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Thyroid-stimulating antibody (TSAb) detected in sera of Graves' patients using human thyroid cell cultures

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SUMMARY

As a homologous system is required to evaluate the effect of thyroid-stimulating antibody (TSAb) present in the serum of Graves' patients, primary cultures obtained from normal human thyroid gland have been used and the stimulatory effect measured as an increase of cAMP intracellular levels.

Monolayer cell cultures were stimulated by IgG purified from sera of Graves' patients or control subjects and compared to the effect of bovine TSH. Bovine TSH produced a dose-dependent increase in cAMP intracellular levels between 0·05 mU and 2·5 mU/ml, reaching a maximal value after 30 min with higher doses. While normal IgG had no effect, IgG prepared from untreated patients with frank Graves' disease elicited a significant increase in cAMP accumulation at a concentration between 0·05 and 0·5 mg/ml within 60 min in thirteen out of fourteen patients. A longer incubation period showed no further increase in cAMP values, even if in one case a higher concentration (5·0 mg/ml) of Graves' IgG had a delayed response. When the cAMP intracellular level modifications produced by Graves' IgG preparations in thyroid cell cultures were compared to those evoked in thyroid slices, an identical percentage (93%) of positive cases was obtained, without a coincidence of negative cases. Using thyroid slices the cAMP intracellular increase above basal levels was higher, if considered as a percentage, but in cultured cells a very low IgG concentration was sufficient to detect the presence of TSAb. No correlation between the two assays was found.

In conclusion, normal human cultured thyroid cells appeared to be a more suitable substrate when compared to human thyroid slices for detecting the presence of TSAb in Graves' disease and for studying its effect on thyroid cells. However, a 100% TSAb positivity was present in our Graves' patient series only when both assays were used.

INTRODUCTION

From the first observations that serum of patients with Graves' disease stimulates the thyroid gland (Adams & Purves, 1956) and that the stimulating substance was identified as an immunoglobulin (Kriss, Pleshakov & Chien, 1964), other procedures have been developed to detect the thyroid-stimulating antibody (TSAb). After the use of non-human substrates (Adams, 1958; McKenzie, 1958; Knight & Adams, 1973), human thyroid slices or plasma membranes were preferred (Onaya *et al.*, 1973; McKenzie & Zakarija, 1976; Orgiazzi *et al.*, 1976). In fact, by using a homologous system

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more emphasis was given to the species specificity of TSAb, and a higher frequency of positive results in sera of Graves' patients was found in comparison to non-human assays.

The aim of the present paper is to study whether normal human thyroid cells in primary culture are more suitable for detecting the presence of TSAb in sera of patients with Graves' disease when compared with thyroid slices. Thyroid-stimulating activity is measured by evaluating intracellular modifications of cyclic AMP (cAMP) induced by immunoglobulins of Graves' patients.

MATERIALS AND METHODS

Patients. Fourteen patients with frank Graves' disease were studied. The diagnosis was based on the following considerations: diffusely enlarged thyroid gland, high levels of circulating thyroid hormones evaluated by radioimmunoassay and abolished response to TRH; the possible presence of eye changes, localized pretibial myxoedema, anti-thyroglobulin and/or anti-microsomal antibodies (Table 1). At the time of the study patients had never received any anti-thyroid drugs, radiant treatment or surgical treatment. Controls consisted of ten subjects less than 35 years old, four of whom were male, with no detectable thyroid disease or any other autoimmune disorders as assessed by clinical and laboratory data.

Thyroid cell cultures. Thyroid tissue was obtained from surgical specimens of normal tissue from the counterside lobe of patients undergoing total thyroidectomy for monofocal cancer. This tissue was minced by a blade into small fragments (0·5–1·0 mm diameter) which, after two washes in phosphate-buffered saline, were suspended in 50 ml/g tissue of Krebs Ringer Bicarbonate (KRB), pH 7·4, containing glucose 1·0 g/l, bovine serum albumin 3·0 g/l (BSA) (Eurobio, Paris, France), deoxyribonuclease I 0·05 g/l (DNA) (Biochemia, Mannheim, West Germany) and supplemented with trypsin 2·5 g/l (Merck, Darmstadt, West Germany). Trypsinization was carried out for 20–30 min in a Bellco spinner flask at 37°C in a 95% O_2–5% CO_2 atmosphere, at a low magnetic stirrer speed. Trypsinized thyroid fragments were washed three times with KRB, centrifuging each time at 500 ***g***, and subsequently washed again three times with KRB free from Ca^{2+} and Mg^{2+}. Thyroid cells were then mechanically dispersed by pipetting with a fine flame-polished and siliconized Pasteur pipette. Dispersed cells were centrifuged through an underlying layer of 4% BSA gradient at room temperature for 15 min at 500 ***g*** to remove debris. Freshly isolated thyroid cells were suspended in McCoy's 5a medium (GIBCO, Grand Island, New York, USA) with sodium bicarbonate 2·2 g/l, supplemented with 20% foetal calf serum (GIBCO, Grand Island, New York, USA) and seeded at a 1×10^5 cell concentration per cm^2 in each well of 2-cm^2 multiwell plates (Linbro, Flow Laboratories Incorporated, Connecticut, USA). Cultures were carried out at 37°C in humidified air with 10% CO_2. Culture medium was changed every 3 days.

Stimulation of adenylate cyclase system. After 6–8 days, thyroid cultured cells were tested for bovine TSH (bTSH) (Ambinon^R, Organon, Oss, Holland) and Graves' IgG or normal IgG responsiveness. The medium was replaced with 0·2 ml KRB enriched with 0·6 mM 3-isobutyl-1-methyl-xanthine (IMX) (Sigma, St Louis, USA) and stimuli were added at different doses and incubation times to monolayer cell cultures. Incubation was carried out at 37°C in air with 10% CO_2 and stopped by adding 0·2 ml of cold absolute ethanol. The plate was stored at −20°C overnight. Thawed, broken cells were detached by means of a rubber policeman and the suspension was centrifuged at 2,000 ***g*** at 4°C. cAMP in the liophylized supernatant and the DNA content in the pellet were determined. The assay, performed in ten runs, showed a variation of sensitivity of 3% or less.

The stimulation of adenylate cyclase system by IgG or bTSH was also studied in thyroid slices 0·5 mm thick, weighing 20 to 30 mg, obtained from normal extranodular tissue using a Stadie Riggs microthome, according to the method of McKenzie & Zakarija (1976) with minor modifications (Tanini *et al.*, 1978). After preincubation at 37°C in an atmosphere of 95% O_2–5% CO_2 in 0·5 ml KRB enriched with 1 mM IMX, the effect of bTSH, Graves' IgG and normal IgG dissolved in 0·5 ml KRB was tested in triplicate. Incubation was stopped at different times by homogenization of the slices with a Polytron homogenizer at medium speed (5/10), with a 10-sec exposure and adding 1·0 ml of cold absolute ethanol. The samples left overnight at −20°C were centrifuged at 2,000 ***g*** at 4°C.

cAMP was determined in duplicate aliquots of the liophylized supernatant and the DNA content was measured in the pellet. The assay, performed in four runs, showed a variation of sensitivity of 4% or less.

Immunoglobulin preparation. Immunoglobulins were prepared from serum according to the method of Baumstark, Laffin & Bardawil (1964) using DEAE-Sephadex (Pharmacia, Uppsala, Sweden) in the chloride form. Purity of each preparation was tested by immunoelectrophoresis.

cAMP and DNA measurement. cAMP intracellular accumulation was measured by saturation analysis using a cytosol protein purified from bovine adrenal cortices (Brown *et al.*, 1971). The DNA content was measured by a fluorometric method based on the reaction of 3,5-diaminobenzoic acid with deoxyribose after complete lipid extraction (Kissane & Robins, 1958). Thyroglobulin showed no interference in the reaction. Fluorescence measured in an Aminco Bowman spectrophotofluorometer was linearly correlated with the DNA content of the cultured cell pellet between 0·6 and 15 μg with an incubation volume of 20 μl and with the DNA content of thyroid slices between 5 and 150 μg with an incubation volume of 200 μl.

Thyroid autoantibodies. Thyroglobulin antibodies were detected by passive haemagglutination, while microsomal antibodies were detected by immunofluorescence.

RESULTS

Cultured cells used by us to test thyroid-stimulating activity of human immunoglobulins were able to grow with an increase in DNA content of 66% in 6 days without addition of bTSH in the medium. When the basal intracellular cAMP accumulation was measured in the different cell preparations used during this study, it ranged from 0·6 to 1·8 pmol cAMP/μg DNA (s.e.m. 1·2 ± 0·3). Furthermore, these cells maintained the capacity to respond to bTSH, measured as cAMP intracellular accumulation, from a dose as low as 250 μU/ml (2·8 pmol cAMP/μg DNA). In fact, a dose-dependent increase in cAMP intracellular levels was observed up to a dose of 2·5 mU/ml of bTSH (21·2 pmol/cAMP/μg DNA), with a maximal value after 30 min (Fig. 1). When a lower bTSH dose of 1·0 mU/ml was used the increase was delayed, reaching a maximal cAMP concentration (11·3 pmol cAMP/μg DNA) after 60 min (Fig. 1).

The basal cAMP content of thyroid slices used to compare the thyroid-stimulatory effect of human IgG ranged from 116 to 204 fmol cAMP/μg DNA (s.e.m. 160 ± 21). After 30 min of incubation, 10 mU/ml of bTSH produced an increase of cAMP levels that ranged from 2·3 to 3·1 pmol/μg DNA (s.e.m. 2·7 ± 0·4), with an increase above basal levels of sixteen-fold, similar to that previously reported by others (Onaya *et al.*, 1973; McKenzie & Zakarija, 1976; Tanini *et al.*, 1978).

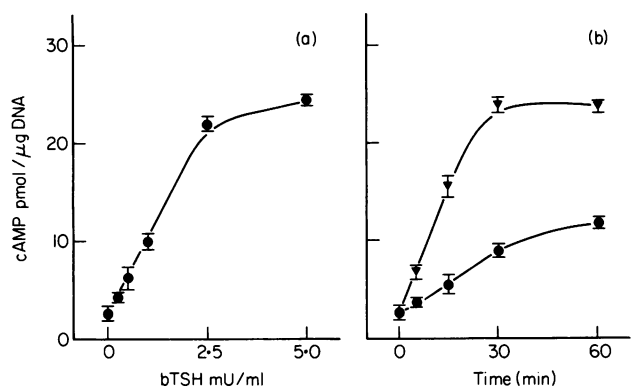

Fig. 1. (a) Effect of bovine TSH in normal human thyroid cultured cells at a concentration ranging from 0·25 to 5·0 mU/ml on intracellular cAMP levels (pmol/μg DNA ± s.e.m.) after 30 min incubation. (b) Time-dependent modifications of intracellular cAMP levels (pmol/μg DNA ± s.e.m.) in normal cultured cells using 2·5 mU/ml (▾——▾) and 1·0 mU/ml (●——●) of bovine TSH.

A pool of IgG purified from sera of four patients affected by Graves' disease produced an increase in cAMP intracellular accumulation of the same magnitude as that obtained when using 250 μU/ml of bTSH. This effect occurred in every case when different amounts of Graves' IgG were used. In fact, in some instances 0·005 mg/ml of IgG were sufficient to exert a significant increase. However, 0·05 or 0·5 mg/ml were more frequently necessary. The effect of Graves' IgG on cAMP accumulation in cultured thyroid cells was very rapid, reaching a maximal value after 30 min, while a longer incubation period elicited no further increase in cAMP levels (Fig. 2).

When the effect of higher Graves' IgG concentrations (from 2·0 to 5·0 mg/ml) on cultured thyroid cell cAMP accumulation was studied, in five cases out of six (Nos 3, 5, 7, 8 and 11) a

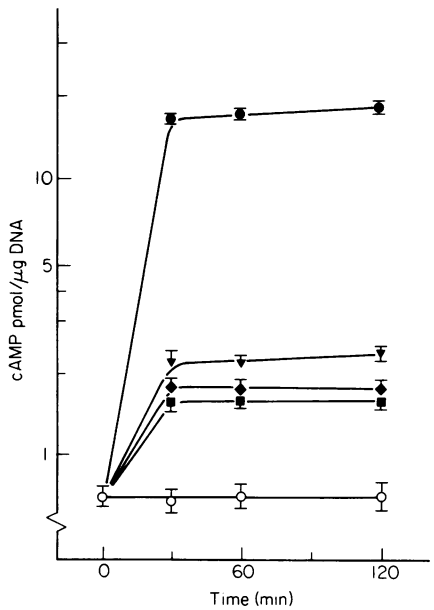

Fig. 2. Time-dependent modifications of intracellular cAMP levels (pmol/μg DNA $\pm$s.e.m.) in normal human cultured cells after the addition of different amounts of Graves' IgG (■——■: 0·05 mg/ml; ◆——◆: 0·5 mg/ml and ▼——▼: 2 mg/ml) in comparison with the time course of a large dose of bovine TSH (●——●: 2·5 mU/ml)

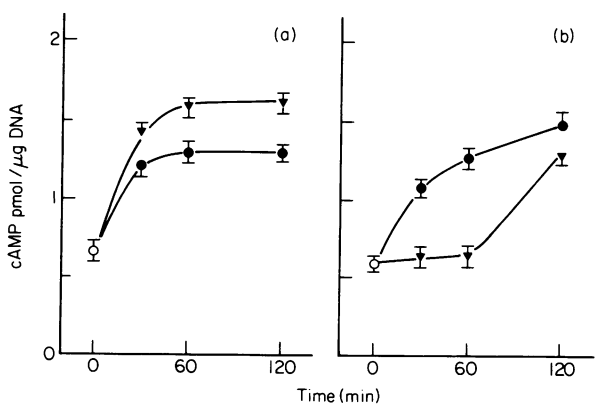

Fig. 3. Time-dependent modifications of intracellular cAMP levels (pmol/μg DNA $\pm$s.e.m.) in normal human cultured cells produced by 0·5 mg/ml (●——●), 5·0 mg/ml (▼——▼) of Graves' disease. (a) Case 7, (b) Case 6. The responses of cases Nos 3, 5, 8 and 11 were similar to that of Case 7. Open circles indicate controls.

Table 1. Effect of IgG prepared from serum of fourteen patients with Graves' disease on cAMP accumulation in normal human thyroid cell cultures and slices

Case No.	Sex	Age (years)	Clinical data: Exophthalmus	Dermatopathy	T3* (ng/ml)	T4† (ng/ml)	TgA‡	TM§	Increase of intracellular cAMP above basal levels: Thyroid cell cultures¶ pmol/μg DNA††	%	Thyroid slices** fmol/μg DNA‡‡	%
1	F	37	−	−	7·0	204	1:5,120	1:80	1·40 ± 0·21	103	215 ± 35	95
2	F	27	−	−	8·2	250	Neg	1:20	1·42 ± 0·18	103	15 ± 1	11
3	F	39	−	−	8·8	260	Neg	1:20	1·33 ± 0·15	71	180 ± 9	82
4	F	36	+	+	8·7	271	1:20	Neg	1·91 ± 0·41	107	68 ± 9	49
5	M	42	+	−	8·5	270	1:640	Neg	1·28 ± 0·19	89	165 ± 6	114
6	F	50	−	−	6·5	170	Neg	Neg	0·71 ± 0·03	59	73 ± 13	52
7	F	49	+	+	19·1	270	1:640	1:40	0·69 ± 0·03	48	100 ± 18	72
8	F	35	−	−	9·2	250	Neg	Neg	0·63 ± 0·04	75	230 ± 25	165
9	F	26	−	−	11·0	245	1:80	Neg	1·00 ± 0·03	62	680 ± 170	309
10	F	28	−	−	9·8	285	1:80	1:80	1·43 ± 0·11	105	180 ± 28	82
11	F	21	+	−	5·4	190	Neg	1:40	1·12 ± 0·14	82	340 ± 51	291
12	F	51	−	−	4·8	231	1:640	Neg	1·65 ± 0·22	100	136 ± 16	98
13	M	55	−	−	6·2	220	1:20	1:40	0	0	380 ± 68	174
14	F	46	+	−	5·7	218	1:320	1:20	1·22 ± 0·18	88	242 ± 36	110

* Determined by radioimmunoassay (normal values: 1·0–2·8 ng/ml).
† Determined by radioimmunoassay (normal values: 40–120 ng/ml).
‡ Anti-thyroglobulin antibody titre.
§ Thyroid anti-microsomal antibody titre.
¶ Incubated for 60 min with IgG (0·5 mg/ml).
** Incubated for 120 min with IgG (10·0 mg/ml).
†† Mean (± s.e.m) value of four cAMP concentration wells.
‡‡ Mean (± s.e.m.) value of cAMP assay duplicates of slice incubation triplicates.

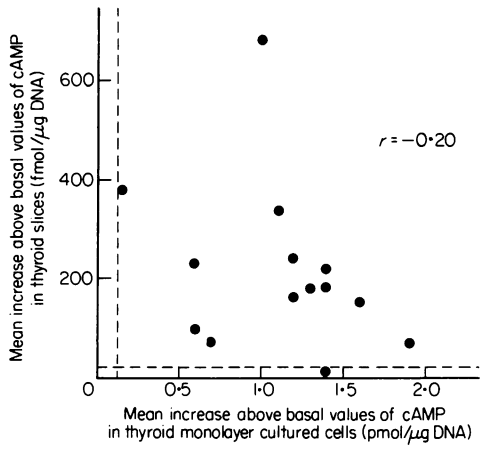

Fig. 4. Correlation according to Spearman's rank coefficient between increase above basal values of cAMP intracellular accumulation in normal human thyroid cultured cells and thyroid slices. Broken line shows upper values of the increase produced by normal IgG.

dose-dependent increase of cAMP intracellular levels was observed. As for the lower doses, high Graves' IgG concentrations did not produce further enhancement of cAMP levels after a longer incubation (Fig. 3a). In the sixth patient (Case 6), while a time-dependent increase using lower IgG doses was observed, high concentrations succeeded in increasing cAMP levels only after 120 min incubation (Fig. 3b).

On the other hand, IgG purified from sera of ten controls and tested at doses ranging from 0·05 to 5·0 mg/ml failed to produce any significant increase of intracellular cAMP accumulation in human cultured thyroid cells. In fact, the increase above basal levels was never more than 0·1 pmol cAMP/μg DNA in cell cultures or more than 21 fmol cAMP/μg DNA in the slices assay.

When all IgG preparations obtained from fourteen patients affected by Graves' disease were tested on cultured thyroid cells, at a concentration of 0·5 mg/ml and for an incubation time of 60 min for a maximal response condition, an increase of cAMP higher than 2 s.d. of controls was observed in thirteen cases (93%) (Table 1). When the same preparations were tested on thyroid slices, using 10 mg/ml of Graves' IgG and an incubation time of 2 hr according to McKenzie & Zakarija (1976), thirteen out of fourteen cases showed an increase of cAMP more than 2 s.d. of controls (Table 1). In both cases no effect on cAMP intracellular accumulation was observed even when a higher IgG concentration and a 120-min incubation were used (i.e. up to 5 mg/ml in cultured cells and 20 mg/ml in slices). It is important to note that the unresponsive IgG in thyroid slices (Case 2) was responsive in cultured thyroid cells, while unresponsive IgG (Case 13) was responsive in the other system. Therefore, detectable TSAb was present in all fourteen patients in our series with untreated frank Graves' disease. However, the increase above cAMP basal levels induced by Graves' IgG in cultured thyroid cells was higher and with a wider range when compared to that produced in thyroid slices. No significant correlation ($r=0{\cdot}20$) was found comparing the increase above cAMP basal values obtained in the two systems (Fig. 4).

When the presence of TSAb in sera of Graves' patients was correlated to other immunological parameters such as anti-microsomal autoantibodies (TMA), no apparent correlation was found. In fact, none of the patients with high TSAb had a high TMA titre, and some of them had no detectable TMA (Table 1).

DISCUSSION

Normal human thyroid cells, prepared and cultured according to the present method, show a good sensitivity to bovine TSH, when measured as cAMP intracellular accumulation. The rapid increase

of cAMP levels after TSH exposure also occurs if the culture medium is not supplemented with an additional dose of bTSH, differently from that observed in dog thyroid cell culture by Wadeleux *et al.* (1978). This sharp sensitivity to TSH permitted us to study the TSH-like activity of human IgG in Graves' disease.

In fact, IgG prepared from patients with Graves' disease is able to stimulate the adenylate cyclase system of human thyroid cell cultures prepared with the present method, while no effect is elicited by normal IgG. Using very low concentrations of Graves' IgG a significant increase of basal cAMP intracellular levels is found and the response is generally dose-dependent. However, as observed in one case of our series, high IgG concentration can exert an independent dose–response only after a longer incubation period. Such behaviour could be due to the simultaneous presence in the Graves' IgG of TSAb and of blocking antibodies whose effect may appear when the IgG concentration increases. However, as the complement is absent in the system used by us, the presence of cytototoxic antibodies against thyroid cultured cells described by Pulvertaft *et al.* (1959) can not be considered.

By comparing the effect of Graves' IgG on the adenylate cyclase system of cultured thyroid cells and that of thyroid slices, cAMP intracellular increases above basal levels are noted to be higher in slices if considered as a percentage increase. However, our results show that cultured cells are able to detect the presence of TSAb also at very low IgG concentrations. In both systems the same number of responsive cases is obtained, but when the two assays are considered together, TSAb was demonstrable in all our patients. The absence of response found in one case on each assay could be due to the presence of an IgG different from TSAb which interferes at different levels in the two systems, as suggested by McKenzie, Zakarija & Sato (1978). The dissociation of response using different assays in different cases seems to exclude the possibility of an autologous specificity, but more likely may be due to different dose–response curves in thyroid cell cultures of single IgG preparations.

In conclusion, thyroid cultured cells appear to be a more sensitive substrate to test TSAb than other systems previously used. TSAb is detectable in almost all patients with frank Graves' disease studied by us, even if it is possible that IgG, not pertaining to TSAb, reacts to the thyroid cell surface without eliciting a stimulatory action but interacting with TSAb itself (Toccafondi *et al.*, 1979).

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REFERENCES

ADAMS, D.D. (1958) The presence of an abnormal thyroid-stimulating hormone in the serum of some thyrotoxic patients. *J. Clin. Endocrinol. Metab.* **18,** 699.

ADAMS, D.D. & PURVES, H.D. (1956) Abnormal responses in the assay of thyrotropin. *Proc. Univ. Otago Sch. Med.* **34,** 11.

BAUMSTARK, J.S., LAFFIN, R.J. & BARDAWIL, W.A. (1964) A preparative method for the separation of 7S gamma globulin from human serum. *Arch. Biochem. Biophys.* **108,** 514.

BROWN, B.L., ALBANO, J.D.M., EKINS, R.P., SGHERZI, A.M. & TAMPION, W. (1971) A simple and sensitive saturation assay method for the measurement of adenosine 3′, 5′-cyclic monophosphate. *J. Biochem.* **121,** 561.

KISSANE, J.M. & ROBINS, E. (1958) The fluorometric measurement of deoxyribonucleic acid in animal tissue with special reference to the central nervous system. *J. Biol. Chem.* **233,** 184.

KNIGHT, A. & ADAMS, D.D. (1973) Infusion of LATS protector and LATS into monkeys. *Proc. Univ. Otago Sch. Med.* **51,** 11.

KRISS, J.P., PLESHAKOV, V. & CHIEN, J.R. (1964) Isolation and identification of the long-acting thyroid stimulator and its relation to hyperthyroidism and circumscribed pretibial myxedema. *J. Clin. Endocrinol. Metab.* **24,** 1005.

MCKENZIE, J.M. (1958) Delayed response to serum from thyrotoxic patients. *Endocrinology*, **62,** 865.

MCKENZIE, J.M. & ZAKARIJA, M. (1976) A reconsideration of a thyroid-stimulating immunoglobulin as the cause of hyperthyroidism in Graves' disease. *J. Clin. Endocrinol. Metab.* **42,** 778.

MCKENZIE, J.M., ZAKARIJA, M. & SATO, A. (1978) Humoral immunity in Graves' disease. *Clinics in Endocrinology and Metabolism* (ed. by R. Volpè), Vol. 7, p. 31. W. B. Saunders, Philadelphia.

ONAYA, T., KOTANI, M., YAMADA, T. & OCHI, Y. (1973) New *in vitro* tests to detect the thyroid stimulator in sera from hyperthyroid patients by measuring colloid droplet formation and cyclic AMP in human thyroid slices. *J. Clin. Endocrinol. Metab.* **36,** 856.

ORGIAZZI, J., WILLIAMS, D.E., CHOPRA, I.J. & SOLOMAN, D.H. (1976) Human thyroid adenyl cyclase

stimulating activity in immunoglobulin G of patients with Graves' disease. *J. Clin. Endocrinol. Metab.* **42,** 341.

PULVERTAFT, R.J.V., DONIACH, D., ROITT. I.M. & HUDSON, R.V. (1959) Cytotoxic effects of Hashimoto serum on human thyroid cells in tissue culture. *Lancet*, **ii,** 214.

TANINI, A., ATERINI, S., FANI, P., ROTELLA, C.M. & TOCCAFONDI, R. (1978) Effects of prostaglandin E_2 and TSH on adenylate cyclase system of intact human thyroid cells. *Prostaglandins*, **5,** 749.

TOCCAFONDI, R.S., ROTELLA, C.M., MEDICI, M.A., BARTALENA, L., CHIOVATO, L., MARCOCCI, C., FENZI, G.F. & PINCHERA, A. (1979) Binding of radioiodinated autoantibodies to intact human thyroid cells: evidence for cell surface antigens. *Symposium on Autoimmune Aspects of Endocrine Disorders* (ed. by A. Pinchera and D. Doniach). Academic Press, London. (In press.)

WADELEUX, P.A., ETIENNE-DECERF, J., WINAND, R.J. & KOHN, L.D. (1978) Effects of thyrotropin on iodine metabolism of dog thyroid cells in tissue culture. *Endocrinology*, **102,** 889.