Production, Characterization, and Cross-Reactivity Studies of Monoclonal Antibodies against the Coccidiostat Nicarbazin

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A cELISA was developed for the coccidiostat nicarbazin. On the basis of previous computer-assisted molecular modeling studies, p-nitrosuccinanilic acid (PNA-S) was selected as a hapten to produce antibodies to 4,4′-dinitrocarbanilide (DNC), the active component of the coccidiostat nicarbazin. Synthesis is described for the hapten [p-nitro-cis-1,2-cyclohexanedicarboxanilic acid (PNA-C)] used in a BSA conjugate as a plate coating antigen. Monoclonal antibodies (Mabs) were isolated that compete with nicarbazin, having IgM$_\kappa$ isotype. Because of the lack of water solubility of nicarbazin, N,N-dimethylformamide (DMF) (3%, v/v) and acetonitrile (ACN) (10%, v/v) were added to the assay buffer to achieve solubility of nicarbazin and related compounds. The Nic 6 Mabs had an IC$_{35}$ value for nicarbazin of 0.92 nmol/mL, with a limit of detection of 0.33 nmol/mL. Nic 6 exhibited high cross-reactivity for PNA-S and PNA-C, and 3-nitrophenol, 4-nitrophenol, and 1-(4-chlorophenyl)-3-(4-nitrophenyl) urea. However, Nic 6 had little or no cross-reactivity with 15 other related compounds.

Keywords: *Broilers; coccidiostat; coccidiosis; DNC; 4,4′-dinitrocarbanilide; ELISA; hapten; immunoassay; monoclonal antibodies; nicarbazin; p-nitro-cis-1,2-cyclohexanedicarboxanilic acid; p-nitrosuccinanilic acid*

INTRODUCTION

Coccidiosis causes significant economic losses to the poultry industry (*1*). The parasites multiply in the intestinal tract and cause tissue damage. This invasion results in the interruption of feeding, digestive processes, and nutrient absorption, dehydration, blood loss, and increased susceptibility to other disease agents (*2*).

Nicarbazin was the first agent found to give satisfactory control of coccidiosis in broiler production (*3, 4*). It is the oldest anticoccidial in use today (*5*). Nicarbazin is used worldwide as a feed additive to prevent outbreaks of cecal and intestinal coccidiosis in poultry (*6, 7*), and it is used to increase the rate of weight gain. Nicarbazin is composed of an equimolar complex of 4,4′-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP) (Figure 1). It was demonstrated in 1967 that nicarbazin inhibited egg production from female adult worms of the parasite *Schistosoma mansoni* in infected mice (*8*). Nicarbazin acts by inhibiting the full development of second-generation meronts, thus reducing the pathogenic effect and numbers of oocysts excreted (*9*). Nicarbazin was one of six out of the 71 compounds screened that completely inhibited cryptosporidial growth at a concentration of 1 μM using a screening method that assessed anticryptosporidial and cytotoxic effects of putative chemotherapeutic compounds (*10*).

Following drug withdrawal, there is no evidence of residual anticoccidial activity with nicarbazin or a
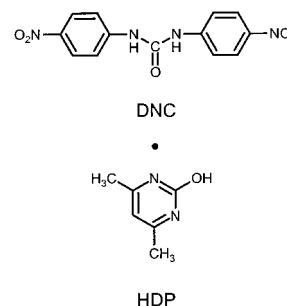
Figure 1. Nicarbazin is an equimolar complex of 4,4′-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP).

number of the other coccidiostats tested (*11*). However, antimicrobial resistance continues to be a problem with respect to animal drug use. Ten field isolates of *Eimeria* sp. obtained from North Germany were studied for sensitivity to various anticoccidials. Many of the isolates (9 out of 10) showed varying resistance to anticoccidials, including nicarbazin (*12*). Battery trials conducted during commercial broiler production in Lower Saxony, Germany, found some isolates that were partially resistant to nicarbazin and other anticoccidials (*13*).

The complex DNC·HDP is 10 times more potent in the control of *Eimeria tenella*, the primary coccidia cecal pathogen, than is DNC by itself. When HDP was used alone it was observed to have no anticoccidial activity (*3*). It was proposed that ultrafine crystals that were obtained as a result of complex formation between DNC and HDP allow for better dissolution of DNC resulting in improved anticoccidial activity (*14*). Chickens also excrete DNC more slowly than they do HDP (*15*). The U.S. Food and Drug Administration has established a

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nicarbazin withdrawal period of 4 days with a tolerance of 4 ppm (*16*).

The Food Safety and Inspection Service (FSIS) uses high performance liquid chromatography (HPLC) with UV detection to analyze for DNC (*17*). The FSIS method, as well as other published methods, uses a variety of organic solvents (e.g., ethyl acetate, acetonitrile, hexane, dimethylformamide, and methanol) for various steps during the analyses of nicarbazin. A number of analytical techniques have been used to quantify nicarbazin. Pulse polarographic determinations were used to quantify nicarbazin in chicken tissues (*18*, *19*). Liquid chromatographic methods have been used to quantify nicarbazin in feeds (*20*, *21*) and chicken tissues (*22*–*25*). Near-infrared reflectance spectroscopy has been used to quantify granulated nicarbazin (*26*), and supercritical fluid extraction followed by HPLC analysis was used for nicarbazin determination in poultry feed, eggs, and muscle tissue (*27*). Liquid chromatography-thermospray mass spectrometry (MS) (*25*), liquid chromatography-atmospheric pressure chemical ionization MS (*28*), and liquid chromatography-electrospray MS (*6*) were used to quantify nicarbazin levels in chicken tissues, eggs and feeds, respectively. All of these methods are time-consuming and result in production of substantial organic solvent wastes.

Our laboratory has had a long-term interest in developing monoclonal antibodies and immunochemical methods for the determination of coccidiostats and antibiotics used in animal production. Detection methods based on immunoassays can greatly increase the rate of sample throughput, allow screening of increased numbers of samples without increasing the cost of analysis, and eliminate or greatly reduce the amount of solvent waste generated during analysis procedures.

We have used computer-assisted molecular modeling as a tool to understand antibody–ligand interactions (*29*–*33*), to demonstrate that different energy state conformations of a hapten can exist and to show that different hapten energy states can influence the outcome of the immune response (*34*), to predict hapten design (*35*, *36*), and to understand physical behavior of molecules (*37*). Some of these molecular modeling applications are discussed in a review on the use of immunoassay for detection of antibiotics in foods and feeds (*38*).

Our original approach to the production of monoclonal antibodies (Mabs) to DNC was to make a hapten from the drug DNC and use it for immunization. This methodology failed to produce Mabs that could compete with "free" DNC, as was discussed by Beier and Stanker (*35*). Computer-assisted molecular modeling was used to study this conjugate as well as to help determine an alternative hapten to use as an antigen (*35*). Figure 2 shows the chemical structure of *p*-nitrosuccinanilic acid (hapten-1). This DNC-mimic was shown by molecular modeling to be a good candidate as a hapten. It was thought that a DNC-mimic–protein conjugate could be used as an immunizing agent for production of antibodies to DNC. A comparison of the computer-assisted electrostatic potential isosurfaces of the molecular models of DNC and *p*-nitrosuccinanilic acid were compared (*35*). A strikingly close similarity was seen in the electrostatic potential isosurfaces of these two compounds.

A preliminary report was published (*39*) on the development of Mabs to nicarbazin where preliminary data from our "fusion 2" products were discussed in a
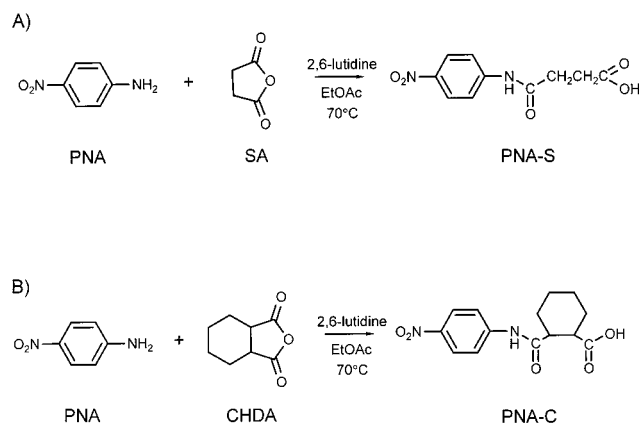
Figure 2. Synthesis and structures of haptens for conjugation to carrier proteins: (A) PNA-S and (B) PNA-C.

symposium. Unfortunately, those early hybridomas did not continue to produce viable antibodies (*39*). Our recent fusions and isolated hybridomas are the subject of this paper.

In this paper, we describe four hybridoma cell lines [two of which (Nic 6, ATCC PTA-2647; and Nic 7, ATCC PTA-2648) have been deposited with the American Type Culture Collection (ATCC)]. These hybridoma cell lines produce Mabs that may be used for the detection of the coccidiostat nicarbazin. Details of production and characterization of these antibodies are reported in this paper. The effect of pH and the use of solvents while carrying out the competitive enzyme-linked immunosorbent assay (cELISA) are discussed.

MATERIALS AND METHODS

Chemicals and Materials. Acetonitrile (ACN), *N*,*N*-dimethylformamide (DMF), ethyl acetate (EtOAc), EM Science methanol (MeOH), and Kieselgel 60 F254 HPTLC plates (EM Science No. 13728) were obtained from VWR Scientific Products Corp. (Suwanee, GA); bovine serum albumin (BSA, No. A-7030), keyhole limpets (*Megathura crenulata*) hemocyanin (KLH, No. H-2133), polyoxyethylene-sorbitan monolaurate (Tween 20), sodium carbonate, sodium bicarbonate, magnesium chloride, NaCl, Na$_2$HPO$_4$, TRIZMA hydrochloride, TRIZMA base, 8-azaguanine (Sigma A-5284 Hybri-Max), pristane (2,6,10,14-tetramethylpentadecane, Sigma P-1403) 95%, HAT media supplement (hypoxanthine, aminopterin and thymidine, Sigma H-0262), HT media 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). Ammonium acetate, 1-benzyl-3-(4-nitrophenyl)urea, 1,3-bis(4-nitrophenyl)urea, 1-(3-chlorophenyl)-3-(2-methoxy-5-nitrophenyl)urea, 1-(3-chlorophenyl)-3-(4-methoxy-3-nitrophenyl)urea, 1-(4-chlorophenyl)-3-(4-nitrophenyl)urea, *cis*-1,2-cyclohexanedicarboxylic anhydride, 1-(2-fluorophenyl)-3-(2-methoxy-4-nitrophenyl)urea, 2,6-lutidine, 1-(3-methoxyphenyl)-3-(3-nitrophenyl)urea, 2-nitroaniline, 3-nitroaniline, 4-nitroaniline, 2-nitrobenzyl alcohol, 3-nitrobenzyl alcohol, 4-nitrobenzyl alcohol, 4-nitrophenethyl alcohol, 2-nitrophenol, 3-nitrophenol, 4-nitrophenol and succinic anhydride were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). *N*-hydroxysulfosuccinimide (Sulfo-NHS; No. 2451) and *N*,*N*-dicyclohexylcarbodiimide (DCC; No. 20320) were obtained from Pierce Chemical Co. (Rockford, IL). Supelcosil LC-ABZ chromatography column was obtained from Supelco (Bellefonte, PA). BALB/c mice and ICR mice were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN). Spectra/Por 4 dialysis membrane tubing, molecular weight cutoff (MWCO) 12 000–14 000 was obtained from Spectrum Medical Industries, Inc. (Los Angeles, CA). Ribi-Corixa adjuvant (No. R700, MPL + TDM emulsion in 2% oil-Tween-80)

was obtained from CORIXA Corp. (Hamilton, MT). Nonfat dry milk (NFDM) Janet Lee instant nonfat dry milk fortified with vitamins A & D$_3$ contained 35.5% protein (Albertson's Inc., Boise, ID, and was obtained from a local grocery store). Iscoves Modified Dulbecco's Medium (Gibco BRL no. 12200-069), penicillin/streptomycin (Gibco BRL No. 15140-122), and L-glutamine (Gibco BRL No. 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 (No. 07-200-90) and Costar 24w plates (No. 3524) were obtained from CoStar Corp. (Cambridge, MA). Nylon cloth sieve w/100 µm pore size (No. 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 using SBA Clonotyping System/AP (No. 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 Microplate Reader, Bio-Rad model 3550 Microplate reader (Bio-Rad Laboratories, Hercules, CA). The Bio-Rad Reader driver software version 1.0 (for Power Macintosh 6100/60) was used at the settings: 8 × 12 format, automix (3 min). Reverse osmosis water, pyrogen free (RO H$_2$O), was produced on site by a reverse osmosis system obtained from Millipore Corp. (Bedford, MA) and used for all cELISA experiments.

Synthesis of *p*-Nitrosuccinanilic Acid [CAS Registry No. 5502-63-6]. *p*-Nitrosuccinanilic acid was synthesized as described by Beier and Stanker (*39*). Briefly, *p*-nitroaniline (0.1 g, 0.724 mmol) and succinic anhydride (0.29 g, 2.898 mmol) were dissolved in EtOAc (4 mL). Addition of 2 drops of 2,6-lutidine started the reaction, and it was held at a temperature of 70 °C overnight (19 h). The reaction mixture was cooled to 4 °C, and the solvent was decanted from the solid products. The products were rinsed with cold EtOAc (1 mL), and a crude product was obtained by removing some impurities by thin-layer chromatography (TLC) using Kieselgel 60 F$_{254}$ HPTLC plates using solvent system CHCl$_3$/EtOAc/MeOH/formic acid, 86:10:4:1%. Following TLC, the material that remained at the origin was eluted with DMF. After removal of DMF with a stream of argon, the product was made basic using NaOH (4 N). The final product was collected from HPLC using a 15 cm × 4.6 mm, 5 µm, Supelcosil LC-ABZ column with a solvent system of MeOH (30%, v/v) at a flow rate of 1 mL/min at 254 nm. Sodium *p*-nitrosuccinanilate (PNA-S) (C$_{10}$H$_9$N$_2$NaO$_5$) was obtained in a yield of 59%. High-resolution mass measurement was obtained by fast atom bombardment mass spectrometry on a double-focusing VG 70-250 EHF spectrometer of the double sodium ion and was observed at *m/z* 283.0326 (calculated for C$_{10}$H$_9$N$_2$Na$_2$O$_5^+$, 283.0307).

Synthesis of *p*-Nitro-*cis*-1,2-cyclohexanedicarboxanilic Acid [CAS Registry No. 17716-19-7]. The desired product was made by dissolving *p*-nitroaniline (0.1 g, 0.724 mmol) and *cis*-1,2-cyclohexanedicarboxylic anhydride (CHDA, 0.446 g, 2.896 mmol) in EtOAc (4 mL). Addition of 2 drops of 2,6-lutidine started the reaction, and it was held at a temperature of 70 °C overnight (19 h). The reaction vessel was cooled to 4 °C, and the solvent was decanted from the solid products. The products were rinsed with cold EtOAc (1 mL). A crude product was obtained by removing some impurities by TLC on Kieselgel 60 F$_{254}$ HPTLC plates using the solvent system CHCl$_3$/EtOAc/MeOH/formic acid, 86:10:4:1%. Following TLC, the material that remained at the origin was eluted with DMF. After removal of the DMF with a stream of argon, the product was made basic using NaOH (4 N). The final product was separated and collected from HPLC using a 15 cm × 4.6 mm, 5 µm, Supelcosil LC-ABZ column with a solvent system of MeOH (30%, v/v) at a flow rate of 1 mL/min at 254 nm. Sodium *p*-nitro-*cis*-1,2-cyclohexanedicarboxanilate (PNA-C) (C$_{14}$H$_{15}$N$_2$-

NaO$_5$) was obtained in a 56% yield. To convert the product to the acid form, the material was extracted from pH 3.00 biphthalate buffer (Micro Essential Laboratory Inc., Brooklyn, NY) with dichloromethane and dried with sodium sulfate. High-resolution mass measurement was obtained by electro-spray MS on a PE-Sciex QSTAR Pulsar mass spectrometer of the M-H$^-$ ion and was observed at *m/z* 291.0978 (calculated for C$_{14}$H$_{15}$N$_2$O$_5^-$, 291.098097).

Preparation of Immunogen and Plate Coating Antigens. The immunogen and plate coating conjugate were synthesized using an *N*-hydroxysuccinimide (NHS)-enhanced, carbodiimide-mediated coupling reaction described by Staros et al. (*40*). These conjugations were conducted in a manner similar to the method used for the coccidiostat salinomycin (*41*).

KLH Conjugate. The immunogen was produced by dissolving Sulfo-NHS (17 mg, 0.078 mmol) in dry DMF (1 mL), followed by addition of a solution of PNA-S (20.1 mg, 0.077 mmol) in dry DMF (0.25 mL). To this solution was added DCC (15.2 mg, 0.074 mmol) and dry DMF for a final volume of 2.5 mL DMF. After the sample was stirred for 2 h at room temperature, the reaction mixture was added to a solution of KLH (42 mg) in H$_2$O (6 mL, pH 8.5). During the addition, NaOH (0.1 N) was used to keep the pH ≅ 8.5. The reaction mixture was stirred overnight at room temperature. The mixture was then dialyzed progressively beginning with a solution of DMF (50%, v/v) in H$_2$O. The concentration of DMF was decreased in three increments until only RO H$_2$O was used, and the dialysis was conducted at 4 °C using Spectra/Por 4 dialysis membrane tubing, MWCO 12 000−14 000.

BSA Conjugate. The plate coating antigen was produced by dissolving Sulfo-NHS (15.4 mg, 0.071 mmol) in dry DMF (1 mL), followed by addition of a solution of PNA-C (22.6 mg, 0.072 mmol) in dry DMF (0.25 mL). To this solution was added DCC (14.1 mg, 0.068 mmol) and dry DMF for a final volume of 2.5 mL DMF. After the sample was stirred for 2 h at room temperature, the reaction mixture was added to a solution of BSA (43.1 mg) in H$_2$O (6 mL, pH 8.5). During the addition, NaOH (0.1 N) was added to keep the pH ≅ 8.5. The reaction mixture was stirred overnight at room temperature. The mixture was then dialyzed against a solution of DMF (20%, v/v) in H$_2$O and three times against RO H$_2$O at 4 °C using Spectra/Por 4 dialysis membrane tubing, MWCO 12 000−14 000.

cELISA Solutions. Detergent wash buffer was made by adding Tween 20 (0.05%, v/v) to RO H$_2$O. Carbonate buffer consisted of Na$_2$CO$_3$ (0.015 M), NaHCO$_3$ (0.035 M), and MgCl$_2$ (0.002 M) in RO H$_2$O, pH 9.6. Phosphate-buffered saline (PBS-9) contained Na$_2$HPO$_4$ (0.01 M) and NaCl (0.15 M) in RO H$_2$O, 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). 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$_2$O adjusted to pH (6.8, 7.0, 7.2, 7.4, 7.6, 7.75) using HCl or NaOH. Part B contained per 95 mL of water: 1 g of NFDM and 0.5 mL Tween-20.

Assay Plate Coatings. Nunc-Immuno plates with a Max-iSorp surface were washed with a solution of Tween-20 (0.05%, v/v) in RO H$_2$O followed by a RO H$_2$O rinse to clean the plates prior to coating. Assay plates were coated overnight at 4 °C with a solution containing nicarbazin (141 ng/100 µL/well) (an equivalent of 100 ng 4,4′-dinitrocarbanilide per 100 µL) in MeOH (50%, v/v) containing Tween-20 (0.02%, v/v) in part A of assay buffer; plate coating 1 (PL-1). The nicarbazin solution was made for coating plates by initially dissolving nicarbazin (2.4 mg) in MeOH (100 mL) with 0.1 mL Tween-20 (Std-1). Five hundred milliliter quantities of the coating solution were made by adding Std-1 (29.4 mL), MeOH (220.6 mL), and assay buffer part A to a 500 mL volumetric flask.

Assay plates also were coated with the PNA-C-BSA conjugate. Washed and rinsed plates were coated overnight at 4 °C with a solution containing 100 ng/100 µL/well PNA-C-BSA in pH 9 carbonate buffer. After the plates were coated overnight, they were washed with RO H$_2$O containing Tween-20 (0.05%, v/v) followed by a RO H$_2$O rinse. Assay plates were incubated

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with blocking buffer (15 g of nonfat dry milk in 500 mL PBS-9) (300 μL/well), for 1 h at room temperature, and then washed and rinsed with RO H_2O.

Cell Growth Media. Iscoves Modified Dulbecco's Medium was used containing the following additives: L-glutamine (100 mL/10 L media) and $NaHCO_3$ (3.7 g/L). The media solution was sterile filtered into 500 mL bottles and incubated overnight at 37 °C to ensure sterility. Media was stored at 4 °C. Complete media 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 concentration of 100 U/mL and 100 μg/mL, respectively. HAT (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 media (480 mL). The concentration of added chemicals in the HAT (2×) media was 2×10^{-4} M hypoxanthine, 8×10^{-7} M aminopterin, and 3.2×10^{-5} M thymidine. The cell suspension in HAT (2×) media 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×. HT media supplement, Hybri-Max, following reconstitution to 10 mL and addition to complete media (490 mL) resulted in a concentration of hypoxanthine and thymidine of 1×10^{-4} M and 1.6×10^{-5} M, respectively.

Solvent Systems Required for Solubility of Nicarbazin. Because of the low solubility of nicarbazin or 4,4′-dinitrocarbanilide in various solvents including H_2O, a study of various concentrations of solvents in assay buffer was undertaken that would lead to the appropriate solubility of nicarbazin, while still allowing the cELISA to work.

Solvent System Used in Titration of Mouse Sera or Media. For titration of mouse sera or media, a solution containing DMF (3%, v/v) and ACN (10%, v/v) in assay buffer was used. This solution also was used in the cELISA control wells.

Nicarbazin Standard and Other Chemicals Used for Competitions. Standards of nicarbazin and all other chemicals used for competition in the cELISA were prepared with a solution containing DMF (12%, v/v), ACN (40%, v/v), and Tween-20 (0.1%, v/v) in part A of assay buffer. These standards were then pipetted (100 μL) into column 2 of the 96-well plates containing 100 μL of assay buffer.

Solvent System Used during Competitions with cELISAs. A solution containing DMF (6%, v/v) and ACN (20%, v/v) in assay buffer was used for serial dilutions of the standards on 96-well plates. For competitions, the mouse sera or media or their dilutions were layered on top of the above diluted standards resulting in solutions with a final solvent concentration of 3% DMF (v/v) and 10% ACN (v/v) in assay buffer.

Competitive ELISA. A cELISA was developed to evaluate antibody specificity and to quantify nicarbazin in solution. Assay plate preparation and coating follow those steps described above under Plate Coating Antigens. Early work during this study utilized nicarbazin (PL-1) as plate coating material, and later work utilized PNA-C-BSA (PL-2) as plate coating material. The competitor (nicarbazin/100 μL) solution in DMF (12%, v/v), ACN (40%, v/v), and Tween-20 (0.1%, v/v) was added to assay buffer (100 μL) in column 2 of the 96-well assay plate. Final solvent concentration in column 2 resulted in DMF (6%, v/v) and ACN (20%, v/v). This solution was diluted 1:2 across the plate in DMF (6%, v/v) and ACN (20%, v/v) in assay buffer (100 μL) to form a concentration gradient of the competitor. Antibody diluted in assay buffer (100 μL) was added to all wells except column 1 (solvent control) making a final concentration of DMF (3%, v/v) and ACN (10%, v/v). Wells in column 1 contain the solvent control, DMF (3%, v/v), and ACN (10%, v/v) in assay buffer. The amount of antibody used was that amount that produced a minimum optical density reading of 0.45 absorbance units after background subtraction. The sample antibody mixture was incubated for 1 h at 37 °C, and the plates were washed with Tween-20 (0.05%, v/v) in RO H_2O 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 Tween-20 (0.05%, v/v) 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 optical density measurements (450 nm) were taken on a Bio-Rad model 3550 microplate reader. The mean of 100 background OD measurements was 0.09 ± 0.008.

Immunization of Mice. The immunogen was prepared for injection by addition of PNA-S-KLH 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 PNA-S-KLH conjugate in Ribi-Corixa adjuvant (75 μg/0.15 mL). BALB/c mice were immunized i.p. with 0.15 mL of the mixture at a minimum of three times at two-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 nicarbazin. The selected mouse received a booster injection i.p. of 75 μg/0.15 mL of only the PNA-S-KLH conjugate (no Ribi-Corixa adjuvant) in sterile isotonic saline 3−4 days prior to the day of fusion.

Monoclonal Antibody (Mab) Production. *Fusion of SP2/0 Myeloma Cells and Spleen Cells.* Some of the fusion and cloning conditions were described by Stanker et al. (*42*), with the following modifications: splenocytes were fused with SP2/0 myeloma cells cultured in 96-well Costar plates containing a macrophage feeder cell layer. Macrophages were obtained from ICR mice treated with pristane. SP2/0 myeloma cells were passed through media containing 8-azaguanine and then grown for 4 to 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 media, and the resulting cell suspension was strained through a sterile nylon cloth sieve w/100 μm pore size into a sterile beaker. SP2/0 myeloma cells were fused to splenocytes by using poly(ethylene glycol) (*43*).

Screening of the Cell Fusion. Cells usually are ready for their initial screening in 10 to 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 (nicarbazin). 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 exhibit competition with the target chemical were then further submitted to hybridoma cloning.

Hybridoma Cloning. Following initial hybridoma screening, HT media was used for cell maintenance until the final picks were made. Screening for antibody producing hybridomas made use of the cELISA described above. Hybridoma cells from wells showing antibody competition to nicarbazin 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 picks were made, those selected cell lines were weaned off HT media and on to complete media.

Mab Production. Rather than producing antibodies by ascites tumor production as described in Stanker et al. (*42*), the Mabs were produced in vitro production. In vitro production of Mabs in high concentration was accomplished in a reusable modular minifermenter for high-density culture of hybridoma cells, the miniPERM bioreactor (*44, 45*). In those cases where the antibody has an isotype of IgG, a protein G column can be used for Mab purification.

Titration Studies with Various Assay Buffer pH Values. Studies were carried out using both PL-1 and PL-2 plate coatings. Assay buffer part A was formulated at various pH values (6.8, 7.0, 7.2, 7.4, 7.6, 7.75) as was described in the

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section titled "cELISA Solutions". Titrations at various pH values were carried out using a solvent mixture similar to that described in the section titled "Solvent System used in Titration of Mouse Sera or Media". Competitions at various pH values were carried out using a solvent mixture similar to that described in the section titled "Solvent System used during Competitions with cELISAs," and the cELISA was accomplished as is described in the section titled "Competitive ELISA."

Characterization of Antibodies. *Isotype and Affinity.* Antibody isotypes were determined using reagents supplied in the commercially available kit SBA Clonotyping System/ AP. Relative affinity of the four Mabs for nicarbazin was measured by determining the 35% inhibition of control values (IC_{35}, center of curve). IC_{35} was used for Nic 6, Nic 8, and Nic 9 Mabs, and IC_{30} was used for the Nic 7 Mabs at an antibody dilution producing an absorbance minus control of 0.45 in the absence of competitor.

Antibody Specificity. Nicarbazin [4,4′-dinitrocarbanilide (DNC) + 2-hydroxy-4,6-dimethylpyrimidine (HDP)], DNC, HDP, PNA-S, and PNA-C were used in cross-reactivity studies. Other chemicals used were arrived at in two different ways. A number of chemicals are structurally similar to PNA-S: 2-nitroaniline, 3-nitroaniline, 4-nitroaniline, 2-nitrobenzyl alcohol, 3-nitrobenzyl alcohol, 4-nitrobenzyl alcohol, 4-nitrophenethyl alcohol, 2-nitrophenol, 3-nitrophenol, and 4-nitrophenol. A number of compounds with structural similarities to 4,4′-dinitrocarbanilide were obtained through a structural fragment-based computer search of available organic compounds from Aldrich Chemical Co., Inc. (Milwaukee, WI): 1-benzyl-3-(4-nitrophenyl)urea, 1,3-bis(4-nitrophenyl)urea (DNC), 1-(3-chlorophenyl)-3-(2-methoxy-5-nitrophenyl)urea, 1-(3-chlorophenyl)-3-(4-methoxy-3-nitrophenyl)urea, 1-(4-chlorophenyl)-3-(4-nitrophenyl)urea, 1-(2-fluorophenyl)-3-(2-methoxy-4-nitrophenyl)urea and 1-(3-methoxyphenyl)-3-(3-nitrophenyl)urea. Each of the compounds studied were dissolved in a solution containing DMF (12%, v/v), ACN (40%, v/v), and Tween-20 (0.1%, v/v) in part A of assay buffer, and examined as potential competitors using the cELISA described above. Structural information regarding these compounds is presented in Table 1.

RESULTS AND DISCUSSION

Synthesis of Mimic Haptens and Conjugates. Antibodies useful for the detection of nicarbazin have not been previously produced. In an initial approach to making antibodies against nicarbazin, we attempted to make the hydrazone of DNC (Figure 1) using 4-hydrazinobenzoic acid. This approach was discussed in Beier and Stanker (*35*). Antibodies obtained from that hapten never competed with "free" DNC. Molecular modeling was used to study this hapten and others to help understand the dynamics of the problem (*35*). Molecular modeling gave the electrostatic potential energy isosurfaces of DNC and other possible haptens. The hapten that had surface charges nearly identical to those of DNC was the mimic succinate derivative of *p*-nitroaniline (referred to as PNA-S, Figure 2). Although this mimic is smaller than DNC, it is identical to each half of DNC bisected at the carbonyl group of the urea. It is well-known that antibodies can be produced to molecules of this size (*46*).

PNA-S was synthesized as described by Beier and Stanker (*39*). Temperature was very critical during the reaction. It was found that heating the reaction mixture to 70 °C overnight resulted in a good product yield. PNA-S was linked to KLH using an NHS-enhanced, carbodiimide-mediated coupling reaction (Figure 3), the conjugate was used as the immunogen and injected into BALB/c mice. However, using the conjugate of PNA-S linked to BSA by the same method resulted in nonspecific binding and "free" nicarbazin or DNC would not

compete. Therefore, another hapten was synthesized (PNA-C, Figure 2). Greirson et al. (*47*) used different bridging moieties during conjugation to overcome unwanted cross-reactivity associated with antibody binding with 13-hydroxylupanine. They used a succinate-KLH derivative of 13-hydroxylupanine as the immunogen and the *cis*-1,2-cyclohexanedicarboxylate derivative with BSA for the plate coating antigen. Likewise, we used *cis*-1,2-cyclohexanedicarboxylic anhydride to react with PNA at the same elevated temperature conditions used with succinic anhydride and produced PNA-C (Figure 2). PNA-C linked to BSA gave a suitable plate coating antigen (Figure 3) that did not suffer from unwanted cross-reactivity.

Production of Monoclonal Antibodies to Nicarbazin. Ten days following the fusion, growing hybridomas were observed in many of the 2880 wells of the seeded 96-well plates. At this time, all wells were tested for anti-nicarbazin activity using nicarbazin (PL-1) bound plates in an ELISA described above. Hybridoma cultures from 120 wells with the highest anti-nicarbazin activity were selected for further evaluation. The hybridomas in these wells were expanded in 24-well plates. The supernatants were titrated on PL-1 coated plates and were evaluated for competition against nicarbazin. Seven picks were made that showed good to poor competition and were grown up after a limiting dilution. Those wells that showed binding to PL-1 were expanded, and titration and competition were completed arriving at picks from three of the initial wells. Selection of clones from these cultures by limiting dilution led to four stable hybridoma cell lines. These monoclonal cultures and their corresponding Mabs were named Nic 6, Nic 7, Nic 8, and Nic 9.

Solubility of Nicarbazin. Nicarbazin is known for its very difficult solubility, both in water and in some organic solvents. It is well understood that some solvent in the ELISA can be tolerated. A general example was the work by Li et al. (*48*). In that study, their Mabs worked well in solutions of acetone or DMSO (2%, v/v) and up to 5% methanol (v/v). Also, the work by Watanabe et al. (*49*) showed the effect of MeOH concentrations on the reactivity and sensitivity of a Mab with various concentrations of imazalil. That work showed a depression of the observed absorbance with 10% or higher MeOH concentrations. They used 10% MeOH (v/v) in their ELISA analysis, which decreased the absorbance by about 20%. That solvent tolerance was sufficient for analysis of their samples. These amounts of solvents in their ELISA seem to be what is most commonly observed, perhaps with 10% MeOH (v/v) being on the high end. In our case, these quantities of solvent in the ELISA were not enough for solubility of nicarbazin.

It was determined that a concentration of 3% DMF (v/v) and 10% ACN (v/v) were required to provide adequate solubility to nicarbazin to allow standard curve applications. Nicarbazin did not have high enough solubility in MeOH buffer solutions to use MeOH in the assay. Standards were made in DMF (12%, v/v), ACN (40%, v/v), and Tween-20 (0.1%, v/v) in part A of assay buffer. These standards were added to equal volumes of assay buffer on the microtiter plates resulting in a solution of 6% DMF (v/v) and 20% ACN (v/v). Concentration gradient dilutions were made on the microtiter

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Table 1. cELISA Inhibition Studies with Nitrophenyls and Other 4,4′-Dinitrocarbanilide Related Compounds
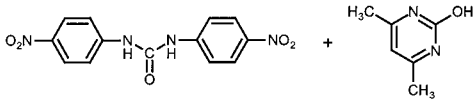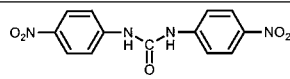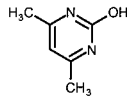
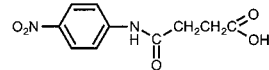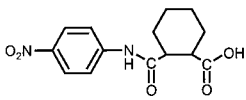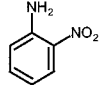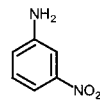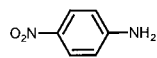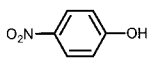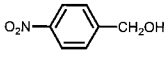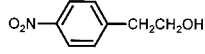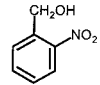| Compound		IC$_{35}$ (nmol/mL) % Cross Reactivity[b]			
Structure	Name Max Conc. (ng/well │ nmol/mL)[a]	Nic 6	Nic 7[c]	Nic 8	Nic 9
	Nicarbazin (DNC + HDP) 2 500 │ 29.32	0.92±0.2 100	1.1±0.3 100	1.2±0.4 100	1.4±0.7 100
	4,4'-dinitrocarbanilide (DNC) 2 500 │ 41.36	7.4±2.8 12	5.9±1.6 19	5.8±1.2 21	7.0±3.4[d] 13[d]
	2-hydroxy-4,6-dimethylpyrimidine (HDP) 2 300 │ 92.64	—[e]	—	—	—
	p-nitrosuccinanilic acid (PNA-S) 2 500 │ 52.48	0.42[f,g] 81[f]	—[e]	C[h]	2.3±1.6[d] 29.2[d]
	p-nitro-cis-1,2-cyclohexanedicarboxanilic acid (PNA-C) 2 500 │ 42.77	0.5±0.2[f] 66[f]	3.9±0.9 28	—[e]	0.73±0.1[d] 90[d]
	2-nitroaniline 2 500 │ 90.49	0.89±0.6[f] 37[f]	—[e]	—	—
	3-nitroaniline 2 500 │ 90.49	1.1±1.0[f] 30[f]	0.62±0.1[i] 65[i]	C[h]	1.2±0.1[j] 40[j]
	4-nitroaniline 2 500 │ 90.49	—[e]	0.33[i,g] 121[i]	1.4[h,g] 24[h]	—
	4-nitrophenol 2 500 │ 89.86	0.48[f,g] 69[f]	—[e]	—	—
	4-nitrobenzyl alcohol 2 400 │ 78.36	—[e]	4.4±2.3[i] 9[i]	5.7±3.1[h] 6[h]	1.6[j,g] 31[j]
	4-nitrophenethyl alcohol 2 500 │ 74.78	38.5[g] 2	—[e]	—	—
	2-nitrobenzyl alcohol 2 500 │ 81.62	—[e]	4.3±1.7[k] 16[k]	2.1[h,g] 16[h]	14.4[j,g] 3[j]

Table 1. (Continued)

Compound		IC$_{35}$ (nmol/mL) % Cross Reactivity[b]			
Structure	Name / Max Conc. (ng/well | nmol/mL)[a]	Nic 6	Nic 7[c]	Nic 8	Nic 9
	3-nitrobenzyl alcohol 2 500 | 81.62	—[e]	C[i]	3.2±1.1[h] 11[h]	5.4±1.8 26
	2-nitrophenol 2 500 | 89.86	C[f]	11.8[k,g] 12[k]	C[h]	2.5[j,g] 20[j]
	3-nitrophenol 2 500 | 89.86	0.43[f,g] 77[f]	11.6[k,g] 6[k]	2.8±0.95[h] 12[h]	1.6±1.0[j] 31[j]
	1-benzyl-3-(4-nitrophenyl)urea 2 500 | 46.08	1.3±1.2[f] 25[f]	2.9[i,g] 14[i]	—[e]	—
	1-(3-chlorophenyl)-3-(2-methoxy-5-nitrophenyl)urea 2 500 | 38.85	3.4±0.3[f] 10[f]	0.64±0.2 172	1.2±0.4[h] 28[h]	8.71[j,g] 6[j]
	1-(3-chlorophenyl)-3-(4-methoxy-3-nitrophenyl)urea 2 500 | 38.85	—[e]	1.03±0.2 107	0.87[h,g] 39[h]	—
	1-(4-chlorophenyl)-3-(4-nitrophenyl)urea 2 500 | 40.63	0.7[f,g] 47[f]	—[e]	—	—
	1-(2-fluorophenyl)-3-(2-methoxy-4-nitrophenyl)urea 2 400 | 39.31	—[e]	—	—	—
	1-(3-methoxyphenyl)-3-(3-nitrophenyl)urea 2 400 | 41.77	2.3±1.1[f] 14[f]	0.48[k,g] 144[k]	—[e]	C[j]

[a] Maximum concentration of compound used in cross-reactivity studies. [b] Percentage of cross-reactivity was calculated according to the formula: (IC$_{35}$ of nicarbazin)/(IC$_{35}$ of other compound) × 100. [c] The IC$_{30}$ was used for Nic 7, which was the center of the curve. [d] The Nic 9 curve was depressed by solvent interactions and values were calculated at the IC$_{20}$, Nic 9-nicarbazin IC$_{20}$ = 0.66 nmol/mL. [e] No cross-reactivity was observed at the concentrations of chemical used. [f] Competition was observed at the limit of detection (LOD) of Nic 6 at IC$_{15}$ = 0.33 nmol/mL nicarbazin. [g] Determined from average of 3 runs. [h] "C" Competition was observed at the LOD of Nic 8 at IC$_{15}$ = 0.34 nmol/mL nicarbazin. [i] Competition was observed at the LOD of Nic 7 at IC$_{10}$ = 0.4 nmol/mL nicarbazin. [j] Competition was observed at the LOD of Nic 9 at IC$_{15}$ = 0.5 nmol/mL nicarbazin. [k] The Nic 7 curve was depressed by solvent interactions, and values were calculated at the IC$_{20}$, Nic 7-nicarbazin IC$_{20}$ = 0.69 nmol/mL.

plates in 6% DMF (v/v) and 20% ACN (v/v). Equal volumes of antibody were then added to the wells resulting in a final concentration of 3% DMF (v/v) and 10% ACN (v/v) used in the cELISA. This concentration of solvent did depress the cELISA results, as observed in attempted trials with less solvent (data not shown). In general, inhibition curves ranged from 0 to 70% inhibition. Therefore, the center of the curves (IC$_{35}$) were

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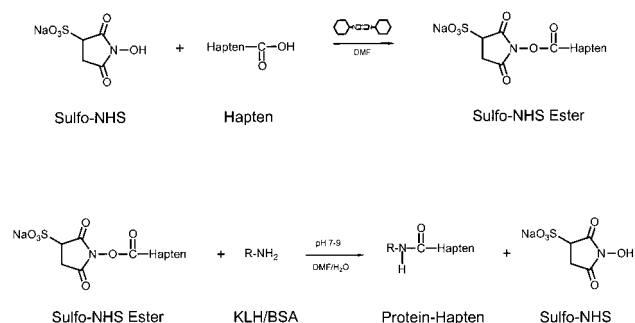
Figure 3. Synthetic pathway for producing the immunogen (PNA-S-KLH) or the plate coating antigen (PNA-C-BSA).
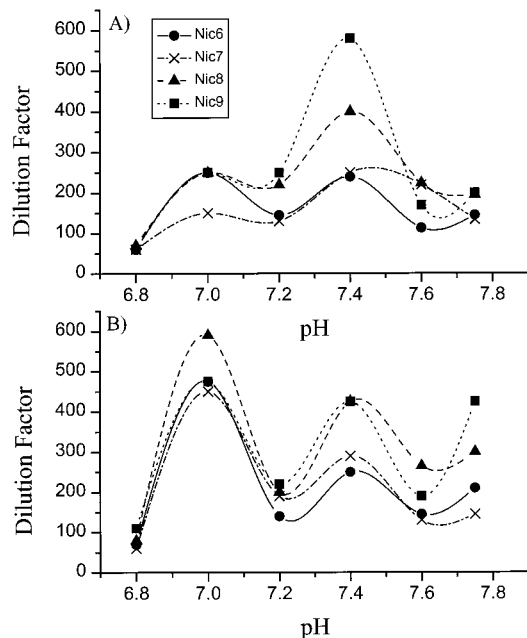

Figure 4. Evaluation of the effect of assay buffer pH on the binding of the Mabs Nic 6–9: (A) PNA-C-BSA is the plate coating antigen, and (B) nicarbazin is the plate coating antigen.

used for analysis points, and the limit of detection (LOD) usually was found at IC_{15}.

Titration Studies with Various Buffer pH Values. Titration studies were carried out with both PL-1 (nicarbazin, Figure 4B) and PL-2 (PNA-C-BSA, Figure 4A) coatings. The assay buffer was formulated at pH values of 6.8, 7.0, 7.2, 7.4, 7.6, and 7.75 with 3% DMF (v/v) and 10% ACN (v/v). The *y*-axis is the dilution of antibody required to produce an OD_{450} reading of 0.45 after subtraction of the solvent background. The four Mabs (Nic 6, Nic 7, Nic 8, and Nic 9) binding on PL-1 plates oscillated across the pH range with peaks at approximately pH 7.0, 7.4, and 7.75. Whereas, use of PL-2 plates resulted in the highest antibody binding at approximately pH 7.3–7.5.

The use of nicarbazin coated directly on the assay plates without the use of a protein conjugate is novel. As seen in Figure 4B, the nicarbazin coating worked well at pH 7.0, 7.4, and 7.75. However, the PNA-C-BSA conjugate coating produced results that were more consistent from plate to plate than that seen on the nicarbazin-coated plates.

cELISA Development. Heterologous assays are well-known to help improve immunoassay sensitivity (*50, 51*) and overcome unwanted cross-reactivity (*47*).
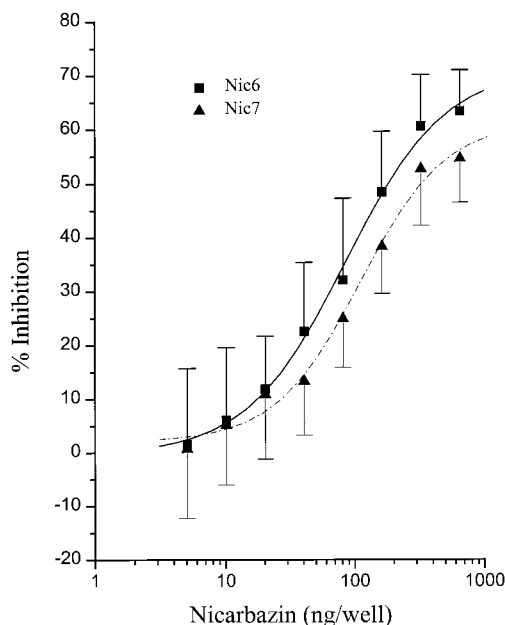
Figure 5. Standard curves for nicarbazin with Mabs Nic 6 and Nic 7 were obtained using assay buffer fortified with DMF (3%, v/v) and ACN (10%, v/v). Each point represents the mean ± standard deviation from 13 and 12 determinations, respectively, over an eight-week period. Each data point was calculated from the absorbance at 450 nm using the equation $(1 - (B/B°)) \times 100$.

We utilized the heterologous assay by using nicarbazin or PNA-C-BSA as a coating antigen and PNA-S-KLH as the immunogen to help overcome unwanted cross-reactivity. Nicarbazin inhibition curves in 3% DMF (v/v) and 10% ACN (v/v) for the Nic 6 and Nic 7 Mabs are shown in Figure 5. Figure 5 shows the inhibition of antibody binding by nicarbazin in the cELISA with PNA-C-BSA coated plates. The IC_{35} for Nic 6 is 78.5 ng/well (0.92 nmol/mL), and the IC_{30} for Nic 7 is 89.7 ng/well (1.1 nmol/mL) (see Table 1).

Characterization of Mabs. *Isotype and Affinity.* Immunoglobulin isotype of the four Mabs Nic 6, Nic 7, Nic 8, and Nic 9 were found to be IgM_{κ}. The concentrations of nicarbazin resulting in 35% inhibition of control activity (i.e., wells with no competitor present) were 0.92, 1.2, and 1.4 nmol/mL for Nic 6, Nic 8, and Nic 9, respectively. The concentration of nicarbazin resulting in 30% inhibition of control for the Nic 7 antibody was 1.1 nmol/mL. The Nic 6 and Nic 7 dosage–inhibition curves are shown in Figure 5.

Specificity. Cross-reactivity studies with all four Mabs yielded similar results in most cases. Table 1 shows all candidate cross-reacting compounds examined. All cross-reactivity studies were carried out using PNA-C-BSA coated plates. The results with nicarbazin was selected to be used as 100% cross-reactivity value since it is nicarbazin that the Mabs will be detecting in feed samples. Percentage of cross-reactivity was calculated according to the formula: (IC_{35} of nicarbazin)/(IC_{35} of other compound) $\times$ 100. The IC_{30} was used for Nic 7 experiments. For those cases where the solvent played a larger role in depressing the inhibition curves, the cross-reactivity was calculated at the LOD for both nicarbazin and the other compound. Only two compounds out of the 21 tested showed no cross-reactivity with nicarbazin; the compound HPD, that is used to form the complex with DNC in nicarbazin, and 1-(2-fluorophenyl)-3-(2-methoxy-4-nitrophenyl)urea. The Mab,

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Nic 6, showed different cross-reactivity patterns than the other Mabs in many cases. Nic 6 showed cross-reactivity with 2-nitroaniline (37%), 4-nitrophenol (69%), 4-nitrophenethyl alcohol (2%), and 1-(4-chlorophenyl)-3-(4-nitrophenyl)urea (47%), and the other Mabs showed no cross-reactivity to these four candidates. Also, the Nic 7 and Nic 8 Mabs showed cross-reactivity to 4-nitroaniline, 4-nitrobenzyl alcohol, 2-nitrobenzyl alcohol, 3-nitrobenzyl alcohol, and 1-(3-chlorophenyl)-3-(4-methoxy-3-nitrophenyl)urea, while the Nic 6 Mabs showed no cross-reactivity to these candidates. Other candidates such as DNC, PNA-S, PNA-C, 3-nitroaniline, 2-nitrophenol, 3-nitrophenol, and 1-(3-chlorophenyl)-3-(2-methoxy-5-nitrophenyl)urea were bound for the most part by all Mabs. Nic 7 was the only Mab that showed better cross-reactivity to some candidates, 4-nitroaniline (121%), 1-(3-chlorophenyl)-3-(2-methoxy-5-nitrophenyl)urea (172%), 1-(3-chlorophenyl)-3-(4-methoxy-3-nitrophenyl)urea (107%), and 1-(3-methoxyphenyl)-3-(3-nitrophenyl)urea (144%), than it did to nicarbazin.

CONCLUSIONS

Mabs were developed to the highly water insoluble coccidiostat nicarbazin. Four Mabs were developed, Nic 6, Nic 7, Nic 8, and Nic 9, and had an isotype of IgM$_k$. These Mabs detected the active component in nicarbazin, DNC, and had no cross-reactivity to the nonactive component, HDP. PNA-C-BSA or nicarbazin used as plate coating antigens overcame unwanted cross-reactivity. Because of the poor solubility of nicarbazin, rather harsh solvent restrictions were placed on the cELISA. A cELISA solvent system of 3% DMF (v/v) and 10% ACN (v/v) was used during these studies. These added solvents did depress the inhibition curves for nicarbazin. It was demonstrated that a pH range from 7.3 to 7.5 showed highest antibody binding using PNA-C-BSA coated microtiter plates. The two Mabs with the best detection for nicarbazin was Nic 6 and Nic 7. The IC$_{35}$ for Nic 6 was 78.5 ng/well (0.92 nmol/mL) and the IC$_{30}$ for Nic 7 was 89.7 ng/well (1.1 nmol/mL). These Mabs are good candidates for the use in a cELISA for the determination of nicarbazin in animal feeds. Nic 6 and Nic 7 have been deposited with ATCC, resulting in ATCC designations of PTA-2647 and PTA-2648, respectively.

ABBREVIATIONS USED

ACN, acetonitrile; ATCC, American Type Culture Collection; BSA, bovine serum albumin; BSA-hapten, BSA conjugated to hapten; CHCl$_3$, chloroform; CHDA, *cis*-1,2-cyclohexanedicarboxylic anhydride; DCC, *N,N*-dicyclohexylcarbodiimide; DMF, dimethylformamide; DNC, 4,4′-dinitrocarbanilide; cELISA, competitive enzyme-linked immunosorbent assay; EtOAc, ethyl acetate; FSIS, Food Safety and Inspection Service; HAT, hypoxanthine, aminopterin, and thymidine; HDP, 2-hydroxy-4,6-dimethylpyrimidine; HPLC, high performance liquid chromatography; HT, hypoxanthine and thymidine; H$_2$SO$_4$, sulfuric acid; i.p., intraperitoneal; KLH, keyhole limpet hemocyanin; KLH-hapten, KLH conjugate to hapten; Mab, monoclonal antibody; MeOH, methanol; MS, mass spectrometry; MWCO, molecular weight cutoff; NFDM, nonfat dry milk; NHS, *N*-hydroxysuccinimide; PBS-9, phosphate buffered saline pH 9; PL-1, plate coating 1 (nicarbazin); PL-2, plate coating 2 (PNA-C-BSA); PNA-C, *p*-nitro-*cis*-1,2-cyclohexanedicar-boxanilate; PNA-S, *p*-nitrosuccinanilate; RO H$_2$O, reverse osmosis water, pyrogen free; NaOH, sodium hydroxide; Sulfo-NHS, sulfo-*N*-hydroxy succinimide; TLC, thin-layer chromatography;

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