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A SIMPLE AND RAPID METHOD FOR DETERMINATION OF HUMAN PLACENTAL LACTOGEN

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Matsuto MOCHIZUKI, Hajime MORIKAWA, Itaru HIRAI, Shimpei TOJO, Nobuhisa OGAWA, Hiroyuki SHINKAI and Hiroshi KOSUZUME. *A Simple and Rapid Method for Determination of Human Placental Lactogen.* Kobe J. Med. Sci. 20, 55-64, June 1974——A newly developed hPL (hCS) HAIR Test Kit with sensitivity of 0.1 μg/ml (2 μg/ml of hPL for the undiluted serum sample) for assay of hPL is described. A doubtful positive reaction may be observed in the presence of the rheumatic factor, but no definite reaction is obtained. Furthermore, the results are not influenced by normal human serum protein or hCG.

Comparison of the serum hPL determination in 250 normal pregnant women with the hPL HAIR Test Kit and by radioimmunoassay (RIA) showed close approximation of the normal ranges with both methods, although slightly higher values were obtained in the early stage of pregnancy by the HAIR method than by RIA.

A serum hPL level of less than 4 μg/ml during the late stage of pregnancy probably indicates insufficiency of placental function.

The low cost, simple procedures and highly accurate estimations requiring only 2 hours with the hPL HAIR Test Kit would make this method useful for routine measurement of maternal hPL serum levels.

INTRODUCTION

Human placental lactogen (hPL, human chorionic somatomammotropin: hCS), a protein hormone secreted from the placenta, is produced in the chorionic villi and released only into the maternal blood. There is a continuous rise of the serum hPL level with progress of pregnancy and, because of its short half-life, any disturbance of placental function is rapidly reflected in the blood level of hPL. Thus, determination of the maternal serum level of hPL is considered one of the useful indices for the evaluation of placental function.

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For the purpose of measuring hPL in maternal serum, many investigators have used radioimmunoassay techniques, using hPL which they themselves had extracted from placenta and purified, because sufficient amounts of purified hPL were not then available. Because of this fact, the purity of the standard sample or that after radioiodination differed among the investigators, and so no valid comparison of the reported values is possible. Moreover, radioimmunoassay methods (RIA) require troublesome procedures and special facilities.

The need for a rapid and simple method with sufficient sensitivity and accuracy for routine clinical application has prompted us to investigate a method for measurement of serum hPL utilizing the principle of hemagglutination inhibition reaction (HAIR). From the results of this investigation, the authors developed a hPL HAIR Test Kit for clinical application. This paper describes the principal conditions and procedures in the developement of this test kit, and its accuracy and clinical applicability are discussed.

MATERIALS AND METHODS

Antigens and Antisera

Antisera against a highly purified hPL (hPL-Kobe)[1, 2] were prepared in male adult rabbits.

Sensitization of Cells

Sensitization of formalin-tannic acid-treated sheep erythrocytes to hPL-Kobe was carried out by Wide's method.[3]

Dilution of Serum Sample and Antiserum

As the hemagglutination inhibition reaction is influenced by the constituents of the serum, the hemagglutination reactions were investigated using various dilutions of non-pregnant serum and various dilutions of anti-hPL serum.

No effect of the serum constituents on the reaction was observed with 1/15 or higher dilutions of the non-pregnant serum, and the dilution of the anti-hPL serum was determined to 1/500, giving a sensitivity of 0.1 μg/ml.[4]

Contents of the hPL HAIR Test Kit and Testing Method

The hPL HAIR Test Kit is composed with three ampoules. That is, diluted anti-hPL serum (freeze-dried powder) is contained in ampoule A, hPL sensitized sheep erythrocytes are in ampoule B and ampoule C contains 0.4 ml of buffer solution for suspending the hPL sensitized erythrocytes.

The testing procedure is as follows: 1. The test sample is reacted with the anti-hPL serum by adding 0.1 ml of either standard hPL-Kobe or a suitable solution of the serum sample to ampoule A, 2. The buffer solution in ampoule C

is added to ampoule B to make a suspension fluid of sensitized erythrocytes, 3. The hPL-sensitized erythrocyte suspension fluid in ampoule B is added to ampoule A and mixed thoroughly by shaking, 4. Ampoule A is then placed in a rack and left standing for 2 hours.

The presence of hPL in the serum sample is judged by the ring formation at the bottom of the ampoule.

Sensitivity and Stability of the hPL HAIR Test Kit

As the rheumatic factor reacts with rabbit gamma-globulin (anti-hPL serum), the effect of the rheumatic factor on the hemagglutination inhibition reaction was tested with a 1/20 dilution of serum samples taken from non-pregnant rheumatic patients.

The influence on the sensitivity of the hemagglutination inhibition reaction of hCG which is similarly secreted from the chorionic villi in a considerable amount was tested with 3000 IU～5 IU/ml solutions of hCG (60,000 IU/ml～100 IU/ml concentrations in the original serum sample) which we extracted from placentae and purified.[5, 6]

The effect of high concentrations of hPL in the serum sample was examined by using a 1/20 dilution of non-pregnant serum in a standard hPL solution, so that the concentration of hPL was 50 μg/ml～1 μg/ml (a concentration of 1 mg/ml when converted to the undiluted serum sample).

The following methods were used to test the stability of the hPL HAIR Test Kit.

1. Ampoule A (anti-hPL serum), ampoule B (hPL-sensitized erythrocytes), and ampoule A and B of different kits were heated at 50°C or 70°C for 24 hours and the sensitivity of each kit was tested.
2. Ampoule A and B were heated at 56°C for periods ranging from 24 to 72 hours and the effect of the duration of heating on the hemagglutination inhibition reaction was observed.
3. Kits that had been stored for 6 months at 37°C or for 3 months at 45°C were tested to observe the effect of storage conditions.

Clinical Application

Serum samples were obtained from 250 cases of normal pregnancy (9～40 weeks), 10 cases of toxemic pregnancy, 3 cases of intrauterine fetal death, and one case each of threatened abortion, Rh negative pregnancy, placenta previa, prolonged pregnancy, twin pregnancy, pregnancy associated with hyperthyroidism and diabetic pregnancy. Dilutions of 1/20, 1/40, 1/60 and 1/80 of each serum sample (giving concentrations of 2, 4, 6, 8 μg/ml for the original serum samples) were simultaneously tested with the hPL HAIR Test Kit and radioimmunoassay (RIA) method according to the double antibody technique of Morgan and Lazurow[7] with minor modifications.[8]

RESULTS

Regarding the influence of the presence of the rheumatic factor or hCG in this reaction system, a concentration of 60,000 IU/ml of hCG was found to have no effect on the sensitivity of the reaction. On the other hand, doubtful postitive reactions were obtained with serum samples from 3 of 18 rheumatic patients.

No influence on the hemagglutination inhibition reaction was observed with an hPL concentration of 50 μg/ml, indicating that normal inhibition of agglutination occurs even in the presence of such a high titer as 1 mg/ml of hPL in the serum.

The results of tests for sensitivity after heating or under various storage conditions are summarized in Table 1, 2 and 3. After heating at 56°C or 70°C for 24 hours, no change in sensitivity of the test kit was observed. When only the anti-serum was heated at 70°C for 24 hours, no change in sensitivity was observed, but increased sensitivity was observed, when only the erythrocytes or the erythrocytes and anti-serum were heated. Storage of the erythrocytes and anti-serum for 72 hours at 56°C, as well as for 6 months at 37°C, produced no change in sensitivity, but storage for 2 months at 45°C increased the sensitivity.

Table 1 Effect of temperature on stability of reaction.

heat treatment	56℃, 24hrs.							70℃, 24hrs.						
	standard hPL (μg/ml)													
	0.5	0.4	0.3	0.2	0.1	0.05	0	0.5	0.4	0.3	0.2	0.1	0.05	0
erythrocytes & anti-serum	○	○	○	○	○	±	−	○	○	○	○	○	○	−
erythrocytes only	○	○	○	○	○	±	−	○	○	○	○	○	±	−
anti-serum only	○	○	○	○	○	±	−	○	○	○	○	○	○	−

reaction temperature: 26°C, reaction time 2 hrs.

Table 2 Effect of duration of heating on the stability of the reaction.

standard hPL (μg/ml)	0.3	0.2	0.1	0
24hrs	○	○	○	−
48hrs	○	○	○	−
72hrs	○	○	○	−

(Both erythrocytes and anti-serum heated at 56°C) reaction temperature: 24°C. reaction time: 2 hrs.

Table 3 Effect of storage period of finished product (kit) on the test result.

storage period	37℃ standard hPL (μg/ml)				37℃ nonpregnant serum						45℃ standard hPL (μg/ml)				45℃ nonpregnant serum					
	0.2	0.1	0.05	0	A	B	C	D	E	F	0.2	0.1	0.05	0	A	B	C	D	E	F
0	○	○	±	−	−	−	−	−	−	−	○	○	±	−	−	−	−	−	−	−
2W	○	○	±	−	−	−	−	−	−	−	○	○	±	−	−	−	−	−	−	−
1M	○	○	±	−	−	−	−	−	−	−	○	○	±	−	−	−	−	−	−	−
2M	○	○	±	−	−	−	−	−	−	−	○	○	○	−	−	−	−	−	−	−
3M	○	○	±	−	−	−	−	−	−	−	○	○	○	±	−	±	−	±	−	−
4M	○	○	±	−	−	−	−	−	−	−										
5M	○	○	±	−	−	−	−	−	−	−										
6M	○	○	±	−	−	−	−	−	−	−										

W: week M: month

reaction time: 2 hrs.
reaction temperature: 26℃

The determinations with the hPL HAIR Test Kit in 250 normal pregnant women are shown in Figure 1. As shown in the graph, consistent measurements of hPL levels were obtained after the 17th week, with a gradual increasing up to the 32nd to 34th week. The serum hPL levels of normal pregnancy, determined both with the hPL HAIR Test Kit and by RIA, are compared in Figure 2. The pattern of increase in hPL level after the 22nd week coincides closely with both methods (correlation coefficient $\gamma=0.869$), and concentrations less than 4 μg/ml after the 32nd week were not obtained with either method.

The serum hPL levels in abnormal pregnancies or when associated with other complications are given in Table 4. The serum levels in pre-eclampsia severe (Cases 1, 3) were at the upper limit of normal, but those in mild toxemia of pregnancy were within normal limits, coinciding with the RIA determinations. The hPL level could not be measured with the HAIR method in the case of intrauterine fetal death, and the RIA method also showed a low level. In the case of threatened abortion, the serum hPL level was 2 μg/ml, which was somewhat lower than that obtained with radioimmunoassay, and premature delivery occurred in the 32nd week. An Rh sensitization case showed a high level of 8 μg/ml. The serum hPL levels in the 41st, 42nd and 43rd weeks in the case of prolonged pregnancy were 8 μg/ml, 8 μg/ml and 4 μg/ml respectively, with eventual delivery. A high concentration of hPL was detected by both assay methods in the cases of twin pregnancy and pregnancy associated with diabetes mellitus.

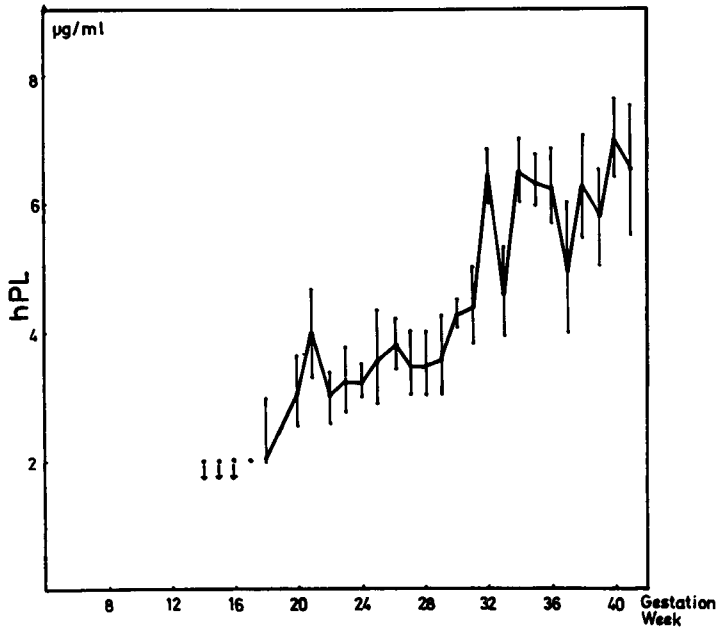

Fig. 1 Graphic representation of measured values and standard deviations of hPL serum levels during normal pregnancy in 250 subjects (HAIR Test Kit).

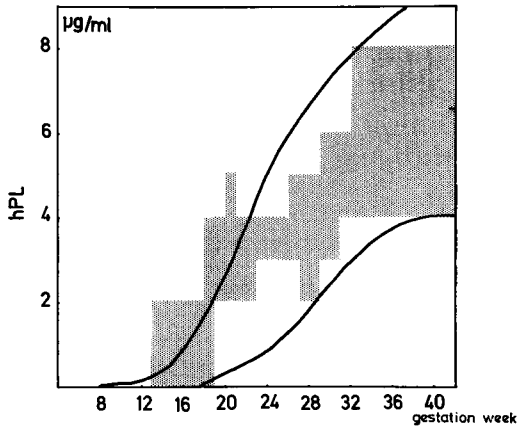

Fig. 2 Normal ranges of maternal serum hPL levels in pregnancy, determined simultaneously with the hPL HAIR Test Kit and by hPL-radioimmunoassay.
▩ : range of values obtained with hPL HAIR Test Kit.
□ : range of values obtained by hPL-Kobe RIA.
Correlation coefficients, $\gamma=0.217$ (1st trimester)
$\gamma=0.869$ (2nd and 3rd trimester)

Table 4 Serum hPL levels in abnormal pregnancy and in pregnancy associated with other complications. (μg/ml)

case	week of gestation	complication	RIA	HAIR
1	40	toxemia	8.2	8
2	40	toxemia	4.8	4
3	39	toxemia	7.5	8
4	39	toxemia	5.6	6
5	39	toxemia	4.9	4
6	37	toxemia	5.7	6
7	37	toxemia	5.2	4
8	36	toxemia	7.2	8
9	32	toxemia	5.0	6
10	31	toxemia	3.6	4
11	28	intrauterine fetal death	0.2	undetectable
12	20	intrauterine fetal death	0.96	undetectable
13	17	intrauterine fetal death	0.3	undetectable
14	27	threatened abortion	3.6	2
15	30	Rh sensitization	5.5	8
16	31	placenta previa	6.8	6
17	20	hyperthyroidism	1.2	2
18	41	prolonged pregnancy	7.3	8
	42	prolonged pregnancy	5.5	8
	43	prolonged pregnancy	5.6	4
19	37	twin	10.0	8
20	39	diabetes mellitus	8.2	8
	40	diabetes mellitus	8.6	8

DISCUSSION

In 1963 Sciarra[9] demonstrated that human placental lactogen was present in the cytoplasm of the syncytio-trophoblast of the placenta. Later investigations by the present authors[10] on the biologic activities of hPL have demonstrated its specific action in augmenting maternal lipolysis, its role in the transport of the increased FFA and glucose in the mother's blood to the fetus, resulting in stimulation of the growth of the fetus. In other words, hPL is considered to be one of the factors concerned with metabolic control in pregnancy, and thus indirectly serve the growth of the fetus.

In this sense, measurement of the serum hPL is considered one of the useful indices for the evaluation of not only placental function but also to surmise the condition of metabolic function of the mother during pregnancy.

For routine assessment of hPL levels by clinicians, simple and accurate methods which are also available for large scale testing have been described by Gusdon (1969, hemagglutination inhibition reaction),[11] by Verma et al. (1970, microcomplement fixation),[12] by Nakamura, R. M. (1970, complement fixation method),[13] by Zuckermann et al. (1970 complement fixation method),[14] and by the present authors (1971, single radial immunodiffusion method[2]). The new method (hPL HAIR Test Kit) described in the present paper is based on the hemagglutination inhibition reaction. As the hPL content of serum is determined, it is much more accurate than estimating the hPL level in urine. However, as the effect of the serum on the hemagglutination inhibition reaction must be considered, the serum sample must be diluted to eliminate this influence. It was found that with dilution of the serum sample to 1/20, no observable effect of the serum protein on the formation of the erythrocyte sedimentation ring occurred and that with higher dilutions the hPL-anti-hPL serum reaction became more specific. A 1/500 dilution of the anti-hPL serum used in the present study was also found to be the most suitable and the end point of the hemagglutination inhibition reaction with this dilution was 0.1 μg/ml of standard hPL. Within the range of titrable antibodies, minute amounts of hPL can be measured by increasing the dilution of the anti-serum, but the end point of the inhibition reaction becomes less distinct with increased sensitivity and reproducibility in poor. Therefore, the anti-serum dilution was determined to give an end point of the reaction within a range of 0.05 μg/ml to 0.1 μg/ml.[4]

The effect of environmental or storage conditions on the stability and sensitivity of the kit was found to be minimal, although some increase in sensitivity was observed after heating at 70°C for 24 hours but not to the extent that false positive results were obtained for negative non-pregnant serum. Also, some increase in sensitivity was observed after storage at 45°C for 2 months but not after 6 months at 37°C.

Furthermore, the principal placental protein hormone, hCG, had no effect on the hemagglutination inhibition reaction, but doubtful positive results were observed with non-pregnant serum samples that showed a positive RA test. This could be obviously anticipated as the rheumatic factor reacts with rabbit gammaglobulin (anti-hPL serum), but none of the reactions could be judged to be definitely positive. On the basis of the above results, the hPL HAIR Test Kit is considered to be a specifically stable measurement system.

Measurements of the serum levels of hPL in the various stages of normal gestation in 250 cases with the hPL HAIR Test Kit revealed that the hPL level could not be detected before the 17th week, although a level of 2 μg/ml was obtained in one case at 13 weeks. Consistent measurements were obtained only from the 17th week, after that the level increased rapidly and then plateaued from the 36th week.

Other than a value in the upper normal range in one case of toxemia of pregnancy, the hPL levels in the remaining cases of abnormal pregnancy were within normal limits. The serum level of hPL which was found to be low with RIA in the case of intrauterine fetal death could not be detected with HAIR. In this case the

death of the fetus was already evident at the time of her initial visit to our hospital. In threatened abortion or prolonged pregnancy, a reduction in the serum hPL levels to low levels was followed by spontaneous abortion or delivery. A high hPL level was obtained in twin pregnancy.

Estimating about the same samples, the value with hPL HAIR Test Kit is closely similar to that of RIA, demonstrating the usefulness of this simplified method for assessing the hPL level in pregnancy. Furthermore, no determinations with the hPL HAIR Test Kit in the 250 normal pregnant cases after the 36th week were below 4 μg/ml. Therefore, a one point determination during this latter period of pregnancy of less than 4 μg/ml might be considered as indicating functional insufficiency of the placenta, as also noted by Spellacy et al.[15, 16] The practicability of serial determinations of hPL during gestation with the hPL HAIR Test Kit would also facilitate assessment of the progress of pregnancy.

In hospitals or clinics without facilities for radioimmunoassay or scintillation counters, the hPL HAIR Test Kit should be useful for determination of hPL to assess placental function.

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