Cytokine response of bovine mammary gland epithelial cells to *Escherichia coli*, coliform culture filtrate, or lipopolysaccharide

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Objective—To define the cytokine response of a cultured mammary gland epithelial cell line (ie, Mac-T) when incubated with *Escherichia coli* or its products.

Sample Population—Mac-T cells and *E coli* from cows with mastitis.

Procedure—Mac-T cells were incubated with *E coli* or its products. The cytokine response of Mac-T cells to these treatments was quantified by measuring mRNA content of interleukin (IL)-1α, IL-1β, IL-8, and tumor necrosis factor (TNF)-α by use of a quantitative reverse transcriptase-polymerase chain reaction assay. The amount of TNF-α secreted was also measured.

Results—Treatment with *E coli* or its products resulted in significant increases in IL-1α, IL-8, and TNF-α mRNA content in Mac-T cells. This increase was reversible when culture filtrate was incubated with polymyxin B. The amount of IL-1β mRNA in Mac-T cells increased only slightly over baseline after treatment with *E coli* or its products, but this increase was not diminished by incubation of *E coli* filtrate with polymyxin B.

Conclusions and Clinical Relevance—Incubation of Mac-T cells with *E coli* or its products resulted in increased amounts of IL-1α, IL-8, and TNF-α mRNA. Inhibition of this response by incubation of culture filtrate with polymyxin B suggested that lipopolysaccharide was the main bacterial product that stimulated the cytokine response. The small increase in IL-1β content in Mac-T cells incubated with *E coli* or its products suggested that this cytokine had a smaller role in the Mac-T cell response to *E coli*. (Am J Vet Res 2005;66:1590–1597)

Mastitis is a costly disease for dairy farmers in North America, costing up to $184/cow/y and up to $1.7 billion annually in the United States. Many cases of acute mastitis are a result of infections with *Escherichia coli* and *Klebsiella* spp, which are important in the initiation of the host inflammatory response to infection. Interleukin-1 (IL) is a form bound to the cell membrane, and IL-1β, which is secreted and involved in activation of distant cells. In cattle, systemic admin-
Irritation of IL-1 can induce some of the clinical signs associated with mastitis, including fever, inappetence, and diuresis. At the tissue level, IL-1 can stimulate human endothelial cells to upregulate adhesion molecules, which increases adherence of neutrophils to the vasculature and their activation. In addition, production of IL-1 stimulates production of IL-8 by cultured bovine epithelial cells. The ability of IL-1 to stimulate neutrophil adherence and the production of other cytokines may be important in initiating inflammation associated with mastitis.

Interleukin-8 is another cytokine that is important in the inflammatory response associated with mastitis. Numerous bovine cells, including macrophages, epithelial cells, and endothelial cells, can produce IL-8 when stimulated. Interleukin-8 is a potent chemotactic for bovine neutrophils, serving as a signal for neutrophil migration into the mammary gland. In addition to its ability to attract neutrophils, IL-8 can also activate neutrophils. Activated neutrophils may serve as an important source of IL-8 in the later stages of E coli mastitis. In 1 study, investigators used a hybridization to document neutrophil production of IL-8 in the lungs of calves experimentally infected with Mannheimia haemolytica.

Tumor necrosis factor is also involved in mammary gland inflammation. Milk content of TNF-α increases after infusion of mammary glands with LPS, and SC injection of TNF-α induces changes in milk yield and fat content that are similar to those seen during coliform mastitis. Injection of TNF-α directly into the teat cistern can cause substantial migration of neutrophils into the mammary gland. Tumor necrosis factor-α can also interact directly with bovine neutrophils to stimulate enhanced phagocytosis, production of reactive-oxygen species, and degranulation. Similar to IL-1 and IL-8, TNF-α is produced by many bovine cell types, including mammary gland macrophages, epithelial cells, and endothelial cells.

The objective of the study reported here was to determine the effects of E coli, E coli culture filtrate, or E coli LPS on IL-1α and β, IL-8, and TNF-α production by bovine mammary gland epithelial cells. We used a cultured bovine mammary gland epithelial cell line (ie, Mac-T) to investigate these effects.

**Materials and Methods**

Bovine mammary gland epithelial cells—A transformed bovine mammary gland epithelial cell line (ie, Mac-T), originally created in another study, was used for the source of mammary gland epithelial cells. Cells were grown in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), 0.1% of cortisone–2-hydroxypropyl-β-cyclodextrin complex/mL, and insulin. Cells were grown to confluence and then detached from the flask by addition of 1 mL of 0.25% trypsin EDTA solution warmed to 37°C. The flask was placed into an incubator (37°C) for approximately 1 minute. The cell layer in the flask was examined by use of an inverted microscope. Once >75% of the cells were detached, the flask was vigorously tapped to loosen the remainder of the attached cells. One hundred microliters of FBS was added to the flask to neutralize the trypsin. Detached cells were transferred to a 15-mL conical tube and centrifuged at 200 X g for 6 minutes. Supernatant was removed and discarded, and the pellet was resuspended in 12 mL of medium warmed to 37°C. Cell monolayers were allocated to 2 or 3 flasks and grown to confluence. Culture medium was changed every 2 days. Trypsin-EDTA solution was used to detach the cells, as previously described.

**Preparation of E coli, E coli culture filtrate, and LPS—**A commercially available E coli–derived LPS solution was used for the LPS treatment experiments. The E coli culture filtrate was derived from 3 clinical isolates (Nos. 832390, 891032, and 891170) of E coli that were initially isolated from cows with mastitis in samples submitted to a state diagnostic laboratory. Each of the isolates was nonhemolytic and had similar results for sugar metabolism, except for isolate 832390, which had negative results for metabolism of rhamnose. For routine culturing, bacteria were streaked onto nutrient agar plates approximately once per month. Plates were incubated at 37°C for 2 days and then stored at 4°C. To prepare the culture filtrate, a single isolated colony of E coli was transferred to 12 mL of RPMI medium and incubated at 37°C on a rotary shaker for 8 hours. The bacterial culture was centrifuged (1,000 X g for 10 minutes) to pellet most of the bacteria, and a 0.22-μm syringe filter was used to remove the remainder of the bacteria from the culture filtrate. The bacterial pellet was resuspended in fresh RPMI medium for use in the experiments. Bacteria numbers were estimated by use of a spectrophotometer to measure the optical density at 600 nm, and then we extrapolated the number of CFUs from a standard curve developed for the organism. Bacteria numbers were confirmed by serially diluting the bacterial suspensions and plating on blood agar; the plates were incubated at 37°C for 24 hours, after which bacterial colonies were counted.

**Study design—**An initial time-course study was performed by incubating Mac-T cells with LPS (1.5 μg/mL) for 0, 0.25, 0.5, 0.75, 1, 2, 4, and 6 hours. Cells were then washed, and mRNA was isolated for quantitative analysis by use of a reverse transcriptase-polymerase chain reaction (RT-PCR) assay. The 1-hour time point was used for the remainder of the quantitative RT-PCR assay experiments because the peak amounts of mRNA for most of the cytokines were detected at that time.

Dose-response experiments were performed by adding E coli–derived LPS (0, 0.15, 0.75, 1.5, and 15 μg/mL) to the Mac-T cells and incubating for 1 hour, after which the cells were washed and mRNA isolated for quantitative RT-PCR assay. Experiments involving E coli culture filtrate were performed in a similar manner by adding 0.1 mL of the culture filtrate to the Mac-T cells and incubating for 1 hour prior to washing the cells and isolating the mRNA. Polymyxin B (5 μg/mL) was added to a portion of the culture filtrate to neutralize the LPS. The polymyxin B was added 15 minutes before the culture filtrate was added to the Mac-T cells. Medium alone was used as a negative control sample. In another series of experiments, Mac-T cells were incubated with 5 X 10⁶ E coli/mL or medium alone for 1.5 hours. Supernatant was removed and filtered through a 0.22-μm filter. Supernatant was analyzed by use of a bioassay to determine TNF-α release from the Mac-T cells. The Mac-T cells on the plate were washed twice, and mRNA was isolated for quantitative RT-PCR assay.

Cells incubated with E coli, culture filtrate, or LPS were isolated from the flask by use of the procedures described previously. These cells were grown in 12-well tissue culture plates with medium that did not contain cortisone and were used for the experiments when the cells were confluent.

**IL-1α, IL-1β, IL-8, and TNF-α mRNA content—**Quantitative RT-PCR assay was used to measure changes in cytokine mRNA content in Mac-T cells after incubation with E coli cells, culture filtrate, or LPS. After incubation, Mac-T...
cells were gently scraped from the flask and transferred to 15-mL conical tubes. Cell suspensions were centrifuged (1,000 × g for 5 minutes), and the supernatant was removed. A commercially available RNA assay kit was used to recover mRNA from the cells. An on-column DNase digestion was performed to remove contaminating genomic DNA. The amount of mRNA recovered was determined by measuring the ratio between the absorbance at 260 and 280 nm by use of a spectrophotometer.1

The mRNA was converted to cDNA by use of a commercially available system. Briefly, 1.5 µg of mRNA recovered from the samples was added to a master mixture consisting of magnesium chloride, RNaase inhibitor, 10× buffer, oligo-DT primers, and reverse transcriptase. The solution was heated at 70°C for 10 minutes, incubated at 42°C for 45 minutes, and finally heated at 95°C for 5 minutes. The cDNA samples were then stored at −20°C until further use.

Primers used to amplify IL-1α, IL-1β, IL-8, TNF-α, and the housekeeping gene β-actin were designed by use of a software program (Appendix). Published bovine sequences for these genes were used to design the primers. All primers were designed to amplify approximately 100 bp of DNA and have a melting point of 60°C. To confirm the uniqueness of the primers to amplify the desired genes, the amplicon was evaluated by use of an online nucleotide search program to determine whether other genes may potentially have been amplified with the primer pairs. The primers were constructed at our university’s biotechnology center.

Primers were reconstituted in H2O to achieve a final concentration of 100µM. Primer dilutions for the various cytokines were initially performed to determine a concentration to use in the reactions that yielded a good sample signal but avoided the formation of primer dimers. This concentration of formers for each primer was combined with a master mixture and cDNA template in a 96-well optical reaction plate. Additional samples containing only H2O and original mRNA samples prior to the RT-PCR assay were added as control samples.

All samples were assayed on a sequence detector system. Cycle variables were 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 0.15 minutes and 60°C for 1 minute. After completion of the PCR cycles, a melting point–dissassocation curve was generated to verify the lack of primer dimer formation and other extraneous double-stranded DNA products. A cutoff of 1 was chosen for the threshold value for the amplicons. The fold increase was calculated by use of a comparative method.

TNF-α secretion—A bioassay was used to determine TNF-α secretion by Mac-T cells after incubation with E coli or medium. The Mac-T cell–conditioned medium was stored at −70°C until assayed by use of a TNF-sensitive cell line (ie, L929 cells). Briefly, L929 cells were grown in medium consisting of DMEM containing 10% FBS, 1% HEPES, and 1% penicillin-streptomycin at 37°C and 5% carbon dioxide. When the cells were between 90% and 100% confluent, they were removed by scraping and then washed twice with addition medium. The L929 cells were added to a 96-well tissue culture plate at a concentration of approximately 150,000 cells/well. Plates were incubated overnight at 37°C and 5% carbon dioxide. Medium was removed from the L929 cells and replaced with medium consisting of DMEM with 2% FBS and 3 µg of actinomycin D/mL. Dilutions of Mac-T cell–conditioned medium or recombinant-human TNF-α standards were also added to the wells to achieve a final volume of 100 µL/well. Plates were incubated overnight at 37°C and 5% carbon dioxide. Twenty microliters of methylthiazol tetrazolium was added to each well, and plates were then incubated at 37°C for an additional 3 hours. Plates were centrifuged at 500 × g for 10 minutes, and medium was carefully removed from the wells. Cells were then resuspended in 100 µL of extraction buffer consisting of 50% N,N-dimethylformamide and 20% SDS in H2O. After a final incubation (37°C for 1 hour), plates were analyzed by use of a microplate reader at a wavelength of 570 nm. Concentrations of TNF-α in the samples were calculated from the standard curve prepared with serial amounts of recombinant-human TNF-α.

Statistical analysis—All data were analyzed by use of a statistical software package. Significant differences were determined by use of a 1-way ANOVA. Values of P ≤ 0.05 were considered significant.

Results

Cytokine response of Mac-T cells after incubation with LPS—Quantitative RT-PCR assay was used to measure cytokine mRNA content in Mac-T cells incubated with E coli LPS. Incubation of Mac-T cells with 1.5 µg of LPS for up to 6 hours caused a rapid increase in IL-1α, IL-1β, IL-8, and TNF-α mRNA content, compared with values for untreated cells (Figure 1). The amount of IL-1β, IL-8, and TNF-α mRNA peaked after incubation with LPS for 1 hour and then remained increased for up to 6 hours. The amount of IL-1α peaked after incubation with LPS for 2 hours and then steadily decreased through 6 hours. Incubating Mac-T cells with various concentrations (0 to 15 µg) of E coli–derived LPS for 1 hour stimulated increases in amounts of IL-1α, IL-8, and TNF-α mRNA (Figure 2). The amount of IL-1β mRNA was only increased over control values when cells were incubated with 0.75, 1.5, and 15 µg of LPS.

Cytokine response of Mac-T cells after incubation with E coli–derived culture filtrate—Quantitative...
RT-PCR assay was used to measure IL-1α and β, IL-8, and TNF-α mRNA content in Mac-T cells incubated with 100 µL of E coli–derived culture filtrate for 1 hour. There were 28-, 13-, and 22-fold increases in IL-1α, IL-8, and TNF-α mRNA content, respectively, in culture filtrate–treated cells, compared with values for nontreated control cells (Figure 3). Addition of polymyxin B (5 µg/mL) to the culture filtrate 15 minutes before the culture filtrate was added to the Mac-T cells caused IL-1α, IL-8, and TNF-α mRNA content to return to amounts similar to those obtained with control cells. Amounts of IL-1β mRNA increased only 3-fold when Mac-T cells were incubated with E coli–derived culture filtrate, and prior treatment of the culture filtrate with polymyxin B did not diminish this response.

Cytokine response of Mac-T cells after incubation with E coli—Quantitative RT-PCR assay was used to assess changes in IL-1α, IL-1β, IL-8, and TNF-α mRNA content in Mac-T cells incubated with 100 µL of E coli–derived culture filtrate for 1 hour. There were 28-, 13-, and 22-fold increases in IL-1α, IL-8, and TNF-α mRNA content, respectively, in culture filtrate–treated cells, compared with values for nontreated control cells (Figure 3). Addition of polymyxin B (5 µg/mL) to the culture filtrate 15 minutes before the culture filtrate was added to the Mac-T cells caused IL-1α, IL-8, and TNF-α mRNA content to return to amounts similar to those obtained with control cells. Amounts of IL-1β mRNA increased only 3-fold when Mac-T cells were incubated with E coli–derived culture filtrate, and prior treatment of the culture filtrate with polymyxin B did not diminish this response.
E coli

...tion with 

(P ...experiments. *Within a time period, value differs significantly...

...have the capability to produce all of these cytokines... 

...with mastitis... 

...produce all of these cytokines... 

...have a more important role in leukocyte migration into the mammary gland in response to initial infection... 

...may suggest that cytokines other than IL-8 are more important for leukocyte migration into the mammary gland in response to E coli infection...
LPS, but the importance of these cytokines to the mammary gland inflammation that follows E coli infection was not determined.

Escherichia coli produces several factors that may stimulate the immune system. The most commonly studied of these is E coli–derived LPS. To determine whether E coli components other than LPS are important in stimulating Mac-T cells, the cells were incubated with E coli–derived culture filtrate or E coli isolates. Similar to results with LPS treatment, IL-1α and IL-8 mRNA content increased rapidly following exposure. In addition, neutralizing the lipid A component of LPS with polymyxin B prior to addition of the culture filtrate to Mac-T cells abrogated the cytokine response. This result supports the importance of LPS in stimulating mammary epithelial cells and the observation that E coli strains isolated from cows with clinical mastitis are usually environmental strains and not strains that produce specific exotoxins. The IL-1β mRNA content increased only slightly following incubation with E coli–derived culture filtrate or E coli organisms, and this increase was resistant to effects of polymyxin B. Therefore, E coli factors other than LPS may be involved in IL-1β stimulation.

Another cytokine that appears to be involved with the inflammatory response seen during coliform mastitis is TNF-α. Concentrations of TNF-α are increased in milk and serum of cattle with naturally acquired or experimentally induced coliform mastitis. In a study, investigators provided evidence that serum and milk concentrations of TNF-α are increased when clinical signs and mortality are observed for cows with experimentally induced E coli mastitis infections. Additional evidence of the importance of TNF in coliform mastitis was documented in a study in which SC injection of recombinant bovine TNF-α resulted in a decrease in milk yield, protein content, and fat concentration. Direct infusion of TNF-α into the teats of cattle causes a substantial migration of neutrophils into the teat cistern.

In the study reported here, incubation of Mac-T cells with E coli–derived LPS resulted in increased amounts of TNF-α mRNA that peaked at 1 hour and remained increased for the duration of the experiment. Similar to the results seen with IL-1α and IL-8, there was a significant increase in TNF-α mRNA content in the cells after incubation with E coli–derived culture filtrate and E coli organisms. The increased amount of mRNA caused by incubation with the culture filtrate was reversible by prior addition of polymyxin B to the filtrate.

To verify that increased mRNA content resulted in increased secretion of TNF-α by the cells, TNF-α concentrations in the cell supernatants were determined by use of a bioassay. Incubation of Mac-T cells with E coli organisms resulted in increased secretion of TNF-α (ie, bioactivity) in the conditioned medium. These results correlate with the in vivo data of another study in which endotoxin infusion into the mammary gland resulted in increasing amounts of TNF-α in the milk at 2 and 4 hours after infusion. Some TNF-α may remain associated with the cell membrane as a type II membrane glycoprotein; thus, concentrations found in milk may represent only a portion of the TNF-α that is actually produced by a cell. In addition, some of the TNF-α from the mammary gland may enter the afferent lymph system and serum because TNF-α concentrations in lymph and serum are increased after intramammary infusion of LPS or naturally acquired coliform mastitis.

Tumor necrosis factor-α can interact directly with endothelial cells, which results in increased expression of P-selectin, E-selectin, and IL-8. In addition, TNF-α causes priming and activation of neutrophils. These events probably increase leukocyte migration into the mammary gland during coliform mastitis. In the study reported here, we documented that bovine mammary gland epithelial cells have the ability to upregulate TNF-α in response to E coli stimulation. Mammary gland epithelial cells may be an important source of increased concentrations of TNF-α in serum and milk of cows with coliform mastitis.

Mammary gland epithelial cells are one of the largest populations of cells in the mammary gland. Similar to their counterparts in the respiratory and intestinal tracts, they form a barrier against potential pathogens. Therefore, mammary gland epithelial cells are probably one of the cell types that is first to be stimulated by E coli and coliform products in infected mammary glands. In addition, the sheer number of epithelial cells in the mammary gland gives them the potential to be a major contributor to the cytokine content in milk and serum from cows with experimentally induced and naturally acquired coliform mastitis. In this study reported here, we documented that mammary gland epithelial cells have the ability to rapidly produce cytokines, such as IL-1α, IL-1β, IL-8, and TNF-α, in response to E coli–derived LPS. In addition, we determined that the LPS component of E coli stimulated most of this response.

a. Dulbecco modified Eagle media, Gibco, Grand Island, NY.
b. Cortisone-2-hydroxypropyl-b-cyclodextrin complex, Sigma Chemical Co, St Louis, Mo.
c. Insulin, Sigma Chemical Co, St Louis, Mo.
d. 0.25% Trypsin-0.1% EDTA solution, Cellgro, Hendon, Va.
e. Lipopolysaccharide (E coli origin), Sigma Chemical Co, St Louis, Mo.
f. Wisconsin Animal Health Laboratory, Madison, Wis.
g. Nutrient agar, Difco, Detroit, Mich.
h. RPMI media, Cellgro, Hendon, Va.
i. Milllex-GP 0.22-μm filter unit, Millipore, Bedford, Mass.
j. SmartSpec 3000, BioRad Laboratories, Hercules, Calif.
k. RNasey, QIAGEN Inc, Valencia, Calif.
l. Reverse transcription system, Promega, Madison, Wis.
m. Primer Express 2.0, Applied Biosystems, Foster City, Calif.
n. BLAST, US National Library of Medicine, Bethesda, Md.
o. Biotechnology Center, University of Wisconsin, Madison, Wis.
p. SYBR Green PCR master mix, Applied Biosystems, Foster City, Calif.
q. ABI Prism, Applied Biosystems, Foster City, Calif.
r. GeneAmp 5700 sequence detector, Applied Biosystems, Foster City, Calif.
s. Penicillin-streptomycin, Cellgro, Hendon, Va.
t. Actinomycin D, Sigma Chemical Co, St Louis, Mo.
u. Tumor necrosis factor-α, Sigma Chemical Co, St Louis, Mo.
v. Methylthiazoletetrazolium, Sigma Chemical Co, St Louis, Mo.
w. EL-312, Bio-Tek Instruments, Winooski, Vt.
**Appendix**

Primers used to amplify genes of cytokines and a housekeeping gene.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>Forward</td>
<td>5’-GCA AGA AAA ATT GTC GCA CAT G-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>3’-GTA AAT AGT AGG CAG ATG CAA GGC TAT T-5’</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Forward</td>
<td>5’-ATT GCC CAG GTT TCT GAA ACA-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>3’-CTG GTC ACT GTA GTA AGC CAT TT-5’</td>
</tr>
<tr>
<td>IL-8</td>
<td>Forward</td>
<td>5’-TGG AAA TTC CAA TCG ATC TGG AAA T-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>3’-AAA CCA AGG CSG ACT TCA A-5’</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward</td>
<td>5’-CCG GTG GTG GGA CTC GAT T-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>3’-CTG GTC GTC TCC CAG CTC AAC A-5’</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward</td>
<td>5’-AAC CGT CGG AAG AAG ATG ACC CAG AT-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>3’-GGG ACA GCA CGA CCT GGA T-5’</td>
</tr>
</tbody>
</table>

IL= Interleukin. TNF = Tumor necrosis factor.

**References**

40. Alhwaimi AM, Leutenegger CM, Farver TB, et al. The


