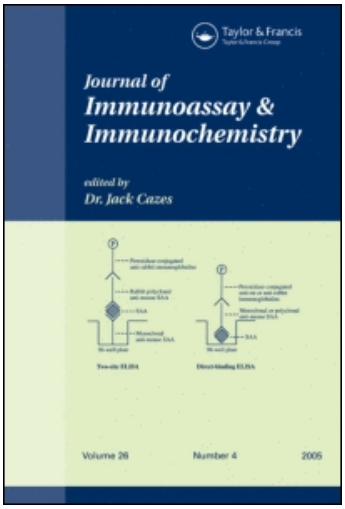

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Monoclonal Antibody-Based Enzyme-Linked Immunosorbent Assay for Glycyrrhizin and Its Aglicon, Glycyrrketic Acid

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MONOCLONAL ANTIBODY-BASED ENZYME-LINKED IMMUNOSORBENT ASSAY FOR GLYCYRRHIZIN AND ITS AGLICON, GLYCYRRHETIC ACID

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ABSTRACT

Monoclonal antibodies (MoAbs) specific for glycyrrhetic acid (GA) were prepared and characterized. Obtained MoAbs (AGA-1, AGA-3, and AGA-6) reacted with GA dose-dependently, but not with glycyrrhizin (GL), carbenoxolone, and steroids. Next, an enzyme-linked immunosorbent assay (ELISA) system using AGA-1 was established. The standard curve showed good linearity between 0.01 and 1000 ng/ml of GA, and the detection limit was 5 pg/ml. Recovery, and intra- and interassay variations of this assay system was satisfactory. GL was also measurable quantitatively after acid-hydrolysis of the samples. The developed ELISA system would be useful to determine GL and GA in biological samples or drugs as an alternative method to high performance liquid chromatography (HPLC).

(KEY WORDS: glycyrrhizin, glycyrrhetic acid, monoclonal antibody, inhibition ELISA)

INTRODUCTION

Glycyrrhizin (GL) is a principal constituent of Glycyrrhizae Radix, which is a naturally occurring Chinese medicine with anti-

inflammatory properties (1). GL and its aglycon, the 18β -glycyrrhetic acid (GA), have anti-ulcerous (2), anti-viral (3-6), anti-protein kinase (7-9), and interferon inducing activities (10). A preparation of GL with glycine and cystein (designated as Stronger Neo-Minophagen C; SNMC) has been widely used in the treatment of chronic hepatitis (11, 12). When patients receive large doses of SNMC over a long period of time, they often exhibit pseudo-aldosteronism with hypertension, edema, and hypokalemia (13). GA has been reported to produce these side-effects because of its aldosterone-like effects (14). To avoid these side-effects while maintaining therapeutic effect, the monitoring of serum GL and GA levels in patients is essential.

High-performance liquid chromatography (HPLC) (15-18) has been widely used to determine GL and GA, though this technique is laborious as well as time consuming. To develop a simple, rapid, sensitive and specific determination system of GL and GA, we prepared monoclonal antibodies (MoAbs) against GA, and obtained three MoAbs (AGA-1, AGA-3, and AGA-6) that specifically react with GA.

In this paper, preparation and characterization of these MoAbs, and establishment of an enzyme-linked immunosorbent assay (ELISA) system for determination of GL and GA are described.

MATERIALS AND METHODS

Chemicals

N-Glycyrrhetylglycine (GA-Gly) was kindly provided by Minophagen Pharmaceuticals Co. Ltd. (Tokyo, Japan). N-Hydroxysuccini-

mide (NHS) was purchased from Wako Pure Chemical (Osaka, Japan). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) was purchased from Dojin Chemical Research (Kumamoto, Japan). Keyhole lympet hemocyanin (KLH) and bovine serum albumin (BSA) were purchased from Sigma (St Louis, MO, USA).

Preparation of Immunogen and Antigen

N-hydroxysuccinimidyl glycyrrhetylglycynate (GA-Gly-NHS) was prepared by the method reported by Kanaoka et al. (19). Briefly, EDC (30 μ mol) was added to a stirred mixture of GA-Gly (20 μ mol), NHS (20 μ mol), and 80% dioxane (2 ml), then the mixture was stirred for 2 hr at room temperature. After the addition of water, the mixture was extracted by ethylacetate (10 ml). The extract was washed with water, dried with sodium sulfate and evaporated in vacuo.

A solution of GA-Gly-NHS in dimethylsulfoxide (0.3 ml) was added to a phosphate buffered-saline (PBS) solution of KLH (carrier protein of immunogen) or BSA (carrier protein of antigen), and the mixture was stirred for 24 hr at 4℃ . The resulting solution was dialyzed for 48 hr against PBS with three changes a day. The dialysate was lyophilized and stored at -20℃ until use.

Immunization and Hybridoma Production

Female BALB/c mice (6 weeks old) were immunized intraperitonealy and subcutaneously with GA-Gly-KLH conjugate (50 μ g protein/mouse) dissolved in PBS emulsified with an equal volume of Freund's complete adjuvant. The same immunization using Freund's

incomplete adjuvant was repeated twice with a 14-days interval. Seven days after the third immunization, the mouse received a booster intravenous injection with 50 μg/mouse of GA-Gly-KLH in 0.2 ml of PBS and was sacrificed 3 days later.

Spleen cells of the mice were fused with Sp2/0-Ag14-K13 myeloma cells in the presence of 50% (w/v) polyethyleneglycol (PEG) 4000. Fused spleen-myeloma cells were selected by culturing in HAT medium (RPMI medium containing 2 mM glutamine, 0.2% glucose, 1 mM pyruvic acid, penicillin at 100 U/ml, streptomycin at 100 μg/ml and 10% heat-inactivated fetal calf serum (FCS) (Standard medium) supplemented with 100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine). Cultures were maintained in a 5% CO_2 incubator at 37℃. When hybridoma colonies appeared, they were expanded and maintained in HT medium (HAT medium without aminopterin) and finally in the standard medium.

The supernatants of hybridoma cell cultures were screened by direct ELISA for the production of anti-GA antibodies. A GA-Gly-BSA conjugate was used as an antigen to exclude the antibodies reactive with KLH. Then selected hybridomas were cloned by a limiting dilution method using mouse thymocytes as feeder cells.

Inhibition ELISA

Aliquot of 100 μl of a GA-Gly-BSA conjugate dissolved in PBS (10 μg protein/ml) was fixed to the polyvinyl chloride microtiter wells (MS-7196 F, Sumitomo Bakelite, Tokyo, Japan) by incubation for overnight at 4℃. The wells were then filled with 100 μl of 1% BSA in PBS. After 1 hr incubation at 37℃, the solution

was discarded and 50 μ l of serially diluted GA solutions or serum specimens were added followed by the addition of an equal volume of AGA-1 solution (5 μ g/ml) and incubated for 1 hr at 4℃ . After the wells were washed 3 times with 0.05% Tween 20-PBS and twice with PBS, the wells were filled with 100 μ l of 1:3000 diluted alkaline phosphatase (ALP) labeled goat anti-mouse IgG (Tago, Burlingame, CA, USA) followed by incubation for 45 min at 4℃ . After the same washing procedure as above, 100 μ l of p-nitrophenylphosphate (Sigma 104 phosphatase substrate) dissolved in 1 M diethanolamine buffer (pH 9.8) (1 mg/ml) was added and incubated for 30 min at 37℃ . The color development was stopped by addition of 2 M sodium hydroxide, and the absorbance of the resultant p-nitrophenol in each well was measured at 405 nm by an EIA reader (Model 2550, Bio-Rad, Richmond, CA, USA).

RESULTS

Specificity of MoAbs

Specificity of MoAbs (all of these MoAbs were IgG_1 type immunoglobulin, and possessed a κ light chain) was determined by the inhibition ELISA (Table 1). The inhibitors used were GA, GL, carbenoxolone, and some steroids. The concentration of GA required for inhibiting 50% of the binding of these MoAbs to GA-Gly-BSA was 1.3 - 30 ng/ml. Other compounds had no inhibitory effect even when they were used at 1000 ng/ml. Next, the cross-reactivity against the compounds, as shown in Fig. 1, that have a structure similar to GA was determined (Table 2). The MoAbs had no or very slight cross-reactivity for GL, Olean-12-ene-3,11-dioxo-30-

TABLE 1

Specificity of Anti-GA Monoclonal Antibodies*

Inhibitor	AGA-1	AGA-3	AGA-6
GA	1.3	22	30
GL	>1000	>1000	>1000
Carbenoxolone	>1000	>1000	>1000
17 β -Estradiol	>1000	>1000	>1000
Progesterone	>1000	>1000	>1000
Testosterone	>1000	>1000	>1000
Aldosterone	>1000	>1000	>1000
Cortisone	>1000	>1000	>1000
Hydrocortisone	>1000	>1000	>1000

*Values are expressed as concentrations (ng/ml) required for 50% inhibition of the binding of MoAb to GA-Gly-BSA.

oic acid (3-oxo GA), and Olean-12-ene-3α -hydroxy-11-oxo-30-oic acid (3α -hydroxy GA), in which the 3β -hydroxy group of GA is substituted by other structures. The MoAbs also had slight cross-reactivity for Olean-12-ene-11-oxo-3β ,30 diol (30-ol GA) and 18α H-Olean-11-oxo -12-ene-30-oic acid (18α -H GA). These results suggest that the MoAbs recognize the entire structure of GA, especially the 3β -hydroxy group of GA.

Establishment of ELISA of GA

ELISA for determination of GA was established. AGA-1 was used because its specificity for GA is the highest among obtained MoAbs. The standard curve showed good linearity between 10 pg/ml and 1000 ng/ml, and the minimum detection limit was approximately

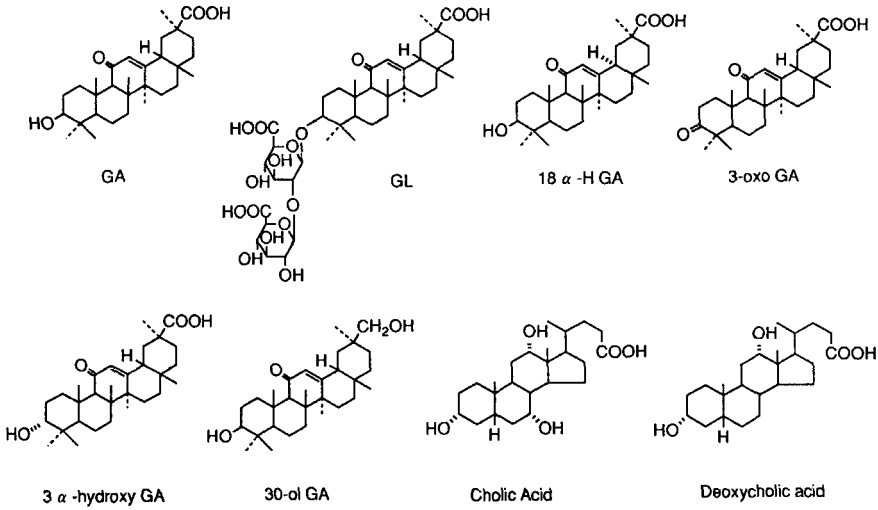

FIGURE 1 Chemical structure of compounds used for analysys of cross-reactivity of anti-GA monoclonal antibodies. 18 α -H GA; 18 α H-Olean-11-oxo-12-ene-30-oic acid, 3-oxo GA; Olean-12-ene-3,11-dioxo-30-oic acid, 3 α -hydroxy GA; Olean-12-ene-3 α -hydroxy-11-oxo-30-oic acid, 30-ol GA; Olean-12-ene-11-oxo-3 β ,30 diol

TABLE 2

Cross-reactivity of Anti-GA Monoclonal Antibodies*

Inhibitor	AGA-1	AGA-3	AGA-6
GA	16	33	44
GL	>1000	>1000	>1000
18 α -H GA[a]	88	88	98
3-oxo GA[b]	832	957	>1000
3 α -hydroxy GA[c]	695	677	793
30-ol GA[d]	437	477	425
Cholic Acid	>1000	>1000	>1000
Deoxy cholic acid	>1000	>1000	>1000

*Values are expressed as concentrations (ng/ml) required for 50% inhibition of the binding of MoAb to GA-Gly-BSA.

[a]18 α -H GA; 18 α H-Olean-11-oxo-12-ene-30-oic acid

[b]3-oxo GA; Olean-12-ene-3,11-dioxo-30-oic acid

[c]3 α -hydroxy GA; Olean-12-ene-3 α -hydroxy-11-oxo-30-oic acid

[d]30-ol GA; Olean-12-ene-11-oxo-3 β ,30 diol

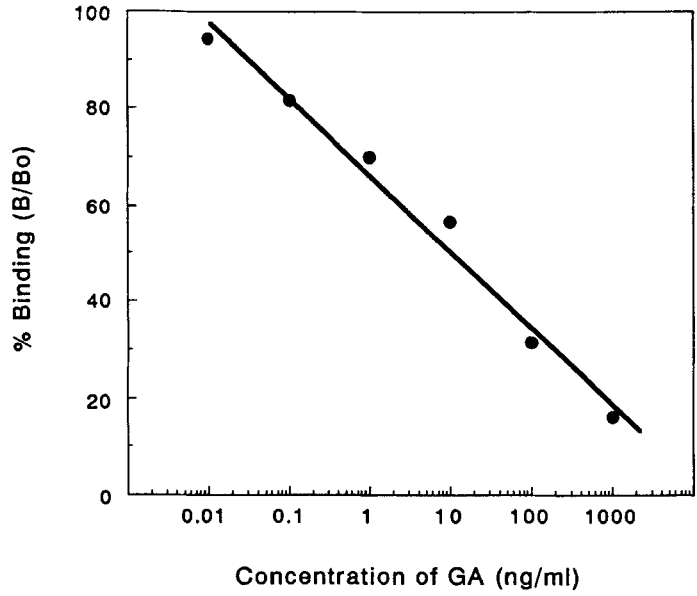

FIGURE 2 Standard curve for quantitation of GA. Data are given in the form of a log-logit plot. Points: mean values of triplicate determination. Absorbancy for no added GA (Bo) was 0.754 ± 0.043.

5 pg/ml (Fig. 2). The coefficients of intra- and inter-assay variations were 7.2 and 9.8%, respectively (data not shown).

Validity of ELISA Applied to Determine GA and GL in Serum

The validity of ELISA was estimated by the recovery of added GA from serum. Samples were extracted with chloroform to avoid the interference with serum components, and GA was determined by the ELISA. The recovery of GA was found to be between 91 and 114% (Table 3). It is known that either acid-hydrolysis or β -glucuronidase digestion of GL generates GA. We tried to deter-

TABLE 3

Recovery of GA from Serum with Chloroform Extraction

Added (ng/ml)	Measured (ng/ml)	Recovery (%)
0	0	-
32	32	100
63	67	106
125	142	114
250	254	102
500	453	91
1000	983	98

mine GL content as an acid-hydrolyzed product using this ELISA. Serum samples which contained exogeneously added GL were acidified with an equal volume of 4 M hydrochloric acid and boiled for 10 min followed by the extraction with chloroform. Recovery of GL extracted with chloroform from serum was found to be between 86 and 114% (Table 4). From these results, it is revealed that this ELISA has an accuracy sufficient for determination of serum GA and GL levels.

Determination of Serum GL and GA Levels after Intravenous Administration of a GL Preparation

To evaluate the usefulness of the developed ELISA system in clinical use, serum GL and GA levels after intravenous administration of a GL preparation (SNMC) was examined. Forty ml of SNMC which contains 80 mg of GL was given intravenously to a healthy

TABLE 4

Recovery of GL from Serum after Acid-Hydrolysis followed by Chloroform Extraction

Added (ng/ml)	Measured[a] (ng/ml)	Recovered[b] (ng/ml)	Recovery (%)
0	0	-	-
32	19	33	104
63	40	70	111
125	81	142	114
250	149	261	104
500	250	438	88
1000	490	858	86

[a]Values are expressed as concentrations of GA determined by the inhibition ELISA.

[b]Values are expressed as concentrations of GL calculated from 1.75 times (the molecular weight ratio of GL and GA) of the "Measured" values.

individual, and serum GL and GA levels were determined at 1, 2, 4, 6, 8, 10, 12, 24, 36, and 48 hr after administration (Fig. 3). Serum GL level decreased rapidly and was eliminated completely by 24 hr after administration. On the other hand, GA began to appear in serum 8 hr after administration and reached to a maximum level (88-98 ng/ml) between 10 and 24 hr, and was eliminated from serum by 36 hr after administration.

DISCUSSION

Although HPLC has been used to determine GL and GA in biological samples or drugs (15-18), this method is laborious, and time

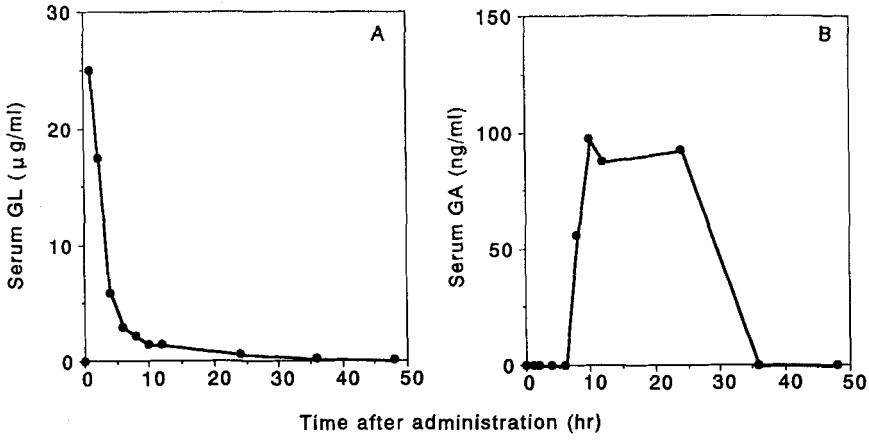

FIGURE 3 Serum GL and GA levels in a healthy individual after intravenous administration of SNMC. Serum GL level (A) was determined after acid-hydrolysis of samples followed by chloroform extraction, and serum GA level (B) was determined after chloroform extraction.

consuming when a large number of samples have to be determined. On the other hand, immunoassay has some advantages such as rapidity and simplicity. Enzyme-immunoassay for GL (20) and GA (21), and radio-immunoassay for GA (22) have been reported previously, though the specificity and sensitivity were not satisfactory.

To develop a more sensitive and specific assay system for GA and GL, we prepared MoAbs against GA. GA-30-glycine was used as a hapten of immunogen to obtain antibodies against GA, because 3β -hydroxy group is the only position that discriminates GA from GL. As a result, MoAbs reactive with GA but not with GL were obtained, and from analysis of cross-reactivity, recognition site is considered to be the 3β -hydroxy group of GA.

As for the ELISA established here, used MoAb had no cross-reactivities to the naturally occurring compounds that have structure similar to GA (e.g. steroids). Five pg/ml of GA was detectable, and the coefficients of intra- and interassay variations were within 10% (data not shown). These results demonstrate that this ELISA is more sensitive and specific than the methods as previously reported (21, 22). Using this ELISA, GA and GL in serum samples were determined accurately, and the change of GL and GA levels after an intravenous admonistration of a GL preparation could be monitored. In addition to the drug monitoring, this ELISA would be applicable for monitoring of GA levels which cause side-effects such as hypokalemia by coadministration of the drug and digitalis, as well as to determine the GL content in Chinese medicine.

ACKNOWLEDGEMENTS

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