Hygromycin B Antibody Production and Characterization by a Surface Plasmon Resonance Biosensor

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Sensitive and accurate methods are needed for the detection of hygromycin B antibiotic in fluids and tissues of farm animals. Sheep antisera were produced from hygromycin B−keyhole limpet hemocyanin and were screened with immunodiffusion, ELISA, and fluorescent latex assays. The antisera were evaluated with the BIAcore, a surface plasmon resonance biosensor, for their binding properties without using signal-generating labels. Hygromycin B was immobilized on the sensor chip, and the capture (binding) of the antibody resulted in a proportional increase in mass. Evaluation of the association (k_a) and dissociation rate (k_d) contants showed that one antibody had an affinity constant (k_a/k_d) of 1.64E+10. The binding capacities and antisera specificity were determined using a competitive binding of the added drug and hygromycin sensor, detecting hygromycin B from 2.5 ng/mL to 5 mg/mL. Neomycin, gentamicin, spectinomycin, dihydrostreptomycin, and streptomycin (1000 times above safe levels) had negligible binding with the antisera. The BIAcore analysis was more rapid and accurate than the immunochemical assays and allow rapid development of methods of hygromycin B analysis in biological samples.

Keywords: *Hygromycin; biosensor; anti-hygromycin; antibody production; surface plasmon resonance; BIAcore; ELISA; fluorescent latex assay*

INTRODUCTION

The surface plasmon resonance (SPR) biosensor can analyze a biochemical interaction in real time, allowing a direct analysis of the binding of an antiserum to an immobilized ligand without the use of labels to generate signals. The binding interaction generates a signal resulting in changes in refractive index on the gold surface and the matrix. This change is proportional to the change in adsorbed mass. The binding interaction results in an increase in molecular mass that the SPR tranducer converts to a light signal, which is detected by a photodiode array. The BIAcore, an automated SPR biosensor system, has been used for real-time analysis of the binding properties of biological compounds. The detailed description of the BIAcore and the principles of its analysis and operation were described by Fagerstram (1991), Fagerstram and Karlsson (1991), Jonsson (1991), Lofas et al. (1991), and Panatayou (1993). The BIAcore has been used for the analysis of antibody−antigen interactions such as binding and dissociation kinetics, active antibody concentrations, specificity of binding molecules, and epitope mapping (Karlsson et al., 1991; Fagerstram et al., 1992; Malmborg et al., 1992; Fagerstram and O'Shannessey, 1993; Malmqvist, 1993). The use of the BIAcore for the analysis of low molecular weight compounds was described for aminotheophylline by Karlsson et al. (1991, 1993), for detection of chemical residues, atrazine (Minunni and Mascini, 1993), and sulfamethazine (Sternesjo et al., 1995).

This study describes methods utilizing an SPR biosensor (BIAcore) in characterizing the antisera against hygromycin B for their affinity, dissociation rate, binding capacity, and specificity. The protocols for antibody production and traditional immunochemcal methods used to screen the production of hygromycin B antisera are also described.

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MATERIALS AND METHODS

Equipment and Reagents. The BIAcore equipped with an autoinjector and software package for system control and evaluation of the data, sensor chip CM5, surfactant P20, amine coupling kit containing *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide (EDC), and ethanolamine hydrochloride were from Pharmacia Biosensor (Piscataway, NJ). The keyhole limpet hemocyanin (KLH) and protein G affinity column were from Pierce (Rockford, IL); hygromycin B was from Calbiochem (La Jolla, CA); sodium periodate, ethylene glycol, Hepes (free acid), *N*-ethyl-N^3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), fluorescein isothiocyanate, isomer I, 98% (FITC), protein A, neomycin, gentamicin, streptomycin, dihydrostreptomycin, and spectinomycin were from Sigma (St. Louis, MO); sodium borohydride was from Aldrich (Milwaukee, WI); PD-10 columns were from Pharmacia (Piscataway); Bindarid was from Binding Site Inc. (La Jolla, CA); 3,3′,5,5′-tetramethylbenzidine (TMB) was from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD); carboxylated polystyrene latex particles (0.87 μm), microtiter wells with membrane bottoms, fluorescent concentration analyzer (FCA) and reader were from Idexx, Inc. (Westbrook, ME). The Bio-Rad protein assay reagent kit was from Bio-Rad (Richmond, CA). The peroxidase-labeled hygromycin B was custom synthesized for ERRC by Diagnostic Specialties (Metuchen, NJ). Antibody production in sheep was contracted with Binding Site, Inc.

Immunogen Preparation. The immunogen was prepared by generation of activated carbonyl groups on the KLH prior to linking with hygromycin B. The immunogen preparation utilized a periodate coupling of carboxyls to amines as reported by Nakane and Kawaoi (1974). This procedure was modified by converting the hydroxyl groups of the KLH to carbonyls, which were then covalently conjugated with the amino groups of the hygromycin B.

The KLH (50 mg) was suspended in 1 mL of 0.3 M sodium bicarbonate buffer, pH 8.1. The suspension was sonicated for 20 s to solubilize the protein. A magnetic bar was added to

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stir the protein solution, and 1 mL of 0.16 M sodium periodate was added drop by drop. The mixture was stirred for 30 min at room temperature, and 1 mL of 0.32 M ethylene glycol was added and gently mixed for 1 h at room temperature. The activated KLH was desalted by passing through a PD-10 column and eluted with a 0.01 M sodium carbonate, pH 9.5. Ten 1 mL fractions were collected and screened at 280 nm. The protein-containing fractions were pooled, and 0.2 mL of an aqueous solution of hygromycin (100 mg) was added drop by drop while the mixture was stirred. Coupling of the hygromycin B to KLH was allowed to progress for 3 h at room temperature. Sodium borohydride (0.5 mL of 0.5 M) was added drop by drop to the reaction tube, and mixing was continued overnight at 4 °C. The mixture was desalted in a PD-10 column and eluted with 0.05 M Hepes−0.15 M NaCl, pH 7. The first 0.3 mL of eluate was collected as void volume, followed by 10 × 1 mL fractions that were screened for protein content. The protein-containing fractions were pooled, and the protein content was measured using a Bi-Rad protein assay and ultraviolet detection at 280 nm. Alternatively, total hygromycin B (before coupling) and the unbound hygromycin B after coupling were measured to determine the coupling efficiency. Underivatized hygromycin B was separated from the protein using a microfuge filter/concentrator with a 10 000 MW cutoff. Hygromycin B was derivatized by addition of fluorescamine at a 1:2 molar concentration ratio, respectively, and the fluorescence signals were measured at 390 nm excitation and 485 nm emission.

Immunization. Three sheep (identity numbers 1595, 1596, and 1597) were immunized with 2 mg of protein equivalent of the immunogen. The animals were reimmunized with 1 mg of protein immunogen in weeks 4, 12, 16, 20, and 24 until an antibody response was measurable at 1:2500 dilution. The animals were bled 10 days after the last immunization. The antisera from sheep 1595 and 1596 were collected in large volume and were evaluated with the BIAcore.

Antibody Screening. The production of antibody was initially screened with a radial immunodiffusion kit (BINDARID) to detect the increase of total IgG over the preimmune serum. The manufacturer's protocol was followed.

An ELISA assay was developed using the peroxidase-labeled hygromycin B. The dilutions of the peroxidase-labeled hygromycin B were optimized such that the chromophoric signals generated were from 0.500 to 1.000 optical density unit. The ELISA wells were coated overnight at room temperature with 100 μL of antisera diluted 100 times. The excess antisera were decanted, and the wells were washed three times with phosphate buffer, pH 7. The peroxidase-labeled hygromycin B, 100 μL, was added and allowed to bind with the antisera for 30 min at room temperature. The excess peroxidase-labeled hygromycin B was washed off with phosphate buffer, pH 7 (4 × 200 μL per well). TMB (100 μL) substrate was added, and the absorbance was measured at 650 nm after 15 min of development. The results of three different antisera were compared.

The antisera were evaluated for specific hygromycin binding with a fluorescent particle concentration assay. To rapidly assess specific antibody production prior to the final bleeding, purified IgG was desired. Affinity column purification of the three antisera was cumbersome; therefore, a protein A−latex particle concentration assay using a fluorescent label was developed. The protein A−latex particles captured the IgG from the serum, which subsequently bound with hygromycin B labeled with FITC. Protein A (0.2 mL of 1 mg/mL in 10 mM sodium acetate buffer, pH 4.5) was immobilized onto 0.2 mL of 5% (w/v) carboxylated polystyrene particles. The carboxylated particles were activated with 2 mg of EDC followed by the addition of protein A. The mixture was incubated at 37 °C for 1.5 h followed by centrifugation for 10 min at 2000*g*. The excess unbound protein in the supernate was measured using a Bio-Rad protein assay and compared to the original concentration prior to coupling. The protein A−latex particles were washed two times with phosphate buffer, pH 7.0, and resuspended in 4 mL of phosphate buffer containing 0.1% sodium azide for storage. The final particle concentration was approximately 0.25% containing 0.43 mg of protein A/10 mL of latex suspension. Hygromycin B was labeled with FITC as described in a previous study (Medina, 1996). The antisera were diluted at 1:500 and 1:1000, while the FITC-labeled hygromycin B was diluted at 1:1000 (2.5 μg/mL) and 1:2500 (1 μg/mL) in phosphate buffer (PBS) at pH 7.0. The assay procedure was as follows: The antisera (20 μL) were transferred to the microtiter wells (with membrane bottoms), followed by the addition of 10 μL of protein A−latex particles and incubated for 30 min. The excess antisera were drained by vacuum, and 20 μL of hygromycin B-FITC was added to each well. The anti-hygromycin B IgG captured by protein A−latex particles and the FITC-labeld hygromycin B mixture were gently mixed and incubated for an additional 20 min. The excess reagents were drained, and the bound mixtures were washed with the binding buffer. The fluorescence signals of FITC-labeld hygromycin B bound to the IgG−protein A−latex particles complex were measured in a fluorescence concentration analyzer at 485 nm excitation and 520 nm emission.

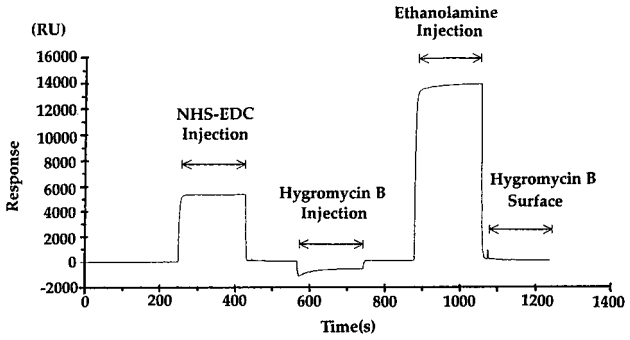

Figure 1. Sensorgram of the immobilization of hygromycin B onto the CM5 sensor chip. A flow rate of 3 μL/min was utilized. A 15 μL volume of all reagents was injected into the BIAcore. The carboxyls were activated with a mixture of NHS and EDC followed by the injection of hygromycin B (0.4 mg/mL). The residual active carboxyls were blocked with ethanolamine.

Characterization of the Antisera by SPR Biosensor. The SPR biosensor (BIAcore) consists of a sensor chip with a gold film mounted on glass. The gold film surface was modified with a carboxylated dextran layer to which the capturing molecule (ligand) is immobilized. The sensor chip has four "flow cells" allowing four separate immobilizations. The sensor chip is housed in a microfluidics cartridge (IFC), which allows the transport of analytes and reagents to the sensor chip. The reactions are monitored continuously, and the binding curve is shown in a sensorgram which is displayed in the monitor screen. The flexible automated system integrated with the biosensing device provides qualitative and quantitative analysis of biospecific interactions.

Preparation of the Sensor Surface. The ligand (hygromycin B) was immobilized on the carboxylated sensor surface, and the antibody was captured by the ligand following procedures recommended by the manufacturer. A continuous flow of HBS (10 mM Hepes, 0.15 M NaCl, 3.4 mM EDTA, and 0.05% surfactant P20, pH 7.4) was maintained over the sensor chip. The carboxyl groups of the dextran layer on the CM5 sensor chip were activated by a mixture of aqueous *N*-hydroxysuccinimide (115 mg/mL) and EDC (750 mg/mL) for 5 min at a rate of 3 μL/min prior to the covalent coupling of the amine containing ligand, hygromycin B (Figure 1). Different concentrations of hygromycin B (0.04, 0.4, and 4.0 mg/mL or 0.076, 0.76, and 7.6 mM) in 10 mM sodium acetate buffer, pH 4.5, were injected into three separate flow cells. The activation of the sensor surfaces (flow cells) and immobilization of the ligand were 5 min each at a flow rate of 3 μL/min. The unbound NHS−ester active sites were blocked with ethanolamine using the same flow rate and volume. The amount of hygromycin B immobilized onto the sensor chip was optimized such that the resonance signals (resonance or response units, RU) generated from the binding of an antisera dilution were between 400 and 1000 RU.

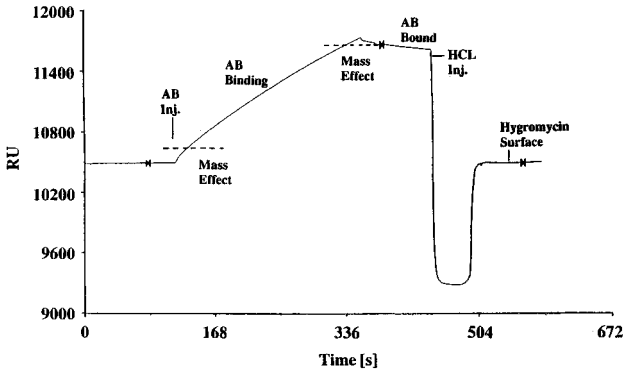

Figure 2. Sensorgram of the capture of anti-hgyromycin IgG by the biospecific sensor surface coated with hygromycin B, a flow rate of 3 μL/min, and 15 μL total volume with HBS (pH 5) running buffer. The bound IgG were desorbed from the chip by a pulse of 4 μL of 100 mM HCl.

Biospecific Interaction with the Polyclonal Antisera. The hygromycin B surface was initially evaluated with a 1:100 dilution of crude antisera. The two antisera (no. 1595 and 1596) were diluted in HBS buffer, pH 7.4, and 25 μL was injected at a rate of 5 μL/min. Following the injection, HBS buffer was allowed to flow over the sensor surface to remove excess sample and a "report point" was designated to show the response (RU) of the captured antibody. The sensor surface was regenerated with a pulse of 100 mM HCl followed by an HBS flow over the sensor chip (Figure 2). The hygromycin B surface was ready for the next analysis. The specificity of the capture of hygromycin-specific antisera was compared to the preimmune serum collected from the same animals. The binding capacity of the hygromycin B surface (0.4 mg/mL) was evaluated using antisera dilutions of 1:1000, 1:2000, or 1:4000. The dissociation rates (k_d) of the crude antisera were fitted from the BIAcore data using the model in the BIAevaluation software. The antisera and the immobilized hygromycin B in buffer solution were then analyzed by the BIAcore for indirect detection of hygromycin B because the BIAcore cannot directly detect the capture of low molecular weight analytes. A competitive binding interaction was utilized wherein the antisera and analyte were allowed to bind in a microtube and the free or unbound antibody was captured by the sensor surface. The binding of the antisera and analyte was carried out "off-line" and were automatically injected into the IFC. (The BIAcore system can automatically mix the samples prior to injection, but this method was not utilized in the competitive inhibition study.) The flow cell surface immobilized with 0.4 mg/mL hygromycin B was utilized to determine the minimum and maximum detectability of hygromycin B. Hygromycin B antiserum (1:4000) was mixed with an increasing amount of hygromycin B from 2.5 ng/mL to 5 mg/mL (0.005 nM to 10 mM) and were automatically injected into the BIAcore system. (The BIAcore can process up to 96 samples in 1 day.) The cross-reactivity or binding of the antisera with other related aminoglycosidic antibiotics was assessed. Using hygromycin B as control, 0.001, 0.01, and 0.1 mM of neomycin, gentamicin, spectinomycin, streptomycin, and dihydrostreptomycin were mixed with antihygromycin B (1:1000; 1:2000, 1:4000), and the mixtures were injected into the BIAcore. The immobilized hygromycin B on the sensor surface captured the free antibody remaining after its interaction with the drugs. The relative amounts of anti-hygromycin captured by the biosensor were determined and evaluated using BIAcore's built-in data processing.

Anti-hygromycin 1596 was purified through a protein G affinity column (Medina and Palumbo, 1996). The IgG isolate was evaluated with the BIAcore system for its kinetic properties using a flow cell immobilized with 0.4 mg/mL hygromycin B. Concentrations of IgG utilized were 12.5 and 25 nM (1.88 and 3.75 μg/mL) in a flow rate of 3 μL/min with an injection volume of 30 μL and binding time of 10 min. The apparent kinetic values were determined using the BIA evaluation system.

Table 1. Protein A−Latex Particle Fluorescence Assessment of Hygromycin B Antisera

anti-serum[a]	1:1000 HAb[b] 1:1000 FITC-hygromycin[c]	1:1000 HAb 1:2500 FITC-hygromycin[d]	1:500 HAb 1:1000 FITC-hygromycin	A:500 HAb 1:2500 FITC-hygromycin
1595	15933[e]	15443	49053	31930
1596	15865	14111	48550	36302
1597	15987	11960	20204	20670
buffer control[f]	8517	7475	8517	7475

[a] Antisera obtained from the 24th immunization week. [b] Anti-hygromycin B dilution. [c] Dilution of FITC-labeled hygromycin (50 ng/20 μL hygromycin). [d] FITC-labeled hygromycin:20 ng/20 μL. [e] Fluorescence units were average of triplicate analysis. [f] FITC-labeled hygromycin was added to wells containing protein A−latex and buffer.

RESULTS AND DISCUSSION

Coupling of Hygromycin B to KLH. The periodate coupling procedure reported by Nakane and Kawaoi (1974) had 70% of the peroxidase enzyme coupled to the IgG molecules, resulting in 99% labeling of the IgG. In this study, determination of the coupling efficiency by protein quantitation was not appropriate. The Bio-Rad protein assay and UV detection at 280 nm showed that the protein contents calculated before and after coupling did not change. However, fluorescence derivatization of the hygromycin B before and after coupling indicated a 50% decrease in fluorescence signals. On the basis of this empirical calculation, the immunogen contained approximately 50 mg of hygromycin B per 50 mg of KLH protein.

Antibody Screening by Traditional Assays. The antisera from three sheep were evaluated for IgG production after 10 and 12 weeks by radial immunodiffusion (RID). The RID showed a slight increase in IgG production compared to the sheep serum prior to immunization. The inhibition results were as follows (sheep number:preimmune/12-week serum): 1595: 132.25/162.56; 1596:121/150; 1597:138/150.06 mm^2 inhibition. These results indicated a slight response, and therefore booster immunizations were continued. The RID test did not show specificity of the IgG to hygromycin B as it detected the total IgG production. The precision of the RID assay was not determined since only a small number of analyses were performed. An ELISA assay was also developed to determine the specific antibody response and was utilized to evaluate the antisera bled in weeks 10, 12, and 21. At 1:100 dilution, the hygromycin B antisera were detectable after 21 weeks of immunization. The ELISA results showed 0.109, 0.122, and 0.096 absorbance units (AU) for antisera 1595, 1596, and 1597, respectively. This low absorbance response indicated a low concentration of hygromycin B IgG. Both RID and ELISA results indicated higher antibody production in sheep 1595 and 1596 than in sheep 1597. The results from sheep 1597 antisera will not be discussed because the antiserum was not collected in large volume due to its low antibody response.

Results (FITC signals) from the fluorescence−protein A−latex assay indicated the relative amounts of IgG captured by protein A. The IgG was captured via its Fc region, leaving the binding sites available for the drug. This assay was more sensitive than that of the ELISA (Table 1). The antisera were also purified by capturing the IgG so that specific binding of FITC-labeled hygromycin B with the captured IgG was detected. The procedure was carried out in 1 h vs overnight assays using RID and ELISA techniques.

Table 2. Estimated Dissociation (k_d) Rates of Hygromycin Antibody Bound by Immobilized Hygromycin B Using HBS as Diluent and Running Buffer[a]

expt no. (flow rate)	anti-serum	dilu-tion	resonance units (RU)	k_d	standard error (k_d)
			A. pH 7[a]		
1 (5 μL/min)	1595	1:1000	477	1.84E−3	1.69E−5
	1595	1:2000	281	1.71E−3	2.43E−5
2 (3 μL/min)	1595	1:1000	570	1.75E−3	1.94E−5
	1595	1:2000	332	1.72E−3	2.00E−5
1 (5 μL/min)	1596	1:1000	474	5.58E−4	7.98E−6
	1596	1:2000	268	5.63E−4	1.54E−5
2 (3 μL/min)	1596	1:1000	540	5.69E−4	1.10E−5
	1596	1:2000	304	5.78E−4	1.59E−5
			B. pH 5[b]		
3 (3 μL/min)	1595	1:1000	2612	3.21E−4	3.46E−6
	1595	1:2000	1268	3.18E−4	6.47E−6
	1595	1:4000	652	3.10E−4	1.71E−5
3 (3 μL/min)	1596	1:1000	1737	5.74E−4	6.67E−6
	1596	1:2000	982	5.70E−4	7.25E−5
	1596	1:4000	518	5.71E−4	1.01E−5

[a] The dissociation rate constant was determined by fitting the line on 100−120 point responses, selected 20 s after the injection (or pulse) of the antisera. Mean k_d values are 1.76E−3 (antiserum1595) and 5.78E−4 (antiserum1596). [b] The dissociation rate constant was determined by fitting the line on 100−120 point responses, selected 20 s after the injection (or pulse) of the antisera. Mean k_d values are 3.16 E−4 (antiserum 1595) and 5.72E−4 (antiserum 1596).

Both fluorescent assay and ELISA techniques require more technical preparation than the BIAcore to screen the antisera production.

SPR Biosensor. Various concentrations of hygromycin B (0.04, 0.4, and 4.0 mg/mL) were immobilized on three separate flow cells on the sensor surface to determine the optimum load of the immobilized ligand. Signals generated were −26, 189, and 652 relative RU, respectively. The flow cell of the sensor chip with −26 RU was not evaluated due to its low response (RU). The flow cells on the sensor chip containing 0.4 and 4.0 mg/mL hygromycin B were evaluated with a 1:100 dilution of the three antisera. The captured antibody by the two flow cells generated 3000 and 12 000 RU, respectively. These responses corresponded to 3 and 12 ng of immunoglobulin/mm^2 of the sensor surface described by Karlsson et al. (1991) and Fagerstram and O'Shannessy (1993). This surface on the flow cell has dimensions of 0.5 × 1.2 × 0.5 mm of width, length, and height. The results suggested that a 0.4 mg/mL surface load of immobilized hygromycin B was sufficient for studying the antibody−ligand interactions. Real-time binding analysis of the sheep antisera 1595 and 1596 indicated that the slower flow rate of (3 μL/min) elicited increased binding response shown by the higher RU in experiment 2 (Table 2A). The data presented in this research are from single replicate analysis. However, the variability between analysis is <10%. A 1:1000 dilution of the antisera generated response units between 400 and 1000, which were desirable for the estimation of the antibody dissociation rate. The dissociation rates were determined by fitting the responses of the bound immunoglobulin to equations available via the BIA evaluation system. Table 2A shows the k_d of two separate analyses of antisera 1595 and 1596 in 1:1000 and 1:2000 dilutions. These results indicate that antiserum 1596 had a slower dissociation rate than antiserum1595, although the latter had higher antibody concentration. When an HBS with a lower pH (5.0) was used, the signals (RU) increased (3.2−4.5 times) vs observed RUs when HBS adjusted to pH 7 was used as running buffer (Table 2B). These results indicated a higher binding of the specific hygromycin antibody to the ligand (hygromycin B). The k_d of bound antiserum 1596 did not change in pH 7 or 5 HBS buffer, but there was a slight change in antiserum 1595, an increase from an average k_d of 1.76E−3 to 3.16E−4. Under these experimental conditions the k_d values indicate relative antibody avidity.

Table 3. Competitive Inhibition Assay Using the BIAcore Shows the Capture of the Free Anti-hygromycin Antibody from the Mixture of Hygromycin B and Antisera[a]

hygromycin B (μg/mL)	RU of bound antibody 1595	% binding of antibody 1595	RU of bound antibody 1596	% binding of antibody 1596
0	723	100	496	100
0.0025	631	87.3	452	91.2
0.005	628	86.9	453	91.3
0.025	617	85.3	447	90.1
0.050	609	84.2	436	87.9
0.500	554	76.6	392	79
5.000	438	60.6	282	56.9
50.000	284	39.3	153	30.8
500.000	107	14.8	49	9.9
5000.000	40	5.5	14	5.5

[a] Hygromycin B (20 μL) diluted in HBS, pH 5, was added to 200 μL of anti-hygromycin B (1:4000) prior to BIAcore analysis. % binding = RU of drug sample/RU at 0 hygromycin. The analysis was carried out two times with <10% variability in results.

Results from the competitive inhibition of the binding of the antibody with the immobilized hygromycin B and the drug in solution showed that with increased dosage of the drug in solution, the amount of the "free" antibody captured by the immobilized drug was proportionally decreased (Table 3). At dilutions of 1:4000, the amount of the free antibody was greater with antibody 1595 than with antibody 1596 shown by the higher RU. The competition of the added hygromycin B was detectable from 2.5 ng/mL through 5 mg/mL. However, with the conditions used in this study, the inhibition of 2.5, 5, and 25 ng/mL may not be significantly different due to a difference of only 5 RU (Table 3). These dosage levels can be accurately determined by reducing the amount of immobilized hygromycin B. Parts A and B of Figure 3 show the plots of the concentration of hygromycin B vs RU of the captured excess antibodies. Saturation of the antibody binding sites was indicated after the addition of 500 μg/mL hygromycin B. The calibration curves derived from these plots showed that antibody 1595 had higher RU than antibody 1595 at 0−5 mg/mL dose, indicating higher amounts of excess specific antibody. The calibration lines for antibodies 1595 and 1596 are $Y = 645.93 - 209.66x + 33.616x^2$, $R^2 = 0.831$, and $Y = 459.55 - 151.43x + 23.184x^2 + 23.184x^2$, $R^2 = 0.938$, respectively. The slopes, saturation points, affinity rates, and dissociation rates derived from the BIAcore analysis may be used in the future to derive formulas to determine applications of the antibody, such as competitive diagnostic assays, immunoprobes, or affinity ligands. The BIAcore provides a more rapid and simple approach than traditional techniques to determine these properties. The binding and regeneration conditions used in the BIAcore also mimic conditions for affinity chomatography (Medina and Palumbo, 1996).

The specificity of the antisera was determined by allowing the antibodies to bind with various aminoglycosidic drugs at 100−10 000 times the "safe level" concentrations determined by the U.S. regulatory agencies. The competitive binding of these drugs was

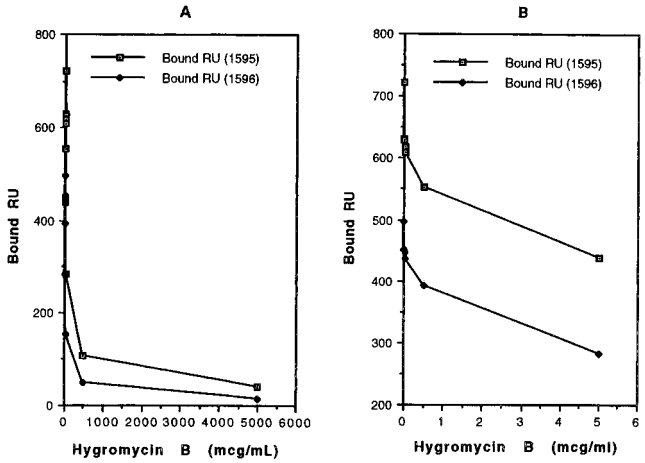

Figure 3. Binding plots of the capture of excess free antihygromycin by the hygromycin sensor surface following an off-line incubation of the antibody with hygromycin B. Percent bound was determined by the ratio of the RU of captured excess antibody in a mixture of hygromycin B and antibody divided by the RU of the antibody solution only, multiplied by 100. The calibration lines of anti-hygromycin 1595 (□) and 1596 (◆) were derived from the plots of RU vs concentration of added drug at doses of (A) 0, 2.5 ng to 5000 μg/mL and (B) 0, 2.5 ng to 5 μg/mL.

Table 4. BIAcore Analysis of the Cross-Reactions of the Aminoglycosides with Anti-hygromycin B To Determine the Specificity of the Antisera

drug	mM	μg/mL (ppm)	% inhibition with 1595	% inhibition with 1596
hygromycin B	0.01	5	37.3	45.3
	0.1	50	60.4	72
	1	500	85.1	90.5
neomycin	0.01	14	13.2	10
	0.1	140	11.7	10.1
	1	1400	8.7	6
gentamicin	0.01	6.73	11.7	11.1
	0.1	67.3	11.7	10.7
	1	673	15	12.1
spectinomycin	0.01	4.05	13.2	11.1
	0.1	40.5	12.7	15.9
	1	405	14.3	13.3
streptomycin	0.01	14.6	11.7	8.8
	0.1	146	12.5	11.1
	1	1460	11.3	9.3
dihydrostreptomycin	0.01	15.3	12.2	9.6
	0.1	153	13.3	11.3
	1	1530	13.2	8.2
mean % NSB[b]			1.2	2.6

[a] The aminoglycosides (20 μL) diluted in HBS buffer, pH 5, were added to 200 μL of anti-hygromycin (1:4000). The mixture was injected into the BIAcore. % inhibition = 100 − % binding. (% binding = RU of drug sample/RU of antisera sample without drug × 100). [b] NSB (nonspecific binding), signals above background which remain on the sensor chip after HCl regeneration. The variability of results between two analyses using different hygromycin sensors was <10%.

compared to that of hygromycin B at the same dose levels (Table 4). The percent binding inhibitions were calculated as 100 − percent binding, where the percent binding = (RU of captured antibody in an antibody−drug mixture/RU of antisera without added drug) × 100. Samples containing 5, 50, and 500 ppm of hygromycin B had 37, 60, and 85% (respectively) inhibition of the free antibody binding with the immobilized ligand. In contrast, the aminoglycosides showed binding inhibition of 9−14% at the same molar concentrations. The average cross-reactions or binding of other aminoglycosidic drugs with anti-hygromycin B 1595 and 1596 were 12 and 10%, respectively, at 0.01 M concentrations

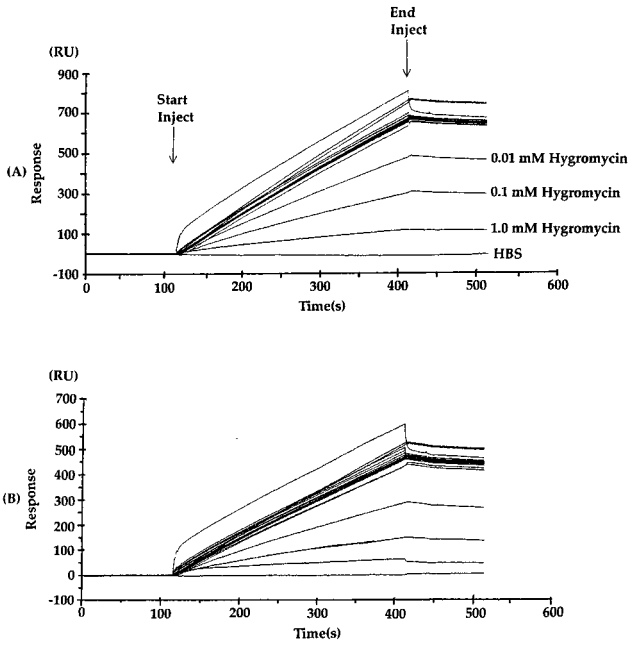

Figure 4. Sensorgrams of the competitive inhibition or binding of aminoglycosidic drugs (hygromycin B, neomycin, gentamicin, spectinomycin, streptomycin, and dihydrostreptomycin) with the antisera. The drugs and antisera were incubated off-line prior to injection into the BIAcore as in Figure 3. The RUs are reported in Table 4. (A) Binding of free antiserum 1595; (B) binding of the excess free antiserum 1596. The top line in the injection phase is a sensorgram generated by a mixture of 1.0 mM neomycin and antiserum. Separate analyses in a different sensor surface show high reproducibility with variability of <10%.

(Table 4). At tolerance or safe levels, these cross-reactivities or competitive inhibitions are near 1%, which is equivalent to nonspecific binding (NSB). The sensorgrams in Figure 4 show the capture of the free antibody by the immobilized hygromycin B. The bottom lines were sensorgrams generated by the injection of an HBS bufffer as baseline control. The top lines of the injection phase were generated by the injection of 1.0 mM (1400 ppm) neomycin and antiserum mixture. The high resonance signals generated by neomycin indicated a high refractive index at this concentration, but this did not enhance or inhibit the binding of the free antibody with the immobilized ligand. Except for the sensorgrams of the HBS and the hygromycin B samples, the sensorgrams from other aminoglycosides were close to the sensorgram of the sample containing the antibody only. These results indicate that the aminoglycosides had minimal interactions with the antisera.

The kinetic properties of the purified anti-hygromycin (1596) IgG were determined using the BIAevaluation system where the association rate, k_a, was obtained from the model $A + B = AB$ and the dissociaton rate, k_d, from $AB = A + B$. The apparent kinetic values are k_a = 1.39E+6 and 1.53E+6 (1/Ms), k_d = 9.55E−5 and 8.32E−5 (1/s) with affinity constants, K = 1.45E+10 and 1.84E+10 (k_a/k_d) for 12.5 and 25 nM IgG concentrations.

CONCLUSIONS

The SPR biosensor (BIAcore) was able to rapidly analyze the binding characteristics of the crude antisera. The binding characteristics of the hygromycin B antisera were determined more rapidly and accurately by the SPR biosensor than with the use of immunodiffusion, enzyme, and fluorescent immunoassay tech-

niques. The estimated dissociation rates of antisera 1595 and 1596 were 1.7E−4 and 5.6E−4, respectively. The binding of the antisera to the immobilized hygromycin B (0.4 mg/mL) showed a curvilinear response when inhibited by the addition of 2.5 ng/mL to 5 mg/mL hygromycin B prior to the biosensor analysis. The cross-reactions or antibody binding competitions of the aminoglycosides with hygromycin B sensor were neglibible (<10% with NSB correction) at 0.01 mM concentrations of neomycin (14 μg/mL), gentamicin (6.7 μg/mL), spectinomycin (4 μg/mL), streptomycin (14.6 μg/mL), and dihydrostreptomycin (15.3 μg/mL). These concentrations are at least 1000 times above safe levels. A diagnostic biosensor system utilizing these antisera and the BIAcore can be optimized for the detection of hygromycin B in biological samples. The detection accuracy and sensitivity of hygromycin B and other aminoglycosides at trace levels are being improved by overcoming the matrix interferences in milk samples.

The BIAcore analytical system provides a more rapid and simple approach than traditional techniques for the assessment of relative antibody concentration in serum, binding capacity, specificity, affinity rate (k_a), dissociation rate (k_d), and affinity constant (K). These properties can be used to derive formulas to determine the application of the antibody in systems, such as competitive diagnostic assays, immunoprobes, or immunoaffinity ligands in flow systems. Development of immunochemical methods for the analysis of hygromycin B in animal tissues and fluids is in progress.

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