Development of a Monoclonal Antibody-Based cELISA for the Analysis of Sulfadimethoxine. 1. Development and Characterization of Monoclonal Antibodies and Molecular Modeling Studies of Antibody Recognition

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Sulfonamide antibiotics are used to treat a variety of bacterial and protozoan infections in cattle, swine, and poultry. Current residue methods for the analysis of sulfonamides in animal-based food products include bioassays, chromatographic methods (HPLC, GLC), and immunoassays. Most immunoassays have employed highly specific polyclonal antibodies. In this paper, we describe the isolation of monoclonal antibodies against sulfadimethoxine (SDM) that vary in their sensitivities and cross-reactivities against a large number of sulfonamides. The most sensitive monoclonal antibody, designated SDM-18, exhibits an IC_{50} value for SDM of 1.53 ppb. Another monoclonal antibody, designated SDM-44, exhibits IC_{50} values for six sulfonamides well below the established threshold level of 100 ppb for animal tissues. Molecular modeling studies of the cross-reactive drugs suggest that, depending on the monoclonal antibody, both steric and electronic features govern antibody binding. Due to the diversity of these monoclonal antibodies, it should be possible to design both compound- and class-specific monoclonal antibody-based immunoassays.

Keywords: *Sulfadimethoxine; sulfonamide; immunoassay; molecular modeling*

INTRODUCTION

Sulfonamides are widely used to treat bacterial and protozoan infections in food animals (Lindsay and Blagburn, 1995). Their pharmacological activity is due to their ability to mimic *p*-aminobenzoic acid (PABA) and inhibit the early stages of folic acid synthesis in bacteria and in various protozoans. They are commonly used in conjunction with dihydrofolate reductase/thymidylate synthase inhibitors, such as the diaminopyrimidine derivatives trimethoprin and ormetoprin, with which they act synergistically.

Sulfonamide residues have been detected in various food products (Franco et al., 1990), and in the United States, the tolerance level for sulfonamides in meat and poultry is 0.1 μg/g (U.S. Department of Agriculture, 1994, 21CFR 500.640). To ensure that residues in animal food products do not exceed this limit, animals undergoing treatment must be withdrawn from medication prior to slaughter. This withdrawal time varies depending on the specific drug, animal, and application.

Conventional residue methods for the detection of sulfonamides in animal tissues include bioassays, thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC) (Aerts et al., 1995). In an effort to increase both method sensitivity and sample throughput, enzyme-linked immunosorbent assays (ELISAs) have been developed for several of the sulfonamides (Fleeker and Lovett, 1985; Dixon-Holland and Katz, 1988; Jackman et al., 1993; Märtlbauer et al., 1994). The majority of immunoassays developed for the sulfonamides have used polyclonal rather than monoclonal antibodies. The use of monoclonal antibodies would have the advantage that a large, renewable, and reproducible supply of a defined immunoreagent would be readily available. This may be particularly important in applications for which large amounts of antibody are used, such as large-scale residue screening programs, immunoaffinity chromatography, and flow-through immunodetection systems that continuously consume antibody. In some cases, the use of monoclonal antibodies may improve immunoassay sensitivity.

In this paper, we describe the development of a panel of murine monoclonal antibodies against the sulfonamide sulfadimethoxine (SDM; Table 1). This sulfonamide is used to control bacterial and protozoan infections in cattle, swine, and poultry. Several mice were immunized with an SDM immunogen, and their antisera were subsequently screened for sensitivity and cross-reactivity toward SDM, sulfamethazine (SMZ), and sulfathiazole (STZ) using both homologous and heterologous ELISAs. Splenocytes from an immunized mouse were used for cell fusion, and a heterologous ELISA was used to screen hybridomas to minimize the unintentional detection and isolation of antibodies exhibiting high affinity for the immobilized antigen but low affinity for the free drug. The monoclonal antibodies that were isolated varied in their relative sensitivities toward

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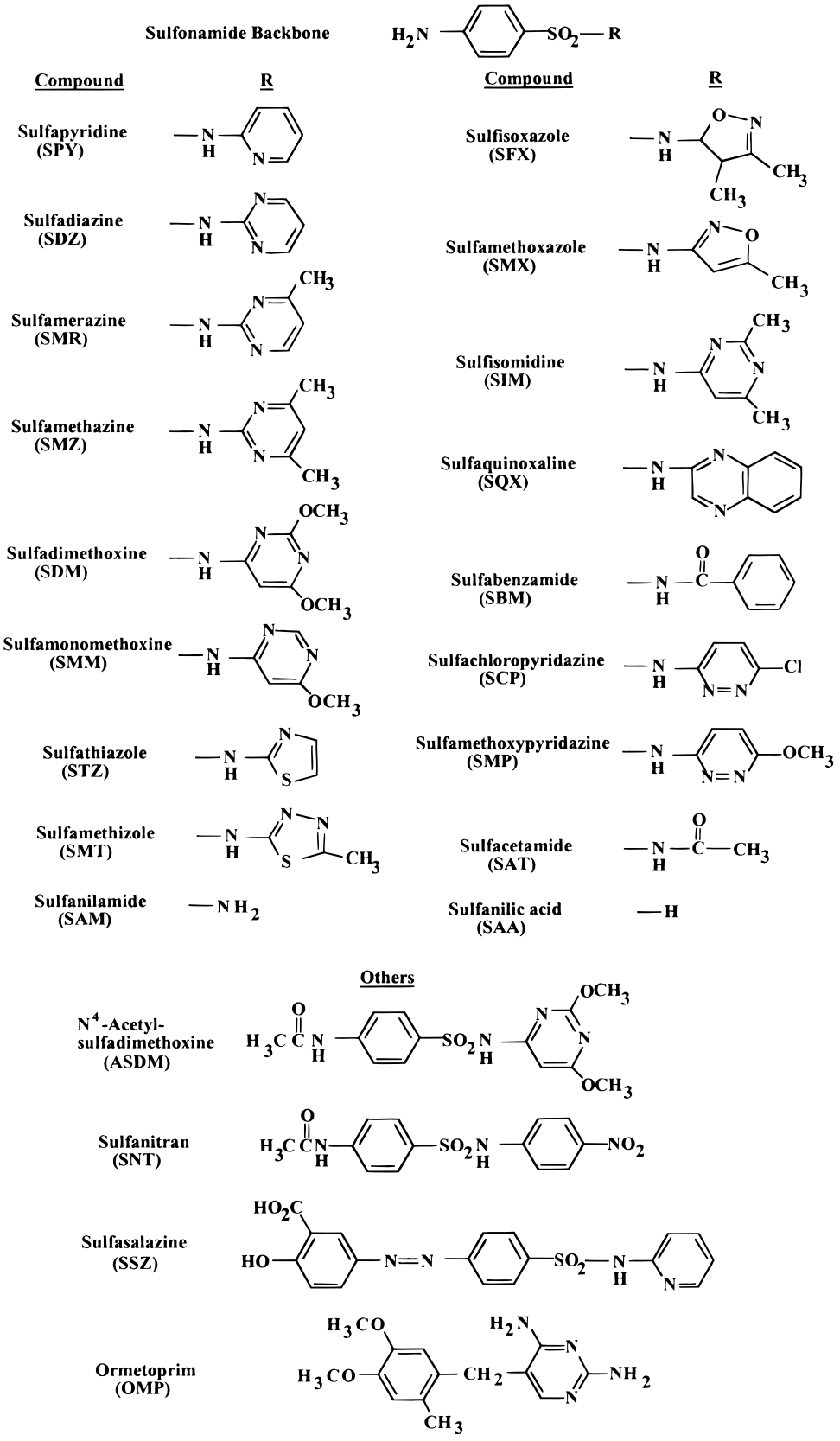
Table 1. Structures of the Sulfonamides Used in This Study



SDM and in their cross-reactivities toward a large panel of other sulfonamides. Molecular modeling studies were performed on the panel of sulfonamides for the purpose of describing the basis of antibody recognition. For the most selective monoclonal antibodies, antibody binding appeared to be dependent on steric factors, whereas for the less selective (broader cross-reactivity) monoclonal antibodies, the electronic configuration of the molecules appeared to significantly contribute to antibody recognition. These results suggest that both highly selective

and broadly cross-reactive anti-sulfonamide monoclonal antibodies can be obtained using a single SDM immunogen.

MATERIALS AND METHODS

Chemicals and Supplies. N^4-Acetylsulfadimethoxine was a gift from Steven A. Barker, Department of Veterinary Physiology, Louisiana State University, Baton Rouge, LA. Ormetoprim (OMP) was a gift from Hoffman-La Roche Inc., Nutley, NJ. Sulfathiazole (STZ), sulfisomidine (SIM), sulfamethazine (SMZ), and sulfisoxazole (SFX) were purchased from Aldrich (Milwaukee, WI). Sulfadimethoxine (SDM) and sulfaquinoxaline (SQX) were purchased from Fluka (Buchs, Switzerland). Sulfamerazine (SMR) and sulfanilic acid (SAA) were purchased from Lancaster Synthesis (Windham, NH). Sulfamonomethoxine (SMM), sulfapyridine (SPY), sulfadiazine (SDZ), sulfacetamide (SAT), sulfanilamide (SAM), sulfamethoxazole (SMX), sulfamethizole (SMT), sulfabenzamide (SBM), sulfachloropyridazine (SCP), sulfanitran (SNT), bovine thyroglobulin (BTG), bovine serum albumin (BSA; fraction V, protease-free, 96−99%), and goat anti-mouse IgG (whole molecule) conjugated to horseradish peroxidase (GαMIgG−HRP) were purchased from Sigma (St. Louis, MO). Sulfasalazine (SSZ) and sulfamethoxypyridazine (SMP) were purchased from ICN Biomedicals (Costa Mesa, CA). K-Blue (enzyme substrate) was purchased from ELISA Technologies (Lexington, KY). RIBI adjuvant R-700 was obtained from RIBI ImmunoChem Research, Inc. (Hamilton, MT). Mouse antibody isotyping kit was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). Nonfat dry milk (NFDM) was obtained from a local grocery store.

Buffers. All buffer components were of cell culture or reagent grade. Assay buffer (pH 7.75) contained, per liter of water, 11.4 g of Tris-HCl, 3.32 g of Tris base, 8.7 g of sodium chloride, 0.01 g of NFDM, and 0.005% (v/v) Tween 20. Coating buffer (pH 9.6) contained, per liter of water, 1.59 g of sodium carbonate, 2.93 g of sodium bicarbonate, and 0.203 g of magnesium chloride. Phosphate-buffered saline (pH 7; PBS-7) contained, per liter of water, 8.0 g of sodium chloride, 0.2 g of potassium chloride, 1.15 g of sodium phosphate (dibasic), and 0.2 g of potassium phosphate. Blocking buffer (pH 9) contained 30 g of NFDM per liter of PBS (pH 9; PBS-9).

Equipment. Cell culture plasticware was from Costar (Cambridge, MA). Microtiter plates used for ELISA studies were flat-bottom Nunc Immunoplate II Maxisorp (Nunc, Roskilde, Denmark). Microtiter plate optical density (OD) measurements were made using a Bio-Rad model 3550 microplate reader (Richmond, CA). Data were collected using a Macintosh II computer with Reader Driver 1.0 and Microplate Manager 1.0 software (Bio-Rad). Other calculations used Excel spreadsheet software (Microsoft Corp., Redmond, WA).

Preparation of the Immunogen (BTG−SDM). SDM (50 mg, 161 μmol) was diazotized and conjugated to bovine thryoglobulin (BTG; 100 mg, 0.15 μmol) according to the procedure of Fleeker and Lovett (1985). Briefly, SDM was dissolved in warm 0.5 N sulfuric acid (4 mL, 70 °C). The solution was then cooled in an ice bath, and an aliquot (1 mL) of freshly prepared sodium nitrate (19 mg/mL) was added dropwise over 3 min and incubated for an additional 3 min. A solution of BTG (100 mg in 4 mL of 0.5 M borate, pH 9.4) was cooled to 0 °C, and the diazotized hapten solution was added to the cooled BTG solution over a period of 15 min. The pH of the solution was maintained between 9.0 and 9.5 using sodium hydroxide (1 M). After 4 h, the reaction mixture was slowly brought to room temperature, exhaustively dialyzed against 20 mM PBS, pH 7.2, and stored at −20 °C prior to use.

Preparation of Coating Antigens (BSA−SDM, −STZ, −SMZ). The BSA−hapten conjugates were prepared according to the procedure described by Singh (1978) using isobutyl chloroformate as the cross-linker. The conjugates were exhaustively dialyzed against 20 mM PBS, pH 7.2, and stored at −20 °C prior to use.

Monoclonal Antibody Production. Five BALB/c mice were injected intraperitoneally (ip) and intramuscularly (im) with 80 and 20 μL of BTG−SDM (1 mg/mL) in RIBI adjuvant, respectively. The mice were boosted on days 17 and 32 with 100 μL (ip) of BTG−SDM in RIBI adjuvant. On day 42, serum titers were determined; subsequently, the sera were tested using a competitive inhibition ELISA (cELISA) to determine which mice were producing antibodies against the sulfonamides. In these studies, BSA−SDM, −SMZ, or −STZ was used as the coating antigen, and SDM, SMZ, and STZ (Table 1) were used as competitors. On day 67, 4 days prior to the cell fusion, the mouse exhibiting the most sensitive antibodies to SDM was immunized a final time with 100 μL (ip) of BSA−SDM (1 mg/mL) in distilled water. The spleen was removed and splenocytes were fused with SP2/O myeloma cells and cultured in 96-well plates using conditions previously described (Stanker et al., 1986).

A direct binding ELISA, described by Stanker et al. (1993) and modified as described below, was used to screen the culture supernatants from the growing hybridomas for antibodies to the sulfonamides. Assay plates were coated with BSA−SMZ (100 ng/well) in coating buffer (100 μL/well) and incubated for 18 h at 4 °C. The plates were exhaustively washed with wash buffer (distilled water containing 0.05% Tween 20) followed by a distilled water rinse. Nonspecific binding was decreased by blocking the wells with blocking buffer for 60 min at room temperature. The plates were either used immediately or stored at −20 °C. After the blocked plates had been washed with wash buffer, 100 μL of the appropriate hybridoma supernatants was allowed to bind to the coated microwells for 60 min at room temperature. Unbound antibody was removed by washing five times with wash buffer. Next, 100 μL of GαMIgG−HRP (1:1000 dilution in assay buffer) was added to each well. After a 60 min incubation at room temperature, the plates were washed, and bound antibody−peroxidase conjugate was determined using K-Blue, a colorimetric substrate. The plates were incubated either for 20 min and optical density measurements made at 450 nm [after the addition of 1 M H_2SO_4)50 μL)] or for 30 min and optical density measurements made at 655 nm. For screening fusion plates, color development was visually monitored.

Hybridoma cells from wells exhibiting a strong positive response in the initial screen were expanded, and the supernatants were rescreened using a cELISA format (described below) with BSA−SMZ as the coating antigen and SDM, STZ, or SMZ as the competitor at a single concentration of 1 μg/mL. Hybridomas that demonstrated inhibition of antibody binding by free sulfonamide were subcloned twice by limiting dilution to ensure their monoclonal origin. Isotype determinations were done by ELISA using mouse heavy- and light-chain-specific antisera.

Competitive Inhibition ELISA (cELISA). Microtiter plates were coated and blocked as previously described. After the blocked plates had been washed, 100 μL of the appropriate sulfonamide (inhibitor) standard in assay buffer and 100 μL of cell supernatant diluted in assay buffer were added to each well and incubated for 60 min at room temperature. The amount of antibody used produced an optical density reading of ∼1.0 absorbency unit and was in the linear portion of the titration curve. After a washing with wash buffer, the assay was completed as described above.

For each experiment, control wells containing all components except the competitor were prepared, and the enzymatic activity (color reaction) in these wells was taken to represent 100% activity. The test wells, which contained various amounts of competitor, were normalized to the 100% activity wells, and percent inhibition was calculated as

$$\% \text{ inhibition} = [1 - (A_{450\text{or}655} \text{ of test}/A_{450\text{or}655} \text{ of control})] \times 100$$

where $A_{450\text{or}655}$ is the absorbance at 450 or 655 nm. The 50% inhibition of control (IC_{50}) values for the various sulfonamides were obtained using the four-parameter curve fitting function in Microplate Manager 1.0.

Characterization of Monoclonal Antibodies for Sulfonamide Recognition. Monoclonal antibodies SDM-18, -19,

-44, -70, and -114 were characterized for reactivity toward SDM, STZ, and SMZ (Table 1) using the cELISA format with either BSA−SDM, −SMZ, or −STZ as the coating antigen. On each plate, nine concentrations of each competitor were analyzed in either triplicate (SDM and SMZ) or duplicate (STZ). Monoclonal antibodies SDM-18, -44, and -114 were further characterized against the entire panel of compounds shown in Table 1. For this study, each plate consisted of nine concentrations of SDM analyzed in duplicate wells and nine concentrations of two competitors each analyzed in triplicate wells. IC_{50} values were obtained as previously described.

Molecular Modeling Studies. *Determination of Minimum Energy Conformations.* Molecular modeling studies were conducted using a CAChe WorkSystem operated on a Macintosh Power PC 9500/200 computer equipped with a stereoscopic display (CAChe Scientific, Inc., Beaverton, OR). Minimum energy conformations of the various structures were calculated using a modification of Allinger's standard MM2 force field parameters (Allinger, 1977). Initial conformation optimization was followed by a sequential search for low-energy conformations in which the dihedral angles of the compound were rotated 360° in 15° increments using the Molecular Mechanics application. Several low-energy conformations were chosen from the sequential search and reoptimized. The lowest energy conformation thus obtained was designated the minimum energy conformation. Potential energy conformation maps were constructed for each compound by re-evaluating the minimal energy conformation in an exhaustive search in which two of the dihedral angles were rotated independently 360° in 15° increments.

Determination of Electronic Properties. The electronic wave function was calculated using the extended Hückel approximation (Hoffmann, 1963). The CAChe tabulator application was used to convert these data into three-dimensional coordinates to visualize electon density and electrostatic potential. The value used for the electron density probability was 0.01 electron/$Å^3$.

RESULTS AND DISCUSSION

Animal Immunization and Antisera Screening. Mice were immunized with BTG−SDM. Following a series of boosts, the mice were bled and the antisera were screened for competitive inhibition using either BSA−SDM, −STZ, or −SMZ as the coating antigen and SDM, STZ, or SMZ as the competitor. This was done to determine antisera sensitivity, selectivity, and heterogeneity for the purpose of selecting an appropriate mouse to use as a source of splenocytes for cell fusion. The results from this screening experiment are shown in Table 2. We observed the highest antisera titers when SDM was used as the ELISA plate coating hapten (homologous ELISA). When either SMZ or STZ was used as the plate coating hapten (heterologous ELISA), higher antisera concentrations were required to obtain equivalent OD values (∼1.0 absorbency unit). This result was not surprising because SDM was the immunizing hapten and most antibodies produced should recognize this hapten with greater affinity than either STZ or SMZ.

The most sensitive assays for free SDM were obtained when BSA−SMZ was used as the coating antigen despite the use of higher antisera concentrations with this heterologous assay (Table 2). This result suggests that antibodies that recognized free SDM were present in the antisera but at relatively low concentrations. Antibodies that recognize the conjugated form of SDM were more prevalent. However, by using a heterologous assay with a higher concentration of antisera, it was possible to detect the population of antibodies that could be inhibited by free drug.

Table 2. Cross-Reactivities of Antisera from BTG−SDM-Immunized Mice toward SDM, STZ, and SMZ Using Different ELISA Haptens

mouse	ELISA hapten	rel antisera concn[b]	% reactivity (SDM = 100%)[a]		
			SDM (IC_{50}, ppb)	SMZ	STZ
1	SDM	1	100 (556.2)	NI[c]	NI
	SMZ	256	100 (418.6)	41.9	NI
	STZ	256	100 (2015)	NI	NI
2	SDM	1	100 (866.1)	NI	NI
	SMZ	32	100 (141.9)	4.7	NI
	STZ	16	100 (1333)	1.3	3332
3	SDM	1	100 (1472)	NI	NI
	SMZ	64	100 (529.8)	17.7	NI
	STZ	16	100 (20000)[d]	20.0	25000
4	SDM	1	100 (780.2)	NI	NI
	SMZ	16	100 (174.7)	8.7	7.0
	STZ	128	100 (891.7)	8.9	2229

[a] % reactivity = ($IC_{50,SDM}/IC_{50,analogue}$) × 100. [b] Relative antisera concentration = antisera dilution used with SDM−BSA (homologous ELISA)/antisera dilution used with designated ELISA hapten. The antisera dilution used with each ELISA hapten was predetermined in a titration experiment using a direct ELISA format (absorbance value of ∼1.0 OD unit in the linear portion of the titration curve). [c] NI, no inhibition at 10000 ppb. [d] Value was extrapolated from standard curve.

We also observed that antisera cross-reactivity toward either SMZ or STZ increased when heterologous assays were used (Table 2). In addition, sensitivities for the respective sulfonamides were greatest when the same sulfonamide was used as the plate coating antigen. For three of four mice, when STZ was used as the plate coating antigen in the cELISA, antisera sensitivity was greater for STZ than for SDM, the immunizing hapten. As changing the coating antigen had such an effect on the antisera sensitivity and cross-reactivity profile, this observation suggests that different populations of antibodies in the antisera preferentially bind to the different coating antigens.

Few examples of heterologous cELISAs for sulfonamide antibiotics have been reported in the literature. In one example, the use of a heterologous cELISA greatly improved assay sensitivity and resulted in some differences in assay selectivity (Sheth and Sporns, 1991). In another example, a decrease in assay sensitivity was reported with no change in assay selectivity when a heterologous cELISA was used (Garden and Sporns, 1994). In these studies, the structural differences between the immunizing hapten and the hapten used in the heterologous cELISA were near the point of attachment to the protein. Therefore, the population of antibodies (polyclonal) that bound the heterologous hapten were probably the same as that which bound the homologous hapten. However, in the current study, both of the plate coating haptens used in the heterologous cELISAs (SMZ and STZ) differ from the immunizing hapten (SDM) in the region of the molecule distal to the point of attachment to the protein (via the primary amine). As a result, the variable region of the sulfonamide molecule, the N^1 ring, was more exposed to the antibody. This may have allowed us to detect a larger repertoire of antibodies.

Cell Fusion and Hybridoma Production. Mouse 4 was selected as the source of splenocytes for cell fusion because the antisera from this mouse exhibited good sensitivity for SDM with each of the coating antigens used in the cELISAs (Table 2). This suggests that, in this particular mouse, a highly varied repertoire of antibody-producing cells existed and therefore it should be a preferred source of splenocytes for cell fusion.

Table 3. Cross-Reactivities of SDM Monoclonal Antibodies toward SDM, STZ, and SMZ Using Different ELISA Haptens

MAb (isotype)	ELISA hapten	rel MAb concn[b]	% reactivity (SDM = 100%)[a]		
			SDM (IC_{50}, ppb)	SMZ	STZ
SDM-18	SDM	1	100 (22.0)	NI[c]	NI
(IgG1, κ)	SMZ	9	100 (1.3)	0.01	NI
	STZ	—[d]	—	—	—
SDM-19	SDM	1	100 (566.0)	19.2	1.0
(IgG1, λ)	SMZ	20	100 (296.1)	30.1	1.2
	STZ	—	—	—	—
SDM-44	SDM	1	100 (16.1)	4.9	81.5
(IgG1, λ)	SMZ	1.2	100 (9.4)	3.5	104.9
	STZ	1.2	100 (9.6)	4.4	145.1
SDM-70	SDM	1	100 (8691.2)	NI	NI
(IgG1, κ)	SMZ	100	100 (898.4)	3.2	2.0
	STZ	—	—	—	—
SDM-114	SDM	1	100 (6.4)	2.5	0.2
(IgG1, κ)	SMZ	1.8	100 (3.5)	2.0	0.09
	STZ	4.5	100 (0.7)	1.8	0.08

[a] % reactivity = ($IC_{50,SDM}/IC_{50,analogue}$) × 100. [b] Relative MAb concentration = cell culture supernatant dilution used with SDM−BSA (homologous ELISA)/cell culture supernatant dilution used with designated ELISA hapten. The cell culture supernatant dilution used with each ELISA hapten was predetermined in a titration experiment using a direct ELISA format (absorbance value of ~1.0 OD unit in the linear portion of the titration curve). [c] NI, no inhibition at 10000 ppb. [d] Dash indicates insufficient antibody binding occurred with these systems.

Following cell fusion and culturing in 30 96-well culture plates, ~90% of the wells contained 1−5 hybridoma colonies. Aliquots of cell culture media from individual wells of the plates (2880) were assayed for antibody-producing hybridomas using a direct heterologous ELISA with BSA−SMZ as the coating antigen. This heterologous coating antigen was chosen for use in the initial screen rather than the homologous coating antigen (BSA−SDM) since previous experiments had demonstrated that most antibodies present in the sera had relatively high titer against BSA−SDM but relatively low sensitivity for the free drug. Therefore, use of the homologous coating antigen at this stage would have most likely screened for these more prevalent antibodies and might have masked the presence of the higher affinity antibodies capable of binding the free drug. Using heterologous ELISA conditions, 120 positive wells were visually selected and expanded into 24-well culture plates. These were rescreened using a cELISA format with SDM, SMZ, and STZ as competitors at a single concentration of 1 ppm. From this screen, 8 of the original 120 positives exhibited binding with at least one sulfonamide. These were subcloned twice by limiting dilution to obtain monoclonal cell lines.

Monoclonal Antibody Characterization. The five most sensitive monoclonal antibodies were titrated on plates coated with BSA−SDM, −SMZ, or −STZ. Next, they were evaluated for cross-reactivity with the agriculturally important sulfonamides SDM, SMZ, and STZ using each of the three coating antigens. These results are presented in Table 3. As previously observed with the mouse polyclonal antisera (Table 2), we observed the highest monoclonal antibody titers using the homologous coating antigen BSA−SDM. Also, in every case, these homologous assays were the least sensitive for SDM. Monoclonal antibody SDM-18 was the most sensitive and selective for the target sulfonamide, SDM. Monoclonal antibody SDM-19 was less sensitive but was the only monoclonal antibody that exhibited significant cross-reactivity with SMZ. Monoclonal antibody SDM-44 was highly sensitive for SDM and exhibited significant cross-reactivity with STZ. Monoclonal antibody SDM-70 was the least sensitive for SDM. Monoclonal antibody SDM-114 was sensitive for SDM and showed some cross-reactivity toward SMZ and weak cross-reactivity toward STZ. Figure 1 shows typical SDM standard curves for each of the monoclonal antibodies using BSA−SMZ as the coating antigen. Three of the monoclonal antibodies, SDM-18, -44, and -114, exhibited IC_{50} values for SDM between 1.0 and 20 ppb, which made these antibodies valuable for use in residue monitoring programs at the tolerance level of 100 ppb. The other monoclonal antibodies, SDM-19 and -70, exhibited IC_{50} values for SDM between 500 and 1000 ppb. Although these would not be useful for residue analysis by ELISA without significant sample concentration, they may be useful for other techniques (such as immunoaffinity chromatography) that can use lower affinity antibodies.

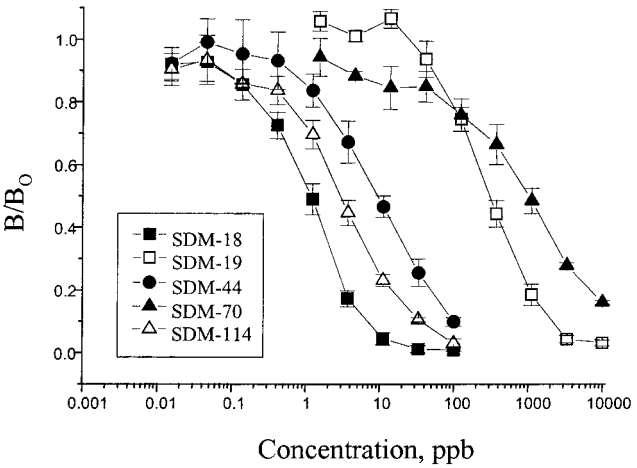


Figure 1. Sulfadimethoxine (SDM) competitive inhibition curves for monoclonal antibodies SDM-18, SDM-19, SDM-44, SDM-70, and SDM-114.

The three most sensitive monoclonal antibodies, SDM-18, SDM-44, and SDM-114, were further characterized using the panel of compounds shown in Table 1 in a cELISA with BSA−SMZ as the coating antigen. The results of this study are shown in Table 4. Monoclonal antibody SDM-18 was the most sensitive (SDM IC_{50} = 1.53 ppb) and the least cross-reactive antibody. However, SDM-18 recognized the major metabolite N^4-acetylsulfadimethoxine (ASDM) to a greater extent than the parent drug. Because the hapten was linked to the carrier protein through the N^4 position for use as the immunogen, some recognition of N^4-substituted SDM was expected. This antibody did not recognize SSZ, which suggests that the major binding epitope for this antibody is in the region of the pyrimidinyl ring. SDM-18 also recognized the 2,6-disubstituted 4-pyrimidinylsulfonamides SMM and SIM. Antibody recognition of SIM was greater than that of SMM, suggesting that this antibody was more sensitive to differences in steric factors (absence of pyrimidinyl ring appendage) than to differences in electronic configuration (absence of oxygen in pyrimidinyl ring appendage).

Monoclonal antibody SDM-44 was the most cross-reactive antibody studied. The percent cross-reactivity for ASDM was >5-fold that of the parent drug. In addition, SDM-44 recognized the N^4-substituted pyrimidinylsulfonamide, SSZ, which possesses an azo bond at this position, the same linkage chemistry used in the immunogen. However, the other pyrindinylsulfonamide tested, SPY, was only slightly recognized. This result

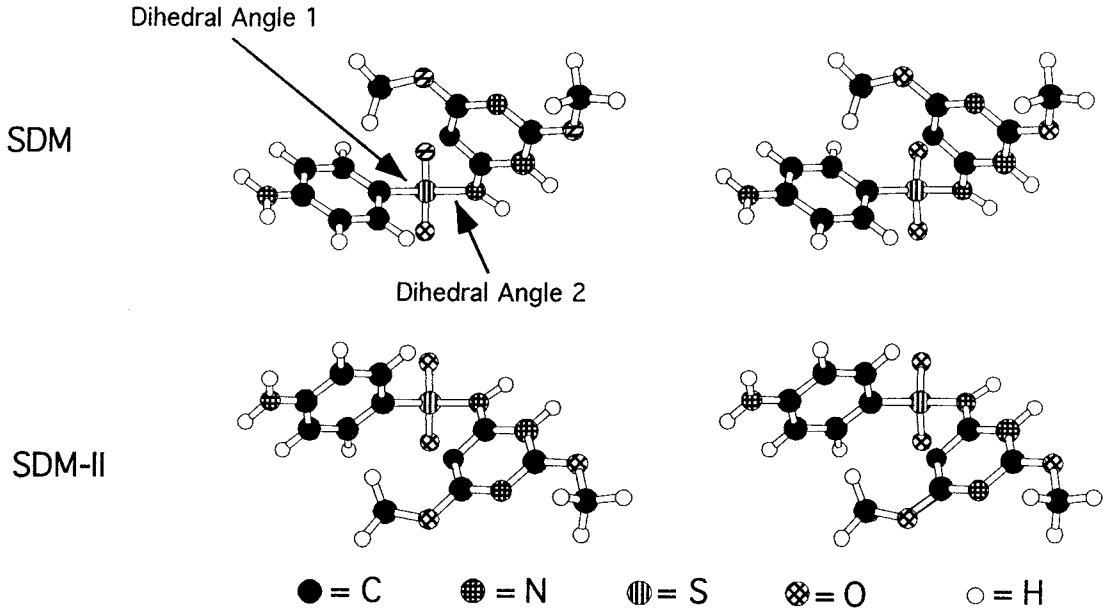


Figure 2. Calculated minimum energy conformation and an alternate low-energy conformation (SDM-II) of sulfadimethoxine (SDM) depicted as stereoscopic pairs of ball and cylinder models.

Table 4. Cross-Reactivities of Monoclonal Antibodies SDM-18, SDM-44, and SDM-114 toward Various Sulfonamides

	% reactivity (SDM = 100.0)		
competitor	SDM-18	SDM-44	SDM-114
SDM (IC_{50}, ppb)	100.0 (1.53)	100.0 (7.62)	100.0 (2.56)
ASDM	136.9	536.9	667.6
SMZ	NI	4.2	2.1
STZ	NI	125.8	0.1
SMR	NI	6.6	3.1
SMM	17.0	0.6	1.5
SPY	NI	2.7	0.1
SDZ	NI	2.2	0.4
SQX	NI	0.3	4.3
SMX	NI	4.1	NI
SIM	118.0	NI	17.2
SFX	NI	NI	NI
SMP	NI	13.6	0.4
SCP	NI	10.5	NI
SSZ	NI	381.2	5.7
SMT	NI	181.5	NI
SBM	NI	NI	NI
SNT	NI	NI	NI
SAM	NI	NI	NI
SAA	NI	NI	NI
SAT	NI	NI	NI
OMP[a]	NI	NI	NI

[a] OMP (ormetoprim) is a dihydrofolate reductase/thymidylate synthase inhibitor and is not a sulfonamide. The relative standard deviations (%CV) for the SDM IC_{50} values (11 curves) obtained for monoclonal antibodies SDM-18, SDM-44, and SDM-114 were 17.5, 18.0, and 17.0%, respectively. NI, no inhibition at 10000 ppb.

suggests that the N^4 linkage is a dominant binding epitope for this antibody. The five-membered heterocycles STZ and SMT were recognized to a greater extent than SDM. Recognition of the other five-membered heterocycles, SMX and SFX, was greatly diminished. These differences in recognition of the five-membered heterocycles may be attributed to the presence of oxygen in the ring systems of SMX and SFX, which resulted in differences in the electronic properties of these compounds. Antibody recognition of the 2-pyrimidinylsulfonamides SMZ, SMR, and SDZ was minimal.

Monoclonal antibody SDM-114 exhibited intermediate cross-reactivity compared with those of SDM-18 and SDM-44. Antibody recognition of ASDM was greatest with SDM-114. Like monoclonal SDM-18, this antibody also recognized SIM, and like monoclonal antibody SDM-44, it recognized SSZ, but to a lesser extent. The other pyridinyl, pyrimidinyl, and five-membered heterocycles were either not recognized or recognized only to a limited extent. None of the monoclonal antibodies recognized the diaminopyrimidine derivative ormetoprim (OMP), which is commonly used in conjunction with SDM.

Molecular Modeling Studies. These studies were conducted to compare the structural and electronic properties of the various sulfonamides and to provide insight for possible mechanisms of antibody recognition. Minimum energy conformations were calculated for the various sulfonamides shown in Table 1. This was performed by rotating the dihedral angles 360° in 15° increments and calculating the resulting free energy at each interval using MM2 force field parameters. Several low-energy conformations were obtained and further optimized, resulting in a minimum energy conformation. Figure 2 shows a stereoscopic view of the minimum energy conformation calculated for SDM ($\Delta E = -4.147$ kcal/mol) and an alternate structure, SDM-II ($\Delta E = -4.101$ kcal/mol), in which the two aromatic rings differ in orientation. In these orientations, the sulfonyl oxygens extend into the page, the N^4 ring is oriented to the left, and the N^1 ring is oriented to the right of the sulfur. The energy barrier for conversion between these two conformational isomers is 4.010 kcal/mol. Because this energy barrier can be overcome under ambient conditions, these conformers may coexist in nearly equal proportions. In both of these low-energy conformations, the two ring systems of SDM were biplanar and appeared to form a cage-like structure. For SDM, the calculated distance from the methoxy carbon of the pyrimidinyl ring to the N^4 nitrogen was 4.63 Å. In the coplanar conformation (not shown), the distance was 12.97 Å. The short atomic distance between the two extremities observed for the minimum energy conformation of SDM may have resulted in the generation of antibodies that were sensitive to changes at both the N^4 position and the N^1 ring.

The electronic properties of the molecules were also studied to describe the basis of antibody recognition.

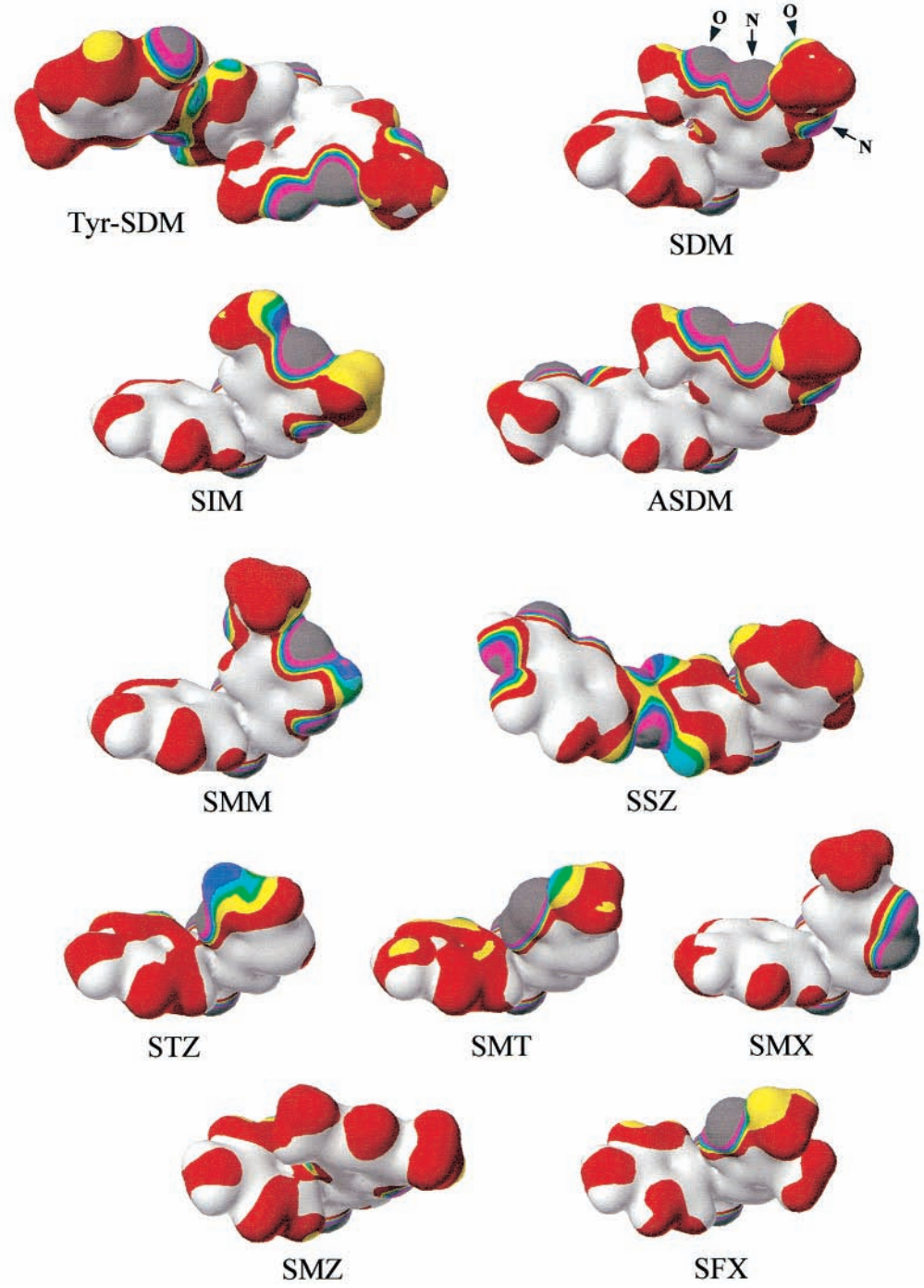


Figure 3. Calculated minimum energy conformations of sulfadimethoxine (SDM), tyrosyl (R group)−sulfadimethoxine (Tyr−SDM), sulfisomidine (SIM), N^4-acetylsulfadimethoxine (ASDM), sulfamonomethoxine (SMM), sulfasalazine (SSZ), sulfathiazole (STZ), sulfamethizole (SMT), sulfamethoxazole (SMX), sulfamethazine (SMZ), and sulfisoxazole (SFX) depicted as electron density surfaces colored by electrostatic potential. The electron density probability value used for all calculations was 0.01 electron/Å^3. The energy values [in atomic units (au)] at each color interface are white-red, +0.09 au; red-yellow, +0.03 au; yellow-green, +0.01 au; green-light blue, 0.00 au; light blue-dark blue, −0.01 au; dark blue-purple, −0.03 au; and purple-black, −0.06 au, where 1 au = 627.503 kcal/mol.

Figure 3 shows the electron density surfaces colored by the electrostatic potential for selected sulfonamides. Dark gray represents regions that are the most electronegative, whereas white and red represent regions that are the most electropositive. As in Figure 2, the sulfonyl oxygens extend into the page (and are partially hidden), the N^4 ring is oriented to the left, and the N^1 ring oriented to the right of the sulfur. Also shown is Tyr-SDM, which is the calculated minimum energy conformation for SDM conjugated to the R group of tyrosine via an azo linkage (which occurs in the immunogen). The minimum energy conformation calculated for Tyr-SDM is similiar to the conformation shown for SDM-II in Figure 2. In all of the sulfonamides, there is a region of high electronegativity associated with the sulfonyl oxygens. The N^1 ring of SDM contains regions of high electronegativity corresponding to the nitrogens (arrows) at the 1 and 3 ring positions and the methoxy oxygens (arrowheads) at the 2 and 6 ring positons.

Binding by monoclonal antibody SDM-18 was dependent, in part, on steric factors (illustrated in Figure 3 by the surfaces shown for SIM and SMM in compari-

son to that of SDM). SIM was recognized to a greater extent than SDM (Table 4) despite the lack of the methoxy oxygen on the ring appendages. However, SMM, which lacks an entire methoxy group, was recognized much less than SDM (Table 4). Furthermore, SMZ, having the N^1 nitrogen linked via position 2 of the pyrimidinyl ring (versus position 4 for SDM), was not recognized by SDM-18, suggesting that the ring nitrogen (and associated electronic properties) opposite the point of attachment to the N^1 nitrogen is required for antibody recognition.

The importance of the electronic contribution to SDM-44 sulfonamide recognition was supported by the observation that this antibody did not recognize SIM or SMM. Differences in SDM-44 recognition of the five-membered heterocycles STZ, SMT, SFX, and SMX (Table 4) may be explained, in part, by the differences in the electronics of these molecules. It can be seen in Figure 3 that for STZ and SMT, which are recognized by the antibody (Table 4), a region of high electronegativity exists on the upper part of the N^1 ring. For SMX and SFX, which are not significantly recognized, this region is either on the opposite side of the ring (SMX) or much reduced in size (SFX). As previously mentioned, N^4 substitution (Tyr-SDM, SSZ, ASDM, and SNT) results in an electronegative region at the point of attachment as well as on the carbons of the N^4 phenyl ring. This appears to improve SDM-44 antibody recognition as most of these compounds were recognized to a greater extent than SDM. However, SNT (Table 1) was not recognized, suggesting that some electronegativity is required in the N^1 ring as well. It is noteworthy that SDM-44 did not recognize SPY (Table 4), which, like SSZ, is a pyridinyl-substituted sulfonamide (Table 1). This suggests that the azo linkage is an important binding epitope for monoclonal antibody SDM-44.

Monoclonal antibody SDM-114 was the most selective; it exhibited cross-reactivity >10% with only three of the compounds (Table 4). This was probably due to both steric and electronic factors. This antibody exhibited significant binding to SIM (in comparison to SDM), suggesting that it tolerated the lack of the methoxy oxygens. However, other changes in the N^1 ring greatly reduced or eliminated antibody recognition. Like SDM-44, there was some recognition of the azo linkage, because SDM-114 showed slight recognition of SSZ and no recognition of SPY.

Conclusions. We have isolated a heterogeneous panel of murine anti-SDM monoclonal antibodies varying in their sensitivities and cross-reactivities against a number of sulfonamide antibiotics. The antibodies were obtained using a single SDM immunogen. Molecular modeling studies aided in determining whether steric factors, electronic properties, or a combination of both contributed most to antibody recognition. The high level of nonspecific antibody binding that was observed with the polyclonal sera was overcome by using a heterologous cELISA for screening hybridomas. Application of this assay strategy may have also had the effect of increasing the likelihood of isolating hybridomas secreting more highly cross-reactive antibodies, such as SDM-44. Due to the diversity of these monoclonal antibodies, it may be possible to devise both SDM-specific and sulfonamide class-specific assays. Class-specific assays may be further enhanced by using a cocktail of selected antibodies; alternatively, molecular biological techniques may be used to alter the antibody binding site of an existing class-specific antibody (i.e., through a point mutation) to allow for even broader cross-reactivity. Use of an antibody exhibiting broad cross-reactivity would decrease the number of assays required to determine total sulfonamide contamination. Further studies will evaluate these antibodies for use in residue analysis of food samples.

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