CHARACTERIZATION OF SPECIFIC MONOCLONAL ANTIBODIES FOR DETECTION OF MEFLOQUINE IN BODY FLUIDS

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Abstract. Specific monoclonal antibodies (MAbs) to mefloquine conjugated to bovine serum albumin (mefloquine-BSA) were produced by hybridoma technology. The mefloquine-BSA was synthesized by converting mefloquine into hemisuccinate followed by convalently linked to bovine serum albumin (BSA) and coupling with N,N′ disuccinimidyl carbonate (DSC). The conjugate was purified by Sephadex G-75 gel filtration using 0.01M PBS pH 7.2. An average of 19.34 molecules of mefloquine were conjugated to each molecule of protein determined by differential UV absorption spectra of hapten and protein carrier. Sixteen monoclones producing antibody specific to mefloquine were screened by indirect ELISA using homologous antigens. The specificity of MAbs was determined by reacting with BSA and the structurally related antimalarial drug, quinine. Three, three, five and two MAbs belonged to IgG_1, IgG_{2a}, IgG_{2b} and IgG_3, respectively. Most of the MAbs slightly reacted with quinine-BSA due to the closely related structure of mefloquine to quinine. The selected MAb designated 11F9(G5)G9 which showed no cross reaction with quinine-BSA gave high reactivity with blood samples from malaria patients previously treated with mefloquine when compared to normal blood by indirect ELISA. The preliminary results indicated that such specific MAb could be used as antibody probe for detection of mefloquine in biological fluids.

INTRODUCTION

Resistance of *Plasmodium falciparum* to existing antimalarial is a major obstacle to malaria control today. Mefloquine is used for treatment of malaria as a replacement for chloroquine in area where chloroquine resistance of *P. falciparum* occurred. However, there has been a decline in sensitivity of *P. falciparum* to mefloquine and the side affects of treatment with mefloquine are being reported with increasing frequency (Rowell, 1990). Furthermore, the successful treatment depends on rapid diagnosis and general clinical assessment of patients. The screening for previous treatment with antimalarials from medical history or drug concentration in biological fluids should be done as this will help in the selection of antimalarial (Karbwang and Harinasuta, 1992). In many areas of the world, patients experience some forms of self-treatment prior to presenting to a health facility. In order to avoid potentially toxic drug levels, and/or known adverse drug interactions, a simple test is needed to ascertain the presence of any of choice and dosage of drug given to the patient compliance for prophylaxis especially in pregnant woman or for treat-ment (WHO/TDR report, 1993).

Although mefloquine can be currently measured by high performance liquid chromatography (HPLC), this method is required sophisticated equipment. A simple, rapid, sensitive and specific assay is the method of choice for detection of antimalarials in blood or urine which is important in a clinical setting and may be useful at the periphery. Therefore, production and characterization of monoclonal antibodies against mefloquine was conducted in this study.

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MATERIALS AND METHODS

Preparation of a mefloquine-BSA conjugate

Covalent coupling of mefloquine to BSA was carried out using the method previously described with some modifications (Ogura *et al*, 1979; Sauer and Morris, 1987). Briefly, 500 mg of mefloquine (Roche, Switzerland) dissolved in 6 ml of methanol was mixed with 6 ml of pyridine (C_5H_5N) (Sigma, USA) and 0.602 g of succinic anhydride (Aldrich, USA). After the mixture was stirred for 4 days at room temperature, 0.309 g of N, N′-disuccimidyl carbonate (DSC) (Sigma, USA) was added followed by stirring at 45ºC for 1 hour. The mefloquine was then conjugated with bovine serum albumin (BSA, Sigma, USA) by gradually adding the solution containing 1.333 g of BSA in

10 ml of 0.2 M sodium carbonate buffer (Na_2CO_3) pH 9.5 and followed by stirring overnight at room temperature. The conjugate was then purified by Sephadex G-75 (Pharmacia, Sweden) gel filtration using 0.01 M PBS pH 7.2. The fraction containing mefloquine-BSA was determined by the absorbance at the wavelength of 222 nm. The results were compared with mefloquine standard curve made by the absorbance of free mefloquine in various concentration at the same wavelength when methanol was used as a solvent and calculated by the formula modified from the method of Erlanger (1980) and Makela and Seppala (1986) [hapten in conjugate = (absorbance of conjugate) x (hapten) / absorbance of hapten] [incorporation ratio = (hapten in conjugate / MW hapten) / (conjugate-hapten in conjugate /MW protein].

To assure the successful conjugation, high performance liquid chromatography (HPLC) previously described (Karbwang *et al*, 1989a) was used to determine the free mefloquine containing in mefloquine-BSA conjugate.

Production of monoclonal antibodies

The inbred strain BALB/c mice (4-6 weeks old) were immunized intraperitoneally with three doses of 60 μg/ml of mefloquine-BSA (MQ-BSA) in normal saline solution (NSS) emulsified in an equal volume of Freund's complete adjuvant initially and incomplete Freund's adjuvant subsequently in an interval of two weeks. The mice were bled two weeks after the fifth injection *via* retroorbital plexus and their sera were tested for specific antibody by an indirect ELISA against MQ-BSA. Three days prior to fusion, the best responder was boost with 100 μg of the respective antigen in 200 μl of NSS.

The hybridization wad done by fusing of murine myeloma line P3-X63-Ag8.653 with splenocytes of immunized mice in a ratio of 1: 10 using 50% W/V polyethylene glycol 4000 (Sigma, USA) in NSS according to the method of Khusmith *et al* (1984). Cells resuspended in hypoxanthine-azaserine selective medium were distributed in 96 well tissue culture plates and grown in humidified 5% CO_2 incubator at 37ºC. The antibody-producing hybrids were cloned twice by limiting dilution technique.

Screening for specific antibody producing hybrids by indirect ELISA

The supernatants from the sufficient growing cells were screened of antibody secreting hybrids by indirect ELISA. Briefly, 150 μl of MQ-BSA (10 μg/ml) was coated in each well of ELISA plates (Dynatech, USA) and incubated overnight at 37ºC until dry. The unbound antigen was removed by washing three time with phosphate buffered saline-tween (PBS-T) and incubated with 200 μl of blocking solution (PBS containing 1% BSA) at 37ºC for 1 hour. After washing three times, 100 μl of undiluted supernatant was added. The hyperimmune mouse serum and medium was used as positive and negative controls, respectively. After incubating at 37ºC for 1 hour followed by three times washing, the plate was reacted with 100 μl of 1:1,000 diluted rabbit antimouse immunoglobulins alkaline phosphatase labeled conjugate (Dakopatts, Denmark), followed by incubation for 1 hour at 37ºC. After final washing, 100 μl of freshly prepared p-nitrophenyl phosphate substrate in diethanolamine buffer was added. The color was allowed to develop in the dark for 1 hour at 37ºC and the reaction was stopped by adding 50 μl of 3N NaOH solution per well and measured as optical density (OD) by an ELISA reader at 405 nm.

Characterization of monoclonal antibodies

Cross-reactivity to related drugs: The monoclonal antibody (MAb) produced were screened for their cross-reactivities against quinine-BSA conjugate and BSA by indirect ELISA as mentioned.

Isotyping of the monoclonal antibodies: The classes and subclasses of MAbs were determined by ELISA using isotype reagents set (Dakopatts, Denmark).

Determination of binding capabiliity of MAb to mefloquine in blood samples: Fifty microliters of blood from malaria patients previously treated with mefloquine were tested with the selected MAbs by indirect ELISA. The normal serum was used as negative control. The Mab which gave higher OD value was selected as antibody probe for detecting mefloquine in body fluids.

RESULTS

Coupling of mefloquine-bovine serum albumin conjugate

After preparation and purification, the amount of mefloquine was determined based on the differential UV adsorption spectra of hapten and carrier

protein. The optical densities of each fraction are shown in Fig 1. The mefloquine standard curve is shown in Fig 2. Mefloquine was measured by UV at 222 nm whereas BSA was detected by UV at 280 nm. An average of 19.34 molecules of mefloquine could couple with one molecule of BSA. It was found that mefloquine-BSA conjugate contained 12% of free mefloquine as determined by high performance liquid chromatography (HPLC).

Specific monoclonal antibodies against mefloquine-BSA conjugate

After single fusion, 384 culture wells (25%) showed growing hybrids and among these, 57 culture wells (14.84%) were positive for antibodies to mefloquine-BSA. After cloning and recloning, 16 monoclones produced MAbs were obtained. All MAbs from these monoclones gave limited reaction with BSA, nine MAbs cross-reacted with quinine while only seven MAbs did not. Reactivity of MAbs with mefloquine-BSA, quinine-BSA and BSA is shown in Table 1. Three, three, five and two MAbs belonged to IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3. The three MAbs reacted with more than one isotypes.

Binding capabilty of MAb to mefloquine in blood samples

The MAb designated 11F9(G5)G9 which gave

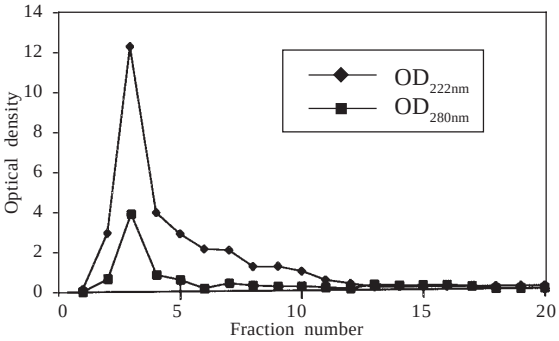

Fig 1–The optical density (OD) of mefloquine-BSA fractions. Mefloquine-BSA was purified by Sephadex G-75 gel filtration. The plots represent the optical density (OD) obtained from each fraction, diluted in 1:100 and measured at the wavelength of 222 nm and 280 nm.

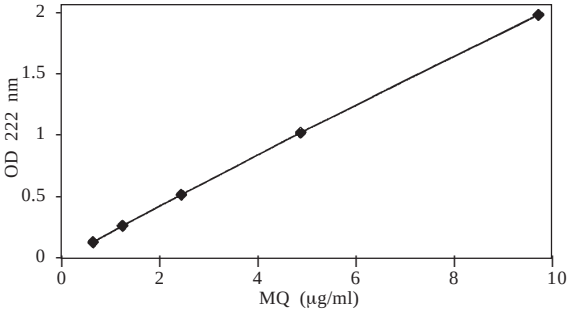

Fig 2–Mefloquine standard curve. The plots represent various optical densities (OD) of mefloquine concentrations at wavelength of 222 nm.

Table 1
Reactivity of MAbs with mefloquine-BSA and BSA determined by indirect ELISA.

MAb	MAb reactivity OD 405 nm		
	Mefloquine-BSA	Quinine-BSA	BSA
2F3(G10)G6	0.320	0.078	0.100
2F3(G10)G11	0.370	0.203	0.201
9B3(G8)E2	0.247	0.054	0.088
11D6(A2)E6[a]	0.558	0.283	0.183
11D6(A2)F7	0.569	0.232	0.236
11F9(B1)E9[a]	0.993	0.574	0.339
11F9(B1)G10[a]	0.906	0.486	0.354
11F9(B1)G12[a]	0.884	0.415	0.172
11F9(E5)G3	0.743	0.400	0.432
11F9(G5)G9	1.364	0.610	0.705
11F9(G5)G11[a]	0.875	0.570	0.191
11F9(E5)E3[a]	0.876	0.606	0.331
14F8(B3)D9[a]	0.363	0.172	0.113
14F8(B3)H3[a]	0.427	0.315	0.244
14F8(C4)E8	0.493	0.223	0.237
14F8(C4)E11[a]	0.358	0.120	0.073

[a]cross-reaction with quinine.

Table 2
Detection of mefloquine in blood using specific MAb 11F9(G5)G9 by indirect ELISA.

Blood	Mefloquine level (μg/ml)	MAb reactivity with mefloquine (OD405nm)
Malaria 1	2.077	0.512
Malaria 2	3.715	0.637
Malaria 3	2.575	0.418
Malaria 4	2.085	0.475
Normal	0.000	0.170

the strongest reaction or the highest OD value with mefloquine-BSA conjugate was selected to detect mefloquine in the blood samples from malaria patients previously treated with mefloquine compared to normal blood. The OD values were also compared to the amount of mefloquine detected by high performance liquid chromatography (HPLC) previously described (Karbwang *et al*, 1989b) (Table 2).

DISCUSSION

Successful production of monoclonal antibodies (MAbs) to drug compound depends greatly upon hapten-protein carrier conjugation in order to induce an immune response. The chemistry used to link the drug to the carrier protein will determine how the drug presented to B cells during the initial phase of immune response and hence, parts of drug molecule are subsequently recognized to induce antibody response. (Rowell, 1990). Previously, the mefloquine conjugate was prepared by reacting mefloquine with succinic anhydride to introduce a succinic linker on the nitrogen of piperidine ring. In the present study, mefloquine-BSA conjugate was prepared using the method previously described with chloroquine and quinine (Freier *et al*, 1986) with some modifications. These included: BSA was used as a carrier of choice for preparation of mefloquine-BSA conjugate; mefloquine should be dissolved in methanol before adding to succinic anhydride to form the mefloquine-succinyl linker for coupling with protein carrier (Wallen *et al*, 1994) due to its low solubility of this drug in most solvents (Mbela *et al*, 1994); N,N′ disuccinimdyl carbonated (DSC) is used to prepare the active esters and peptide instead of carbodiimide (Ogura *et al*, 1979; Sauer and Morris,1987) which are useful for coupling compounds with a variety of functional groups including carboxylic acids, amines, alcohols, *etc*.

The appropriate test system for screening antibodies with required specificity is important. Despite to the fact that the immunization of hapten-protein conjugate, the antibodies could be possibly recognize either the hapten or carrier protein or the combination of the two (Danilova, 1994). Since mefloquine and quinine are structurally-related antimalarials and the quinine-BSA conjugate was prepared by using the similar procedure, so they may show cross-reaction with the bridge or carrier protein. Given a large enough panel of unrelated test antigens, sooner or later a cross-reaction might be found (Goding, 1980). Therefore, specific MAbs to mefloquine were selected based on their high reactivity with mefloquine-BSA conjugate and low reactivity with BSA and quinine-BSA. All MAbs showed cross-reaction with BSA and quinine-BSA, but only 7 MAbs did not cross-react with quinine.

In order to determine whether the MAb could react with mefloquine in blood samples, the binding capability by ELISA was performed using the selected MAb against mefloquine-BSA, 11F9(G5)G9 as a detective reagent. Four blood samples from patients previously treated with mefloquine and containing mefloquine ranging from 2.077-3.715 μg/ml as detected by HPLC and one normal blood was tested. This preliminary test showed higher reactivity was obtained when such MAb reacted with blood sample containing mefloquine when compared to that of normal blood. The results indicated that such MAb may be useful to be further used as antibody probe for detection of mefloquine in body fluid. However, more blood samples from patients previously treated with mefloquine or other drugs should be tested in order to evaluate the more definite specificity and binding capabilities of this MAb as well as the sensitivity of the test for further application.

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