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Monoclonal Antibody to 2-Amino-3-Methylimidazo(4,5-F)quinoline, a Dietary Carcinogen

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ABSTRACT

In order to investigate relationships between human carcinogenesis and dietary carcinogens, one hybridoma cell line secreting a monoclonal antibody against 2-amino-3-methylimidazo(4,5-f)quinoline (IQ), a dietary carcinogen, was produced by fusing splenocytes from Balb/c mice immunized with IQ-Lysine(Lys)-Ascaris protein conjugate. The subclass of monoclonal anti-IQ antibody was determined by double immunodiffusion using culture medium and identified as IgG_1. Monoclonal anti-IQ antibody was purified from ascites fluids of Balb/c mice with affinity chromatography on Protein A-Sepharose CL4B and analyzed concerning its cross-reactivity and sensitivity with RIA. Finally, we showed that our monoclonal antibody recognized IQ, 2-amino-3,4-dimethylimidazo(4,5-f)quinoline(MeIQ) and several β-carbolines more intensely and that the sensitivity to IQ was 23 nmol in 50% displacement.

Index Entries: Monoclonal antibody; dietary carcinogen; dietary comutagen; low molecular weight substance; immunology.

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INTRODUCTION

Mutagenic heterocyclic amines, a group of carcinogens and 1-methyl-9H-pyrido(3,4-b)indole (harman) and 9H-pyrido(3,4-b)indole (norharman) of β-carbolines, comutagens have been reported to be present in amino acid pyrolysis products, cooked foods, and cigaret smoke condensate (*1–6*). Mutagenic heterocyclic amines require metabolic activation, most probably by a cytochrome P-450, to exert their mutagenicity (*2,7*). Recently, most mutagenic heterocyclic amines have shown carcinogenic effects in animal experiments (*2,8,9*). Harman and norharman are nonmutagenic in the Salmonella test system. However, they have been reported to be comutagenic compounds that enhance the mutagenicity of mutagens and induce the mutagenic activity of nonmutagens (*10–12*).

Dietary components have been indicated as major causes of human carcinogenesis (*13,14*). In order to investigate the relationship between human cancer and dietary factors, we have evaluated the actual exposure levels of carcinogenic heterocyclic amines in the dialysate and plasma of patients with uremia, of whom the incidence of malignancy has been reported to be significantly higher than of normal subjects (*15–17*). By employing a high-performance liquid chromatography (HPLC) method, we demonstrated that several kinds of carcinogenic heterocyclic amines were actually present in the dialysate of patients with uremia (*18–22*) and showed that the levels of carcinogenic glutamic acid pyrolysis products, 2-amino-6-methyldipyrido(1,2-a:3′,2′-d) imidazole (Glu-P-1) and 2-amino-dipyrido(1,2-a:3′,2′-d)imidazole (Glu-P-2), and IQ-type heterocyclic amines, 2-amino-3-methyl-imidazo(4,5-f)quinoline (IQ) and 2-amino-3,8-dimethylimidazo(4,5-f)quinoxaline (MeIQx), in the plasma of uremic patients before induction of hemodialysis treatment were significantly higher than normal subjects (*22,23*).

In this paper, we show a technique available for development of antibodies to low molecular weight substances. At the same time, we report the production of a monoclonal antibody against IQ using the technique to investigate further the correlation between carcinogenesis and these carcinogens and that our antibody also recognizes 2-amino-3,4-dimethylimidazo(4,5-f)quinoline (MeIQ), harman and norharman.

MATERIALS AND METHODS

Materials

MeIQ, MeIQx, 2-amino-3,4,8-trimethylimidazo(4,5-f)quinoxaline (4,8-DiMeIQx), 2-amino-3,7,8-trimethylimidazo(4,5-f)quinoxaline (7,8-DiMeIQx), 2-amino-9H-pyrido(2,3-b)indole (Aα C), and 2-amino-3-methyl-9H-pyrido(2,3-b)indole (MeAα C) were kindly provided by Shigeaki Sato, National Cancer Center Research Institute (Tokyo, Japan). IQ, Glu-P-1

hydrochloride, Glu-P-2 hydrochloride, 3-amino-1,4-dimethyl-5H-pyrido-(4,3-b)indole (Trp-P-1) acetate, 3-amino-1-methyl-5H-pyrido(4,3-b)indole (Trp-P-2) acetate, quinoline, quinoxaline, 4-azabenzimidazole, L-lysine, L-tryptophan, L-glutamic acid (monosodium salt), and silica gel C-300 were purchased from Wako Pure Chemical Industries (Osaka, Japan); harman hydrochloride, norharman hydrochloride, 3,4-dihydro-1-methyl-9H-pyrido(3,4-b)indole-7-ol (harmalol) hydrochloride, 2,3,4,9-tetrahydro-1H-pyrido(3,4-b)indole (noreleagnine), bovine serum albumine (BSA), and hypoxanthine/aminopterin/thymidine (HAT) from Sigma Chemical Co. (St. Louis, MO); N^{α}, N^{β}-dicarbobenzoxy-L-lysine (Z·Lys(Z)) and water soluble carbodiimide (WSCD) from Peptide Institute, Inc. (Osaka, Japan); Freund's complete adjuvant from Difco Laboratories (Detroit, MI); polyethylene glycol (PEG) (mw 1,500) from Boehringer Mannheim (West Germany); peroxidase-goat anti-mouse immunoglobulin from Zymed Laboratories, Inc. (San Francisco, CA); and 1-methyl-7-methoxy-3,4-dihydro-9H-pyrido-(3,4-b)indole (harmaline) hydrochloride dihydrate, 7-methoxy-1-methyl-9H-pyrido(3,4-b)indole (harmine) hydrochloride hydrate, 1-methyl-9H-pyrido(3,4-b)indole-7-ol (harmol) hydrochloride dihydrate, and pristane from Aldrich Chemical Company, Inc. (Milwaukee, WI). All other chemicals were from commercial sources and of reagent grade quality.

Synthesis of Immunogen

In the first coupling step, peptide synthesis was carried out between the amino group of IQ and the carboxylic acid of Z·Lys(Z). As shown in Fig. 1, Z·Lys(Z) (1) (124 mg, 0.0003 mol) was dissolved in dry methylene chloride (3 mL) containing triethylamine (60 mg, 0.0006 mol). Diphenylphosphinous chloride (70 mg, 0.0003 mol) was added to the solution at 0°C under an argon atmosphere via a syringe. The reaction mixture was stirred at 0°C for 30 min. IQ (3) (60 mg, 0.0003 mol) was added to the mixture, and stirring was continued at 0°C for 30 min and then at room temperature overnight. Water was added to the mixture and then extracted with ethyl acetate (×3). The extracted solution was washed with a saturated NaCl solution and dried over with $MgSO_4$. The extract was evaporated to dryness and purified by silica gel C-300 (4g) chromatography. The solvent system was 5% methanol–chloroform (v/v). The purified sample (4) was dried by an evaporator and then confirmed to be IQ-Z·Lys(Z) by ^{1}H-NMR spectra (not shown). Z groups of Z·Lys(Z) were removed by using vanadium black.

The second coupling step was done between the amino group of lysyl residues of IQ-Lys and the carboxylic acid of Ascaris protein (Otsuka Assay Laboratories, Tokushima, Japan) (24–26) using WSCD. IQ-Lys (2.4 mg, 7.35 μmol) and Ascaris protein (2.5 mg, molecular weight not determined) were mixed well and then WSCD (12 mg, 60 μmol) was slowly added dropwise. The reaction mixture was stirred at room temperature

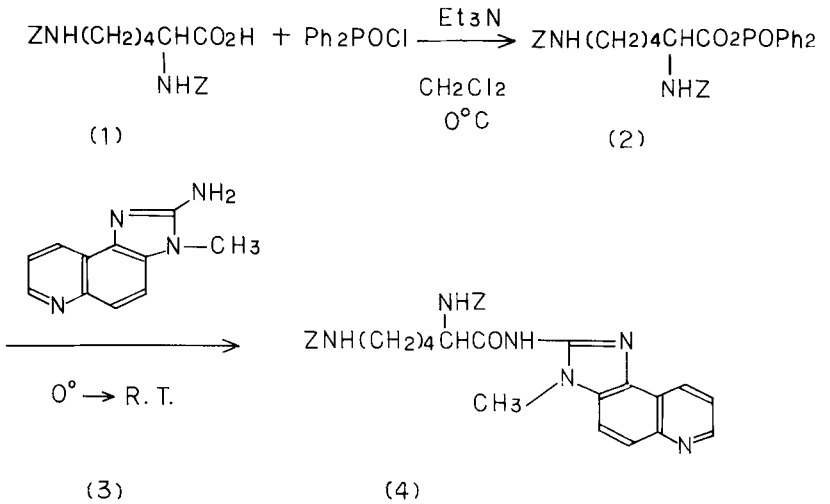

Fig. 1. Peptide synthesis procedure of IQ-Z·Lys(Z). (1), (2), (3), and (4) show Z·Lys(Z), diphenylphosphinous Z·Lys(Z), IQ, and IQ-Z·Lys(Z), respectively. Abbreviations: Z·Lys(Z), N^{α},N^{β}-dicarbobenzoxy-L-lysine; Ph_2POCl, diphenylphosphinous chloride; Et_3N, triethylamine; CH_2Cl_2, methylene chloride; IQ, 2-amino-3-methylimidazo(4,5-f)quinoline; RT, room temperature.

for 3 h around ph 7.0. After the reaction, free IQ-Lys and WSCD were dialyzed with saline at 4°C overnight using cellophane seamless tube (a mol wt cutoff of 3,000) (Union Carbide Co., Chicago, IL).

Immunization and Cell Fusion

Balb/c mice (8-wk-old) (Shizuoka Agricultural Cooperative for Experimental Animals, Shizuoka, Japan) were subcutaneously immunized three, five, or eight times every two weeks with IQ-Lys-Ascaris protein conjugate containing 16.6 μg of IQ in 100 μL of saline emulsified with an equal volume of Freund's complete adjuvant. Three days after the last injection, cell fusion was carried out between splenocytes (10^8) and myeloma cells (P3U1, 2×10^7) (Otsuka Assay Laboratories) in 1 mL of PEG 1500 according to Köhler and Milstein (27) with a slight modification. The fused cells were distributed to 96 well flat-bottomed microtiter plates (Toyobo Engineering Co., Ltd., Osaka, Japan) and cultured in HAT (hypoxanthine/aminopterin/thymidine) medium at 37°C with 5% CO_2 in a humidified atmosphere. When visible hybridoma clones appeared, supernatants of growing cultures were assessed for the production of antibodies.

Screening for Antibody Formation and Cloning

IQ-Lys-BSA conjugate was generated according to the method of IQ-Lys-Ascaris protein conjugate synthesis. Screening for antibody formation was done by using an enzyme-linked immunosorbent assay (ELISA).

IQ-Lys-BSA conjugate (100 ng) was coated on each well of 96 well microtiter plates and shaken with 50 μL of supernatants of hybrid cultures at room temperature for 2 h. After shaking, 96 well microtiter plates were washed with distilled water three times and then reacted with 50 μL of goat anti-mouse immunoglobulin conjugated peroxidase (×5000) at room temperature for 1.5 h. After the reaction, 96 well microtiter plates were washed with distilled water three times. One hundred microliters of phosphate citrate buffer (23.9 g/L $Na_2HPO_4 \cdot 12H_2O$, 7.0 g/L citric acid) containing 2.5 mg/ml O-phenylenediamine and 0.015% H_2O_2 were added to each well and then allowed to stand for 15 min. The reaction was finally terminated with 100 μL of 1*M* H_2SO_4. The expression of antibody activities was determined by measuring the absorbance at 490 nm using a Titertek Multiscan NCC (a Joint Venture Company of Labsystems and Flow Laboratories, West Germany). The hybridomas expressing antibody activities were cloned by the limiting dilution technique. Cloning was performed to 0.5 hybridoma per well by this method. A 96 well microtiter plate coated with BSA (100 ng) was used as a negative control.

Immunoglobulin Subclass Determination of Monoclonal Anti-IQ Antibody

The subclass of monoclonal anti-IQ antibody in the supernatant of the hybrid culture was determined by double immunodiffusion against mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM, and IgA.

Generation and Purification of Ascites Fluid

Ascites fluid was generated by injecting the hybridoma cells (1.0×10^7) into Balb/c mice (8-wk-old) that had been pretreated intraperitoneally with pristane (0.5 mL) 3–14 d before. Seven to 10 d after the injection, ascites fluid was tapped off repeatedly. The purification of monoclonal anti-IQ antibody was carried out with a Monoclonal Antibody Purification System (Bio-Rad Laboratories, Richmond, CA). Ascites fluid (5 mL) was added to an equal volume of binding buffer and then filtered with a 0.22 μM filter. The diluted ascites fluid (10 mL) was applied to a column of Protein A-Sepharose CL4B (size, 10×70 mm). Unbound materials were washed out with the binding buffer, and then, the bound antibody was eluted with 50 mM sodium citrate buffer, pH 5.5. Cold ammonium sulfate (pH 7.0) was added to the antibody until 50% saturation and centrifuged at 8000 rpm at room temperature for 10 min. The precipitate was collected and redissolved in 500 μL of 0.1*M* borate saline, pH 8.2. The redissolved antibody was purified further with gel filtration on Sepharose 6B (size, 16×450 mm, Pharmacia, Uppsala, Sweden). The solvent system was 0.1 mol/L borate saline, pH 8.2. The concentration of the further purified antibody was determined by the absorbance at 280 nm using a Shimadzu UV 260 spectrophotometer (Shimadzu, Kyoto, Japan), compared with 1 mg/mL mouse IgG.

RIA for Mutagenic Carcinogenic Heterocyclic Amines, Their Similar Compounds, and Their Related Compounds

Twenty-five kinds of inhibitors were dissolved and adjusted to various concentrations (original solution $x3^0$, $x3^1$,·····$x3^6$) in 50 m*M* Tris–HCl buffer, pH 7.0, containing 150 m*M* NaCl, 0.5% BSA, 1% normal mouse serum (NMS) and 0.05% NaN_3 (RIA-buffer). Monoclonal antibody was radiolabeled with ^{125}I (New England Nuclear, Boston, MA) by the Iodogen (Pierce Chemical Co., Rockford, IL) method. Unreacted radioiodine was separated from antibody-bound ^{125}I by passage over a Sepharose 6B column (size, 22×750 mm) equilibrated in 50 m*M* phosphate-buffered saline, pH 7.4, containing 0.1% BSA and 0.05% NaN_3. Inhibition assay was performed between a polystyrene ball (Sekisui Chemical Industries, Co., Ltd., Tokyo, Japan) coated with IQ-Lys-Ascaris protein conjugate, and each inhibitor using the ^{125}I-rabeled monoclonal antibody.

Briefly, 100 μL of the ^{125}I-labeled antibody (150,000 cpm), 100 μL of various concentrations of each inhibitor solution, and a polystyrene ball coated with IQ-Lys-Ascaris protein conjugate were added to a tube and shaken for 2 h at room temperature. The balls were then washed with distilled water three times and transferred to new RIA tubes. ^{125}I-radioactivities of the RIA tubes were counted in triplicate in an Aloka Auto Well Gamma System (Aloka, Tokyo, Japan). Polystylene balls coated with Ascaris protein were used as blank tests.

RESULTS

Production of Monoclonal Anti-IQ Antibody

Fusions were performed between myeloma cells (P3U1) and spleen cells from mice immunized three, five, or eight times with IQ-Lys-Ascaris protein conjugate. Hybridomas from mice immunized three or five times did not exhibit anti-IQ antibody activity. Eight days after the fusions, hybridomas from mice immunized eight times appeared in 148 of 196 wells, and one of those only expressed anti-IQ antibody activity, as judged by ELISA. The hybridomas exhibiting the antibody activity were cloned by the limiting dilution technique. Cloning was performed to 0.5 hybridoma/well by this method. Finally, only one hybridoma cell line secreting monoclonal anti-IQ antibody was established.

Immunoglobulin Subclass Determination of Monoclonal Anti-IQ Antibody

The subclass of monoclonal anti-IQ antibody was determined by double immunodiffusion. The monoclonal antibody was identified as IgG_1.

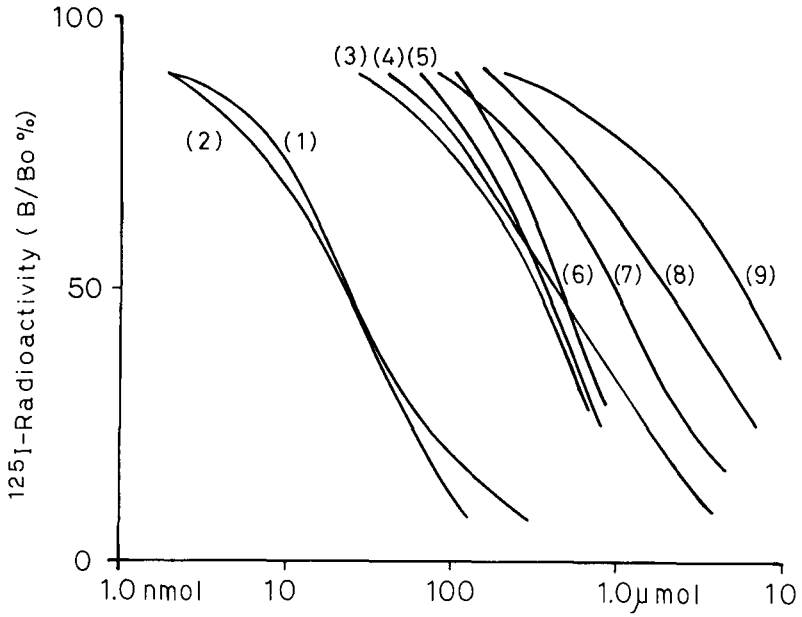

Fig. 2. Cross-reactivity of IQ-type heterocyclic amines, their similar compounds, and their related compounds. Inhibition assays were performed between IQ-Lys-Ascaris protein beads and various inhibitors: (1), IQ; (2), MeIQ; (3), MeIQx; (4) ,7,8-DiMeIQx; (5), imidazole; (6), 4,8-DiMeIQx; (7), quinoline; (8), 4-azabenzimidazole; and (9), quinoxaline, with RIA using monoclonal anti-IQ antibody in a final vol of 200 μL in a RIA-buffer. Abbreviations: MeIQ, 2-amino-3,4-dimethylimidazo(4,5-f)quinoline; MeIQx, 2-amino-3,8-dimethylimidazo(4,5-f)quinoxaline; 7,8-DiMeIQx, 2-amino-3,7,8-trimethylimidazo(4,5-f)quinoxaline; 4,8-DiMeIQx, 2-amino-3,4,8-trimethylimidazo(4,5-f)quinoxaline.

Purification of Monoclonal Anti-IQ Antibody in Ascites Fluid

Ascites fluid from Balb/c mice injected into the hybridoma cells intraperitoneally was purified with both affinity chromatography on Protein A-Sepharose CL4B and gel filtration on Sepharose 6B. The concentration of the purified monoclonal antibody was 2.44 mg/mL, compared with 1 mg/mL mouse IgG by the absorbance at 280 nm.

Cross-Reactivity and Sensitivity Determination for Monoclonal Anti-IQ Antibody with RIA

As shown in Figs. 2–5, cross-reactivities of IQ, its similar compounds, and its related compounds were determined in triplicate in the inhibition assay. MeIQ showed a sigmoid curve as similar as IQ and was nearly identical to IQ in 50% displacement. Half of the reversibly bound ^{125}I-labeled monoclonal anti-IQ antibody was displaced by 23 nmol of IQ and

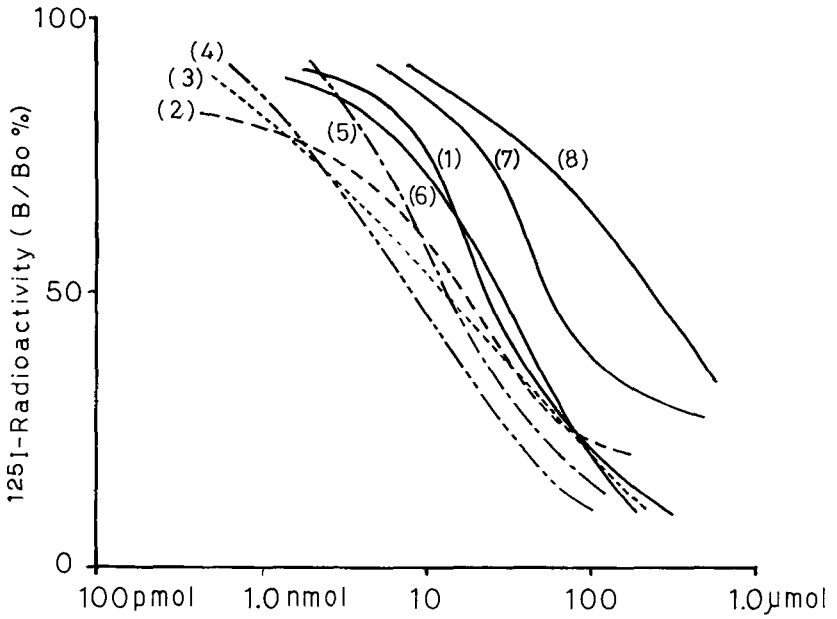

Fig. 3. Cross-reactivity of IQ and β-carbolines. Inhibition assay were performed between IQ-Lys-Ascaris protein beads and various inhibitors: (1), IQ; (2), harmol; (3), harmalol; (4), harmaline; (5), harmine; (6), harman; (7), norharman; and (8), noreleagnine, with RIA using monoclonal anti-IQ antibody in a final vol of 200 μL in RIA-buffer. Abbreviations: harmol, 1-methyl-9H-pyrido(3,4-b)indole-7-ol; harmalol, 3,4-dihydro-1-methyl-9H-pyrido(3,4-b)indole-7-ol; harmaline, 1-methyl-7-methoxy-3,4-dihydro-9H-pyrido(3,4-b)indole; harmine, 7-methoxy-1-methyl-9H-pyrido(3,4-b)indole; harman, 1-methyl-9H-pyrido(3,4-b) indole; norharman, 9H-pyrido(3,4-b)indole; noreleagnine, 2,3,4,9-tetrahydro-1H-pyrido(3,4-b)indole.

22 nmol of MeIQ, respectively (Fig. 2). β-Carbolines exhibited high cross-reactivities to IQ, and the contents of 50% displacement of harmaline, harmalol, harmine, harmol, harman, and norharman were 8, 12.5, 13, 17, 30, and 45 nmol, respectively (Fig. 3). The reactivities of MeIQx, DiMeIQx, quinoline, imidazole, Glu-P-1, and Glu-P-2 were about 13–44 times lower than IQ in terms of the attainment of 50% displacement (Figs. 2 and 4). The solubility of Aα C and MeAαC in RIA buffer was too low to examine until the attainment of 50% displacement. In the range of the concentration examined, the cross-reactivity of AαC to IQ was nearly identical to Glu-P-1. In addition, the cross-reactivity of MeAαC was a little lower than Glu-P-1 (Fig. 4). The slopes of curves of Trp-P-1 and Trp-P-2 were gentler than IQ, though we could not clarify the cause. Therefore, we could not determine whether the antibody actually recognized Trp-P-1 and Trp-P-2 (Fig. 4). The reactivities of the other compounds, 4-azabenzimidazole, quinoxaline, L-lysine, L-tryptophan, and L-glutamic acid, were over 100 times lower than IQ in 50% displacement (Figs. 2 and 5). Chemical structures of IQ and the compounds showing high cross-reactivities to IQ were presented in Fig. 6.

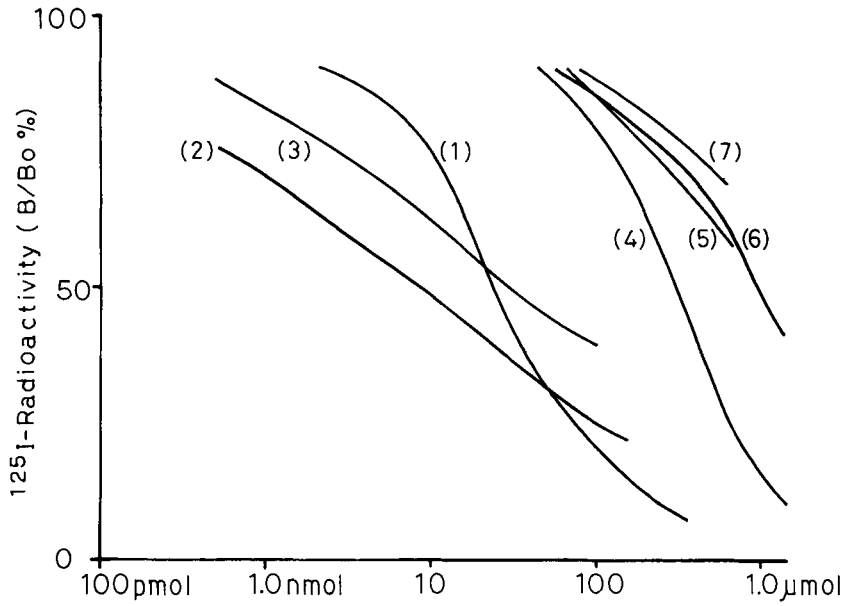

Fig. 4. Cross-reactivity of IQ and non-IQ-type heterocyclic amines. Inhibition assays were performed between IQ-Lys-Ascaris protein beads and various inhibitors: (1), IQ; (2), Trp-P-2; (3), Trp-P-1; (4), Glu-P-2; (5), AαC; (6), Glu-P-1; and (7), MeAαC, with RIA using monoclonal anti-IQ antibody in a final vol of 200 μL in RIA-buffer. Abbreviations: Trp-P-1, 3-amino-1,4-dimethyl-5H-pyrido(4,3-b)indole; Trp-P-2, 3-amino-1-methyl-5H-pyrido(4,3-b)indole; Glu-P-1, 2-amino-6-methyldipyrido(1,2-a:3′,2′-d)imidazole; Glu-P-2, 2-amino-dipyrido (1,2-a:3′,2′-d)imidazole; AαC, 2-amino-9H-pyrido(2,3-b)indole; MeAαC, 2-amino-3-methyl-9H-pyrido(2,3-b)indole.

DISCUSSION

In this experiment, Balb/c mice were subcutaneously immunized with IQ-Lys-Ascaris protein conjugate three, five, or eight times at the interval of two weeks. Finally, only a hybrid cell line from the Balb/c mouse immunized with the immunogen eight times exhibited monoclonal anti-IQ antibody activity, but hybridomas from the other mice did not produce antibodies to the immunogen. As shown in our investigation, more immunizations were required for the generation of antibodies against IQ. The result suggests that it is very difficult to produce antibodies against IQ because of a low molecular weight substance (mol wt 198) and might, furthermore, suggest that the antigenicity of IQ is lower. Therefore, it would be worth reporting to have developed a monoclonal antibody against a low molecular weight substance such as IQ.

We examined reactivities among our monoclonal antibody and 25 kinds of compounds, including IQ, its similar compounds, and its related compounds, in order to determine specific recognition sites of our antibody (Figs. 2–5). Our monoclonal antibody showed higher reactivities to

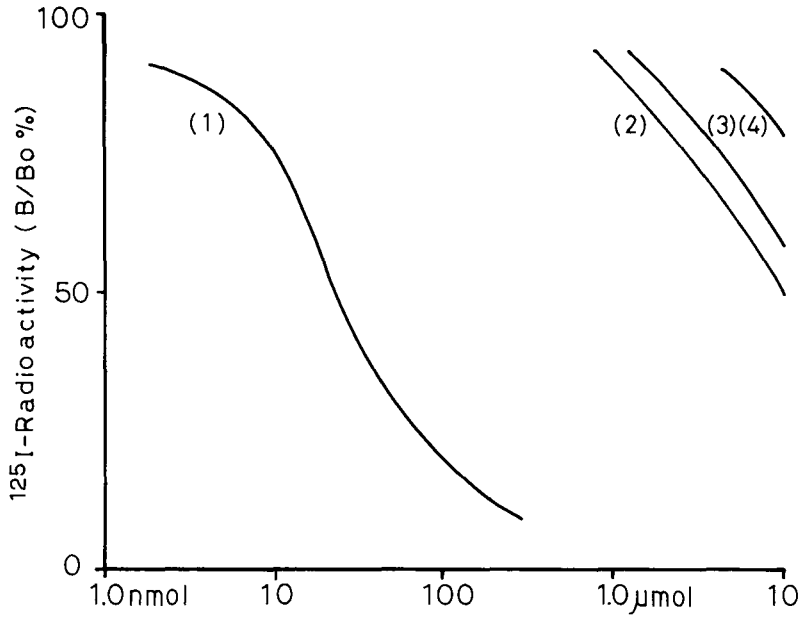

Fig. 5. Cross-reactivity of IQ and its related amino acids. Inhibition assays were performed between IQ-Lys-Ascaris protein beads and various inhibitors (1), IQ; (2), L-lysine; (3), L-tryptophan; and (4), L-glutamic acid, with RIA using monoclonal anti-IQ antibody in a final vol of 200 μL in RIA-buffer.

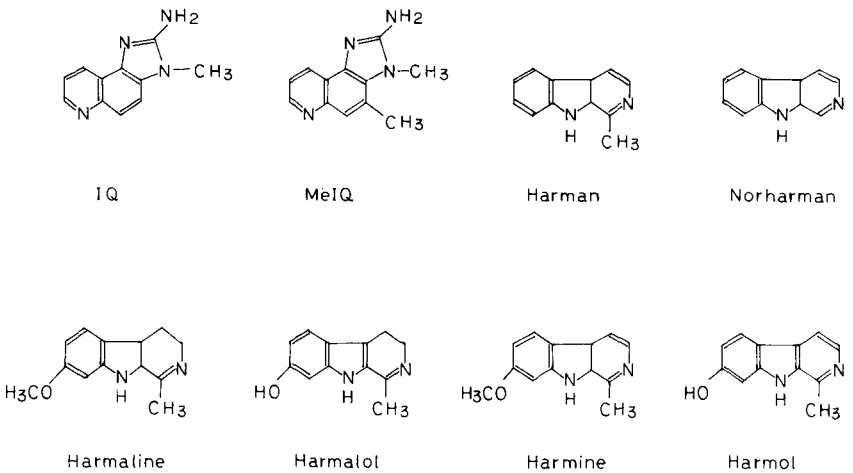

Fig. 6. Chemical structures of IQ and the compounds showing high cross-reactivity to IQ.

IQ, MeIQ, and several kinds of β-carbolines. The affinities to IQ and MeIQ showed essentially no difference in terms of the activity for 50% displacement (Fig. 2). β-carbolines, excluding norharman and noreleagnine, were higher or equally effective in the displacement, compared with IQ and MeIQ (Figs. 2 and 3). Especially, the affinity to harmaline was about three times higher than those to IQ and MeIQ (Figs. 2 and 3). Although the affinities to imidazole and quinoline were approximately 20 and 45 times lower than IQ and MeIQ in 50% displacement, respectively, these compounds that composed IQ and MeIQ were also recognized by our antibody, and the affinity to imidazole was about 2.25 times higher than quinoline (Fig. 2). These results indicate that IQ and MeIQ occupy most of the antibody binding sites and that our antibody recognizes imidazole moiety more intensely than quinoline moiety in chemical structures of IQ and MeIQ. However, we could not determine the reason why our antibody recognized some β-carbolines more intensely than IQ and MeIQ. Further studies are necessary for specific recognition sites of our antibody.

Recently, we investigated whether humans were actually exposed to carcinogenic heterocyclic amines, dietary carcinogens, in order to examine relationships between human carcinogenesis and the carcinogens. As a result, we demonstrated that humans were actually exposed to carcinogenic heterocyclic amines (*18–23*) and that the exposure levels of Glu-P-1, Glu-P-2, IQ, and MeIQx in patients with uremia before induction of hemodialysis treatment were significantly higher than in normal subjects (*22,23*). Harman and norharman of β-carbolines, as well as carcinogenic heterocyclic amines, have even been reported to be obtained from amino acid pyrolysates, cigaret smoke condensate, and cooked foods (*1–6*), and harmine and harmaline from the vine, *Banisteria caapi, Penganum harmala,* and other South American plants (*28–30*). Harmol and harmalol have been reported to be metabolites of harmine and harmaline when the compounds are given to rats (*29,30*). More recently, harman and norharman, comutagens were showed to be present in the dialysate of patients with uremia (*31*). The presence of the other β-carbolines in humans has not been demonstrated although those compounds have been investigated on a broad spectrum of pharmacological actions, such as the cardiovascular system, the central nervous system, and monoamine oxidase inhibition (*28–30,32*). However, to our regret, our antibody would not be available for the evaluation of human exposure levels of dietary carcinogens and/or comutagens, such as IQ, MeIQ, harman, and norharman, because of low affinities.

In general, it is difficult to develop antibodies against low molecular weight substances. In this study, we clearly showed a methodology of production of a monoclonal antibody to a low molecular weight substance (IQ). Our method would be useful as one of the techniques of generation of antibodies against low molecular weight substances. However, further studies are necessary for the development of antibodies to low molecular weight substances.

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