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Thyroid growth stimulating activity in highly purified IgG-fractions of patients with nonimmune thyroid diseases[1]

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ABSTRACT. Two different proliferation assays have been used to measure the proliferative potential of IgG-fractions from 57 patients with nontoxic goiter of an iodine-deficient area: primary human thyroid epithelial cells (TEC) and the thouroughly investigated FRTL-5 cell line. IgG-fractions from patients with nontoxic goiter (n = 30), nontoxic recurrent goiter (n = 8), toxic-nodular goiter (n = 15) and carcinoma of the thyroid (n = 4) were highly purified on DEAE-Sepharose and additionaly Protein A-Sepharose in some cases. The two proliferation assays gave contradictory results: primary cultures of human thyroid epithelial cells (TEC) could not be stimulated by any of the patient's IgG-fractions nor by bTSH. The FRTL-5 cells, however, were stimulated with 10 μU/ml bTSH by 326% $\pm$ 96% (range: 222% - 497%, $p < 0.001$). In one experimental series, 72% of all patients exceeded mean + 2 SD of normal controls, when the stimulation index was referred to the effect of bTSH (NTG: 77%, Rec. G.: 88%, Tox. G.: 53%, Ca. thyroid: 75%). With a different method of calculation - stimulation index referred to the basal value - the number of patients above mean + 2 SD of normal controls decreased to 30% (NTG: 33%, Rec. G.: 12,5%, Tox. G.: 33%, Ca. thyroid: 25%). Statistical analysis, however, of results of different patient groups compared to the normal control group failed to show any significance.

INTRODUCTION

When growth stimulating activity on thyroid cells *in vitro* was first described by Drexhage in 1980 (1) and ascribed to immunoglobulin G action (TGI), an intriguing concept to explain goiter formation was created. TGI has been reported not only in autoimmune thyroid diseases such as Graves' disease (GD), but also in patients with nontoxic (euthyroid) goiter (2-4). This concept would unify almost all thyroid diseases in an autoimmune etiology. The existence, however, of this growth stimulating immunoglobulin in patients with nonimmune thyroid diseases is still a matter of controversy. A variety of assay systems have been applied to measure TGI, but results have not been consistent (Table 1). This is in part due to different sources of thyroid cells and might reflect different growth responses in diverse *in vitro* systems. Regulation of cell growth has been shown to be a highly complex matter in both human and animal thyroid cells (14-23). Furthermore, Gärtner showed that the thyroid growth stimulating activity of crude IgG-fractions prepared by ammonium sulfate precipitation was due to the contamination with epidermal growth factor (EGF) and could be abolished by subsequent purification with DEAE- and Protein A-Sepharose (24). Moreover, TGI has not yet been convincingly detected with assay systems using human thyroid cells, as shown by Wenzel (25).

Our interest was to reassess the suggested role of TGI in nonimmune goitrous patients from an iodine deficient area. We used FTRL-5 cells for the measurement of the proliferative potential of IgG-fractions (26). Results in this animal system were compared to a system using human thyroid epithelial

[1] Abbreviations used:
AC-activity, activity of the adenylate-cyclase; bTSH, bovine thyroidea stimulating hormone; Ca. thyroid, Carcinoma of thyroid; FRTL-5 cells, Fischer's rat thyroid cell line 5; GD, Graves' disease; ^{3}H-TdR, ^{3}H-Thymidine; IBMX, 3-isobutyl-1-methyl-xanthine; MAB, microsomal antibody; NTG, non-toxic goitre; PBS, phosphate buffered saline; Rec. goiter, recurrent goiter; SD, standard deviation; SI, stimulation index; TAB, thyroglobulin antibody; TBII, TSH-binding inhibiting immunoglobulin; TGI, thyroid growth stimulating immunoglobulin; Tox. goiter, toxic goiter, TSI, thyroid stimulating immunoglobulin.

Key-words: Non-immune thyroid disease, thyroid growth stimulating antibody (TGI), FRTL-5 cells, human thyroid epithelial cells (TEC).

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cells (TEC) to account for species-specific differences of growth control. Furthermore we studied the stimulation of the second messenger cAMP in a TSI assay. Thereby we looked closely at our methods to identify possible pitfalls in the measurement of TGI activity in clinical studies as summarized by Dumont (27).

MATERIALS AND METHODS

Patient profile

IgG-fractions were collected from 57 consecutive patients with nonimmune thyroid diseases attending the thyroid clinic at the Medical University of Lübeck. Diagnosis was based on clinical assessment and confirmed by an euthyroid state and the absence of TBII. Thirty patients had a nontoxic goiter (NTG), 8 patients had a nontoxic recurrent goiter (Rec. G.), 15 had a toxic-nodular goiter (Tox. G.) and 4 patients suffered from carcinoma of thyroid (Ca. thyroid); 78% of patients were female (n = 46). Age ranged from 16-77 yr (mean: 47 yr, median: 50 yr); 23% were weakly positive for MAB (range: 1:1600 - 1:6400), 18% weakly positive for TAB (range: 1:160 - 1:1280). Goiter size was estimated clinically and measured sonographically: 47% had WHO grade I, 40% grade II and 13% grade III with symptoms due to local obstruction. The iodine-deficiency of the region is manifested by a low urinary iodine excretion of 84 μg I/g creatinine as compared to 170 μg I/g creatinine in Sweden (28).

Control group

Control sera for the normal control were obtained from 11 healthy individuals without evidence of thyroid disease, as confirmed by clinical examination, estimation of thyroid hormones and the absence of thyroid autoantibodies. IgG-fractions were treated and assayed individually. The IgG of a patient with Graves' disease who was newly diagnosed served as positive control (pos. control). She had high titers of microsomal, thyroglobulin and TSH-receptor antibodies (89% inhibition of TSH-binding). In addition IgG-fractions from 3 patients with active Graves' disease and from 5 patients with Graves' disease in remission were measured in the TSI assay.

IgG Preparation

IgG-fractions were prepared from serum by single step affinity chromatography (DEAE Affi-Gel Blue®) and followed by protein A-sepharose chromatography (Protein A-Sepharose CL-4B®) in some cases.

For DEAE Affi-Gel Blue chromatography 3 ml of serum were dialysed against 20 mM phosphate buffer (pH 8.0) for 12 h. The serum was applied to a column and the IgG-peak eluted with 20 mM phosphate buffer. The column was regenerated with 2 M Guanidine-HCl. Eluated fractions were pooled according to the protein profile monitored by UV-absorption at 280 nm and dialyzed against PBS, pH 7.4, for further 12 h.

For Protein A-Sepharose a small column was packed with 3 ml of swollen gel and equilibrated with PBS (pH 7.2). One ml of serum was diluted with 1 ml of PBS and applied to the column. First all serum components except IgG 1, 2 and 4 were eluted with PBS. The release of the bound IgG was performed by an elution with acetic acid (pH 3.0).

Table 1 - *Summary of the results of* in vitro *measurements of TGI in the serum of patients with Graves' disease, nontoxic goiter and recurrent goiter by different research groups. Percentage of TGI-positive patients are given in brackets.*

Graves' disease	Nontoxic goiter	Recurrent goiter	Authors
9/11 (82%)	4/10(40%)		Drexhage HA. 1982 (5)
16/43 (37%)	1/40 (3%)	3/9 (33%)	Chiovato L. 1983 (6)
17/27 (63%)	0/7 (0%)		Valente WA. 1983 (2)
0/10 (0%)	0/10 (0%)		Risdall J.E. 1984 (7)
	43/62 (69%)		Drexhage H.A. 1986 (8)
	22/36 (61%)		Smyth P.P.A. 1986 (9)
	51/91 (56%)		Rotella C.M. 1986 (10)
	20/72 (27%)	10/26 (38%)	Schalz H. 1986 (4)
	33/52 (63%)		Halpern A. 1986 (11)
	0/10 (0%)		Gartner R. 1986 (12)
3/19 (16%)	4/15 (27%)		Goretzki P.E. 1987 (13)

Eluated fractions were pooled according to the protein profile. They were neutralized and desalted immediately by a second run through a Sephadex G-25® column with Tris buffer (pH 7.4). The Protein A-Sepharose column was regenerated by elution with Tris buffer, sodium acetate and reequilibrated with PBS.

Finally IgG fractions were concentrated by dry dialysis against Polyethylenglycol (MW 20,000) and IgG content was measured by radial immunodiffusion using a commercially available kit. They were stored in aliquots in liquid nitrogen until use. At time of experiment all IgG were diluted in assay medium to a standard concentration of 0.1 mg/ml and finally sterilized by passage through a Millipore 22-μm filter. This method of purification yielded a highly purified IgGs as was demonstrated by immunoelectrophoresis studies.

FRTL-5 cells

Stock culture: FRTL-5 cells were obtained from A. Pinchera, Pisa. They are a continuously growing, expanded normal rat-thyroid cell strain derived from the fisher rat thyroid cell line, which is dependent on TSH. The cells have been in culture in our laboratory since 1985 and still exhibit the characteristic growth pattern of FRTL-5 cells: withdrawal of TSH results in a resting phase without evidence of cell damage. Proliferation is restored upon readdition of TSH. Cells are cultured according to the methods of Ambesi-Impiombato (26). Briefly, cells are grown in a humidified atmosphere of 5% CO_2/95% air at 37 C in Coon's modified F-12 medium supplemented with: 5% calf serum, glutamin (2 mmol/l), steptomycin-penicillin (100 mg/1-10^5 U/l), 33 ml/l $NaHCO_3$- (7.4%), insulin (10 μg/l) cortisol (0,4 μg/l), transferrin (5μg/l), glycyl-L-histidyl-L-lysin acetat (10 ng/l), somatostatin (10 ng/l) ("5H-culture medium) and 1 mU/ml bTSH (6H-culture medium). They are seeded into culture flasks (4 x 10^6 cells/flask) and split weekly in a ratio of 1:3 (approximate doubling time: 30 h). Medium is changed twice weekly. On this occasion morphology of cells is assessed. In regular intervals the medium is checked for contaminations with mycoplasma.

For the proliferation assay FRTL-5 cells were seeded into 96-well flat bottom microtiter plates (2 x 10^4 cells/well), for the TSI assay into 24-well plates (2 x 10^5 cells/well). They were grown in 6H-medium for 3 to 5 days (80%-confluency), when they were switched to 5H-medium and maintained in this resting phase for 7 to 10 days.

Human thyroid epithelial cells (TEC)

Short term cultures of TEC were established from thyroid tissue obtained at operation from patients with GD or NTG. Cells were cultured as previoulsy described (25, 29). In brief, thyroid tissue was minced, washed and digested enzymatically with Dispase II® (2.4 U/ml) two times for 90 min at 37 C. Cells were washed, separated from debris and erythrocytes by density centrifugation, washed again and plated in Iscove medium containing the 5H-mixture described above, glutamine (2 mmol/l), antibiotics and 2.5% fetal calf serum ("Iscove culture medium").

For the proliferation assay TEC were either seeded into 96-well plates (5 x 10^4 cells/well) to obtain primary cultures or grown in culture flasks first and passaged once or twice before seeded into 96-well plates. Cells adhered to the flat bottom in semifollicle-like structures ("domes") displaying "right-side-out" polarity. The differentiated function of TEC was verified by their ability to secrete thyroglobulin. After 3 days cultures were used for experiments. For the TSI assay 10^5 cells/well were seeded into 24-well plates and used after 3 days.

Proliferation assay

Assay protocols for both cell systems, FRTL-5 cells and TEC, were comparable. At time of experiment cells were washed and medium was replaced by medium + dissolved stimulators ("5H culture medium" for FRTL-5 cells and "Iscove culture medium" for TEC as described above). Incubations were carried out in quadruplicates (final volume 200 μl/well for both systems). In preliminary dose-finding experiments FRTL-5 cells were incubated with different doses of bTSH (range: 0.1μU/ml - 1000 mU/ml) or IgG from the positive control (range: 0.05 mg/ml - 2.0 mg/ml). In further experiments FRTL-5 cells on each plate were incubated with medium only (basal value), with bTSH (0.01 mU/ml) or other stimulators such as EGF or IGF-1. All IgGs, i.e. pos. IgG, control IgG and unknown IgG samples, were used at a final concentration of 0.1 mg/ml. 10 μl of ^{3}H-Thymidine was added to each well to a final concentration of 0.5 μCi/well. After 72 h of incubation cultures were trypsinized and harvested with use of a semiautomatic cell harvester (washing sequence: distilled water, TCA and alcohol). Radioactivity incorporated into DNA of cells (rinsed

onto cellulose acetate filter disks) was counted in a liquid scintillation counter. The proliferative effect of bTSH or IgG was routinely evaluated by calculating a stimulation index I (SI) as follows:
cpm (sample)/ cpm (basal) x 100 = SI in %.
With a different calculation method the IgG-effect on the ^{3}H-Thymidine incorporation into cells was referred to the bTSH effect and a further stimulation index II was calculated as follows:
cpm (sample)/ cpm (bTSH) x 100 = SI in %.

TSI assay

For the TSI assay the stimulators (bTSH, IgG) were dissolved in "5H culture medium" for FRTL-5 cells and "Iscove culture medium" for TEC, both supplemented with 0.5 mmol/l IBMX. IgGs were measured at a standard concentration of 0.1 mg/ml. Incubations were performed in duplicate wells (200 μl/well). After 24 h at 37 C the supernatant was taken of the cells. Twenty μl of 1 M Hepes-buffer was added to the supernatant and samples were stored at -70 C. For the time course the supernatants were taken of the cells at time intervals indicated. Extracellular cAMP content was measured in duplicates with a commercially available competitive protein binding assay kit using 8-(3H) cAMP as tracer (detection range: 0.2-16 pmol cAMP/50μl; between-assay variation: 7.3%). Separation of protein-bound from free cAMP was performed by charcoal absorption. An aliquot of the protein-bound fraction was counted in a liquid scintillation counter. Results were expressed as pmol cAMP / well (mean of duplicate wells) or stimulation indices were calculated as pmol cAMP (sample)/ pmol cAMP (basal) x 100 = SI in %.

Statistical evaluation

Results of normal controls were checked for normal distribution, which proved to be the case for both proliferation and TSI assay. The distribution of TGI and TSI activities of control and patient groups was evaluated by the U-test of Mann and Whitney for unpaired data and the Wilcoxon rank test for paired data. The level of significance was chosen as $p < 0.05$.

Reagents

TRAK-assay was purchased form Henning Berlin, FRG. Deae Affi-Gel Blue was obtained from Bio-Rad Laboratories GmbH, München FRG, and Protein A Sepharose CL-4B from Pharmacia Laboratories, Uppsala, Sweden. LC-Partigen-IgG and protein Standard-Serum LC-V were from the Behring Insituts, Frankfurt, FRG. Dispase II was from Boehringer, Mannheim. FRG. Coon's modified F-12 medium was purchased from Gibco, Paisley, UK. Insulin was obtained from Hoechst, Frankfurt, FRG, bTSH (Thyreostimulin) from Organon, München, FRG. Somatostatin, hydrocortisone, transferrin, glycyl-histidyl-l-lysin-acetate and IBMX were all purchased from Sigma Chemie GmbH, Deisenhofen, FRG. Iscove medium, IGF-1 and calf serum were from Biochrom KG, Berlin, FRG. EGF was purchased from Genzyme, München, FRG. (Methyl-^{3}H)-Thymidine was obtained from New England Nuclear, NEN Research Products, Dreieich, FRG. The cAMP protein binding assay kit was purchased from Amersham Buchler, Braunschweig, FRG. A semi-automatic cell harvester, Multimash 2000 AM 74, from Dynatech, Denkendorf, FRG and a LKB Wallac 1219 Rackbeta liquid scintillation counter from LKB Instrument GmbH, Gräfelfing, FRG were used.

RESULTS

Standardization of the proliferation assay

In an initial experiment the dose response curve of bTSH-deprived FRTL-5 cells to re-addition of bTSH was established. As shown in Figure 1 A, FRTL-5

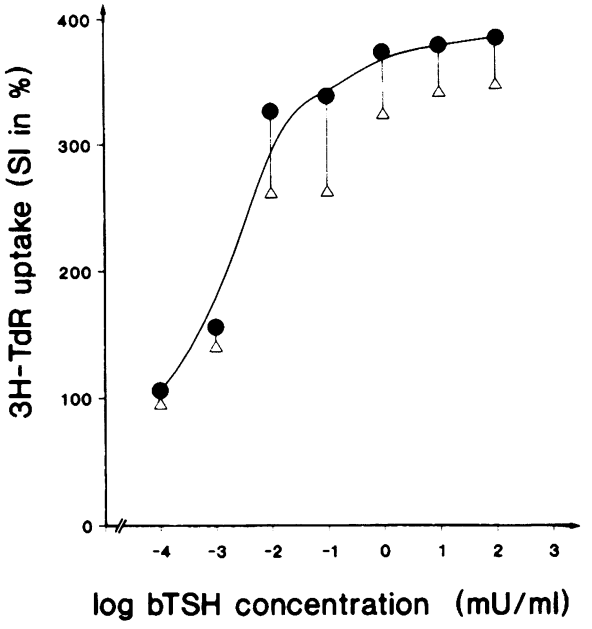

Fig. 1 (A) - *Dose response curve of FRTL-5 cells in 96-well plates stimulated with bTSH. Results are expressed as SI in % of basal Mean + SD of 6 experiments are depicted.*

stimulation with 10 μU/ml bTSH resulted in a significantly increased radioisotope incorporation: the SI (compared to the basal value = 100%) was about 300%. The dose of 1 mU/ml bTSH gave a maximal response, a plateau was reached at higher concentrations. Hence the submaximal dose of 10 μU/ml bTSH was used as the TSH control on each plate.

In a similar way the dose-response curve of FRTL-5 cells to human IgG-fractions was established. The positive control (pos. control) stimulated ^{3}H-TdR incorporation maximal at a concentration of 0.12 mg/ml (Fig. 1B). It was a less potent stimulator at 0.05 mg/ml. Concentrations exceeding 1 mg/ml, however, resulted in a variable degree of cell damage and reduced radioisotope incorporation. In contrast, IgG-fractions of healthy individuals (control IgG) did not show this bell-shaped dose response curve: ^{3}H-TdR incorporation was only slightly increased compared to basal incorporation of resting cells at all concentrations investigated (data not shown). Thus the concentration of 0.1 mg/ml was adopted as standard condition for all subsequent studies described in this report.

Furthemore the possible influence of the IgG-purification process on the FRTL-5 cell response was investigated. No significant difference was found between IgG-fractions purified with DEAE Affi-Gel Blue chromatography as compared to Protein A-Sepharose. Analysis of possible contaminations in both IgG-preparations showed only traces of TSH (< 0.22 mU/l), whereas TBII content was fully preserved (e.g. serum: 29% inhibition of TSH-binding, following DEAE Affi- Gel Blue chromatography: 37%, following Protein A-Sepharose chromatography: 28%).

In order to establish a normal range, we measured the IgG-fractions of 11 healthy controls individually at the beginning and at the end of our main experimental series, i.e. using different subcultures of cells. The SI ranged from 109% to 187% with respect to control cultures (basal value). Results showed a standard distribution (Fig. 2): mean (142%)

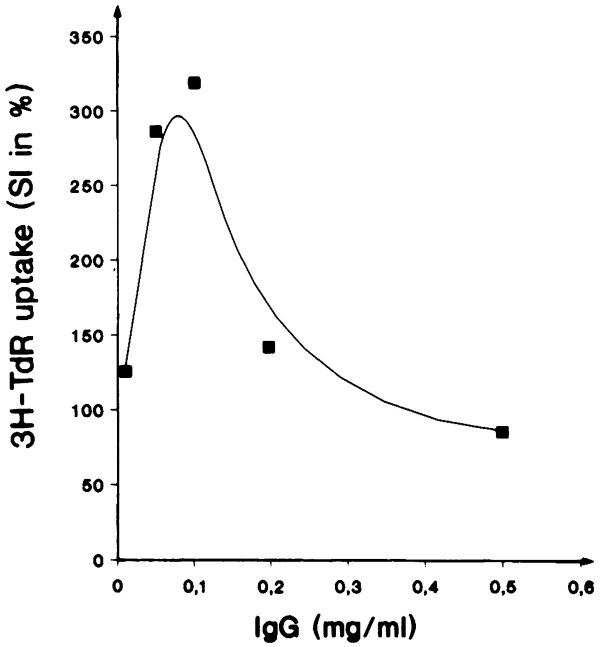

Fig. 1 (B) - *Dose reponse curve of FRTL-5 cells in 96-well plates stimulated with IgG from a patient with active Graves' disease. Results are expressed as SI in % of basal. A typical curve is shown.*

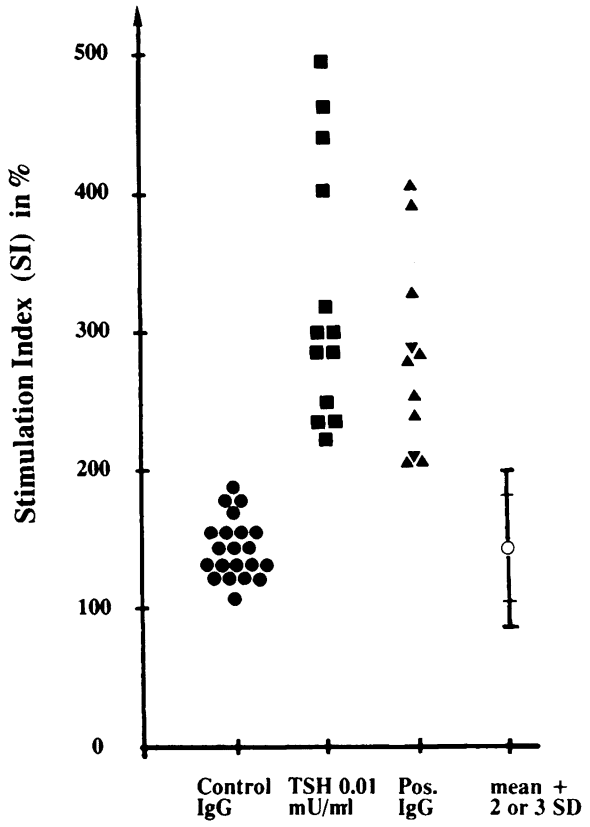

Fig. 2 - *Left: SI of IgG-fractions of normal control, measured in two separate assays. Right: SI of controls during one experimental series (n = 13 plates measured in 5 separate assays). Each symbol represents the result of bTSH and positive IgG on one plate.*

and SD (21%) was calculated. We defined the normale range as mean + 2 SD with a cut-off line of 184% (95%-percentile), and also a confidence range as mean + 3 SD (99.7%-percentile): Cut-off line was now 205%.

The results of the controls on each 96-well plate were used to assess the reproducibility of our proliferation assay. Table 2 summarizes range, mean, intra-and inter assay variation of repetitive results of two given IgG-fractions and bTSH in the standard concentration of 0.01 mU/ml during one experimental series. This series concluded thirteen 96-well plates measured in five separate assays, i.e. using five different FRTL-5 cell preparations, within six weeks. The intraassay variation (coefficient of variation: 8% to 13%) was low, whilst inter assay variation was considerably high with a coefficient of variation of almost 30%.

Application of the same standardization protocol to TEC proved to be impossible. We failed to induce a growth response of TEC towards bTSH (0.01 - 10 mU/ml) or to other growth stimulators such as EGF (10 ng/ml) or IGF-1 (10 ng/ml). None of our IgG-fractions resulted in any significant increase of ^{3}H-TdR incorporation (e.g. medium: 1410 cpm/well, pos, IgG, 1717 cpm/well). We did not achieve proliferation by use of different sources of thyroid tissue nor by subculturing cells prior to experiments. In contrast, the same batches of TEC were responsive to bTSH and IgG-fractions in respect to AC-activity measured as increase of extracellular cAMP in the TSI-assay (Fig. 3B, Table 3).

Standardization of the TSI assay

The stimulation of FRTL-5 cells grown in 24-well plates resulted in an increase of extracellular cAMP detectable after as little as 30 min. Stimulation of the AC-activity by bTSH was dose-dependent in

Table 2 - *Range, mean and median of stimulation indices (in %) during one experimental series (n = 13 plates measured in 5 separate assays).*
**Average of coefficients of variation of samples on one plate*
***Coefficient of variation of between-assay-variation* # $p < 0.001$

	Contr. IgG	pos. IgG	bTSH
Range (SI)	90 - 147#	205 - 408#	222 - 497
Mean (SI)	113	266	326
Median (SI)	110	267	299
Intraassay variation of SI*	11%	13%	8%
Interassay variation of SI**	15%	29%	29%

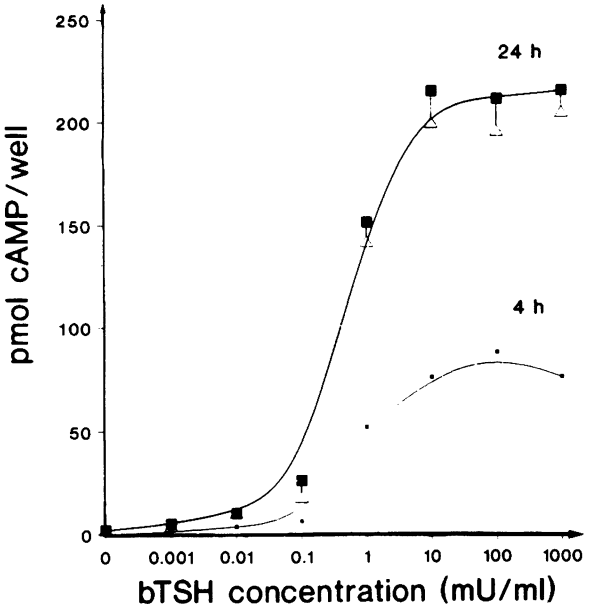

Fig. 3 (A) - *Dose-response curve of FRTL-5 cells stimulated with bTSH as measured by the increase of extracellular cAMP after 4 h and 24 h of incubation.*

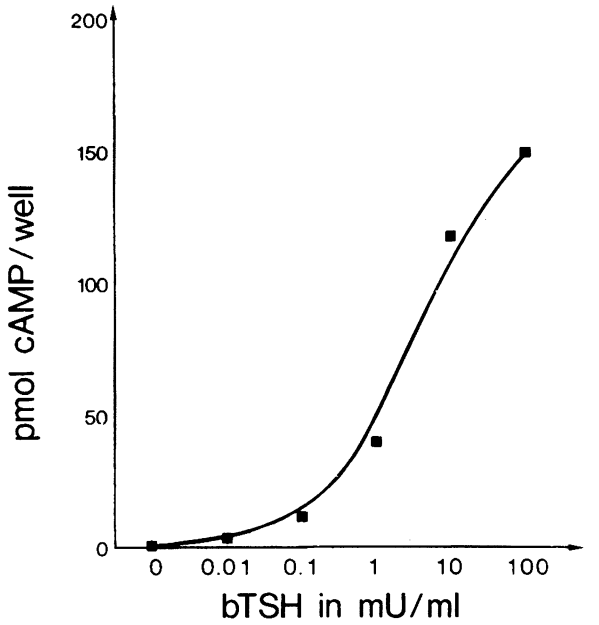

Fig. 3 (B) - *Dose-response curve of human TEC stimulated with bTSH as measured by the increase of extracellular cAMP after 24 h of incubation.*

Table 3 - *Standardization of the TSI assays with FRTL-5 cells and TEC in 24-well plates. Stimulation of AC-activity by IgG-fractions of normal control, positive control and unknown samples from patients with active GD, GD in remission and non-immune thyroid diseases. Results are given in pmol cAMP/well (mean of duplicate) or expressed as SI in % of basal.*

	FRTL-5 cells	TEC
● Controls		
Basal value		
- Range (pmol cAMP/well)	8.1-9.6	1.0-1.4
- Mean ± SD (pmol cAMP/well)	8.9 ± 1.8	1.2 ± 0.2
Control IgG (n = 11)		
- Range (pmol cAMP/well)	5.0-9.2	0.8-1.6
- Mean ± SD (pmol cAMP/well)	6.6 ± 1.8	1.3 ± 0.27
- Mean ± SD (as SI in %)	75% ± 20%	108% ± 23%
● Definition of normal range		
- Mean + 2 SD (in pmol cAMP/well)	10.2	1.84
- Mean + 2 SD (as SI in %)	115%	154%
● Patient samples (range as SI in % of basal)		
Active Graves' disease (n = 4)	44%-511%	117%-433%
Graves' disease in remission (n = 5)	33%-100%	100%-134%
Nontoxic goiter (n = 16)	50%-110%	100%-133%

FRTL-5 cells (Fig. 3A) as well as TEC (Fig. 3B). It seemed from these few experiments that TEC response did not result in a plateau, whereas in FRTL-5 cells 0.5 mU/ml bTSH yielded a maximal response. IgG-fractions exhibited a slower and less pronounced effect than bTSH, as shown in the time-course in Figure 4. IgG-fractions from the control group failed to stimulate AC-activity significantly. From these results a confidence range was calculated as given in Table 3.

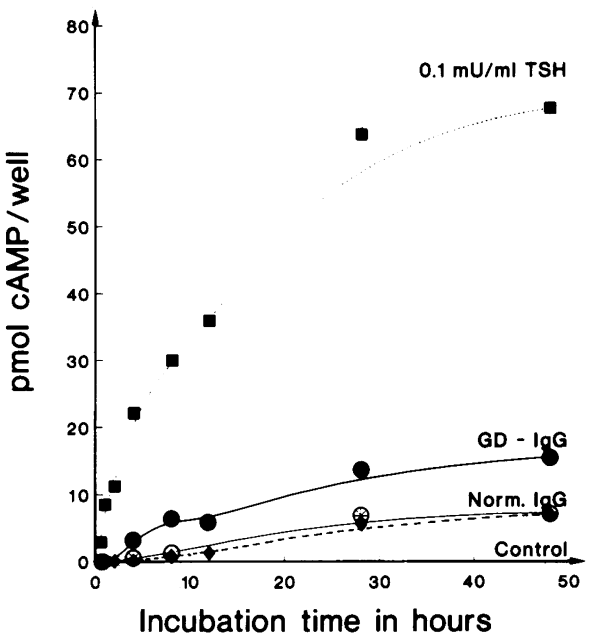

Fig. 4 - *Time course of adenylate-cyclase activation of FRTL-5 cells by bTSH and different IgG-fractions as measured by the increase of extracellular cAMP. Standard concentration of IgG was 0.1 mg/ml.*

Patient studies

All 57 samples were measured in one experimental series as described above: all samples were processed on thirteen plates in five separate assays within six weeks. The results of the patients are given graphically in Figure 5: each symbol represents the result of one patient within his group of diagnosis. The percentages of patients above the cutt-off line of the normal range are: NTG = 33%, Rec. G. = 12.5%, Tox. G. = 33% and Ca. thyroid = 25%. Marked differences of results can be achieved when the definition of the confidence range is applied. The percentages decrease to NTG = 20%, Tox G. = 20% and even Ca. thyroid = 0%.

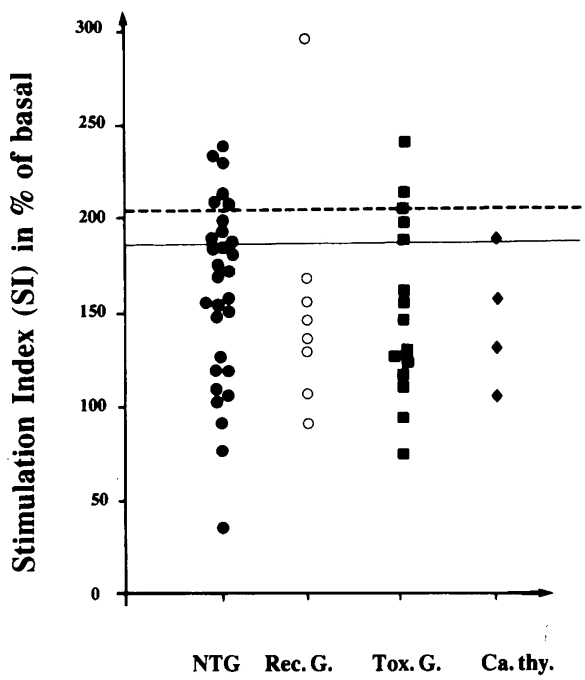

Fig. 5 - *^{3}H-Thymidine incorporation of FRTL-5 cells incubated with IgG from patients with nonimmune goitrous disease: NTG = nontoxic goiter (n= 30), Rec. G. = recurrent goiter (n = 8), Tox. G. = toxic-nodular goiter (n = 15), Ca. thyroid = Carcinoma of thyroid (n = 4). Cut-off line was defined as mean of normal controls + 2 SD (normal range: -) and mean of normal control + 3 SD (confidence range: --).*

The next step was to alter the method of evaluation completely. Results of samples were expressed as percent of bTSH-effect and the stimulation index II was calculated as described in Materials and Methods. Although the same raw data were used as before, far higher frequencies of "TGI-positive" IgGs can be achieved by this procedure (Fig. 6). Percentages exceeding mean + 3 SD are now: NTG = 73%, Rec. G. = 88%, Tox. G. = 47% and Ca. thyroid = 75%; these figures are still higher by looking at samples exceeding mean + 2 SD: NTG = 77% and Tox. G. = 53%.

In all cases, however, statistical analysis of the distribution of either patient group showed no difference from the normal control group. In contrast, the response of the pos. IgG did differ significantly from the normal control group ($p < 0.001$). Likewise statistical analysis failed to show any correlation between results of single patients in the proliferation assay and clinical data such as goiter size, level of autoantibodies, therapy or onset of disease.

Finally TSI was measured in the bioassay described above. None of the patients with nonimmune goitrous disease were found to be TSI-positive. A small group of Graves' disease patients with high titers of TBII were measured as control group in this additional assay: only patients with active disease (hyperthyroid state) could stimulate the AC-activity. The TGI-positive IgG-fraction was also TSI-positive (FRTL-5: SI = 211% of basal, TEC: SI = 433%). Two IgG-fractions from patients with Graves' disease were found to be TSI-positive without stimulating the proliferation of FRTL-5 cells (SI in the TSI assay with FRTL-5 cells: 144% and 511% respectively, SI in the proliferation assay with FRTL-5 cells: 129% and 118% respectively). The contrary, however, TGI-positive but TSI-negative IgG-fractions were not encountered in this limited study. Results obtained in the TSI-assay using human TEC confirmed the results of the FRTL-5 cell assay.

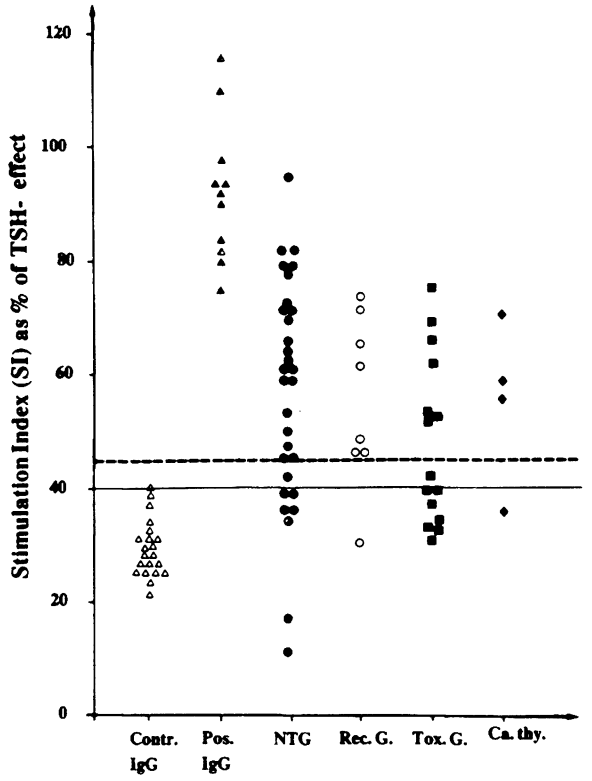

Fig. 6 - ^{3}H-Thymidine uptake of FRTL-5 cells incubated with IgG from patients with non-immune goitrous thyroid diseases. SI is now related to the TSH-effect in the proliferation assay as described in Materials and Methods (normal range: —— confidence range - - -).

DISCUSSION

In our study we intended to establish a reliable assay system to assess proliferative potential of human IgG-fractions from patients with nonimmune goitrous diseases. In contrast to most studies we used highly purified IgG-fractions which were virtually free of potientially contaminating growth factors. We could confirm that this way of purification does not abolish biological activity of IgG, since IgG-fractions from GD-patients were still capable of stimulating the adenylate-cyclase activity of FRTL-5 cells as well as TEC. Our positive control, too, was able to stimulate ^{3}H-Thymidine incorporation of FRTL-5 cells to a degree which was comparable to the effect of 10 μU/ml bTSH.

We could clearly demonstrate that the method of data calculation alters the outcome of the study. Using the same raw data but changing the reference point, we got 0/4 TGI-positive patients with carcinoma of the thyroid (Fig. 5) as well as 3/4 (Fig. 6)! Further studies showed that TSH - and IgG-effect have different kinetics (Fig. 4) and TSH should therefore not be used as reference. This is very consistent with a study of Bidey (30). In a TSI-assay using primary monolayer cultures of human thyroid cells 3 of 23 untreated patients with active Graves' disease were shown to be TSI-negative when their IgG was initially tested. With a different cell prep-

aration these IgG-fractions induced a significant stimulation of intracellular cAMP accumulation. TSH, however, stimulated the AC-activity in both cell preparations by more than 300% of basal. Bidey concluded that there may be no consistent correlation between TSH and TSI-responsiveness.

Another source of divergent results of patient studies is the need to draw a reliable cut-off line to label IgG-fractions as being TGI-negative or TGI-positive. In most studies the standard definition of "upper limit of normal range" is employed, i.e. mean + 2 SD, but Goretzki (13) f.ex. uses 99.9% cut-off line (mean + 5 SD). We decided to apply two different operational definitions (mean + 2 SD, mean + 3 SD) to illustrate the effect on the results of our study. Further more, there are different ways of measuring normal control IgG-fractions: in many studies IgG-fractions are pooled prior to incubation or even prior to purification procedures. This leads to idiotype-antiidiotype complexing and makes biological activities incompatible (33). In consequence an IgG-preparation is obtained with a comparably narrow range of results in consecutive proliferation assays which itself results in a relatively low upper limit of normal range and hence a high proportion of "TGI-positive" samples.

Furthermore, looking at quality criteria of an experimental system like intra- and interassay variation our FRTL-5 cell strain unfortunately showed a high variability between subcultures even within one experimental series, expressed by an inter assay variation of 29%. Although most studies do not quote the figures of between-assay-variability, it is well known to be a problem. Bidey and Marshall looked at this general problem of bioassays. They evaluated both human thyroid cells in primary culture (30, 31), and FRTL-5 cells (32) with a particular interest in precision characteristics. The interassay variation of the cAMP-response of seven FRTL-5 subcultures to TSH was 25%. This is similar to our finding in 14 FRTL-5 subcultures (29%). This result was considered to be in marked contrast to equivalent data for primary cultures of human thyroid cells, when an interassay variation of over 70% was observed.

There are a number of theoretical reasons, why this high variability should exist:

— IgG-fractions could gradually loose biological activity. This is unlikely, because there was no systematic decline in the proliferative potential of positive and normal control during our experimental series.

— SI of cells show a mirror-image dependency on basal ^{3}H-Thymidine incorporation (not published). This basal incorporation itself could be influenced by a different number of cells per well. This is unlikely, because high precision pipettes are used in laboratories. We could not, however, rule this out, since our method with use of a semiautomatic cell harvester makes DNA assays or cell counting impossible.

— The FRTL-5 cell strain is a mixture of different subcultures of cells. it is known, that long term cultures *in vitro* change their genotype or de-differentiate (15). This could lead to differences in receptor density for TSH or IgG or in different cell metabolism rates thus influencing basal ^{3}H-Thymidine incorporation.

We favor the latter, but did not look at this topic in detail. Further studies need to be conducted to look closer at the cells on a biochemical level.

The application of FRTL-5 cells in proliferation assays is further limited by what looks like species-specific control mechanism of proliferation. We confirmed once again (25, 29, 34) that TSH is not a growth factor for human TEC in monolayer culture. Recent studies suggest an involvement of EGF, insulin and insulin-like growth factor I (34), but the exact mechanisms of proliferation control are not yet known (35). Our human cell system was not responsive to EGF which is in contrast to other studies (13, 36). One explanation could be that EGF was unable to bind to its receptor since human TEC grow as a monolayer. Westemark, however, demonstrated recently that human thyroid cells possess EGF-receptors which were found to be located asymmetrically at the basal cell surface (35). Roger investigated the influence of different serum content of culture media on responsiveness of cells towards TSH and EGF (36). Proliferation of human thyroid cells could be stimulated by TSH only when cells were grown in insulin-supplemented low serum (1%) or even under nonphysiological serum-free conditions.

The growth regulation in FRTL-5 cells is obviously different from human thyroid cells *in vitro* and results may lead to erroneous conclusions (27): bTSH stimulates growth of FRTL-5 cells and cAMP is at least one mediator for that action. In human TEC, bTSH does not influence growth but increases activity of the adenylate-cyclase (25). Hence, TSI could be a growth promotor in the FRTL-5 cell assay acting via increased production of cAMP, whilst this TSI would

only exhibit stimulation of function, but not of growth in the human TEC assay.

In summary we conclude that the proliferation assay can be readily used to detect substances with a pronounced stimulatory effect on FRTL-5 cells, e.g. bTSH or GD-IgG. Its use, however, for substances with a rather low potential to stimulate FRTL-5 cell proliferation as would be expected in patients with non immune thyroid disease, is limited due to high inter assay variation. This could only be circumvented by multiple measurements using different subcultures of cells and by increasing numbers of controls and patients. Standardization of the proliferation assay would help to make results of different laboratories comparable. We conclude that only IgG-fractions with a consistent growth stimulating effect should in future be labelled "TGI-positive".

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