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Development of Monoclonal Antibodies Bearing the Internal Image of the Gizzerosine Epitope and Application in a Competitive ELISA for Fish Meal

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ABSTRACT

Gizzerosine (GZ), a derivative of histamine, is a biogenic amine found in fish meal, and one of the causative agents of black vomit, a poultry disease. We describe here the preparation of anti-idiotype antibodies to the anti-GZ monoclonal antibody (anti-GZ 3H4) and their possible application to an immunoassay. BALB-c mice were immunized with anti-GZ 3H4 antibody coupled to hemocyanin from *Concholepas concholepas*. Using somatic cell fusion between NSO/2 cells and splenic lymphocytes from the immunized mice, we obtained 34 potential anti-idiotype antibodies. They were characterized by passive agglutination with supernatants from hybridoma cultures and latex particles conjugated to the idiotype. Anti-idiotype antibodies were analyzed by a competitive RIA, to determine their ability to dissociate the interaction between ^{125}I-GZ and the anti-GZ 3H4-idiotype antibody. They were also characterized by GZ inhibition of latex passive agglutination assay. Three anti-idiotypes named 2D11, 2H6, and 3A12, all of the IgG isotype, were obtained. They were evaluated by a competitive ELISA, in which GZ competes with the tracer (HRP-idiotype). All presented sensitivity in the range of 0.1–10 μg/mL of GZ; and the 3A12 anti-idiotype antibody showed the best performance. An ELISA was developed using the idiotype bound to the solid phase and the anti-idiotype 3A12–HRP as the tracer. The assay showed a similar sensitivity and cross-reactivity with histamine was only observed at concentrations over 10 μg/mL. Lysine and histidine did not interfere with the assay up to 500 μg/mL. An experiment was conducted with fish meal contaminated with synthetic GZ. The results are promising, and showed that no other compounds of the fish meal interfere with the ELISA system; however the extraction procedure of the sample needs to be improved. From the results presented here, we conclude that the idiotype anti-idiotype ELISA would be an appropriate method to determine GZ in fish meal.

INTRODUCTION

GIZZEROSINE (2-amino-9-[4-imidazolil]-7azanonanoic acid) (GZ, Mr 240) is a biogenic amine found in some kinds of fish meal. This compound causes the disease called "black vomit" or "gizzard erosion," observed in chicks.[9,12,21,22,25,40,41] Gizzerosine formation has been related to high histamine levels and to overheating in the processing of fish meal rich in lysine.[20]

It has been postulated that gizzerosine results from the reaction of the ε-amino group of lysine with the histamine ethylimidazole group during heating processes in the manufacture of fish meal. One of the bioactive substances in fish meal has been shown to be histamine, but a relatively large amount of histamine in feed (usually over 500 μg/mL) is needed to induce gizzard erosion.[11,20,34]

Gizzerosine is a histamine H2-receptor agonist, and its erosive effect on chicken gizzard has been demonstrated.[22,12,15] However, gizzerosine is about 1,000 times stronger than histamine and has a longer lasting effect.[20] Sugahara et al.[33,34] reported that gizzerosine is lethal to chickens at concentrations over 1 μg/mL, and recently Tisljar et al.[38] reported that 0.65 ppm of gizzerosine induced histopathological lesions in gastrointestinal organs of broiler chicks. Scarce information is available on the effect of gizzerosine on other animal species fed with fish meal, although it has been reported to affect salmonids.[8]

At present, no rapid and sensitive *in vitro* specific assays are available for gizzerosine determination in fish meal. The tradi-

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tional gizzerosine test is a bioassay with 4-day-old chicken fed with the fish meal under study. After 7 days, the gizzard is removed and subjective scores are assigned to determine its degree of erosion. This bioassay is sensitive and reliable, but it is expensive and slow and gives a semi-quantitative estimation of the gizzerosine content.

Gizzerosine determination would be simplified by using an analytical method. In the past, procedures using HPLC have been applied but they lack sensitivity and reproducibility.[15,24] We believe that assays like ELISA, based on gizzerosine monoclonal antibody-immunodetection could be adequate. One great advantage of the hybridoma technology is that the humoral immune response of the experimental animal can be dissected to each one of its components.[4,18] This property facilitates the search of low cross-reaction to histamine antibodies and other structurally related compounds.

We have described the development of monoclonal antibodies to gizzerosine and their use in a competitive ELISA[3] that showed sensitivity in the range of 0.1–10 μg/mL of gizzerosine. No cross-reaction was observed with histamine, histidine, or lysine at the same concentrations. However, some limitations may hinder its application because the assay depends exclusively on the availability of synthetic gizzerosine (for standards and tracer preparations), which is quite expensive. Presently Ajinomoto (Japan) elaborates it exclusively. We believe that an idiotype-anti-idiotype system[16] could be used instead: the antibody (Ab1) that represents the inner image of an antigen's epitope, can produce several anti-antibodies (AB2α, AB2β, and AB2γ) when it is used as an antigen; of which AB2β has an idiotype region similar to the antigen, and is capable of inhibiting its binding to Ab1.

In recent years, anti-idiotype monoclonal antibodies (MAbs) directed to anti-hapten antibodies have been successfully generated. The assays do not depend on the availability of the hapten, and the tracer is an antibody which can be readily labeled. This system has been applied to herbicides,[32] T4 thyroid hormone,[2] mycotoxins,[5,13] phycotoxins,[5] okadaic acid,[31] dinitrophenol,[19] gibberellin,[35] and 11-deoxycortisol.[17] From the biotechnological viewpoint, the use of this system to determine GZ in fish meal is particularly justified since the supply of gizzerosine is limited and expensive.

MATERIALS AND METHODS

Development of anti-idiotype monoclonal antibodies

Antigen preparation. Anti-GZ 3H4 monoclonal antibody was coupled to hemocyanin from *Concholepas concholepas*[26] (Blue Carrier, developed by Biosonda S.A., Santiago, Chile) using the protocol described by Becker et al.[3] and Mura et al.[23] with minor modifications. Briefly, 2 mg of the anti-GZ 3H4 antibody previously purified with a Sepharose-Protein-G column (Pierce, Rockford, IL) as described by Harlow and Lane[10] were dissolved in 0.5 mL of distilled water and slowly added to 2 mg of hemocyanin dissolved in 2 mL of 0.1 M borate buffer pH 10. Then 1 mL of 0.3% glutaraldehyde (Polysciences, Warrington, PA) was added. The mixture was incubated for 2 h in darkness at 25°C, then dialyzed at 4°C against phosphate buffer saline (PBS; 0.1M NaCl, 2.5 mM KCl, 1.5 mM KH_2PO_4, pH 7.2).

Mouse immunization. BALB/c mice were immunized with the anti-GZ 3H4 monoclonal antibody as follows. On day 1, they received intraperitoneally 75 μg of antibody coupled to hemocyanin plus 75 μg of uncoupled antibody emulsified in complete Freund's adjuvant (Pierce); on days 16, 54, and 79, the same immunization was carried out, but emulsified with incomplete Freund's adjuvant. A booster was given on day 90 with an intraperitoneal and intravenous injection of the antigen in PBS. Mice were bled prior to the immunizations, to obtain pre-immune control serum. Ten days after each injection, the animals were bled by the tail to obtain serum, and the humoral response to anti-GZ 3H4 antibody was determined using latex bead particles coated with anti-GZ 3H4 monoclonal antibody.

Preparation of latex particles coated with monoclonal antibodies. The procedure described by Becker et al.[2] with minor modifications was employed. Briefly, 2 mL of 10% 0.8-μm polystyrene latex beads (Sigma, St. Louis, MO) were dialyzed against PBS pH 7.2. Then the anti-GZ 3H4 monoclonal antibody or anti-Acro C5F10 as control,[39] previously purified with a Protein G Sepharose column, were added separately to a final concentration of 200 μg/mL of 1% latex. The mixture was incubated for 3 h at 37°C, then left overnight at 4°C with stirring. The mixture was extensively washed by centrifugation. The supernatant was discarded, and the pellet was resuspended in 1% BSA glycine-saline buffer pH 8.2. Finally, the activated latex was spectrophotometrically adjusted to 1% final concentration.

Determination of the anti-idiotype antibodies titer by passive latex agglutination. Drops of 15 μL of PBS-BSA were placed on a 96-well U-bottom plate and serial dilutions of the sera from experimental mice were made. Then we added 15 μL of latex activated with the anti-GZ 3H4 antibody or with the anti-Acro C5F10[39] antibody as negative control. The preparation was incubated for 1 h at 37°C and the intensity of the agglutination was observed using an arbitrary scale from +1 to +3, as follows:

(+3) fully agglutinated latex and transparent supernatant
(+2) agglutinated latex and turbid supernatant
(+1) weakly agglutinated latex and turbid supernatant
(−) latex without agglutination

Somatic fusion. The general procedure described by Köhler and Milstein[18] with minor modifications was employed.[1,2] Briefly, 1 week before the somatic fusion, NSO/2 cells (provided by Dr. C. Milstein, from MRC Laboratory of Molecular Biology, Cambridge, UK) were cultured at 37°C in a 10% CO_2 atmosphere and 100% humidity in D-MEM complete medium (D-MEM medium supplemented with 10% fetal calf serum, Hyclone, Logan, UT) and antibiotics. An intravenous and intraperitoneal booster were given to the mouse with the highest titer of antibodies to the antigen; three days after, it was sacrificed by cervical dislocation. The spleen was isolated in a sterile form and placed on a Petri dish with 10 mL of D-MEM. Lymphocytes were liberated mechanically by pressing the spleen with a spatula. Both cell populations were separately washed with D-MEM and centrifuged at 1,000 rpm for 10 min. The splenic lymphocytes pellet, free from supernatant was then

washed with NSO/2 cells. After discarding the supernatant, the pellet was gently removed, and 0.5 mL of 50% PEG 4,000 (Merck, Darmstat, Germany) was used for the fusion. The pellet containing the hybridomas was finally resuspended in 70 mL of complete D-MEM containing HAT medium, and seeded on sterile 96-well culture plates that were incubated at 37°C under a 10% CO_2 atmosphere. At 7 days after fusion, 2 drops/well complete D-MEM medium plus HT was added to the plates.

Starting at day 10, the hybridomas potentially secreting anti-idiotype antibodies were selected by passive latex agglutination. Fifteen μl of each hybridoma supernatant and 15 μL of latex-Anti GZ 3H4 or latex-anti-Acro C5F10 as control were mixed. Hybridomas identified as possible anti-idiotypes were expanded on a 24-well plate and seeded on 1 mL of D-MEM medium complete with HT, allowing their growth up to confluence.

The determination of the isotype of the selected monoclonal antibodies was performed with a commercial ELISA kit from Pierce (Immunopure monoclonal antibody isotyping kit II).

Characterization of the anti-idiotype antibodies by RIA. Two procedures were employed: in the first, all the components of the reaction mixture were co-incubated (equilibrium condition) and in the second the components were sequentially added (non-equilibrium condition). Gizzerosine (Ajinomoto, Tokyo, Japan) was labeled with ^{125}I (Comisión Chilena de Energía Nucler, Santiago, Chile) as described by Torres et al.[37]

Equilibrium condition: 0.1 mL of non-diluted supernatant of the anti-GZ 3H4 monoclonal was incubated for 24 h at 4°C with 0.1 mL of 1% BSA borate buffer at pH 9.0 plus 0.1 mL of ^{125}I-GZ (10,000 cpm) and 100 μL of supernatant of clones producing anti-idiotype antibodies. The mixture was then precipitated with a solution containing: 100 μL of anti mouse IgG goat serum diluted 1:15 in 1% BSA borate buffer plus 100 μL of 2% mouse pre-immune serum in 1% BSA borate buffer and 500 μL of 8% PEG 8,000 in 1% BSA borate buffer. It was vigorously stirred, incubated for 2 h at 4°C, and centrifuged for 45 min at 2,800 rpm. The supernatant was discarded and radioactivity was measured in a liquid scintillation γ counter.

Non-equilibrium condition: 0.1 mL of undiluted supernatant from anti-GZ 3H4 monoclonal was incubated for 24 h at 4°C with 0.1 mL of 1% BSA borate buffer pH 9.0 and 100 μL of supernatant of the clones likely to produce anti-idiotype antibodies. After incubation, 0.1 mL of ^{125}I-GZ (10,000 cpm) was added and re-incubated for 1 h; then the mixture was precipitated, and the radioactivity determined as before.

Characterization of the anti-idiotype antibodies by inhibition of passive latex agglutination. Fifteen μL of a PBS solution containing different concentrations of GZ or histamine (0–100 μg/mL), and 15 μL of latex activated with the anti-GZ 3H4 monoclonal were co-incubated for 30 min at 37°C in 96-well U-bottom plates. Then 15 μL of each anti-idiotype hybridoma supernatant, previously diluted (from 1:5 to 1:10), was added and incubated for 1.5 h. It was observed visually and arbitrary values ranging from +1 to +3 were assigned as previously described.

Development of a competitive ELISA with synthetic gizzerosine

ELISA using as conjugate the idiotype labeled with HRP

Labeling of anti-GZ 3H4 monoclonal antibody with peroxidase. Labeling of anti-GZ 3H4 antibody with peroxidase was performed with a Pierce commercial assay according to the provider's instructions. Briefly, one mg/ml anti-GZ 3H4 antibody was incubated in PBS in the presence of 2-mercaptoethylamine (MEA) for 1.5 h at 37°C. It was run in a polyacrylamide column to extract the salts, and the protein concentrations were measured to 280 nm to identify the fractions containing the antibody. Then 1 mL of the antibody fraction was added with 1 mg/mL peroxidase activated with maleinimide and incubated for 1 h at room temperature. It was dialyzed against PBS and the anti-GZ 3H4–HRP conjugate was finally stored at −20°C with Super Freeze Conjugate Stabilizer (Pierce) for peroxidase.

Titration of the anti-GZ-3H4-HRP conjugate antibody. Commercial plates coated with mouse anti-IgG (Pierce) were incubated for 30 min at 37°C with 50 μL of the anti-GZ 3H4-HRP conjugate diluted on base 2 in BSA borate buffer pH 9.0 or PBS-BSA pH 7.2. The plate was washed with 0.02% PBS-Tween-20 and enzyme activity was revealed with 100 μL/well of Pierce's TMB-H_2O_2 system, incubating for 30 min at room temperature. The reaction was stopped with 100 μL of 3N sulfuric acid and OD was read at 450 nm.

Titration of the supernatants of anti-idiotypes 2D11, 2H6, and 3A12 with the 3H4–HRP conjugate. Commercial polystyrene plates coated with mouse anti-IgG were added with 50 μL/well of dilutions on base 2 of the supernatants of each anti-idiotype in PBS-BSA, incubating for 2 h at 37°C. The plate was washed with 0.02% PBS-Tween 20 and saturated overnight at 4°C with an irrelevant IgG (anti-Acro C5F10 antibody). The plate was washed again and different dilutions of the anti-GZ 3H4-HRP were added (1:50, 1:100, 1:200, 1:400) followed by incubation for 30 min at 37°C; the plate was revealed and read as described above.

Competitive ELISA for synthetic gizzerosine using the anti-idiotype-idiotype-HRP system. Commercial polystyrene plates coated with mouse IgG were added with 100 μL/well of the supernatant from hybridomas secreting anti-idiotype antibodies of the following dilutions in 1% BSA borate buffer: 2D11 1:50; 2H5 1:200; 3A12 1:50. The plate was washed and saturated as described before. A 1:1 mixture of anti-GZ 3H4-HRP and GZ or histamine was incubated in Eppendorf tubes at the following concentrations: 10, 0.1, 0.01, and 0.001 μg/mL. The mixture was added to the plate (100 μL/well) and incubated for 1 h at 37°C; then, enzyme activity was revealed as described above.

ELISA using as conjugate the anti-idiotype antibody labeled with HRP

Titration of the 3A12-HRP anti-idiotype antibody. Labeling of the 3A12 anti-idiotype antibody with HRP was performed by means of a Pierce assay, following two labeling protocols.

The first protocol using MEA was described previously. The second protocol was as follows: 20 μL of *N*-succinymidil *S*-acetylthioacetate solution (SATA) was added to 1 mg/mL 3A12 monoclonal antibody in PBS. The mixture was incubated for 30 min at room temperature. Then 1 mL of 1 mg/mL SATA-antibody was added to the de-acetylation solution (hydroxylamine-HCl), and incubation was carried out for 2 h at room temperature. The preparation was filtered through a polyacrylamide column in order to desalt, and the protein concentrations were measured to identify the fractions containing the antibody. Then 1 mg/mL peroxidase activated with maleinimide was added to 1 mL of the antibody fraction. The mixture was incubated for 1 h at room temperature, dialyzed against PBS and finally stored at −20°C with Super Freeze Conjugate Stabilizer (Pierce) for peroxidase.

Titration of the anti-idiotype 3A12-HRP conjugate antibody. Commercial plates coated with mouse anti-IgG were incubated for 30 min at 37°C with 100 μL of BSA-borate buffer pH 9 or 1% PBS-BSA pH 7.2 for performing serial double dilutions of the anti-idiotype 3A12-G5-HRP conjugate via MEA or SATA. The plate was washed with 0.02% PBS–Tween-20 and HRP activity was revealed with 100 μL/well of TMB-H_2O_2, incubating for 30 min at room temperature. The reaction was stopped with 100 μL 3N sulfuric acid, and the OD of the preparation was determined at 450 nm.

Determination of antibody dilutions to be used in the ELISA. Polystyrene plates coated with rabbit anti-IgG serum were incubated overnight at 4°C with different concentrations of the anti-GZ 3H4 antibody (2.5, 5, 10, 15, and 20 μg/mL) and washed with 0.02% PBS-Tween-20. The plates were saturated for 1 h at 37°C with ascitic fluid, diluted 1:50 of a non-related IgG (anti-Acro C5F10 monoclonal antibody); washing and different dilutions of the anti-idiotype 3A12-HRP conjugate were added (1:100; 1:1,000; 1:10,000; 1:25,000; 1:50,000; 1:100,000). The plates were incubated for 30 min at 37°C and revealed as described before.

Competitive ELISA for determination of gizzerosine using the idiotype-anti-idiotype-HRP system. Plates coated with mouse anti-IgG were incubated at 4°C overnight with 100 μL/well of 5 μg/mL anti-GZ 3H4 antibody in 1% BSA borate buffer. The plates were washed and saturated as described above, then 100 μL/well of a 1:1 mixture of 3A12-HRP conjugate was added, as well as solutions of gizzerosine, histidine, lysine, or histamine (0.01–500 μg/mL), incubating for 1 h at 37°C. Finally, they were washed and revealed as above.

The result were plotted in a semilogarithmic graph as the mean values of the absorbance obtained for the different amines and amino acid versus each hapten concentration added in solution.

Idiotype anti-idiotype ELISA with a sample of fish meal contaminated with synthetic gizzerosine

Sample preparation. The fish meal sample (brown fish meal no. 119, provided by Fundación Chile, Santiago, Chile) was processed according to Okasaki et al.[25] with modifications. The histamine and fat were extracted by vigorous stirring from 5 g of fish meal with 5 mL of methanol. Then the sample was centrifuged at 2,500 rpm for 20 min, the supernatant was discarded, and the pellet dried at 25°C for 24 h. Subsequently, 200 μg of fish meal were placed in a glass ampoule filled with 1 mL of 6N HCL (Pierce) and contaminated with known concentrations of GZ: 1, 5, 10 and 20 μg/mL. The ampoule was sealed in N_2-saturated atmosphere and the sample was incubated for 24 h at 110°C, then centrifuged for 20 min at 2,500 rpm and neutralized with 25% ammonia (Merck). The mixture was dried and resuspended, adding 500 μL sodium borate buffer pH 9.0. It was then centrifuged for 10 min at 12,000 rpm. The supernatant was stored and the pellet was washed three times with 100 μL of the buffer used before. Supernatants were used to determine the percentage of gizzerosine recovered in each one.

Idiotype anti-idiotype competitive ELISA. The competitive ELISA for determining GZ was performed by mixing 50 μL/well of the sample obtained as described above with 50 μL of the anti-idiotype 3A12-HRP conjugate diluted 1:25,000 in plates previously coated with 5 μg/mL of anti-GZ 3H4 monoclonal antibody. In parallel, a standard curve in duplicate was prepared with known concentrations (between 0 and 100 μg/mL) of gizzerosine in borate buffer pH 8.0. The plate was incubated 1 h at 37°C and then washed three times with 0.02% PBS-Tween-20; enzyme activity was revealed with 100 μL/well of TMB-H_2O_2, incubating for 30 min at 37°C. The reaction was stopped with 100 μL of 3N sulfuric acid, and the optic density was read at 450 nm.

The GZ concentration in the samples were read from the calibration curve in a semilogarithmic graph where the mean values of the absorbance obtained for the standards were plotted versus GZ concentration.

To calculate the percentage of GZ recovery in fish meal the following formula was used:

$$\% \text{ of GZ recovery} = \frac{(GZ - S_c) - (GZ - S_u)}{(GZ - S_a)} \times 100$$

where $GZ - S_c$ is GZ concentration in sample (S) contaminated with synthetic GZ; $GZ - S_u$ is GZ concentration in uncontaminated fish meal; and $GZ - S_a$ is GZ concentration added to the fish meal.

RESULTS

Development of anti-idiotype anti-GZ 3H4 antibodies

To develop hybridomas secreting anti-idiotype monoclonal antibodies, BALB/c mice were immunized with the anti-GZ 3H4 antibody coupled to hemocyanin from *Concholepas concholepas*, using glutaraldehyde. The humoral immune response was determined by means of passive agglutination in microwell plates, containing latex particles coated with the idiotype (latex anti-GZ 3H4) and serum from immunized animals. As control we used latex coated with a non-related monoclonal (latex-anti-Acro C5F10) of the same idiotype as the antibody under study (IgG_1). The titer of serum antibodies, defined as the last dilution of the serum showing agglutination (assigned as +1), was $1:2^{12}$ dilutions. This result was considered adequate and the development of hybridomas secreting anti-idiotypes was started.

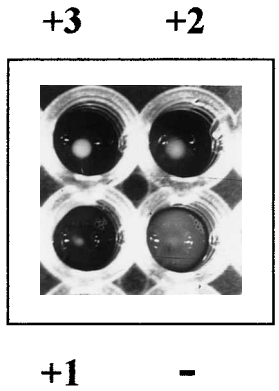

FIG. 1. Passive latex agglutination assay. Photography of an area of a U-bottom plate, showing the agglutination reaction of latex using the idiotype-anti-idiotype system. It was scored visually, assigning an arbitrary scale from +1 to +3 and (−) as follows: (+3) fully agglutinated latex and transparent supernatant; (+2) agglutinated latex and turbid supernatant; (+1) weakly agglutinated latex and turbid supernatant; (−) without agglutination.

For the primary selection of hybridomas secreting anti-idiotype anti-GZ 3H4 antibodies, the agglutination procedure described before was used. The agglutination capacity of 566 hybridoma supernatants was determined using latex-anti GZ 3H4 and latex-anti Acro C5F10 as control; 34 positive supernatants showed specific activity with intensities between +1 to +3, as shown in Figure 1 for some supernatants.

As a first approach to select the anti-idiotype antibodies for a competitive ELISA to gizzerosine, i.e., anti-idiotype antibodies whose binding to the anti-GZ 3H4 monoclonal would be displaceable by GZ in solution, the 34 antibodies under study were analyzed by RIA, co-incubating the components of the reaction mixture (equilibrium conditions). Results are presented in Figure 2, as a plot of the percentage of inhibition of the binding of ^{125}I-GZ to the anti-GZ 3H4 antibody with each anti-idiotype antibody. Twenty-four antibodies (gray bars) did not inhibit the binding of gizzerosine to the specific antibody (spotted gray bar), while 10 (black bars) showed variable degrees of inhibition (48.5%–96.1%). These anti-idiotype supernatants were subsequently evaluated in a RIA, preincubating the idiotype and anti-idiotype (non-equilibrium condition) and the results were confirmed for six of them, named 2D11, 2HG, 3A12, 3B3, 5C2, and 5C8. From this panel, we selected the anti-idiotype antibodies 2D11, 2H6, and 3A12, because they exhibited a high percentage of inhibition and a rapid and stable growth after recloning.

Thereafter, the three anti-idiotype antibodies selected were characterized using an inhibition assay (passive latex agglutination), where we evaluated the capacity of gizzerosine (0.001–10 μg/mL) to inhibit the binding of each anti-idiotype to latex activated with anti GZ 3H4 idiotype. Another assay was carried out as control, by adding similar concentrations of histamine, an amine structurally related to GZ that is also found in fish meal. The results of this assay are shown in Table 1. The three anti-idiotype monoclonals showed a dose-response specific inhibition of the passive agglutination reaction with GZ. The addition of increasing concentrations of histamine had no effect whatsoever.

Establishment of a competitive ELISA for gizzerosine based on the idiotype-anti-idiotype system

To develop this ELISA and to set the conditions of the assay, a tracer was needed, either the idiotype or the anti-idiotype coupled to an enzyme.

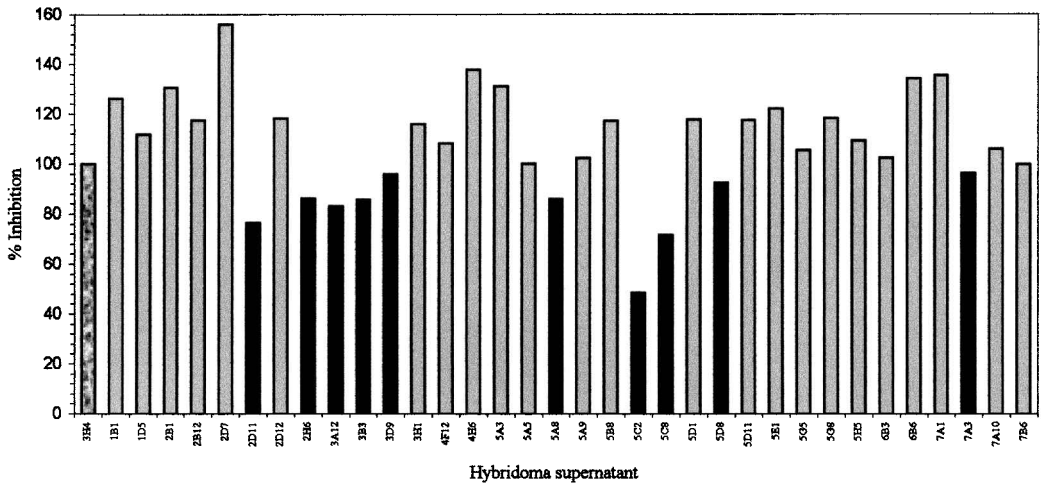

FIG. 2. Competitive RIA co-incubating ^{125}I-GZ and the supernatants of the hybridomas secreting potential anti-idiotype antibodies. The specific monoclonal antibody (anti-GZ 3H4) was incubated overnight with ^{125}I-GZ and the anti-idiotypic hybridoma supernatants. Bars represent duplicates. One hundred % was assigned to the positive control (spotted gray bar), corresponding to the anti-GZ 3H4 incubated with ^{125}I-GZ. In the majority of cases, inhibition did not occur (gray bars); inhibition was observed in the range of 45–98% in the case of supernatants represented by black bars.

TABLE 1. INHIBITION OF THE PASSIVE LATEX AGGLUTINATION ASSAY BY ANTI-GZ 3H4 ANTI-IDIOTYPE MABS

		Concentration (μg/mL)				
Anti-Idiotype antibody	*Hapten*	*0*	*0.4*	*4*	*40*	*100*
2D11	Gizzerosine	+2	+2	+2	−	−
	Histamine	+2	+2	+2	+2	+2
3A12	Gizzerosine	+2/+3	+2/+3	+2	−	−
	Histamine	+2/+3	+2/+3	+2/+3	+2/+3	+2/+3
2H6	Gizzerosine	+3	+3	+3	+2	+1
	Histamine	+3	+3	+3	+3	+3

Fifteen μL of a PBS solution containing increasing concentrations of gizzerosine or histamine and 15 μL of latex coated with the anti-GZ 3H4 monoclonal antibody were co-incubated for 30 min at 37°C in 96-well U-bottom plate. Then 15 μL of each anti-idiotype hybridoma supernatant was added and incubated for 1.5 h. The results of agglutination reaction were read visually, assigning an arbitrary scale from +1 to +3, as follows: (+3) fully agglutinated latex and transparent supernatant; (+2) agglutinated latex and turbid supernatant; (+1) weakly agglutinated latex and turbid supernatant; (+) latex without agglutination.

ELISA using the anti-idiotype antibody coupled to a solid phase and the idiotype antibody as a tracer (anti-GZ 3H4-HRP)

The anti-GZ 3H4 antibody was conjugated with HRP by means of a commercial kit that uses MEA. To establish the working dilution, the titer was determined by a direct ELISA in plates coated with mouse anti-IgG. The reaction was performed in borate buffer pH 9.0 and in PBS pH 7.2. A titer of about 1:64 dilutions was found for both buffers. In the same way, to determine the working concentration of the supernatant of each anti-idiotype antibody, the titers for ELISA were also determined, which were 1:50, 1:200, and 1:50 for the anti-idiotypes supernatants 2D11, 2H6, and 3A12, respectively.

To develop the ELISA for GZ, increasing concentrations of GZ, 0.01–10 μl/mL, were co-incubated with the tracer (anti-GZ 3H4-HRP) diluted 1:50. Then the supernatant of each anti-idiotype was added at the pre-established dilution. The specificity control was a similar assay with histamine. Results are presented in Figure 3, where the OD was plotted at 450 nm with respect to the concentration of each hapten under study. The three anti-idiotype antibodies showed a specific dose-response with GZ, which was linear between 0.1 and 10 μg/mL GZ; the most sensitive anti-idiotype antibody was 3A12, as shown by its higher slope.

ELISA using the idiotype antibody bound to the solid phase and the anti-idiotype antibody as the tracer (3A12 HRP anti-idiotype)

The 3A12 anti-idiotype monoclonal antibody was conjugated to peroxidase, by means of a kit that uses MEA and also by an alternative procedure that uses SATA. As described above, the dilution of each conjugate was first determined in borate buffers pH 9.0 and PBS pH 7.2. The results presented in Figure 4 show that in both buffers the titer of the conjugate using SATA was much higher (1:120,000) than that obtained by labeling with MEA (1:250 dilutions).

To determine the concentration of the antibodies needed to further develop the idiotype anti-idiotype ELISA, plates with increasing concentrations of the anti-GZ 3H4 antibody (2.5, 5, 15, and 20 μg/mL) were activated and then revealed with increasing dilutions of the SATA labeled 3A12-HRP anti-idiotype (1:10,000, 1:25,000, 1:50,000, 1:100,000). The optimal conditions found were 5 μg/mL of idiotype antibody and a conjugate dilution of 1:25,000.

Once the aforementioned basic parameters had been standardized, a competitive ELISA with GZ was developed; histidine, lysine and histamine were included as controls. Results are presented in Figure 5, where the OD was plotted versus the concentrations of the different haptens. A specific dose-response effect was observed with GZ, which was linear between

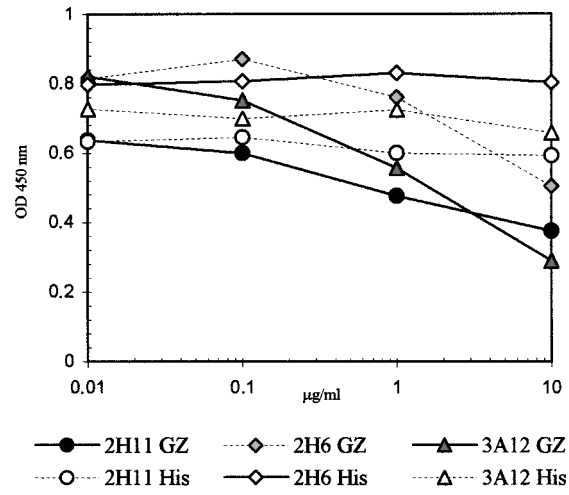

FIG. 3. Competitive ELISA based on the idiotype-anti-idiotype system. Plates coated with mouse anti-IgG were incubated overnight at 4°C with supernatant of the anti-idiotypic antibodies (2H11, 2H6 and 3A12) previously titrated. Subsequently, gizzerosine (GZ) or histamine (His) was co-incubated for 30 min with the idiotype-conjugate (anti-GZ 3H4–HRP antibody), and the preparation was added to the plate to start competition. Color was developed during 30 min with TMB and H_2O_2. The reaction was stopped with 100 μL of 3N sulfuric acid, and the optic density was read at 450 nm. Each point in the curves represents duplicates expressed as a mean, with a variation of less than 10%.

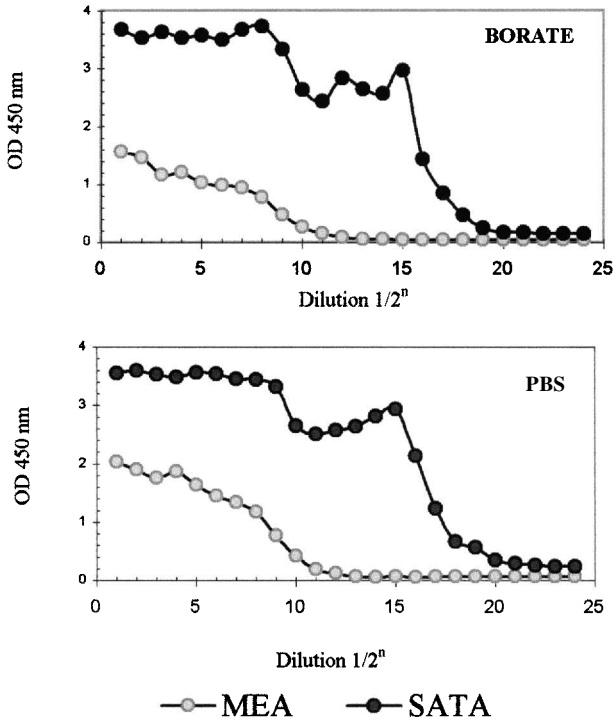

FIG. 4. Direct ELISA evaluation of the conjugate 3A12 anti-idiotypic antibody coupled to HRP via SATA and via MEA. Plates of 96-wells coated with mouse anti-IgG were incubated at 37°C for 30 min with serial dilutions of each conjugate and after washing with 0.02% PBS-Tween 20; color was developed during 30 min with TMB and H_2O_2. The reaction was stopped with 100 μL of 3N sulfuric acid, and the optic density was read at 450 nm.

0.1 and 10 μg/mL. When using histamine, a cross-reaction at concentrations over 10 μg/mL was observed. Lysine and histidine had no effect on the whole range of concentrations studied.

Evaluation of the idiotype-anti-idiotype-HRP ELISA with a sample of fish meal contaminated with gizzerosine

Gizzerosine at known concentrations was added to a brown fish meal sample, prior to the acid extraction treatment and then treated by acid hidrolysis as described in Materials and Methods. Subsequently, the percentage of recovery was determined by the assay described here. The results presented in Table 2 show percentages of GZ recovery corresponding to 198%, 90%, 55.8%, and 53% for each GZ concentration added (0, 1, 5, 10, and 20 μg/mL, respectively) indicating that the fish meal used in this experiment had a basal GZ concentration around 4.86 μg/mL.

DISCUSSION

At present, it is not possible to determine gizzerosine quantitatively, and it has been effectively diagnosed only by means of an indirect bioassay that uses Broiler chicks. It is known that ELISA assays based on monoclonals have enormously simplified diagnoses in clinics and they are being successfully used for the determination of food toxins and residues.[4,7]

We have developed a competitive ELISA based on the anti-GZ 3H4 monoclonal antibody,[3] but this assay has some limitations, because it depends on the availability of synthetic GZ, which is quite expensive and difficult to obtain. Further, the small size of the molecule and the presence of a secondary amino group complicate its labeling with enzymes. This led us to design a competitive ELISA based on an idiotype-anti-idiotype system which could solve some of these problems: it does not depend exclusively on synthetic gizzerosine because the tracer is an antibody coupled to an enzyme. At present, a number of highly stable and reproducible commercial kits for labeling immunoglobulins are readily available.

A simple system using latex particles activated with the idiotype antibody was employed to evaluate the sera and to select the anti-idiotype antibodies. Anti-idiotype antibodies are detected by a passive agglutination reaction, a system that has been successfully used to select anti-idiotype antibodies against the T4 thyroid hormone.[2] Using this procedure, 34 potential anti-idiotype antibodies were selected which were subsequently tested on a competitive RIA, often used for the selection and characterization of anti-idiotype antibodies.[2,6,27,30] In this way, three antibodies were selected by means of an inhibition reaction of the agglutination, adding gizzerosine in solution and, in parallel, histamine at the same concentrations. It was found that gizzerosine inhibits the agglutination between the latex bound idiotype and the anti-idiotype in solution in a dose-dependent manner. Taking together the latex bead inhibition of agglutination and the RIA results, we concluded that the anti-idiotype antibodies may have the gizzerosine image at their active site.

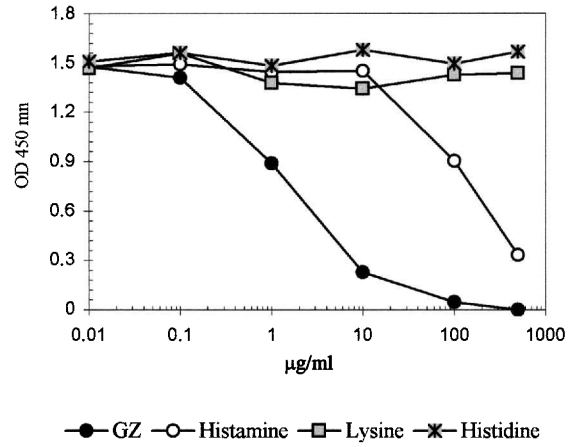

FIG. 5. Competitive ELISA based on the idiotype-anti-idiotype-HRP system. A plate coated with mouse anti-IgG was incubated overnight at 4°C with 5 μg/mL of the idiotypic antibody (anti-GZ 3H4). Then 3A12-HRP anti-idiotypic antibody diluted 1:25.000 was added with histamine, histidine, and lysine solutions at known concentrations. Color was revealed by incubating for 30 min with TMB and H_2O_2. The reaction was stopped with 100 μL of 3N sulfuric acid, and the optic density at 450 nm was read. The points in the curves represent duplicates expressed as a mean, with a variation of less than 10%.

TABLE 2. GIZZEROSINE DETERMINATION FROM A SAMPLE OF FISH MEAL BY IDIOTYPE ANTI-IDIOTYPE COMPETITIVE ELISA

Amount of GZ added (μg/mL)	*GZ detection (μg/mL) in ELISA*	*Percentage GZ recovered*
0[a]	4.86	0
1	6.84	198%
5	9.36	90%
10	10.44	55.8%
20	15.46	53%

A fish meal sample from which histamine was previously extracted, was contaminated with gizzerosine and submitted to acid hydrolysis. The recovery of gizzerosine was determined in the present assay as described in Materials and Methods. Samples were run in duplicate with an experimental error lower than 10%.

[a]Basal GZ concentration of the fish meal used in this experiment.

It is worth noting that the inhibition assay of latex passive agglutination could be a useful tool to determine gizzerosine during the processing of fish meal, since it is rapid and easy to achieve, and does not call for sophisticated instruments.

To develop the ELISA based on the idiotype-anti-idiotype system for GZ determination, one of the antibodies had to be labeled with an appropriate enzyme to be used as the tracer. We used peroxidase for labeling, because it has higher sensitivity and therefore it is more specific than other enzymes such as alkaline phosphatase and β-galactosidase Portsman et al.[28]

The results of the anti-idiotype-idiotype-HRP ELISA showed that gizzerosine produced a linear displacement of the binding between the tracer and the anti-idiotypes, in a range of 0.1–10 μg/mL. Although this range was similar for the three anti-idiotypes, the 3A12 antibody was selected for its highest sensitivity. An ELISA was done in the opposite way, i.e., we labeled the 3A12 anti-idiotype with peroxidase and the idiotype was bound to the solid phase, and the same range of sensitivity was found with synthetic gizzerosine. However, a better reading was found, which is explained because the labeling system of the antibodies was optimized using SATA, thus adding sulphydril groups to the antibody to increase the rate of enzyme molecules per antibody.

The sensitivity obtained in the present assay is consistent with that reported by Sugahara et al.,[33] who showed toxic GZ levels around 1 μg/mL, in experiments where synthetic GZ was added in known concentrations to the Broiler chickens diet. Wagener et al.[40] developed a HPLC method to determine gizzerosine, and obtained a sensitivity of 10 mg/K. Notwithstanding, they analyzed 17 samples of fish meal hydrolyzed with HCl—some of which had caused severe erosion in the chicken gizzard—and did not find gizzerosine in any of them. This suggests that the fish meal causing gizzard erosion would have gizzerosine levels under 10 mg/K. Rosselot et al.,[29] who determined GZ in fish meal by RIA, found that this compound was present in a range of 1.6–26 μg/mL in fish meal, with a score between 1 (no erosions) and 5 (severe erosions), respectively.

To determine the specificity of the ELISA based on the idiotype-anti-idiotype antibodies, we tested other substances structurally related to the gizzerosine, and also present in fish meal, such as histidine, lysine and histamine. We found that only histamine exhibited cross-reaction, in concentrations over 10 μg/mL. It is important to consider this cross-reaction because histamine is found in fish meal in large amounts in relation to gizzerosine, hence histamine may be easily extracted with routine techniques, because histamine is soluble both in PBS and methanol.[36]

Gizzerosine is mostly bound to proteins, since it was isolated by treating fish meal with acid hydrolysis.[25] Therefore, to develop the assay proposed in this work, after extracting histamine with methanol, the sample was hydrolyzed and then neutralized. We believe that the neutralization process of the sample has to be improved, because a fine precipitate was generated that affects the results, as shown by the recovery experiments. Nonetheless, the results showed that acid hydrolysis neither destroys nor modifies the gizzerosine and, apparently, no other compounds of the fish meal interfere with the system.

Although the results of GZ recovery obtained in this ELISA are promising, they show that the extraction procedure of the sample needs to be improved, and experiments are in progress. Also, for optimal recovery analysis, white fish meal is required, because it is regarded as relatively non-toxic in the development of gizzard erosion. On the other hand, brown fish meal, as is the case of our sample, is often associated with the formation of gizzerosine, due to a higher histidine content. Our results in the recovery experiment are comparable to those of Hsu and Chu[13] for aflatoxin with an idiotype-anti-idiotype ELISA. Furthermore, commercial assays of the ELISA format, currently used for determining histamine in fish meal samples and some fermented products (R-Biopharm, Darmstadt, Germany), show variable recovery rates, that fall within the range of our results. We think that such variations may be largely explained by the complexity of the food samples, as in the case of fish meal.

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