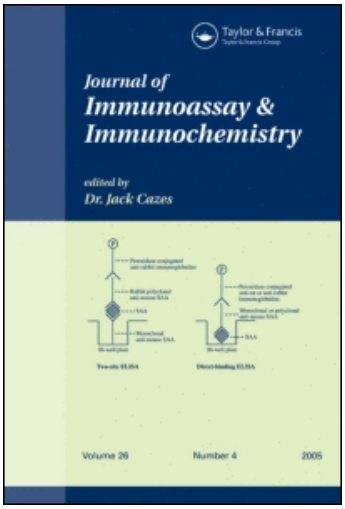

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DEVELOPMENT OF CONCANAVALIN A-ENZYME IMMUNOSORBENT ASSAY FOR GLYCATED HAPTOGLOBIN USING POLYCLONAL AND MONOCLONAL ANTIBODIES

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(KEY WORDS: Haptoglobin, Concanavalin A, ELISA)

Abbreviations used: Hp, haptoglobin; Con A, Concanavalin A; anti-Hp-HRP, anti-haptoglobin antibodies conjugated with horse-radish peroxidase; PEG, polyethylene glycol 6000; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid buffer; PBS, phosphate buffered saline.

ABSTRACT

Two-site lectin-haptoglobin-enzyme immunosorbent assay (L-Hp-ELISA), is described. Haptoglobin binding to Concanavalin A, immobilized to polystyrene microtiter plate, was estimated by anti-haptoglobin polyclonal and monoclonal antibodies conjugated with horse-radish peroxidase. The range of haptoglobin binding to Concanavalin A, measured by the L-Hp-ELISA was 25 to 300 ng/ml using polyclonal, and 50 to 600 ng/ml using monoclonal anti-haptoglobin antibodies, respectively.

INTRODUCTION

Haptoglobin (Hp) is an α_2-acid glycoprotein, which irreversibly binds hemoglobin. Hp is an acute phase protein, its level in blood is increased in inflammatory reaction, cancer, tissue necrosis, burns etc.(1).

Recently, many papers have focused on the fact that increased concentrations of acute phase serum glycoproteins have been often associated with altered binding to lectins as a result of differences in oligosaccharide structures (16, 17). Changes in lectin-glycoprotein complexes have been studied by means of affinity chromatography (15), affinity immunoelectrophoresis (2, 5, 8), electroimmunoblotting (22), enzyme-immunosorption (12, 13), and immunofluorimetric (21) techniques.

Our previous immunoenzymatic assay for human Hp was based on two binding sites on the Hp molecule, namely for hemoglobin and for the specific antibody (11). The present paper describes a Hp-ConA-enzyme immunosorbent assay taking advantage of the reaction of a lectin with the mannobiosyl core of Hp.

MATERIALS AND METHODS

Materials

Normal sera were obtained from healthy volunteers. Cancer sera, cord sera, cerebrospinal fluids, and pleu-

ral effusions were kindly donated from Clinics of the University Medical School in Wrocław (Poland).

Human Hp type 2-1 was isolated from ascitic fluid of a patient with ovarian carcinoma, as previously described (10). The preparation formed one band in crossed immunoelectrophoresis.

Antiserum directed against human Hp was produced in a goat (9). Goat antibody conjugated with horse-radish peroxidase was prepared by the periodate method (18).

Monoclonal antibodies were produced in BALB/c/J/I i W mice with human Hp 2-1. Spleen cells were fused with SP 2/0-Ag 14 hybrid plasmacytoma cells. Selected clones were expanded in vitro and injected into BALB/c mice. Monoclonal antibodies 7.60.66.55 and 18.4.40.80 (both of IgG_1 class) were used in further experiments (10).

Concanavalin A (Con A), the lectin from Canavalia ensiformis was purchased from Serva (F.R.G.) in lyophilised, research grade form (control A 6).

Lectin-Haptoglobin-Enzyme-Linked Immunosorbent Assay (L-Hp-ELISA)

Principle. The assay is based in the specific interaction of Hp with immobilized Con A. Con A-Hp complex is detected by means of either polyclonal or monoclonal anti-Hp antibodies conjugated with horse-radish

peroxidase. The sandwich assay was as described by Engvall and Perlman (4).

Reagents.

1) Coating buffer - acetate buffer 0.1 mol/L, pH 6.0, containing 1 mmol/L of each cation: Ca, Mg, Mn.
2) Washing buffer - HEPES (Hopkin and Williams, England) 0.005 mol/L, pH 7.2, containing 0.15 mol/L NaCl, 0.5 ml/L Tween 20, 02 g/L Thiomersal (BDH Chemicals Ltd, England), and 1 mmol/L of Ca, Mg, Mn.
3) Washing buffer containing 20 g/L polyethylene glycol 6000 (PEG).
4) Con A - 0.1 g/L in the coating buffer.
5) Hp standard - prepared from ovarian cancer ascitic fluid (ref.10). Serum containing Hp can also be used.
6) Anti-Hp-HRP - goat or monoclonal antibodies conjugated with horse-radish peroxidase, were stored at -20^{o}C in small aliquots in PBS buffer, pH 7.3, containing 50% glycerol, 0.2 g/L Thiomersal, 10 g/L bovine serum albumin. A stock solution of the conjugate was diluted 2000-fold with washing buffer containing PEG.

Test procedure

Coating with Con A; washing; blocking. To each well in amicrotiter plate, 0.1 g/L Con A (200 µl per well) in

coating buffer was added and incubated at 37°C for 3 h. The coated plate was washed 4 times with washing buffer, once fast and 3 times with 5 min intervals between, followed by blocking with 5 g/L of bovine serum albumin, incubation for 1 h at 37°C, and overnight at 4°C.

Binding of haptoglobin; washing. Fifty µl of Hp standard dilutions (2-150 ng/well), and diluted patient and control samples were added to the wells and incubated for 1 h at 37°C. A 1:5000 dilution of serum usually sufficed. When Con A-bound Hp per well exceeded 60 ng, the sample had to be more diluted. The plate was washed 4 times with HEPES buffer and once with PEG buffer.

Three control samples were included: cord serum without detectable Hp (i.e. less than 5 ng/ml measured by Hp-ELISA); washing buffer; and serum with known Con A binding ability. All analyses were done in triplicate. Standard curves were prepared with each series of analyses.

Binding of conjugate; washing. Anti-Hp-HRP was added and incubated for 30 min at 37°C. Four cycles of washing with PEG-buffer were carried out.

Development of peroxidase activity. 200 µl of substrate solution (0.5 mg p-phenylenediamine in 1 ml citrate buffer, pH 5.0 was added to each well. After incubation

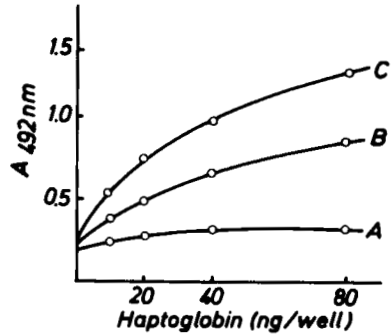

FIGURE 1. Effect of pH on Con A binding to the polystyrene microtiter plate. 3 h coating was carried out with 20 ug/well Con A in: **A** - 0.1 mol/L carbonate/bicarbonate buffer, ph 9.2; **B** - HEPES buffer, 0.005 mol/L, pH 7.2, with NaCl and cations, without Tween 20; **C** - acetate buffer, 0.1 mol/L, pH 6.0, with cations. Other details as in Methods.
The points represent mean values from 4 estimations.

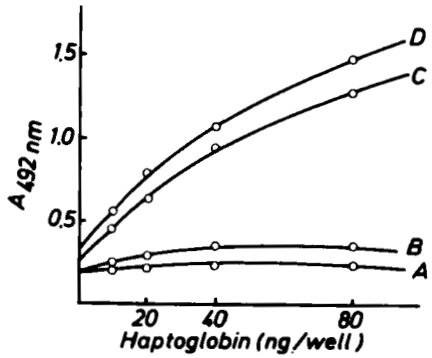

FIGURE 2. Con A binding to the polystyrene microtiter plate.
Con A 0.2 ug/well in **A**, 2 ug/well in **B**, 20 ug/well in **C**, 200 ug/well in **D**, resp., in acetate buffer 0.1 mol/L, pH 6.0, with cations. Other details as in Methods.
The points represent mean values from 4 estimations.

in the dark at room temperature for 30 min, 50 µl of 12.5% H_2SO_4 was added and the absorbance was read at 492 nm in a stripreader Microelisa system (Organon Teknika, Holland).

The amount of Hp bound to Con A was read from the standard curve.

RESULTS

Assay conditions

The pH-dependence of Con A binding to the polystyrene surface of the microtiter plate, is shown in Fig.1. There was no binding of Con A at pH 9.2. The highest binding was achieved at pH 6.0, and 20 µg of Con A per well was sufficient for the assay (Fig. 2). Finally, the highest absorbances of the Hp-Con A interaction were obtained with 1 h incubation at 37°C and pH 6.0.

Standardization. Recovery. Precision.

Table 1 shows results of a variety of blanks. Absorbances of 0.26-0.31 in the absence of the antigen indicated some interaction between Con A and anti-Hp-HRP conjugate. Nevertheless, such a background was satisfactory for further experiments.

In Table 2 are shown results of Hp determinations in sera by L-Hp-ELISA using one polyclonal and two monoclonal antibodies.

TABLE 1. Effect on absorbance of omitting various components from the Con A-binding assay mixture.

Omitted components	n	Absorbance at 492 nm
1. Con A, serum,*	6	0.010 ± 0.002
2. Con A,*	6	0.018 ± 0.005
3. Serum, anti-Hp-HRP,*	6	0.000
4. Anti-Hp-HRP,*	6	0.005 ± 0.002
5. Serum	12	0.267 ± 0.056
6. Serum,*	12	0.312 ± 0.04

Means ± S.D. of control samples were based on measurements from repeated experiments, carried out on several days, with different microtiter plates.
*Cord serum without Hp as checked by Hp-ELISA (11) was omitted from these tubes.

Calibration curves for purified Hp preparation (A,C) and serum containing Hp (B) with polyclonal (A, B) and monoclonal (C) anti-Hp antibodies, are shown in Fig. 3. The curves A and B are almost identical in the range of 2-80 ng Hp per well, while the curve C, a little lower than A and B, but parallel to them reaches plateau at values higher than 150 ng of Hp per well.

Sera with low, medium, and high content of Hp bound to Con A, were analysed for within-day and between-day

TABLE 2. Haptoglobin determinations in serum by L-Hp-ELISA with the use of polyclonal or monoclonal anti-Hp antibodies.

Concanavalin A-bound haptoglobin (g/L)		
Polyclonal antibodies	Monoclonal antibodies 7.60.66.55.	18.4.40.80
0.17	0.12	0.13
0.086	0.05	0.065
0.67	0.57	0.62
13.40	9.50	10.50
0.60	0.48	0.52
1.19	0.87	1.10
3.13	2.00	2.68
8.20	7.20	7.40
6.60	4.00	6.10
2.20	1.55	1.71
9.25	7.20	7.80
1.50	1.02	1.20

Mean values of 5 independent experiments are given.

precision (Table 3). CV's within-day were 4 to 7 %, and between-day 6 to 9 %.

Analytical recoveries were calculated after adding 10 or 20 ng of Hp standard to diluted normal serum. The average recovery ranged from 94 to 105 %. The binding of 20 ng Hp to Con A was also determined in the presence of 10-1000 ng of purified orosomucoid and 10 µl

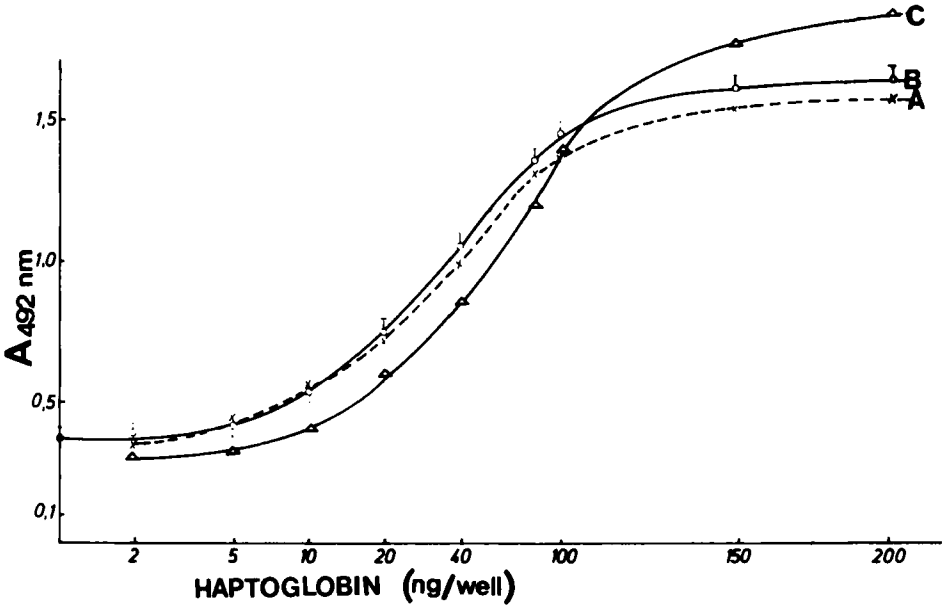

FIGURE 3. Calibration curves for the L-Hp-ELISA. Purified Hp preparation (**A**, **C**) and serum containing Hp (**B**), were taken as standards. In **A** and **B** polyclonal, in **C** monoclonal anti-Hp antibodies, were used. Curve **C** represents mean values obtained from the use of monoclonal antibodies 18.4.40.80 and 7.60.66.55.
Each point represents mean value ± S.D. from 10 measurements, carried out with different plates on several days.

of 2000-fold diluted cord serum devoid of Hp, but containing other glycoproteins able to react with Con A. In both cases the recovery of Hp bound to Con A was higher than 97 %. In order to avoid the effect of competitive glycoproteins, values only in the range of 5

TABLE 3. Precision of the L-Hp-ELISA

Sample	Concanavalin A-bound Hp (mg/L)	
	Within day*	Between day**
Cerebrospinal fluid	3.5 ± 0.2 (6)	not determined
Serum (hemolysis)	109 ± 7 (7)	107 ± 10 (9)
Normal serum	1050 ± 42 (4)	1060 ± 60 (6)
Pleural exudate	1820 ± 120 (7)	1860 ± 120 (6)
Cancer serum	8840 ± 610 (7)	8540 ± 760 (9)

The values are means ± S.D. from 10 estimations, using polyclonal antibodies. CV is shown in parenthesis.
*Calculated from repeated assays on the same day with different microtiter plates.
**Calculated from repeated assays with different microtiter plates, on several days, each time with own standard curve.

to 60 ng of Hp bound to Con A per well, were considered valid. The minimum detectable concentration, calculated as 3 S.D. for the mean of 10 determinations of a blank sample (washing buffer) was 5 ng/well (Table 1, nos. 5, 6), with polyclonal antibodies. With monoclonal antibodies the respective values were: 10-120 ng/well and 10 ng/well minimum detectable concentration.

TABLE 4. Con A-bound haptoglobin in biological fluids.

Sample	Con A-bound Hp (mg/L)
1. Normal sera	1000
	1050
	775
2. Pathological sera, cancer excluded	700
	109
	3050
Ovarian cancer	6600
	4000
	8840
	5600
3. Cord sera (Rh immunisation)	0
	0
	0.05
	0.03
4. Cerebrospinal fluid	
Cancer of the brain	18.9
Hydrocephalus	2.5
Aneurysm following surgery	1.7
Protrusion of pulpy nucleus	0
5. Pleural exudates	
Tuberculosis	760
Inflammation	92
Cancer	330

Measurements of Con A-bound haptoglobin

The amount of Hp binding to Con A in some biological fluids was measured by the L-Hp-ELISA (Table 4). Values of Con A-bound Hp in sera from healthy adults ranged from 780 to 1000 mg/L. Con A binding was extremely high

in most ovarian cancer sera, but one showed very low Hp content 109 mg/L. Four cord sera derived from complicated pregnancies (Rh immunisation) showed no or traces of reactivity with Con A, in spite of Hp content 65-480 mg/L, as measured by Hp-ELISA (11). Reaction of Hp with Con A was low in cerebrospinal fluids from patients with non-inflammatory neurological diseases, whereas whole Hp derived from a patient with tumor of the brain was found to be Con A-bound i.e. measurements carried out by Hp-ELISA (11) and L-Hp-ELISA gave the same results.

DISCUSSION

Human Hp, independently of the phenotype, is known to bind mannosyl specific lectin from Canavalia ensiformis (6, 7, 15). However, in crossed affino-immunoelectrophoresis with Con A, Hp forms one strongly binding peak, making this technique difficult for interpretation and useless as an additional test in the laboratory diagnosis of cancer and inflammatory disease (7). Also Hp chromatography on Con A-Sepharose did not produce a change in the Con A-binding pattern (15). Therefore, we developed a lectin-Hp immunoenzymatic solid phase assay for the quantitative characterization of reactivity of human Hp with Con A. The assay is

based on the reaction of two independent binding sites on the Hp molecule, namely for antibody and for the lectin (6).

The proposed method is quicker (results may be obtained in a few hours) and less expensive than affinity-immunoelectrophoresis or affinity-chromatography, because approximately 100 times less lectin is used per one assay. Moreover, the sensitivity of L-Hp-ELISA is higher than that of affinity-immunoelectrophoresis by an order of 1000.

Characteristics of the method was established by the use of polyclonal and monoclonal anti-Hp antibodies. Monoclonal antibodies 7.60.66.55 and 18.4.40.80 are specific for the β (heavy) chain of Hp, the common subunit in three main genetic types of Hp (10). The standard curve obtained with monoclonal antibodies resembled that with a polyclonal one (Fig. 3), with a slightly different working range.

In the present sandwich assay, undesired interaction between horse-radish peroxidase and Con A, could occur. The peroxidase, being rich in carbohydrate, is known to form a complex with Con A (3, 20). However, hydroxyl groups of peroxidase glycans, some of them necessary for the binding with Con A (3), are oxidized by sodium

periodate during conjugation with antibodies (18). Therefore, absorbance of blank samples confirms this.

Our previous haptoglobin assay (polystyrene-hemoglobin-Hp-anti-Hp antibody) differs from the present one (polystyrene-Con A-Hp-anti-Hp antibody). Recent work suggests that the polymeric form of Hp from cancer patients is immunochemically analogous to the fetal and neonatal Hp, which in contrast to adult Hp, does not form an active complex with hemoglobin (19). The protein expressed as the product of the Hp-related protein gene (also with no reaction with hemoglobin) displays similar functions in pregnancy and neoplasia (14). Abnormally glycated variants of Hp, with differences in lectin binding, have been found in sera from cancer and rheumatoid arthritis (22). Therefore, in some diseases Hp may bind to lectin with different avidity and affinity. Hp which does not bind hemoglobin may be present in cancer sera. On the other hand, the fetal glycation type of neoplastic Hp would reduce Con A ability to bind Hp in the L-Hp-ELISA.

We believe that a comparison of Hp measurements in biological fluids by different assays might reveal differences in cancer patients. These studies are under way in our laboratory.

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