Development of a Monoclonal Antibody-Based ELISA for the Anthelmintic Hygromycin B

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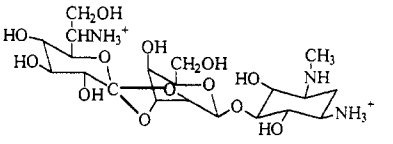
Monoclonal antibodies were prepared against hygromycin B, a Food and Drug Administration (FDA) approved feed additive used for the control of worms in swine and poultry. Splenocytes from mice immunized with a hygromycin B–ovalbumin conjugate were fused with SP2/0 myeloma cells, and hybridomas secreting antibodies against hygromycin B were selected and cloned. An assay using the antibody with the highest affinity for hygromycin B is described. The monoclonal antibody is specific for hygromycin B and does not cross-react with structurally similar aminoglycoside antibiotics commonly added to medicated feed. When tissue samples were spiked with hygromycin B, the average percent recoveries at 1, 2, and 4 ppm were 83, 82, and 91%, respectively. The ELISA described here can be used to rapidly screen porcine kidney samples for the presence of hygromycin B residues.

INTRODUCTION

The anthelmintic hygromycin B (HB) is an FDA-approved feed additive used to control roundworms, nodular worms, and whipworms in swine and large roundworms, cecal worms, and capillary worms in poultry. It is often used in conjunction with other drugs to increase the rate of weight gain and feed efficiency. Although approved as a feed additive, a zero tolerance level for hygromycin B in swine and poultry products (i.e., tissues, eggs) has been set by the FDA (21 CFR 556.330). For enforcement purposes, a residue limit of 1.4 ppm of the parent compound in the kidney has been established, and these limits are used by the U.S. Department of Agriculture Food Safety Inspection Service (USDA/FSIS) to detect adulterated products. To comply with the regulations, medicated feed must be withdrawn 15 days and 3 days prior to slaughter in swine and poultry, respectively.

Conventional methods for detection of hygromycin B in tissues include a microbiological assay (Johnston et al., 1981) and a high-performance liquid chromatography (HPLC) method (McLaughlin and Henion, 1992). The microbiological assay is nonspecific and requires long incubation periods. The HPLC method is labor intensive, has a multistep sample cleanup, and requires expensive equipment. A polyclonal-based radioimmunoassay (RIA) to detect hygromycin B in feed has been described (Foglesong and LeFeber, 1982). This RIA overcomes some of the disadvantages of the previous methods, but it suffers from the problems associated with the need to continually synthesize unstable radiotracers and dispose of radiological waste. The disadvantages associated with these assay methods have prevented their being used for routine screening of large numbers of samples.

Enzyme-linked immunosorbent assays have been successfully developed as alternatives to the conventional microbiological or chemical methods for detection of pesticides (insecticides and herbicides), drug residues, and undesirable natural products (Azcona-Olivera et al., 1992; Candlish et al., 1988; Degand et al., 1992; Groopman et al., 1984; Hu et al., 1984; Jung et al., 1989; Plhak and Sporns, 1992; Roseman et al., 1992; Shelby et al., 1992; Wong and Ahmed, 1992; Woychik et al., 1984; Xu et al., 1988). In contrast to microbiological assays, immunoassays are highly specific (Stanker et al., 1987; Vanderlaan et al., 1988; Van Emon et al., 1986), and unlike conventional chemical assays, they require minimal sample preparation procedures (Monroe, 1984).



Hygromycin B

Figure 1. Structure of hygromycin B.

We report here the development and characterization of monoclonal antibodies to hygromycin B, as well as the development of a competitive indirect enzyme-linked immunosorbent assay (CI-ELISA). Preliminary studies demonstrate the applicability of the CI-ELISA in detecting hygromycin B in porcine kidney.

MATERIALS AND METHODS

Materials. Sulfosuccinimidyl 4-(*p*-maleidophenyl)butyrate (sulfo-SMPB), phosphate-buffered saline (PBS; 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2), bovine serum albumin (BSA), ovalbumin (OVA), and 2-iminothiolane were obtained from Pierce (Rockford, IL). Hygromycin B (Figure 1), goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase, Tween 20, 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS), hydrogen peroxide (30% solution), tylosin (8 mg/mL), spiramycin, amikacin, poly(ethylene glycol) (PEG 4000), hypoxanthine, aminopterine, thymidine, and pristane were obtained from Sigma (St. Louis, MO). RIBI adjuvant was obtained from RIBI ImmunoChem Research, Inc. (Hamilton, MT). Iscoves media, fetal bovine serum, gentamycin (10 mg/mL), penicillin/streptomycin solution, and kanamycin (10 mg/mL) were obtained from Gibco (Grand Island, NY). Tissue culture plasticware was obtained from Intermountain Scientific Corp. (Bountiful, UT). Microtiter plates were purchased from Costar (Cambridge, MA). Mice (BALB/c) were purchased from Harlan/Sprague Dawley (Houston, TX).

Hapten Synthesis. Three conjugation methods were used to prepare hygromycin B–carrier protein conjugates, all of which couple an amine on the hapten to a reactive group on a carrier protein or a modified carrier. The hapten was coupled by (1) direct carbodiimide-activated coupling to the carrier (no spacer), (2) oxime formation with a glutaraldehyde spacer arm on the glutarated carrier (5-atom spacer), or (3) amide formation with the *N*-hydroxysuccinimide moiety of the heterobifunctional cross-linker and attachment of the hapten–cross-linker through the maleimide moiety of the cross-linker to sulfhydryl groups on the thiolated carrier (16-atom spacer).

(1) *Carbodiimide Method.* Hygromycin B (70 mg) was mixed with 10 mg of carrier protein in 1.5 mL of PBS, and to this mixture was added 14 mg of carbodiimide (EDC). The reaction mixture was stirred at room temperature overnight, and then the sample was dialyzed against PBS.

(2) *Glutarated Carrier Method.* To 98 mL of PBS was added 2.5 mL of 8% glutaraldehyde and 1 mL of a 10 mg/mL solution of carrier protein. The mixture was stirred overnight at 4 °C, and then the volume was reduced to 15 mL by ultrafiltration using a 30 000 MWCO stirred cell (Amicon, Beverly, MA). Hygromycin B (50 mg) was added to 5 mg of the glutarated carrier protein, the reaction mixture was stirred overnight at 4 °C, and the sample was then dialyzed against PBS.

(3) *Thiolated Carrier Method.* Six milligrams of the protein modifier 2-iminothiolane hydrochloride (Traut's reagent) was added to 44 mg of carrier protein in 2.2 mL of PBS/0.1 M EDTA, pH 8.0. The EDTA was added to the buffer to prevent disulfide bond formation. After 24 h, 200 μL of 1 M glycine was added to stop the addition of 2-iminothiolane hydrochloride to the carrier protein. The samples were then dialyzed against PBS/0.1 M EDTA, pH 8.0. A 5-mg sample of the thiolated carrier in 0.5 mL of PBS/0.1 M EDTA, pH 8.0, was mixed with 35 mg of hygromycin B. The cross-linker, sulfo-SMPB (0.5 mg), was added and the solution stirred overnight at room temperature. The sample was then dialyzed against PBS. The BSA and OVA conjugates produced by this method were designated HB-SMPB-S-BSA and HB-SMPB-S-OVA, respectively.

Monoclonal Antibody Production. Two immunization schedules were used to produce hygromycin B antibodies. In the short immunization schedule, female BALB/c mice (6–8 weeks of age) were injected intraperitoneally (ip) with 75 μg of HB-SMPB-S-OVA (in 0.2 mL of physiological saline/RIBI adjuvant). The mice received a second ip injection after 3 weeks. One week after the second injection, the mice were bled and serum titers were determined. Five weeks after the initial immunization (3 days before the fusion), a third 75-μg ip injection of the immunogen in PBS (without adjuvant) was administered.

In the extended immunization schedule, female BALB/c mice were immunized with 75-μg ip injections every 2 weeks for a total of three injections. On day 35, the mice were bled so that serum titers and antibody specificities could be determined. No injections were administered for 1 month, and then a final 75-μg ip dose of the immunogen in PBS (no adjuvant) was administered 4 days prior to the fusion.

Hybridoma Production. Splenocytes from an immunized mouse were fused with SP2/0 myeloma cells using PEG (Stanker et al., 1986). Fused cells were resuspended in HAT medium (Iscove's modified Dulbecco's medium containing 36 mM $NaHCO_3$, 2 mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 5% fetal bovine serum, 100 mM hypoxanthine, 0.2 mM aminopterin, and 8 mM thymidine) and pipetted into the wells of 96-well plates containing a feeder layer of mouse macrophages. After 10–14 days, hybridoma supernatants were analyzed by indirect ELISA for the presence of antibodies against hygromycin B. Cells in fusion wells with supernatants exhibiting inhibition were transferred from the fusion plates to 24-well plates. Cultures showing the highest percentage of inhibition were cloned twice by limiting dilution (Goding, 1983). For each cloning procedure, the cells were plated in HT medium on a layer of macrophage feeder cells, and final clones were obtained from wells that had been seeded with one (or fewer) cell per well.

Indirect ELISA. Wells of polystyrene microtiter plates were coated with 100 μL of the coating antigen, HB-SMPB-S-BSA (1 μg/mL), and air-dried overnight at 40 °C. The BSA conjugate was used as the solid-phase antigen to avoid detection of antibodies to the OVA carrier used to immunize the animals. Nonspecific binding was decreased by blocking the wells with 3% (w/v) nonfat milk solution in deionized water for 30 min at 37 °C. After five washes with 0.05% Tween 20 in distilled water, 100 μL of the appropriate serum or cell culture supernatant dilutions was allowed to bind to the coated microwells for 90 min at 37 °C. Unbound antibody was removed by washing five times with the 0.05% Tween 20/water solution. Next, 50 μL of goat anti-mouse IgG–peroxidase conjugate (1:500 dilution) was added to each well. After a 60-min incubation at 37 °C, the plates were washed 10 times with 0.05% Tween 20, and bound antibody–peroxidase conjugate was determined using the colorimetric substrate, 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS). Absorbance was read at 405 nm.

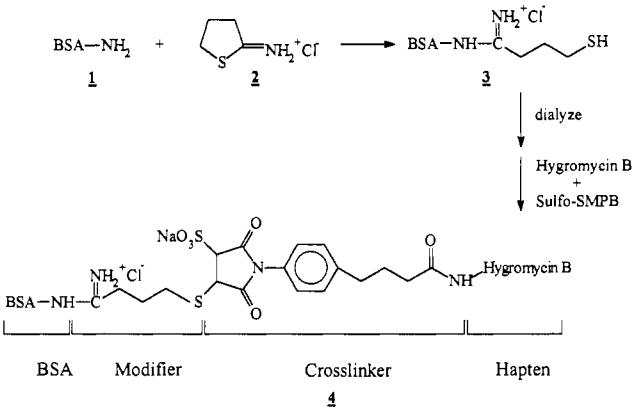


Figure 2. Synthetic route to obtain hygromycin B–carrier protein conjugate using 2-iminothiolane to derivatize the carrier and sulfo-SMPB to link hygromycin B. The BSA conjugate produced using this scheme is designated HB-SMPB-S-BSA. (1) BSA; (2) 2-iminothiolane; (3) thiolated BSA; (4) HB-SMPB-S-BSA conjugate.

A competitive indirect ELISA (CI-ELISA) was used to determine the sensitivity and specificity of the monoclonal antibodies. Microtiter plates were coated with HB-SMPB-S-BSA (400 ng/well) and blocked with 3% nonfat milk solution as described above. After five washings with 0.05% Tween 20 in distilled water, 100 μL of hygromycin B standard in assay buffer (0.1 M Tris, 0.15 M NaCl, 0.05% Tween 20, 1% BSA, pH 7.5) and 100 μL antiserum (diluted 1:60 in assay buffer) were added to each well and incubated for 90 min at 37 °C. After five washings with 0.05% Tween 20, the assay was completed as described above.

Kidney Extract Preparation. Minced samples (5 g) of hygromycin-free kidney (obtained from the Texas A&M University swine center) were spiked with hygromycin B at concentrations of 1, 2, or 4 ppm (μg/g) and were kept frozen until analysis. To prepare spiked samples for analysis, the samples were digested with 5 mL of 2 N NaOH for 45 min at 37 °C, diluted with 8.2 mL of deionized water, and neutralized to a final pH of 6.5–7.0 with 1.8 mL of 6 N HCl. Following centrifugation at 14000*g* for 45 min at 4 °C, the supernatants were added directly to the microtiter plates and analyzed by CI-ELISA.

RESULTS

Hapten Synthesis. Hygromycin B is a small molecule which is not itself immunogenic and, therefore, must be conjugated to a carrier protein. Three conjugation methods were used, each of which used a reactive amine group on hygromycin B to conjugate the hapten to the carrier protein. In the first method, hygromycin B was conjugated directly to carrier proteins using carbodiimide. An excess of hygromycin B was used to minimize intraprotein cross-linking and maximize hapten attachment. In the second method, carrier proteins were modified with glutaraldehyde to introduce reactive aldehyde groups into the proteins. The glutarated proteins were dialyzed prior to addition of an excess of the hapten to minimize the possibility that free glutaraldehyde would attach to the second amine on hygromycin B. In the third method, carrier proteins were modified with 2-iminothiolane to introduce reactive free sulfhydryl groups into the protein. After removal of excess modifier by dialysis, hygromycin B and the heterobifunctional cross-linker sulfosuccinimidyl 4-(*p*-maleidophenyl)butyrate (sulfo-SMPB) were added to the modified protein (see conjugation scheme in Figure 2). An excess of hygromycin B (compared to both the cross-linker and modified protein) was also used with this conjugation method to minimize the number of

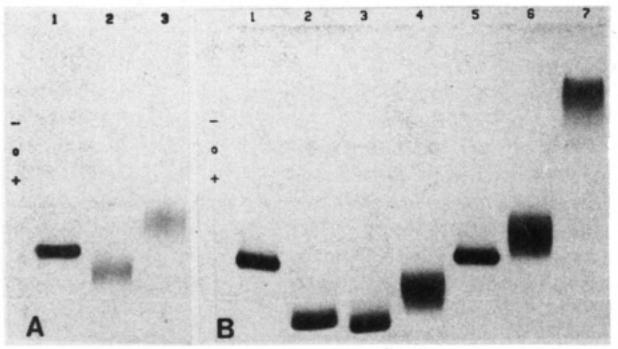


Figure 3. Nondenaturing gel analysis of the conjugates using three different conjugation methods. (A) Lane 1, BSA; lane 2, glutarated BSA; lane 3, hygromycin B–glutaraldehyde–BSA conjugate. (B) Lane 1, BSA; lane 2, thiolated BSA; lane 3, thiolated BSA treated with the cross-linker (SMPB) alone; lane 4, HB-SMPB–S-BSA conjugate; lane 5, BSA; lane 6, BSA treated with the cross-linker (carbodiimide) alone; lane 7, HB–(carbodiimide)–BSA conjugate.

hygromycin B molecules that would be derivatized with two molecules of the cross-linker.

Since hygromycin B possesses no unique UV–vis absorbing group and since it cannot be easily removed from the carrier for quantitative analysis, qualitative analysis of the conjugates by nondenaturing gel electrophoresis (Kamps-Holtzapple et al., 1993) was performed to demonstrate that hygromycin B had been successfully conjugated. The migration patterns of the BSA conjugates are shown in Figure 3. In each case, the migration of the HB–BSA conjugate is distinct from that of the carrier protein [i.e., BSA (lane 1, panels A and B); glutaraldehyde-modified BSA (lane 2, panel A); or 2-iminothiolane-modified BSA (lane 2, panel B)] and from that of the carrier protein treated with cross-linker alone. No cross-linker was used with the glutaraldehyde-modified BSA since the aldehyde groups react directly with the amines on hygromycin B.

Serum Titer Determination. Although qualitative analysis of the conjugates demonstrated that hygromycin B had been successfully conjugated to the carrier proteins, none of the mice treated with hygromycin B conjugated directly to the carrier protein (using carbodiimide) or with hygromycin B conjugated to glutaraldehyde-treated carrier protein produced antibodies to free hygromycin B (data not shown). In contrast, mice immunized with hygromycin B conjugated to 2-iminothiolane-modified carrier using the cross-linker sulfo-SMPB resulted in antibody serum titers greater than 20 000 (Figure 4). In these experiments, the titer was defined as the reciprocal of the dilution that results in an absorbance value that is twice that of background.

Hybridoma Production. Splenocytes from a BALB/c mouse immunized with HB-SMPB–S-OVA were fused with SP2/0 myeloma cells and the resulting hybridomas were cultured in 23 96-well culture plates. Growing hybridomas were observed in greater than 90% of the wells 10 days after fusion. The supernatant from each well was screened for the presence of antibodies against the immunogen using an indirect ELISA. Hybridoma cells from wells that were positive (80 wells) in the above ELISA were expanded, and the supernatants were analyzed by CI-ELISA for antibodies capable of binding native hygromycin B. Only 7 of the original 80 picks resulted in stable hybridomas secreting antibodies against free hygromycin B. Cells from these seven were subcloned, and monoclonal cell lines secreting anti-hygromycin B antibodies were established. All of the monoclonal antibodies were determined to be IgG1 antibodies with κ light chains.

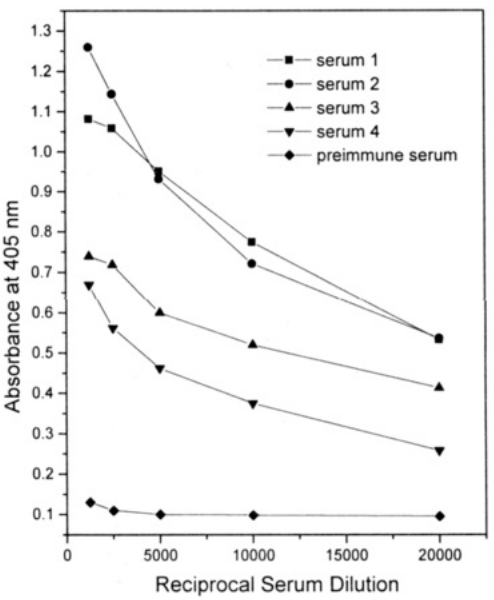


Figure 4. Titration curves of sera from mice immunized with HB-SMPB–S-OVA. Titers were determined by indirect ELISA using HB-SMPB–S-BSA as the solid-phase coating antigen.

Immunization Protocol. A number of immunization schedules may be used to produce antibodies in mice. In attempting to produce antibodies to hygromycin B, we found that when the immunization protocol originally recommended by the adjuvant manufacturer was used (injections on days 0 and 21, serum titer determination day 26, and a final injection day 35, 3–4 days before fusion), the monoclonal antibodies that were produced exhibited low affinity for hygromycin B (IC_{50} ranging from 10 to 100 ppm). This same protocol has been used to successfully produce high-affinity antibodies (IC_{50} ranging from 0.1 to 20 ppb) to other haptens being investigated in our laboratory (unpublished data). However, higher affinity antibodies to hygromycin B were obtained after an extended immunization protocol was used (injections on days 0, 14, and 28, serum titer determination day 35, 1 month of no injections, and an injection 3–4 days before fusion).

Representative competition ELISA curves for the three most sensitive antibodies obtained using the two immunization schedules are shown in Figure 5. Antibody HB-13 (the most sensitive antibody obtained using the extended immunization protocol) was chosen for development of an ELISA to detect hygromycin B.

Assay Development. The optimum conditions for a competition ELISA were determined as follows. The plate-coating antigen (HB-SMPB–S-BSA) was serially diluted across a microtiter plate so that each well contained between 0.05 and 0.8 μg. HB-13 antibody (unpurified culture supernatant) was added to the wells and the concentration of immobilized antigen that resulted in 80% of maximum activity was determined to be 0.4 μg/well. HB-13 antibody was then titrated against immobilized antigen (0.4 μg/well), and a final dilution of 1:120 resulted in 50% of maximum activity. Typical absorbance readings (405 nm) in control wells (antibody added with no competitor) were between 0.4 and 0.5 AU, while background absorbance readings were typically 0.09 AU.

Antibody Characterization. A representative inhibition curve for HB-13 obtained in the CI-ELISA, using hygromycin B as competitor, is shown in Figure 6. Each point represents the average of six replicates. The average IC_{50} observed for hygromycin B was 76.2 ng/well (0.762 ppm). The mean intraassay IC_{50} coefficient of variation

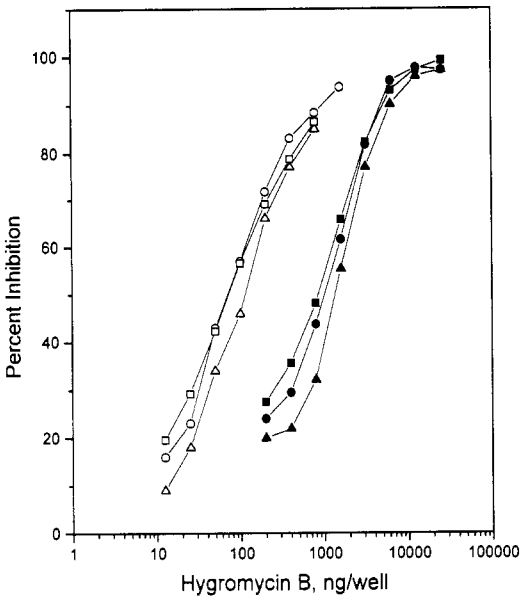


Figure 5. Representative inhibition curves of the three most sensitive monoclonal antibodies obtained using the short immunization protocol (solid symbols) or the extended immunization protocol (open symbols). Inhibition curves were generated using a competitive indirect ELISA.

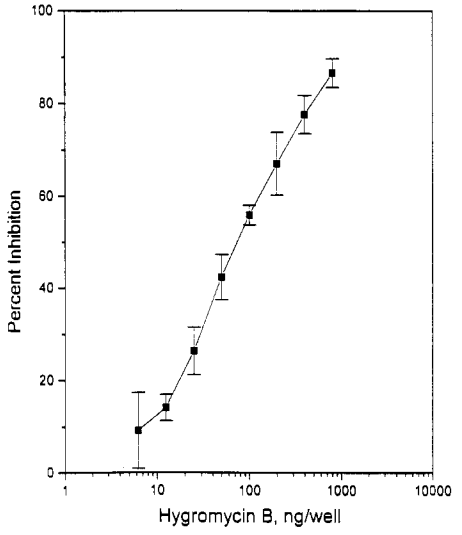


Figure 6. Competitive indirect ELISA standard curve for hygromycin B using monoclonal antibody HB-13. The solid-phase antigen was HB-SMPB–S-BSA. Error bars represent ±1 standard deviation.

(CV) was 7.8% (n = 3), while a mean interassay IC_{50} coefficient of variation of 7.0% (n = 3) was observed. The detection limit of the assay (defined as the lowest concentration of hygromycin B exhibiting OD readings that were greater than that of the zero standard by twice the standard deviation of the zero standard) was 25 ng/well or 250 ng/mL (0.25 ppm).

HB-13 was also characterized as to its ability to recognize related aminoglycoside antibiotics. None of these compounds (Figure 7) were observed to cross-react with the HB-13 antibody in the competitive indirect ELISA (using a competitor concentration of 50 ppm).

Immunoassay of Hygromycin B in Porcine Kidney. The CI-ELISA was used to detect hygromycin B in porcine kidney extracts. Kidney samples spiked at 1, 2, and 4 ppm (μg/g) gave recoveries of 83, 82, and 91%, respectively (Table 1). Figure 8 demonstrates the correlation between the spike level of hygromycin B and the level detected by ELISA.

DISCUSSION

Immunoassay procedures to detect contaminants in feeds and foods have gained widespread popularity (Jung et al., 1989; Azcona-Olivera et al., 1992; Van Emon et al., 1989). Various strategies have been employed to increase the probability that high-affinity antibodies to the hapten will be produced. These strategies include (i) maintaining the structure of potentially important determinant groups on the hapten (Sheth and Sporns, 1991), (ii) using or extending the length of a linker arm (McAdam et al., 1992), and (iii) varying the immunization protocol (i.e., mode of injection, number of injections, or length of immunization schedule) (Vallejo et al., 1982).

The factors that exactly control generation of a strong immune response are not clearly understood. Thus, in this study, we employed three conjugation methods using linker arms of various lengths and followed two immunization schedules in an attempt to obtain antibodies with sufficiently high affinities for hygromycin B. Of the three conjugation methods used, the only conjugate that resulted in a positive immune response to free hygromycin B in our mice was the conjugate obtained using the heterobifunctional cross-linker sulfo-SMPB to link hygromycin B to a thiolated carrier protein. The length of the linker arm in this case was 16 atoms. Linkage of hygromycin B directly to the carrier (no linker arm) and linkage to glutarated carrier proteins (five atom linker arm) failed to elicit specific antibodies in the sera of immunized mice as determined by CI-ELISA using a hygromycin B concentration of 500 ppm. Others also have reported effects of the linker arm length on the specificity and the sensitivity of the resulting antibodies (Wei and Hammock, 1984; Vallejo et al., 1982; Harrison et al., 1991; Sheth and Sporns, 1991). In general, for small haptens, extending the hapten away from the protein mass has been helpful or even critical to the production of high-affinity antibodies (Hastings et al., 1988; Paxton et al., 1976). It is possible that hygromycin B, when linked directly to the carrier, was masked to a large extent by the surrounding protein. It is unclear why the hygromycin B conjugates with a five-atom linker failed to generate an immune response. Since hygromycin B contains two reactive amines, many of the hapten molecules may have been conjugated by two reactive aldehyde groups on the glutarated carrier protein. It is clear, however, that the sulfo-SMPB-linked hapten possessed the necessary design for raising antibodies to hygromycin B.

In addition to the length of the bridging group, the number of injections and the time between injections affected the sensitivity of the resulting monoclonal antibodies. Two immunization schedules were used in an effort to obtain antibodies sensitive to hygromycin B. The schedule recommended for mice by the adjuvant manufacturer (short schedule) resulted in the production of hygromycin B-specific monoclonal antibodies, but these were of relatively low affinity. Extending the immunization protocol appears to have allowed for maturation of the immune response and production of higher affinity antibodies. Since the residue limit for hygromycin B in pork kidney is 1.4 ppm, the monoclonal antibodies produced using the short schedule resulted in unacceptably high IC_{50} values (i.e., IC_{50} values of 10–20 ppm) using the CI-ELISA. It is not clear why high-affinity antibodies against hygromycin B were not detected using this immunization schedule. However, adding an additional injection (containing adjuvant) and incorporating a 1-month rest period into the immunization protocol

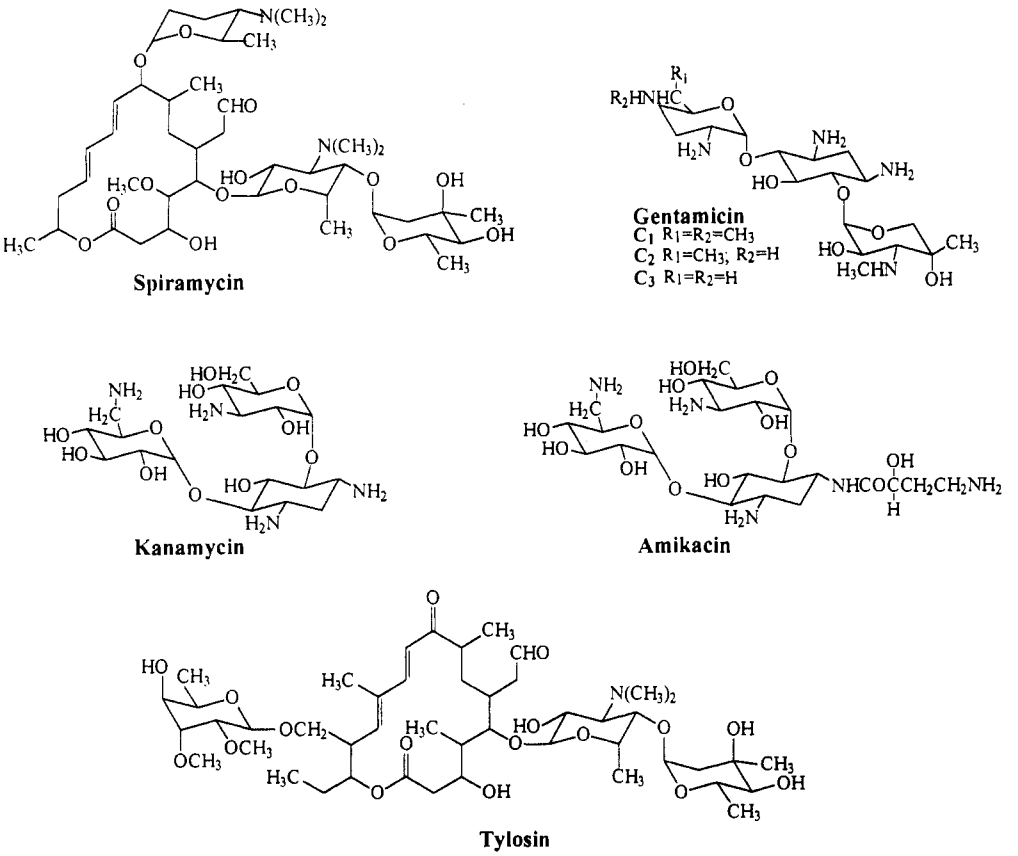


Figure 7. Structures of aminoglycosides tested for cross-reactivity.

Table 1. Recovery of Hygromycin B from Spiked Kidney by Competitive Indirect ELISA

hygromycin B added, ppm (μg/g)	sample[a]	recovery ppm (μg/g)[b]	recovery %[c]
0	A	nd[d]	
0	B	nd	
0	C	nd	
1	A	1.1 ± 0.4	110
1	B	0.6 ± 0.1	60
1	C	0.8 ± 0.2	80
2	A	1.6 ± 0.5	80
2	B	1.5 ± 0.4	75
2	C	1.8 ± 1.0	90
4	A	3.3 ± 0.6	83
4	B	3.5 ± 1.1	88
4	C	4.1 ± 1.8	103

[a] Samples were spiked separately in triplicate and assayed on separate days (A–C). [b] Mean recoveries ($n = 3$) for samples containing 1, 2, or 4 ppm (μg/g) of hygromycin B were 0.8, 1.6, and 3.6 ppm, respectively. [c] Mean percent recoveries ($n = 3$) for 1, 2, and 4 ppm were 83, 82, and 91%, respectively. [d] None detected (less than 0.25 ppm, the detection limit of this assay).

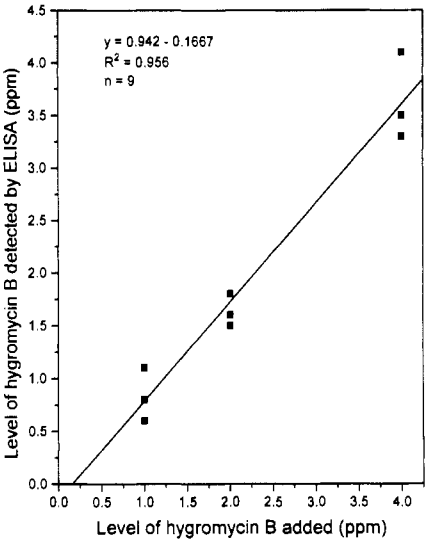


Figure 8. Correlation between the spike level of hygromycin B and the level detected by ELISA.

resulted in production of antibodies with a 12-fold increase in sensitivity to hygromycin B (i.e., IC_{50} values of 0.7–1 ppm).

The increase in antibody affinity that is seen in the immune response to most haptens (affinity maturation) occurs primarily in the IgG class of immunoglobulins (Golub, 1981). The antigen selection hypothesis proposed by Siskind and Benacerraf (1969) suggests that the change in affinity occurs as a result of the selective proliferation of B-cells having the highest-affinity receptors. However, Griffiths et al. (1984) reported that affinity maturation is not due solely to an increase in the proportion of cells having high-affinity receptors but that it also involves introduction of point mutations in the hypervariable regions of the antibody molecules. Cells expressing mutated antibodies having greater affinity for the antigen are thought to possess a proliferative advantage. The repetitive injections and waiting periods that are incorporated into immunization schedules are necessary to allow sufficient affinity maturation to occur such that high-affinity antibodies can be obtained.

For many highly immunogenic haptens, an immunization protocol consisting of three or four injections within a 2-month period may be sufficient for eliciting high-affinity antibodies. For other less immunogenic molecules, longer protocols may be required to allow further affinity maturation of the antibodies. In the case of hygromycin B, an additional injection combined with a rest period before the final injection resulted in the production of

antibodies with affinities capable of detecting residues in porcine kidney at regulatory levels.

In conclusion, a monoclonal antibody specific for hygromycin B was produced and used to develop a CI-ELISA capable of detecting hygromycin B in porcine kidney. The assay has the advantages of being more specific than the microbiological assay and more rapid and less expensive than HPLC. It can be incorporated into a residue monitoring program as a rapid initial screen to eliminate samples that do not contain violative levels of hygromycin B. Therefore, use of this method to detect hygromycin B in kidney has the potential to increase sample throughput and to decrease costs associated with sample analyses.

ABBREVIATIONS USED

BSA, bovine serum albumin; OVA, ovalbumin; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; EDC, 1-ethyl-3-[(3-dimethylamino)propyl]carbodiimide hydrochloride; SMPB, sulfosuccinimidyl 4-(*p*-maleidophenyl)butyrate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDA, ethylenediamine; MW, molecular weight.

LITERATURE CITED

Azcona-Olivera, J. I.; Abouzied, M. M.; Plattner, R. D.; Pestka, J. J. Production of monoclonal antibodies to the mycotoxins fumonisins B_1, B_2 and B_3. *J. Agric. Food Chem.* **1992**, *40*, 531–534.

Candlish, A. A. G.; Stimson, W. H.; Smith, J. E. Determination of ochratoxin A by monoclonal antibody-based enzyme immunoassay. *J. Assoc. Off. Anal. Chem.* **1988**, *71*, 961–964.

Degand, G.; Bernes-Duyckaerts, A.; Maghuin-Rogister, G. Determination of clenbuterol in bovine tissues and urine by enzyme immunoassay. *J. Agric. Food Chem.* **1992**, *40*, 70–75.

Foglesong, M. A.; LeFeber, S. Radioimmunoassay for hygromycin B in feeds. *J. Assoc. Off. Anal. Chem.* **1982**, *65*, 48–51.

Golub, E. S. *The Cellular Basis of the Immune Response*, 2nd ed.; Sinauer Associates: Sunderland, MA, 1981.

Griffiths, G. M.; Berek, C.; Kaartinen, M.; Milstein, C. Somatic mutation and the maturation of immune response to 2-phenyl oxazolone. *Nature* **1984**, *312*, 271–275.

Groopman, J. D.; Trudel, L. J.; Donahue, P. R.; Marshok-Rothstein, A.; Wogan, G. N. High affinity monoclonal antibodies for aflatoxins and their application to solid-phase immunoassays. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 7728–7731.

Harrison, R. O.; Goodrow, M. H.; Hammock, B. D. Competitive inhibition ELISA for the s-triazine herbicides: Assay optimization and antibody characterization. *J. Agric. Food Chem.* **1991**, *39*, 122–128.

Hastings, K. L.; Tulis, J. J.; Dean, J. H. Production and characterization of a monoclonal antibody to aflatoxin B_2. *J. Agric. Food Chem.* **1988**, *36*, 404–408.

Hu, W. J.; Woychik, N.; Chu, F. S. ELISA of picogram quantities of aflatoxin M_1 in urine and milk. *J. Food Prot.* **1984**, *47*, 126–127.

Johnston, R. W.; Reamer, R. H.; Harris, E. W.; Fugate, H. G.; Schwab, B. A new screening method for the detection of antibiotic residues in meat and poultry tissues. *J. Food Prot.* **1981**, *44*, 828–831.

Jung, F.; Meyer, H. H. D.; Hamm, R. T. Development of a sensitive enzyme-linked immunosorbent assay for the fungicide fenpropimorph. *J. Agric. Food Chem.* **1989**, *37*, 1183–1187.

Kamps-Holtzapple, C.; Carlin, R. J.; Sheffield, C.; Kubena, L.; Stanker, L.; DeLoach, J. R. Analysis of hapten-carrier protein conjugates by nondenaturing gel electrophoresis. *J. Immunol. Methods* **1993**, *164*, 245–253.

McAdam, D. P.; Hill, A. S.; Beasley, H. L.; Skerritt, J. H. Mono- and polyclonal antibodies to the organophosphate fenitrothion. 1. Approaches to hapten-protein conjugation. *J. Agric. Food Chem.* **1992**, *40*, 1466–1470.

McLaughlin, L. G.; Henion, J. D. Determination of aminoglycoside antibiotics by reversed phase ion-pair high-performance liquid chromatography coupled with pulsed amperometry and ion spray mass spectrometry. *J. Chromatogr.* **1992**, *59*, 195–206.

Monroe, D. Enzyme immunoassay. *Anal. Chem.* **1984**, *56*, 920a–931a.

Paxton, J. W.; Rowell, F. J.; Ratcliffe, J. G. Production and characterisation of antisera to diphenylhydantoin suitable for radioimmunoassay. *J. Immunol. Methods* **1976**, *10*, 317–327.

Plhak, L. C.; Sporns, P. Enzyme immunoassay for potato glycoalkaloids. *J. Agric. Food Chem.* **1992**, *40*, 2533–2540.

Roseman, D. M.; Wu, X.; Milco, L. A.; Bober, M.; Miller, R. B.; Kurth, M. J. Development of a class-specific competitive enzyme-linked immunosorbent assay for the detection of pyrrolizidine alkaloids in vitro. *J. Agric. Food Chem.* **1992**, *40*, 1008–1014.

Shelby, R. A.; Kelley, V. C. Detection of ergot alkaloids from *Claviceps* species in agricultural products by competitive ELISA using a monoclonal antibody. *J. Agric. Food Chem.* **1992**, *40*, 1090–1092.

Sheth, H. B.; Sporns, P. Development of a single ELISA for detection of sulfonamides. *J. Agric. Food Chem.* **1991**, *39*, 1696–1700.

Siskind, G. W.; Benacerraf, B. Cell selection by antigen in the immune response. *Adv. Immunol.* **1969**, *10*, 1–50.

Stanker, L. H.; Branscomb, E.; Vanderlaan, M.; Jensen, R. H. Monoclonal antibodies recognizing single amino acid substitutions in hemoglobin. *J. Immunol.* **1986**, *136*, 615–622.

Stanker, L. H.; Watkins, B.; Rogers, N.; Vanderlaan, M. Monoclonal antibodies for dioxin: Antibody characterization and assay development. *Toxicology* **1987**, *45*, 229–243.

Vallejo, R. P.; Bogus, E. R.; Mumma, R. O. Effects of hapten structure and bridging groups on antisera specificity in parathion immunoassay development. *J. Agric. Food Chem.* **1982**, *30*, 572–580.

Vanderlaan, M.; Watkins, B. E.; Hwang, M.; Knize, M. G.; Felton, J. S. Monoclonal antibodies for the immunoassay of mutagenic compounds produced by cooking beef. *Carcinogenesis* **1988**, *9*, 153–160.

Van Emon, J.; Hammock, B.; Seiber, J. Enzyme-linked immunosorbent assay for paraquat and its application to exposure analysis. *Anal. Chem.* **1986**, *58*, 1866–1873.

Van Emon, J. M.; Seiber, J. N.; Hammock, B. D. Immunoassay techniques for pesticides analysis. In *Analytical Methods for Pesticides and Plant Growth Regulators: Advanced Analytical Techniques*; Sherman, J., Ed.; Academic Press: New York, 1989; Vol. 17, pp 217–263.

Wie, S. I.; Hammock, B. D. Comparison of coating and immunizing antigen structure on the sensitivity and specificity of immunoassays for benzoylphenylurea insecticides. *J. Agric. Food Chem.* **1984**, *32*, 1294–1301.

Wong, R. B.; Ahmed, Z. H. Development of an enzyme-linked immunosorbent assay for imazaquin herbicide. *J. Agric. Food Chem.* **1992**, *40*, 811–816.

Woychik, N. A.; Hinsdill, R. D.; Chu, F. S. Production and characterization of monoclonal antibodies against aflatoxin M_1. *Appl. Environ. Microbiol.* **1984**, *48*, 1096–1099.

Xu, Y.-C.; Zhang, G.-S; Chu, F. S. Enzyme-linked immunosorbent assay for deoxynivalenol in corn and wheat. *J. Assoc. Off. Anal. Chem.* **1988**, *71*, 945–948.

Received for review August 9, 1993. Revised manuscript received December 17, 1993. Accepted December 28, 1993.[⊗] Mention of a tradename, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

[⊗] Abstract published in *Advance ACS Abstracts*, February 1, 1994.