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

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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-K1 cells. CHO-K1 cells transfected 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

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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\textsuperscript{TM} (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\textsuperscript{TM} and CHO-K\textsubscript{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 \( \mu l \) of the test samples were first precipitated with polyethylene glycol (PEG) (Wako, Chuo-Ku, Osaka, Japan). The precipitates were reconstituted in 250 \( \mu l \) 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 \(^\circ\)C, then cAMP content in the medium was measured with a commercial radioimmunoassay kit (Yamasa Shoyu, Chiba, Japan).

Cell culture of CHO-K\textsubscript{1} cells

CHO-K\textsubscript{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\% fetal calf serum and 0.5 mg/ml G418 (Wako, Chuo-Ku, Osaka, Japan). Cells were routinely cultured in 10 cm dishes at 37 \(^\circ\)C in an atmosphere of 95\% air, 5\% CO\textsubscript{2} in a humidified incubator.

Measuring TSAb activity with CHO-K\textsubscript{1} cells

TSAb assay with CHO-K\textsubscript{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 \( \mu l \) of samples were precipitated with PEG as described previously and diluted with 250 \( \mu l \) of HBSS containing 0.5 mM isobutylmethylxanthine. 200 \( \mu l \) prepared samples were incubated with CHO-K\textsubscript{1} cells in a 96 well plate for 2 h at 37 \(^\circ\)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\textsubscript{1} cells (frozen-cell method)

Cultured CHO-K\textsubscript{1} cells were harvested with a trypsin-EDTA mixture and diluted with Cell Banker\textsuperscript{TM}. 10 \( \mu l \) 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 \(-70 \)^{\circ}\text{C} until use. The cells were incubated in a water bath to be thawed for 3 min at 37 \(^\circ\)C, then quickly placed on ice. The survival rate of CHO-K\textsubscript{1} cells determined with trypan blue after they were frozen for 2 months was 92\%. They were incubated at 37 \(^\circ\)C after addition of 200 \( \mu l \) 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
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with frozen stocks of CHO-K1 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).

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.
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-K1 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-K1 cells had almost the same sensitivity to bovine TSH as those of continuous culture, although they were less sensitive than FRTL-5 cells that could detect 0.3 μU/ml 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/ml TSH was added, which suggests the cells once frozen have lower maximum ability to produce cAMP than those of continuous culture. This may, however, not be a big problem because with 1.0 or 10 μU/ml 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-K1 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/ml. Because transfected CHO-K1 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.

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