Endocrine Journal 1997, **44** (3), 431–435

NOTE

Detection of Thyroid-Stimulating Antibody Using Frozen Stocks of Chinese Hamster Ovary Cells Transfected with Cloned Human Thyrotropin Receptor

TORU TAKANO, HIROMI SUMIZAKI, AND NOBUYUKI AMINO

Department of Laboratory Medicine, Osaka University Medical School, Osaka 565, Japan

Abstract. To measure thyroid-stimulating antibody (TSAb) in sera from patients with Graves' disease, we developed a new assay system with using frozen stocks of CHO-K_1 cells. CHO-K_1 cells trasfected with cloned thyrotropin (TSH) receptor on a 96 well plate were frozen in Cell Banker™ and stored at −70 °C. Three days before the assay, they were thawed in the culture medium and allowed to grow in a monolayer until use. The medium was replaced with medium containing IgGs from the patients, then after 2 h, it was collected and concentrations of adenosine 3'-5'-cyclicmonophosphate (cAMP) were measured. This method is sensitive enough to detect TSAb and it is simpler and easier than the methods which use FRTL-5 cells.

Key words: Thyroid-stimulating antibody, CHO, Graves' disease, Cyclic AMP

(*Endocrine Journal* **44**: 431–435, 1997)

THYROID-stimulating antibody (TSAb) binds to thyrotropin (TSH) receptors in thyroid follicular cells and stimulate thyroid adenylate cyclase to produce thyroid hormone [1]. The measurement of TSAb is clinically useful in confirming the diagnosis of Graves' disease. TSAb have been most commonly measured by using the rat thyroid cell line (FRTL-5). This cell line is a non-transformed, differentiated but continuous cell line that has been well characterized in many reports [2, 3], but many factors make these cells less than ideal for the assay of TSAb. First, they are slow in growing and it is more difficult to be cultured than other fast growing cell lines [4]. A well-trained technician is therefore always needed and the use of sterilized materials and reagents increases the total cost of the assay. Second, the characteristics of this cell line, including the rate of production of adenosine 3'-5'-cyclicmonophosphate (cAMP) in response to TSH, can change easily during the continuous culture [5, 6]. The use of frozen stocks of FRTL-5 cells is required to obtain the stable results, although frozen cells have to be cultured more than one month before to ensure sufficient sensitivity of the assay. Furthermore, the characteristics of FRTL-5 cells still vary among the laboratories, so that comparison of the results obtained at various institutions is quite difficult. Third, patients' IgGs do not always react in the same way with rat TSH receptors as with human TSH receptors [7–9], so that the use of the cell line with human TSH receptors is better than that with rat TSH receptors.

Avoiding continuous culture of cells can solve some of the above problems and all of the procedures used in detecting TSAb become much easier and simpler if there is no need for cell culture for a long period. It is, however, not always easy to use the frozen stocks of cells immediately after they are thawed because the damage caused by freezing can affect their growth rate and production of cAMP. Vitti *et al.* succeeded to detecting TSAb

Received: August 21, 1996
Accepted: December 12, 1996
Correspondence to: Dr. Toru TAKANO, Department of Laboratory Medicine, Osaka University Medical School, 2–2 Yamadaoka, Suita, Osaka 565, Japan

using Chinese hamster ovary (CHO) cells transfected with human TSH receptor, which grow much faster than FRTL-5 cells [7, 10]. Furthermore, a newly developed reagent, Cell Banker™ (Nihonzenyaku, Koriyama, Fukushima, Japan), allows CHO cells to survive in frozen conditions at a 10 to 30 percent higher rate than other materials such as dimethyl sulfoxide (DMSO) (personal communications). By using Cell Banker™ and CHO-K_1 cells transfected with human TSH receptor, we investigated whether the immediate use of the frozen stocks in TSAb assay (frozen-cell method) is possible.

Subjects and Methods

Subjects

Sera were obtained from three patients with newly diagnosed Graves' disease. Sera were also obtained from ten healthy subjects with no personal or family history of autoimmune disease.

Materials

Bovine TSH and other chemicals were obtained from Sigma (St. Louis, MO).

Measuring TSAb activity with FRTL-5 cells

Serum TSAb activity was measured as the activity to stimulate cAMP increase in FRTL-5 cells, as described previously [11]. Briefly, 250 μl of the test samples were first precipitated with polyethylene glycol (PEG) (Wako, Chuo-Ku, Osaka, Japan). The precipitates were reconstituted in 250 μl of modified Hanks' solution (HBSS) without NaCl containing 0.5 mM isobutylmethylxanthine. The prepared samples were incubated with FRTL-5 cells in a 24 well dish for 2 h at 37 °C, then cAMP content in the medium was measured with a commercial radioimmunoassay kit (Yamasa Shoyu, Chiba, Japan).

Cell culture of CHO-K_1 cells

CHO-K_1 cells transfected with human TSH receptor were kindly provided by Dr. T. Endo and Dr. T. Onaya (University of Yamanashi Medical School, Yamanashi, Japan) [12]. They were cultured in Ham's F-12 medium containing 10% fatal calf serum and 0.5 mg/m*l* G418 (Wako, Chuo-Ku, Osaka, Japan). Cells were routinely cultured in 10 cm dishes at 37 °C in an atmosphere of 95% air, 5% CO_2 in a humidified incubator.

Measuring TSAb activity with CHO-K_1 cells

TSAb assay with CHO-K_1 cells was performed with some modifications to the previous description [7]. In brief, cells were harvested using a trypsin-EDTA mixture and seeded (10,000 cells/well) in 96 well plates (Costar, Cambridge, MA). The cells were fed fresh culture medium 24 h after seeding and used for the assay of TSAb after 48 h. 250 μl of samples were precipitated with PEG as described previously and diluted with 250 μl of HBSS containing 0.5 mM isobutylmenthylxanthine. 200 μl prepared samples were incubated with CHO-K_1 cells in a 96 well plate for 2 h at 37 °C, then cAMP content in the medium was measured. Results are expressed as the percentage of cAMP released in the extracellular medium with respect to the basal value obtained with the normal pooled serum [11].

Measuring TSAb activity with frozen stocks CHO-K_1 cells (frozen-cell method)

Cultured CHO-K_1 cells were harvested with a trypsin-EDTA mixture and diluted with Cell Banker™. 10 μl of cells (10,000 cells/well) were placed on the bottom of each well of 96 well plates, then the cells were frozen and stored at −70 °C until use. The cells were incubated in a water bath to be thawed for 3 min at 37 °C, then quickly placed on ice. The survival rate of CHO-K_1 cells determined with trypan blue after they were frozen for 2 months was 92%. They were incubated at 37 °C after addition of 200 μl of culture medium. The next day, the cells were fed fresh culture medium. They were incubated for additional 48 h then used for the assay of TSAb as previously described.

Results

First we determined the sensitivity of the assay

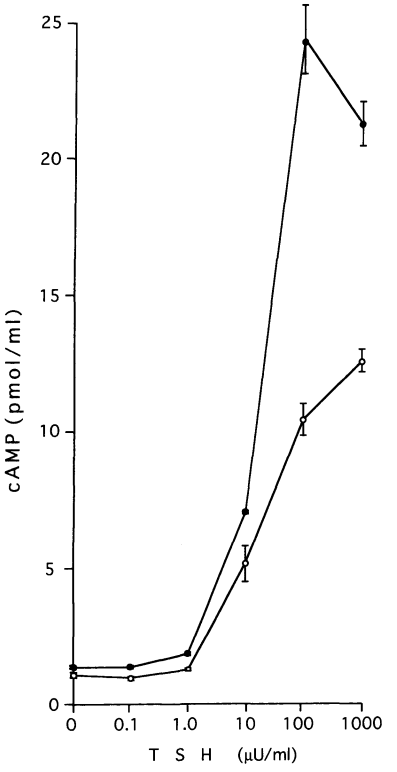

Fig. 1. Dose-response curves of cAMP production in frozen stocks (open circles) and cells of continuous culture (closed circles) stimulated by bovine TSH. The assay was performed as described in Materials and Methods, measuring extracellular cAMP after 2 h of incubation. The points represent the mean ± SD for triplicate determinations.

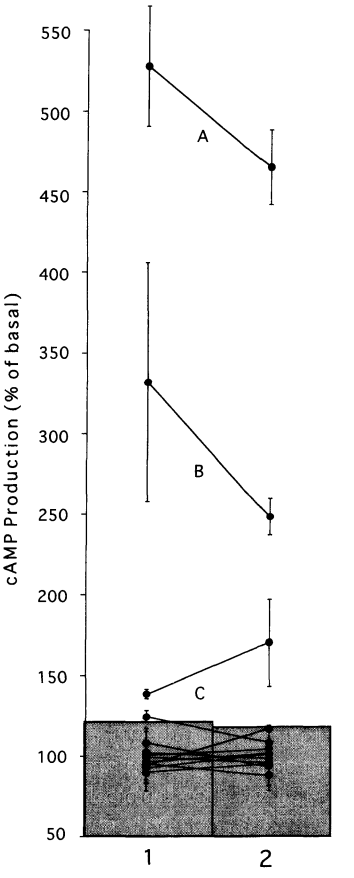

Fig. 2. Results obtained by frozen-cell method using sera form three patients with Graves' disease (A, B, C) and ten healthy subjects. Results are expressed as the percentage of cAMP released in the extracellular medium with respect to the basal value obtained with the normal pooled serum. Columns 1 and 2 show the results obtained when using cells immediately and two months, respectively, after being frozen. The shaded area indicates the mean ± 2SD (120.6% for lane 1 and 119.0% for lane 2) of results obtained with sera from normal subjects. The points represent the mean ± SD for duplicate determinations.

with frozen stocks of CHO-K_1 cells compared with that with the continuous culture cells. An increase in the cAMP concentration was detectable at 1 μU/ml bovine TSH by both methods, but as the concentration of TSH increased, the production of cAMP by the frozen-cell method does not increase as much as in the method with the continuous culture cells (Fig. 1).

We next estimated the reproducibility of the results of the frozen-cell method. Sera from ten healthy subjects and three patients with Graves' disease (A, B and C, TSAb activity measured with FRTL-5 cells were 715, 267 and 1254%, respectively) were used in this experiment. The results of the assay performed immediately after preparing the frozen stocks were compared with that performed after two months. All the results for three samples from patients were above the range of the mean ± 2SD of normal samples in both assays. Although by duplicate determinations, the results of TSAb activity varied about 20% in these three samples, no obvious decrease in the production of cAMP was observed (Fig. 2).

Discussion

Continuous cell culture is always required for most of bio-assays including TSAb, but its cost and difficulty prevent these assay from being commonly used in many laboratories. The use of frozen stocks of cells is desired, although in many cases, it reduces the sensitivity of the assay. In this study, we performed the assay of TSAb activity with the frozen stocks of CHO-K_1 cells transfected with human TSH receptor. In this method, after frozen stocks on 96 well plates are prepared, there is no need for cell passage, and cell culture for only three days is needed. Because of the short period of cell culture and G418 in the culture medium, the use of a clean bench is not required. For these reasons, all the procedures in the frozen-cell method are much easier than those with continuous culture cells. Furthermore, if many plates of frozen cells are prepared, assays with cells with the same characteristics can be performed in different laboratories.

The sensitivity of the assay is the most important problem when using frozen stocks. Probably because of the use of Cell Banker™, frozen stocks of CHO-K_1 cells had almost the same sensitivity to bovine TSH as those of continuos culture, although they were less sensitive than FRTL-5 cells that could detect 0.3 μU/m*l* TSH (data not shown). We did not use the hypotonic solution described by Vitti *et al.* [7], since this medium rather reduced the sensitivity of the TSAb assay in these cells.

Frozen stocks of cells produced a smaller amount of cAMP than cells of continuous culture, especially when 100 or 1000 μU/m*l* TSH was added, which suggests the cells once frozen have lower maximum ability to produce cAMP than those of continuos culture. This may, however, not be a big problem because with 1.0 or 10 μU/m*l* TSH, which is the usual range we use for measuring TSAb activity (about 100–500% of basal activity), the two showed almost the same increase.

A new assay kit using frozen porcine thyroid cells was recently developed [13]. Its procedures are as easy as our method with frozen CHO-K_1 cells, although it can detect TSAb in only 53% of the sera from patients with untreated Graves' disease and the sensitivity to TSH was 3.0 μU/m*l*. Because transfected CHO-K_1 cells have higher sensitivity to TSH and they express human TSH receptor, our methods probably provide better sensitivity to the patients' sera than frozen porcine cells. Further study on a large number of patients' sera is needed.

Frozen cells kept at −70 °C provided almost the same results after two months. The effect of a longer storage period on the TSAb assay has to be determined, but Cell Banker™ is known to allow CHO cells to survive in the frozen condition at least two years (personal communication). We may therefore be able to maintain the similar cell conditions for more than a half year which is long enough for the commercial use. Although we have not examined it in this study, the use of a programmed freezer or plates with high heat conductivity may help to improve the conditions of the frozen cells.

Acknowledgments

This work was supported by a Grant-in-Aid for Encouragement of Young Scientists (to T. T.; No. 07770829) from the Ministry of Education, Science, Sports and Culture of Japan. We thank Dr. T. Endo and Dr. T. Onaya (University of Yamanashi Medical School, Yamanashi, Japan) for providing us with CHO-K_1 cells.

References

1. Smith BR, McLachan SM, Furmania KJ (1988) Autoantibodies to the thyrotropin receptor. *Endocrine Rev* 9: 106–120.
2. Ambesi-Impiombato FS, Parks LAM, Coon HG (1980) Culture of hormone-dependent functional epithelial cells from rat thyroid. *Proc Natl Acad Sci USA* 77: 3455–3459.
3. Vitti P, Rotella C, Valente W, Cohen J, Aloj S, Laccetti P, Ambiesi-Imbiombato F, Grollman E, Pinchera A, Toccafondi R, Kohn L (1983) Characterization of the optimal stimulatory effects of Graves' monoclonal and serum immunoglobulin G on adenosine 3':5'-monophosphate production in FRTL-5 thyroid cells: A potential clinical assay. *J*

Clin Endocrinol Metab 57: 782–791.

4. Takano T, Takada K, Tada H, Nishiyama S, Amino N (1994) Abundant exisitence of 40kD CDC2-related protein in rat thyroid cells. *Endocrine Res* 20: 127–137.
5. Bellur S, Tahara K, Saji M, Grollman EF, Kohn LD (1990) Repeatedly passed FRTL-5 rat thyroid cells can develop insulin and insulin-like growth factor-I-sensitive cyclooxygenase and prostaglandin E2 isomerase-like activities together with altered basal and thyrotropin-responsive thymidine incorporation into DNA. *Endocrinology* 127: 1526–1540.
6. Takano T, Sumizaki H, Liu G, Amino N (1995) Thyroid stimulating hormone and N_6-2′-O-dibutyryladenosine 3′-5′-cyclic monophosphate decrease 110,000–130,000 Mr tyrosine-phosphorylated substrate in rat thyroid cells. *Cell Signal* 7: 519–525.
7. Vitti P, Elisei R, Tonacchera M, Chiovato L, Mancusi F, Rago T, Mannoli C, Ludgate M, Vassart G, Pinchera A (1993) Detection of thyroid-stimulating antibody using Chinese hamster ovary cells transfected with cloned human thyrotropin receptor. *J Clin Endocrinol Metab* 76: 499–503.
8. Persani L, Tonacchera M, Beck-Peccoz P, Vitti P, Mammoli C, Chiovato L, Elisei R, Faglia G, Ludgate M, Vassart G (1993) Measurement of cAMP accumulation in Chinese hamster ovary cells transfected with the recombinant human TSH receptor (CHO-R): A new bioassay for human thyrotropin. *J Endocrinol Invest* 16: 511–519.
9. Michelangeli VP, Munro DS, Poon CW, Frauman AG, Colman PG (1994) Measurement of thyroid stimulating immunoglobulins in a new cell line transfected with a functional human TSH receptor (JPO9 cells), compared with an assay using FRTL-5 cells. *Clin Endocrinol* 40: 645–652.
10. Van-Sande J, Raspe E, Perret J, Lejeune C, Maenhaut G, Vassart G, Dumont J (1990) Thyrotropin activates both the cyclic AMP and the PIP2 cascades in CHO cells expressing the human cDNA of TSH receptor. *Molecular and Cellular Endocrinology* 74: 1–6.
11. Tamaki H, Amino N, Aozasa M, Masao M, Tanizawa O, Miyai K (1987) Serial changes in thyroid-stimulating antibody and thyrotropin binding inhibitor immunoglobulin at the time of postpartum occurrence of thyrotoxicosis in Graves' disease. *J Clin Endocrinol Metab* 65: 324–330.
12. Haraguchi K, Peng X, Kaneshige M, Anzai E, Endo T, Onaya T (1993) Thyrotropin-dependent desensitization by Chinese hamster ovary cells that express the recombinant human thyrotropin receptor. *J Endocrinol* 139: 425–429.
13. Asahi K, Takeoka K, Sumizaki H, Takano T, Fushimi R, Tada H, Hidaka Y, Amino N (1996) Fundamental and clinlcal evaluation of determination of thyroid stimulating antibody (TSAb) by Yamasa TSAb kit. *Horumon-to-rinsyo* 44: 401–409.