemical and quantitative PCR investigations. The mean SCC in the single late-infused quarter rose from a normal level of 0.02 ± 0.05 × 10⁶ cells/mL to reach a peak of 8.9 ± 2.4 × 10⁶ cells/mL 48 h after treatment. In the double-infused quarter, the mean SCC before the second infusion was already elevated at 2.8 ± 1.2 × 10⁶ cells/mL and showed a further moderate increase to 5.60 ± 3.60 × 10⁶ cells/mL 48 h after treatment. However, the single early-infused quarter also showed a small increase and was at 5.3 ± 3.0 × 10⁶ cells/mL at the end of the study period having been 2.6 ± 1.6 × 10⁶ cells/mL on D012. The mean SCC in the within-cow control quarters (which were not infused with *S. aureus* at either occasion) rose from 0.05 ± 10⁶ cells/mL to 0.20 ± 0.05 × 10⁶ cells/mL. The mean SCC in 3 of the quarters in the control cows was <1.2 × 10⁶ cells/mL at all times sampled. The mean SCC in the fourth quarter (LH) of the control cows was elevated with a SCC of 5.3 ± 3.9 × 10⁶ cells/mL.

The SCC were significantly increased in the bacterially infused quarters (single- or double-infused) compared with the control quarter in the same cow (Wilcoxon signed ranks test; all *P* < 0.05).

**APP in Milk and Serum.** The second infusion with *S. aureus* led to increases in the mean M-SAA concentration in quarters infused with *S. aureus* for either the first or second time (Table 3). The mean M-SAA3...
concentration in the single late-infused quarter rose from 2.1 ± 1.9 μg/mL to reach a peak of 43.2 ± 19.4 μg/mL on the second day posttreatment. In the double-infused quarter, the mean M-SAA3 concentration before this second infusion was 2.2 ± 1.4 μg/mL and showed an increase to 16.5 ± 6.2 μg/mL on d 2I-2. The mean M-SAA3 concentration in 3 of the quarters in the control cows was <1.0 μg/mL at all times, but the mean M-SAA3 concentration in milk from the fourth (LH) quarter was 4.8 ± 4.6 μg/mL at euthanasia. The single, early-infused quarter had mean M-SAA3 concentrations rising from 3.2 ± 2.7 μg/mL up to 5.6 ± 3.2 μg/mL. The mean M-SAA concentration in the within-cow control quarters was 0.3 ± 0.1 μg/mL at the end of the experiment. The M-SAA3 concentrations were significantly increased in the single- and double-infused quarters compared with the control quarter in the same cow (Wilcoxon signed ranks test; all P < 0.05).

The second infusion of quarters with *S. aureus* led to increases in the mean milk Hp concentration 2 d after infusion with *S. aureus* for either the first or second time (Table 3). The mean Hp concentration in the single late-infused quarter rose from 1.7 ± 1.6 μg/mL to reach 11.5 ± 3.1 μg/mL 48 h posttreatment. In the double-infused quarter, the mean Hp concentration before this second infusion was 12.3 ± 8.5 μg/mL and showed an increase to 25.0 ± 16 μg/mL after 2 d. The single, early-infused quarters had a mean Hp concentration that started at 25.7 ± 24.7 μg/mL, showed fluctuation, but was 21.7 ± 15.8 μg/mL at euthanasia. The mean Hp concentration in the within-cow control quarters was 0.5 ± 0.1 μg/mL. The mean Hp concentration in 3 quarters in the control cows was <0.7 μg/mL, but in the fourth (LH) quarter was 3.6 ± 3.1 μg/mL. The elevated mean values of SCC, M-SAA3, and Hp were caused by raised levels of all 3 analytes in this quarter of only one of the control cows and was presumed to be caused by incidental IMI.

Milk Hp concentrations were significantly increased in the single- and double-infused quarters during the second study period compared with the control quarter in the same cow (Wilcoxon signed ranks test; both P < 0.05). There was no significant difference between the control quarter and the single-infused quarter that had been infused at the start of the first study period.

In the second study period, the mean serum concentrations of SAA in the *S. aureus*-infused cows on the day of the second infusion was 33.2 ± 26 μg/mL and increased to 63.2 ± 20 μg/mL on d 2I-2 (Table 4). In the control cows, the mean SAA concentration was 2.1 ± 1.3 μg/mL on the day of treatment but rose to 19.2 ± 11.0 μg/mL on d 2I-2. The mean serum Hp concentration in the *S. aureus*-infused cows was undetectable on the day of treatment (<0.01 mg/mL) but rose to 0.31 ± 0.10 mg/mL on d 2I-2 (Table 4). In the control cows, the mean serum Hp concentration was <0.01 mg/mL before the second infusion but did rise to 0.06 ± 0.03 mg/mL 48 h after the infusion.

The mean serum Hp concentration was significantly (P < 0.05) higher in the *S. aureus*-infused animals than in controls, whereas the mean SAA concentration tended to be significantly higher in the infused animals compared with the controls (P = 0.07; Mann-Whitney test).

**APP in Mammary Tissue by Immunocytochemistry.** Immunocytochemistry using antisera that cross-reacts to both SAA and M-SAA3 (McDonald et al., 2001) was used to identify M-SAA3 within mammary tissues. As shown in Figure 3, M-SAA3 was present in secretory epithelium, gland cistern, and, to a lesser extent, teat canal. Staining associated with secreted milk was detected in the lumen of alveoli and ducts and on the apical (milk-facing) membrane of secretory alveolar cells. Scoring data are in Table 5, which quantifies total detection of M-SAA3 (i.e., in the section as a whole) as well as detection at 5 subcellular

### Table 3. Somatic cell count (mean ± SEM), mammary-associated serum amyloid A3 (M-SAA3; mean × SEM), and haptoglobin (Hp; mean ± SEM) in milk from cows 2 d after the second infusion (D0/2) with saline or *Staphylococcus aureus*

<table>
<thead>
<tr>
<th>Group</th>
<th>Quarter1</th>
<th>Designation</th>
<th>D0 treatment</th>
<th>D0/2 treatment</th>
<th>n</th>
<th>SCC, ×10⁶ cells/mL</th>
<th>Hp, μg/mL</th>
<th>M-SAA3, μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>LF</td>
<td>Control Saline</td>
<td>8</td>
<td>0.3 ± 0.1</td>
<td>0</td>
<td>3.0 ± 0.1</td>
<td>4.8 ± 4.6</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>RH</td>
<td>Control</td>
<td>Saline</td>
<td>8</td>
<td>5.3 ± 3.9</td>
<td>3.6 ± 3.1</td>
<td>4.8 ± 4.6</td>
<td>3.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>RH</td>
<td>Control</td>
<td>Saline</td>
<td>8</td>
<td>1.0 ± 0.9</td>
<td>0</td>
<td>3.0 ± 0.1</td>
<td>4.8 ± 4.6</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>RH</td>
<td>Control</td>
<td>Saline</td>
<td>8</td>
<td>1.1 ± 0.6</td>
<td>0.6 ± 0.6</td>
<td>3.0 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus-infused</td>
<td>LF</td>
<td>Within-cow control</td>
<td>8</td>
<td>0.2 ± 0.05</td>
<td>0.5 ± 0.1</td>
<td>3.0 ± 0.1</td>
<td>4.8 ± 4.6</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>LH</td>
<td>Single late-infusion quarter</td>
<td>S. aureus</td>
<td>8</td>
<td>8.8 ± 2.4**</td>
<td>11.5 ± 3.1*</td>
<td>43.2 ± 19.4*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RF</td>
<td>Single early-infusion quarter</td>
<td>S. aureus</td>
<td>8</td>
<td>5.3 ± 3.0**</td>
<td>21.7 ± 15.6**</td>
<td>5.6 ± 3.2**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RH</td>
<td>Single late-infusion quarter</td>
<td>S. aureus</td>
<td>8</td>
<td>5.6 ± 3.6**</td>
<td>25.0 ± 16.0**</td>
<td>16.5 ± 6.2**</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1Quarter: LF = left front; LH = left hind; RF = right front; RH = right hind.

*P < 0.05; **P < 0.01; NS=Nonsignificant, P > 0.05.
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Table 4. Mean serum amyloid A (SAA; mean × SEM) and haptoglobin (Hp; mean × SEM) in serum from cows 2 d after second infusion (D0₂) of either saline or *Staphylococcus aureus* Group D0 treatment D0₂ treatment Hp, μg/mL SAA, μg/mL

<table>
<thead>
<tr>
<th></th>
<th>D0 treatment</th>
<th>D0₂ treatment</th>
<th>Hp, μg/mL</th>
<th>SAA, μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Saline</td>
<td>Saline</td>
<td>0.06 ± 0.03</td>
<td>19.3 ± 11.0</td>
</tr>
<tr>
<td><em>S. aureus</em>-infused</td>
<td><em>S. aureus</em></td>
<td><em>S. aureus</em></td>
<td>0.31 ± 0.10**</td>
<td>63.2 ± 20.0NS</td>
</tr>
</tbody>
</table>

**P < 0.05; NS Nonsignificant (P = 0.07).

locations. Irrespective of the location, M-SAA3 was consistently detected at lower levels in tissue from control cows than in tissue from infected cows. These differences were significant by ANOVA for all locations except cytoplasm. Within infused cows, there was little difference between tissue from *S. aureus*-infused quarters and tissue from saline-infused (control cows) quarters. The teat canal consistently exhibited less staining for M-SAA3 than either secretory epithelium or gland cistern, which had similar patterns of staining.

**Figure 3.** Examples of immunocytochemical detection of mammary-associated serum amyloid A3 (M-SAA3) in the gland cistern (A and B) and secretory epithelium (C and D) of infected cows. Positive staining associated with secreted milk was detected in the lumen of alveoli and ducts and membrane-associated staining was detected on the apical (milk-facing) surface of secretory alveolar cells. Staining was absent from control cows.
These differences among sites within the mammary gland were significant for total detection and for the alveolar lumen and apical membrane only. At the cellular and subcellular level, M-SAA3 was detected at higher levels in the alveolar lumen and apical membrane than at other locations (P < 0.001). This difference was evident irrespective of treatment and site within the gland.

Attempts to stain mammary tissue for Hp using an ovine antiserum to bovine Hp (Eckersall and Conner, 1990) were not successful.

**APP mRNA Expression.** Considerable among-cow variation was evident in the real-time RT-PCR data such that differences between mean APP mRNA expression were not significant (P > 0.05) between control and *S. aureus*-infused samples. However, trends were apparent in the results for both M-SAA3/SAA and Hp with, in mammary tissue, increasing effects observed with single and double infusions of *S. aureus* (Figure 4, A and B). The expression of mRNA for Hp and M-SAA3 in mammary tissue relative to β-actin in mammary gland tissue increased in the samples taken 48 h after a single infusion of *S. aureus*, and increased further in quarters that received a double infusion of bacteria. The expression of Hp and SAA mRNA in liver were similarly increased after infection (Figure 4, C and D).

In mammary tissue, average levels of M-SAA3 mRNA expression increase were highest in the double-infused quarter compared with the single, late-infused quarter or tissue from a quarter from a control cow. The double-infected quarter showed an 11-fold increase of relative expression of M-SAA3 to β-actin over the control, and a 3.6-fold increase over the single late-infused quarter (Figure 4A). Similarly, Hp mRNA expression was highest in the double-infused quarter (64-fold that of control) compared with the single, late-infused quarter (13-fold that of control) or control quarter (Figure 4B).

In all groups, the expression of M-SAA3 was greater than that of Hp; in the control group the relative expression of mRNA for M-SAA3 was 362-fold greater than the relative expression of Hp (P < 0.001); following single late-infusion, the difference was 101-fold (P < 0.01) and in double-infused expression of M-SAA3 was 64-fold greater than that of Hp (P < 0.001).

In liver tissue, mean levels of SAA mRNA expression increased following infusion of *S. aureus*. Mean levels of SAA mRNA relative to the expression of β-actin were 2.3-fold higher in the infected liver than control liver (Figure 4C), whereas the relative increases in expression of mRNA for Hp was 3.4-fold higher than in the control samples (Figure 4D).

The expression of mRNA for Hp was significantly higher (21-fold) than the expression of SAA in liver samples from infected cows (P < 0.001). There was no significant difference in expression of mRNA for Hp and for SAA in liver samples from the control cows.

Although real-time RT-PCR was attempted with primers for bovine cytokines, expression of mRNA for IL-6 and TNF-α in mammary tissue and liver taken 48 h after infusion of *S. aureus* was quite variable among individuals within groups such that no significant differences were detected between groups.

**DISCUSSION**

One objective of this investigation was to induce subclinical mastitis, which is more difficult to diagnose than the clinical form of the disease; hence, a low-virulence field strain of *S. aureus* was selected for infusion. Infusion of *S. aureus* induced subclinical mastitis (defined as SCC > 2.0 × 10^6 cells/mL) in 8 of 10 cows. That 2 of the original 10 animals failed to develop...
Figure 4. The expression of mRNA for A) mammary-associated serum amyloid A3 (M-SAA3) in mammary tissue; B) haptoglobin (Hp) in mammary tissue; C) serum amyloid A (SAA) in liver; and D) Hp in liver in noninfused (control) quarters, single-infused quarters (infused 1×), and double-infused quarters (infused 2×). The results (mean ± SEM) are relative to the expression of β-actin.

subclinical mastitis (no bacteriological isolation of S. aureus or other pathogen associated with clinical mastitis and no rise in SCC) indicated that they were resistant to infection with the strain of the pathogen used; results from these cows were therefore excluded from the study.

Results from the first study period demonstrated the pathophysiological response to the infection. The SCC in milk from infected quarters, the established marker for the disease, showed a rapid increase, demonstrating a well established pattern of intramammary cellular immune response to bacterial invasion (Sears et al., 1993; Sears and McCarthy, 2003).

The concentrations of both M-SAA3 and Hp in milk from the infected quarter rose by the first sampling time point at 15 h, indicating that there was either a rapid production of these proteins locally or marked leakage from the circulation. The rapid increases measured in the concentration of milk APP are consistent with previous studies (Gronlund et al., 2003; Pedersen et al., 2003). However, the finding here that the earliest times for detection of increased serum concentrations of both of these proteins was at 24 h postinfusion demonstrated that blood was unlikely to be the source of the immediate increase in milk. This conclusion is supported by the earlier and higher peak in milk M-SAA3 compared with SAA in serum recently reported by Lehtolainen et al. (2004). The mean concentration of M-SAA3, Hp, and SCC in both the within-cow control quarter and the quarters in the control cows increased slightly after treatment with sterile saline, but were not significantly different from the preinfection levels. Although an APR to an infection in a specific quarter did lead to a systemic APR, with increases in serum Hp and SAA, other quarters in the same cow were
unaffected, confirming findings from cases of naturally occurring clinical mastitis (Nielsen et al., 2004).

Serum concentrations of APP, and of Hp in particular, give further support that the experimental model was successful in the induction of subclinical *S. aureus* mastitis. The concentration of Hp in serum can rise to a level of 3 mg/mL in clinical cases of mastitis and other acute bacterial infections (Horadagoda et al., 1999). In our study, there were no samples with serum Hp of >1.0 mg/mL, confirming that only a mild to moderate APR had been stimulated. These increases in serum concentrations of SAA and Hp indicate that a mediator of the APR, probably a proinflammatory cytokine of mammary origin stimulated production of these proteins in liver. Interesting differences were also evident in the relative concentrations of the 2 APP in serum and milk. The peak concentration of M-SAA3 in milk was one-sixth that of the peak concentration of SAA in serum, whereas the peak concentration of Hp in milk was only one-sixteenth of the amount obtained in serum.

The second study period of the experimental model, in which measurement of APP and SCC was assessed in milk from all 4 quarters of *S. aureus*-infused and control cows, was undertaken to determine if the pathophysiological responses of individual quarters were affected by prior infection. Between the time of the second infusion and euthanasia there was considerable variation among individuals in SCC, SAA, and serum Hp. No significant differences were found between samples from quarters infused with *S. aureus* illustrated by the SCC and protein concentrations found in the milk sample taken before euthanasia. Thus, there was no difference in the levels of these analytes in quarters that had been infected once either 48 h or 30 d before euthanasia or twice (both 48 h and 30 d prior). When compared with the intramammary control quarter, which had not been infused on any treatment day, the mean SCC and milk concentrations of both M-SAA3 and Hp were significantly raised in all infected quarters.

In quarters that received 2 infusions of bacteria, there was a tendency for the level of M-SAA3 and Hp to be greater after the second infusion than after the first infusion. Maximum mean M-SAA3 was 40 μg/mL in the second infusion compared with 20 μg/mL after a single infusion, whereas SCC in the second infusion peaked at 1.7 × 10^6 cells/mL compared with 1.0 × 10^6 cells/mL in quarters that had only a single infusion. These results provide support for the use of M-SAA3 and milk Hp as biomarkers of mastitis, which can provide a quantitative estimate of the severity of the condition (Eckersall et al., 2001b; Gronlund et al., 2003; Nielsen et al., 2004; Petersen et al., 2004) although further investigation is warranted to aid the interpretation of the APP results and in particular to determine if changes in the ratio between the APP in milk can provide diagnostic information on the state of the disease.

The results of serum and milk APP provide circumstantial evidence that the origin of both APP in milk is not from the circulation but from production in the mammary gland. Confirmation of this possibility for M-SAA3 was derived from the results of immunocytochemistry, which showed that this protein was present in the secretory epithelium, gland cistern and, at a significantly lower level, in the teat canal. The finding of staining significantly higher in tissue from *S. aureus*-infused quarters than in that of control cows, in the alveolar lumen (i.e., in secreted milk) and associated with the apical (milk-facing) membrane was of great interest. Taken together with the relative absence of staining on the basal membrane, this suggests that the origin of M-SAA3 in milk may be synthesis within secretory epithelial cells and cells lining the gland cistern. There was no evidence that this was a direct and local response to the presence of *S. aureus* pathogen. Surprisingly, similar levels of M-SAA3 were detected at the same subcellular locations in quarters of infected cows that had not been infused. This is an unexpected observation, because milk M-SAA3 concentration was only elevated in *S. aureus*-infused quarters. Normally the teat canal is the first line of defense against invasion by pathogen. The experimental model used here involved infusion of pathogen directly into the gland cistern, effectively bypassing the teat canal. The fact that less M-SAA3 was detected in the cells lining the teat canal may reflect this aspect of the model, rather than resulting from lack of synthetic capability.

Although Hp synthesis in mammary tissue induced by the *S. aureus* infusion was not detected by immunocytochemistry, the probability of local production of both M-SAA3 and Hp mRNA in the mammary gland was further supported by quantitative RT-PCR. Although *S. aureus* infusion only induced a change that tended to be significant, the findings agree with the conclusion that M-SAA3 (Molenaar et al., 2002) and Hp (Hiss et al., 2004) present in milk are synthesized in the mammary gland. The finding that upregulation of M-SAA3 relative to β-actin was greater than that of Hp in mammary epithelia, with the reverse being true in liver, is interesting, and is in agreement with their relative serum and milk concentrations during an APR (serum Hp > serum SAA; M-SAA3 > milk Hp). These differential alterations in expression of Hp and M-SAA3 mRNA in liver and mammary tissues following *S. aureus* infusion might indicate differences in
the control of synthesis between the systemic APR of the liver and the mucosal APR in the mammary gland.

The lack of significant findings in the levels of expression of the proinflammatory cytokines following infection may be due to the timing of the sample collection because they were taken at the last sampling time point—48 h after infection. Although TNFα could be measured by ELISA in milk whey up to 5 d after experimental Streptococcus uberis infection (Rambeaud et al., 2003), no difference in expression of TNFα could be found between infected and control tissues 9 h or more after an LPS stimulation of mastitis (Schmitz et al., 2004).

The findings of this study provide strong evidence for production of significant amounts of APP in milk during IMI. This result and the previous discovery that M-SAA3 is present in colostrum (McDonald et al., 2001) prompt the question as to the likely functions of APP in milk. It has been found that the isoform of M-SAA3 has specific antibacterial functions in intestinal cells, stimulating secretion of mucus (Larson et al., 2003b; Mack et al., 2003). The ability to stimulate the neonatal intestinal cells to begin or increase secretion of mucus that can reduce bacterial colonization of intestines would be very valuable in milk. In addition, SAA has been shown to bind with high affinity to gram-negative bacteria (Hari-Dass et al., 2005), presumably with a defensive antibacterial action. If M-SAA3 has the same activity, then this could also be a major function for M-SAA3 in milk. Haptoglobin may have a similar useful bioactivity in milk, possibly related to its known antioxidant actions (Lim et al., 1998), which might be valuable in countering bacterial invasion of neonatal intestine. Such activities would be useful in colostrum and milk to improve the resistance to disease of the neonate and could thus represent a passive transfer of innate immunity to the calf.

The evidence presented in this study shows that significant extrahepatic production of APP occurs in the mammary gland during bovine mastitis. Because these mediators of the innate immune response have a potentially major role in defense against infection, the extrahepatic production and activities of the APP in the mammary gland and their role in passive transfer of innate immunity to neonatal animals should be investigated.

ACKNOWLEDGMENTS

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