Peroxidase Labelled Monoclonal Antibody Against Light Chains of Human Cardiac Myosin

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Abstract. Monoclonal antibody against light chains of human cardiac myosin (MLC) was labelled with horseradish peroxidase. The conjugation was performed by two different methods with glutaraldehyde and periodate respectively. The binding activities of the conjugates were tested by enzyme linked immunosorbent assay (ELISA) on the microtitration plates with immobilized MLC (1—1000 ng per well). A comparison of both methods revealed their universal suitability for the preparation of conjugates as well as their applicability. The use of conjugates shortens the time needed and improves the ELISA method for MLC estimation. Specific advantages of the glutaraldehyde and the periodate method concern diverse details.

Key words: Monoclonal antibody — Myosin light chains — Horseradish peroxidase — Conjugation

Introduction

Among the best known methods for conjugation of enzymes with antibodies are glutaraldehyde methods of Avrameas (1969) and of Avrameas and Ternynck (1971). However, for the conjugation of horseradish peroxidase (HRPO) with antibody preference has been given to the periodate method of Nakane and Kawoi (1974) as simplified and optimized by Tijssen and Kurstak (1984). Henning and Nielsen (1987) showed that replacement of sodium borohydride by ascorbic acid in the final step of the periodate method produced a monoclonal antibody (MAb) — HRPO conjugate with a high level of stable activity.

Measurement of serum concentrations of human cardiac myosin light chains (MLC) may allow a non-invasive estimation of infarct size (Khaw et al. 1978; Haber et al. 1982; Katus et al. 1984; Katus et al. 1988). Cardiac MLC represent a heart specific antigen. Hence, monoclonal antibodies may be utilized for MLC estimation. The aim of this study was to prepare a conjugate of MAb against light chains of human cardiac myosin (anti-MLC MAb) with HRPO. Moreover, the glutaraldehyde and the periodate method for the preparation of the conjugate were compared and the conjugate activity was evaluated by the enzyme linked immunosorbent assay (ELISA).

Materials and Methods

Chemicals

The chemicals used in this study were purchased from commercial suppliers as indicated below: glutaraldehyde, horseradish peroxidase, bovine serum albumin, ethylmercury thiosalicylic Na-salt (thimerosal), Tween-20 (SERVA, FRG), Sephadex G-50 (Pharmacia, Sweden); other chemicals were purchased from Lachema, Czechoslovakia.

Preparation of antigen

Myosin light chains were isolated from human heart ventricle by the methods of Richards et al. (1967) and Perrie and Perry (1970) modified as follows: After initial extraction of homogenized cardiac muscle with solution containing KCl, $Na_4P_2O_7$ and Mg^{2+} ions the supernatant was precipitated with cold water. The sediment was dissolved in 0.3 mol/l KCl and centrifugated. The obtained supernatant was once again precipitated with cold water. Guanidine hydrochloride was added to the sediment to a final concentration of 5 mol/l. Then ethanol was added to the solution to a final concentration of 64 %, and the myosin light chains were extracted in the supernatant. The purity of MLC was checked by PAGE-electrophoresis.

Preparation of anti-MLC monoclonal antibody

Female BALB/c mice, approx. 12 weeks old, were prepared as spleen cell donors by intraperitoneal injections (100 μg mixture of MLC in Freund's complete adjuvans) 102 and 98 days before fusion. The booster injections (100 μg MLC in PBS) were given on days 7, 3, 2, 1 before fusion. The splenocytes from the immunized mice and Sp2/0 myeloma cells were used as parental lines, and hybridomas were constructed by the method of Köhler and Milstein (1975). Hybridomas were tested by ELISA test to estimate the production of antibodies. Positive hybridomas were twice cloned by the limiting dilution technique. The production clone 7A11/10 was cultivated in vivo in mice, and the ascites fluid was obtained.

Purification of monoclonal antibody

The monoclonal antibody was purified by the method of McKinney and Parkinson (1987). The method involves purification of IgG fraction from ascites fluid by a combination of caprylic acid

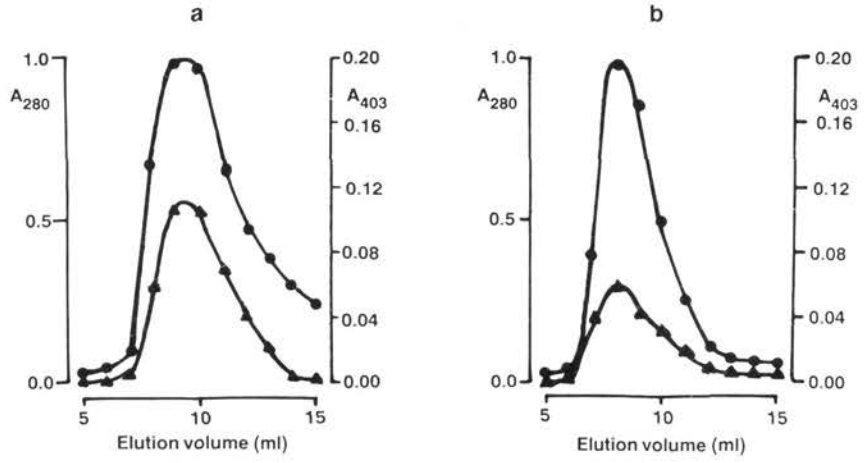

Fig. 1. Purification of conjugates prepared by: *a.* the glutaraldehyde method; *b.* periodate method on Sephadex G-50 column. Samples prepared by the described methods in a volume 0.5 ml in Tris. HCl buffer were applied on the column (12.5 ml) and the fractions were collected and measured at 280 nm ((●), total proteins) and 403 nm ((▲), the heme-component of horseradish peroxidase).

and ammonium sulfate precipitation. The purity of the antibody was checked by SDS-PAGE and its specifity was verified by "Western blotting".

Preparation of conjugates

a. The glutaraldehyde method of Avrameas and Ternynck (1971) was modified as follows:

Peroxidase (RZ = 3), 10 mg was dissolved in 0.2 ml phosphate buffer (0.15 mol/l KH_2PO_4, 0.15 mol/l Na_2HPO_4) pH 6.8 with 1.25 % glutaraldehyde. The mixture was kept overnight at room temperature and then applied to a Sephadex G-50 column. The protein fractions were eluted with phosphate buffer and the most active fractions (according to the absorbances at 280 nm and 403 nm) were pooled. To 1800 μl of the pooled fraction were added 0.1 ml 1 mol/l NaCl, 0.1 ml carbonate--bicarbonate buffer (pH 9.5) and 5 mg of anti-MLC antibody in 0.1 ml. The mixture was gently stirred and kept at 4 °C overnight. The reaction was stopped by adding 0.1 ml of 0.2 mol/l ε-aminocapronic acid, and the mixture was allowed to stay at room temperature for 2 hours. After precipitation with saturated ammonium sulfate solution the precipitate was collected by centrifugation (6000 *g*, 20 min), then twice washed with 5 ml of 50 % ammonium sulfate. The pellet was dissolved in 0.5 ml Tris. HCl buffer (pH 7.2) and fractionated on Sephadex G-50. The fractions containing the conjugate were conserved by bovine serum albumin (1 %) and thimerosal (0.01 %) and stored at 4 °C in darkness.

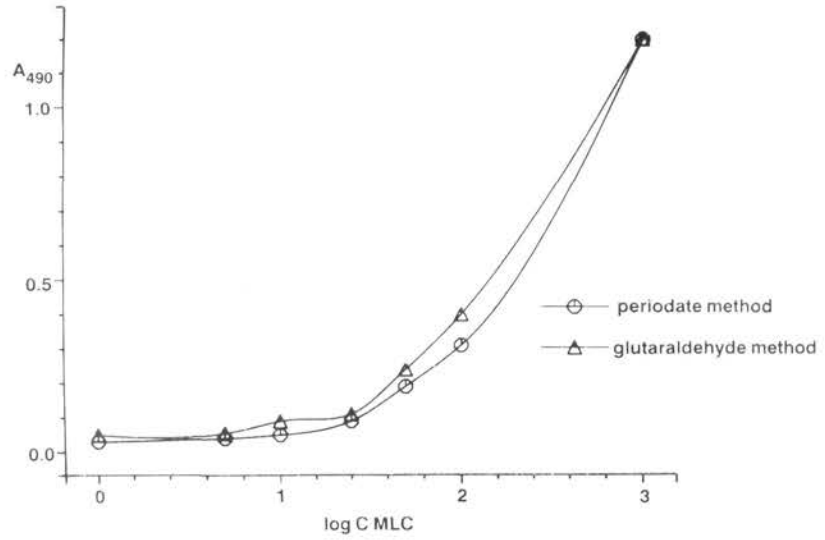

Fig. 2. Comparison of MLC binding activity of conjugates prepared by the glutaraldehyde (△) and the periodate method (○). The activity was determined by ELISA-test, and expressed as absorbance at 490 nm. Log c MLC is the logarithm of concentrations of myosin light chains (ng) bound to the surface of the microtitration plate. The values are means of triplicate assays.

b. The periodate method of Tijssen and Kurstak (1984) was modified as follows:

Peroxidase (RZ = 3), 4 mg was dissolved in 0.5 ml 0.1 mol/l carbonate buffer pH 8.4 and mixed with 0.5 ml of 0.016 mol/l natrium periodate (freshly prepared). The mixture was kept at room temperature in the dark for 2 hours, and subsequently applied to a Sephadex G-50 column equilibrated with 0.001 mol/l acetate buffer pH 4.4. Proteins were eluted with the same buffer and the most active fractions (according to the absorbances at 280 and 403 nm) were selected. Two ml of the fraction was mixed with 24.5 mg anti-MLC monoclonal antibody in 0.4 ml 0.1 mol/l carbonate-bicarbonate buffer (pH 9.2). The mixture was gently stirred and kept at room temperature in the dark for 2 hours. The reaction was stopped by addition of 1/20 volume of fresh 5 mg/ml ascorbic acid solution. The procedure was repeated after 1 hour with addition of 3/20 volume of the same freshly prepared solution and left at 4 °C for 1 hour. Precipitation with ammonium sulfate, application on Sephadex G-50, conservation and storage was the same as described for method under *a*).

ELISA

The wells of polystyrene microtitration plates (Novogen, ÚMG ČSAV) were coated with 0.1 ml of 1 – 1000 ng MLC per well in phosphate buffered saline (PBS, pH 7.4) overnight at 4 °C. The coated

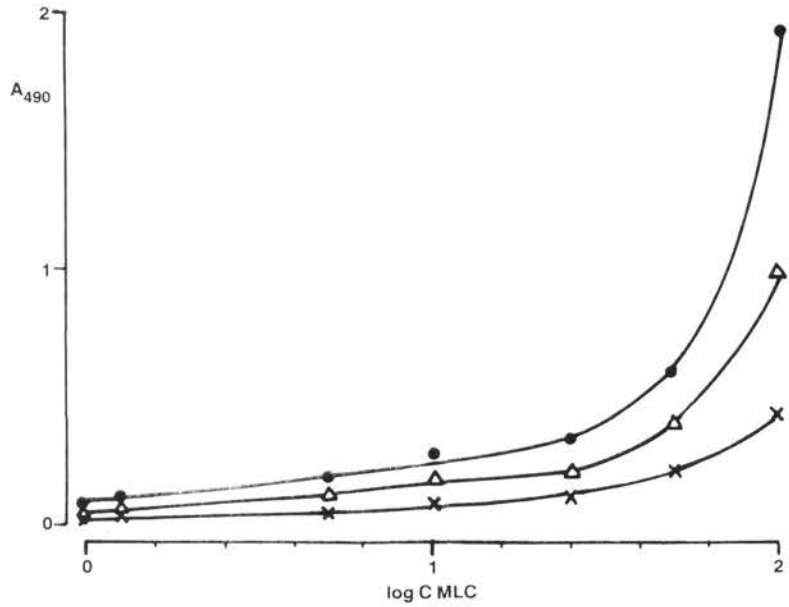

Fig. 3. The binding activity of conjugate prepared by the periodate method at different dilutions (1:25 – ●; 1:50 – △; 1:100 – x) as determined by ELISA test (absorbance at 490 nm). Log c MLC is the logarithm of MLC (ng) bound to the microtitration plate. Values are means of triplicate assays.

plates were washed three times with a wash buffer (PBS containing 0.05 % Tween 20). Non-specific binding sites were blocked with 10 % bovine serum in PBS for 1 hour at room temperature. The plates were washed three times with a wash buffer, and 0.1 ml anti-MLC MAb-HRPO conjugate was added to each well and incubated 1 hour at 37 °C. After the incubation and five washings with the wash buffer, 0.1 ml substrate was added (5 mg *o*-phenylenediamine dissolved in 10 ml phosphate-citrate buffer pH 5.0, and mixed with 4 μl of 30 % H_2O_2). Twenty min after the addition of the substrate the reaction was stopped with 0.05 ml 10 % H_2SO_4. The resulting optical density was measured at 490 nm on an ELISA Reader (Dynatech, FRG).

Results

The elution profiles of conjugates obtained from the Sephadex G-50 column are shown in Fig. 1. On the elution curves the activity of HRPO is illustrated by absorbance at 403 nm representing the heme, which is the key component of HRPO. The absorbance values at 280 nm indicate the total protein content (mainly the immunoglobulins). The elution profiles at 403 nm show that with

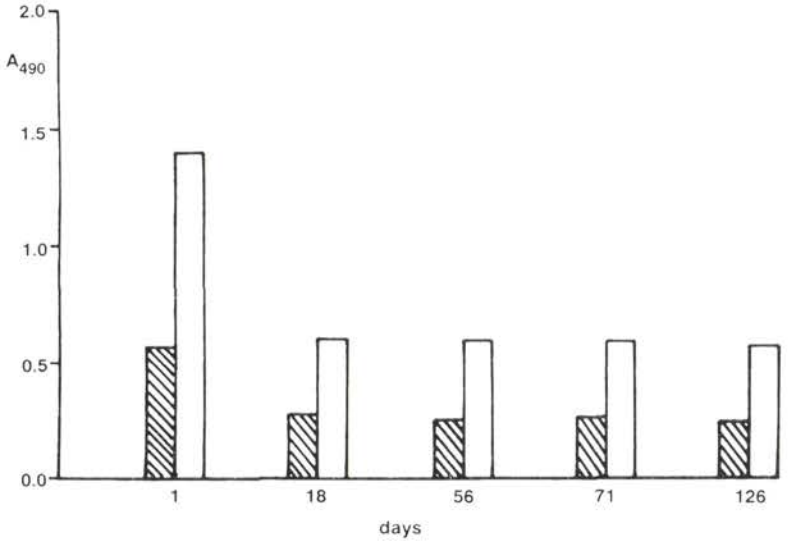

Fig. 4. The stability of the conjugate prepared by the periodate method. The preserved conjugates were stored at 4 °C in the dark. The binding activity was measured by ELISA-test and expressed as absorbance at 490 nm for MLC concentrations of 10 and 50 ng bound to the well surface within 1—126 days after the conjugate preparation. Values are means of triplicate assays. ▨ — 10 ng MLC, □ — 50 ng MLC.

the glutaraldehyde method the HRPO concentration was higher than with the periodate method. This corresponds with the quantities of HRPO used for the conjugation with the two methods.

The MLC binding efficacy of conjugates prepared by the glutaraldehyde and the periodate method is illustrated in Fig. 2. The binding activity was determined by ELISA-test and expressed as absorbance at 490 nm versus log concentration of MLC. At lower concentrations, (1—10 ng per well), a very moderate increase of activity of both conjugates can be seen; a marked increase of activity occurred at MLC concentrations higher than 10 ng per well.

In order to estimate the optimal conjugate concentration for MLC detection by ELISA test; the conjugate activity was measured at different dilutions (Fig. 3.). The curves in Fig. 3. were obtained for conjugate prepared by periodate method. The curves obtained for the glutaraldehyde preparation were similar.

Fig. 4. illustrates the stability of the conjugate at 4 °C in darkness at various time intervals as tested by the ELISA method. A comparison of the stability obtained by the glutaraldehyde method with that obtained by the periodate method did not show any significant differences.

Discussion

In present work two methods commonly used for the preparation of HRPO-anti MLC MAb conjugates were modified and compared to each other: the glutaraldehyde method of Avrameas and Ternynck (1971) and periodate method (Tijssen and Kurstak 1984; Henning and Nielsen 1987). The conjugation experiments using the periodate method showed that the sodium periodate concentration is critical for an efficient conjugation and preservation of the enzyme activity. Low oxidation prevented an efficient conjugation, whereas strong oxidation resulted in the formation of carboxyl groups instead of aldehyde groups. Futhermore in inactivation of enzymes, in the formation of polymers, strongly oxidized HRPO acted as a bridging molecule between two IgG molecules (Tijssen and Kurstak 1984). In the experiments sodium periodate at 16 mmol/l was selected; this concentration appeared suitable for the preparation of active HRPO-MLC MAb conjugate.

The MLC binding activity of the conjugates was verified by ELISA-test on microtitration plates with immobilized MLC. The results confirmed comparable activities of conjugates prepared by the two methods.

The procedure of conjugate preparation may affect their stability. Henning and Nielsen (1987) showed that upon replacing sodium borohydride by ascorbic acid or ethanolamine in the final step of the periodate method of conjugating MAbs with HRPO, the antibody-enzyme conjugates maintained a high level of stability. Therefore in the present work ascorbic acid (5 mg/ml) was chosen as the reduction agent for the conjugate preparation by the periodate method. The common procedure for enzyme-antibody complexes using bovine serum albumin and thimerosal (Weston et al. 1980) was employed for the storage of HRPO-anti MLC MAb at 4 °C in the dark.

The activity of the conjugate prepared by the periodate method was tested by ELISA test at different MLC concentrations and at various time intervals. At 100 and 1000 ng MLC per well the activity of the conjugate after 126 days was still at a high level (over the measurable maximum). At 50 ng and 10 ng per well, the activity decreased after 18 days of storage, and remained constant thereafter.

It can be concluded that both methods are suitable for the preparation of conjugates of HRPO with monoclonal antibody against MLC. A main advantage of the glutaraldehyde method seem to be the small amounts of monoclonal antibody required in comparison with the periodate method. On the other hand, the periodate method offers products of good stability and binding activity, and requires small concentrations of HRPO. Nevertheless, the periodate method should be optimalized as for the $NaIO_4$ concentration required to obtain active conjugate.

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