Antimicrobial-induced endotoxin and cytokine activity in an in vitro model of septicemia in foals

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Objective—To determine which antimicrobials that are used to treat neonatal foals with septicemia attributable to *Escherichia coli* will minimize endotoxin release from bacteria and subsequent activity of inflammatory mediators while maintaining bactericidal efficacy.

Sample Population—Blood samples from 10 healthy foals.

Procedure—*Escherichia coli* isolates A and B were isolated from 2 septicemic foals, and minimal inhibitory concentrations (MIC) were determined for 9 antimicrobials. Five of these antimicrobials were tested in vitro at 2 and 20 times their respective MIC. Whole blood or mononuclear cells grown in tissue-culture media were incubated with 10^5 colony-forming units of *E coli* and each antimicrobial or saline (0.9% NaCl) solution. After 6 hours, number of viable bacteria remaining was determined, and supernatant was tested for endotoxin and tumor necrosis activity.

Results—Testing in whole blood was compromised by bactericidal effects of the blood itself. In mononuclear cell suspensions, each antimicrobial significantly reduced the number of viable bacteria to low or undetectable amounts. Antimicrobials did not differ significantly in efficacy of bacterial killing. Amikacin used alone or in combination with ampicillin resulted in significantly less endotoxin activity than did ampicillin, imipenem, or ceftiofur alone. There was a correlation between TNF-α and endotoxin activity.

Conclusions and Clinical Relevance—Aminoglycosides appear less likely to induce endotoxemia and TNF-α synthesis during bactericidal treatment of *E coli* septicemia, compared with β-lactam antimicrobials. Use of ampicillin, imipenem, or ceftiofur in the treatment of septicemic neonatal foals should be accompanied by appropriate treatment for endotoxemia. (*Am J Vet Res* 2002;63:660–668)

Septicemia is the most common cause of morbidity and mortality in neonatal foals, accounting for approximately a third of all deaths in the first week after birth.[1-3] The infectious agents most commonly isolated from septicemic foals are gram-negative bacteria, with *Escherichia coli* accounting for at least half of all isolates.[1,3-6] Furthermore, foals with gram-negative sepsis are less likely to survive than foals infected with gram-positive organisms.[7] Endotoxin, the **lipopolysaccharide (LPS)** component of the outer membrane of the cell wall in gram-negative bacteria,[8] is released during rapid bacterial proliferation or bacteriolysis. Thus, endotoxemia is a common sequela of gram-negative sepsis in foals, because endotoxin is released directly into the circulation as bacteria multiply within the blood.[9] Clinical signs of disease and fatalites attributable to gram-negative septicemia may be directly associated with systemic effects of endotoxin.[8,10] Evidence of the deleterious effects of endotoxemia can be seen in the fact that foals with positive sepsis scores have higher plasma concentrations of endotoxin than do foals with negative sepsis scores.[11] At least half of septicemic foals are endotoxemic at the time of admission to referral hospitals,[7] and endotoxemia in septicemic foals is associated with an unfavorable outcome.[11]

Endotoxin is a critical bacterial factor in the pathogenesis of shock in gram-negative sepsis.[8,9] Once released into the circulation, LPS is rapidly bound to LPS-binding protein, which serves as a transport protein to increase delivery of LPS to mononuclear phagocytes.[12] The LPS then binds specifically to CD14, a pattern-recognition receptor on the surface of monocytes, which in turn activates a toll-like receptor.[13-15] Activation of monocytes by LPS culminates in the synthesis and secretion of many inflammatory mediators, including **tumor necrosis factor-α (TNF-α)**, interleukins, eicosanoids, platelet-activating factor, thromboplastin, tissue plasminogen activator, and plasminogen activator inhibitor.[9,16] Tumor necrosis factor-α is an important early mediator during endotoxemia.[17] Blood concentrations of TNF-α are increased in endotoxemic foals[7] and are correlated with clinical criteria of sepsis, including tachycardia, petechiae, neutropenia, increased band-to-segmented neutrophil ratio, toxic leukocytes, and fatalities.[18] Serum TNF-α concentrations also are increased in foals experimentally infused with LPS and are correlated with severity of clinical signs.[19] The ultimate consequences of unchecked endotoxemia and release of inflammatory mediators are circulatory shock, multi-systemic organ failure, and death.[16]

Because of the rapid progression of septicemia in neonates, the key to a successful outcome is early

Received Sep 26, 2001.
Accepted Dec 4, 2001.
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Supported by a grant from the Veterinary Medical Experimental Station, University of Georgia.
Presented in part at the 19th Annual Forum of the American College of Veterinary Internal Medicine, Denver, Colo, May 24, 2001.
The authors thank Drs. Richard E. Wooley and Larry Benchat for technical assistance.
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antimicrobial treatment to halt bacterial replication as well as specific therapeutic regimens aimed at controlling endotoxemia.[3,9] Although antimicrobials decrease the number of proliferating bacteria, administration of some antimicrobials does not improve survival rates of patients with gram-negative sepsis.[20] This may be attributable to the fact that aggressive use of bactericidal antimicrobials can result in liberation of large amounts of endotoxin.[21-25] Because the use of antimicrobials is essential in the treatment of septic animals, it would be advantageous to know whether equally effective bactericidal antimicrobials differ with respect to liberation of endotoxin and subsequent generation of inflammatory mediators. Antimicrobials that liberate smaller amounts of biologically active endotoxin may be superior to those that liberate large amounts of endotoxin.

The purposes of the study reported here were to determine the efficacy of the most commonly used or purportedly effective antimicrobials against a native isolate of *E coli* by use of an in vitro model of septicemia in neonatal foals, quantify the amount of endotoxin released during bactericidal treatment, and determine the inflammatory response elicited by each antimicrobial by measuring subsequent production of TNF-α. The hypotheses of this study were that the amount of endotoxin released during treatment of foals with gram-negative bacterial septicemia depends on the mechanism of action of the antimicrobial used and that amounts of endotoxin released during bacterial killing determine the extent of activation of the inflammatory cascade as represented by synthesis of TNF-α.

Materials and Methods

Sample population—Ten healthy Quarter Horse foals that were between 4 and 22 days old were randomly selected for use in the study. These foals had been born at the University of Georgia horse breeding farm between Mar 8 and Jun 9, 2000. Blood was collected once from each foal. Foals were selected on the criteria of a full-term gestation, typical parturition, adequate passive transfer of immunoglobulins, and lack of overt illness. A CBC was performed on each foal; foals with clinical or hematologic evidence of sepsis were excluded from the study.

Determination of antimicrobial susceptibility—Antimicrobial susceptibility patterns of bacteria isolated from 2 septicemic foals were determined. In addition, the **minimal inhibitory concentration (MIC)** of each antimicrobial that would subsequently be used for in vitro incubation with whole blood or mononuclear cells obtained from healthy foals was determined.

Two isolates of *E coli*, designated A and B, were cultured from the blood of 2 septicemic foals that had been examined at our facility's veterinary medical teaching hospital between Feb 26 and May 5, 1999. The bacteria were stored frozen in stock medium (1% peptone, 15% glycerol) at –70 C until use. For each experiment, the frozen cultures were inoculated onto MacConkey agar and incubated overnight at 37 C. One colony of each isolate was then inoculated into 3 ml of sterile **Roswell Park Memorial (RPMI)** media and incubated overnight for 15 to 18 hours at 37 C. Immediately prior to use, the suspension was vortexed, and **optical density (OD)** was measured to determine that the culture was in logarithmic-phase growth (OD < 0.6).

Stock solutions of penicillin G sodium[a] (500,000 U/ml), gentamicin sulfate[b] (50 mg/ml), amikacin sulfate[c] (250 mg/ml), ceftiofur sodium[d] (50 mg/ml), enrofloxacin[e] (100 mg/ml), a product[f] that contained ticarcillin (200 mg/ml) with clavulanic acid (6.7 mg/ml), and ceftizoxime[g] (95 mg/ml) were prepared in accordance with manufacturers' instructions and stored at –70 C. Solutions of ampicillin sodium[h] (250 mg/ml) and imipenem-cilastatin[i] (25 mg/ml) were reconstituted immediately prior to each experiment.

The MIC for each antimicrobial alone was determined by use of the broth-dilution test tube method.[26] Effects of combinations of penicillin or ampicillin with gentamicin or amikacin on each isolate of *E coli* were measured by use of a 2-dimensional microtiter checkerboard technique, using Mueller-Hinton broth.[27,28] Serial 2-fold dilutions of each antimicrobial (50 µl) were inoculated into each well of a sterile flat-bottom 96-well microtiter plate.[j] An equal volume (50 µl) of the *E coli* isolate being tested (obtained from the logarithmic-phase growth after 15 to 18 hours of culture) was added to each well. Appropriate positive-control (*E coli* alone) and negative-control (broth alone) wells were included on each plate. Plates were incubated overnight at 37 C, and each well was visually classified (growth or no growth) at 20 to 22 hours after onset of incubation.

Fractional inhibitory concentration (FIC) indices were calculated for each of the antimicrobial combinations to determine whether the combined effects of the 2 antimicrobial agents for inhibition of bacterial growth were antagonistic, indifferent-additive, or synergistic.[27,29] The FIC index is equal to the sum of the values of FIC for each of the specific drugs, as determined by use of the following equation:

$$FIC = \frac{\text{MIC of drug A with drug B}}{\text{MIC of drug A alone}} + \frac{\text{MIC of drug B with drug A}}{\text{MIC of drug B alone}}$$

A value for the FIC index of > 1 indicates antagonism, and a value of 1.0 indicates an indifferent-additive effect. The FIC must be ≤ 0.5 for the combined effect of the antimicrobials to be considered synergistic.[29] The MIC for antimicrobial combinations were determined on the basis of the ratio that yielded the lowest repeatable FIC of ≤ 0.5 in duplicate tests. When the exact same ratio was not obtained, the higher concentration of the 2 duplicate tests was used.

In vitro studies—For each experiment, each *E coli* isolate was streaked onto MacConkey agar plates and incubated overnight. The day before incubation with whole blood or mononuclear cells, 1 colony of each *E coli* isolate was inoculated into 3 ml of RPMI media and incubated overnight for 15 hours at 37 C. The following day, the OD of bacteria in logarithmic-phase growth was measured; on the basis of the OD, the culture suspension was diluted to a final stock concentration of 2×10^6 **colony-forming units (CFU)**/ml in RPMI-1640.

A volume of 25 to 50 ml of blood was aseptically collected from each of 4 foals by venipuncture of the jugular vein. Blood samples were collected into sterile tubes that contained sodium heparin (10 U of heparin/ml of blood). Aliquots of 0.9 ml of whole blood were dispensed into sterile 1-ml screw-top polypropylene tubes. To each tube, we added 50 µl of an antimicrobial, antimicrobial combination, or saline (0.9% NaCl; control) solution. Antimicrobials tested in vitro included amikacin, a combination of amikacin and ampicillin, ampicillin, imipenem, and ceftiofur. Antimicrobials were prepared as described previously.

On the basis of results of antimicrobial susceptibility testing, antimicrobials were added to the culture tubes to achieve final concentrations of **2 times the MIC per milliliter of blood (2X MIC)** and **20 times the MIC per milliliter of blood (20X MIC)**. An aliquot (50 µl) of logarithmic-phase bacterial suspension (2×10^6 CFU/ml of RPMI-1640) of *E coli*

isolate A or isolate B then was added to each tube to achieve a concentration of 1×10^5 CFU/ml of blood. For each foal, a control tube that did not contain bacteria (ie, 50 µl of RPMI-1640 with 50 µl of saline solution) also was included in the study. Tubes were incubated at 37 C with gentle rocking for 6 hours. After incubation, 20-µl aliquots of blood were used for quantitation of viable bacteria, and plasma was obtained from each tube, using centrifugation, and filtered through a 0.22-µm filter. Plasma from each tube was diluted 1:10 in pyrogen-free water in pyrogen-free borosilicate glass tubes, heated for 10 minutes at 70 C, and then frozen at –20 C until assayed for endotoxin content, using the **limulus amebocyte lysate (LAL)** assay. Aliquots of filtered plasma were frozen at –70 C in polypropylene bullet tubes until assayed for TNF-α activity, using the WEHI bioassay.

Mononuclear cells were isolated from 50 ml of whole blood collected from the other 6 healthy foals. Mononuclear cells were collected by use of density-gradient centrifugation.[30,k] Mononuclear cells (1×10^6 cells) were suspended in 0.9 ml of RPMI-1640 media in sterile 1-ml polypropylene culture tubes. Antimicrobials or saline solution were added in 50-µl aliquots to each tube, as described for the assay of whole blood. To determine the effect of plasma alone on bacterial killing, a tube was prepared that contained mononuclear cells suspended in 0.9 ml of autologous plasma, rather than RPMI-1640. We then added 50 µl of saline solution to the tube. A 50-µl volume of a suspension of *E coli* isolate A (2×10^6 CFU/ml of RPMI-1640) was then added to each tube, and mononuclear cells were incubated for 6 hours at 37 C. At the end of incubation, 20 µl was removed for quantitation of viable bacteria. The supernatant was filtered through a 0.22-µm filter and stored as described previously until analyzed by use of the LAL and WEHI bioassays.

The micromethod dilution was performed to verify a quantitative viability count for each bacterial isolate prior to addition to suspension of whole blood or mononuclear cells. Using RPMI-1640, dilutions of 10^{-4}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} CFU/ml of culture suspension were made from the original stock suspension that contained 2×10^6 CFU/ml as determined on the basis of OD values. Three aliquots (10 µl/aliquot) of the original culture suspension and each of the dilutions were plated onto brain-heart infusion agar and incubated at 37 C overnight. Colony counts were performed the following day and used to verify the initial concentration of bacteria that had been introduced into the whole blood or mononuclear cells.

The most-probable number scheme[31] was used to determine the concentration of viable bacteria remaining after incubation of whole blood or mononuclear cells with antimicrobials. This scheme uses a mathematical model to infer the number of viable bacteria in a sample on the basis of the percentage of multiple cultures that do not have growth in a series of dilution tubes in culture media. Briefly, a sterile flat-bottom 96-well microtiter plate was used to set up the dilutions. Suspensions of whole blood or mononuclear cells (10 µl) were removed from each tube after incubation for 6 hours, and four 10-fold serial dilutions were made. Each dilution was then immediately divided into 3 samples (30 µl), each of which was added to 200 µl of brain-heart infusion broth. The final dilution factor of each well ensured that the final antimicrobial concentration was less than the MIC. Another 10 µl of whole blood or mononuclear cells also was removed immediately after the 6-hour incubation and spread directly on brain-heart infusion agar to determine CFU counts that were less than the detection range of the most-probable number assay. Colony forming units were counted on plates after incubation for 15 hours at 37 C. Microtiter plates were incubated overnight (15 hours) at 37 C, then each well was visually classified (growth or no growth). Data for each plate was entered into a computer algorithm[31] to determine the most-probable number of viable bacteria remaining in each sample after incubation. When the most-probable number disagreed with the colony counts on the plates, the higher of the 2 values was used. Bacterial killing for each sample was then determined by subtracting the concentration of viable bacteria after incubation from the initial concentration of bacteria added to the tubes containing whole blood or mononuclear cells.

Plasma or mononuclear cell ultrafiltrate prepared as described previously was thawed, and endotoxin activity of each sample was determined by use of a chromogenic LAL assay,[l] in accordance with the manufacturer's directions. Lower limit of detection of this assay was 0.01 endotoxin units/ml.

Activity of TNF-α in each sample of plasma or mononuclear cell supernatant was determined, using a modified in vitro cytotoxicity bioassay with WEHI 164 clone-13 murine fibrosarcoma cells.[18,19] Human recombinant TNF-α[m] was used as a standard. Activity of TNF-α was expressed in units per milliliter; 50% killing (the amount of cytotoxic activity necessary to lyse 50% of the cells) was defined as 1 unit of TNF-α activity/ml.

Statistical analysis—Statistical analysis was performed by use of statistical software.[n] Endotoxin and TNF-α data were logarithmically transformed prior to statistical analysis. Comparison of bacterial killing, endotoxin concentration, and TNF-α activity among various antimicrobial treatments and concentrations were made by use of an ANOVA. Means of interest were compared, using the Student-Newman-Keuls test for multiple comparisons. Significance was defined as values of $P < 0.05$.

Results

Antimicrobial susceptibility testing—Bacterial growth was not detected in negative-control cultures to which we did not add *E coli*, and typical bacterial growth was detected in positive-control cultures that contained *E coli* but that did not contain an antimicrobial. The MIC for penicillin, ampicillin, gentamicin, amikacin, ceftiofur, enrofloxacin, timentin, ceftizoxime, and imipenem were determined for the 2 *E coli* isolates (**Table 1**). Isolates A and B were susceptible to all antimicrobials tested, except for penicillin.

The FIC for combinations of penicillin-gentamicin, penicillin-amikacin, ampicillin-gentamicin, and ampicillin-amikacin were determined for both iso-

Table 1—Minimal inhibitory concentrations (MIC) of antimicrobials against *Escherichia coli* isolates A and B cultured from the blood of 2 septicemic foals

	MIC (µg/ml)	
Antimicrobial (U/ml)	**Isolate A**	**Isolate B**
Penicillin	40.00	40.00
Ampicillin*	3.12	3.12
Gentamicin	3.12	3.12
Amikacin*	12.50	12.50
Combination of ampicillin and amikacin*	0.39 and 3.12	0.78 and 3.12
Ceftiofur*	0.39	0.39
Enrofloxacin	0.04	0.04
Ticarcillin-clavulanic acid	3.12	0.78
Ceftizoxime	0.04	0.05
Imipenem*	0.16	0.16

*Antimicrobials selected for subsequent use in incubations of whole blood and mononuclear cells.

lates. All antimicrobial combinations resulted in synergistic indices, (ie, FIC ≤ 0.5) for both *E coli* isolates, except for penicillin-gentamicin against isolate A (FIC, 0.625). Although FIC were determined for penicillin combinations, these combinations were ultimately excluded from the in vitro studies, because the concentration of penicillin required to reach the MIC was considerably higher than that attainable in plasma after in vivo administration of the recommended dose. On the basis of these antimicrobial susceptibilities, 5 of the 9 antimicrobials or antimicrobial combinations were chosen because of their efficacy or common clinical usage for inclusion in the subsequent in vitro studies involving the use of whole blood and mononuclear cells.

Results for whole blood—Effects of 5 antimicrobials at 2X and 20X MIC on *E coli* isolates A and B were tested in whole blood collected from 4 healthy foals. Efficacy of bacterial killing, endotoxin released during bacterial killing, and inflammatory response (TNF-α activity) were assessed for each sample.

The initial inoculum of *E coli* contained a mean of 0.84×10^5 CFU/ml. Bacterial growth was not detected in any of the control tubes to which we did not add bacteria. Comparison between *E coli* isolates A and B did not reveal significant differences in bacterial killing in response to the antimicrobial treatments (**Table 2**). For both *E coli* isolates, viable bacteria remaining after incubation without antimicrobials were decreased by 2 to 4 logarithms or were undetectable, revealing that the blood itself was killing bacteria. A significant effect of antimicrobial concentration was detected, with greater bacterial killing at 20X MIC than 2X MIC for both isolates. At 2X MIC, none of the antimicrobials completely killed all bacteria. For both *E coli* isolates at 20X MIC, ampicillin-amikacin and amikacin alone did not have detectable viable bacteria remaining after incubation. There was not a significant difference in bacterial killing between each antimicrobial tested at 2X MIC and control solutions for both isolates of *E coli*. However, a significant difference was detected between bacterial killing by antimicrobials at 20X MIC, compared with values for the control solutions. For both isolates of *E coli*, there was not a significant difference among antimicrobials in efficacy of bacterial killing.

Table 2—Antimicrobial-induced killing of bacteria in whole blood from healthy foals after incubation with an *E coli* isolate for 6 hours

Antimicrobial	Concentration of antimicrobial	Viable bacteria* (log CFU/ml)	
		Isolate A	Isolate B
Control solution†	None	3.1 ± 0.6	2.4 ± 0.4
Ampicillin-amikacin	2X MIC	1.0 ± 1.1	2.0 ± 0.0
	20X MIC	0	0‡
Amikacin	2X MIC	0.5 ± 1.0	0.5 ± 1.0
	20X MIC	0	0
Ampicillin	2X MIC	0.6 ± 1.2	0.5 ± 1.0
	20X MIC	0.5 ± 1.0	0.6 ± 1.1
Ceftiofur	2X MIC	2.2 ± 1.5	1.3 ± 1.5
	20X MIC	0.5 ± 1.0	0
Imipenem	2X MIC	1.8 ± 1.3	1.8 ± 2.1
	20X MIC	0‡	0.5 ± 1.0

Values reported are mean ± SD.
*Number of viable bacteria remaining from initial inoculum of 5.0 log colony-forming units (CFU)/ml. †Saline (0.9% NaCl) solution. ‡Within each antimicrobial, value differs significantly ($P < 0.05$) from value for 2X MIC.
Each value for 20X MIC differed significantly ($P < 0.05$) from the value for the control solution.

Mean endotoxin activity in whole blood without added bacteria or antimicrobials was 0 U/ml. Mean ± SD endotoxin concentration in control solutions without added antimicrobials was 1.0 ± 0.3 and 2.5 ± 2.3 U/ml for isolates A and B, respectively. Antimicrobial killing of isolate B released significantly more endotoxin than killing of isolate A. For isolate A, mean endotoxin activity for all antimicrobial treatments was 1.0 ± 0.3 U/ml, compared with a mean of 1.6 ± 0.5 U/ml for isolate B. We did not detect significant differences in endotoxin activity among antimicrobials used at either concentration.

Mean TNF-α activity in whole blood without added bacteria or antimicrobial was 2 ± 0 U/ml. In control solutions that did not contain antimicrobials, isolates A and B elicited TNF-α activity of 714 ± 373 and 510 ± 473 U/ml, respectively. The 2 isolates of *E coli* also did not differ in antimicrobial-induced TNF-α activity. For isolate A, mean TNF-α production for all antimicrobials was 537 ± 333 U/ml, whereas mean TNF-α production was 449 ± 378 U/ml for isolate B. There was not a significant difference in TNF-α activity among antimicrobials or antimicrobial concentrations or between any antimicrobial and the control solution.

Results for mononuclear cells—Using mononuclear cells isolated from the blood of 6 healthy foals as the target for endotoxin activity, the effects of 5 antimicrobials or antimicrobial combinations were tested at 2X MIC and 20X MIC on *E coli* isolate A. Each sample was assessed for efficacy of antimicrobial treatment (viable bacteria remaining), endotoxin activity induced during bacterial killing, and inflammatory response of the mononuclear cells, as reflected by TNF-α activity.

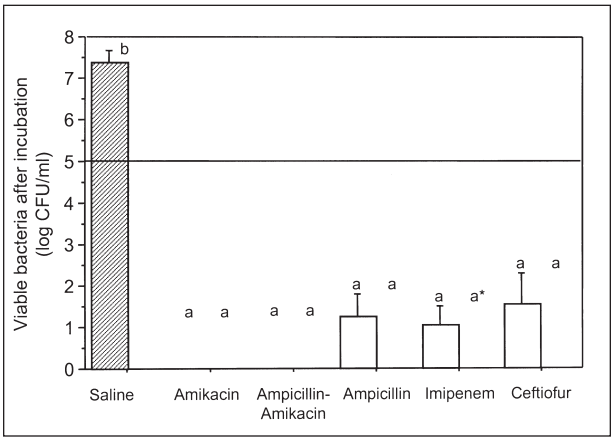

Figure 1—Mean ± SE number of viable bacteria remaining after mononuclear cells obtained from 6 healthy foals were incubated for 6 hours with each antimicrobial at 2 or 20 times the minimal inhibitory concentration (2X MIC (white bars) or 20X MIC (black bars), respectively) or saline (0.9% NaCl; control) solution (gray bar). The horizontal line indicates the concentration of the initial inoculum of *Escherichia coli* (1×10^5 colony-forming units [CFU]/ml). *Value differs significantly ($P < 0.05$) between concentrations for that antimicrobial. [a,b]Values with different letters differ significantly within that concentration ($P < 0.05$).

Number of viable bacteria remaining from a mean initial inoculum of 0.92×10^5 CFU/ml after incubation for 6 hours was determined by the most probable number scheme and colony plate counts (**Fig 1**). Plasma had a bactericidal effect, because there were significantly fewer remaining viable bacteria (5.1 ± 0.3 log CFU/ml) after incubation with mononuclear cells in plasma, compared with cells incubated only in RPMI-1640 (7.3 ± 0.8 log CFU/ml). Bacteria incubated with mononuclear cells suspended in RPMI-1640 without any antimicrobial had exponential growth of approximately 2 logarithms to 5×10^7 CFU/ml after 6 hours. At 2X MIC of each antimicrobial, there were significantly fewer viable bacteria remaining, compared with the control solution. Ampicillin-amikacin as well as amikacin alone had the most efficacious bacterial killing at 2X MIC (viable bacteria were not detected). At 20X MIC, bacterial killing was complete for all antimicrobials tested. The difference in efficacy of bacterial killing between the 2X MIC and 20X MIC concentrations was significant only for imipenem. There was not a significant difference in efficacy of bacterial killing among the antimicrobials tested.

Endotoxin activity was determined after mononuclear cells were incubated for 6 hours with *E coli* isolate A and each antimicrobial at 2X MIC or 20X MIC (**Fig 2**). In control solutions with added *E coli* and without antimicrobials, endotoxin activity was high, reflecting endotoxin that resulted from logarithmic bacterial proliferation. At 2X MIC, significantly less endotoxin activity was generated after treatment with amikacin alone than with any other antimicrobial or the control solution. Treatment with 2X MIC of ampicillin-amikacin induced significantly more endotoxin activity than amikacin alone; however, ampicillin-amikacin or amikacin alone induced significantly less (one-tenth to one-twentieth) endotoxin activity than ampicillin alone, ceftiofur, imipenem, or control solution. There was not a significant difference in the amount of endotoxin activity induced by the control solution or by ampicillin, ceftiofur, or imipenem at 2X MIC.

When the antimicrobial concentration was increased to 20X MIC, there was a significant decrease in the amount of endotoxin activity induced for ampicillin, ampicillin-amikacin, and imipenem. The difference between treatment with 2X MIC and 20X MIC was most pronounced for imipenem, with a 10-fold decrease in the amount of endotoxin activity at the higher concentration. However, for ceftiofur, there was not a significant difference in endotoxin production at 20X MIC, compared with 2X MIC, and neither concentration was significantly different from values for the control solution. Ceftiofur induced significantly more endotoxin activity at 20X MIC than any other antimicrobial tested. Endotoxin activity after treatment with amikacin at 20X MIC did not differ significantly from amikacin at 2X MIC. At 20X MIC, amikacin alone or in combination with ampicillin induced significantly less endotoxin activity than the other 3 antimicrobials or the control solution.

Tumor necrosis factor-α activity was determined after mononuclear cells were incubated for 6 hours with *E coli* isolate A and each antimicrobial at 2X MIC or 20X MIC (**Fig 3**). Tumor necrosis factor-α activity in the control solution was not significantly different than that induced by any antimicrobial treatment. At 2X MIC, treatment with amikacin alone resulted in significantly less TNF-α activity than treatment with ampicillin, ceftiofur, or imipenem. After treatment with 2X MIC of ampicillin-amikacin, TNF-α activity was not significantly different from amikacin alone or ceftiofur but was significantly less than ampicillin alone or imipenem. There was not a significant difference between the TNF-α activity induced by treatment with ampicillin, imipenem, or ceftiofur at 2X MIC.

At 20X MIC, amikacin and ampicillin-amikacin induced the lowest amount of TNF-α activity; these values were significantly less than those for ceftiofur. Ceftiofur, the antimicrobial treatment that caused the largest release of endotoxin, also caused the greatest TNF-α activity at 20X MIC. Unlike the effects on endotoxin activity, there was not a significant difference in

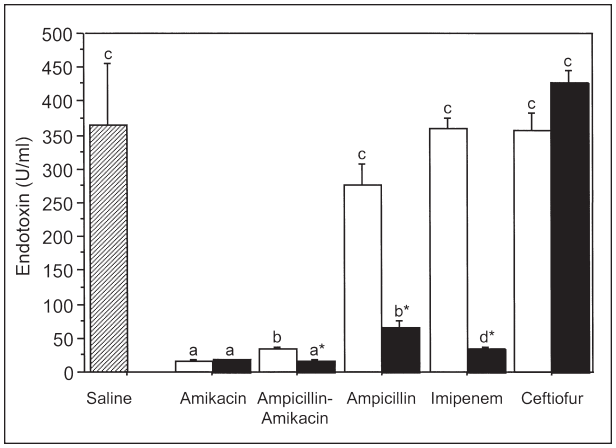

Figure 2—Mean ± SE endotoxin activity of mononuclear cells isolated from 6 healthy foals that were incubated for 6 hours with *E coli* isolate A and each antimicrobial at 2X MIC (white bars) or 20X MIC (black bars) or saline solution (gray bar). [a-d]Values with different letters differ significantly within that concentration ($P < 0.05$). *See* Figure 1 for remainder of key.

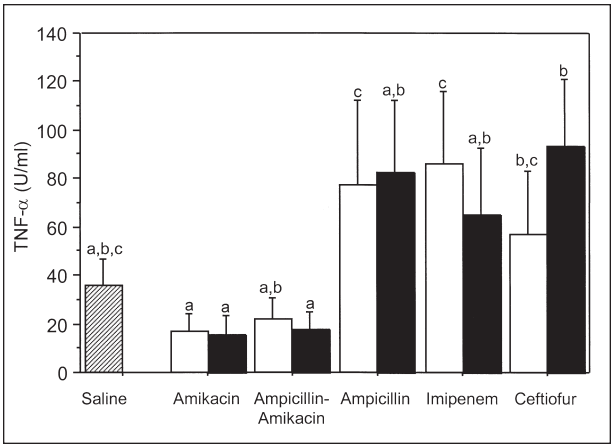

Figure 3—Mean ± SE tumor necrosis factor-α (TNF-α) activity released from mononuclear cells isolated from 6 healthy foals and that were incubated for 6 hours with *E coli* isolate A and each antimicrobial at 2X MIC (white bar) or 20X MIC (black bars) or saline solution (gray bar). [a-c]Values with different letters differ significantly within that concentration($P < 0.05$).

TNF-α activity between antimicrobial concentrations.

Tumor necrosis factor-α activity was correlated significantly with endotoxin activity for the 2X MIC ($P < 0.001$) and 20X MIC ($P = 0.007$) concentrations.

Discussion

The primary goal of the study reported here was to determine whether there was an effect of class of antimicrobial on bactericidal efficacy and endotoxin and cytokine activity during treatment of *E coli* isolates in an in vitro model of septicemia in foals. Results of this study were consistent with the hypothesis that the amount of endotoxin released and subsequent inflammatory reaction, as reflected by TNF-α activity, was dependent on the mechanisms of action of the antimicrobial used, despite similar degrees of bacterial killing.

In this study, it was determined that equine whole blood had a bactericidal effect on the native *E coli* isolates that had been obtained from septicemic foals; thus, we also used an alternate model that involved isolated mononuclear cells. When mononuclear cells were incubated with bacteria in autologous plasma, a bactericidal effect was still evident, necessitating the use of tissue culture media for cellular suspension. Although whole blood may better represent the physiologic environment in animals with endotoxemia,[32] the mononuclear cells allowed identification of the effect of endotoxin production on the main effector cells that produce TNF-α in vivo (ie, monocytes). Suspension of the mononuclear cells in tissue culture media also removed the confounding factor of bacterial killing by blood components, allowing for less ambiguous evaluation of the effect of each antimicrobial. We only tested antimicrobials to which each *E coli* isolate was susceptible at MIC consistent with serum concentrations achievable in vivo.[33-36] Each antimicrobial was tested at 2X MIC and 20X MIC, because administration of antimicrobials in accordance with some dosing regimens may achieve serum concentrations considerably greater than the MIC (eg, once-daily administration of amikacin).[35]

In the mononuclear cell study, treatment of *E coli* with the 3 β-lactam antimicrobials (ie, ceftiofur, ampicillin, and imipenem) generated 2 to 25 times more endotoxin activity than did treatment with amikacin, an aminoglycoside, despite similar degrees of bacterial killing. At both concentrations, amikacin alone or in combination with ampicillin induced the least amount of endotoxin activity, whereas ceftiofur induced the greatest release of endotoxin. Other experimental models of gram-negative bacterial sepsis have revealed that antimicrobials active against the cell wall of gram-negative bacteria promote substantial release of endotoxin by causing a large amount of endotoxin exposure during bacterial killing, compared with antimicrobials that have other mechanisms of action.[21,22,24,37] Within the class of β-lactams, antimicrobials differ significantly in the amount of biologically active endotoxin that is generated, depending on the affinity of the antimicrobial for various **penicillin-binding proteins (PBP)**, which are enzymes located in the bacterial plasma membrane that catalyze assembly of the peptidoglycan network of the bacterial cell wall.[24,37-40] Inhibition of PBP-1a or -1b of *E coli* induces rapid killing and bacteriolysis, whereas PBP-2 binding leads to conversion of bacilli to round cells (ie, spheroplasts) followed by loss of viability with minimal degradation of the cell wall.[38,39] Selective PBP-3 binding, however, causes inhibition of bacterial septation, which leads to formation of long filaments and a large increase in nonviable biomass.[24,38,41] Antimicrobials that bind PBP-3 can induce substantially greater endotoxin activity during bacterial killing than those that primarily bind PBP-2.[23,24,37,40,42,43] Cephalosporins primarily bind PBP-3, whereas imipenem primarily binds PBP-2.[37,39,44] Specific research has not been performed with ceftiofur, but other third-generation cephalosporins have similar binding affinities for PBP-3, although they differ in their affinities for the other PBP.[44,45] Ampicillin binds to all the major PBP of *E coli*, but it has higher affinity for PBP-3.[46] Preferential binding of PBP-3 by ceftiofur and ampicillin would cause filament formation and increased endotoxin activity, explaining the high amounts of endotoxin activity generated by these antimicrobials in the study reported here.

Imipenem and ampicillin induced a large amount of endotoxin activity at 2X MIC but an intermediate amount at 20X MIC. The high endotoxin activity detected after 2X MIC treatment with imipenem was in contrast to other reports[23,24,47] in which imipenem induced significantly less endotoxin activity and TNF-α synthesis than cephalosporins. Differences in endotoxin release during bacteriolysis caused by β-lactam antimicrobials have been associated with their differential binding affinities for PBP at various concentrations.[24] Imipenem binds PBP-2 at lower concentrations, causing spheroplast formation, but it binds PBP-1b with greater affinity at higher concentrations.[44,48] More rapid bacterial killing caused by PBP-1b binding could explain the lower endotoxin activity induced by imipenem at 20X MIC, compared with 2X MIC. Cephalosporins bind PBP-1a with greater affinity at higher concentrations[38,41] but may still release substantial amounts of endotoxin over a wide range of concentrations,[23,24] as was seen with ceftiofur at 2X MIC and 20X MIC in the study reported here. Greater release of endotoxin after treatment with β-lactams may also be attributable to latency in killing during which additional bacterial growth is allowed, with increasing biomass and continued release of endotoxin before onset of lysis.[23,42] In our study, ampicillin and imipenem had some bacteria that remained viable at 2X MIC, which may explain the reason that endotoxin activity was higher than after treatments at 20X MIC in which no viable bacteria remained.

Aminoglycosides such as amikacin cause rapid killing without lysis or morphologic change, do not bind to PBP, and induce minimal increases in endotoxin activity, compared with other antimicrobials.[21,24,47,49-51] Amikacin concentration had minimal effect on endotoxin activity, compared with the strong concentration-dependent effect of some β-lactams, which has been reported in other studies.[24,43] When ampicillin was combined with amikacin in our study, significantly less endotoxin activity was induced than

with ampicillin alone, a phenomenon attributed to the effect of the aminoglycoside.[40,42] The low amount of endotoxin activity identified with amikacin may also be partly related to the putative abilities of aminoglycosides to bind endotoxin and inhibit endotoxin synthesis.[21,52,53]

In the study reported here, TNF-α activity was significantly correlated with endotoxin release, except for some minor inconsistencies. Amikacin alone or in combination with ampicillin induced the lowest amount of TNF-α activity, compared with ampicillin alone, ceftiofur, or imipenem, which did not differ significantly. Ceftiofur at 20X MIC generated the most endotoxin activity, which was accompanied by the highest TNF-α production. Results of studies[24,42,43,54,55] that involved the use of human and murine whole blood or isolated human mononuclear cells also indicate that cell wall-active antimicrobials such as cephalosporins release more biologically active endotoxin than imipenem and aminoglycosides when killing bacteria and that these differences correlate with cytokine production (eg, TNF-α and interleukin-6). It also has been documented[24] that the addition of polymyxin B inhibits TNF-α synthesis, indicating that differences in cytokine production between antimicrobials are attributable to differences in the amount of biologically active endotoxin.

Tumor necrosis factor-α activity did not always parallel endotoxin activity in the study reported here. For example, the control solution had relatively high amounts of endotoxin activity, but this was not accompanied by substantial production of TNF-α. The biological activity of endotoxin as well as the amount of endotoxin dictates the inducible inflammatory response; differences in biological activity between endotoxin released from proliferating bacteria, compared with antibiotic-treated bacteria, and between free and cell-bound endotoxin may explain discrepancies in TNF-α production.[43,56,57] Ampicillin can induce more exposure of endotoxin than gentamicin,[58] which may explain the reason that endotoxin activity induced by ampicillin at 20X MIC was relatively low but TNF-α activity was high. Because TNF-α activity can be induced by free endotoxin or biologically active membrane-bound endotoxin, TNF-α activity may be a better assessment of true differences in the amount or type of endotoxin released or exposed by antimicrobials with differing mechanisms of action than measurement of free endotoxin by the LAL assay. Overall, analysis of the TNF-α data still supports the conclusion that amikacin or amikacin-ampicillin induces less biologically active endotoxin and less synthesis of inflammatory mediators during bacterial killing than ceftiofur.

Results for TNF-α activity could also have been affected if the bacterial inoculum or the antimicrobials themselves had a toxic effect on the mononuclear cells, causing loss of physiologic activity or death and, therefore, decreased TNF-α production. Clinical studies of animals with gram-negative sepsis revealed that bacterial inocula can vary from as low as 10 CFU/ml of blood[59,60] to as high as 3×10^8 CFU/ml of cerebrospinal fluid.[61] To provide a model for bacteremia, in vivo susceptibility studies typically are performed with an inoculum of 1×10^4 to 1×10^8 CFU/ml.[45,62,63] The inoculum of bacteria used in the study reported here was comparable to that used in similar studies[43,55] of antibiotic-induced endotoxin and cytokine activity. In that sense, it allows equivalent comparisons of results. Antimicrobials could also induce toxic effects in the mononuclear cells, particularly at 20X MIC. However, even at 20X MIC, concentrations of imipenem and ceftiofur were well within serum concentrations attained at the recommended dose in vivo.[34,36] Only the concentration of amikacin at 20X MIC (250 µg/ml) exceeded the nontoxic peak serum concentration achieved with once-daily dosing regimens in foals (21 mg/kg; 130 µg/ml).[35] However, because the results for endotoxin content and TNF-α activity after treatment with amikacin at 2X MIC and 20X MIC were virtually identical, it seems unlikely that the higher concentration of amikacin had a toxic effect on mononuclear cells that significantly affected TNF-α production.

Analysis of results of the study reported here indicated that amikacin or amikacin-ampicillin was least likely to cause endotoxin release and TNF-α activity in vitro, compared with ampicillin, imipenem, or ceftiofur. Furthermore, unlike other studies, imipenem was not particularly effective in reducing endotoxin release and subsequent TNF-α activity when used at 2X MIC. Results of this study can be applied clinically to help make effective antimicrobial choices for the treatment of animals with gram-negative sepsis that will minimize endotoxemia and improve the chance for survival. However, questions remain as to whether the differences in endotoxin release and inflammatory mediator activity induced by antimicrobials with differing mechanisms of action seen in vitro in this and other similar studies are applicable in vivo. Furthermore, if so, it could be questioned as to whether this would truly affect clinical outcome. Several in vivo studies in laboratory animals[23,25] as well as clinical trials in people[64,65] have suggested that antimicrobials that cause different amounts of endotoxin release in vitro also have differing degrees of protection in vivo, with good correlation between endotoxin activity, TNF-α activity, and morbidity, despite similar bacterial killing. Additional studies in foals will be required to determine whether these antimicrobials have the same effects on endotoxin and TNF-α activity in vivo in foals with infections attributable to gram-negative pathogens other than *E coli* and whether outcome is improved by use of antimicrobials that minimize endotoxin activity. Nonetheless, the study reported here indicated that antimicrobials that bind PBP may be more likely to induce endotoxemia and subsequent inflammation than aminoglycosides and, therefore, should be accompanied by appropriate treatments for endotoxemia when being used to treat bacterial infections in septicemic foals.

[a]Penicillin G Sodium, Marsam, Cherry Hill, NJ.
[b]Gentocin, Schering-Plough, Kenilworth, NJ.
[c]Amiglyde-V, Fort Dodge Animal Health, Fort Dodge, Iowa.
[d]Naxcel, Pharmacia & Upjohn, Kalamazoo, Mich.
[e]Baytril 100, Bayer, Shawnee Mission, Kan.
[f]Timentin, SmithKline Beecham, Philadelphia, Pa.
[g]Cefizox, Fujisawa, Deerfield, Ill.
[h]Ampicillin, Apothecon, Princeton, NJ.

[i]Primax IV, Merck, Rahway, NJ.
[j]96 Well Cell Culture Cluster, Costar, Cambridge, Mass.
[k]Percoll, Sigma Chemical Co, St Louis, Mo.
[l]Chromogenic LAL-QCL-1000, BioWhittaker, Walkersville, Md.
[m]Human recombinant TNF α, Endogen, Woburn, Mass.
[n]StatView, SAS Institute Inc, Cary, NC.

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