

Effect of tilmicosin on chemotactic, phagocytic, and bactericidal activities of bovine and porcine alveolar macrophages

Gordon W. Brumbaugh, DVM, PhD; James D. Herman, DVM, PhD; Julianne S. Clancy, BS; Kyland I. Burden, MS; Tracie Barry, DVM; R. B. Simpson, DVM, MS; Héctor Sumano López, MVZ, PhD, MC

Objective—To evaluate chemotactic, phagocytic, and bactericidal activities of bovine and porcine alveolar macrophages (AM) exposed to tilmicosin.

Animals—12 healthy calves and 12 healthy pigs.

Procedures—Lungs were obtained immediately after euthanasia; AM were collected by means of bronchoalveolar lavage and density gradient centrifugation. Chemotactic activity was evaluated by exposing AM to lipopolysaccharide or macrophage inhibitory peptide during incubation with tilmicosin. Phagocytic activity was evaluated by incubating AM with tilmicosin for 24 hours and then with tilmicosin-resistant *Salmonella* serotype Typhimurium. Bactericidal activity was evaluated by incubating AM with tilmicosin (0, 10, or 20 µg/ml for bovine AM; 0 or 10 µg/ml or 10 µg/ml but washed free of tilmicosin for porcine AM) and then with *Mannheimia haemolytica* (bovine AM) or with *Actinobacillus pleuropneumoniae* or *Pasteurella multocida* (porcine AM).

Results—Tilmicosin had no significant effects on chemotactic or phagocytic activities of bovine or porcine AM. The time-course of bactericidal activity was best described by polynomial equations. Time to cessation of bacterial growth and area under the time versus bacterial number curve were significantly affected by incubation of AM with tilmicosin.

Conclusions and Clinical Relevance—Results show that bactericidal activity of bovine and porcine AM was enhanced by tilmicosin, but not in proportion to the reported ability of AM to concentrate tilmicosin intracellularly. With or without exposure to tilmicosin, the time-course of bactericidal activity of bovine AM against *M haemolytica* and of porcine AM against *A pleuropneumoniae* or *P multocida* was too complex to be reduced to a simple linear equation. (*Am J Vet Res* 2002;63:36–41)

Tilmicosin phosphate is a semisynthetic macrolide antimicrobial drug¹ indicated for treatment of respiratory tract disease associated with *Mannheimia haemolytica* infection in cattle and with *Actinobacillus pleuropneumoniae* or *Pasteurella multocida* infection in

pigs. Alveolar macrophages (AM) from cattle and pigs accumulate tilmicosin intracellularly^{2,3,a,b} and, therefore, may deliver high concentrations of the drug to sites of infection or inflammation.^{3-12,a,b} In addition, bacteria phagocytosed by AM that have accumulated tilmicosin are potentially exposed to high concentrations of the drug.

Intracellular accumulation of tilmicosin is beneficial only if the drug does not detrimentally alter normal functions of those cells. Anti-inflammatory effects of tilmicosin have been documented in calves with experimentally induced *M haemolytica* pneumonia,¹³ but effects of tilmicosin on functions of AM have not, to our knowledge, been determined. The purpose of the study reported here was to evaluate chemotactic, phagocytic, and bactericidal activities of bovine and porcine AM exposed to tilmicosin.

Materials and Methods

Animals—The protocol for this study was approved by the Texas A&M University Laboratory Animal Care Committee. Nonanimal alternatives would not have provided AM directly from the animals' lungs, as needed for this study. Cultured AM would have been too far removed from physiologic influences on their function in vivo. Twelve calves (6 steers and 6 heifers) and 12 pigs (6 barrows and 6 gilts) were used in the study. Calves were obtained from a local producer. They were overtly healthy crossbred commercial weanling steers and heifers that were matched by age (approx 4 to 6 months) and weight (approx 160 to 227 kg) and had not received any medications during the 4 weeks immediately prior to the study. Pigs were obtained from the Swine Center at the College of Agriculture and Life Sciences, Texas A&M University. They were overtly healthy crossbred commercial feeder pigs that were matched by age (approx 3 to 4 months) and weight (approx 25 to 30 kg) and had not received any medications for at least 2 weeks prior to the study.

Recovery of alveolar macrophages—Calves were immobilized with a captive-bolt device and euthanatized by exsanguination; pigs were euthanatized by means of electrocution. Both lungs, with trachea attached, were removed from each animal and examined by visual inspection and manual palpation for evidence of disease. None of the lungs had any visible or palpable lesions.

Alveolar macrophages were recovered from the lungs of each animal, using bronchoalveolar lavage (BAL). A modification of described techniques^{14,15} was used. Briefly, phosphate-buffered saline solution (PBSS) with sodium heparin (10 units/ml) at 4 C in approximately 60-ml aliquots was infused gently into the mainstem bronchi. Lungs were gently massaged, and lavage fluid was poured from the lungs into sterile 500-ml bottles. This process was repeated until

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From the Departments of Veterinary Physiology and Pharmacology (Brumbaugh, Herman, Burden) and Veterinary Pathobiology (Clancy, Barry, Simpson), College of Veterinary Medicine, Texas A&M University, College Station, TX 77843; and the Departamento de Farmacología y Fisiología, Facultad de Medicina Veterinaria, Universidad Nacional Autónoma de México, México DF, México (López).

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at least 250 ml of BAL fluid was collected from the lungs of each animal.

Alveolar macrophages were recovered from the BAL fluid by density gradient centrifugation. Bronchoalveolar lavage fluid was filtered through sterile nylon mesh^c into 50-ml centrifuge tubes (number of tubes was dependent on volume of BAL recovered), and tubes were centrifuged at $190 \times g$ for 5 minutes at 4 C. The supernatant was decanted, and cells were resuspended in PBSS. Aliquots (30 ml) of the suspended cells were layered over 20 ml of polysucrose and sodium diatrizoate^d in 50-ml centrifuge tubes that were centrifuged at $454 \times g$ for 45 minutes at 27 C. The buffy coat was aspirated and placed in 50-ml centrifuge tubes with a sufficient quantity of PBSS at 4 C to produce a final volume of 50 ml. Tubes were centrifuged at $190 \times g$ for 10 minutes at 4 C, and the supernatant was decanted. Five milliliters of RBC lysing buffer^e was added to the cell pellet, and tubes were incubated at 4 C for 5 minutes. Alveolar macrophages in the pellet were resuspended to a volume of 50 ml with PBSS, and the cellular suspension was centrifuged for 5 minutes at $190 \times g$ at 4 C. Supernatant was decanted, and cells were re-suspended in exactly 50 ml of complete RPMI solution (for bovine AM, incomplete RPMI solution^f with 10% fetal calf serum; for porcine AM, incomplete RPMI solution with 10% heat-inactivated porcine serum). Ten microliters of the cellular suspension from each animal was placed in a hemacytometer, and using trypan blue as a vital stain, viable macrophages were counted.

Chemotactic activity—Chemotaxis of AM was evaluated after 2 hours and after 24 hours of incubation with tilmicosin,⁸ using a modification of a described procedure.¹⁶ Aliquots of 5×10^5 viable AM from each animal were suspended in 4 ml of complete RPMI solution and placed in Petri dishes (60 mm in diameter) that had a grid of 1-cm² squares molded onto the bottom of the dishes. Twelve Petri dishes were prepared with AM from each calf; 8 Petri dishes were prepared with AM from each pig.

Six Petri dishes with AM from each calf and 4 Petri dishes with AM from each pig were used to evaluate the effect of incubation with tilmicosin for 2 hours on chemotaxis. For each calf, tilmicosin was added to 2 Petri dishes at a concentration of 20 µg/ml and to an additional 2 dishes at a concentration of 10 µg/ml; the remaining 2 dishes were used as controls with no tilmicosin. For each pig, tilmicosin was added to 2 Petri dishes at a concentration of 10 µg/ml, and the remaining 2 dishes were used as controls with no tilmicosin. For 1 of each pair of Petri dishes, a capillary tube containing lipopolysaccharide (LPS)^b and a second capillary tube containing complete RPMI solution were inserted through separate holes in the lid of the dish. For the other Petri dish in each pair, a capillary tube containing macrophage inhibitory peptide (MIP)ⁱ and a second capillary tube containing complete RPMI solution were inserted through separate holes in the lid of the dish. All Petri dishes were incubated for 2 hours at 37 C in an atmosphere of 5% CO₂ with 95% relative humidity. At the end of the incubation period, 2 slide photographs were taken of cells adhered to the bottom of each Petri dish at the ends of the capillary tubes.

The remaining 6 Petri dishes with AM from each calf and the remaining 4 Petri dishes with AM from each pig were used to evaluate the effect of incubation with tilmicosin for 24 hours. Tilmicosin was added to pairs of dishes at concentrations of 0 (control), 10, and 20 µg/ml (bovine AM) or 0 and 10 µg/ml (porcine AM). Petri dishes were incubated for 22 hours, as described. At that time, a capillary tube with LPS and a second capillary tube with complete RPMI solution were inserted through separate holes in the lid of 1 Petri dish of each pair, and a capillary tube with MIP and a second capillary tube with complete RPMI solution were inserted

through separate holes in the lid of the remaining dish of each pair. Petri dishes were incubated for an additional 2 hours, and slide photographs were taken.

A slide projector was positioned so that slide photographs could be projected onto graph paper; photographs were projected so that each 1-cm² square in the Petri dish was magnified to 400 cm² (20 cm \times 20 cm). The 1-cm² square of the Petri dish that was in contact with the tip of the capillary tube containing LPS, MIP, or RPMI solution was used as a reference point, and the eight 1-cm² squares that surrounded the reference square were examined. One of these 8 squares was obscured by superimposition of the end of the capillary tube; therefore, AM in the obscured square and in the reference square were not counted. Four of the remaining 7 squares were randomly selected, and AM in those 4 squares were counted and multiplied by 2 to calculate the number of AM in the 3 \times 3-cm area around the tip of the capillary tube. For each Petri dish, number of AM within the 3 \times 3-cm area around the tip of the capillary tube containing LPS was divided by the number of AM in the 3 \times 3-cm area around the tip of the capillary tube containing RPMI or the number of AM within the 3 \times 3 cm area around the tip of the capillary tube containing MIP was divided by the number of AM in the 3 \times 3 cm area around the tip of the capillary tube containing RPMI. These ratios were used for statistical analysis.

Phagocytic and bactericidal activities—Bovine AM were suspended in complete RPMI solution containing tilmicosin at a concentration of 0, 10, or 20 µg/ml; porcine AM were suspended in complete RPMI solution containing tilmicosin at a concentration of 0 or 10 µg/ml. Suspensions were incubated in tissue culture flasks for 24 hours at 37 C in an atmosphere of 5% CO₂ with 95% relative humidity. An aliquot of porcine AM incubated with tilmicosin (10 µg/ml) for 24 hours was washed free of the drug. Suspensions of AM were then inoculated with bacteria to produce targeted final concentrations of 3.33×10^5 AM/ml and 4×10^6 bacteria/ml (12.01 colony-forming units [cfu]/AM).

Tilmicosin-resistant *Salmonella* serotype Typhimurium was used to evaluate phagocytic activity of bovine and porcine AM. *Mannheimia haemolytica* was used to evaluate bactericidal activity of bovine AM. *Pasteurella multocida* and *Actinobacillus pleuropneumoniae* were used to evaluate bactericidal activity of porcine AM. Organisms were diluted in sterile saline (0.9% NaCl) solution, and the number of cfu per milliliter was determined by plating aliquots of each solution on agar plates and counting colonies.

Flasks that contained suspensions of AM and bacteria were positioned at an angle to prevent expulsion of contents and were incubated at 37 C for 2 hours on a rotator table (144 RPM). At the end of the 2-hour incubation, extracellular organisms were removed by washing the AM 3 times with incomplete RPMI solution (AM were centrifuged at $400 \times g$ for 5 minutes at 4 C after each washing). Macrophages were then resuspended in the same concentration of tilmicosin used during the 2-hour incubation period in a sufficient quantity of complete RPMI solution to yield a volume of 20 ml in a 50-ml centrifuge tube. Dilution resulted in approximately 2.5×10^5 AM/ml.

For evaluation of phagocytic activity, suspensions of AM incubated with *S* Typhimurium were plated immediately after resuspension of the inoculated AM. For evaluation of bactericidal activity, suspensions of AM incubated with *M haemolytica*, *P multocida*, or *A pleuropneumoniae* were plated immediately after resuspension of the inoculated AM and after further incubation on a rotator table for 1, 2, 3, 4, 6, and 8 hours.

Prior to plating of suspensions, AM were lysed by adding cold (4 C) sterile distilled water to the suspension

and placing it on ice for 15 minutes. Suspensions were then centrifuged at $900 \times g$ for 20 minutes at 4 C. Supernatant was aspirated, using a 10-ml pipette, until the quantity left in the tube was 2.5 ml. Tubes were vortexed to resuspend bacteria. Serial dilutions of bacterial inoculum obtained from the lysed AM were then prepared in duplicate, using sterile saline solution. Plates of blood agar (for isolation of *M haemolytica*, *P multocida* and *S Typhimurium*) or chocolate agar (for *A pleuropneumoniae*) were prepared in duplicate and labeled according to the dilution that was to be inoculated onto the plate. Plates were incubated for 24 hours, and the number of colonies was counted. Appropriate calculations were made for dilution, and number of cfu per 2.5×10^5 AM was recorded. To distinguish direct antimicrobial effects of tilimicosin from cellular bactericidal effects, bacteria were incubated in triplicate with tilimicosin at concentrations of 0, 10, or 20 $\mu\text{g/ml}$, but without AM.

Bactericidal activity was evaluated by constructing timed-killing curves for each suspension. These timed-killing curves were constructed by plotting the natural logarithm of the number of cfu per 2.5×10^5 AM on the Y-axis and incubation time on the X-axis. End-points used for analysis were the time until cessation of bacterial growth and the area under the curve. Time of cessation of bacterial growth was defined as the time when the natural logarithm (ln) of the number of cfu per 2.5×10^5 AM (or ln of the number of cfu in the same volume of medium for suspensions without AM) was ≤ 0.1 .

Statistical analyses—Aliquots of AM from each animal were the experimental units. A randomized complete block design was followed, with each animal serving as a block; therefore, results could not be compared among animals. Ratios obtained with the chemotactic assay for each concentration of tilimicosin were compared with the Wilcoxon rank-sum test.¹⁷

Data for phagocytic activity were analyzed by use of regression ANOVA. The number of cfu of *S Typhimurium* per 2.5×10^5 AM after 2 hours of incubation was the response variable, and the independent variable was concentration of tilimicosin to which AM were exposed (0, 10, or 20 $\mu\text{g/ml}$ for bovine AM; 0 or 10 $\mu\text{g/ml}$ or 10 $\mu\text{g/ml}$ but washed free of tilimicosin for porcine AM).

Bactericidal activity was assessed by evaluating change in the natural logarithm of the number of cfu per 2.5×10^5 AM during the 8-hour incubation period. For each animal, a polynomial mathematical model¹⁸ was fitted to the data with natural logarithm of the number of cfu per 2.5×10^5 AM as the dependent variable and time as the independent variable. Coefficients and exponents of the polynomial mathematical model were evaluated by regression analysis to determine the effect of concentration of tilimicosin to which AM were exposed (0, 10, or 20 $\mu\text{g/ml}$ for bovine AM; 0 or 10 $\mu\text{g/ml}$ or 10 $\mu\text{g/ml}$ but washed free of tilimicosin for porcine AM). Area under the timed-killing curve was calculated by use of the trapezoidal method; regression analysis¹⁹ was used to determine the effects of tilimicosin. Polynomial equations were also used to predict the time at which bacterial growth would cease. Because there is no value for the natural logarithm of 0, the time that the natural logarithm of the number of cfu was ≤ 0.10 was used as the end point. For all analyses, values of $P \leq 0.05$ (type-I error) were considered significant.

Results

Chemotactic activity—Incubation of bovine and porcine AM with tilimicosin for 2 or 24 hours did not significantly affect chemotactic activity. There was no effect of concentration at each time nor of time at each concentration.

Phagocytic activity—Incubation of bovine and porcine AM with tilimicosin for 24 hours did not significantly affect phagocytic activity, regardless of whether porcine AM were washed free of tilimicosin prior to inoculation with the bacteria.

Bactericidal activity of bovine AM—Fourth-degree polynomial models were needed to adequately describe the timed-killing curves constructed following incubation of *M haemolytica* with tilimicosin (0 or 10 $\mu\text{g/ml}$) and bovine AM (Fig 1). The curve constructed following incubation of *M haemolytica* with the highest concentration of tilimicosin (20 $\mu\text{g/ml}$) and bovine AM was best described by a single-exponential polynomial, but the exponent was not significantly different from the first exponent of the polynomial that described the timed-killing curve following incubation with bovine AM and tilimicosin at a concentration of 10 $\mu\text{g/ml}$. Timed-killing curves constructed after incubation of *M haemolytica* with tilimicosin (0, 10, or 20 $\mu\text{g/ml}$) but without bovine AM were best described by a bi-exponential polynomial.

Areas (mean \pm SD) under the timed-killing curve following incubation of *M haemolytica* with bovine AM and tilimicosin at a concentration of 10 $\mu\text{g/ml}$ ($3.01 \times 10^4 \pm 2.919 \times 10^4 \text{ cfu} \cdot \text{h}/2.5 \times 10^5 \text{ AM}$) or 20 $\mu\text{g/ml}$ ($2.73 \times 10^4 \pm 3.484 \times 10^4 \text{ cfu} \cdot \text{h}/2.5 \times 10^5 \text{ AM}$) were significantly less than area under the curve following incubation of *M haemolytica* with bovine AM

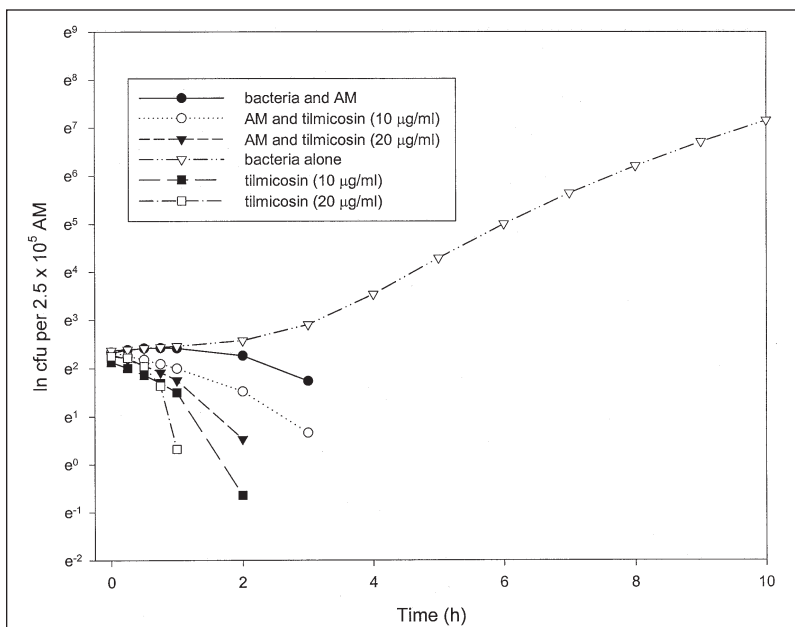


Figure 1—Least-squares, best-fit, polynomial curves of bacterial numbers (natural logarithm of number of colony-forming units [cfu] per 2.5×10^5 alveolar macrophages [AM]) following incubation of *Mannheimia haemolytica* alone or with tilimicosin at a concentration of 10 or 20 $\mu\text{g/ml}$ and following incubation of *M haemolytica* with bovine AM alone or with bovine AM that had been treated with tilimicosin at a concentration of 10 or 20 $\mu\text{g/ml}$.

without tilmicosin ($1.49 \times 10^5 \pm 1.017 \times 10^5$ cfu · h/ 2.5×10^5 AM). Areas under the timed-killing curve following incubation of *M haemolytica* without AM but with tilmicosin at a concentration of 10 µg/ml ($1.96 \times 10^3 \pm 1.537 \times 10^3$ cfu · h/ 2.5×10^5 AM) or 20 µg/ml ($2.33 \times 10^3 \pm 2.145 \times 10^3$ cfu · h/ 2.5×10^5 AM) were not significantly different from areas following incubation of the bacteria with bovine AM treated with tilmicosin at the same concentrations. Area under the timed-killing curve following incubation of *M haemolytica* without tilmicosin or AM ($2.36 \times 10^3 \pm 0.659 \times 10^3$ cfu · h) was significantly greater than area following incubation of the bacteria with bovine AM but without tilmicosin ($1.49 \times 10^5 \pm 1.017 \times 10^5$ cfu · h/ 2.5×10^5 AM).

Time to cessation of bacterial growth was shortest when *M haemolytica* was incubated with 10 or 20 µg tilmicosin/ml without bovine AM. The time to cessation of bacterial growth for *M haemolytica* incubated with bovine AM treated with 20 µg tilmicosin/ml was not significantly different from times following incubation of the bacteria with tilmicosin at a concentration of 10 or 20 µg/ml without bovine AM (Table 1).

Bactericidal activity of porcine AM—Third- and fourth-degree polynomial models were required to describe the timed-killing curves constructed follow-

Table 1—Time to cessation of bacterial growth during incubation of various bacterial organisms with bovine or porcine alveolar macrophages (AM) with or without tilmicosin

Incubation conditions	Time to cessation of growth (h)	
	Mean	SD
<i>Mannheimia haemolytica</i>		
With bovine AM		
Tilmicosin 0 µg/ml (7)	5.01 ^a	0.963
Tilmicosin 10 µg/ml (6)	4.64 ^a	2.794
Tilmicosin 20 µg/ml (4)	2.64 ^{a,b}	0.113
Without bovine AM		
Tilmicosin 0 µg/ml (2)	4.86 ^a	0.149
Tilmicosin 10 µg/ml (2)	2.35 ^{a,b}	0.021
Tilmicosin 20 µg/ml (3)	1.32 ^b	0.019
<i>Actinobacillus pleuropneumoniae</i>		
With porcine AM		
Tilmicosin 0 µg/ml (5)	6.55 ^a	0.666
Tilmicosin 10 µg/ml (7)	2.92 ^b	1.131
Tilmicosin 10 µg/ml (5)*	6.57 ^a	1.026
Without porcine AM		
Tilmicosin 0 µg/ml (2)	4.10 ^b	0.396
Tilmicosin 10 µg/ml (3)	3.83 ^b	0.150
<i>Pasteurella multocida</i>		
With porcine AM		
Tilmicosin 0 µg/ml (9)	8.25 ^a	1.728
Tilmicosin 10 µg/ml (10)	7.33 ^{a,b}	5.812
Tilmicosin 10 µg/ml (10)*	8.59 ^a	1.094
Without porcine AM		
Tilmicosin 0 µg/ml (1)	10.23 ^a	NA
Tilmicosin 10 µg/ml (2)	2.04 ^b	0.177

*Alveolar macrophages were incubated with tilmicosin for 24 hours and then washed free of the drug before addition of bacteria.

**For each organism, values with different superscript letters were significantly ($P < 0.05$) different.

NA = Not applicable.

Time to cessation of bacterial growth was defined as the time when the natural logarithm (ln) of the number of cfu per 2.5×10^5 AM (or ln of the number of cfu in the same volume of medium for suspensions without AM) was ≤ 0.1 .

Numbers in parentheses are the number of samples (n) used to calculate the value reported.

ing incubation of *A pleuropneumoniae* and *P multocida* with tilmicosin with or without porcine AM (Fig 2).

Area ($4.25 \times 10^5 \pm 3.886 \times 10^5$ cfu · h/ 2.5×10^5 AM) under the timed-killing curve following incubation of *A pleuropneumoniae* with porcine AM treated with tilmicosin at a concentration of 10 µg/ml was not significantly different from area following incubation of the bacteria with porcine AM without tilmicosin ($3.23 \times 10^7 \pm 3.097 \times 10^7$ cfu · h/ 2.5×10^5 AM). Area following incubation of *A pleuropneumoniae* with porcine AM treated with tilmicosin at a concentration of 10 µg/ml from which the antimicrobial was then removed ($4.00 \times 10^7 \pm 3.715 \times 10^7$ cfu · h/ 2.5×10^5 AM) was not significantly different from area following incubation of *A pleuropneumoniae* with porcine AM without tilmicosin but was significantly larger than area following incubation of *A pleuropneumoniae* with porcine AM treated with tilmicosin at a concentration of 10 µg/ml. Area under the timed-killing curve following incubation of *A pleuropneumoniae* without porcine AM or tilmicosin ($1.40 \times 10^6 \pm 1.397 \times 10^6$

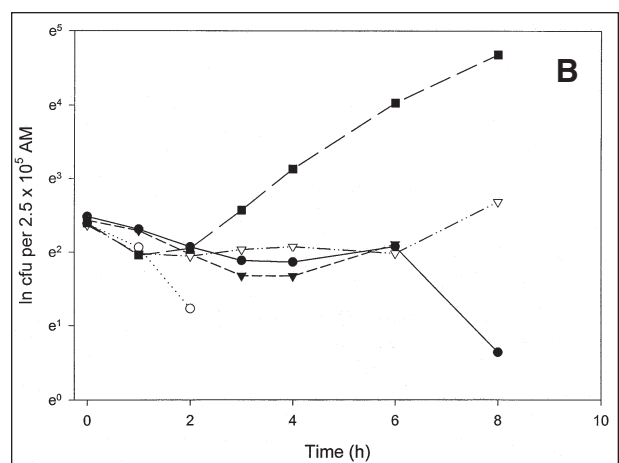
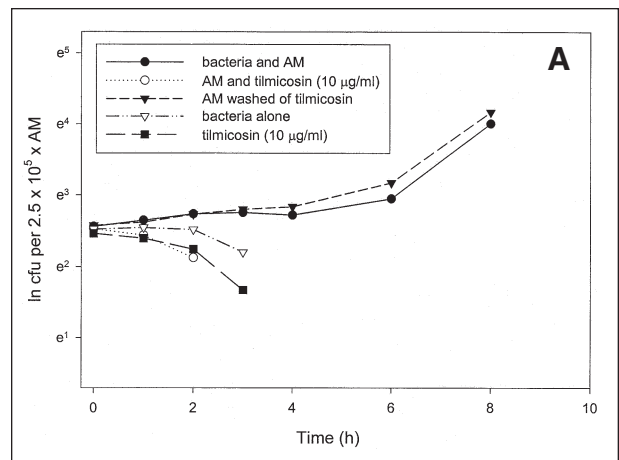


Figure 2—Least-squares, best-fit, polynomial curves of bacterial numbers following incubation of *Actinobacillus pleuropneumoniae* (A) or *Pasteurella multocida* (B) alone or with tilmicosin at a concentration of 10 µg/ml and following incubation of the bacteria with porcine AM alone, with porcine AM that had been treated with tilmicosin at a concentration of 10 µg/ml, and with porcine AM that had been treated with tilmicosin at a concentration of 10 µg/ml and had then been washed free of the drug.

cfu · h/2.5 × 10⁵ AM) and area following incubation of the bacteria with tilmicosin at a concentration of 10 µg/ml without porcine AM (2.114 × 10⁵ ± 1.181 × 10⁵ cfu · h/2.5 × 10⁵ AM) were not significantly different from values obtained when bacteria were incubated with AM and the same concentrations of tilmicosin.

Area (4.03 × 10⁴ ± 2.437 × 10⁴ cfu · h/2.5 × 10⁵ AM) under the timed-killing curve following incubation of *P multocida* with porcine AM treated with tilmicosin at a concentration of 10 µg/ml was significantly less than the value obtained following incubation of the bacteria with porcine AM not treated with tilmicosin (2.30 × 10⁵ ± 1.484 × 10⁵ cfu · h/2.5 × 10⁵ AM) but was not significantly different from area obtained following incubation of the bacteria with porcine AM that had been washed free of the drug. Area (1.55 × 10⁵ ± 1.392 × 10⁵ cfu · h/2.5 × 10⁵ AM) following incubation of the bacteria with porcine AM that had been treated with tilmicosin at a concentration of 10 µg/ml and then washed free of the drug was not significantly different from area obtained following incubation of bacteria with porcine AM without tilmicosin. Area (4.31 × 10⁴ ± 1.827 × 10⁴ cfu · h/2.5 × 10⁵ AM) obtained following incubation of *P multocida* without AM or tilmicosin was not significantly different from value (2.96 × 10⁴ ± 0.9719 × 10⁴ cfu · h/2.5 × 10⁵ AM) obtained when the organism was incubated with tilmicosin (10 µg/ml) without AM.

Times to cessation of growth of *A pleuropneumoniae* were shortest when the bacteria were incubated with porcine AM treated with tilmicosin at a concentration of 10 µg/ml, with tilmicosin (10 µg/ml) without AM, and without tilmicosin or AM (Table 1). Time to cessation of growth of *P multocida* was shortest when bacteria were incubated with tilmicosin (10 µg/ml) without AM.

Discussion

Results of the present study indicate that tilmicosin does not adversely affect the chemotactic or phagocytic activities of bovine AM, that bovine AM inherently kill *M haemolytica*, and that tilmicosin enhances killing of *M haemolytica* by bovine AM. A concentration-related effect was suggested, because bovine AM killed *M haemolytica* more quickly when they were incubated with tilmicosin at the highest concentration used (20 µg/ml).

Results further indicated that tilmicosin does not adversely affect the chemotactic or phagocytic activities of porcine AM and that tilmicosin enhances killing of *A pleuropneumoniae* and *P multocida* by porcine AM. However, killing of *A pleuropneumoniae* and *P multocida* may have been a result of direct antibacterial activity of the drug rather than a result of alterations in the bactericidal activity of the porcine AM. Removal of tilmicosin from porcine AM that had been exposed to the drug at a concentration of 10 mg/ml for 24 hours reduced bactericidal activity of those porcine AM.

In this study, bactericidal activity was not enhanced in proportion to the ability of bovine and porcine AM to concentrate tilmicosin intracellularly.^{a,b} Variations in time-course of the bactericidal activity

were not explained by results of this study, and organisms in this study did not all respond the same. The implication is that generalizations cannot be made, and each organism should be evaluated separately.

Time-courses of bacterial growth and bactericidal activity in the present study were not linear and were best described by polynomial equations. Because the time-course was a first-order process, transformation of the data to natural logarithms for analysis was appropriate.²⁰ However, the polynomial shape of the timed-killing curves was not anticipated and cannot be explained. Results of this study call to question the legitimacy of the linear evaluation that is universally used for bactericidal assays. Results of bactericidal assays may be used to predict times for cessation of bacterial growth under given experimental conditions. It was necessary, however, to develop an end-point that could be used following conversion of numbers of cfu to natural logarithms. Time to cessation of bacterial growth differed with the organism used, whether viable AM were present, and the concentration of tilmicosin used. In clinical situations, host defenses, species of bacteria, size of inoculum, concentration of medication, and microenvironmental conditions will also differ among patients. Thus, this method of estimating time to cessation of bacterial growth may be more applicable for development of dosage regimens to be evaluated in controlled prospective clinical investigations than are other methods such as time that a drug exceeds the minimum inhibitory concentration against a particular organism or time to peak concentration of the drug in plasma.

^aFossler SC, Moran JW, Thomson TD. Pharmacologic mechanism for tilmicosin in the control of cattle pneumonia (abstr), in *Proceedings*. 79th Annu Conf Res Workers Anim Dis, 1998;82.

^bBlais J, Chamberland S. Intracellular accumulation of tilmicosin in primary porcine alveolar macrophages, in *Proceedings*. 13th IPVS Congr, 1994;331.

^cWomens' nylon hosiery, Hanes, Rural Hall, NC.

^dHistopaque, Sigma Chemical Co, St Louis, Mo.

^eSigma red cell lysing buffer, Sigma Chemical Co, St Louis, Mo.

^fRPMI (Rosewell Park Memorial Institute), Sigma Chemical Co, St Louis, Mo.

^gLilly Research Laboratories, Greenfield, Ind.

^hLPS, Sigma Chemical Co, St Louis, Mo.

ⁱMIP, Sigma Chemical Co, St Louis, Mo.

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