One-Step Sandwich Enzyme Immunoassay for Serum Myoglobin with Three Monoclonal Antibodies to Different Epitopes

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Enzyme-linked sandwich immunosorbent assay for serum myoglobin with 3 monoclonal antibodies to different epitopes is described. Antibodies 13G6 and 18H2 were adsorbed on a carrier, and 14D6 antibodies were labeled with horseradish peroxidase. One-step incubation with standard myoglobin solution yielded double sandwich: adsorbed antibodies—myoglobin—labeled antibodies; the intensity of peroxidase reaction was directly proportional to myoglobin concentration. The sensitivity of the proposed method for serum myoglobin was below 5 ng/ml. The sensitivity of the method increases due to myoglobin binding with two antibodies adsorbed on the carrier. Fluctuations within and between series did not exceed 10%.

Key Words: *myoglobin; monoclonal antibodies; myocardial infarction; myoglobin enzyme immunoassay; sandwich assay*

Serum concentration of myoglobin (MG) is often measured in clinics for early diagnostics of myocardial infarction and the monitoring of thrombolytic therapy [4,8,12]. MG appears in the blood as soon as 2-4 h after heart attack [4,13], while other myocardial infarction markers (creatine kinase MB, troponins T and I) appear later [3]. Radioimmunoassays, enzyme immunoassays, turbidimetric and agglutination methods based on polyclonal antibodies have been proposed for MG measurements [12]. Only few publications describe methods of MG determination based on monoclonal antibodies (MAB) [1,5,9]. We developed a one-step enzyme immunoassay for human serum MG with 3 MAB to different epitopes of MG. Two of these MAB are adsorbed on a carrier and the third MAB was labeled with horseradish peroxidase.

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MATERIALS AND METHODS

MG from human myocardium was isolated as described elsewhere [14]. Experiments were carried out with MG of higher then 99% purity. Hybridomas producing anti-MG antibodies were prepared as described previously [6] by fusing splenocytes from MG-immunized mouse with X63-AG8.653 myeloma cells. MAB-producing clones were identified by enzyme immunoassay. Microtitration plates for immunoassay were saturated with MG for 40-48 h at 2°C (5-10 μg/ml in 0.05 M carbonate buffer, pH 9.5, 100 μl per well), other procedures were described in previous reports [2,7,8]. Ascitic fluid was obtained as described previously [2,7,8], antibodies from ascitic fluids were isolated by affinity chromatography on MG-Sepharose [2]. Conjugate of 14D6 MAB with horseradish peroxidase was prepared using standard methods [11]. For sandwich MG assay, rows of microtitration plates (Institute of Medical Polymers, Moscow) were saturated with anti-MG antibodies (clones 13G6 and

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18H2) for 40-48 h at 2°C (5 μg/ml MAB of each clone in 0.05 N carbonate buffer, pH 9.5; 200 μl per well). The wells were washed with distilled water and 175 μl peroxidase-labeled MAB (clone 14D6) diluted 1:8000 and 25 μl test serum or standard MG solution containing 5, 10, 20, 40, 80, 160, and 320 ng/ml MG for calibration curve were added. To this end, purified MG was diluted in MG-free human serum. MG was removed from the serum by affinity chromatography on BrCN Sepharose, conjugated with anti-MG MAB (clones 18H2 and 13G6). The plates were incubated for 2.5 h (until equilibration) at 37°C, washed 4-5 times with distilled water, and 200 μl orthophenylenediamine (1 mg/ml in 0.05 M citrate-phosphate buffer (pH 5) containing 0.03% H_2O_2) was added. The plates were incubated for 30 min at room temperature and the reaction was stopped by adding 2 M HCl (25 μl per well). Absorption was measured in a Titertek Multiskan spectrophotometer (Flow Lab.) at 472 nm.

RESULTS

Fusion of splenocytes from immune mice with X63-AG8.653 myeloma cells yielded 69 immunopositive clones (A_{472}>0.3 by the data of enzyme immunoassay), which constituted about 11% of the total number of clones (640). Five clones: 19B5, 20C10, 14D6, 13G6, and 18H2 produced MAB characterized by strong positive reaction in the enzyme-linked immunosorbent assay even after the supernatants were diluted 100-1000-fold. After cloning these hybridomas were used for reproduction of ascitis. MAB isolated from ascitic fluids were conjugated with horseradish peroxidase. Then we analyzed various combinations of MAB that formed sandwich in the presence of MG. To this end, purified MAB (capturing antibodies) were adsorbed on microtitration plates. The plates were incubated with MG and peroxidase-labeled MAB of other clone. If the capturing (adsorbed) and labeled antibodies bind

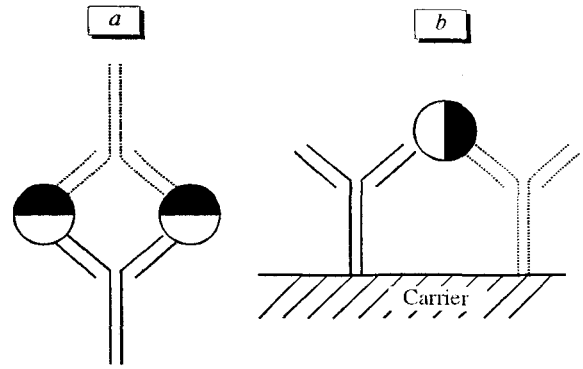

Fig. 1. Cyclic complexes formed by two monoclonal antibodies (MAB) to different epitopes of the antigen molecule (circle) and two antigen molecules (*a*), and complexes formed on the carrier surface by two adsorbed MAB to different antigen epitopes and one antigen molecule (*b*).

to different epitopes of MG, a double sandwich is formed: adsorbed antibodies—MG—labeled antibodies, which can be detected by its peroxidase activity.

We analyzed all possible combinations of capturing and labeled MAB for 5 clones. For each combination of MAB with MG forming a sandwich, the corresponding calibration curve was constructed using a standard MG solutions with concentrations of 5-320 ng/ml. A variant when clones 18H2 and 13G6 were used as capturing MAB and clone 14D6 as labeled MAB was the optimum combination. It should be noted that clones 18H2 and 13G6 interact with different MG epitopes, because these antibodies form sandwich with MG. Moreover, clone 14G6 in the presence of MG forms sandwich with both clone 18H2 and 13G6 antibodies. Thus, the optimized sandwich analysis uses MAB to 3 different epitopes of MG.

The combined use of 2 MAB to different epitopes as capturing antibodies considerably increased the sensitivity of this method (Table 1), which can be attributed to simultaneous interaction of some MG molecules with two adsorbed MAB clones 13G6 and 18H2 yielding a cyclic complex: 13G6 clone MAB—MG—

TABLE 1. Calibration Curves of MG Assay in Sandwich-Method with MAB (Clones 13G6, 18H2, and Their Combination) Adsorbed to a Carrier (*M*±*m*, *n*=5)

Concentration of MG, ng/ml	Optical density on antibody saturation		
	13G6, 10 μg/ml	18H2, 10 μg/ml	13G6, 5 μg/ml+18H2, 5 μg/ml
0	0.127±0.016	0.195±0.034	0.186±0.021
5	0.137±0.018	0.206±0.017	0.285±0.020**
10	0.146±0.007	0.249±0.010*	0.448±0.019
20	0.153±0.022	0.282±0.025	0.654±0.014
40	0.186±0.015**	0.432±0.050	1.161±0.065
80	0.265±0.030	0.843±0.084	1.799±0.114
160	0.446±0.071	1.467±0.086	?2

Note. Incubation mixture also obtained MG and peroxidase-labeled MAB (clone 14D6. *p<0.01, **p<0.001 compared with zero MG standard.

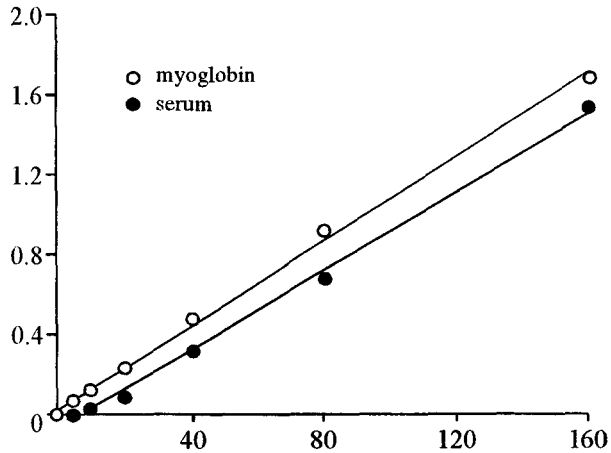

Fig. 2. Standard calibration curve constructed with purified myoglobin and experimental curve obtained by adding increasing concentrations of myoglobin-rich serum to the incubation mixture. Ordinate: optical density; abscissa: myoglobin, ng/ml; serum, μl×13.

18H2 clone MAB attached to the plate surface (Fig. 1, *b*). Complexes between 2 MAB to different epitopes and 2 antigen molecules can be formed in the solution [10] (Fig 1, *a*). The use of 2 MAB in competitive analysis considerably increased the sensitivity of the assay [10]. Thus, the affinity of MAB mixture far surpasses that of individual MAB, because dissociation of MG from the complex is improbable.

In the next experimental series we proved the identity of purified MG isolated from the heart and immunoreactive substance in pooled serum from patients with myocardial infarction (with high MG content). Pooled serum was diluted 2-, 4-, 8-fold and more with MG-free serum. The obtained concentration curve practically coincided with the calibration curve constructed with purified MG (Fig. 2). This confirmed the identity of immunoreactive serum MG and purified MG used as the standard.

The proposed method allows to measure serum MG in the concentration range of 5-80 ng/ml. Interserial fluctuations and fluctuations between series (n=5) did not exceed 10%. Detection of MG in this concentration range was 96.5-119.8%.

Concentrations of MG were measured in 59 donor sera (age and sex were not taken into consideration). In 27 samples MG were not detected (the concentration was taken to be equal a half-minimal concentration: 2.5 ng/ml). In 32 samples MG concentration varied from 5 to 165 ng/ml. The mean MG concentration in the serum was 12.5±2.28 ng/ml.

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