Acta Medica et Biologica
Vol. 36, No. 2, 85-94, 1988

A NEWLY DEVELOPED ENZYME IMMUNOASSAY FOR THE DETECTION OF HUMAN CHORIONIC GONADOTROPIN AND ITS SIGNIFICANCE IN THE FOLLOW-UP OF TROPHOBLASTIC DISEASE

YOSHIAKI SATO, YUHETSU SUDO
AND
SHOSHICHI TAKEUCHI

Department of Obstetrics and Gynecology
Niigata University School of Medicine
1-757 Asahimachi-Dori Niigata, Japan

(Received October 13, 1988)

ABSTRACT

We developed an assay that measures human chorionic gonadotropin (hCG) at levels as low as 0.2 mIU/ml and that is roughly 25 and 10 times as sensitive as the hemagglutination (5 mIU/ml) and β-hCG radioimmunoassay (2 mIU/ml) methods, respectively. Cross-reaction in this assay system with human luteinizing hormones, human follicle-stimulating hormones, human thyroid-stimulating hormones, and α-hCG was very low; however, human menopausal gonadotropin showed a 4% crossreaction. In normal women, the hCG secretion tended to increase with age; the maximum value was 3.2 mIU/ml. This method is useful for long-term follow-up of trophoblastic diseases and early detection of recurrent choriocarcinomas.

Human chorionic gonadotropin (hCG) is a glycoprotein hormone secreted by trophoblastic cells. As trophoblastic tumors also secrete hCG, it is regarded as a valuable tumor marker in trophoblastic disease. This hormone has α- and β-subunit which are biologically inactive as free subunits. The α-subunits of the hCG, the luteinzing hormone (LH), the follicle-stimulating hormone (FSH), and the tyroid-stimulating hormone are nearly identical, but the amino acid sequences of the β-subunits differ and account for the unique immunologic and biologic activities of each glycoprotein hormone. Nevertheless, the α-subunits of the 4 hormones have considerable homology, especially hCG and LH,

which are similar in biologic activity. The β-subunits of hCG, however, contains an additional unique CTP of 30 amino acids that is absent in LH. The production of antiserum against the hormone-specific β-subunit,which possesses a greater affinity for hCG than for LH, has enabled us to develop the hCG assay that is more specific at low concentrations.

This report compares the efficacy of the hCG RIA and this newly developed enzyme immunoassay (EIA) in measuring serum hCG activity in patients with molar pregnancies and choriocarcinomas.

PATIENTS AND METHODS

Patients: We examined the sera of 9 patients with a hydatidiform mole, 4 with an invasive mola, and 7 who had a choriocarcinoma. Sera from 100 healthy, nonpregnant women were used as controls.

Follow-up and treatment: The patients' blood was taken by venipuncture before treatmest, and serial samples were obtained at weekly intervals.

Assay method: Urinary hCG was determined by Hi-gonavis, and serum hCG was evaluated by β-hCG radioimmunoassay. Hi-gonavis (Mochida Pharmaceutical Co., Ltd., Japan) is an immunochemical test designed to detect LH/hCG in urine by a hemagglutination reaction.

The minimum detectable amounts of LH and hCG with this method are 12.5 IU/L and 5 IU/L, respectively, which are similar to those detected by radioimmunoassay.

Sandwich enzyme immunoassay for human chorionic gonadotropin: We developed a highly sensitive and specific colorimetric sandwich EIA for hCG measurement. The Fab' fragment of a rabbit anti-whole hCG antibody was conjugated with horseradish peroxidase (HRP) by using the *N*-hydroxysuccinimide ester of *N* (4-carboxycyclohexylmethyl)

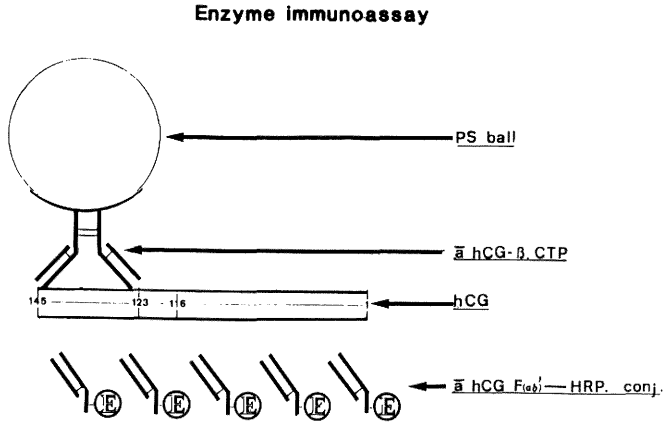

Fig. 1

maleimide. Polystyrene balls were coated with the hCG-specific antibody directed against the carboxy-terminal peptide (CTP) of β-hCG, which was purified from anti-β-hCG bovine thyrogloblulin serum by sequential affinity chromatography. This assay for hCG, using specific antibodies against the CTP of the β-subunit, was performed as previously described by Suzuki[4]. The assay method is illustrated in Fig.1.

A 0.25-ml sample of a solution of standard hCG in buffer B (0.02 mol/liter of phosphate-buffered saline, pH 7.0, containing 1% bovine serum albumin, 10% sheep serum, and 0.002% merthiolate) was mixed 0.05 ml of sheep serum, and 0.05 ml of a human serum sample was diluted with 0.25 ml of buffer B. A polystyrene ball (4.8 mm in diameter; Precision Plastic Ball Co., Chicago, I ll.) coated with the specific anti-hCG antibody was added to the hCG standard or the test serum. After overnight incubation, the ball was washed twice with 5 ml of water and added to 15 nm of the anti-hCG Fab'-HRP conjugate in 0.3 ml of buffer C (0.2 mol/liter of potassium phoshate, pH 7.5, containing 0.1% bovine serum albumin and 0.002% merthiolate). After incubation for 3-h, the ball was washed twice with 5 ml of water and transferred to a clean test tube. The HRP bound to the ball was assayed by incubating it for 40 min. with 0.5 ml of a chromogen solution (0.1 mol/liter of citrate buffer, pH 4.9, containing 0.2% *O*-phenylenediamine, 0.02% h_2O_2, and 0.002% merthiolate). We stopped the enzyme reaction by adding 1.5 ml of 1 M H_2SO_4, and the absorption was measured at 492 nm by spectrophotometry. All steps of the assay were carrided out at room temperature.

RESULTS

The recovery and dilution test results and the reproducibility were found to be satisfactory, measuring hCG in serum at levels as low as 0.2 mIU/ml. The β-hCG reaction with this assay was 10.4% and the human LH, FSH, tyroid-stimulating hormone, α-hCG, and β-hCG showed very low cross-reaction; however, human menopausal gonadotropin gave a 4% cross-reaction (Fig. 2). The results obtained with this assay system correlated well those from the β-hCG RIA system (Fig. 3).

In clinical tests of this method, the hCG levels in normal women were all within the limit of detection (Fig. 4). However, the hCG levels were higher and thus more detectable in older women; the highest hCG level (3.2 mIU/ml) was found in a postmenopausal woman.

On trophoblastic disease patients, we performed serial determinations of serum hCG levels. The "good-course" hCG regression pattern of a woman with a hydatidiform mole is illustrated in Figure 5. Serum hCG levels fell to the limit of detection in 4 or 8 weeks according to the β-hCG RIA system or the sandwich-EIA method, respectively, after falling to 20 IU/liter in the hemagglutination assay (Hi-Gonavis).

In serial assays, we determined that urine hCG levels fell to the LH level (20 IU/liter) in an average of 6.7 weeks. Reaching the limit of detection in the β-hCG RIA system or

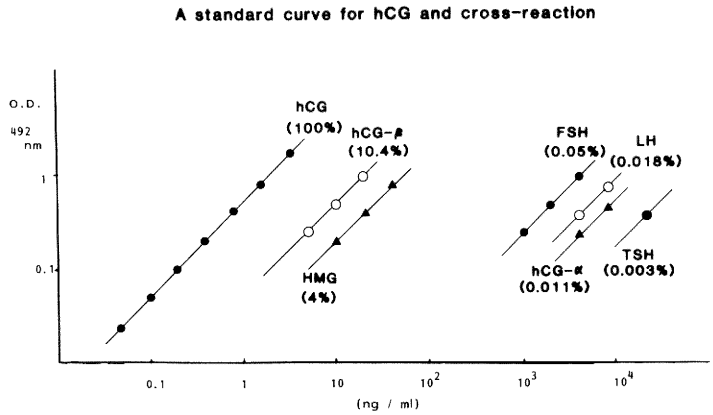
A standard curve for hCG and cross-reaction
O.D.
492
nm
1
0.1
hCG
(100%)
hCG-β
(10.4%)
HMG
(4%)
FSH
(0.05%)
LH
(0.018%)
TSH
(0.003%)
hCG-α
(0.011%)
0.1
1
10
10²
10³
10⁴
(ng / ml)

Fig. 2

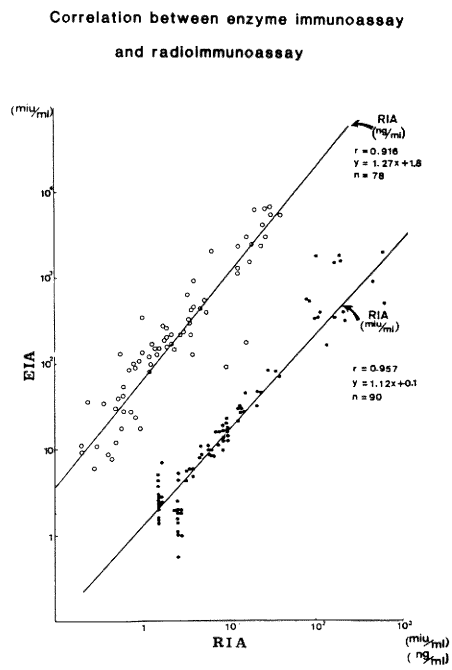
Correlation between enzyme immunoassay
and radioimmunoassay
(miu/ml)
RIA
(ng/ml)
r = 0.916
y = 1.27x + 1.8
n = 78
RIA
(miu/ml)
r = 0.957
y = 1.12x + 0.1
n = 90
EIA
RIA
(miu/ml)
(ng/ml)

Fig. 3

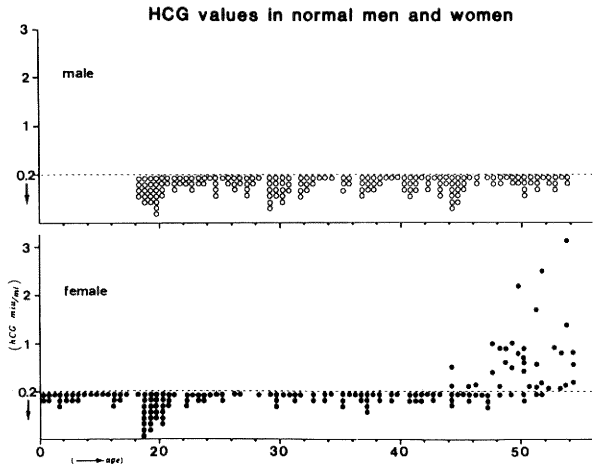
HCG values in normal men and women
male
female
(hCG mIU/ml)
0
20
30
40
50
(→ age)

Fig. 4

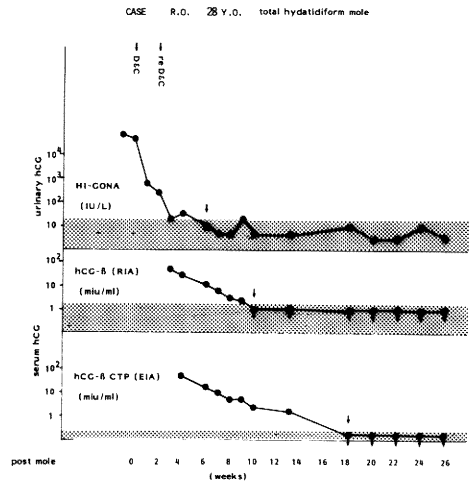
CASE R.O. 28 Y.O. total hydatidiform mole
D&C
re D&C
urinary hCG
HI-CONA
(IU/L)
serum hCG
hCG-ß (RIA)
(miu/ml)
hCG-ß CTP (EIA)
(miu/ml)
post mole
0 2 4 6 8 10 12 14 16 18 20 22 24 26
(weeks)

Fig. 5

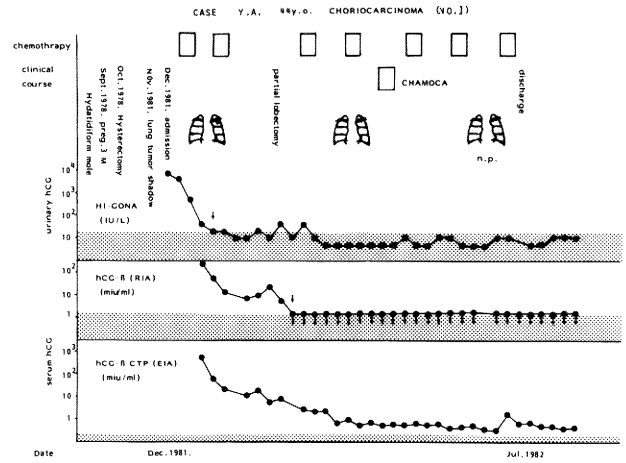
CASE Y.A. 44y.o. CHORIOCARCINOMA (NO.1)
chemothrapy
clinical course
Hydatidiform mole
Sept.1978. preg.3 M
Oct.1978. Hysterectomy
Nov.1981. lung tumor shadow
Dec.1981. admission
partial lobectomy
CHAMOCA
discharge
n.p.
urinary hCG
HI-GONA (IU/L)
hCG ß (RIA) (miu/ml)
serum hCG
hCG ß CTP (EIA) (miu/ml)
Date
Dec.1981.
Jul.1982

Fig. 6a

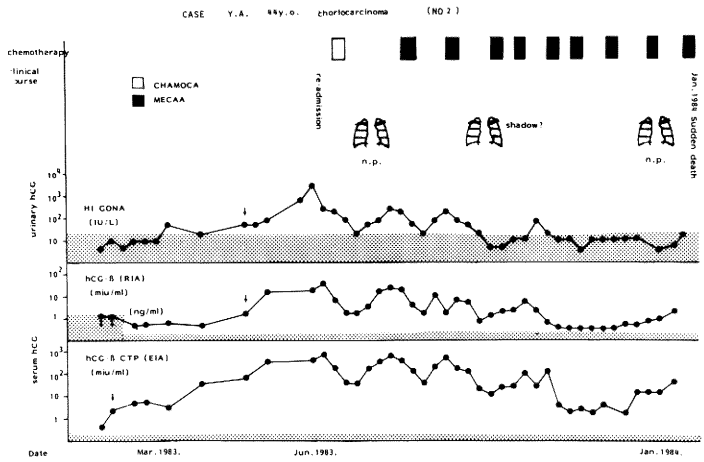
CASE Y.A. 44y.o. chortocarcinoma (NO 2)
chemotherapy
clinical course
CHAMOCA
MECAA
re admission
shadow?
Jan.1984. Sudden death
n.p.
n.p.
urinary hCG
HI GONA (IU/L)
hCG ß (RIA) (miu/ml)
(ng/ml)
serum hCG
hCG ß CTP (EIA) (miu/ml)
Date
Mar.1983.
Jun.1983.
Jan.1984.

Fig. 6b

		weeks after evacuation (mean± SD)	lag weeks (mean± SD)
urinary hCG	(HI-GONA)	6.7±4.9 (n=9)	6.4±3.8 (n=6)
serum hCG	hCG-ß (RIA)	13.1± 5.6 (n=6)	9.7±3.8 (n=6)
	hCG-ß CTP (EIA)	24.5±4.4 (n=6)	

HCG regression periods after evacuation of hydatidiform moles to the LH level (20IU/L) in urinary hCG and to the limit of detection in serum hCG (hCG-ß and hCG-ß CTP).

Table 1

		weeks after treatment (mean±S.D.)	lag weeks (mean±S.D.)
urinary hCG	(HI-GONA)	9.2± 7.3 (n=6)	11.8 (n=3)
serum hCG	hCG-ß (RIA)	21.8±14.9 (n=5)	9.5 (n=2)
	hCG-ß CTP (EIA)	28.0 (n=2)	

HCG regression periods after treatment of choriocarcinomas to the LH level (20IU/L) in urinary hCG and to the limit of detection in serum hCG (hCG-ß and hCG-ß CTP).

Table 2

the sandwich-EIA method required 2 or 4 times as long, respectively. The mean time for urinary hCG to fall to the LH-level took 6.4 and 9.7 times by the RIA and EIA methods, respectively, as it did by Hi-Gonavis (Table 1).

The clinical course of a patient with histopathologically confirmed choriocarcinoma is illustrated in Figure 6a. In 1978, a simple abdominal hysterectomy was performed because of persistent hCG secretion after evacuation of a hydatidiform mole. In 1981, examination revealed a lung shadow, and choriocarcinoma was clinically diagnosed. After combination chemotherapy, a partial lobectomy was performed, and the patient was given additional courses of chemotherapy. Urinary hCG quickly fell to the LH level according to the hemagglutination assay method and to the limit of detection 7 weeks later, according to the β-hCG RIA system; however, the EIA method continued to detect hCG (Fig. 6b). About one year later, this method detected a significant increase in the urinary hCG level 16 weeks before the other assay methods did.

In choriocarcinoma patients, hCG levels reached the LH level in 9.2 weeks, 21.8 weels, and 28 weeks according to the hemagglutination, β-hCG RIA, and EIA methods, respectively (Table 2). Although hCG levels fell to the limit of detection in all hydatidiform mole patients, 5 of 7 choriocarcinoma patients showed elevated levels despite the absence of clinical evidence indicating recurrence; 1 patient had re-elevated titers in all assays. We do not know at the moment whether this finding indicates the persistence of trophoblastic cells or the influence of hCG-like substances, such as those often found in menopausal subjects.

Discussion

It is now possible to measure low levels of hCG by several methods. In the HAR assay (Hi-Gonavis), urinary hCG/LH can be measured within 2 h with a sensitivity of 5 mIU/ml.

It is well known that the measurement of β-hCG provides a better predictive index of trophoblastic disease regression than assays of whole hCG. Human chorionic gonadotropin has the same biologic activity as LH and a very similar structure, differing mainly in the 30 additional carboxy-terminal residues of its β-subunit and the consequent difference in carbohydrate. The specific and sensitive sandwich-EIA described here allowed us to detect low serum levels of hCG in the presence of human LH, and we showed that the antibodies used in this assay recognize the polypeptide chain and that their reaction are not affected by the carbohydrate moieties at the β-hCG carboxy terminus. Our absorbed anti-β-hCG serum seems better able than antiserum to discriminate between hCG and LH. Because α-hCG partially cross-reacts with β-hCG, we conclude that antigenic similarities between the α- and β-subunits of FSH and hCG are very slight.

In sandwich-EIA, the limit of detection of hCG was 0.01 mIU/tube, corresponding to

0.2 mIU/ml in serum. No significant cross-reaction was recognized with human LH at a concentration of 200 mIU/ml. Therefore, our method is 3-8 times more sensitive for hCG 10-40 times less cross-reaction with human LH than other methods.

Our investigation revealed similarly low hCG levels in normal subjects. We also observed that the hCG levels in the sera of females increased with age, these results agreeing well with those reported by other investigators[4),6)]. Positive hCG levels in the sera of older women may result from the hCG- like substance, but the regulation of this substance in normal nonpregnant subjects is not understood. The concentration of the hCG-like substance substantially increases in postmenopausal women. The significance of tihis immunoreactive substance in the sera of postmenopausal women was not clear until the present study. It has been suggested that it is a precursor of LH in that removal of the CTP of the β-subunit generates human LH, but this hypothesis has not been substantiated. Hartree[7)] reported that hCG is produced in the human pituitary gland at levels 25- to 50-fold lower than the concentration of human LH. Whatever its function may, further analysis of this hCG substance and its implications for clinical follow-up of trophoblastic disease is necessary.

Although the β-hCG assay is specific to hCG and sensitive at low levels, there is some concern about the clinical significance of a negative result in determining complete remission during follow-up. Theoretically, according to Bagshawe[1)], the β-subunit becomes undetectable by current assay methods when the tumor load is reduced to 10^4-10^5 cells. In the method described here, serial CTP levels were found to provide a more accurate asessment of the clinical course of trophoblastic disease than β-hCG did. When β-hCG becomes undetectable by sandwich-EIA, the tumor burden may be reduced to 10^3-10^4 cells.

In conclusion, this method is useful for long-term follow-up of trophoblastic disease and early detection of recurrent choriocarcinomas.

References

1) Bagshawe KD: Choriocarcinoma: The Clinical Biology of the Trophoblast and Its Tumors. London: Arnold, 1969.
2) Swaminathan N, Bahl OP: Dissociation and recombination of the subunits of human chorionic gonadotropin. *Biochem Biophys Res Commun* **40**: 422-427, 1970.
3) Kurosky A, Markel DE, Peterson JW, et al: Primary structure of cholera toxin β-chain: A glycoprotein hormone analog? *Science* **195**: 299-301, 1977.
4) Suzuki N, Konishi E, Kondo E, et al: A highly sensitive colorimetric sandwich-enzyme immunoassay for human chorionic gonadotropin using specific antibodies against the carboxy-terminal portion of the β-subunit. *Clin Chem Acta* **150**: 247-253, 1985.
5) Chen HC, Matsuura S, Hodgen GD, et al: A human chorionic gonadotropin-like substance distinct from human luteinzing hormone. *In* Novel Aspects of Reproductive Physiology (Spilman CH, Wilks JW, eds). New York: Spectrum, 1978, pp 289-308.
6) Borkowski A, Muquardt C: Human chorionic gonadotropin in the plasma of normal, nonpregnant subjects. *N Engl J Med* **301**: 298-302, 1979.
7) Hartree AS, Shownkeen RC, Stevens VC, et al: Studies of the human chorionic gonadotropin-like

substance of human pituitary glands and its significance. *J Endocrinol* **96**: 115-126, 1983.
8) Braunstein GD: Human chorionic gonadotropin in non-trophoblastic tumors and tissue. *In* Recent Advances in Reproduction and Regulation of Fertility (Talwar GP, ed). Amsterdam: Elsevier, 1979, pp 389-397.