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Generation of Group-Specific Antibodies against Sulfonamides

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To develop a sulfonamide-specific ELISA, different attempts were made to obtain monoclonal antibodies specific for the common structure of sulfonamides. In a first approach, sulfanilamide was linked to albumins using glutaraldehyde or a succinimide ester as cross-linker. A weak immune response or none at all was induced after immunization of mice with those conjugates. High antibody titers were obtained with conjugates where sulfanilamide was linked to albumins or casein (azocasein) with a diazotation reaction. However, the antibodies were only highly specific for the bound sulfanilamide molecule. In a second approach, sulfonamide-protein conjugates were used in which the sulfonamide molecule is linked at its side chain, leaving the common structure of sulfonamides unchanged. Three sulfonamide derivatives (S, TS, and PS, previously described in the literature) containing a carboxyl group in their side chain were linked to proteins using a carbodiimide mediated reaction. Immunization with the S-conjugates led to high antibody titers, but the antibodies were only highly specific for the bound S-molecule. Group-specific antibodies were obtained after immunization with the PS- and TS-conjugates. It was described that immunization with PS-conjugates lead to the recognition of other sulfonamides (sulfamethazine, -merazine, -diazine, and -dimethoxine) that are not well recognized by antibodies induced after immunization with TS-conjugates. Therefore, we tried to guide the immune response in the direction of recognition of the common structure of sulfonamides by immunizing the animals alternately with PS- and TS-conjugates. The polyclonal antibodies of the mice indeed had a broader specificity, but the specificity of the monoclonals obtained after fusion experiments was not influenced. Immunization with TS-conjugates seemed sufficient to obtain sulfonamide-specific monoclonal antibodies. With the best monoclonal (mAb 3B5B10E3) two competitive inhibition (ci) ELISA's were developed: one coated with antigen and the other coated with the monoclonal antibody. Sulfadiazine, -dimethoxine, -thiazole, -pyridine, and -methoxazole were detected in both ELISA's at their MRL-value (100 ppb) in buffer solution. Sulfadiazine, sulfathiazole, and sulfamethoxazole could even be detected at 10 ppb.

KEYWORDS: Sulfonamide; monoclonal antibody; ELISA

INTRODUCTION

Sulfonamides are chemotherapeutical reagents widely used in human as well as in veterinary medicine for the treatment of bacterial infections. They are also used as growth-promoting feed additives (*1*). As a consequence, sulfonamides have appeared in food products from animal origin (*2*). To protect consumers from risks related to drug residues, maximum residue limits (MRL) are determined by law. In Europe, Canada, and the United States, the MRL for total sulfonamides in edible tissues and milk is 100 ppb (*3*, *4*), whereas it is 20 ppb in Japan. In Europe, at least nine sulfonamides are allowed to be used in veterinary medicine (sulfamethazine, sulfadiazine, sulfadimethoxine, sulfanilamide, sulfathiazole, sulfadoxine, sulfamethoxazole, sulfatroxazole, sulfachloropyridazine...). Screening methods for sulfonamides include bioassays, immunoassays, thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC, LC-MS/MS). Liquid chromatography is a sensitive and specific assay, but is also very laborious and expensive. The method is more suitable for confirmation than for screening of

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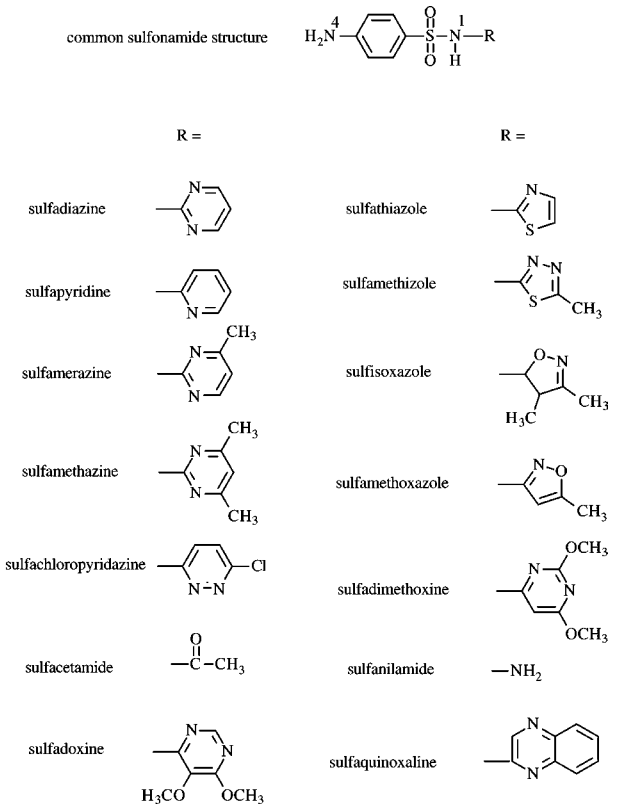

Figure 1. Structures of the sulfonamides.

large amounts of samples. A rapid, sensitive, and specific assay is needed to pick up positive samples in routine analyses, which then can be confirmed for the presence of sulfonamides by liquid chromatography. Therefore, during the past 10 years, a variety of immunoassays were developed, each highly specific for an individual sulfonamide (*5*−*11*). However, it would be more efficient to have one immunoassay able to detect all sulfonamides instead of several immunoassays, each specific for an individual sulfonamide.

The sulfonamides share a common *p*-aminobenzoyl ring moiety with an aromatic amino group at the *N*4-position and differ in the substitution at the *N*1-position (**Figure 1**). For the group-specific detection of sulfonamides, antibodies against the aromatic amino group are needed. Sheth and Sporns (*12*) were the first who reported the development of sulfonamide-specific antibodies. They immunized rabbits with a sulfathiazole derivative (N1-[4-(carboxymethyl)-2-thiazolyl]-sulfonamide, TS) linked at its side chain to limphet haemocyanin (TS-LPH). The polyclonals recognized nine sulfonamides showing 50% inhibition at a concentration of less than 5 μg/mL. Assil et al. (*13*) synthesized another sulfonamide derivative with a larger side chain (N1-[4-methyl-5-[2−4-carboxyethyl-1-hydroxyphenyl)]-azo-2-pyridyl]-sulfanilamide, PS). The polyclonal serum they obtained was purified by affinity chromatography and the purified fraction showed 50% inhibition with seven sulfonamides at concentrations less than 10 μg/mL. The first published study about sulfonamide-specific monoclonal antibodies (mAb) was from Muldoon et al. (*14*). After immunization with a N-sulfanilyl-4-aminobenzoic acid−protein conjugate, only one mAb was obtained that recognized eight sulfonamides at levels less than 10 μg/mL. Haasnoot et al. (*15*, *16*) used the sulfonamide derivatives of Sheth and Sporns (*12*) and Assil et al. (*13*) to induce mAb. The best mAb showed 50% inhibition with 18 tested sulfonamides at values less than 10 μg/mL, and with eight at a concentration of less than 0.1 μg/mL. Unfortunately, the relevant sulfonamides sulfamethazine, sulfatroxazole, and sulfachloropyrazine were not detected at the MRL value (100 ppb). Spinks et al. (*17*) carried out molecular modeling studies on the sulfonamide structure revealing that the molecule has a characteristic bend around the tetrahedral $-SO_2-$ grouping. Recognition of the common structure would be maximal in those drugs where the bend had the greatest angle. They deduced that cross-reactive antibodies could possibly be obtained using a sulfonamide as hapten with a more planar structure (sulfacetamide) or a greater bend (sulfachloropyridazine). Despite this interesting hypothesis, immunization with such conjugates did not lead to antiserum with a broad specificity for sulfonamides. Finally, Li et al. (*18*) reported the detection of sulfonamides in swine meat by immunoaffinity chromatography using cross-reactive polyclonal antibodies (pAb) induced with three different sulfonamide haptens: N1-(4-carboxyphenyl)sulfanilamide (H1), N1-(4-carboxyphenyl)-N4-(4-aminobenzenesulfonyl)sulfanilamide (H2) and N1-(6-carboxyhexyl)sulfanilamide (H3). Sulfonamides were best recognized by the antibodies induced with H2-protein conjugates, slightly lesser with H1-protein and worst with H3-protein conjugates. None of the reported mAbs or pAbs (except those of Li et al. (*18*), in which cross-reactivity values were not mentioned) were able to detect all of the relevant sulfonamides (sulfamethazine, -doxine, -chloropyridazine, -quinoxaline, sulfatroxazole, ...) at the MRL.

In this study, different strategies for the development of sulfonamide-specific mouse antibodies are compared. In a first approach, sulfanilamide was chosen for the construction of hapten-protein conjugates because this molecule is the common structure of the sulfonamides. Antibodies against sulfanilamide should therefore be group-specific. The conjugations were achieved using glutaraldehyde or succinimide ester as cross-linker, or using a diazotation reaction. In a second approach, sulfonamide−protein conjugates were used in which the sulfonamide molecule is linked at its side chain, leaving the common structure of sulfonamides unchanged and thus free for the induction of group-specific antibodies. In previous studies, it was demonstrated that the specificity of antibodies obtained after immunization with PS-protein conjugates was different from the specificity of antibodies induced with TS-protein conjugates (*15*, *16*). Therefore, mice were immunized alternately with PS- and TS-conjugates in order to induce antibodies with a broader specificity.

MATERIALS AND METHODS

Reagents and Chemicals. Sulfamethazine, sulfamerazine, sulfathiazole, sulfadiazine, sulfadimethoxine, sulfamethoxazole, sulfachloropyridazine, sulfapyridine, sulfisoxazole, sulfanilamide, bovine serum albumin (bsa), thyroglobulin, ovalbumin (ova), 1-cyclohexyl-3-(2-morpholino-ethyl)carbodiimidemetho-*p*-toluenesulfonate (MEDC), 1-ethyl-3-(3-(dimethylamino)-propyl)carbodiimide (EDAC), *s*-acetylmercaptosuccinic anhydride (SAMSA), 3-maleimidobenzoic-*N*-hydroxysuccinimide ester (MBS), Ellman's reagent (= 5,5′-dithio-bis(2-nitrobenzoic acid), citraconic anhydride, bicinchoninic acid (BCA), cupper(II)sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), kaolin, OPI supplement media and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (Bornem, Belgium). The TMB substrate solution was prepared by adding 3.3 mg TMB in 250 μL DMSO to 25 mL of phosphate-citrate buffer (0.1M citric acid + 0.2M Na_2HPO_4; pH 4.3) containing 3.25 μL of a 30% H_2O_2 solution. Biotin-LC-PEO-amine and keyhole lympet hemocyanin (klh) were purchased from Pierce, (Perbio, Erembodegem-Aalst, Belgium). Poly(ethylene glycol) 1500 (PEG), ABTS (2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonate)) tablet and ABTS buffer were obtained from Roche Diagnostics (Brussels, Belgium). The ABTS substrate solution was prepared by dissolving 1 ABTS tablet (5 mg) in 50 mL of ABTS buffer. Complete Freund's

adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were provided by Difco Laboratories (Biotrading, Bierbeek, Belgium). Tween 20 (polyoxyethylene sorbitan monolaurate) and Tween 80 (polyoxyethylene sorbitan monooleate) were purchased from Merck-Belgolabo (Overijse, Belgium). Rabbit anti-mouse immunoglobulins (code no Z0259) and rabbit anti-mouse immunoglobulins conjugated to peroxidase (code no P0260) were obtained from DAKO Diagnostica (Prosan, Ghent, Belgium). Dialysis tube VIKING (12 000−14 000 MW cut off) was provided by ROTH (Fiers, Kuurne, Belgium). Dimethylformamide (DMF) was supplied by SERVA (Polylab, Antwerp, Belgium). Dimethyl sulfoxide (DMSO) was from VWR (Leuven, Belgium), ELISA microtiter plates (maxisorp) from NUNC (Life technologies, Merelbeke, Belgium), tissue culture plates from Greiner (Wemmel, Belgium). Dulbecco modified Eagle's medium (DMEM), glutamine, gentamycin, sodium pyruvate, fetal calf serum (FCS) and hypoxanthine, aminopterin, and thymidine supplement (HAT) were purchased from GibcoBRL (Life technologies, Merelbeke, Belgium). All other chemicals were of reagent grade or better. HAT-selection medium consisted of DMEM containing 20% FCS, 1% glutamine, 0.1% gentamycin, 1% sodium pyruvate, 1% OPI supplement media, and 2% HAT.

Commercially Available Sulfonamide-Protein Conjugates. Azocaseïne was purchased from Sigma-Aldrich (Bornem, Belgium), sulfamethazine-bovine gamma globulin antigen conjugate (smt-bgg) was obtained from Chemicon International INC. (Biognost, Wevelgem, Belgium).

Synthesis of Sulfanilamide-Protein Conjugates. *Conjugation by Diazotation*. Sulfanilamide was conjugated to bsa according to Fleeker and Lovett (*5*). Briefly, sulfanilamide (34.5 mg) was diluted in 0.5 N H_2SO_4 by heating. After cooling, 1 mL of $NaNO_2$ (19 mg/mL) was added over 3 min. The solution was stirred for another five minutes, whereafter it was added over 10 min to a cooled bsa solution (100 mg in 4 mL of sodium carbonate 1M pH 10) and subsequently incubated during 4 h at room temperature, sulfa-bsa.

Conjugation with Glutaraldehyde According to Van Regenmortel et al. (19). Briefly, 7.6 mg of sulfanilamide was diluted in 1 mL of PBS and added to 5 mL of ova solution (1 mg/mL of PBS) (ratio sulfanilamide/ovalbumin = 400:1). Next, 4 mL of glutaraldehyde (0.5%) was added in drops to the mixture, whereafter the reaction mixture was stirred for 3 h at room temperature, sulfa-glut-ova (1).

Conjugation with Glutaraldehyde According to Märtlbauer (20). Briefly, 0.4 mmol of sulfanilamide was diluted in 8 mL of dimethylformamide and added to a solution of 0.003 mmol of bsa or ova in 16 mL of phosphate buffered saline (PBS, 0.15 M pH 7.4) (ratio sulfanilamide/ovalbumin = 133/1). Next, 0.15 mL of glutaraldehyde (25%) was added in drops to the reaction mixture and subsequently stirred for 3 h at room temperature, sulfa-glut-bsa (2) and sulfa-glut-ova (2).

All conjugates were dialyzed during 3 days against several changes of PBS before they were stored at −20 °C.

To determine the amount of sulfonamide molecules bound to the carrier protein (molar incorporation), the protein concentrations were first determined with the BCA assay (*21*). Sulfonamides do not react with the BCA components. Next, the amount of bound sulfonamide in the conjugate was determined by measuring the absorbance at 280 nm. Because the carrier-protein and the bound sulfonamide in the conjugate both show absorbance at 280 nm, the absorbance of a sample containing only the carrier protein at a concentration equal to the one in the conjugate was also measured and subtracted from the absorbance of the conjugate to obtain the absorbance of the sulfonamide. A calibration curve for the sulfonamide was established plotting the concentration of a standard dilution of the sulfonamide against the absorbances at 280 nm and was used to extrapolate the concentration of sulfonamide in the product, and consequently to calculate the amount of sulfonamide molecules per carrier molecule.

Conjugation with a Succinimide Ester. Conjugation with a succinimide ester followed the method described by Kitagawa et al. (*22*) and van de Water (*23*):

(a) Introduction of sulfhydryl groups on the carrier-protein (acetylthio-carrier-protein): 4.08 mmol *s*-acetylmercaptosuccinic anhydride (SAMSA) was added slowly to 0.077 mmol carrier-protein dissolved in 15 mL of 0.1M potassium phosphate buffer pH 7.3 while maintaining the pH between 7 and 7.5. Once all SAMSA was added, the pH was lowered to pH 6 by adding 1N HCl. The solution was dialyzed during 1 week against distilled H_2O, whereafter the conjugate was lyophilized.

(b) Removal of the acetyl group of acetylthio-carrier protein: 10 μL of deoxygenated 0.1M hydroxylamine was added to 20 mg of lyophilized acetylthio-carrier-protein in 500 μL of deoxygenated 0.1M phosphate buffer pH 7.3. The solution was then mixed under N_2 until no further increase in number of sulfhydryl groups could be observed. The number of sulfhydryl groups was determined using the Ellman standard method (*24*).

(c) 3-Maleimidobenzoic-*n*-hydroxysuccinimide ester (0.015 mmol MBS) in 0.5 mL of tetrahydrofuran was added to 0.015 mmol sulfanilamide dissolved in 1 mL of 0.05 M sodium phosphate buffer pH 7. The mixture was then incubated during 1 h, while gently stirring. Subsequently, tetrahydrofuran was removed by mixing the solution under N_2 and the excess of MBS by extraction with 3 × 5 mL of methylenechloride/ether (1:2; vol/vol). The aqueous phase contained the MBS-coupled sulfanilamide (sulfa-MBS) and was used in the next step.

(d) The thio-carrier-protein solution was added to the sulfa-MBS solution and incubated for 2 h at 25 °C. The mixture was dialyzed against PBS during 3 days. Aliquots of the conjugates sulfa-MBS-ova and sulfa-MBS-bsa were stored at −20 °C.

The molar incorporation was established by determining the number of free sulfhydryl groups left after coupling (*24*) and subtracting this amount from the number determined in step b. Because one sulfanilamide molecule only binds to one sulfhydryl group, the amount of bound sulfanilamide molecules equals the amount of reacted sulfhydryl groups:

$$\text{number of sulfanilamide molecules} = [(\text{Ev-En})/\text{Em}] \times f \times N_A$$

where Ev = absorbance at 412 nm of the thio-carrier solution after reaction with Ellman's reagent, En = absorbance at 412 nm of the final product after reaction with Ellman's reagent, Em = molar extinction coefficient for the Ellman's reagent at 412 nm (13600), N_A = number of Avogadro, and f = dilution factor.

The amount of carrier-protein molecules was determined by measuring the absorbance at 280 nm of the thio-carrier-protein solution before adding sulfa-MBS. The molar incorporation is expressed as the number of sulfanilamide molecules bound to one carrier molecule in the final product.

Conjugates with Sulfonamide Derivatives. The synthesis of N-sulfanyl-4-aminobenzoic acid (S) was described by Muldoon et al. (*14*). For the coupling of S to bsa and ova, the aromatic amino group of S was protected with citraconic anhydride. Hereto, 20 mg of S was diluted in 1 mL of DMSO, then 1 mL of distilled water was added. The pH of this solution was adjusted to pH 8.5 using 1 M NaOH. Then, 2 mL of citraconic anhydride solution (3.2 mg citraconic anhydride/mL of distilled water) was added in drops and 1-cyclohexyl-3-(2-morpholino-ethyl)carbodiimidemetho-*p*-toluenesulfonate (MEDC, 24.2 mg in 200 μL distilled water) was added to the mixture for 10 min at room-temperature, maintaining the pH at 8.5. Meanwhile, the carrier protein (39 mg bsa or 26 mg ova) was diluted in 2 mL of distilled water, and the pH was adjusted to pH 8.5. The diluted protein was then added to the reaction mixture and incubated for 2 h at room temperature. Citraconic anhydride was removed from the aromatic amino group of S by dialysis of the reaction mixture against 100 mM sodium acetate for 3 h at room temperature. Then, the mixture was dialyzed against PBS. The conjugates S-ova and S-bsa were stored at −20 °C. The coupling efficiency was determined as done for the conjugations with glutaraldehyde.

The synthesis of the sulfathiazole derivative (N1-[4-(carboxymethyl)-2-thiazolyl]sulfanilamide (TS) was described by Sheth and Sporns (*12*). TS was coupled to klh, bsa, and ova according to Haasnoot et al. (*15*). Briefly, TS (60 mg), N-hydroxysuccinimide (NHS, 35 mg), and 1-ethyl-3-(3-(dimethylamino)-propyl)carbodiimide (EDAC, 37.6 mg) were diluted in 1.5 mL of DMF and incubated overnight at 4 °C. The dicyclohexylurea precipitate was removed by centrifugation (12000*g*, 10 min) and 0.5 mL of the supernatant was added to 1 mL of the cooled protein solution (5 mg/mL in PBS), then the pH of the mixture was

Table 1. Methods Used for Sulfonamide–Protein Conjugation

sulfonamide–protein conjugate	coupling method	hapten/protein ratio
sulfa–glut–ova(1)	glutaraldehyde, method van regenmortel	165/1
sulfa–glut–bsa(2), sulfa–glut–ova(2)	glutaraldehyde, method martlbauer	ND[a]
sulfa–bsa	diazotation	ND
azocaseïn, smt–bgg	commercial conjugates	no data available
sulfa–MBS–bsa	succinimide ester	10/1
sulfa–MBS–ova	succinimide ester	ND
S–ova, S–bsa	carbodiimide	3/1–8/1
PS–ova, PS–bsa, PS–klh	carbodiimide	ND
TS–ova,	carbodiimide	37/1
TS–klh	carbodiimide	ND

[a] ND = not determined.

adjusted to 7.6. After stirring overnight at 4 °C, the reaction mixture was dialyzed against 8 M urea, then against 0.5 M ammonium bicarbonate and finally against 0.25 M ammonium bicarbonate (41). The conjugates TS-ova, TS-bsa, and TS-klh were stored at −20 °C. The coupling efficiency was determined as done for the conjugations with glutaraldehyde, except for the klh-conjugates.

N1-[4-methyl-5-[2−4-carboxyethyl-1-hydroxyphenyl)]-azo-2-pyridyl]-sulfanilamide (PS) was synthesized and conjugated to klh, ova, and bsa as described (*13*, *15*).

Biotinylation of TS and PS. TS (1.8 mg) or PS (2.6 mg), N-hydroxysuccinimide (NHS, 1.25 mg), and MEDC (4.8 mg) were diluted in 300 μL of DMSO and incubated overnight at room temperature while shaking. The mixture was added to a biotin−LC−PEO−amine solution (4.6 mg diluted in 400 μL of PBS). After shaking overnight at room temperature, the reaction mixture was brought to 1 mL with PBS, and aliquots of TS-bio and PS-bio were stored at −20 °C. It was not necessary to separate the unreacted biotin from the biotin-labeled sulfonamide, because no background signals were observed when using TS-bio or PS-bio in the antibody coating ciELISA.

Immunization and Production of mAb. Balb/c mice (minimum 10 weeks old) were immunized with an intraperitoneal injection of 50 μg of sulfonamide-protein conjugate (emulsified in 100 μL of sterile PBS and 100 μL of CFA), followed 4 weeks later by a second (and third) intraperitoneal injection with the same amount of conjugate (emulsified in sterile PBS and IFA). Blood samples were collected from the tail vein of the mice 2 weeks after each immunization and treated with kaolin, as previously described (*25*), before analysis in ELISA for the presence of sulfonamide-specific antibodies. For the final booster injection, an intravenous immunization is preferred above an intraperitoneal one (*26*). However, to our experience, intravenous injection can sometimes fail, probably because it is a delicate manipulation and some of the solution is injected intradermally or subcutanously. To overcome this risk, the final boost consisted of an intravenous (100 μg of conjugate in sterile PBS) and an intraperitoneal injection (100 μg of conjugate emulsified in sterile PBS and IFA), and fusion was performed 4 days later instead of three (for intravenous injection) or five (for intraperitoneal injections). In our hands, this procedure works excellently.

Hybridomas were obtained by poly(ethylene glycol)-mediated fusion of $SP_2OAG/14$ mouse myeloma cells with splenocytes from immunized mice. The isolation of lymphocytes, the culturing of the myeloma cells, the poly(ethylene glycol)-mediated fusion and the cloning of the hybridomas were performed according to the procedures previously described (*26*, *27*). The supernatants of the hybridomas were tested twice in the screening ELISA coated with TS-ova. Hybridomas exhibiting absorbances higher than 2.0 were expanded to 24-well culture plates and tested for their specificity in the antigen coating ciELISA-coated with PS-ova. The hybridomas with the highest specificity and sensitivity were cloned twice.

Screening ELISA. Microtiter plates were coated overnight at 4 °C with sulfonamide-protein conjugate (100 μL/well) diluted in bicarbonate coating buffer (0.05 M; pH 9.4). The plates were washed three times with PBS containing 0.05% Tween 20 between each incubation step. Free binding sites were blocked with 200 μL of 5% glycine in coating buffer for 2 h at 37 °C. Subsequently, 100 μL/well of an appropriate dilution of sera or hybridoma supernatant in dilution buffer (PBS containing 3% bsa and 0.05% Tween20), was added. The plates were incubated for 1 h at 37 °C. Then, 100 μL/well of rabbit anti-mouse immunoglobulins conjugated to peroxidase in dilution buffer was added for 1 h at 37 °C. Subsequently, 50 μL/well enzyme substrate ABTS solution was added. After incubation at 37 °C, the absorbance was measured at 405 nm.

Antigen-Coating Competitive Inhibition (ci) ELISA. The only difference between the antigen-coating ciELISA and the screening ELISA was that in the former, the samples (sera and hybridoma supernatant) were incubated in the wells of the coated plate with a sulfonamide mixture (10 μg/mL) containing sulfamethazine, sulfadiazine, sulfadimethoxine, and sulfathiazole, or with serial dilutions (concentrations ranging from 10 to 0.001 μg/mL) of a sulfonamide.

The competition in the ELISA between a free sulfonamide in the sample and the coated sulfonamide was calculated with the formula: competition (%) = $[1 - (A/A_0)] \times 100$, with A = absorbance of a tested sample solution and A_0 = the absorbance of a similar solution without sulfonamide.

Antibody-Coating Competitive Inhibition (ci) ELISA. Microtiter plates were coated overnight at 4 °C with rabbit anti-mouse immunoglobulins (0.1 μg/100 μL/well) diluted in bicarbonate coating buffer. Between each step, the plates were washed 3 times with PBS containing 0.05% Tween 20. Free binding sites were blocked with 5% Tween 80 in coating buffer for 2 h at 37 °C. Then, 100 μL/well of an appropriate dilution of the monoclonal antibody in PBS (or only PBS for background measurements) was added for 1 h at 37 °C. Subsequently, sulfonamides in dilution buffer were added and incubated for 1 h at 37 °C. Without washing the plates, a biotinylated sulfonamide in dilution buffer was added for 30 min at 37 °C. After washing, 100 μL/well of streptavidine-peroxidase in dilution buffer was added for 30 min at 37 °C. Finally, the plates were washed again, and TMB solution (100 μL/well) was added. The absorbance was measured at 650 nm.

RESULTS

Sulfonamide Immunogens and Antibody Response. Different sulfonamide-protein conjugates were used for immunization (**Table 1**). Smt-bgg and azocasein were commercially available. Sulfanilamide was coupled to albumins using glutaraldehyde or a succinimide ester as cross-linker and using a diazotation reaction. Three sulfonamide derivatives containing a carboxyl group in their side chain (PS, TS, and S; **Figure 2**) were synthesized and were linked to proteins using a carbodiimide coupling method. In those conjugates, the common structure of sulfonamides was left unchanged, so that they could induce group-specific antibodies.

Blood samples were collected two weeks after each immunization and were tested in ELISA for the presence of anti-sulfonamide antibodies (**Tables 2** and **3**).

No sulfanilamide-specific antibodies were induced after immunization with the glutaraldehyde (sulfa−glut−albumin (1) and (2)) or succinimide (sulfa−MBS−albumin) conjugates. Sera

Table 2. Immunogenicity of Different Sulfonamide–Protein Conjugates

			antigen coating ciELISA				
immunogen	number of mice	number of injections	dilution serum	coating antigen	absorbance[a] average (SD)	competitor 10 μg/mL	competition[b] average (SD) %
sulfa-glut-bsa(1)	2	4	100	sulfa–MBS–ova	–		
sulfa-glut-ova(2)	2	4	100	sulfa–MBS–bsa	–		
sulfa–mbs–bsa	2	4	100	Sulfa-glut-ova(1)	–		
sulfa–bsa	2	9	1000	azocaseïn	+++ (+)	Smix[c]	<20%
azocaseïn	4	7	1000	sulfa–bsa	+++ (++)	Smix	<20%
smt–bgg	3	7	1000	S–albumin	+ (+)	sulfanilamide	<20%
			1000	TS–ova[d]	++++	Smix	80%
S–albumin	12	4	1000	S–albumin[e]	++++ (+)	Smix	<20%
TS–klh	6	3 (2)	10000	TS–ova	+++ (++)	Smix	79 (10)
			10000	PS–ova	+ (+)	Smix	44 (36)
TS– & PS–klh	4	4	10000	TS–ova	++ (++)	Smix	65 (12)
			10000	PS–ova	++ (++)	Smix	60 (32)

[a] Scores are given for the average of the absorbance obtained in the antigen coating ciELISA for the sera of a number of mice in absence of competitor: – = <0.200; + = 0.200–0.500; ++ = 0.500–1.000; +++ = 1.000–1.800; ++++ = >1.800. [b] Competition (%) = (100 – (absorbance in the presence of competitor/ absorbance in absence of competitor)) × 100. [c] Smix = mixture of four sulfonamides (sulfamethazine, sulfadiazine, sulfadimethoxine, and sulfathiazole) at a final concentration of 10 μg/mL. [d] Sera of the best responding mouse. [e] The albumin of the coating antigen was different from the one in the immunogen.

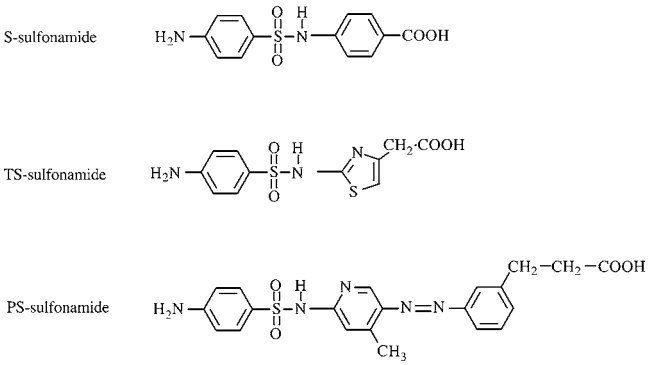

Figure 2. Structures of the sulfonamide derivatives. S-sulfonamide = N-sulfanyl-4-aminobenzoic acid; PS-sulfonamide = N1-[4-methyl-5-[2-4-carboxyethyl-1-hydroxyphenyl)]-azo-2-pyridyl]-sulfanilamide; TS-sulfonamide = (N1-[4-(carboxymethyl)-2-thiazolyl]sulfanilamide.

of mice immunized with sulfa−bsa showed high absorbances in the ELISA coated with azocasein (**Tables 2** and **3**). However, the binding of those antibodies to azocasein could only be slightly inhibited by adding sulfonamides (**Table 2**). This was independent of the number of immunizations. Similarly, high absorbances were also detected for blood samples collected after immunizations with azocasein and analyzed in the ELISA coated with sulfa−bsa, and again only a slight inhibition was observed in the presence of sulfonamides (**Table 2**). So again, competition average remained low, independent of the number of immunizations.

Three mice were immunized with smt-bgg. The sera of the best responding mouse reacted with azocasein, sulfa−bsa, S−ova, and TS−ova (**Table 3**), and showed 80% inhibition in the presence of 10 μg/mL of a mix of sulfonamides in the ciELISA coated with TS-ova (**Table 2**). This means that the antibodies recognized a common structure. Unfortunately, this mouse died. The antibody titers of the other mice did not become high enough, even after seven immunizations.

Immunizations with the S-conjugates led to high antibody responses, highly specific for the S-conjugates (**Table 3**). However, the free S-molecule was only weakly recognized (data not shown) as were the four sulfonamides in the competitor mixture (at a concentration of 10 ppm, less than 20% inhibition was observed, **Table 2**). Fusion experiments with the splenocytes of mice immunized with these conjugates did not result in any group-specific mAb.

Table 3. Reactivity[a] of Sera on Different Coating Antigens in a Screening ELISA[b]

immunogen	azocasein	sulfa-bsa	S-ova	TS-ova	ova	bsa
Smt–bgg	++	+++	++	++++	–	–
azocaseïn	++++	++++	–	–	–	–
sulfa–bsa	+++	++++	–	–	–	–
S–bsa	+	+	++++	–	–	+
TS– & PS–klh	–	–	–	++++	–	–
TS–klh	–	–	–	++++	–	–

[a] Scores are given for the absorbance obtained in the screening ELISA for 1/1000 diluted sera: – = <0.200; + = 0.200–0.500; ++ = 0.500–1.000; +++ = 1.000–1.800; ++++ = >1.800. [b] The results of the sera obtained from the best responding mouse of each group immunized with different immunogens are presented.

High antibody responses were also obtained after immunizations with the PS- and TS-klh conjugate. Sera tested in the antigen coating ciELISA had titers from 100 000 to 300 000 on TS-ova coating (**Table 3**). Furthermore, those antibodies could be inhibited in the ELISA with different sulfonamides (**Table 4**). Lower titers (20 000−50 000) were obtained on PS-ova coating (**Table 2**). No response was measured in the ELISA coated with azocasein, sulfa−bsa, or S−ova (**Table 3**).

Production of mAb. Three mice (m90, m98, and m95, **Table 4**) differently immunized with TS- and PS-conjugates were selected for fusion experiments. In **Table 4**, the cross-reactivities are given for different sulfonamides detected in the antigen coating ciELISA with sera of the three mice. The three sera had the highest affinity for sulfachloropyridazine and sulfathiazole, a lower affinity for sulfadiazine, sulfadimethoxine, and sulfapyridine, and the lowest affinity for sulfamethazine, sulfamerazine, and sulfisoxazole. The sensitivity of the three sera for sulfonamides was higher in the ELISA coated with PS-ova as compared to TS-ova.

Antigen-Coating ciELISA. The cross-reactivities of the mAb were determined in the antigen-coating ciELISA coated with PS-ova (**Table 5**). The antibodies could be divided into three groups. The mAb of group I had a very high affinity for sulfathiazole, sulfamethoxazole, and sulfachloropyridazine, but also recognized sulfadiazine, sulfadimethoxine, and sulfapyridine at an acceptable level. The antibodies were at least 10 times less reactive to sulfamethazine and sulfamerazine. The antibodies of group II had the same reactivities as group I, except that the sensitivity for sulfamethazine and sulfamerazine was at least

Table 4. Cross-Reactivities[a] (IC50, ng/mL) for Different Sulfonamides of Three Mice Sera (m90, m95, and m98) in the Antigen-Coating CiELISA (Coated with PS–ova)

mouse	m90		m95		m98	
immunogen	TS–klh (2×), PS–bsa (2×)[b]		TS–klh (2×), PS–klh (1×)[b]		TS–klh (3×)	
coating	PS–ova	TS–ova	PS–ova	TS–ova	PS–ova	TS–ova
dilution serum	1/5000	1/20000	1/8000	1/20000	1/8000	1/20000
sulfonamide (sulfa-)	IC50 (ng/mL)					
-chloropyridazine	7	100	30	400	30	600
-thiazol	7	85	30	150	3	100
-diazine	70	600	500	800	300	7000
-dimethoxine	400	3000	500	3000	600	1500
-pyridine	150	900	800	4000	1000	4000
-methazine	1000	>10000	10000	>10000	10000	>10000
-merazine	600	>10000	4000	>10000	10000	>10000
sulfisoxazole	3000	3000	10000	4000	>10000	>10000

[a] The cross-reactivities = concentration of a sulfonamide (ng/mL) required to obtain 50% inhibition (IC50) in the antigen-coating ciELISA. [b] Alternated injected with TS-klh and PS-bsa or PS-klh

Table 5. Cross-Reactivities[a] (IC50, ng/mL) for Different Sulfonamides of Different Monoclonal Antibodies in the Antigen-Coating CiELISA (Coated with PS–ova)

group	I					II		III	IV
mouse	m95	m95	m98	m98	m98	m98	m90	m95	Haasnoot[b]
monoclonal antibody	6H12H3	18E1D9	17D11E6	11F5D5B12	1C12B11G5	3A10E3	3B5B10E3	14D6D6 14D6C9	27G3A9B10
sulfonamide (sulfa-)									
-thiazole	20	7	5	10	15	<20	30	350	10
-methoxazole	20	7	9	10	15	<20	9	1000	150
-chloropyridazine	30	10	15	40	30	<20	<20	600	4
-diazine	1000	300	250	500	1000	60	30	>10 000	80
-dimethoxine	1000	150	200	500	1000	200	100	>10 000	250
-pyridine	1000	300	300	1000	2000	400	350	3000	30
-methazine	>10 000	4000	8000	>10 000	>10 000	1050	1050	4000	8000
-merazine	>10 000	4000	8000	>10 000	>10 000	700	600	4000	500
sulfisoxazole	>10 000	10 000	6000	>10 000	>10 000	400	350	>10 000	250

[a] The cross-reactivities = concentration of a sulfonamide (ng/mL) required to obtain 50% inhibition (IC50) in the antigen-coating ciELISA. [b] Haasnoot et al. (*15*)

four times higher. The mAb also recognized sulfadiazine at least four times better than group I. Group III showed better recognition of sulfamethazine and sulfamerazine as compared to sulfadiazine and sulfadimethoxine. However, the recognition of both latter was at least four times lower as for group II. The antibodies seemed to recognize sulfamethazine and sulfamerazine in the same way. However, Mab 27G3 (group IV) (*15*) obtained after TS-klh immunization, had higher affinity for sulfamerazine as compared to sulfamethazine.

Antibody-Coating ciELISA. Monoclonal antibody 3B5B10E3 of group II was selected to develop an antibody-coating ciELISA. Therefore, the mAb was captured on an ELISA plate precoated with mouse-specific polyclonal antibodies. Furthermore, competition of sample sulfonamide for binding to the mAb was done using TS-biotin, because with PS-biotin, no or very little absorbances were obtained. The cross-reactivity values (IC50) and limit of detection (LOD) (*28*) were determined for different sulfonamides in buffer solution analyzed in the antigen-coating ciELISA and in the antibody-coating ciELISA (**Table 6**). Most of the sulfonamides were recognized with the same sensitivity (LOD) in both ELISA's.

Table 6. Cross-Reactivity[a] (IC50, ng/mL) and Limit of Detection[b] (LOD, ng/mL) for Different Sulfonamides in Buffer Solution Detected with mAb 3B5B10E3 in the Antigen-Coating CiELISA (Coated with PS-ova) and in the Antibody-Coating CiELISA

	antigen-coating ciELISA		antibody-coating ciELISA	
	LOD (ng/mL)	IC50 (ng/mL)	LOD (ng/mL)	IC50| (ng/mL)
sulfathiazole	5	30	3	50
sulfamethoxazole	<1	9	<1	10
sulfachloropyridazine	<1	<20	ND	ND
sulfadiazine	6	30	3	50
sulfadimethoxine	50	100	30	500
sulfapyridine	80	350	70	1050
sulfamethazine	300	1050	400	4000
sulfamerazine	200	600	ND	ND
sulfisoxazole	80	350	200	2000

[a] The cross-reactivities = concentration of a sulfonamide (ng/mL) required to obtain 50% inhibition (IC50) in the antigen-coating ciELISA. [b] Limit of detection = concentration read from the calibration curve at a response (absorbance) minus 3 standard deviation from the mean ($n = 12$) for the zero standard (buffer sample without sulfonamides).

DISCUSSION

The aim of this study was the production of mAb specific for all sulfonamides. With such antibodies, a sensitive, group-specific immunoassay could be developed for the detection of sulfonamides in food products.

Our first approach was the use of sulfanilamide as hapten because it is the common structure of all sulfonamides and it does not have a "disturbing" side group. Antibodies against sulfanilamide should be group-specific. Different coupling

methods were applied to link sulfanilamide to proteins using glutaraldehyde (sulfa−glut−albumin (1) and (2)) or a succinimide (sulfa−MBS−albumin) as cross-linker or by diazotation (sulfa−bsa and azocasein). No immune response was obtained after immunization with the sulfa−glut−albumin or sulfa−MBS−albumin. For the glutaraldehyde coupling with the procedure of Van Regenmortel (*19*), a sulfa−glut−ova (1) conjugate was developed with a molar incorporation of 165/1 (**Table 1**). The reaction of glutaradehyde with proteins involves mainly lysine residues, as well as the α-amino group and sulfhydryl group of cysteine residues of the protein. Ovalbumin has 20 lysine and 4 cysteine residues accessible for glutaraldehyde conjugation, and therefore the highest molar incorporation possible is 24/1, if a one-to-one ratio is respected. However, glutaraldehyde can form polymers (*19*, *29*). This property in combination with the high ratio of sulfanilamide/ovalbumin (400:1) mixture used for coupling could explain the high coupling ratio of 165:1. The absence of immune response using this sulfa−glut−ova (1) conjugate could be due to the high load of hapten on the carrier protein. Ideally, a hapten/protein ratio of 5:20 is needed to obtain a good immunogen (*19*). Therefore, the glutaraldehyde procedure according to Märtlbauer (*20*) was applied (sulfa−glut−albumin (2)). Märtlbauer (*20*) reported sulfonamide conjugates using this method with a molar incorporation of 7:1 and high antibody titers after immunization. However, our conjugates were still not immunogenic. Unfortunately, the hapten/protein ratio was not determined. Haasnoot et al. (*16*) also did not determine the molar incorporation ratio and obtained only low titers after immunization with several sulfonamide−protein conjugates linked by glutaraldehyde. In previous work (*26*), we immunized mice with ampicillin coupled to albumins using the glutaraldehyde procedure according to Märtlbauer (coupling efficiency of 8−16) and also found the conjugates to be weak immunogens.

The molar incorporation of our sulfa−MBS−albumin conjugates (10/1, **Table 1**) was in accordance with other studies (*22*, *23*, *26*). In previous work, we constructed ampicillin−MBS−albumin conjugates with a hapten/protein ratio of 8:1 to 13:1 but obtained only a moderate immune response (*26*).

High antibody titers were obtained after immunization with the diazotation conjugates sulfa−bsa and azocasein. However, those antibodies could only be slightly inhibited in ELISA by free sulfonamides or even sulfanilamide. This means that the antibodies were highly specific for the bound sulfanilamide molecule. The diazotation reaction was used by others for the development of antibodies specific for sulfamethazine (*5*), sulfamerazine (*8*), and sulfathiazole (*30*), leading in all cases to antibodies able to recognize the respectively free sulfonamide molecule. Sulfanilamide does not have a side chain like sulfamethazine, sulfamerazine, and sulfathiazole, and this could be the reason only low amounts of sulfanilamide specific antibodies were obtained. The available epitope in the sulfanilamide conjugates is probably built up by the linkage at the aromatic amino group (N4) and the benzene ring (**Figure 1**).

Our second approach was the use of sulfonamide−protein conjugates in which the sulfonamide is linked at its side chain, leaving the common group unchanged. In the commercially available smt-bgg conjugate, sulfamethazine was linked through its pyrimidin ring to bovine gamma globulin (bgg). Polyclonal antibodies obtained after immunization with this antigen are also commercially available. Those antibodies recognized several sulfonamides, and therefore the antigen was believed to induce group-specific antibodies. One of the three mice immunized with this smt-bgg in particular indeed produced sulfonamide-specific antibodies. Unfortunately, no fusion experiments could be performed with the splenocytes of this mouse.

Immunizations with the N-sulfanilyl-4-aminobenzoic acid−protein conjugates (S-conjugates) led to a large production of antibodies (high absorbances), but all of them were highly specific for the conjugates. Fusion experiments with the splenocytes of mice immunized with the S-albumin conjugates did not result in any group-specific antibody. Muldoon et al. (*14*) used the same sulfonamide derivative coupled to klh. Only one of five immunized mice produced high titers of antibodies able to recognize other sulfonamides. Fusion experiment resulted in only one monoclonal antibody specific for the group of sulfonamides.

Using the sulfonamide derivatives of Sheth and Sporns (*12*) and Assil et al. (*13*) was more successful. Haasnoot et al. (*15*, *16*) also used the same approach and found out that immunization of mice with PS-klh led to the recognition of sulfonamides (sulfamethazine, sulfamerazine, sulfadiazine, and sulfadimethoxine) that were not well recognized after TS-klh immunization. Consequently, we tried to guide the immune response in the direction of recognition of the common sulfonamide structure by immunizing the animals alternately with TS− and PS− protein conjugates. All mice immunized with TS−klh showed high titers in the ELISA with TS−ova coating. As expected from the results of Haasnoot et al. (*15*, *16*), immunization with PS−bsa or PS−klh did not induce a high increase of the titer. However, the mouse only immunized with TS−klh had a lower antibody binding to sulfonamides containing a pyrimidinyl group (sulfamethazine, sulfamerazine, or sulfadiazine) or a pyridinyl group (sulfapyridine) than the alternately immunized mice (**Table 4**). The detection of the sulfonamides was improved when the ELISA was coated with PS−ova as compared to TS−ova.

In contrast to the group-specific serum antibodies of the mouse immunized with smt-bgg, serum of the mouse immunized with TS−klh did not bind to coated azocasein, sulfa−bsa or S−ova (**Table 3**). Probably, the major epitope recognized by the TS−klh antibodies is more positioned toward the side chain of the sulfonamides, and therefore binding of these antibodies to the conjugated sulfanilamide or S-molecule could be sterically hindered by the carrier protein. The major epitope recognized by the smt-bgg antibodies is probably more located at the side of the common sulfonamide structure, and antibody binding is therefore less influenced by the side chain.

The mAb obtained after fusion experiments with the splenocytes of mice immunized with TS-klh could be divided, based on their cross-reactivities, in three groups, independently from which mouse they were deduced. This means that, alternately, immunization with two different immunogens will improve the broad specificity of the polyclonal serum but not of the individual mAb. Consequently, to obtain broad specific mAb, it would be sufficient to immunize with TS-klh and to screen the hybridomas in ELISA coated with PS-ova.

Two ELISA systems, an antigen-coated and an antibody-coated ciELISA, were constructed with mAb 3B5B10E3. Almost all tested sulfonamides were detected with the same sensitivity in both ELISA. Because the sensitivity of the antigen-coated ciELISA was improved when PS-ova was used as coating antigen compared to TS−ova, the sensitivity of the antibody-coated ciELISA would probably be improved using the biotinylated PS−sulfonamide PS−bio instead of TS−bio. However, very little absorbances were obtained with PS−bio.

Most sulfonamides, except sulfamethazine and sulfamerazine, can be detected in buffer solution at the MRL (100 ppb) in the

antigen-coating ciELISA with mAb 3B5B10E3. On the other hand, many sulfamethazine-specific immunoassays already exist. Most of the time, such assays cross-react with sulfamerazine. Due to the diversity of the sulfonamide side chain, a broad, specific immunoassay for sulfonamides using one monoclonal antibody is probably not possible. Most likely, two or three immunoassays, each with a different monoclonal, have to be used for the screening of food products (*9*, *15*, *31*, *32*). Haasnoot et al. (*31*) could detect sixteen sulfonamides in the BIAcore 2000 biosensor using a mixture of three group-specific monoclonal antibodies. However, a group-specific sulfonamide ELISA based on the use of several monoclonals has not been described yet. In further work, the detection of sulfonamides in meat samples using the antigen-coating ciELISA with Mab 3B5B10E3 will be investigated.

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