Novel *in vitro* assay for direct quantification of stimulating TSH receptor autoantibodies in Graves‘ disease

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Introduction

We demonstrate an *in vitro* assay for direct detection of TSH receptor (TSHR) stimulating autoantibodies (Ab) causing hyperthyroidism (HT) in Graves' disease (GD, Morbus Basedow). TSHR is a seven membrane receptor coupling to the cAMP signaling cascade. It contains a large extracellular domain presenting epitopes for stimulating as well as blocking autoantibodies. TSHR plays a central role in the pathogenesis of GD.

Until now commercially available methods measure TSHR autoantibodies only indirectly by the displacement of labeled TSH from the TSHR. The competitive receptor assays determine a heterogenous pool of TSHR Ab. However, the TSH binding covers only 20 to 30 % of the epitopes for both stimulating and blocking TSHR Ab.

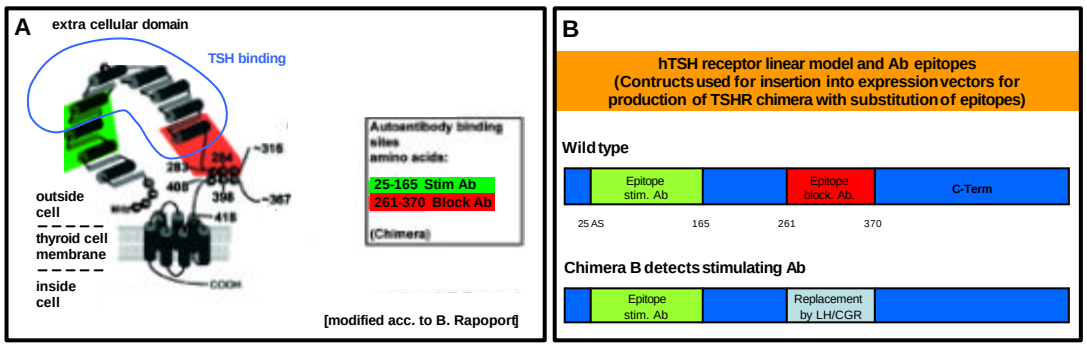

Figure 1: Model of TSH receptor illustrating epitopes for stimulating and blocking TSHR autoantibodies as well as binding of TSH to the extracellular domain (A);
Linear models of TSHR and the TSHR B chimera construct showing autoantibody epitopes (B).

Materials & Methods

Chimera of human TSHR in which the epitope for blocking TSHR Ab is replaced by neutral LH/CG receptor sequence and which at the C-terminal is fused to firefly Luciferase (Luc), was stably expressed in HEK293 cells. TSHR chimera fusion proteins were extracted from cells by triton (1%) and immunoprecipitation was tested using TSHR Ab standard (90/672), normal sera, and sera from GD patients, respectively. After incubation (3h, 4 °C) of 20 µl serum with TSHR chimera immunocomplexes were collected either by Protein G sepharose beads or Protein A Pansorbin cells and quantified by measuring Luc activity (LUMAT LB 9507, Berthold Technologies).

Results & Discussion

The range of detection was established by using the WHO standard for Thyroid-Stimulating Antibody 90/672. High sensitivity of the assay was demonstrated: the lower limit of detection was assessed below 1 IU/l. Linear correlation between relative light units (RLU) and Thyroid-Stimulating Antibody standard concentrations was shown up to 30 IU/l. Good reproducibility of values was obtained using selected patient sera for determining within-run coefficient of variation (CV, for instance 7.1 %) and between-run CV (example: 7.2 %). Reproducibility was further confirmed by running data recovery experiments of serum dilutions: selected serum shows minimal deviations (1.8–14.0 %). In active GD stimulating TSHR Ab levels correlated with disease activity, whereas controls were negative. Values obtained in GD patients by our novel assay correlate only in part with those measured by commercial competitive TSHR Ab assays. Competitive assays measure the displacement of TSH from the TSHR by all pathological Ab (both stimulating and blocking Ab) giving rise to higher values in these assays for patients with both types of Ab. Furthermore TSH binds only partially to the epitopes of both Ab types what might be the reason for lower values in competitive assays. Similar to the bioassay, we directly measure only stimulating Ab which cause HT in GD. However, compared to the bioassay our novel assay is not such time consuming and not expensive. It is planned to adapt this method of direct detection of TSHR Ab to a simple system for usage in a routine laboratory.

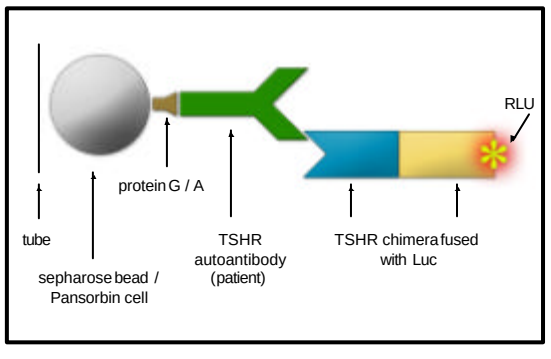

Figure 2:
Direct detection of TSHR stimulating autoantibodies: schematic representation of the assay principle
(Luc – firefly Luciferase)

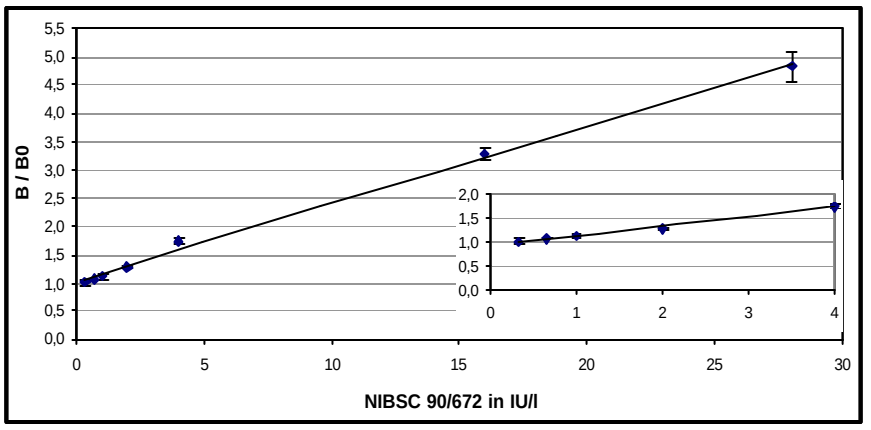

Figure 3: Representative standard curve using Pansorbin cells (protein A) and WHO standard 90/672 for immunoprecipitation of stimulating TSHR Ab. B/B0: mean RLU of each sample relating to mean RLU of zero standard (n = 2).

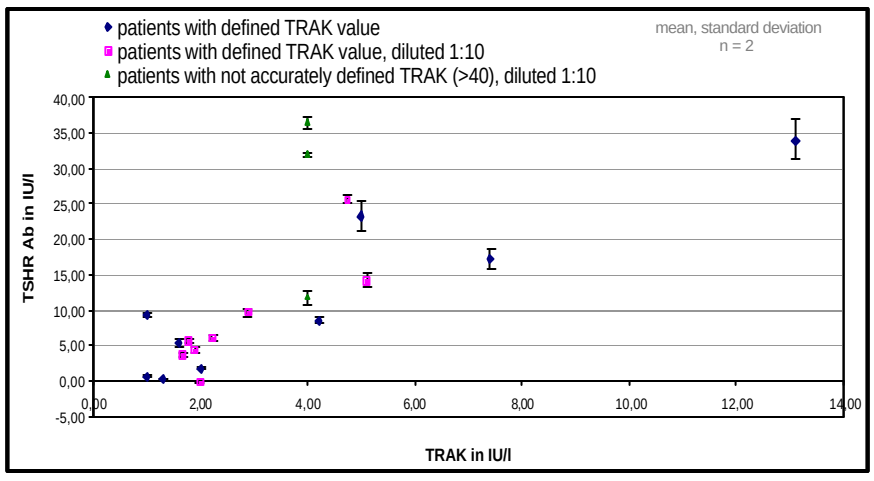

Figure 4: Comparison of direct measured stimulating TSHR Ab and values obtained by competitive receptor assay (TRAK®). Values correlate only partially. Stimulating TSHR Ab were measured by immunoprecipitation, related to zero standard (pool of negative sera) and expressed in IU/l compared to WHO standard 90/672.

sample	within-run	mean [IU / l]	% CV of rough data	% CV	runs
P 1:25		7.11	4.25	7.12	n=9
Sample	**between-run**	**mean [IU / l]**	**% CV (within-run, n=2)** range / mean CV	**% CV**	**runs**
	TRAK				
P 1	*20.1 IU/l*	51.26	0.33 – 6.54 / 2.96	7.18	n=9
P 2	*1.6 IU/l*	13.62	0.08 – 4.29 / 1.35	23.96	n=9
P 3 1:12,5	*>200 IU/l*	24.84	1.41 – 13.51 / 5.05	17.06	n=6
		S: standard	P: patient		

Figure 5: Determination of within-run and between-run coefficients of variation (CV) using selected patient sera. For between-run CV's patient samples were measured in duplicate per run.

TSHR Ab P 1 51.26 IU / l (mean, n=9)		observed TSHR Ab in IU / l	extrapolation IU / l	% mean / observed value
dilution	1	44.95	44.95	**114.0**
	1:2	26.11	52.22	**98.2**
	1:5	10.77	53.85	**95.2**
	1:10	5.45	54.50	**94.1**
	1:20	2.44	48.80	**105.0**

Figure 6: Recovery: Dilutions of a patient's sera were measured in duplicate, means were related to the WHO standard 90/672 and extrapolated to the original values. Extrapolated values were compared to a mean value of this patient's sera attained out of nine independent runs.

Literature

Kim TY, Park YJ, Park DJ, Chung HK, Kim WB, Kohn LD and Cho BY (2003). Epitope Heterogeneity of Thyroid-Stimulating Antibodies Predicts Long-Term Outcome in Graves' Patients Treated with Antithyroid Drugs. *J Clin Endocrinol Metab* **88**:117-124