STUDY ON ANTIGENECITY OF HUMAN CHORIONIC GONADOTROPIN (hCG) FACTORS WHICH EFFECT ON hCG IMMUNOHISTOCHEMISTRY¹

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Qualitative as well as quantitative study on antigenic stability of hCG and its subunits showed that fixatives tested were more or less injurious and that heating at 60°C or xylol treatment significantly affected the antigenicity. Fixation with 10% formalin or with Zamboni's solution had less effect. It was concluded that frozen sections were more suitable for detection of small amounts of hCG antigen than paraffin-embedded materials.

Human chorionic gonadotropin (hCG) is a glycoprotein hormone produced by syncytiotrophoblast of the human placenta (1, 5, 8, 10, 13, 17, 21, 22), and is known to be composed of alpha and beta subunits (2, 3, 5, 13). It is also produced by some tumors including both trophoblastic and non-trophoblastic types (4, 6, 12, 13, 14, 17, 18, 23, 24). During immunohistochemical study on the hCG producing tumors, we experienced not infrequently cases which demonstrated negative immune staining in spite of a high value of hCG or its subunits in urine, serum, and tissue extract. That led us to investigate the stability of hCG antigenicity for immune staining.

MATERIALS AND METHODS

The hCG and hCG subunits: hCG (Lot 41 H, potency of 5,900 i.u. per mg) was purified from human placenta by the method described in our previous reports (15, 16). hCG-alpha (Lot 32 alpha) and hCG-beta (Lot 30-31 beta) were obtained from human placental hCG treating with urea by the modified method of Swaminathan and Bahl (20).

Insolubilized hCG: Sheep red blood cells coupled with native hCG (SRBC-hCG) (Mochida Pharmaceutical Co. Ltd.) were used as an insoluble hCG.

Tissue hCG: Human placentas in the first, the second and the third trimesters were used as dry ice acetone frozen, five micron thick cryostat sections.

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Incubation of soluble hCG and hCG subunits with fixatives: 13 mg of hCG was dissolved in 2.6 ml of phosphate buffered saline (PBS, pH 7.2), 29,500 i.u. per ml, and 2.6 mg of hCG-alpha and hCG-beta were dissolved in 2.6 ml of PBS respectively. 0.2 ml from each solution was mixed with one of the following fixatives [1) to 5)] in the test tubes and incubated at 4°C or 60°C for one or 24 hr. Then the mixture was transferred to the cellulose tubes, and dialyzed against distilled water at 4°C for 24 to 48 hr. The fixatives used were; 1) 10% buffered formalin, 2) Zamboni's solution (19, 25), 3) 100% ethanol, 4) 100% xylol, and 5) PBS (control). Before dialysis xylol-treated solution was immersed in ethanol to remove xylol. After dialysis, each sample was tested for the immunoreactivity and bio-activity by hemagglutination inhibition reaction (HI), radioimmunoassay (RIA) and biological assay (BA) by Diczfalussy (7).

Incubation of insolubilized hCG with fixatives: SRBC-hCG, 11.5×10^5 per ml, were suspended in 0.2 ml of the fixatives [1) to 5)] and incubated at 4°C for one or 24 hr. Then SRBC-hCG were washed five times by cold PBS, and incubated with 0.1 ml of anti-hCG rabbit antisera diluted 1 : 400 in PBS. After incubation the SRBC-hCG were washed five times with cold PBS, and 0.1 ml of horseradish peroxidase labeled anti-rabbit gamma globulin goat gamma globulin (9) prepared in this laboratory and incubated at 37°C for one hr was added. The enzyme activity was assayed by incubation with 3 ml of 0.06% 5-aminosalicylic acid with 0.003% H_2O_2 in 0.01 M PBS at 18°C for one hr. The enzyme reaction was stopped by adding 30 μ l of 0.5% nitrogen oxide. The optical density of the supernatant at 500 nm was read by the spectrophotometer (Hitachi Model 200-20).

Treatment of tissue sections: The frozen sections of the placenta were treated at 4°C or 60°C for one hr with, 1) 10% buffered formalin, 2) Zamboni's solution, 3) 100% ethanol, 4) 100% xylol, 5) PLP fixative (11), 6) 2.5% glutaraldehyde, and 7) PBS (control). Then the tissues were washed with cold PBS for 30 min changing three times. Indirect immunofluorescence technique was applied to detect hCG and its subunits immunoreactivity by incubating firstly with anti-hCG or its subunits at 37°C for 60 min, followed by incubation with FITC-labeled anti-rabbit gamma globulin goat gamma globulin prepared in this laboratory at 37°C for 60 min.

RESULTS

1) *Effects of the fixatives on soluble hCG, hCG-alpha and hCG-beta* The effect on hCG is shown in Figs. 1 and 2. hCG activity treated with Zamboni's solution at 4°C was preserved at 70% of the control, and with 10% buffered formalin at 4°C was decreased to 50% (RIA). By HI method hCG treated with 100% ethanol at 4°C preserved 72% activity. Under the incubation temperature at 60°C, hCG activity showed remarkably decreased (Fig. 2). Fig. 3 shows the influence on hCG-alpha and hCG-beta subunits as assayed by RIA. Except for Zamboni's solution similar effects were observed in both immuno- and bio-activity of hCG. The hCG-beta immunoreactivity was mostly well preserved in 10% buffered formalin and showed 86% of control. The alpha-subunit reactivity treated with the fixatives was lower than that of beta-subunit (Fig. 3).

2) *Effects of the fixatives on insoluble hCG* Effect of the fixatives on insoluble hCG is shown in Fig. 4. On the contrast to soluble hCG, no remarkable difference

FIG. 1. Effects of fixatives on soluble hCG immuno- and bio-activities.

FIG. 2. Effect of fixatives and incubation temperature on hCG immunoreactivity.

FIG. 3. Effects of fixatives on hCG and subunits immunoreactivity.

in the effect by the fixatives on immunoreactivity was found, and the activity was preserved in the range from 79 to 88 percent of the control.

3) *Effects of the fixatives on immune staining* Table 1 summarizes the result of

FIG. 4. Effects of fixatives on insolubilized hCG immunoreactivity.

TABLE 1. *Effects of Fixatives on Placental hCG Subunits Immunofluorescence*

Treatment	First trimester		Second trimester		Third trimester	
	hCG- α	hCG- β	hCG- α	hCG- β	hCG- α	hCG- β
PBS	(++)	(++) to (++)	(++)	($\pm$)	(++)	(+)
10% Buffered formalin	(+) to (++)	(++)	(+) to (++)	($\pm$)	(+) to (++)	($\pm$) to (+)
Zamboni's solution	(+) to (++)	(++) to (++)	(+) to (++)	($\pm$)	(+) to (++)	(+)
100% Ethanol	($\pm$) to (+)	(+)	(+)	(-) to ($\pm$)	($\pm$)	(-)
100% Xylol	($\pm$)	($\pm$)	n.e.	n.e.	n.e.	n.e.
PLP	(+)	(+) to (++)	(+)	($\pm$)	(+) to (++)	(+)
2.5% Glutaraldehyde	(-)	(-)	(-)	(-)	(-)	(-)

n.e.: not examined

FIG. 5. hCG-beta immunostaining of human placental tissue unfixed. Intensive fluorescence of hCG-beta in syncytiotrophoblast is demonstrated. $\times 200$

FIG. 6. hCG-beta immunostaining of human placental tissue treated with 10% buffered formalin. Specific fluorescence for hCG-beta is clearly observed. $\times 200$

FIG. 7. hCG-beta immunostaining of human placental tissue treated with 2.5% glutaraldehyde. No positive staining is obtained in placental tissue. $\times 200$

immune staining of hCG-alpha and hCG-beta subunits in placental sections treated with various fixatives. Although the immunofluorescence was most intensive in unfixed sections (Fig. 5), the specimens treated with 10% buffered formalin or Zamboni's solution also demonstrated satisfactory fluorescence (Fig. 6). On the contrary, 2.5% glutaraldehyde resulted in completely negative staining in all materials (Fig. 7). The sections treated with 100% ethanol or PLP fixatives showed frequently decreased fluorescence compared with that of 10% formalin or Zamboni's solution treatment.

DISCUSSION

A qualitative study on the antigenetic stability of hCG has already been done by Midgley and Pierce (10) in 1962, where hCG in solution or placental tissue was

shown immunochemically resistant to 10% formalin treatment.

The present study disclosed that the antigenicities of hCG and its subunits were considerably affected as expected by incubation time and temperature, although the effects depended on whether the hormone was soluble or insoluble, native or in the form of subunits, extracted or *in situ* in the placental tissue and so on.

All fixatives tested decreased more or less the antigenic activity of hCG as compared with the untreated one. Treatment with 10% formalin or Zamboni's solution was less harmful to native hCG, however 2.5% glutaraldehyde impaired it seriously. Also heating soluble hCG at 60°C as well as treatment with xylol caused significant damage on its antigenicity, indicating that in order to detect a small amount of hCG antigen in tissues paraffin-embedded material was less advantageous than frozen sections.

It was demonstrated that native hCG was more stable in antigenicity than its subunits, and that, comparing alpha subunit with beta, the latter was less affected by heating treatment with fixatives than the former. However, the results do not necessarily prove that the beta subunit which is implied in the native hCG molecule should also possess more stable antigenicity than alpha. Our final aim is an effective conditioning for immunohistochemical detection of hCG and its subunits in tumors, and the present study was conducted as a preliminary experiment. Examination on the extracted tumor hormone is to be further undertaken.

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REFERENCES

1. Bahl, O. P.: Human chorionic gonadotropin. *J. Biol. Chem.* 244; 567, 1969.
2. Bahl, O. P.: Human chorionic gonadotropin. *J. Biol. Chem.* 244; 575, 1969.
3. Bellisario, R., Carlsen, R. B. and Bahl, O. P.: Human chorionic gonadotropin. *J. Biol. Chem.* 248; 6796, 1973.
4. Braunstein, G. D., Vaitukaitis, J. L., Carbone, P. P. and Ross, G. T.: Ectopic production of human chorionic gonadotropin by neoplasms. *Ann. Intern. Med.* 78; 39, 1973.
5. Canfield, R. E., Morgan, F. J., Kammerman, S., Bell, J. J. and Agosto, G. M.: Studies of human chorionic gonadotropin. *Recent Progr. Hormone Res.* 27; 121, 1971.
6. Cottrell, J. C., Becker, K. L. and Moore, C. F.: Immunofluorescent studies in gonadotropin-secreting bronchogenic carcinoma. *Amer. J. Clin. Path.* 50; 422, 1968.
7. Diczfalussy, E. and Loraine, J. A.: Sources of error in clinical bioassay of serum chorionic gonadotropin. *J. Clin. Endocr.* 15; 424, 1955.
8. Gau, G. and Chard, T.: Location of the protein hormone of the placenta by the immunoperoxidase technique. *Brit. J. Obstet. Gynec.* 83; 876, 1976.
9. Nakane, P. K. and Kawaoi, A.: Peroxidase-labeled antibody. A new method of conjugation. *J. Histochem. Cytochem.* 22; 1084, 1974.
10. Midgley, A. R. and Pierce, G. B.: Immunohistochemical localization of human chorionic gonadotropin. *J. Exp. Med.* 115; 289, 1962.
11. McLean, I. W. and Nakane, P. K.: Periodate-lysine-paraformaldehyde fixative for immunoelectron microscopy. *J. Histochem. Cytochem.* 22; 1077, 1974.

12. Muggia, F. M., Rosen, S. W., Weintraub, B. D. and Hansen, H. H.: Ectopic placental proteins in nontrophoblastic tumors. *Cancer* 36; 1327, 1975.
13. Naughton, M. A., Merrill, D. A., McManus, L. M., Fink, L. M., Berman, E., White, M. J. and Martinez-Hernandez, A.: Localization of the beta chain of human chorionic gonadotropin on human tumor cells and placental cells. *Cancer Res.* 35; 1887, 1975.
14. Okano, T.: Immunohistochemical and clinical study of hCG-producing tumors. *Jap. J. Clin. Oncol.* 9; 215, 1979.
15. Okumura, H., Namba, S. and Matsushima, S.: An improved purification of human chorionic gonadotropin. *Endocr. Jap.* 20; 67, 1973.
16. Okumura, H., Sudo, T. and Sawai, M.: A specific radioimmunoassay of a hCG-beta subunit in the presence of native hCG, and immunological, biological and chemical characterization of hCG and its chemically modified subunits. *Folia Endocr. Jap.* 55; 38, 1979.
17. Pierce, G. B. and Midgley, A. R.: The origin and function of human syncytiotrophoblastic giant cells. *Amer. J. Path.* 43; 153, 1963.
18. Rees, L. H. and Ratcliffe, J. G.: Review article. Ectopic hormone production by non-endocrine tumours. *Clin. Endocr.* 3; 263, 1974.
19. Stefani, M., Martino, C. D. and Zamboni, L.: Fixation of ejaculated spermatozoa for electron microscopy. *Nature* 216; 173, 1967.
20. Swaminathan, N. and Bahl, O. P.: Dissociation and recombination of the subunits of human chorionic gonadotropin. *Biochem. Biophys. Res. Commun.* 40; 422, 1970.
21. Thiede, H. A. and Choate, J. W.: Chorionic gonadotropin localization in the human placenta by immunofluorescent staining. *Obstet. Gynec.* 22; 310, 1963.
22. Thiede, H. A., Choate, J. W. and Bindschadler, D. D.: Chorionic gonadotropin localization in the human placenta by immunofluorescent staining. *Obstet. Gynec.* 22; 433, 1963.
23. Vaitukaitis, J. L.: Immunologic and physical characterization of human chorionic gonadotropin (hCG) secreted by tumors. *J. Clin. Endocr. Metab.* 37; 505, 1973.
24. Weintraub, B. D. and Rosen, S. W.: Ectopic production of the isolated beta subunit of human chorionic gonadotropin. *J. Clin. Invest.* 52; 3135, 1973.
25. Zamboni, L. and Martino, C. D.: Buffered picric acid-formaldehyde: A new, rapid fixative for electron microscopy. *J. Cell Biol.* 35; 148A, 1967.