MONOCLONAL ANTIBODIES TO QUININE

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Abstract. Monoclonal antibodies (MAbs) to quinine conjugated to a carrier protein were produced. Quinine was converted into a hemisuccinate prior to covalently linked to bovine serum albumin (BSA) by reacting with *N,N'*-disuccinimidyl carbonate (DSC). Coupling ratio of quinine-BSA was 13:1 calculated by spectrophotometry and 14:1 by calculation from quinine standard curve. This immunogen was used for both monoclonal antibody production and for screening test, indirect ELISA. The specificity of quinine-BSA MAbs was examined by checking the cross reactivity with BSA and the structurally related antimalarial drug, mefloquine. Six MAbs belonging to IgG_1 were obtained. These MAbs slightly reacted with mefloquine-BSA because of closely related structure of mefloquine to quinine and similar conjugate preparation procedure used for conjugation. One selected MAb against quinine-BSA, showed higher reactivity with blood samples from patients previously treated with quinine when compared to normal blood. This preliminary test indicated that MAbs obtained may be useful to be used as the probe for detection of quinine in biological fluids.

INTRODUCTION

Quinine is still an appropriate drug for severe and complicated falciparum malaria. Quinine has narrow therapeutic range and became progressively narrower since the efficacy of the drug has gradually declined against malaria strains in various parts of Southeast Asia as documented by RIII type of quinine resistance case report (Karbwang *et al,* 1994). Prescribing treatment in patients who have had previous medication may produce adverse effect of overdosage and contradicted regimens while unprescribing may not control the disease. Thus, there is an increasing need to know the levels of patients' current medication for monitoring the effectiveness of severe malaria treatments.

Although quinine can be currently measured by HPLC, this method is required sophisticated instruments and carefully maintenance. Owing to its simplicity and sensitivity, immunoassay using monoclonal antibody is the method of choice. Therefore, production and characterization of monoclonal antibodies against quinine was conducted.

MATERIALS AND METHODS

Antigen preparation

Covalent coupling of quinine to BSA was carried out based on the methods of Sauer and Morris (1987) with some modifications. Briefly, 500 mg of quinine (Sigma, USA) was dissolved in 6 ml of pyridine (Sigma, USA) and to this mixture 1 g of succinic anhydride (Sigma, USA) was added. After the mixture was stirred at room temperature for 2 hours, 0.4 g *N,N'*-disuccinimidyl carbonate (DSC, Sigma, USA) was added following by stirring at 45 °C for 1 hour. The quinine was then conjugated with bovine serum albumin (BSA, Sigma, USA) by gradually adding of one milliliter of 0.2 M sodium carbonate buffer pH 9.5 containing 334 mg of BSA following by stirring overnight at room temperature. The conjugate was then fractionated in sephadex G-75 with 0.01 M PBS pH 7.2. Fraction containing quinine-BSA conjugate determined by A_{280} for BSA and A_{330} for quinine were pooled and lyophilized. About 600 mg of quinine-BSA conjugate was obtained. Quinine-BSA molar ratio was 13:1 determined by spectrophotometry and 14:1 by using calculation based on quinine standard curve. To assure the successful conjugation, high performance liquid chromatography (HPLC), according to the method of Karbwang *et al* (1989) was done. It was found that quinine-BSA conjugate contained 7.43 % of free quinine.

Production of monoclonal antibodies

Immunization procedure : Two female BALB/c mice (6 weeks old) were immunized with four intraperitoneal injections of 24 μg (expressed in

hapten) suspended in 0.2 ml of normal saline solution (NSS) emulsified in an equal volume of Freund's complete adjuvant initially and incomplete adjuvant subsequently at an interval of 2 weeks. Mice were bled from retroorbital plexus a week after the third immunization and their sera were tested for the presence of antibody activity to quinine. Three days prior to fusion, the best responder was boostered intravenously with 6 μg of quinine in BSA-conjugated form in 0.2 ml of NSS.

Hybridization : Approximately 12.8 x 10^7 immune splenocytes were fused with 12.8 x 10^6 P3-X63-Ag 8.653 myeloma cells in the presence of 50 % polyethylene glycol-4000 (Sigma, USA) according to the method described by Khusmith *et al* (1984). Cells resuspended in hypoxanthine azaserine selective medium were distributed in eleven 96 well tissue culture plates and grown at 37 °C in a humidified 5% CO_2 incubator.

The antibody-producing hybridomas were transferred into 24 well tissue culture plates. The hybridomas that still retained their reactivity were cloned twice by limiting dilution technic.

Screening for specific antibody-producing hybrids by indirect ELISA

After 2 weeks, the supernatants of the sufficiently grown cells were screened for the antibody secreting hybridomas by indirect ELISA. Briefly, each well of ELISA plate (Dynatech, USA) was coated with 150 μl of 10 μg/ml of quinine-BSA conjugate in coating buffer and incubated until dry at 37 °C overnight. Unbound antigen was removed by washing with PBST-0.5% BSA three times, and 200 ml of blocking solution (PBST-1% BSA) was added in each well. The plate was incubated at 37 °C for 1 hour After washing three times, 100 μl/well of undiluted supernatants were used. The plate was incubated for 1 hour at 37 °C followed by washing three times with PBST-0.5% BSA. Rabbit antimouse alkaline phosphatase labeled conjugate (Dakopatts, Denmark) diluted (1:1,000) in PBST-0.5% BSA was added (100 μl/well) followed by incubation for 1 hour at 37 °C. After a final wash, 100 μl of p-nitrophenyl phosphate substrate in substrate buffer was added to each well. Color was allowed to develop in the dark for 1 hour at 37 °C and the reaction was stopped by adding 50 μl of 3 N NaOH per well. The optical density (OD) was measured by an ELISA reader at 405 nm.

Characterization of monoclonal antibodies

Cross reactivity checking : The antibodies produced were checked for their cross reactivities against mefloquine-BSA conjugate and BSA by indirect ELISA as described. The concentrations of BSA, quinine-BSA conjugate and mefloquine-BSA conjugate used were 10 mg/ml. The desired monoclonal antibodies should give the optical density (OD) values in the well of quinine-BSA conjugate higher than those of mefloquine-BSA conjugate and BSA.

Isotyping of monoclonal antibodies : Isotypes of the MAbs were determined by ELISA using isotyping reagents set (Dakopatts, Denmark).

Binding capability test of selected monoclonal antibody with quinine in blood samples : Indirect ELISA was performed as mentioned. The culture fluid which gave relatively high OD value was selected as detective reagent of quinine in the blood samples. Fifty microliters of the blood samples containg quinine were used as an antigen. The normal blood was used as control.

RESULTS

From single fusion of P3-X63-Ag 8.653 myeloma cells with splenocytes from mouse immunized with quinine-BSA conjugate, 333 hybridomas were obtained. Six monoclones from 16 positive hybrids namely 7D8F10-1, 7D8F10-2, 7D8F12-1, 7D8F12-4, 7D8F12-7 and 7D8F12-8 were finally obtained after cloning and recloning. All MAbs obtained were of IgG_1 isotype. Reactivity of MAbs with quinine-BSA, mefloquine-BSA and BSA is shown in Table 1.

Binding capability test of selected MAb, 7D8F10-1 with quinine in blood samples showed higher reactivity with blood samples from patients previously treated with quinine compared to normal blood (Table 2).

DISCUSSION

Successful production of monoclonal antibodies to drugs depends greatly on hapten-carrier

Table 1

Reactivity of MAbs with quinine-BSA, mefloquine-BSA and BSA determined by indirect ELISA.

MAb	MAb reactivity (OD 405 nm)		
	Quinine-BSA	Mefloquine-BSA	BSA
7D8F10-1	0.633	0.316	0.184
7D8F10-2	0.519	0.342	0.184
7D8F12-1	0.308	0.155	0.123
7D8F12-4	0.293	0.192	0.108
7D8F12-7	0.331	0.170	0.109
7D8F12-8	0.324	0.223	0.151

Table 2

Binding capability of selected MAb, 7D8F10-1 with quinine in human blood samples determined by indirect ELISA.

Human blood	Quinine level * (μg/ml)	MAb reactivity with quinine (OD 405 mm)
Normal	0	1.616
Sample 1	6.600	2.647
Sample 2	5.110	2.562
Sample 3	4.903	2.674
Sample 4	4.649	2.598
Sample 5	3.947	2.631

* The amount of quinine was measured at Clinical Pharmacology Unit, Faculty of Tropical Medicine by HPLC.

conjugation. The chemistry used to attach the drug to the carrier will determine how the drug is presented to the B-cells during initiation of the immune response and, hence, which parts of the drug molecule are subsequently recognized resulting antibodies (Rowell, 1990). In the present study, quinine-bovine serum albumin (BSA) conjugate was prepared by the adaptation of the available coupling reagent and simple reaction combination. Because two available methods are impractical due to the complexity and costs involved in preparing the quinine-protein conjugate (Robin *et al,* 1984; Sidki *et al,* 1987).

The appropriate test system for screening antibodies with required specificity is important. According to the fact that the immunization of conjugated antigen, the antibodies obtained may possibly recognize either the carrier protein, the hapten, or a site combining parts of both. Generally, the conjugates used for monoclonal antibody production and screening test should be made different both the carrier protein and methods of conjugation (Freier *et al,* 1986; Pauillac *et al,* 1993). In contrast, quinine-BSA conjugate was used for both purposes leading to extensive screening to yield truly superior antibodies.

MAbs were selected on the basis of their high reactivity with quinine-BSA conjugate and the lowest reactivity with BSA and mefloquine-BSA conjugate. As may be expected, it is obvious that all MAbs obtained showed cross reactivity with BSA and mefloquine-BSA conjugate, because mefloquine and quinine have closely related structure and mefloquine-BSA conjugate was prepared by using the similar procedure as described for quinine. However, MAbs obtained appeared to be

more specific for quinine-BSA than mefloquine-BSA which can be seen from considerable difference in optical density (OD) value measured at 405 nm. This implies that the specificity of MAbs recognizing quinine-BSA was influenced by the closely related structure of mefloquine to quinine and the linkage used for coupling to a protein carrier.

In order to know whether the MAbs against quinine-BSA conjugate could react with quinine in human blood samples, a binding capability test was done using selected MAb, 7D8F10-1 as a detective reagent. Five blood samples from patients previously treated with quinine which containing quinine measured by HPLC ranging from 3.497-6.6 μg/ml and one normal blood sample were tested. Although the irrelevant proportion of MAb reactivity and quinine level due to samples use without making an appropriate dilution was observed. This preliminary test showed higher reactivity with blood samples containing quinine comparing with normal blood, indicating that MAb produced may be useful for detection of quinine in biological fluids.

Whether MAbs against quinine-BSA conjugate can be successfully developed to use in the assay for detection of quinine in biological fluids, further work needs to be done. Establishment of MAbs with definite specificities against quinine-BSA would provide useful tools for studies in both malarial treatment and research fields. To exploit the full potential of MAbs produced, they should be characterized more clearly by binding assaying with free quinine and other related compounds. Moreover, to improve the specificity of these MAbs, quinine conjugated to different carriers should be used to eliminate cross reactivity against carrier protein.

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