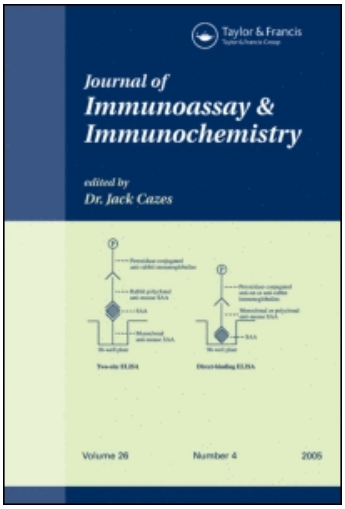

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QUANTITATIVE ELISA FOR HUMAN LACTATE DEHYDROGENASE ISOENZYME 5

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ABSTRACT

Four monoclonal antibodies (Mab) derived from mice immunized with lactate dehydrogenase 5 (LDH5) react strongly with LDH5, but weakly with LDH2 which contains a single subunit of type M. Experimental evidence suggests that these antibodies are directed to an antigenic determinant in the interface between two subunits of type M. A sandwich ELISA procedure was devised, using these Mabs to identify and quantify LDH5. The procedure involves immobilization of one of these Mabs by its adsorption onto polyclonal anti-mouse IgG coated polystyrene plates, adsorption of LDH5, its identification by the same Mab as that used in the immobilization step, and finally color development by an enzyme labeled rabbit anti-mouse IgG antiserum. The method enables LDH5 to be assayed at a concentration range of 0-5 µg/ml.
(KEY WORDS: Lactate Dehydrogenase - monoclonal antibodies - ELISA)

INTRODUCTION

The five lactate dehydrogenase (LDH) isoenzymes in vertebrates are tetramers composed of two types of subunits designated H and M. Although the amino acid composition differs in each of the subunits and in every species, there is considerable homology among the subunits and particularly between

subunits of the same type in different species (e.g. porcine and human). Each subunit (34000 Mr) is noncovalently bound to its neighbor subunit and possesses a binding site for nicotinamide adenine dinucleotide (NAD) and an active site. The tertiary structure of all LDH isoenzymes so far examined is similar and shows perfect 222 symmetry. In figure 1 a color nomenclature is used to show the way in which the subunits are arranged in association (1).

The diagnostic importance of LDH levels in the serum is widely recognized. Raised serum levels of LDH isoenzymes are associated with many disease states, in particular those involving the heart, skeletal muscles, the liver, erythrocytes and tumors (2). The distribution pattern of the five LDH isoenzymes is affected by various disease conditions. In myocardial infarction, for example, LDH1 and LDH2 levels are increased by 2- to 10- fold; in hepatitis, liver congestion, and myopathies of skeletal muscle, LDH5 and LDH4 levels are raised by 1- to 2- fold. In healthy subjects, LDH5 and LDH4 each constitute approximately 6% of the total LDH activity (260 IU/ml) (3). Current methods for the separation of LDH isoenzymes are based on differential heat stability, substrate analogs and differential inhibitors. Of the direct visualization procedures on agar or agarose, the most accurate appears to be agarose electrophoresis followed by fluorescence densitometry (4). All of these methods are tedious and the results are usually expressed as ratios between the activities of the isoenzymes rather than as their concentrations.

This paper describes a quantitative ELISA for LDH5. The assay (see fig. 2) is based on the concept that a Mab specific for an antigenic determinant in the interface between two subunits can be used first as a capture antibody and subsequently as a specific labeling antibody for a given LDH isoenzyme. The method was tested with four different Mabs and the dose response range was between 0 and 5 µg of LDH5/ml.

MATERIALS AND METHODS

Human LDH isoenzymes: LDH1 (H4), LDH2 (H3M), LDH3 (H2M2), LDH5 (M4) in 2.1 M $(NH_4)_2SO_4$ suspensions were obtained from Sigma, USA. Chemicals used for buffers were of analytical grade and were purchased from Merck, Darmstadt and from Frutarom, Haifa. Other materials used were Tween 20, bovine serum albumin (BSA), p- nitrophenylphosphate (Sigma, USA), diethanolamine (DEA) (Fluka AG, Buchs), Dulbecco modification of Eagle's medium (DMEM) and horse serum (Beit Haemek Israel). Phosphate buffer saline (PBS) pH 7.4 contained 0.15 M NaCl and 30 mM phosphate.

Monoclonal Antibodies

The four clones of Mabs against porcine LDH5 used in these experiments (2/66 (IgG1), 40 µg/ml; 4/32 (IgG2a), 15 µg/ml; 5/43 (IgG1), 14 µg/ml; 7/54 (IgG2a), 17 µg/ml) were prepared by Hollander (6). Mab H/06 was from Bio-Yeda, Rehovot. The antibodies were obtained from the supernatants of cell culture media which consisted of DMEM with 10% (v/v) horse serum.

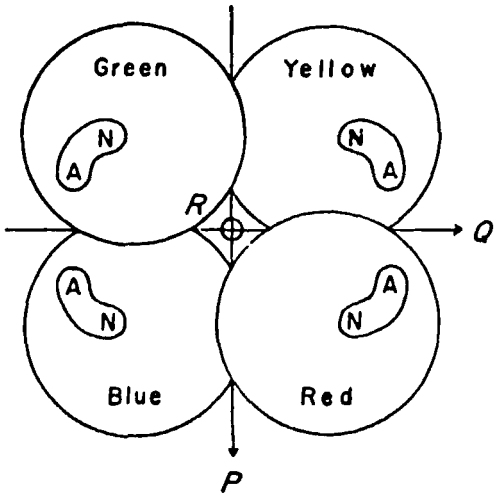

FIGURE 1. Diagrammatic representation of LDH (5). The binding site of the NAD cofactor is indicated on each subunit by the letters A N.

Determination of Mab Specificity by ELISA

Polystyrene microtiter plates (Costar, Holland) were coated for one hour at 37°C with 0.1 ml of LDH isoenzyme solution (LDH5, LDH2, or LDH1, 100 µg/ml) containing 50 µg of BSA/ml in PBS. The plates were blocked with 200 µl of 5 mg BSA/ml in PBS. Undiluted Mab (0.1 ml of each of the four clones) was added and the mixture incubated for one hour at 37°C. Washing procedures, incubation conditions and labeling with rabbit antiserum against mouse IgG conjugated to alkaline phosphatase were as described below.

Quantitative ELISA for LDH5

Polystyrene microtiter plates were coated overnight at 4° C or for one hour at 37°C with 0.1 ml of rabbit antiserum (Bio-Yeda,

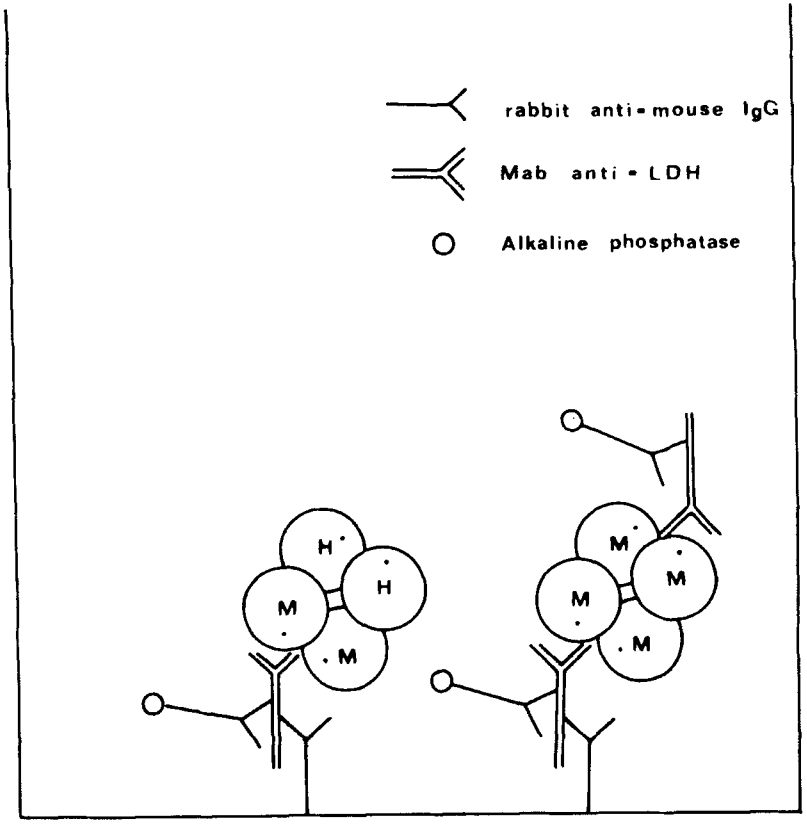

FIGURE 2. Configuration of the quantitative ELISA developed for LDH5. From left to right, assay with LDH3 and with LDH5. The binding site of the NAD cofactor, marking the relative orientation of the LDH subunits, is indicated by a dot.

Rehovot) against mouse IgG (H+L) at 1:1000 dilution in PBS pH 7.4. The plates were washed three times with PBS containing 0.5% Tween 20 and once with PBS, and were then blocked with 200 µl of 5mg BSA/ml in PBS. The following solutions (100 µl) were then added sequentially and incubated in each case for one hour at 37°C: Mab

against LDH, undiluted or at 1:10 dilution in PBS; LDH diluted in 5 mg BSA/ml in PBS; Mab against LDH (same solution as described above); rabbit antiserum against mouse IgG (H+L) conjugated to alkaline phosphatase (Bio-Yeda, Rehovot) at 1:1500 dilution in PBS containing 0.5% Tween 20. The plates were washed with 0.1 M DEA pH 9.8 prior to the addition of the enzyme substrate p-nitrophenylphosphate (1 mg/ml) in 10% DEA pH 9.8, 0.4×10^{-3} M $MgCl_2$. The absorbance at 410 nm (O.D.) was read after 45 min at $37^{o}C$ in an SLT210 ELISA reader (SLT-Labinstruments, Austria). All assays were performed at least in triplicate. Control wells were prepared without the addition of LDH. The net absorbance at 410 nm for each sample was calculated by subtracting the average value obtained for control wells from the average for the test wells.

RESULTS

Characterization of the Monoclonal Antibodies

The results of the screening assay for the Mabs appear in table 1. As expected on the basis of previous results (6), the four Mabs tested reacted weakly with LDH2, while a strong reaction was observed in the control wells where a Mab was directed against the H subunit. The specificity of the ELISA was tested using the four LDH isoenzymes commercially available. The weak response of the four Mabs to LDH3 (table 2) relative to their response to LDH5 demonstrates the specificity of the assay towards LDH5. Raising the concentration of LDH1 to 50 ug/ml did not affect the results .

TABLE 1

Determination of Mab Specificity by ELISA.

Mab\LDH	LDH1	LDH2	LDH3	LDH5	LDH3-LDH1	LDH2-LDH1
2/66	0	N.D.	.083	.440	.083	N.D.
	.011	.018	N.D.	.141	N.D.	.007
4/32	0	N.D.	.210	.910	.210	N.D.
	.013	.074	N.D.	.209	N.D.	.061
5/43	.008	.039	N.D.	.129	N.D.	.031
7/54	0	N.D.	.108	.820	.108	N.D.
	.017	.044	N.D.	.149	N.D.	.027
H/06	.351	.521	N.D.	.009	N.D.	N.D.

The net absorbance values at 410 nm are the results of two separate experiments. Microtiter plates were coated with 100 µg/ml of the indicated LDH isoenzymes. The Mabs were applied without dilution. The second antibody was a rabbit anti-mouse IgG-alkaline phosphatase conjugate at a dilution of 1:1500. A Mab specific to the H subunit, H/06 , was used as a positive control for LDH5 and LDH2 coating. The differences in net absorbance values at 410 nm between the plates coated with LDH3 and LDH1 and those coated with LDH2 and LDH1 are given in the last two columns.

TABLE 2

Specificity of the ELISA for the detection of LDH5.

Mab\LDH	LDH5	LDH3	LDH2	LDH1
2/66	.088±.030	0 ±.010	0 ±.030	0 ±.040
7/54	.109±.030	.019±.016	.022±.012	.043±.015
5/43	.103±.016	.023± 0	.039±.016	.018±.008
4/32	.190±.006	0 ±.018	0 ±.020	0 ±.007

Values represent the mean net results +/- S.D. obtained in the ELISA developed for the detection of LDH5. Mabs were diluted 1:10 in PBS. All LDH isoenzymes were at 10 µg/ml in 5mg of BSA/ml.

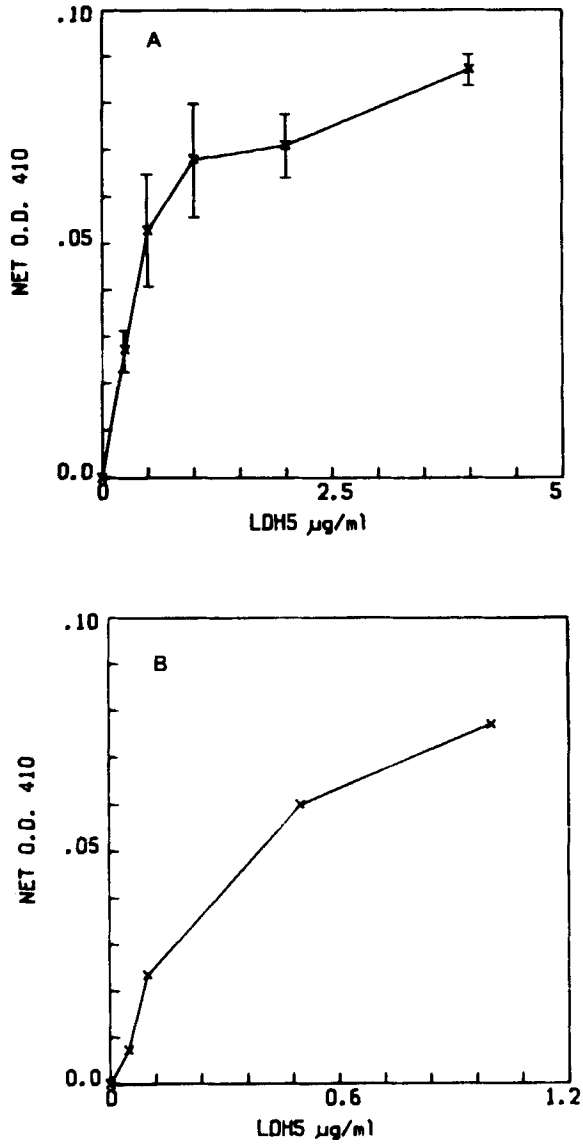

FIGURE 3. Quantitative ELISA developed for LDH5: standard curves for LDH5 with two different Mabs at 1:10 dilutions. (A) Mab 5/43; (B) Mab 4/32.

Quantitative ELISA for LDH5

The standard curves obtained for ELISA of LDH5 with Mabs 5/43 and 4/32 are presented in figure 3. A similar dose-response relationship to LDH5 was obtained with Mab 2/66 and 7/54, and also when the antigen was diluted in horse serum previously heated to 56°C for 30 minutes. The present assay was found to be sensitive at 50 ng of LDH5/ml (fig. 3B). Under the assay conditions employed here, the beginning of a plateau at 2 µg/ml of LDH5 suggests a binding capacity of 200 ng LDH5 per well. The use of purified Mab for coating the wells would probably result in an increase in the binding capacity of the wells for LDH5 and an extension of the linear part of the curve.

DISCUSSION

Attempts to develop an immunoassay for the quantitative determination of LDH isoenzymes are being pursued by medical diagnostic companies. The strategy adopted by many workers is to produce monoclonal antibodies specific for the various LDH isoenzymes. So far these efforts have apparently been unsuccessful, and to the best of my knowledge there is no direct quantitative immunoassay available for the various LDH isoenzymes. Recently a better understanding of the interactions between Mabs and proteins at the molecular level has been reached through the elucidation of the three-dimensional structure of the complex between Fab D1-3 and lysozyme from hen (7). These studies showed that the interaction between the antibody and the enzyme takes place over a surface of 20x25Å. Such an area would not be large

enough to include amino acid residues from all four subunits of LDH, and the feasibility of raising specific Mab to the various LDH isoenzymes is therefore called into question. Moreover, analysis of the contact residues between the four subunits (8) indicates that most contacts take place between residues on two of the subunits while there are no contacts between the four subunits within 5Å.

In the assay presented here, my object was to find monoclonal antibodies which are specific for an epitope in the contact region between two subunits only. Mabs reacting with intersubunit antigenic determinants of other proteins have been described (9). Since the association between the four subunits of LDH occurs in an ordered and well-defined way, there are theoretically only two identical antigenic determinants of this kind per tetramer. This is illustrated in figure 1, where the relative orientation of the subunits is indicated by the cofactor binding site (A-N). The diagram shows that the contact area between identical subunits, color-coded green and blue, differs from the contact area between the green and yellow subunits. A whole molecule of LDH would therefore accommodate only two Mab molecules directed to the intersubunit contact region. The specificity of the assay should thus depend on the type of intersubunit antigenic determinant recognized by the Mab, as shown in table 3. One might then expect that only a Mab reacting with an intersubunit region between two subunits could ensure specificity to a given LDH isoenzyme in the ELISA described here. Experimentally it was

TABLE 3

Expected Recognition of LDH Isoenzymes by Mabs with assumed specificities.

Mab specificity	LDH1	LDH2	LDH3	LDH4	LDH5
H subunit	+	+	+	-	-
M subunit	-	-	+	+	+
HM intersubunit	-	-	+	-	-
MM intersubunit	-	-	-	-	+
HH intersubunit	+	-	-	-	-

This speculative table shows that a Mab directed to one of the subunits would not confer specificity on the ELISA assay, while a Mab directed to an intersubunit region would discriminate between LDH1, LDH3 and LDH5.

found that the four Mabs reacted to different extents with the LDH isoenzymes and that a higher specificity for LDH5 was achieved when using Mabs 2/66 and 4/32 (table 2). Evidence that the four Mabs used in the assay are directed to antigenic determinants in the interface of two subunits comes from the screening assay and from an analysis of the size of the Mab-LDH5 complexes. The screening assay (table 1) shows that the binding of these monoclonal antibodies to LDH2, which contains a single subunit of type M, is weaker than the binding to LDH3 which contains two M subunits (the differences in net absorbance at 410 nm between the binding of the Mab to LDH2 and LDH1 or to LDH3 and LDH1 were compared). The weak binding with subunit M is interpreted as a result of interaction between the antibody with only half of the antigenic determinant contributed by the single subunit.

Complexes of molecular weight of 300000 and 450000 dalton were detected in a sucrose gradient under rate zonal

centrifugation conditions (6) with tenfold molar excess of Mab over LDH5. Such molecular species correspond to a 2:1 molar ratio of antibody bound per LDH. If the antibodies were directed to an antigen on a single subunit of type M, complexes of 750000 daltons would have been found (i.e., a 4:1 molar ratio of antibody bound per LDH5).

Some of the characteristic features of the assay described here are as follows : 1) direct quantitative assay of LDH5 without needing to isolate LDH5; 2) binding of the antigen occurs in solution; 3) use of the same Mab in the sandwich assay described. The use of purified Mab can be expected to improve the sensitivity of the assay. It would also allow the procedure to be shortened, as the polyclonal plate coating and the polyclonal labeling step could be omitted. It should be noted that in this immunoassay a single Mab plays two different roles: in its immobilized form as a capture antibody and thereafter as a specific label of the bound antigen. Using the same principle it is probable that a quantitative ELISA for LDH1 and LDH3 could be developed.

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