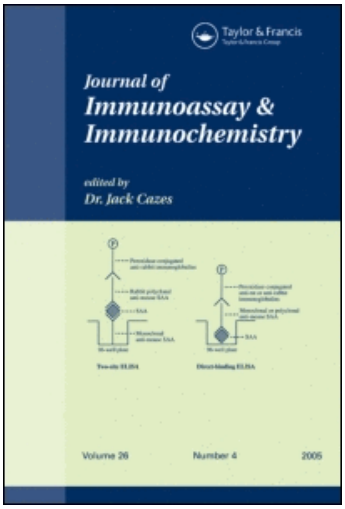

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A Monoclonal Antibody-Based Immunoradiometric Assay for Low Molecular Weight Insulin-Like Growth Factor Binding Protein/Placental Protein 12

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A MONOCLONAL ANTIBODY-BASED IMMUNORADIOMETRIC ASSAY FOR LOW MOLECULAR WEIGHT INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN/ PLACENTAL PROTEIN12.

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ABSTRACT

We describe a sensitive immunoradiometric assay for insulin-like growth factor binding protein/Placental Protein12 (IGF-BP/PP12) using monoclonal antibodies. This assay has a detection limit of 0.25 µg/l IGF-BP/PP12. Parallel dose response curves were obtained with purified IGF-BP/PP12, amniotic fluid, decidual cytosol extract, and serum . The assay is reproducible (intra-assay variation 4.3-8.2% and interassay variation 9.7-11.1%) and fast (< 5 hours). A crossreactivity of less than 0.01% for all other proteins tested reflects high specificity. Using this method the mean serum IGF-BP/PP12 concentration in healthy women was 5.2 µg/l. During pregnancy, the mean IGF-BP/PP12 at 7-11 weeks was 43.3 µg/l, and at 36-40 weeks 121 µg/l. After early pregnancy termination the serum IGF-BP/PP12 decreased rapidly reaching a mean level of 8 µg/l within 4 days. (KEY WORDS: immunoradiometric assay, monoclonal antibodies, IGF binding protein, placental protein 12.)

INTRODUCTION

Insulin-like growth factors (IGFs) are bound to specific binding proteins in blood and body fluids(1). The physiological role of IGF binding proteins (IGF-BP) has not been defined. The main binding proteins in

human serum are the 125-150K growth hormone dependent species and the 27-40K species. The 125-150K IGF-BP circulates saturated with IGF, whereas the 27-40K IGF-BP, which is abundant in amniotic fluid, contains unsaturated IGF binding sites (1,2). Amniotic fluid IGF-BP is identical to placental protein 12 (PP12)(2,3,4). The estimated molecular weight of IGF-BP/PP12 varies in the range of 27-34K as determined by mobility on sodiumdodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The predicted molecular mass from the cDNA sequence is 25,293 Da (4). The major sites of synthesis for IGF-BP/PP12 are decidualized endometrium and liver (4). The endometrial production of this protein is dependent on progesterone and its circulating levels are regulated by insulin (4,5). We have developed monoclonal antibodies (Mab) to the IGF-BP/PP12 (6) and used these antibodies to establish a highly sensitive immunoradiometric assay (IRMA).

MATERIALS AND METHODS

IGF-BP/PP12 was provided by Behringwerke AG, Marburg, West Germany. It was purified from soluble extract of term human placenta/decidua (7). Development of monoclonal antibodies to IGF-BP/PP12 was previously described (6). Mab 6305 is one of the 11 established clones (subclass IgG 2b), recognizing IGF-BP/PP12 in biological fluids. This Mab was selected as the catching antibody since its affinity was highest of all established IGF-BP/PP12 antibodies (Ka 1 x $10^{10}M^{-1}$). The most sensitive assay was achieved using Mab 6303 (Ka 5 x $10^{9}M^{-1}$) as the detecting antibody. Both antibodies specifically detect the IGF-BP/PP12 in biological fluids as tested by Western blotting and autoradiography (8). Mab 6303 was labeled by the chloramin T method (9) to a specific activity of 15-30 μC/μg. The label was stable for at least 2 months at 4C.

Polystyrene Maxisorb tubes (Nunc, Roskilde, Denmark) were coated with 300 µl Mab 6305, 10 µg/ml, in 0.1 M sodiumcarbonate buffer, pH 9.0, at room temperature for 18 hours. The tubes were then washed 3 times with 1 ml phosphate buffered saline (PBS), and blocked with 0.5% bovine serum albumin (BSA) in PBS containing 0.1% NaN_3 for 2 hours at room temperature. The antibody-coated and BSA-blocked tubes were either used directly or stored in blocking solution at 4C for up to 2 weeks. Before use the blocked tubes were washed twice with 1 ml PBS. Then, 250 µl assay buffer (50 mM sodiumphosphate, 50 mM NaCl, 10 mM EDTA, 0.1% NaN_3, 0.3% BSA, 0.03% tween, pH 7.4), and 50 µl testsubstance or standard in heat inactivated sterile filtered horse serum were added. After 2 hours incubation on a horizontal shaker, the tubes were aspirated and washed twice with 3 ml of washing buffer (10 mM Tris, 0.1% BSA, 0.1% NaN_3, pH 7.4). Then, ^{125}I labeled Mab 6303 (100,000 cpm) in 300 µl assay buffer was added and the tubes were further incubated for 2 hours on a horizontal shaker. The tubes were washed twice with 3 ml washing buffer and radioactivity bound to the tubes was measured in a gammacounter. To determine whether this assay detects IGF-BP/PP12 equally in its free and bound form, IGF-I (10 ng/ml - 5 µg/ml) was added to the incubation mixtures containing 10 µg/ml and 100 µg/ml purified IGF-BP/PP12. All determinations were done in duplicate. Part of the samples were assayed both by IRMA and by PP12 radioimmunoassay (RIA) using a polyclonal antibodies (10).

Serum samples were obtained from 38 healthy nonpregnant women, 35 women at 7 to 11 weeks' gestation, 18 women at 12 to 16 weeks' gestation and 33 women at 36 to 40 weeks' gestation. In addition, serum samples from 7 women having legal abortion of normal early pregnancy for social reasons were obtained before and after the abortion. Serum samples were also obtained from 10 normal healthy women having a 75

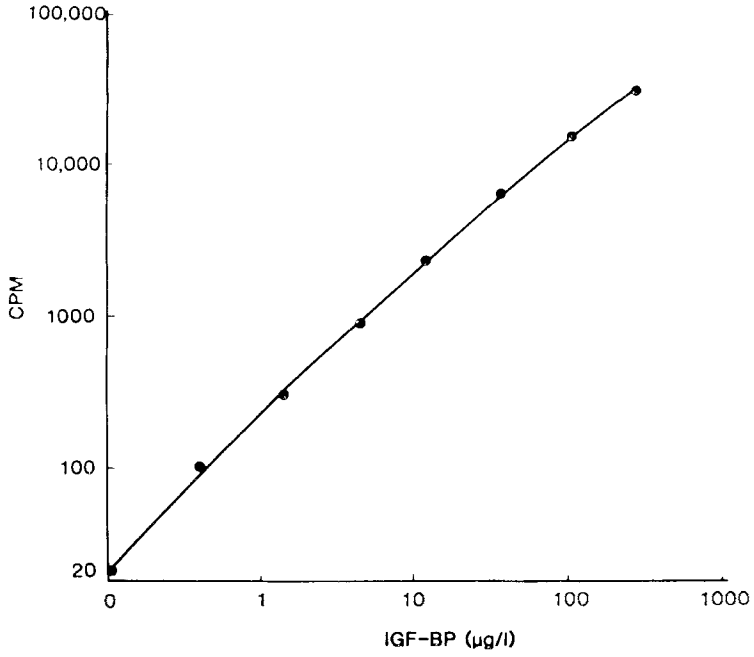

FIGURE 1: Standard curve of IRMA for purified IGF-BP/PP12. Mean of duplicate determinations.

g, 3 hour, oral glucose tolerance test (OGTT). Amniotic fluid was obtained from second trimester amniocentesis. Decidual tissue was obtained at cesarean section after term pregnancy. Cytosol fractions were prepared by homogenisation and differential centrifugation as earlier described (11). For statistical analysis, Student's t test and analysis of variance were used.

RESULTS

The dose response curve of IGF-BP/PP12 in the IRMA was almost linear in the range of 0.25-250 µg/l (Fig. 1). Parallel dose response

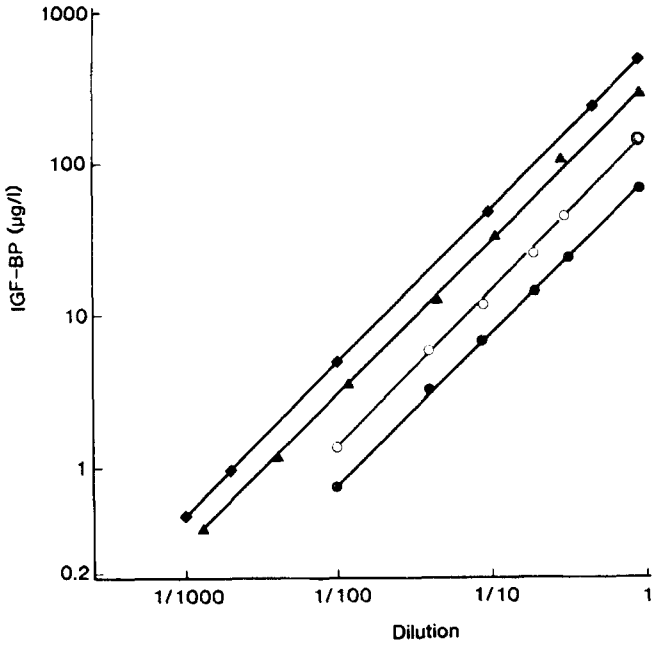

FIGURE 2: Dilution-response curves for purified IGF-BP/PP12 , amniotic fluid , decidual cytosol fraction ,and serum . All samples were diluted in inactivated sterile horse serum. Mean of duplicate determinations.

curves were obtained with purified IGF-BP/PP12, amniotic fluid, decidual cytosol extract, and serum (Fig. 2). Addition of IGF-I (10 ng/ml to 5 µg/ml) did not change the amount of IGF-BP/PP12 detected in the IRMA (data not shown).

The detection limit of 0.25 µg/l was defined as the amount of IGF-BP/PP12 corresponding to the mean counts per minute in 20 replicates of a zero sample (plain heat inactivated horse serum) plus 2 SD. The intra-assay variation was 4.6%-8.2% and the interassay variation was 9.7-11.1%. The analytical recovery was 97-106%, when serum samples with IGF-BP/PP12 levels in the range of 5 to 140 µg/l were diluted 1:2 with 3.8 µg/l, 23 µg/l or 110 µg/l IGF-BP/PP12 standards (Table 1). Less than

TABLE 1

Characteristics of IGF-BP/PP12 IRMA

Interassay variation				
	IGF-BP (μg/l)	n	CV (%)	
	2.4	17	10.3	
	25	17	9.7	
	128	10	11.1	
Intra-assay variation				
	5	10	4.3	
	40	10	8.2	
Recovery			recovery (%)	SD (%)
	110	8	97	9.5
	23	8	104	7.8
	3.8	8	106	4.5

Crossreaction	(%)
human chorionic gonadotropin	< 0.001
α-fetoprotein	< 0.001
placental lactogen	< 0.001
retinol binding protein	< 0.001
sex-hormone-binding globulin	< 0.001
prolactin	< 0.01
IGF-I	< 0.01
IGF-II	< 0.01
placenta protein 14	< 0.01
transforming growth factor α	< 0.01

0.01% crossreactivity was observed with human placental lactogen (UCB Bioproducts, Braine-l'Alleud, Belgium), human chorionic gonadotropin (Organon, Os, Holland), prolactin (International Laboratory Services, London, UK), α-fetoprotein (Aalto Bio Reagents, Dublin, Ireland), sex-hormone-binding globulin (Purified from pregnancy serum)(12), IGF-I (Amgen Biologicals, Thousand Oaks, Ca), IGF-II (Bissendorf Biochemicals, Torrance, CA), placental protein 14 (Behringwereke AG,

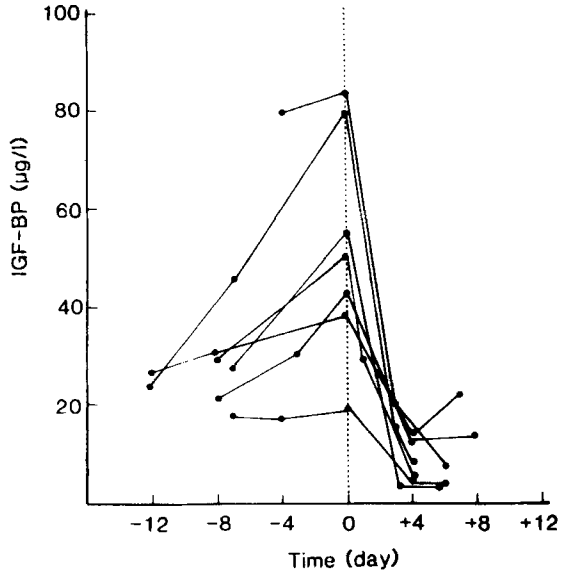

FIGURE 3: Serum IGF-BP/PP12 levels in 7 healthy women in early pregnancy before and after legal abortion . The day of the procedure is day 0. Mean of duplicate determinations.

TABLE 2

IGF-BP/PP12 in Nonpregnant and Pregnant Women.

	n	mean (µg/l)	SD (µg/l)	range (µg/l)
nonpregnant	38	5.2	4.4	0.6-20.0
pregnancy				
week 7-11	35	43.3	53.9	4.4-330
week 12-16	18	118.1	62.8	32-246
week 36-40	33	121.1	94.0	30-426

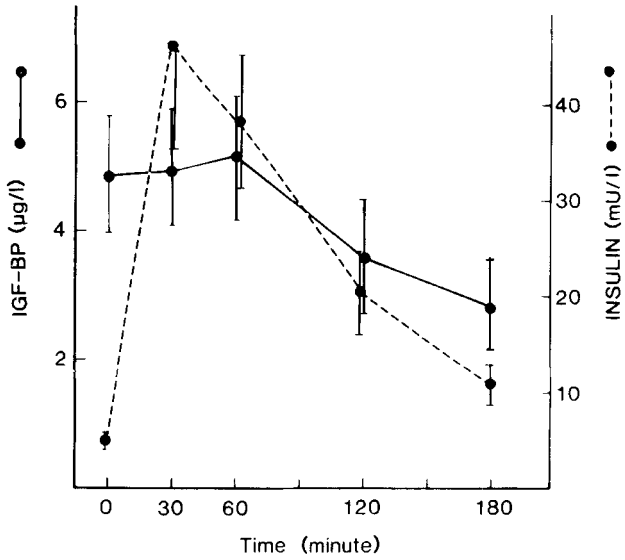

FIGURE 4: Serum IGF-BP/PP12 and insulin responce during a 75 gram oral glucose tolerance test in 10 healthy women. Mean ± SD.

Marburg, West Germany), α-transforming growth factor and retinol binding protein (Chemicon International, El Segundo, Ca) (Table 1).

In nonpregnant women, the mean serum IGF-BP/PP12 (±SD) was 5.23 ± 4.43 µg/l, the range being 0.6-20.0 µg/l. In early pregnancy, the IGF-BP/PP12 increased steadily (Fig. 3, Table 2). Between 7 and 11 weeks' gestation, the mean IGF-BP/PP12 level(±SD) was 43.3 ± 53.9 µg/l (range 4.4-330 µg/l). At 12 to 16 weeks' gestation, the level was 118 ± 62.8 µg/l (range 32-246 µg/l), and at 36 to 40 weeks it was 121 ± 94 µg/l (range 30-426 ug/l) (Table 2). After termination of first trimester pregnancy, the IGF-BP/PP12 decreased rapidly from a mean level of 47 µg/l on the day of pregnancy termination to a level of 8 µg/l 4 days later (Fig. 3). During an oral glucose tolerance test, followed by a secondary increase in insulin, the serum IGF-BP/PP12 decreased 44% ($p < 0.001$)(Fig. 4).

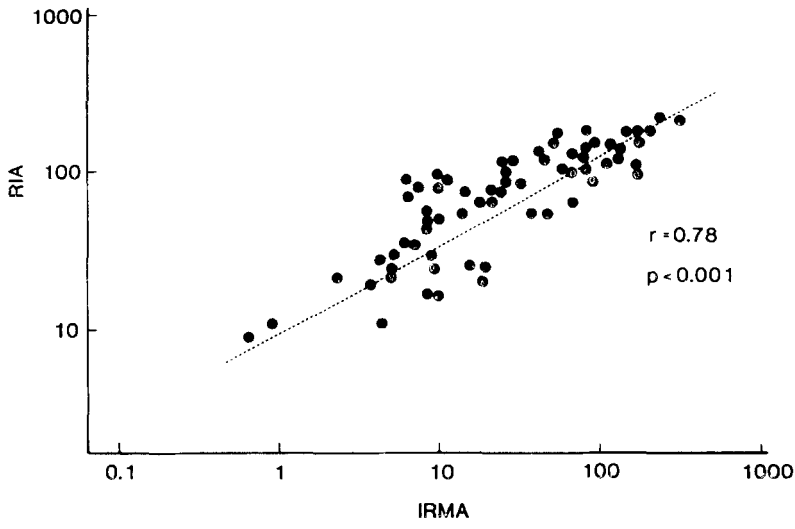

FIGURE 5: Correlation between serum IGF-BP/PP12 levels measured by monoclonal IRMA and polyclonal RIA.

The concentrations of IGF-BP/PP12 in serum from 67 pregnant and nonpregnant women were compared by RIA and IRMA. Only serum samples with IGF-BP/PP12 detectable in RIA were included. The correlation coefficient was $r = 0.77$, $p < 0.001$ (Fig 5).

DISCUSSION

The immunoradiometric assay using Mabs raised against IGF-BP/PP12 is highly sensitive, permitting accurate quantification in serum and body fluids. The sensitivity of the assay is 20-40 times improved as compared to conventional RIA (10). One of the drawbacks with conventional RIA:s is, in addition to the limited sensitivity, the need for purified iodinated antigens. In the case of IGF-BP/PP12 this has been a

major problem, since purified IGF-BP/PP12, upon storage and when iodinated, easily is degraded. With the IRMA method, where a monoclonal antibody is used as tracer, this problem is avoided. The ^{125}I labeled anti IGF-BP/PP12 Mab is stable for at least 2 months. An other advantage is the unlimited availability of Mabs.

Using IRMA technique with Mabs and purified IGF-BP/PP12 as standard, the serum levels during pregnancy were in accordance with those earlier measured by RIA (10). The mean level of 5 µg/l IGF-BP/PP12 in nonpregnant women, as measured by IRMA, is lower than that earlier described by RIA:s and by an immunofluorometric assay using polyclonal antibodies (2,10,13,14,15). The reason for this discrepancy is not evident. The antigen used in raising both monoclonal and polyclonal antibodies is IGF-BP/PP12 purified from pregnancy tissue. Thus, the comparable levels during pregnancy suggest that monoclonal and polyclonal antibodies recognize the same antigen population in pregnancy serum. The lower IRMA (monoclonal) level in nonpregnant subjects as compared to RIA (polyclonal) levels might suggest that the antibodies recognize different antigenic IGF-BP/PP12 populations in nonpregnant subjects, possibly due to IGF-BP/PP12 microheterogeneity. An other explanation might be that some unknown substancies in nonpregnant serum crossreact in the RIA. Although the mean level of IGF-BP/PP12 in nonpregnant subjects was lower when measured by monoclonal IRMA than when measured by assays using polyclonal antibodies, the physiological regulation of the protein was equally detected by both monoclonal and polyclonal antibodies. Thus glucose loading caused a decrease in serum IGF-BP/PP12 (IRMA), results comparable to those earlier observed by RIA (5,16), and serum IGF-BP/PP12 (IRMA) rapidly decreased after termination of early pregnancy. The decrease was more dramatic than that observed after spontaneous term delivery as measured by RIA (17). One explanation for this difference in results may be that decidual tissue, which during pregnancy

produces high amounts of IGF-BP/PP12 (18,19), is removed more efficiently by vacuum curettage than in normal term delivery.

The performance characteristics for IGF-BP/PP12 measurements in IRMA were the same for serum, purified protein, amniotic fluid and decidual cytosols. This stability of the assay, irrespective of what kind of body fluids were measured, grants reproducible results and enables comparison of IGF-BP/PP12 levels in serum and tissue extracts. In conclusion, the IGF-BP/PP12 IRMA using Mabs is a convenient and useful addition to the earlier available assays using polyclonal antibodies. The increasing evidence for an active role of IGF-BP/PP12 in female reproductive tissues (11, 20), and the observation of its insulin dependency calls for assays that reproducibly and specifically measure small amounts of IGF/BP in different body fluids.

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