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Improving Broad Specificity Hapten Recognition with Protein Engineering

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Sulfa antibiotics (sulfonamides) are derivatives of *p*-aminobenzenesulfonamide that are widely used in veterinary medicine. Foods derived from treated animals may be contaminated with these drugs. However, current immunobased sulfonamide detection methods are unfit for screening of products because they are either too insensitive or specific for a few compounds only. An immunoassay capable of detecting all sulfas in a single reaction would be ideal for screening. For development of a binder capable of binding all sulfas, a protein engineering approach was chosen and the properties of monoclonal antibody 27G3 were improved with mutagenesis followed by selection with phage display. Several different mutant antibodies were isolated. The cross-reaction profile of the best mutant antibody was significantly improved over that of the wild-type antibody: it was capable of binding 9 of the tested 13 sulfonamides within a narrow concentration range and also bound the rest of the sulfas, albeit within a wider concentration range.

KEYWORDS: Drug residues; group specificity; phage display; protein engineering; sulfonamides; time-resolved fluoroimmunoassay

INTRODUCTION

Sulfa antibiotics (sulfonamides) are a group of antimicrobial agents that are used in veterinary and human medicine for the treatment and prevention of bacterial infections and also as animal feed additives, because of their growth-promoting properties. Antimicrobial sulfonamides (**Figure 1**) are N^1-substituted derivatives of *p*-aminobenzenesulfonamide (sulfanilamide), and they are thus structurally related. As a result of their use, foodstuffs (for example, meat and milk) derived from treated animals may be contaminated with sulfonamide drugs. These residues may cause adverse effects in some humans, as indicated by the fact that ∼5% of the patients receiving sulfonamide therapy experience some kind of unwanted effect (*1*). The maximum residue limit (MRL) for sulfonamides has been set to 100 μg/kg in the United States and the European Union, but, for example, in Japan it is as low as 20 μg/kg (*2*, *3*). The current sulfonamide detection technologies are based on bacteriological growth inhibition (*4*, *5*) or chromatographic methods (*6*−*10*). In addition, single sulfonamide drugs have been measured with immunochemical methods (*11*, *12*). These methods are either laborious or slow for mass screening or, in the case of immunoassays, capable of detecting only a single analyte per each assay reaction.

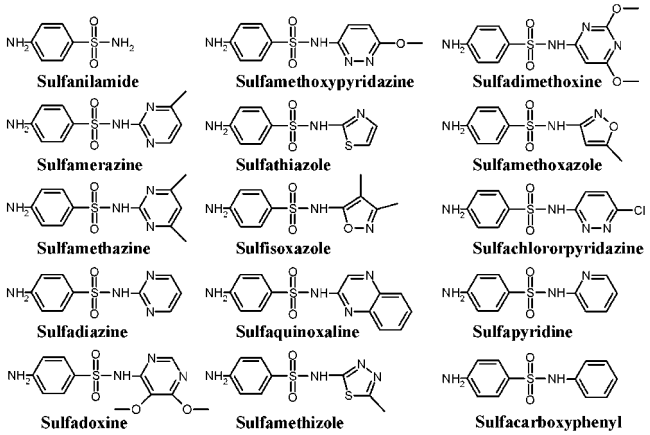

Figure 1. Structures of selected sulfonamides.

A rapid immunoassay capable of detecting nearly all sulfonamides in one reaction would be very useful for the mass screening of foodstuffs. The development of this kind of immunoassay has, however, proven to be difficult; despite several efforts (*1*−*3*, *13*, *14*), a completely generic assay is still not available. The main obstacle is the development of an antibody capable of binding all different sulfonamides with an affinity yielding sufficient assay sensitivity. It seems that the obtained antibodies recognize the used immunogen and the

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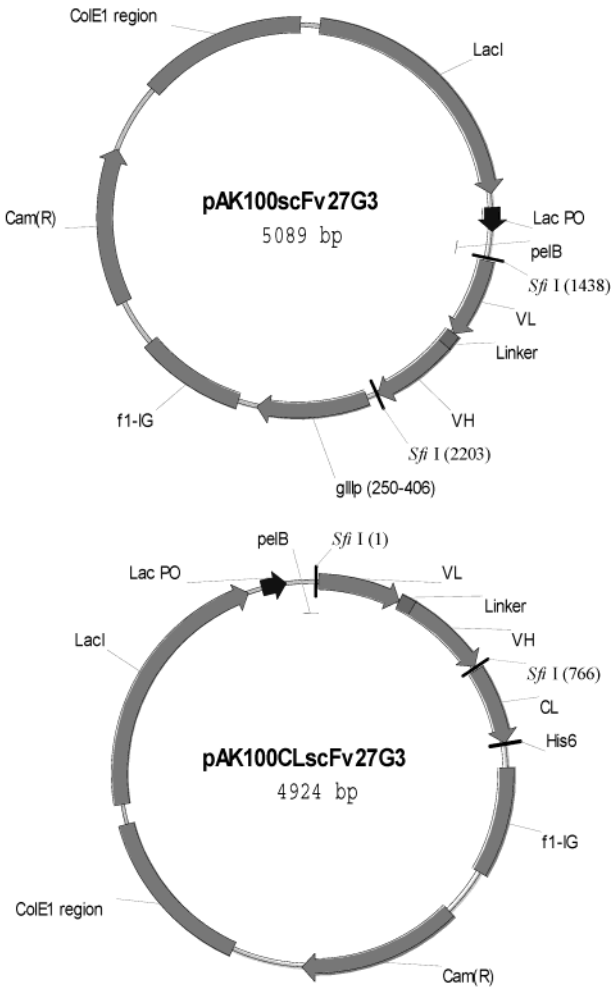

Figure 2. Plasmid vectors used. Two different plasmids of the pAK series (*16*) were used as the gene-carrying vectors in this work. Marked in the maps are the *E. coli* propagation signal *ColE1*, the M13 phage packing signal *f1-IG*, and chloramphenicol resistance selection marker *Cam(R)*. The scFv (V_L, V_H, and linker domains are marked) expression from both plasmids is controlled by the *lac* promoter (*lac PO*) repressed by the *lac* inhibitor (*lacI*). The signal sequence (*pel B*) activates transport of scFv to periplasm for folding.

structurally alike sulfonamides with greater affinity than the sulfonamides, which are structurally more divergent from the immunogen.

The aim of our study was to use protein engineering to modify previously described (*2*) monoclonal antibody (Mab) 27G3A9B10 so that it would recognize a wider range of structurally different sulfonamides with similar affinities. We chose 27G3 because the cross-reactivity profile of this antibody was one of the best of the available antibodies. Antibody 27G3 was cloned as a single-chain Fv fragment (scFv), and random mutant libraries were created with error-prone PCR over the whole scFv coding region. The libraries were screened with phage display (for a recent review, see ref *15*) using alternating panning ligands for the enrichment of mutants with improved genericity. The binding properties of the mutants were evaluated in a competitive time-resolved fluoroimmunoassay.

MATERIALS AND METHODS

Strains, Plasmids, Reagents, and Instruments. The bacterial host used throughout the work was *Escherichia coli* K12 strain XL1-Blue (Stratagene, La Jolla, CA). Most of the vectors used (**Figure 2**) either belonged to or were derived from the pAK series of vectors (*16*), and they were obtained as gifts from the laboratory of Andreas Plückthun (Biochemisches Institut, Universität Zürich, Zurich, Switzerland). The pGEM-T -vector was purchased from Promega (Madison, WI). The helper phage used in the phage production was VCS-M13 (*KanR*, Stratagene). Monoclonal cell-line 27G3A9B10 (*2*) was a gift from the laboratory of Willem Haasnoot (DLO−State Institute for Quality Control of Agricultural Products, Wageningen, The Netherlands).

All reagents used in the organic synthesis were commercially available and were of reagent grade or better. The DELFIA-Eu-N1-ITC labeling reagent (catalog no. 1244-301), rabbit anti-mouse IgG-coated microtiter plates, DELFIA assay buffer, DELFIA wash solution, and DELFIA enhancement solution were obtained from Perkin-Elmer Life Sciences (Turku, Finland). The biotinylation reagents Biotin-XX-hydrazine (catalog no. 203110) and biotin-XX-NHS (catalog no. 203114) were purchased from Calbiochem (San Diego, CA). The LB agar plates, SB medium, and SOC medium were prepared as described previously (*17*). The antibiotics used in the cultures were obtained from Sigma-Aldrich (Helsinki, Finland). The concentrations of the antibiotics were as follows: ampicillin, 100 mg/L; chloramphenicol, 25 mg/L; tetracycline, 5 mg/L; and kanamycin, 50 mg/L. Isopropyl β-D-thiogalactopyranoside (IPTG) was purchased from Promega. The different sulfa antibiotics (**Figure 1**) were purchased from Sigma-Aldrich.

All sequencing was done using an ABI PRISM 377 dye terminator cycle sequencer (Applied Biosystems, Foster City, CA). ^{1}H NMR spectra were recorded at 400 MHz on a JNM-GX-400 spectrometer (JEOL, Peabody, MA) or at 200 MHz on a AM200 spectrometer (Bruker, Täby, Sweden). LC/ESI-MS analyses were performed on a Sciex API 365 LC/MS/MS triple-quadruple mass spectrometer (Perkin-Elmer, Boston, MA). Time-resolved fluorescence was measured with a Victor 1420 multilabel counter (Perkin-Elmer Life Sciences).

***N*^{1}-[4-(Carboxymethyl)-2-thiazolyl]sulfanilamide** was synthesized as previously described (*1*): ^{1}H NMR (400 MHz, DMSO) δ 3.50 (s, 2H, CH_2), 5.83 (br s, 2H, NH_2), 6.51(s, 1H), 6.54−6.59 (s, 4H, Ar H), 12.49 (br s, 1H, COOH).

Synthesis of *N*^{1}-{[4-(5-Amino)pentyl]-2-pyrimidyl}sulfanilamide Monohydrochloride. *(6-N-tert-Butoxycarbonylamino)caproic Acid (**A**).* 6-Aminocaproic acid (4.8 g) was dissolved in water (30 mL) and in dioxane (30 mL). NaOH (2 M, 37 mL) was added followed by di-*tert*-butyldicarbonate (12 g) in small aliquots. After ~5 h of stirring, the reaction mixture was evaporated, and dichloromethane/H_2O (100 mL/100 mL) was added. The pH of the water phase was adjusted to 3−4 with 6 M HCl and extracted with dichloromethane (3 × 150 mL). The combined organic phases were dried (Na_2SO_4) and evaporated, and the product was purified with flash chromatography (FC) using silica gel and 10% MeOH/dichloromethane.

(6-N-tert-Butoxycarbonylamino)caproic Acid α*-N-Methoxy-N-methylamide (**B**).* A solution of **A** (3.9 g) in tetrahydrofuran (THF) was cooled to −15 °C, and 4-methylmorpholine (1.85 mL) was added followed by isobutyl chloroformate (2.19 mL). A cooled (−15 °C) mixture of *N*,*O*-dimethylhydroxylamine hydrochloride (1.6 g) and triethylamine (2.58 mL) in dimethylformamide (DMF, 60 mL) was then added to the reaction mixture, which was stirred for ~30 min in an ice−salt bath and then at room temperature overnight. The reaction mixture was evaporated, dissolved in ethyl acetate (200 mL), washed with aqueous 1 M H_3PO_4 (2 × 100 mL) and aqueous $NaHCO_3$ (3 × 100 mL), and dried (Na_2SO_4), after which it was evaporated to dryness. The product was purified with FC (silica gel, 50% ethyl acetate/petroleum ether).

*(8-N-tert-Butyloxycarbonylamino)oct-1-yn-3-one (**C**).* Ethynylmagnesium bromide (43.4 mL, commercially available as 0.5 M in THF) was added dropwise to a cooled (−78 °C) solution of **B** (1.19 g) in dry ether (100 mL). The reaction was stirred for 2 h before being warmed to room temperature and stirred overnight. The reaction mixture was poured onto a vigorously stirred mixture of Et_2O (150 mL), 1 M KH_2PO_4 (300 mL), and ice. The phases were separated, and the water phase was extracted with Et_2O (3 × 100 mL). The combined organic phases were washed with aqueous 1 M KH_2PO_4 (2 × 100 mL), saturated aqueous sodium bicarbonate solution (2 × 100 mL), and brine (2 ×

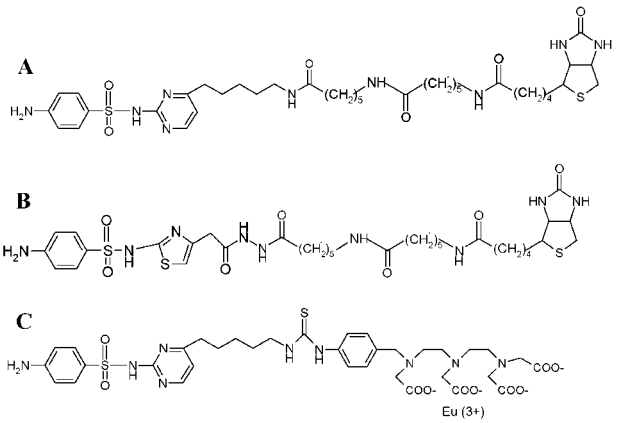

Figure 3. Sulfonamide derivatives synthesized for use in phage display and immunoassays: (A) biotinylated sulfamerazine derivative; (B) biotinylated sulfathiazole derivative; (C) N1-europium chelate labeled sulfamerazine derivative.

100 mL), dried (Na_2SO_4), and evaporated. The product was purified with FC (silica gel, 30% ethyl acetate/petroleum ether).

*{2-Amino-[4-(5-N-tert-butyloxycarbonylamino)pentyl]}pyrimidine (**D**).* A mixture of **C** (0.53 g) and guanidium nitrate (0.325 g) in ethanol was heated in an oil bath until the guanidium nitrate was dissolved. A solution of NaOH in water (0.213 g/0.70 mL) was added. The reaction mixture was refluxed for 3 h and allowed to cool before evaporation. The product was purified with FC (silica gel, 3% MeOH/dichloromethane).

*N^4-Acetyl-{N^1-[4-(5-N-tert-butoxycarbonylamino)pentyl]-2-pyrimidyl}-sulfanilamide (**E**).* *N*-Acetylsulfanilyl chloride (0.220 g) was dissolved in dry pyridine and cooled in an ice bath. A cooled solution of **D** (0.240 g) in dry pyridine was added. The reaction mixture was stirred overnight at room temperature, evaporated, and dissolved in dichloromethane (20 mL). The solution was washed once with brine (10 mL), dried (Na_2SO_4), and evaporated. The product was purified with FC (silica gel, first 3/5/1 = ethyl acetate/petroleum ether/triethylamine and finally the product 10% MeOH/dichloromethane).

*N^1-{[4-(5-N-tert-Butoxycarbonylamino)pentyl]-2-pyrimidyl}sulfanilamide (**F**).* **E** (0.039 g) was dissolved in 2 M NaOH (25 mL) and stirred overnight at room temperature. NaCl was added to the reaction mixture followed by dichloromethane (35 mL). The phases were separated, and the water phase was extracted with dichloromethane (2 × 40 mL). The combined organic phases were dried (Na_2SO_4) and evaporated. The product was purified with FC (silica gel, 10% MeOH/dichloromethane).

N^1-{[4-(5-Amino)pentyl]-2-pyrimidyl}sulfanilamide Monohydrochloride. **F** (0.024 g) was dissolved in 1 M HCl and stirred overnight at room temperature. The reaction mixture was evaporated, and the product was used without further purification: ^{1}H NMR (200 MHz, D_2O) δ 1.12 (t, 2H, CH_2), 1.42 (t, 4H, 2 × CH_2), 2.53 (t, 2H, CH_2), 2.78 (t, 2H, CH_2), 6.75 (d, 2H, ArH), 6.87 (d, 1H, ArH), 7.67 (d, 2H, ArH), 8.07 (d, 1H, ArH).

Biotinylation and Labeling of the Sulfonamide Derivatives. N^1-[4-(Carboxymethyl)-2-thiazolyl]sulfanilamide (3.24 mg) was first activated with carbonyldimidazole (1.80 mg) in dry DMF (0.20 mL). The mixture was stirred for 2 h and then added to a solution of Biotin-XX-Hydratzide (4.81 mg) in dry DMF (0.30 mL). The reaction mixture was stirred overnight at room temperature. The product was purified with RP-HPLC using the LC/MS/MS instrument with a 150 × 4.6 mm, 5 μm HyPurity Elite C18 column (Thermo Hypersil, Runcorn, U.K.), gradient from aqueous 0.1% TFA to acetonitrile in 30 min, flow rate of 1.0 mL/min, and detection at 265 nm. The peaks were analyzed using the mass spectrometer function of the instrument. The peak at 12.54 min found 780.6 $[M + H]^+$, $C_{33}H_{43}N_9O_7S_2$ + H requires 780.2917. The final product (**Figure 3**, referred to as biotinylated sulfathiazole from here on) was dissolved in 1 mL of DMF.

Biotin-XX-NHS (5.88 mg) in dry DMF (0.4 mL) was added to the solution of N^1-{[4-(5-amino)pentyl]-2-pyrimidyl}sulfanilamide (3.85 mg) in dry DMF (0.2 mL). Diisopropylethylamine (2 μL) was added to the reaction mixture and stirred overnight at room temperature. The product was purified with RP-HPLC as above. The peak at 14.35 min found 788.6 $[M + H]^+$, $C_{37}H_{57}N_9O_6S_2$ + H requires 788.3873. The final product (**Figure 3**, referred to as biotinylated sulfamerazine from here on) was dissolved in 1 mL of DMF.

DELFIA-Eu-N1-ITC (10.1 mg) was dissolved in aqueous 50 mM CO^{3-} (0.20 mL) and added to the solution of N^1-{[4-(5-amino)pentyl]-2-pyrimidyl}sulfanilamide (4.9 mg) in aqueous 50 mM CO^{3-} (0.4 mL). The reaction mixture was stirred overnight at room temperature. The product was purified with RP-HPLC using a 250 × 4 mm, 5 μm Hypersil ODS C 18 column (Thermo Hypersil), gradient from 95% A and 5% B to 100% B in 30 min, flow rate of 1.0 mL/min, and detection at 280 nm (A = aqueous 20 mM TEEA and B = 20 mM TEEA + 50% acetonitrile). The peaks were analyzed using the mass spectrometer function of the instrument. The peak at 17.91 min found 968.2 $[M + H]^+$, $C_{35}H_{43}EuN_9O_{10}S_2$ + H requires 967.1787. The final product (**Figure 3**, referred to as Eu-sulfamerazine from here on) was dissolved in 1 mL of DMF.

Cloning of Mab27G3 to scFv Format. Messenger RNA was isolated from a frozen pellet of 10^7 hybridoma cells of the 27G3A9B10 line (*2*) using a Quickprep Micro mRNA purification kit (Amersham Pharmacia Biotech, Espoo, Finland) as instructed by the manufacturer. Complementary DNA was synthesized with a First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech) following the random hexamer primer protocol of the kit. The variable domains of antibody 27G3 were amplified in PCR reactions using *Pfu* polymerase (Promega) and the primer mixes provided in an RPAS mouse scFv module kit (Amersham Pharmacia Biotech) as instructed by the manufacturer. PCR products were cloned using the pGEM-T vector system (Promega) by following the standard protocols provided with the kit. Several individual clones of each variable chain were sequenced, and the correct terminus sequences were deduced by comparison to the corresponding germline gene sequences, which were retrieved from the GenBank sequence database of the U.S. National Center of Biotechnology Information. Primers matching the most closely related germline genes were designed, and the variable chains were amplified again from cDNA in PCR reactions containing 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 0.1% Triton X-100, 0.1 mg/mL bovine serum albumin (BSA), 200 μM dNTP, 5 μL of the cDNA preparate, and 0.04 unit/μL of *Pfu* polymerase in a total volume of 50 μL. For the amplifications of the heavy chain, 5 pmol of HeavyFor and HeavyRev primers were used, and for the case of the light chain, 5 pmol of LightFor and LightRev primers were used (**Table 1**). The PCR program was 95 °C for 5 min, 7 cycles of 94 °C for 1 min, 63 °C for 30 s, 58 °C for 50 s, and 72 °C for 1 min, and finally 23 cycles of 94 °C for 1 min, 63 °C for 30 s, and 72 °C for 1 min.

The obtained PCR products were connected to form a full-length scFv gene [synthetic $(Gly_4Ser)_4$-linker and V_L-V_H orientation] in a strand overlap extension PCR reaction (*18*). The reaction tube contained 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 0.1% Triton X-100, 0.1 mg/mL BSA, 200 μM dNTP, 20 ng of both amplified variable chains, and 0.04 unit/μL of *Pfu* polymerase in a total volume of 50 μL. A PCR program of 95 °C for 3 min and 2 cycles of 94 °C for 1 min, 63 °C for 30 s, 58 °C for 50 s, and 72 °C for 1 min was completed. Five picomoles each of *Sfi*I site containing primers ScFor and ScRev (**Table 1**) was added to the reaction vessel, and the PCR was continued with the following program: 5 cycles of 94 °C for 1 min, 63 °C for 30 s, 58 °C for 50 s, and 72 °C for 1 min and 23 cycles of 94 °C for 1 min, 63 °C for 30 s, and 72 °C for 1 min. The resulting scFv27G3 gene was cloned to the pAK series of vectors (**Figure 2**) using the introduced *Sfi*I sites.

Primary Libraries. The primary libraries were constructed using random mutagenesis by error-prone PCR (*19*, *20*). The scFv27G3 gene fragment was amplified in PCR reactions containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 0.01% gelatin, 2.5 mM $MgCl_2$, 20 μM each ATP and GTP, 100 μM each TTP and CTP, 0.5 mM $MnCl_2$, 0.4 nM ScFor- primer, 0.4 nM ScRev primer (**Table 1**), and 0.05 unit/μL Taq polymerase in a total reaction volume of 50 μL. A high amount (600 ng) of template was used to obtain the low mutation frequency library (library 1), and a low amount of template (6 ng) was used to obtain the high mutation frequency library (library 4). This was done by assuming that the mutation frequency is dependent on

Table 1. Sequences of the PCR Primers

primer	sequence[a]
LightRev	5′-**GGAGCCGCCGCCGCC**(AGAACCACCACCACC)$_2$ACGTTTTATTTCCAGCTTGG-3′
LightFor	5′-GCCCAGCCGGCCATGGCGGAYRTTKTGATGACCCARAC-3′
HeavyRev	5′-GGAATTCGGCCCCCGAGGCCGCAGAGACAGTGACCAGAGT-3′
HeavyFor	5′-**GGCGGCGGCGGCTCC**GGTGGTGGTGGATCCCAGRTCCAGCTGCAGCARTC-3′
ScRev	5′-GGAATTCGGCCCCCGAGGCC-3′
ScFor	5′-TTACTCGCCGGCCAGCCGGCCATGGCG-3′

[a] Complementary sequences for SOE-PCR are in bold and *Sfi*I sites are underlined.

Table 2. Type of Panning Antigen and Dilution Used during Each Panning Round[a]

round	oanning antigen	dilution
1	biotinylated sulfathiazole	1:500
2	biotinylated sulfamerazine	1:2000
3	biotinylated sulfamerazine	1:4000
4	biotinylated sulfamerazine	1:10000

[a] Concentration of biotinylated products could not be determined due to low synthesis yields.

the number of amplifications in the PCR reactions (*21*). The PCR program used was 94 °C for 3 min, 25 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 3 min, and the final extension of 72 °C for 6 min.

All PCR products (~2 μg per library) were cloned to pAK100 phagemid vector (**Figure 2**) using the *Sfi*I sites, which enabled cloning without phosphatase digestion of the open vector. The ligation reactions were precipitated with ethanol and electroporated to electrocompetent *E. coli* XL1-Blue cells in nine separate electroporations each, yielding roughly 1×10^8 transformants per library (determined with platings on LB/chloramphenicol agar). Electroporated cells were diluted to 27 mL total volume in SOC medium, and they were incubated in shake flasks at 37 °C and 200 rpm for 1 h. Cultures were diluted to 150 mL with SB medium containing tetracycline, chloramphenicol, and 0.2% glucose. Growth was continued at 37 °C and 240 rpm, until the optical density (OD_{600nm}) of the cultures reached 0.5.

The cultures were infected with 3.8×10^{11} pfu of VCS-M13 helper phage each for 15 min at 37 °C without shaking. Cells were pelleted with centrifugation (3000*g*, 10 min, 4 °C) and resuspended in 20 mL of SB medium containing tetracycline and chloramphenicol. After 2 h of growth at 37 °C and 240 rpm, IPTG and kanamycin were added to the cultures to final concentrations of 100 μM and 50 mg/L, respectively. Phage production was continued overnight (26 °C, 220 rpm). After removal of bacteria by centrifugation, the phages were precipitated twice by adding polyethylene glycol 8000 (Sigma-Aldrich) to 5% and NaCl to 4%. Phages were finally dissolved in 400 μL of TBS/BSA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Sigma-Aldrich fraction V BSA). The recombinant phage libraries were stored at 4 °C.

Enrichment of the Libraries. Phage libraries were diluted in TBT-0.05 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Sigma-Aldrich fraction V BSA, 0.05% Tween-20) to a final concentration of 1×10^{12} pfu/mL. Each diluted library was divided into two 200 μL aliquots. Appropriate dilution of the selected biotinylated panning antigen (**Table 2**) was added to one of the replicas. Tubes were agitated in a rotamix for 1 h at 25 °C. Dynabeads M-280 magnetic streptavidin particles (Dynal Biotech, Oslo, Norway) were prewashed three times with 200 μL of TBT-0.05, and ~7000 beads was transferred to each phage dilution tube in a volume of 10 μL. The tubes were incubated in a rotamix for 30 min at room temperature. Afterward, the beads were washed five times in 200 μL aliquots of TBT-0.5 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Sigma-Aldrich fraction V BSA, 0.5% Tween-20) using a magnetic concentrator. The particles were finally suspended in 192 μL aliquots of 0.1 M glycine buffer (pH 2.2) and incubated in a rotamix for 15 min at room temperature. The particles were removed, and the eluate was neutralized with 8 μL of 2 M Tris-HCl buffer, pH 9.

One milliliter of fresh XL1-Blue cells was mixed with each eluate batch. Tubes were incubated for 30 min at 37 °C without agitation. Serial dilutions of each tube were plated on LB/chlormaphenicol agar. Shake flasks containing 20 mL of SB medium with tetracycline, chloramphenicol, and 0.2% glucose were inoculated with 1 mL of infected cell suspension from the replicas that had the biotinylated antigen added to them. The flasks were shaken at 37 °C and 300 rpm until the OD_{600nm} of the cultures reached 0.4. The cells were then infected with 3.1×10^{11} pfu of VCS-M13 helper phage, and the recombinant phage preparations were produced as described above.

Isolation of Active Clones. Fresh XL1-Blue cells were infected with the enriched phage libraries as above. Plasmid DNA was isolated from overnight cultures. ScFv genes were cut out with a *Sfi*I digestion and cloned to pAK200CL vector (**Figure 2**), which has a mouse IgG C_L domain fused downstream and in-frame with the scFv. The ligation products were electroporated to XL1-Blue cells, which were then plated on LB/chloramphenicol agar. Individual colonies were picked from the plates for activity measurements. Selected clones were grown (37 °C, 300 rpm) in 5 mL aliquots of SB medium with chloramphenicol and tetracycline until the OD_{600nm} of the cultures reached 0.8. IPTG was added to a final concentration of 100 μM, and growth was continued overnight (26 °C, 250 rpm). The cells were separated with centrifugation, and the supernatants were used for the activity determinations.

Binding activity was determined using a time-resolved, dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA; *22*). Rabbit anti-mouse IgG-coated microtiter plates were prewashed once with wash solution. A 100 μL aliquot of assay buffer was applied to each well followed by 100 μL of the supernatant sample. The plates were incubated for 1 h at room temperature with gentle shaking and washed four times as above. To each well was applied 200 μL of assay buffer containing 30 ng/mL of Eu-sulfamerazine. The plates were incubated for 1 h at room temperature with gentle shaking and washed four times. A 200 μL aliquot of enhancement solution was added to each well. After 5 min of incubation with gentle shaking, time-resolved fluorescence was measured.

Characterization of Active Clones. Active clones were produced in various scales to be used in competitive assays. Cells were grown (37 °C, 300 rpm) in 5−100 mL aliquots of SB medium containing chloramphenicol and tetracycline, until the OD_{600nm} of the cultures reached 0.8. IPTG was added to a final concentration of 100 μM, and growth was continued overnight (26 °C, 250 rpm). The cells were separated with centrifugation and resuspended to an aliquot of lysis buffer (30 mM Tris-HCl, 2 mM EDTA, 20% sucrose, and 2 mg/mL Sigma-Aldrich hen egg white lysozyme) 0.2 times the volume of the original culture. The suspensions were incubated at room temperature for 10 min. Cell debris was removed with centrifugation, and the lysate was diluted with an aliquot of assay buffer 0.6 times the volume of the original culture.

The cross-reaction profiles of the active antibody mutants for different sulfonamide antibiotics were determined. Rabbit anti-mouse IgG-coated microtiter plates were prewashed once with wash solution. Aliquots of the cell lysate samples (200 μL) were applied to the wells. The strips were incubated for 1 h at room temperature with gentle shaking and washed four times as above. To each well was applied 100 μL of assay buffer containing 100 ng/mL Eu-sulfamerazine followed by 100 μL of assay buffer containing various concentrations of different sulfonamides (**Figure 1**). The plates were incubated for 1 h at room temperature with gentle shaking and washed four times. A 200 μL aliquot of enhancement solution was added to each well. After

5 min of incubation with gentle shaking, time-resolved fluorescence was measured. Six different concentrations were measured for each sulfonamide to obtain datapoints for the whole range of inhibition, and sigmoidal curves were fitted to these data. The R^2 values obtained from the comparison of the fitted curves to the raw data varied between 1 and 0.97, being almost always >0.98.

Selected clones were characterized also with respect to their binding affinity toward the sulfamerazine tracer. Rabbit anti-mouse IgG-coated microtiter plates were prewashed once with wash solution. Assay buffer (100 μL) was applied to each well followed by 100 μL of the different supernatant samples. The strips were incubated for 1 h at room temperature with gentle shaking and washed four times as above. A 200 μL aliquot of assay buffer containing a series of concentrations of Eu-sulfamerazine was applied to each well. The strips were incubated for 1 h at room temperature with gentle shaking and washed four times. Finally, 200 μL of enhancement solution was added to each well. After 5 min of incubation with gentle shaking, time-resolved fluorescence was measured. Affinities were determined using a Scatchard plot (*23*).

RESULTS

Synthesis of Sulfonamide Derivatives. Sulfonamide derivatives with linker arms were synthesized and labeled with either biotin or an N1-europium chelate for the purposes of phage display and competitive immunoassays (**Figure 3**). To our knowledge, N^1-{[4-(5-amino)pentyl)-2-pyrimidiyl}sulfanilamide monohydrochloride has not been synthesized before, but we found that alkynyl ketones have been used in the formation of pyrimidin-4-yl (*24*), so we adapted this knowledge to our synthesis. N^1-(4-Carbonylmethyl-2-thiazolyl)sulfanilamide was synthesized as previously described (*1*).

Cloning of Mab27G3 to ScFv Format. To clone Mab27G3 to a single-chain Fv (scFv) format for protein engineering purposes, mRNA was isolated from hybridoma cell-line 27G3A9B10, and cDNA was synthesized. The antibody variable domains were then amplified with commercial "universal" primer mixes. The cloning and sequencing of the PCR products yielded the sequences of the two variable domains of Mab 27G3. Unfortunately, when assembled to scFv format with strand overlap extension PCR (*18*) utilizing the complementary overhang sequences of the universal primers, an scFv gene producing an inactive recombinant antibody was obtained. A comparison of the 27G3 variable domain sequences to mouse germline gene sequences revealed an extensive amount of mutations located in the 5′-termini of both sequences, probably caused by PCR amplification events with slightly mismatching primers from the primer mixes. Because these terminal sequences can be important for the activity of the antibody (*25*, *26*), new primers (**Table 1**) were designed with sequences matching the 5′ termini of the most closely related mouse germline genes. These primers amplified the variable domains from the cDNA with good efficiency, and after assembly to scFv format with SOE-PCR, a gene yielding active recombinant scFv27G3 antibody was obtained.

Primary Libraries. To introduce random mutations over the whole scFv27G3 gene, it was amplified with error-prone PCR (*19*, *20*). The average number of mutations introduced per gene was tuned by adjusting the quantity of template in the EP-PCR reactions (*21*). On the basis of the amount of DNA in the reactions before and after amplification, it was estimated that the low mutation frequency library (library 1) contained roughly two amino acid changes per gene and the high mutation frequency library (library 4) contained roughly eight amino acid changes per gene (*21*). The smaller mutation frequency was assumed to keep many of the mutants in the respective library in active form, thereby providing a large pool of active mutants. The high mutation rate library was constructed because there are hypotheses of this kind of approach being beneficial despite the resulting high number of inactive clones in the library (*27*). Both PCR products were cloned to a pAK100 phagemid vector (**Figure 2**). The scFv mutants were produced as filamentous phage coat protein III fusions from this vector, enabling phage display. After transformation to *E. coli* XL1-Blue cells, both libraries contained $\sim 1 \times 10^8$ independent clones.

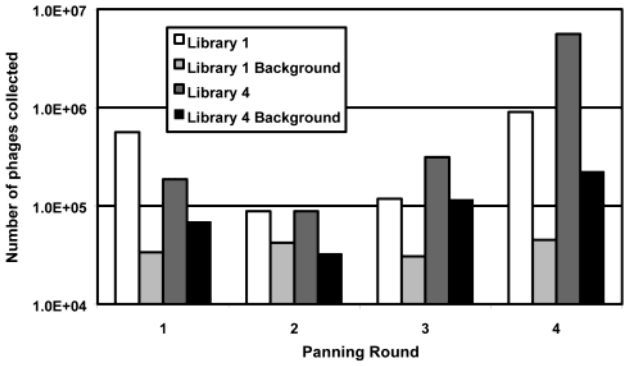

Figure 4. Number of phages collected during each panning round. Shown for libraries 1 and 4 is the number of phages collected from the input pool of $\sim 2 \times 10^{11}$ phages with and without (background) the panning antigen during each round of panning.

Enrichment of Libraries. Libraries 1 and 4 were panned using four rounds of phage display, where sulfa binders were repeatedly enriched by collecting phages with biotinylated sulfonamides attached to streptavidin-coated paramagnetic beads followed by phage amplification in vivo. The antigen type and dilution for each round are shown in **Table 2**. It was assumed that in order to favor mutants displaying improved broad recognition of sulfonamides, panning antigens should be switched between enrichment rounds. In the first round all active mutants were collected with biotinylated sulfathiazole, because the wild-type scFv27G3 has a good affinity for this sulfonamide. In the subsequent rounds, mutants with a broader specificity profile were selected with a decreasing concentration of biotinylated sulfamerazine for which wild-type 27G3 has a lot poorer affinity.

Figure 4 shows how many phages were collected from each library with the antigen during each round of panning and how many of those were collected by unspecific background binding. The increasing output of both libraries after the switch of panning antigen at round 2 indicates that enrichment has happened in the libraries even after the change of selection antigen.

Isolation of Active Clones. To isolate and characterize active clones, phagemid DNA was produced in *E. coli* cells. ScFv genes were cut out with *Sfi*I digestion and cloned to pAK100CL vector (**Figure 2**). From this vector, scFv antibodies are produced as mouse IgG constant light domain fusions, which enables immobilization on anti-mouse IgG-coated surfaces. From each enriched library, 20 individual clones were grown in minicultures and sulfonamide binding activity was measured. Of the clones isolated from library 1, 14 showed measurable (S/N > 2) sulfonamide binding activity, whereas 13 of the library 4 clones also did so. It can be assumed that a random mutagenesis of a whole scFv gene with error-prone PCR produces a library containing mostly inactive mutants, and because over half of the isolated clones in each enriched library were active, it is likely that the panning procedure had resulted in enrichment of active binders.

Characterization of Active Clones. Sequencing revealed that there were five different mutants in the enriched library 1 and

Clone	<LF1> 1 5 15	<LC1> 27D 30	<LF2> 36 49	<LC2> 50 53	<LF3> 58	Ka(x10^7 M^{-1})
27G3	D T I	H K	L Y	L K	V	0.33
Lib1.1	- - -	- -	- -	Q -	-	n.d.
Lib1.9	V - -	- M	M -	- -	A	1.27
Lib1.3	- A -	- -	M -	Q -	-	n.d.
Lib1.14	- - -	R -	M H	Q -	-	n.d.
Lib1.20	- - -	- -	M -	- N	-	1.08
Lib4.2	- - L	- -	M -	- -	A	1.12
Lib4.11	- - -	- -	- -	- -	-	1.42

Clone	<HF1> 10 29	<HC1> 35	<HF2> 46	<HC2> 52A 54	<HF3> 71 73 82A 82B	<HF4> 105	Ka(x10^7 M^{-1})
27G3	D F	N	K	T T	L T N L	Q	0.33
Lib1.1	- -	T	-	A -	- - - -	-	n.d.
Lib1.9	- -	-	-	- -	- - D I	-	1.27
Lib1.3	- -	-	-	- -	- - - -	-	n.d.
Lib1.14	- L	-	-	- -	S - - -	-	n.d.
Lib1.20	- -	-	T	A -	- - - -	-	1.08
Lib4.2	V Y	-	E	- S	- A - -	R	1.12
Lib4.11	- -	T	Q	A -	- - - -	-	1.42

Figure 5. Isolated mutant sequences. The mutations present in light chain framework (LF1–LF4) and CDR (LC1–LC3) regions and in heavy chain framework (HF1–HF4) and CDR (HC1–HC3) regions are illustrated. The numbering of the residues follows that devised by Kabat (*30*). The affinity for a sulfamerazine derivative used as a tracer in the immunoassays is also shown for some clones.

Table 3. Performance of Mutants in a Competitive Sulfonamide Assay for Three Different Sulfonamides

	IC_{50} concn as nmol L^{-1}		
mutant	sulfamethazine	sulfathiazole	sulfamethoxazole
27G3	15000	35	280
Lib1.1	10000 (1.5)[a]	70 (0.5)	170 (1.7)
Lib1.9	2300 (6.5)	12 (2.9)	79 (3.5)
Lib1.3	5000 (3.0)	16 (2.2)	150 (1.9)
Lib1.14	5000 (3.0)	8 (4.4)	55 (5.1)
Lib1.20	4300 (3.5)	16 (2.2)	79 (3.5)
Lib4.2	3600 (4.2)	12 (2.9)	36 (7.8)
Lib4.11	15000 (1.0)	78 (0.5)	79 (3.5)

[a] Number in parentheses indicates relative improvement over wild type.

two different mutants in the enriched library 4 (**Figure 5**), as could be expected considering that there are probably a lot fewer different active mutants in a high mutation frequency library of which even fewer are enriched by the selection procedure. One clone representing each mutant sequence was picked and grown in cultures of various scales. Cell lysates were tested with a time-resolved competitive sulfa fluoroimmunoassay. **Table 3** shows the concentration of selected sulfonamides needed to inhibit 50% of the tracer binding for each active mutant antibody (IC_{50}). Almost all IC_{50} values determined for the mutants were improved over that of the wild-type antibody. The panning scheme also efficiently enriched mutants with improved cross-reaction profiles with respect to broad specificity recognition, because the IC_{50} values for sulfamethazine and sulfamethoxazole were improved more than those for sulfathiazole with at least five different mutants.

Lib4.2, Lib1.9, and Lib1.20 were chosen for further characterization because these mutants had clearly become more generic sulfa binders than the wild-type antibody. Lib4.11 was also studied further, because this mutant seemed to recognize sulfathiazole and sulfamethoxazole with equal affinities (same IC_{50} values): Compared to wild-type, sulfathiazole recognition had diminished and sulfamethoxazole recognition had improved. Although Lib1.1 had an improved genericity, this mutant was not characterized further because it gave low signals in immunoassays (probably due to low affinity for the sulfamerazine tracer).

Table 4. Performance of Selected Clones in a Competitive Sulfonamide Assay for 13 Different Sulfonamides

	IC_{50} concn as μg L^{-1}				
sulfonamide[a]	27G3	Lib1.9	Lib1.20	Lib4.2	Lib4.11
SMT	1.2	0.44	0.25	0.33	0.84
STZ	8.7	2.5	4.2	3.2	20
SCP	9	3.5	3.7	3.4	15
SMP	19	4.8	3.1	5.7	39
SDO	22	11	7.5	8.5	21
SPY	35	5.1	20	7.5	70
SMX	69	17	16	13	21
SDZ	160	40	76	50	34
SMZ	310	54	59	85	98
SFX	420	110	200	110	40
SQX	760	220	320	330	2600
SDM	1100	390	390	330	1900
SHZ	4200	630	1200	990	4300

[a] Sulfonamides are abbreviated as follows: SMT, sulfamethizole; STZ, sulfathiazole; SCP, sulfachlororpyridazine; SMP, sulfamethoxypyridazine. SDO, sulfadoxine; SPY, sulfapyridine; SMX, sulfamethoxazole; SDZ, sulfadiazine; SMZ, sulfamerazine; SFX, sulfisoxazole; SQX, sulfaquinoxaline; SDM, sulfadimethoxine; SHZ, sulfamethazine.

The performance of the selected mutants in a competitive sulfa assay for 13 different sulfonamides is shown in **Table 4**. The affinity constants of these mutants for the sulfamerazine tracer are shown in the right-hand part of **Figure 5**. The results show that the mutants recognized different sulfonamides within a narrower range of affinities as compared to the wild-type antibody. The IC_{50} values of mutants Lib4.2, Lib1.9, and Lib1.20 had a slightly narrower range of variation than the wild-type antibody, but still the IC_{50} values were distributed evenly across this range. Mutant Lib4.11, however, seems to recognize 9 of the 13 tested sulfas with very similar affinities. As for the other four sulfas, sulfamethizole is recognized with a markedly higher affinity, whereas the group of sulfamethazine, sulfaquinoxaline, and sulfadimethoxine is bound with a poorer affinity than the rest of the sulfas tested.

DISCUSSION

Mutant Cross-Reaction Profiles. A lot of effort has been put into the development of generic sulfa binders (*1*−*3*, *13*, *14*), and several authors have discussed the chemical nature of sulfonamides. Studies have been made to model the minimum energy conformations of different sulfonamides and to determine the steric and electrostatic properties of these conformations (*3*, *14*). These seem to differ considerably from each other, which suggests that there is an obstacle to generic sulfa recognition. Still, our mutant Lib4.11 bound nine sulfonamides with very similar affinities despite the fact that these sulfas have been shown to differ from each other structurally and electrostatically in their minimum energy conformations. Thus, generic sulfa recognition of Lib4.11 may rely on binding some higher energy conformation of each sulfonamide, which the antibody stabilizes with favorable interactions of binding. It has been pointed out (*3*) that sulfonamides have rather flat potential energy maps of rotation around the dihedral angles, and there are a number of available conformations that are only slightly more energetic than the minimum energy conformation for each sulfonamide.

However, some sulfas seem to be problematic for all of the isolated mutants. Sulfamethizole is recognized by all mutants with a much better affinity than any other compound tested. Sulfamethizole differs structurally very little from, for example, sulfathiazole, and the only major difference is the presence of

an additional nitrogen in the N^1 ring. It is possible that this nitrogen forms some favorable interactions with the binding cavities of the mutant antibodies, accounting for the increase in affinity. Explaining why sulfamethazine, sulfaquinoxaline, and sulfadimethoxine were poorly recognized by all mutants is not any easier. However, these sulfonamides are the ones with the bulkiest N^1 rings, so the reason for lower affinity may be that they do not fit properly to the binding cavity of the antibodies.

All of the enriched mutants had an improved affinity toward the sulfamerazine tracer and, therefore, less tracer could be used in the immunoassays. This improvement could be expected because sulfamerazine was one of the panning antigens used. The best mutant Lib4.11 had an affinity $K_a = 1.4 \times 10^7\ M^{-1}$ for the sulfamerazine tracer, which is a 4-fold improvement over that of the wild-type antibody.

Observed Mutations. At least mutations L:D1, L:T5, L:I15, H:D10, H:K46, H:N82a, H:L82c, and H:Q105 found in the isolated mutant antibodies (**Figure 5**) were located far away from the binding site and near the surface of scFv 27G3 as judged from generalized antibody schematics (*28*). These mutations probably did not markedly affect the binding of sulfas and probably had no major role in the enrichment of the mutants that had them. However, some of these mutated residues may still affect the folding speed and the overall stability of the mutants. For example, mutation L:I15L present in clone Lib4.2 is located right next to a β-sheet turn and could thus affect the folding rate of this mutant.

None of the sequenced mutants had any mutations in the area of the H-CDR3 or the L-CDR3 loop. This implies that the CDR3 areas of scFv 27G3 are so well adapted to sulfonamide binding that a single substitution causes a significant loss of affinity. On the other hand, the other CDR loops had a number of residues that had mutated in the isolated clones. Most of these mutations probably did not affect the general canonical structure (*29*) of the CDR loops because they were not in the key loop residues. However, mutation H:F29Y in Lib4.2 probably disturbed the general morphology of the H-CDR1 loop as did mutation H:L71S in Lib1.14 for the H-CDR2 loop. Both of these mutations are in general loop conformation maintaining residues. Clones Lib4.2 and Lib1.14 still bound sulfonamides, which might indicate that the role of these two CDR loops in binding is not a major one.

The most frequent mutation in the isolated clones was L:L36M, which was present in five different mutants. This residue is in the framework region and thus probably located in proximity to the bottom of the binding cavity of 27G3. The mutation seems to affect the binding of sulfas in a positive manner. Mutations L:L50Q and H:T52A were both present in three different clones. These residues are located in the L-CDR2 and H-CDR2 loops, respectively, and are thus probably near the binding cavity walls. Also, these mutations seem to affect the binding of sulfas.

In the mutant Lib1.9, which bound the bulkiest sulfas with the best affinity, the frequent mutation L:L36M was combined with L:K30M and L:V58A plus some other mutations. Residue L:K30 is located in the L-CDR1 loop and L:V58 in the framework. These mutations in clone Lib1.9 seem to change the conformation of the antibody so that sulfonamides with bulky N^1 rings fit slightly better to the binding cavity.

The best mutant Lib4.11 had only three mutations, all of them in the heavy chain. These mutations significantly alter the cross-reaction profile of this mutant, making it the best broad specificity binder. It could be interesting to combine some of the point mutations from the other mutants to Lib4.11 to see whether the properties of Lib4.11 would be enhanced even further.

Conclusions. Despite many attempts, hybridoma technology has been unable to produce a broad specificity antibody capable of binding all sulfonamides with sufficient affinity for the purpose of developing a group specific immunoassay (*1*−*3*, *13*, *14*). This can partly be explained by the notion that the immune systems of animals have evolved to produce specific antibodies as opposed to what is wanted here. It has been pointed out (*3*) that protein engineering might be the answer to this problem. We proved the validity of this hypothesis by showing that protein engineering could be used to develop a sulfa binder such as Lib4.11 with an improved cross-reaction profile by using a monoclonal antibody as the starting point. The diverse selection options available allow the optimization of phage display for selection of broad specificity binders in the manner shown in this study. Our mutants showed significant improvement over the wild-type antibody but were not yet completely generic with respect to the three bulky sulfonamides.

ABBREVIATIONS USED

MRL, maximum residue limit; Mab, monoclonal antibody; *KanR*, kanamycin resistance; IPTG, isopropyl β-D-thiogalactopyranoside; FC, flash chromatography; THF, tetrahydrofuran; DMF, dimethylformamide; BSA, bovine serum albumin; OD_{600nm}, optical density at 600 nm; SOE-PCR, strand overlap extension PCR; IC_{50}, inhibiting concentration 50%; CDR, complementarity determining region.

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