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Production and Characterization of Monoclonal Antibodies against the Antibiotic Tilmicosin

ROSS C. BEIER,*,† LAWRENCE C. CREEMER,‡ RICHARD L. ZIPRIN,† AND DAVID J. NISBET†

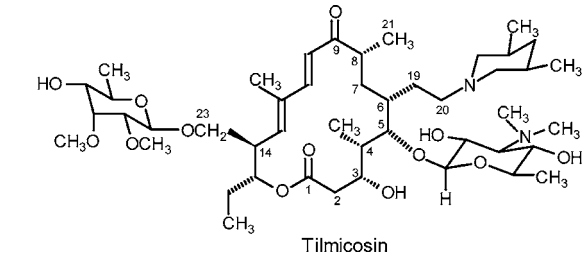
Southern Plains Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, 2881 F&B Road, College Station, Texas 77845-4998, and Elanco Animal Health, A Division of Eli Lilly and Company, 2001 West Main Street, Greenfield, Indiana 46140

Monoclonal antibodies (Mabs) were developed that specifically bind tilmicosin. Keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) conjugates were used for the immunogen and plate coating antigen, respectively. The conjugates were synthesized by different methods, resulting in different linkages. Six hybridoma cell lines were isolated that produced Mabs that competed with tilmicosin, and have IgG1 isotype. The Til-1 and Til-5 Mabs had IC_{50} values for tilmicosin of 9.6 and 6.4 ng/well (48 and 32 ng/mL), respectively, and limits of detection at IC_{20} of 1.84 and 0.89 ng/well (9.2 and 4.45 ng/mL), respectively. The Mabs demonstrated high cross-reactivity to the macrolides containing 3,5-dimethylpiperidine at C20 and the amino sugar at C5. No cross-reactivity was observed for tylosin and other macrolides that did not contain 3,5-dimethylpiperidine. A competitive enzyme-linked immunosorbent assay (ELISA) was developed for the antibiotic tilmicosin by use of the developed Mabs. These Mabs may be excellent candidates for the determination and immunolocalization of tilmicosin.

KEYWORDS: Antibiotic; ELISA; immunoassay; Mabs; macrolide antibiotics; monoclonal antibodies; tilmicosin; tylosin

INTRODUCTION

20-Deoxo-20-(3,5-dimethylpiperidin-1-yl)desmycosin (tilmicosin) is a semisynthetic macrolide broad-spectrum antibiotic derived from the macrolide antibiotic tylosin (*1*, *2*) (**Figure 1**), which is produced by fermentation of *Streptomyces fradiae*. As a result of its long half-life, it is effective for the treatment of respiratory diseases in cattle (*3*−*5*). A single subcutaneous injection in cattle of 10 mg/kg tilmicosin can result in pulmonary tissue tilmicosin levels above the minimum inhibitory concentration (MIC) for *Pasteurella haemolytica* (*6*), a cause of pneumonia in cattle (*3*). Most antimicrobial agents have limited ability for cellular penetration, but tilmicosin accumulates in polymorphonuclear neutrophilic leukocytes (PMN) and macrophage cells in bovine lung tissue (*7*). Tilmicosin is effective for treating cattle with leptospirosis caused by *Leptospira borgpetersenii* serovar Hardjo (*8*), which can cause mastitis, decreased milk production, weak calves, abortion, and infertility (*9*). Tilmicosin is approved for veterinary use in swine (*10*) and poultry (*11*) to combat respiratory diseases and also is used for treating sheep in various areas of the world for mastitis and respiratory disease (*12*−*14*).



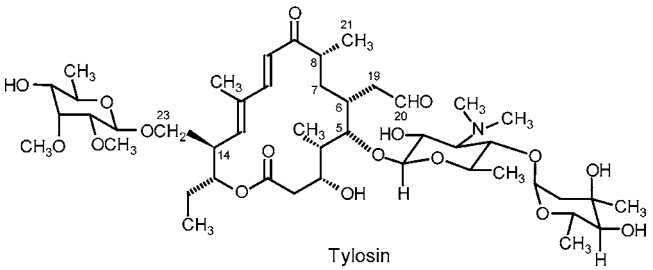


Figure 1. Chemical structures of the macrolide antibiotics tilmicosin and tylosin.

Attempts have been made to produce antibodies to tylosin-related macrolides for use in determining residues of tilmicosin and tylosin in feeds and tissues. Jackman et al. (*15*) reported preparation of polyclonal antibodies against desmycosin, conjugated through the C20 aldehyde group; however, such

* To whom correspondence should be addressed: phone 979-260-9411; fax 979-260-9332; e-mail rcbeier@ffsru.tamu.edu.
† Southern Plains Agricultural Research Center.
‡ Elanco Animal Health.

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polyclonal antibodies also had 96% cross-reactivity with tylosin (*16*). Wicker et al. (*17*) reported the use of polyclonal antibodies to tylosin in a fluorescence immunoassay for detecting tylosin in premix and feeds. The antibodies produced to tylosin also cross-reacted with tilmicosin. Yao and Mahoney (*18*) produced polyclonal antibodies to 23-deoxy-23-amino-*O*-mycaminosyltylonolide; however, these antibodies demonstrated reactivity with 12-, 14-, and 16-membered macrolides that contained amino sugar moieties, regardless of the presence of neutral sugar residues.

We required Mabs that had high specificity to investigate potential tissue binding sites of tilmicosin. This paper describes six hybridoma cell lines, two of which (Til-1, ATCC PTA-3715; and Til-5, ATCC PTA-3714) have been deposited with the American Type Culture Collection (ATCC). These hydridoma cell lines produce Mabs that may be used for the detection of the antibiotic tilmicosin. Details of conjugation, production, and characterization of these antibodies are reported in this paper.

MATERIALS AND METHODS

Chemicals and Materials. Bovine serum albumin (BSA, A-7030), dimethyl sulfoxide (DMSO), ethylenediaminetetracetic acid (EDTA), 2-(*N*-morpholino)ethanesulfonic acid (MES), polyoxyethylene-sorbitan monolaurate (Tween 20), sodium azide, sodium bicarbonate, sodium carbonate, sodium phosphate, Na_2HPO_4, Trizma Hydrochloride, Trizma Base, 8-azaguanine (Sigma A-5284 Hybri-Max), pristane (2,6,10,14-tetramethylpentadecane, Sigma P-1403) 95%, HAT medium supplement (hypoxanthine, aminopterin and thymidine, Sigma H-0262), HT medium supplement (hypoxanthine and thymidine, Sigma H-0137), and goat anti-mouse IgG (whole molecule) peroxidase conjugate (Sigma A-5278) were obtained from Sigma Chemical Co. (St. Louis, MO). Argon gas was obtained from Bailey Oxygen & Tool Co., Inc., Bryan, TX. Tylosin, 5-*O*-mycaminosyltylonolide (OMT), demycinosyltylosin (DMT), tilmicosin, 20-deoxo-20-(3,5-dimethylpiperidin-1-yl)-χ-tylosin (Chem 354415), 20-deoxo-20-(3,5-dimethylpiperidin-1-yl)macrocin (Chem 354477) (*2*), 20-deoxo-20-(3,5-dimethylpiperidin-1-yl)-2″-demethyllactenocin (Chem 354478), 20-deoxo-20-(3,5-dimethylpiperidin-1-yl)lactenocin (Chem 354830) (*2*), *N*-demethyltilmicosin (Chem 303540), and 20-deoxo-20-[3,5-di-(trifluoromethyl)piperidin-1-yl]desmycosin (Chem 307645) (*19*) were supplied by Elanco Animal Health R&D, 2001 West Main St., Greenfield, IN. 3,5-Dimethylpiperidine, *cis*-2,6-dimethylpiperidine, 1-, 2-, 3-, and 4-methylpiperidine, piperidine, 1-piperidineethanol, and sodium chloride (NaCl) were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 22980), 2-iminothiolane hydrochloride (Traut's reagent, 26101), and Imject maleimide-activated immunogen conjugation kit with mcKLH (77607) were obtained from Pierce Chemical Co. (Rockford, IL). NAP 5 Columns were obtained from Amersham Biosciences Corp., Piscataway, NJ. BALB/c mice and Hsd:ICR(CD-1) outbred mice were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN). Ribi-Corixa adjuvant (R700, MPL + TDM emulsion in 2% oil−Tween-80) was obtained from Corixa Corp. (Hamilton, MT). Nonfat dry milk (NFDM) was Janet Lee brand instant nonfat dry milk fortified with vitamins A and D_3 containing 35.5% protein (Albertson's Inc., Boise, ID) and was obtained from a local grocery store. Iscoves modified Dulbecco's medium (Gibco BRL 12200-069), penicillin/streptomycin (Gibco BRL 15140-122), and L-glutamine (Gibco BRL 25030-081) were obtained from Gibco/Life Technologies (Grand Island, NY). Fetal bovine serum was obtained from JRH Biosciences (Lenexa, KS). Sample dilutions were made with a portable pipet-aid, electrically actuated (Drummond Scientific Co., Broomall, PA). A multichannel pipettor was used to conduct the cELISA [Finnpipet, digital multichannel 50−300 μL pipet (LSI North America, Needham Heights, MA)]. CoStar 96w microtiter plates (07-200-90) and CoStar 24w plates (3524) were obtained from CoStar Corp. (Cambridge, MA). Nylon cloth sieve with 100 μm pore size (34-1800-04) and Nunc-Immuno plates F96 MaxiSorp and Nunc lids (Nunc 439454) used for immunoassays were obtained from PGC Scientifics Corp. (Frederick, MD). K-Blue substrate was obtained from Elisa Technologies (Lexington, KY). Antibody isotype was determined by use of SBA Clonotyping System/AP (5300-04, Southern Biotechnology Associates, Inc., Birmingham, AL). The miniPERM bioreactor (IV-76001059) was obtained from Sartorius (New York, NY). Optical densities of developed assays were read with a 96-well Spectra Max 340PC microplate reader (Molecular Devices, Sunnyvale, CA). Software was used at the settings: 8 × 12 format, automix (3 s). Reverse osmosis water, pyrogen-free (RO H_2O), was produced on site by a reverse osmosis system obtained from Millipore Corp. (Bedford, MA), and used for all cELISA experiments.

Preparation of Immunogen and Plate Coating Antigens. The hapten used for the immunogen and plate-coating conjugate were synthesized by use of 23-demycinosyl-23-deoxy-23-(3-aminoprop-1-yl)aminotilmicosin (hapten) (*20*). Since heterologous assays can improve sensitivity (*21*, *22*) and overcome unwanted cross-reactivity (*23*), KLH and BSA conjugates were used for the immunogen and plate-coating antigen, respectively, and the conjugates were synthesized by different methods.

KLH Conjugate. The immunogen was produced by completing the linker arm of the hapten and conjugating the product to maleimide-activated mcKLH. The free amino terminus (NH_2) of the hapten was reacted with Traut's reagent to terminate the hapten with a reactive -SH group (**Figure 2**). The hapten (86% purity, 1.82 mg, 2.423 μmol) was dissolved with DMSO (30 μL) and then 190 μL of maleimide conjugation buffer (0.083 M sodium phosphate buffer, 0.1 M EDTA, 0.9 M NaCl, and 0.02% sodium azide, pH 7.2) was added. (Note: All solvents used, maleimide conjugation buffer, DMSO, and H_2O were deaerated with argon gas and stored in a septum-sealed flask under argon gas.) Traut's reagent (0.3 mg, 30 μL of a 1 mg/100 μL solution, 2.35 μmol) was added. The solution was stirred for 1 h in the absence of light to afford the activated sulfhydryl-containing hapten (**Figure 2**). Maleimide-activated mcKLH (2 mg) was dissolved with argon-treated H_2O (200 μL), and the mcKLH solution was transferred to the above completed Traut's reaction (two 25 μL aliquots of the maleimide conjugation buffer was used to make the transfer). The reaction mixture was stirred for 2 h at room temperature. The reaction mixture was purified to remove the salts and unreacted material by using a NAP 5 column. The NAP 5 column was equilibrated with 10 mL of 0.083 M sodium phosphate, 0.9 M NaCl, pH 7.2 (purification buffer), and after the purification buffer completely entered the column bed, the sample (0.5 mL) was placed on the column. When the sample completely entered the bed, it was eluted with purification buffer, and the first 1 mL aliquot was collected. This immunogen solution (1.165 mg/mL) was added to Ribi-Corixa adjuvant and used for immunization.

BSA Conjugate. The plate-coating conjugate was produced by the Pierce protocol for EDC-assisted hapten coupling to carrier proteins (Pierce Biotechnology, Inc., Rockford, IL). BSA (3 mg, approximately 1.34 μmol sites) was added to 300 μL of 0.1 M MES, pH 5 (EDC coupling buffer). The hapten (5.856

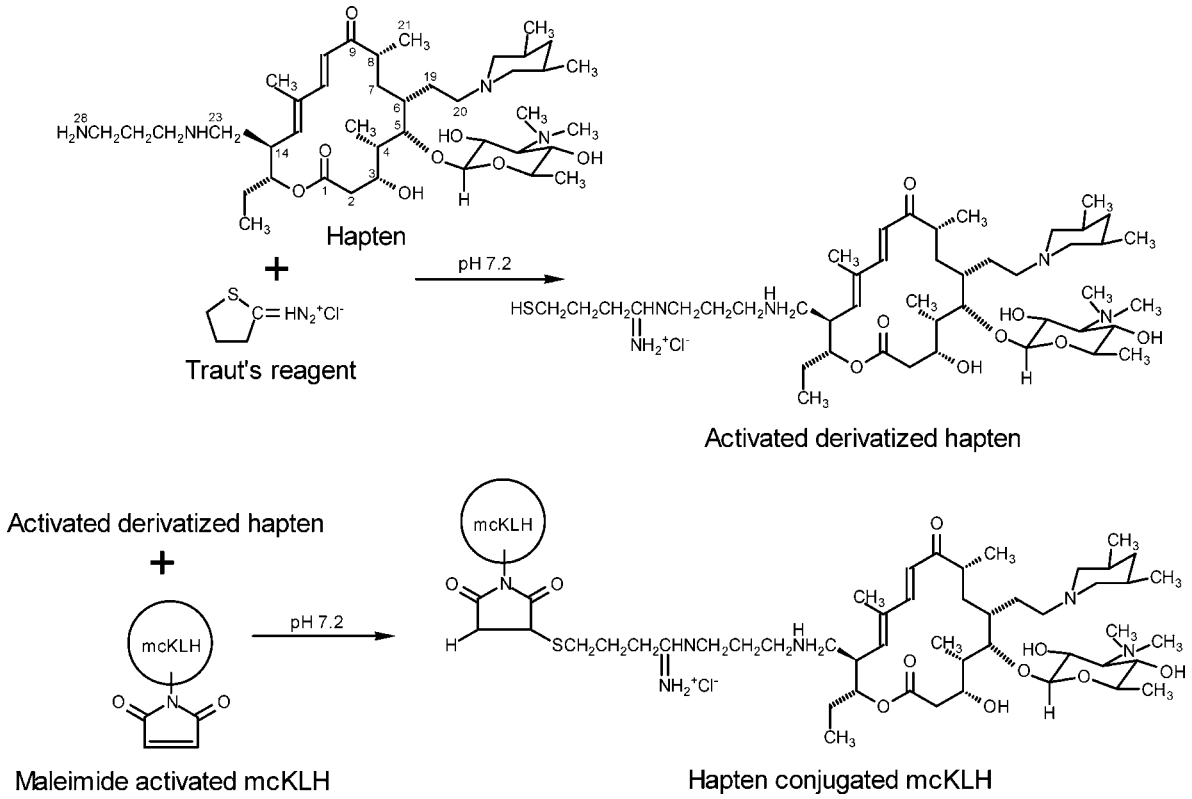


Figure 2. Synthetic pathway for producing the immunogen with mcKLH as carrier protein.

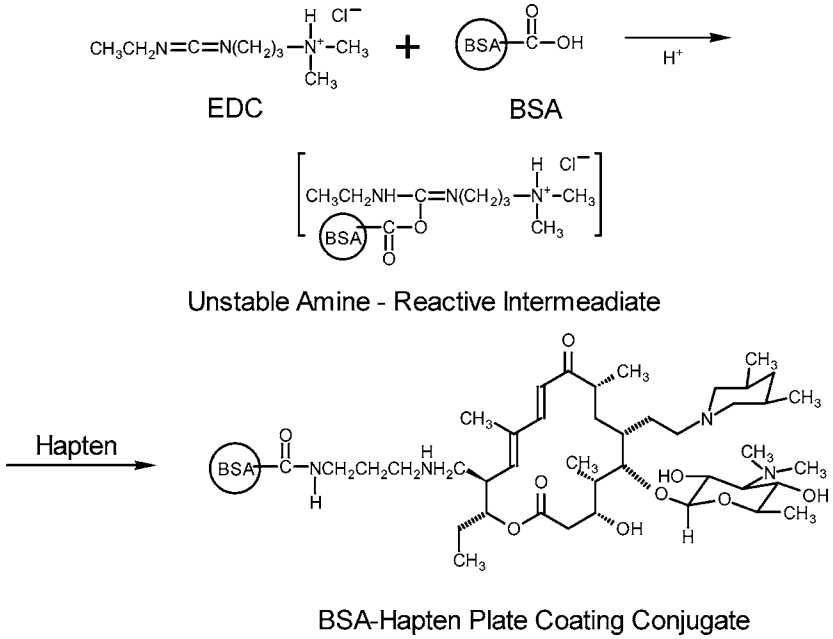


Figure 3. Synthetic pathway for producing the plate coating antigen by use of BSA.

mg, 7.8 μmol) was dissolved in coupling buffer (550 μL). The BSA solution was added to the hapten solution. EDC (12.8 mg) was dissolved in the EDC coupling buffer (1.28 mL), and 150 μL of the solution was immediately added to the reaction mixture (**Figure 3**). The reaction was stirred for 2 h at room temperature. The reaction mixture was purified by gel filtration on two NAP 5 columns that were previously equilibrated with purification buffer (each column was filled three times for a total of 10 mL each). The reaction mixture was split into two 0.5 mL portions. Each portion was allowed to fully enter the column bed of the respective NAP 5 column. Each column was eluted with the purification buffer, and the first 1 mL was collected. The combined solutions resulted in an approximate protein concentration of 1.83 mg/mL. This plate-coating antigen was used in 96-well plates at 75 ng of protein/well.

cELISA Solutions. Detergent wash buffer was made by adding Tween 20 (0.05% v/v) to RO H_2O. Carbonate coating buffer consisted of Na_2CO_3 (0.015 M), $NaHCO_3$ (0.035 M), and $MgCl_2$ (0.002 M) in RO H_2O, pH 9.6. Phosphate-buffered saline (PBS-9) contained Na_2HPO_4 (0.01 M) and NaCl (0.15 M) in RO H_2O, pH 9. Blocking buffer consisted of NFDM (3% w/v) in PBS-9. Assay buffer consisted of adding part A (495 mL) and part B (5 mL) solutions together. Part A contained (per 1 L of water) 11.4 g of Trizma hydrochloride, 3.32 g of

Trizma base, and 8.7 g of NaCl in RO H_2O adjusted to pH 7.4 with HCl or NaOH. Part B contained (per 95 mL of water) 1 g of NFDM and 0.5 mL of Tween-20.

Assay Plate Coatings. Nunc-Immuno plates with a MaxiSorp surface were washed with detergent wash buffer followed by a RO H_2O rinse to clean the plates prior to coating. Assay plates were coated overnight at 4 °C with a solution containing the BSA−hapten conjugate (75 ng per 100 μL per well) in carbonate coating buffer. After coating overnight, plates were washed with detergent wash buffer followed by a RO H_2O rinse. Assay plates were incubated with blocking buffer (300 μL/well), for 1 h at room temperature and rinsed three times with RO H_2O.

Cell Growth Media. Iscoves modified Dulbecco's medium was used containing the following additives: L-glutamine (100 mL/10 L of medium) and $NaHCO_3$ (3.7 g/L). The medium was sterile-filtered into 500-mL bottles and incubated overnight at 37 °C to ensure sterility. Medium was stored at 4 °C. Complete medium was used for growing the SP2/0 myeloma cells and for all other cell maintenance applications and consisted of the addition of fetal bovine serum (FBS, 25 mL/500 mL) and a penicillin/streptomycin (Pen/Strep) solution (5 mL/500 mL) resulting in a final concentration of 100 units/mL and 100 μg/mL, respectively. HAT medium (2×) was used only for the newly fused cells to select against unfused cells. Two vials were reconstituted to 10 mL each and added to complete medium (480 mL). The concentration of added chemicals in the HAT (2×) medium was 2×10^{-4} M hypoxanthine, 8×10^{-7} M aminopterin, and 3.2×10^{-5} M thymidine. The fused cell suspension in HAT (2×) medium was pipetted (100 μL/well) into 30 macrophage-coated 96-well Costar 96w plates. Final concentration of HAT on the fusion-coated plates was 1×. Addition of HT medium supplement, Hybri-Max, following reconstitution to 10 mL, to complete medium (490 mL) resulted in concentrations of hypoxanthine and thymidine of 1×10^{-4} M and 1.6×10^{-5} M, respectively.

Competitive ELISA. A cELISA was developed to evaluate antibody specificity and to quantify tilmicosin in solution. Assay plate preparation and coating follow those steps described above under Assay Plate Coatings. The competitor (tilmicosin) solution in assay buffer (200 μL) was added in column 2 of the 96-well assay plate. This solution was diluted 1:2 across the plate with assay buffer (100 μL) to form a concentration gradient of the competitor. Antibody from one of six isolated hybridomas was diluted in assay buffer and was added (100 μL) to all wells except column 1 (solvent control). Wells in column 1 contained only assay buffer (100 μL). The amount of antibody used was that amount which produced a minimum optical density reading of 0.45 absorbance unit after background subtraction. The sample−antibody mixture was incubated for 1 h at 37 °C, and the plates were washed with detergent wash buffer and rinsed with RO H_2O. Then, goat anti-mouse IgG (whole molecule) horseradish peroxidase conjugate diluted 1:500 in assay buffer, plus NFDM (2% w/v), was added (100 μL) to each well. Following a second 1-h incubation at 37 °C, the plates were washed with detergent wash buffer and rinsed with RO H_2O. K-Blue substrate (100 μL/well) was added to each well and incubated at room temperature for 30 min. A stop solution of 2 N H_2SO_4 (50 μL) was added to each well, and absorbance measurements (450 nm) were read with a 96-well Spectra MAX 340PC microplate reader. The mean absorbance of 96 reaction solution background measurements from 12 96-well plates was 0.08 ± 0.03.

Immunization of Mice. The immunogen was prepared for injection by addition of hapten-conjugated mcKLH (**Figure 2**) and sterile isotonic saline to the contents of one vial of Ribi-Corixa adjuvant system for a final volume of 2 mL. The mixture was emulsified to produce a solution of hapten-conjugated mcKLH conjugate in Ribi-Corixa adjuvant (75 μg/0.15 mL). BALB/c mice were immunized ip with 0.1 mL and im with 0.05 mL of the mixture at a minimum of three times at 2-week intervals. One week after the third injection, blood was collected from the tail vein to determine which mouse had the highest anti-hapten titer and best competition for tilmicosin. The selected mouse received a booster injection ip of 75 μg/0.15 mL of only the hapten-conjugated mcKLH (no Ribi-Corixa adjuvant) in sterile isotonic saline 3−4 days prior to the day of fusion.

Monoclonal Antibody Production: *Fusion of SP2/0 Myeloma Cells and Spleen Cells.* Portions of the fusion and cloning conditions were described previously by Stanker et al. (*24*) with further modifications by Beier et al. (*23*). Macrophage cells were obtained from Hsd:ICR(CD-1) outbred mice treated with pristane. SP2/0 myeloma cells were passed through medium containing 8-azaguanine and then grown for 4−5 days immediately before the fusion. On the day of fusion, the mouse (that received the booster injection) was sacrificed by cervical dislocation and the spleen was removed aseptically. The spleen was disrupted through a tissue sieve into serum-free Iscoves modified Dulbecco's medium, and the resulting cell suspension was strained through a sterile nylon cloth sieve with 100 μm pore size into a sterile beaker. Splenocytes were fused with SP2/0 myeloma cells by using poly(ethylene glycol) (*25*) and were cultured in 96-well Costar plates containing a macrophage feeder cell layer.

Screening of the Cell Fusion. Cells usually are ready for their initial screening in 10−14 days. In the initial screen, the supernatant from each well of 30 fusion-coated plates was evaluated for antibody binding to the coating conjugate. Cells from positive wells (approximately 144) were transferred to 24-well Costar plates to allow expansion of cells and production of enough antibody to further test for anti-hapten titer and complete competition studies with the target chemical (tilmicosin). After a grow-out period of 7−10 days, the supernatants from the 24-well Costar plates were checked for antibody titer levels, followed by competition studies. Those wells that exhibited competition with tilmicosin and were negative for competition with tylosin were then further submitted to another hybridoma cloning.

Hybridoma Cloning. Following the initial hybridoma screening, HT medium was used for cell maintenance until the final selections were made. Screening for antibody-producing hybridomas made use of the cELISA described above. Hybridoma cells from wells showing antibody competition to tilmicosin were expanded and subcloned three times by limiting dilution. The wells picked for expansion were viewed under a microscope to confirm the presence of a single cell source in the well, ensuring their monoclonal origin. After the final selections were made, the concentration of HT medium was gradually reduced from the selected cell lines and they were placed in complete medium.

Mab Production. Rather than producing antibodies by ascites tumor production as described in Stanker et al. (*24*), the Mabs were produced by in vitro production similar to Mabs produced against nicarbazin (*23*). In vitro production of Til-1 and Til-5 Mabs in high concentration was accomplished in a reusable modular minifermenter for high-density culture of hybridoma cells, the miniPERM bioreactor (*26*). These Mabs were purified by protein G chromatography.

Characterization of Mabs: *Isotype and Affinity.* Antibody isotypes were determined by use of reagents supplied in the

commercially available kit SBA Clonotyping System/AP. Relative affinity of the six Mabs (Til-1, Til-2, Til-3, Til-4, Til-5, and Til-6) for tilmicosin was measured by determining the 50% inhibition of control values (IC_{50}). An antibody dilution was used that produced an absorbance minus control of 0.45 in the absence of competitor.

Antibody Specificity. Tilmicosin, tylosin, 5-*O*-mycaminosyltylonolide (OMT), demycinosyltylosin (DMT), and 23-demycinosyl-23-deoxy-23-(3-aminoprop-1-yl)aminotilmicosin (hapten) (*20*) were used in cross-reactivity studies. Other chemicals used were chosen in two different ways. A number of chemicals were structurally similar to tilmicosin or tylosin: Chem 354415, Chem 354477, Chem 354478, Chem 354830, Chem 303540, and Chem 307645. Also, a number of compounds have structural similarities to 3,5-dimethylpiperidine, which is the moiety synthetically added to help convert tylosin to tilmicosin (*1*,*2*): piperidine, 1-, 2-, 3-, and 4-methylpiperidine, 1-piperidineethanol, *cis*-2,6-dimethylpiperidine, and 3,5-dimethylpiperidine. Each of the compounds studied was dissolved in assay buffer (pH 7.4) and examined as a potential competitor by use of the cELISA described above. Structural information regarding these compounds is presented in **Table 1**.

Statistical Analysis. Mab binding results was analyzed with the Kruskal−Wallis test (nonparametric ANOVA) and Dunn's multiple comparison test. These were each accomplished by using GraphPad's Instat software, version 3.01, San Diego, CA.

RESULTS AND DISCUSSION

Synthesis of Antigen and Plate-Coating Conjugate. Previous polyclonal antibodies produced to a conjugate through the C20 aldehyde group of tylosin (*15*) had about equal cross-reactivity with tylosin as they did with tilmicosin (*16*). Polyclonal antibodies produced to tylosin also cross-reacted to tilmicosin (*17*), and polyclonal antibodies produced to 23-deoxy-23-amino-*O*-mycaminosyltylonolide cross-reacted with various sized macrolides that possessed an amino sugar (*18*). In an attempt to produce antibodies specific for tilmicosin that did not bind tylosin, we developed this specificity by synthesizing the hapten 23-demycinosyl-23-deoxy-23-(3-aminoprop-1-yl)-aminotilmicosin (**Figure 2**) (*20*).

The hapten was reacted with Traut's reagent to produce a free -SH group on the linker arm that readily reacted with maleimide-activated mcKLH to produce the immunogen and was then injected into BALB/c mice. It is well understood that different bridging moieties can overcome unwanted cross-reactivity associated with antibody binding (*23*, *27*, *28*). Therefore, the plate-coating conjugate was produced differently than the immunogen by coupling the hapten to BSA by an EDC reaction (**Figure 3**).

Production of Monoclonal Antibodies to Tilmicosin. Ten days following the fusion, growing hybridomas were observed in many of the 2880 wells of the seeded 96-well plates. Supernatants of all wells were tested for anti-tilmicosin activity by use of BSA−hapten-coated plates in the ELISA described above. Hybridoma cultures from 120 wells with the highest anti-tilmicosin activity were selected for further evaluation. The hybridomas in these wells were expanded in 24-well plates. The supernatants were titrated on BSA−hapten-coated plates and were evaluated for competition against tilmicosin and tylosin. Six selections were chosen that exhibited good competition with tilmicosin and no competition with tylosin, and these were grown up following a limiting dilution. The wells were expanded, titrated to determine antibody levels, and followed by competition with tilmicosin. Selection of clones from these cultures by limiting dilution led to six stable hybridoma cell lines. These monoclonal cultures and their corresponding Mabs were named Til-1, Til-2, Til-3, Til-4, Til-5 and Til-6.

cELISA Development. Heterologous assays are well-known to help improve immunoassay sensitivity (*21*, *22*), and overcome unwanted cross-reactivity (*23*). We utilized the heterologous assay by using the BSA−hapten conjugate as a coating antigen and mcKLH−hapten as the immunogen (both were synthesized by different methodologies) to help overcome unwanted cross-reactivity (nonspecific binding).

Characterization of Mabs: *Isotype and Affinity*. The immunoglobulin isotype of all the Mabs Til-1, Til-2, Til-3, Til-4, Til-5 and Til-6 was found to be IgG1. The concentrations of tilmicosin at the IC_{50} (50% inhibition of control activity) (i.e., wells with no competitor present) for Til-1, Til-2, Til-3, Til-4, Til-5, and Til-6 were 9.6, 8.7, 10.8, 7.5, 6.4, and 7.6 ng/well (48, 43.5, 54, 37.5, 32, and 38 ng/mL), respectively (**Table 1**). The tilmicosin inhibition curve for the Til-1 Mab with BSA−hapten-coated plates is shown in **Figure 4**.

Specificity. **Table 1** shows all candidate compounds examined in cross-reactivity studies. All cross-reactivity studies were carried out with the BSA−hapten-conjugate coated plates. Kruskal−Wallis statistical analysis was used to test for associations of the six different Mabs with tilmicosin binding and determined that there were no statistically significant differences in the binding of these Mabs with tilmicosin. The competition result for tilmicosin with each Mab was selected to be used as the 100% cross-reactivity value for that Mab since tilmicosin was the target compound that the Mabs were detecting. Percentage of cross-reactivity was calculated according to the formula (IC_{50} in picomoles of tilmicosin per milliliter)/(IC_{50} in picomoles of the competitor per milliliter) × 100. Ten compounds out of the 19 tested showed no binding to the six Mabs. The three macrolides (tylosin, OMT, and DMT) that do not contain a 3,5-dimethylpiperidine group at the C20 carbon of the macrolide and the various piperidines (piperidine, 1-, 2-, 3-, and 4-methylpiperidine, 1-piperidineethanol, and *cis*-2,6-dimethylpiperidine) all exhibited no binding to these six Mabs (**Table 1**). The macrolides that demonstrated binding to the six Mabs contained the 3,5-dimethylpiperidine group at the C20 carbon (tilmicosin, hapten, Chem 354415, Chem 354477, Chem 354478, Chem 354830, Chem 303540, and Chem 307645), and free 3,5-dimethylpiperidine also demonstrated binding (**Table 1**).

Til-1 and Til-5 performed the best overall in the cELISA (the day to day performance of Til-1 and Til-5 in the assay was more consistent), and these hybridomas were deposited with the American Type Culture Collection (ATCC). Til-1 and Til-5 demonstrated a limit of detection value for tilmicosin at the IC_{20} of 1.84 and 0.89 ng/well (9.2 and 4.45 ng/mL), respectively. The Kruskal−Wallis statistical analysis test determined that free 3,5-dimethylpiperidine exhibited a statistically significant difference in cross-reactivity with the Til-1 and Til-5 Mabs compared to tilmicosin ($P < 0.01$ and $P < 0.01$, respectively), Chem 354478 ($P < 0.05$ and $P < 0.05$, respectively), and Chem 354830 ($P < 0.001$ and $P < 0.001$, respectively), and 3,5-dimethylpiperidine also showed a significant difference in cross-reactivity with the Til-1 Mabs compared to Chem 354415 ($P < 0.05$). The Kruskal−Wallis test determined a statistically significant difference in cross-reactivity of Chem 307645 with the Til-1 and Til-5 Mabs compared to tilmicosin ($P < 0.001$ and $P < 0.001$, respectively), Chem 354415 ($P < 0.01$ and $P < 0.01$, respectively), Chem 354478 ($P < 0.01$ and $P < 0.001$, respectively), and Chem 354830 ($P < 0.001$ and $P < 0.001$,

Table 1. cELISA Inhibition Studies with Various Macrolides and Other Piperidine Related Compounds[a]

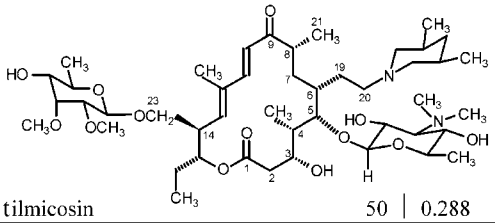
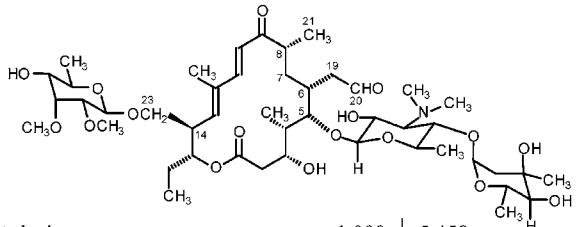
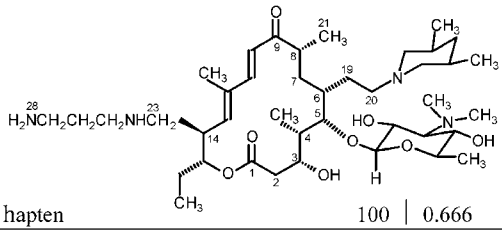
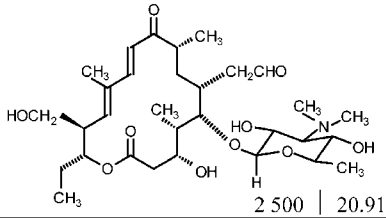
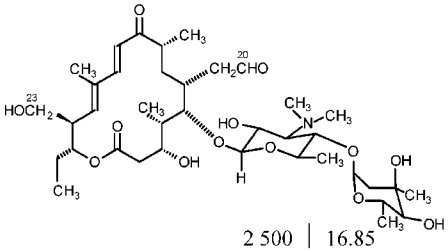
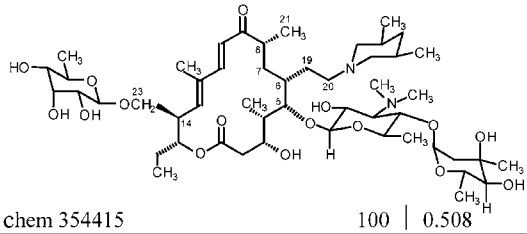
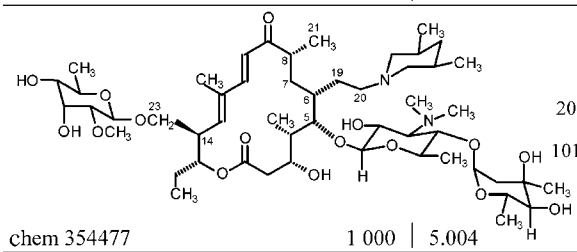
compound		IC_{50} (ng/well ±STD)[b,c] IC_{50} (pmol/mL ±STD)[d] % cross reactivity[e]					
name	max concn (ng/well | nmol/mL)[f]	Til-1	Til-2	Til-3	Til-4	Til-5	Til-6
		9.6[b] ±4.2	8.7 ±1.1	10.8 ±8.3	7.5 ±1.9	6.4 ±2.5	7.6 ±2.6
		55.4[d] ±24.2	50.1 ±6.6	62.4 ±47.8	43.1 ±10.7	36.7 ±14.5	43.9 ±15.0
tilmicosin	50 | 0.288	100[e]	100	100	100	100	100
		—[g]	—	—	—	—	—
		—[g]	—	—	—	—	—
tylosin	1 000 | 5.458	—[g]	—	—	—	—	—
		20.7[b] ±4.6	21.7 ±5.5	22.7 ±3.4	16.0 ±5.2	17.0 ±4.4	13.9 ±6.4
		138.0[d] ±30.7	144.6 ±36.8	151.4 ±22.6	106.5 ±34.4	113.1 ±29.5	92.7 ±42.9
hapten	100 | 0.666	40.1[e]	34.6	41.1	40.6	32.5	47.2
		—[g]	—	—	—	—	—
		—[g]	—	—	—	—	—
OMT	2 500 | 20.91	—[g]	—	—	—	—	—
		—[g]	—	—	—	—	—
		—[g]	—	—	—	—	—
DMT	2 500 | 16.85	—[g]	—	—	—	—	—
		13.4[b] ±2.1	12.2 ±2.0	14.8 ±2.9	16.7 ±3.1	12.8 ±3.0	12.4 ±2.5
		68.1[d] ±10.7	62.2 ±10.2	75.4 ±14.9	84.9 ±15.5	64.7 ±15.2	63.1 ±12.8
chem 354415	100 | 0.508	81.2[e]	80.8	82.7	50.9	56.6	69.5
		20.3[b] ±12.4	12.3 ±4.9	23.7 ±22.0	17.6 ±6.3	22.2 ±16.4	14.4 ±5.2
		101.7[d] ±62.0	61.7 ±24.3	118.6 ±109.9	87.9 ±31.5	111.3 ±82.3	71.9 ±26.2
chem 354477	1 000 | 5.004	54.3[e]	81.3	52.4	49.0	33.1	60.6

Table 1 (Continued)

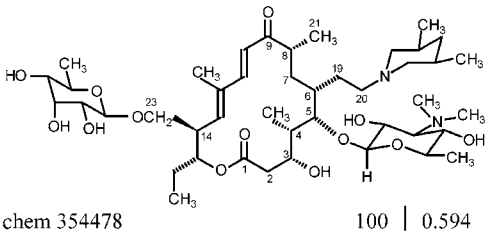
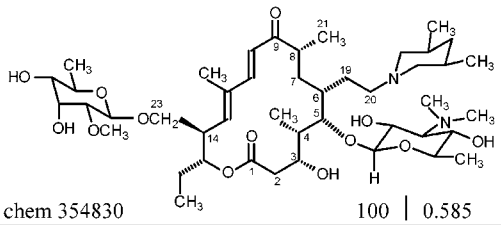
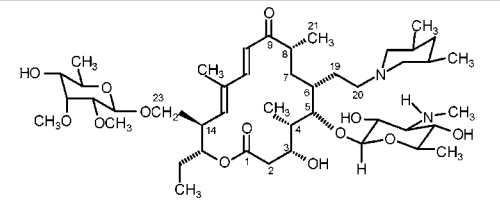
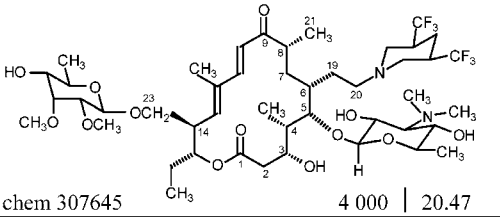
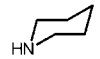
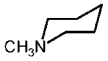
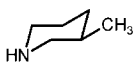
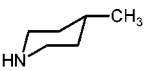
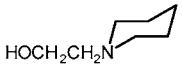
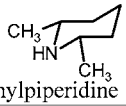
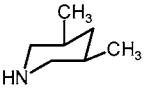
compound			IC_{50} (ng/well ±STD)[b,c] IC_{50} (pmol/mL ±STD)[d] % cross reactivity[e]					
name	max concn (ng/well)	nmol/mL)[f]	Til-1	Til-2	Til-3	Til-4	Til-5	Til-6
 chem 354478	100	0.594	11.7[b] ±2.1 69.6[d] ±12.5 79.3[e]	7.8 ±1.9 46.1 ±11.5 107.8	11.6 ±5.1 69.0 ±30.3 90.0	8.7 ±2.7 51.9 ±15.8 83.6	7.6 ±1.1 45.0 ±6.8 81.4	6.2 ±1.6 36.7 ±9.4 118.4
 chem 354830	100	0.585	4.8[b] ±1.8 27.9[d] ±10.8 196.4[e]	4.7 ±2.5 27.4 ±14.4 181.8	5.7 ±2.9 33.1 ±17.3 186.5	7.5 ±2.3 43.9 ±13.3 98.4	5.3 ±2.1 31.0 ±12.5 118.7	5.8 ±2.4 34.1 ±14.1 128.9
 chem 303540	500	2.924	16.5[b] ±5.1 96.7[d] ±30.0 57.2[e]	35.7±12.5 208.6 ±73.2 24.0	48.1±12.8 281.3 ±75.0 22.1	22.7±8.3 132.7 ±48.6 32.6	39.3±9.9 229.6 ±57.9 16.0	20.2±5.5 118.1 ±32.4 37.0
 chem 307645	4 000	20.47	293[b] ±60 1 497[d] ±308 3.7[e]	267±113 1 367 ±580 3.7	681±114 3 485 ±582 1.8	606±89 3 098 ±454 1.4	365±85 1 868 ±434 2.0	308±80 1 574 ±409 2.8
 piperidine	2 500	146.8	—[g] —[g] —[g]	— — —	— — —	— — —	— — —	— — —
 1-methylpiperidine	2 500	126.0	—[g] —[g] —[g]	— — —	— — —	— — —	— — —	— — —
 2-methylpiperidine	2 500	126.0	—[g] —[g] —[g]	— — —	— — —	— — —	— — —	— — —
 3-methylpiperidine	4 000	201.6	—[g] —[g] —[g]	— — —	— — —	— — —	— — —	— — —
 4-methylpiperidine	2 500	126.0	—[g] —[g] —[g]	— — —	— — —	— — —	— — —	— — —

Table 1 (Continued)

compound name	max concn (ng/well | nmol/mL)[f]	IC$_{50}$ (ng/well ±STD)[b,c] IC$_{50}$ (pmol/mL ±STD)[d] % cross reactivity[e] Til-1	Til-2	Til-3	Til-4	Til-5	Til-6
		—[g]	—	—	—	—	—
		—[g]	—	—	—	—	—
1-piperidineethanol	2 500 | 126.0	—[g]	—	—	—	—	—
		—[g]	—	—	—	—	—
		—[g]	—	—	—	—	—
cis-2,6-dimethylpiperidine	2 500 | 110.4	—[g]	—	—	—	—	—
		7.7[b] ±1.7	5.3 ±1.5	5.5 ±1.3	4.3 ±0.8	4.6 ±0.9	4.0 ±1.0
		340[d] ±75	234 ±64	242 ±56	192 ±33	202 ±42	175 ±43
3,5-dimethylpiperidine (*cis*/*trans* mix)	100 | 4.417	16.2[e]	21.4	25.6	22.7	18.1	24.7

[a] Each data point is the average of eight experimental evaluations. [b] IC$_{50}$, nanograms perwell ± STD (standard deviation). [c] IC$_{50}$, nanograms per mL = nanograms per well × 5. [d] IC$_{50}$, picomoles per milliliter ± STD (standard deviation); IC$_{50}$ in picomoles is the value used for calculating the percent cross-reactivity. [e] Percentage of cross-reactivity was calculated according to the formula: (IC$_{50}$ in picomoles of tilmicosin per milliliter)/(IC$_{50}$ in picomoles of another compound per milliliter) × 100. [f] Maximum concentration of compound used in cross-reactivity studies. [g] No cross-reactivity was observed at the concentration of chemical used.

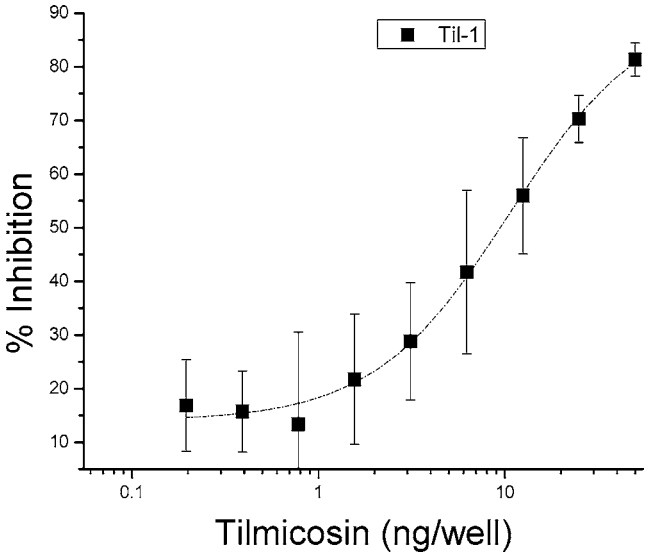


Figure 4. Standard curve for tilmicosin with Mab Til-1. Each point represents the mean ± standard deviation from eight determinations. Each data point was calculated from the absorbance at 450 nm by use of the expression $[1 - (B/B_0)] \times 100$, where B_0 is the absorbance of the sample blank and B is the absorbance of the standard tilmicosin sample. The mean B_0 of 96 measurements from 12 96-well plates was 0.08 ± 0.03.

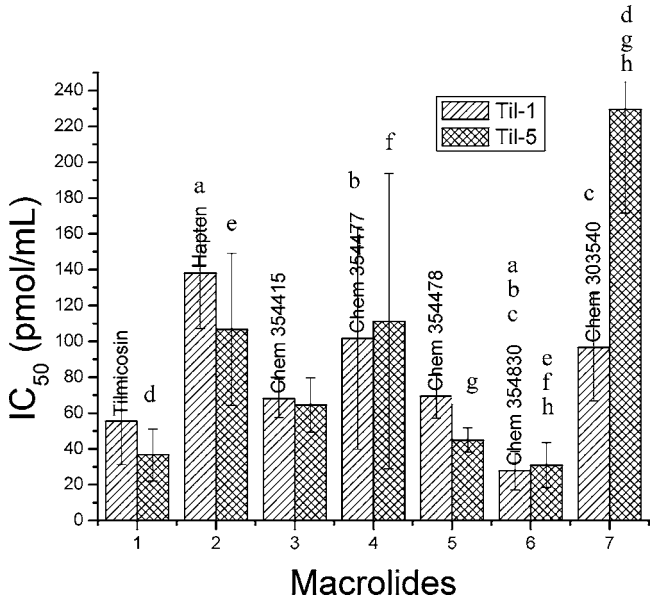


Figure 5. Til-1 and Til-5 Mab IC$_{50}$ values for the antibiotic tilmicosin and six related macrolides. Columns with the same letter are significantly different. For Til-1, the Kruskal–Wallis test determined a significant difference between the binding of (a) the hapten and Chem 354830, $P < 0.001$; (b) Chem 354477 and Chem 354830, $P < 0.01$; and (c) Chem 354830 and Chem 303540, $P < 0.01$. For Til-5, the Kruskal–Wallis test determined a significant difference between the binding of (d) tilmicosin and Chem 303540, $P < 0.001$; (e) the hapten and Chem 354830, $P < 0.05$; (f) Chem 354477 and Chem 354830, $P < 0.05$; (g) Chem 354478 and Chem 303540, $P < 0.01$; and (h) Chem 354830 and Chem 303540, $P < 0.001$.

respectively), and Chem 307645 also was significantly different in cross-reactivity with the Til-1 Mabs compared to Chem 354477 ($P < 0.05$). Chem 307645 had a low cross-reactivity with Til-1 and Til-5 Mabs of 3.7% and 2.0%, respectively (**Table 1**). The piperidine group in Chem 307645 had been altered. The hydrogens on the two methyl groups of 3,5-dimethylpiperidine were substituted with fluorines. Apparently, that alteration resulted in poor binding of the macrolide to the Mabs.

Figure 5 shows the distribution of the IC$_{50}$ values of the Til-1 and Til-5 Mabs for tilmicosin, hapten, Chem 354415, Chem 354477, Chem 354478, Chem 354830, and Chem 303540. The Kruskal−Wallis statistical analysis test was used to test for differences in binding between these chemicals and the two Mabs. Til-1 Mabs had a significant difference between the binding of the hapten and Chem 354830 ($P < 0.001$), between Chem 354477 and Chem 354830 ($P < 0.01$), and between Chem 354830 and Chem 303540 ($P < 0.01$). Til-5 Mabs were significantly different between the binding of tilmicosin and Chem 303540 ($P < 0.001$), between the hapten and Chem

354830 ($P < 0.05$), between Chem 354477 and Chem 354830 ($P < 0.05$), between Chem 354478 and Chem 303540 ($P < 0.01$), and between Chem 354830 and Chem 303540 ($P < 0.001$).

The decrease in binding of Chem 303540 to Til-5 is hypothesized to be a result of the substitution of hydrogen for a methyl on the dimethylamine of the mycaminose sugar at C5 (**Table 1**). We have clearly seen that the 3,5-dimethylpiperidine moiety at C20 appears to be of primary importance in antibody binding. Additionally, even free 3,5-dimethylpiperidine exhibited a low level of binding without the base macrolide structure. We demonstrated that altering a methyl group on the adjacent mycaminose also affects the binding characteristics (**Table 1**). **Figure 5** shows the comparison between the IC_{50}s of tilmicosin and the hapten. These two chemicals are not significantly different in their binding to Til-1 and Til-5; however, there appears to be a tendency for tilmicosin to demonstrate better binding than the hapten. We hypothesize that the difference in binding may result from the diaminopropyl linkage arm extension at C23 (**Table 1**). Perhaps the amino group on the free hapten has some interaction with the antibody resulting in slightly reduced antigen binding. The interaction of the free amine would be expected to be different than with the conjugated hapten.

Conclusions. Mabs were developed to the antibiotic tilmicosin. Six antibodies (Til-1, Til-2, Til-3, Til-4, Til-5, and Til-6) were developed, and they all had the IgG1 isotype. The Mabs did not cross-react with the antibiotic tylosin. The hapten−BSA conjugate used for plate coating was synthesized by a different method than the hapten−mcKLH conjugate that was used as the immunogen, thus overcoming unwanted cross-reactivity (nonspecific binding). The six different Mabs reacted similarly with tilmicosin showing no statistical difference in binding. Two Mabs, Til-1 and Til-5, had the best overall consistent performance in the cELISA. The IC_{50}s for Til-1 and Til-5 were 9.6 ng/well (48 ng/mL, 55.4 pmol/mL) and 6.4 ng/well (32 ng/mL, 36.7 pmol/mL), respectively. The Mabs appeared to react slightly better with tilmicosin than with the hapten, which may be due to the free amino group of the hapten interfering with antibody binding. The Mabs do bind other related noncommercial laboratory macrolides similar to tilmicosin containing a synthetically added 3,5-dimethylpiperidine moiety. The Mabs did not bind other macrolides that did not contain the 3,5-dimethylpiperidine moiety. Structural changes in the 3,5-dimethylpiperidine moiety or the adjacent mycaminose sugar of the macrolide can alter the binding to the Mabs. Til-1 and Til-5 hybridomas have been deposited with the ATCC, resulting in ATCC designations of PTA-3715 and PTA-3714, respectively. These Mabs may be excellent candidates for the determination and immunolocalization of tilmicosin.

ABBREVIATIONS USED

ATCC, American Type Culture Collection; BSA, bovine serum albumin; BSA−hapten, bovine serum albumin conjugated to hapten; Chem 303540, *N*-demethyltilmicosin; Chem 307645, 20-deoxo-20-[3,5-di(trifluoromethyl)piperidin-1-yl]desmycosin; Chem 354415, 20-deoxo-20-(3,5-dimethylpiperidin-1-yl)-χ-tylosin; Chem 354477, 20-deoxo-20-(3,5-dimethylpiperidin-1-yl)macrocin; Chem 354478, 20-deoxo-20-(3,5-dimethylpiperidin-1-yl)-2″-demethyllactenocin; Chem 354830, 20-deoxo-20-(3,5-dimethylpiperidin-1-yl)lactenocin; DMSO, dimethyl sulfoxide; DMT, demycinosyltylosin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EDTA, ethylenediaminetetraacetic acid; cELISA, competitive enzyme-linked immunosorbent assay; HAT, hypoxanthine−aminopterin−thymidine; HT, hypoxanthine−thymidine; im, intramuscular; ip, intraperitoneal; mcKLH, keyhole limpet (*Megathura crenulata*) hemocyanin; LTB_4, leukotriene B_4; mcKLH−hapten, keyhole limpet (*Megathura crenulata*) hemocyanin conjugated to hapten; Mab, monoclonal antibody; MES, 2-(*N*-morpholino)ethanesulfonic acid; MIC, minimum inhibitory concentration; NFDM, nonfat dry milk; OMT, 5-*O*-mycaminosyltylonolide; PBS-9, phosphate-buffered saline, pH 9; PMN, polymorphonuclear neutrophilic leukocytes; RO H_2O, reverse osmosis water, pyrogen-free; NaCl, sodium chloride; tilmicosin, 20-deoxo-20-(3,5-dimethylpiperidin-1-yl)-desmycosin; Traut's reagent, 2-iminothiolane hydrochloride; Tween 20, polyoxyethylene-sorbitan monolaurate.

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