INVITED COMMENTARY

Autoimmunity and thyroid growth. Where do we stand?

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This issue of European Journal of Endocrinology contains two articles which have in common the study in humans of the involvement of TSH receptor antibodies (TSH-R-ab) and T cells in goitrous conditions such as Graves’ disease and sporadic goitre.

Aust et al. (1) describe activated, i.e. HLA-DR+ T cells not only in Graves goitres but also—and in the same proportions—in goitrous conditions such as non-toxic multinodular goitre (NTG) and thyroid autonomy (TA). Forty per cent of these activated T cells show an intracellullar staining for IFN-γ, whereas half of them co-express another activation marker, i.e. CD69. Both NTG and TA are generally regarded as conditions in which the immune system is not activated (“non-autoimmune”), and hence the presence of activated T cells—although clearly in lower numbers as compared to Graves goitres—was surprising to the authors. Because the activation marker CD69 has also been implicated in tolerance induction in the thymus, the authors explain their data by assuming that the activated T cells are involved in tolerance induction (“fail-safe mechanisms”) and not in the stimulation of effector or helper T cells.

There are, however, other possibilities to explain the presence of activated T cells in “non-autoimmune” goitres, and one of them is highlighted by the report of Kashima et al. (2) in this issue. These authors describe thyrocytes in Graves’ goitres adjacent to areas of lymphocytes (focal thyroiditis; in fact these lymphocytic areas represent intrathyroidally developed lymphoid tissue (3)) that are not only expressing HLA-DR molecules but also show signs of proliferation, i.e. c-myc and PCNA expression. Kashima et al. (2) interpret their data such that in these areas of the Graves’ gland the accumulated lymphocytes act on thyrocyte proliferation via a cytokine action. The authors additionally show that there are other areas in the Graves’ gland where thyrocytes show signs of thyrocyte proliferation (c-myc and PCNA expression) in the absence of HLA-DR expression and a lymphocytic infiltration. Because similar changes (no lymphocytic infiltration, yet signs of thyrocyte proliferation) could be observed in rabbits injected with either TSH or Graves’ immunoglobulins (positive for TSH-R-ab) as well as in rabbits immunized with a TSH receptor peptide (AA 172-202), the authors hold TSH-R-ab—the serological hallmark of Graves’ disease—responsible for the observed signs of thyrocyte proliferation in the absence of adjacent lymphocytic infiltration. In their discussion they express the currently generally accepted view that these TSH-R-ab stimulate both the cell proliferation and function of the follicular cells via the action of the 2nd messenger system adenylyl cyclase.

The report of Atwa et al. (4), to be published in a forthcoming issue, shows that in cultured human thyrocytes, not only c-AMP can act as a 2nd messenger system after TSH-R-ab stimulation, but Graves’ IgGs are also capable of stimulating other 2nd messenger systems such as the phospholipase A2 and C systems. Similar data are obtained by Atwa et al. (4) using rat FRTL-5 cells.

The subject of the three studies, i.e. thyroid growth, autoimmunity and a possible involvement of TSH-R-ab triggering non-classical signal transduction pathways, has been a much debated area in thyroid research over the past 10 years (5—10).

In the early 1980s, two studies were published showing that ammonium sulphate precipitated Ig preparations (Igs) purified from Graves’ sera were capable of stimulating the Feulgen and glucose-6-phosphate dehydrogenase (G-6-PD) staining in guinea pig thyroid segments kept in organ culture (11, 12). Though staining reactions were maximal after 24 and 48 h of exposure, reactivity was already visible after 5 h. The data were taken as evidence for the existence of Graves’ Igs that could stimulate thyrocyte proliferation both in vitro as well as in vivo, on the basis of the following: similar Feulgen and G-6-PD staining reactions were found in growing tissues (13, 14); the exposure to Graves’ Igs also raised the frequency of nuclei labelled with [3H]thymidine in the guinea pig thyroid explants (11); and stimulating Igs were found in Graves’ patients with large goitres but were absent in those with impalpable goitres (11). The concept of “thyroid growth stimulating Igs” was further strengthened by the finding of Kohn’s group, of Igs in Graves’ patients that could stimulate [3H]thymidine uptake in the rat thyroid FRTL-5 cell line (15). Graves’ Igs that could stimulate thyroid hormone synthesis—the so-called TSI or TS-ab—were already known in the early 1980s for over 15 years (16). It was also known that these TSI were directed to the TSH-receptor and were able to stimulate cAMP. It was hypothesized that thyroid growth induced by the Graves’ Igs also reflected an antibody activity to the TSH receptor (11, 15), because TSH itself was able to induce Feulgen and...
G-6-PD staining in the guinea pig thyroid explants or [3H]thymidine uptake in FRTL-5 cells. Because the “thyroid Feulgen-stimulating Igs”, the “G-6-PD-stimulating Igs” and the “thyminde uptake-stimulating Igs” did not correlate in activity with the “cAMP-stimulating Igs” in the same Graves’ sera (11, 15), it was hypothesized that there were distinct sets of TSH receptor-reactive Igs, and the operational term of “thyroid growth immunoglobulins” (TGI or TG-ab) was introduced for the set of antibodies that could stimulate thyrocyte growth in the absence of CAMP stimulation. The concept was that the TGIs represented an antibody activity to another part of another immunological form (shape) of the receptor. It could, however, not be excluded that some of the Graves’ Igs induced their Feulgen or [3H]thymidine uptake-stimulating effects via other receptors relevant for thyrocyte stimulation and proliferation, such as the type 1 IGF receptor. In 1993, type 1 IGF receptor antibodies were described in Graves’ patients (17), but recent extensive attempts of our group to detect such Igs in Graves’ sera have failed after initial encouraging results (18).

Also in the early 1980s, the blocking variants of the TSH-R-ab were found in primary myxoedema (19, 20). The main messages (21) from all these observations were: the thyroid autoimmune response is polyclonal; each thyroid autoimmune patient probably produces a heterogeneous variety of TSH-R-ab of different specificities, sometimes with variable and/or opposing effects on thyrocyte 2nd messengers and thyroid function and growth; and thyroid autoimmune diseases form a broad spectrum, including Graves’ disease, Hashimoto goitre and primary myxoedema.

The “Feulgen and G-6-PD-stimulating Igs” were detected in the so-called cytotoxic biochemical assay (CBA). The CBA had been developed in the 1970s as a very sensitive method for measuring TSH (22), and the method made use of the integrated organ structure of the guinea pig thyroid. Thyrotrophin was measurable in the CBA in amounts as low as 0.1 μU/ml culture fluid. Employing the sensitive CBA, “Feulgen and G-6-PD-stimulating Igs” could surprisingly also be found in the sera of patients with “non-autoimmune” goitres, such as non-toxic (11, 23) and toxic nodular goitres (24), albeit with considerably lower activity. Smyth et al. (25, 26) (also using a CBA) had on an earlier occasion reported lysosomal naphthylamidase (LNaSe)-stimulating Igs in these goitrous conditions. Brown et al. (27) had reported low levels of TSH-R-ab in non-toxic and toxic goitre using a competition assay with improved sensitivity, i.e. with less stringent assays, viz particulate thyroid membranes without NaCl added to the buffer. Also, the polyendocrine autoimmune-prone BB rat proved to be positive for “Feulgen-stimulating Igs” (28); it should be noted that the BB rat often shows a small non-toxic colloid goitre in the absence of any lymphocytic infiltration (28).

We interpreted these observations in the sense that “a high proportion of individuals with so-called non-autoimmune, non-toxic, sporadic goitres had in fact disorders of immune regulation and self-recognition” on the level of the thyroid (23). Carefully avoided was the term “autoimmune colloid goitre” (which was unfortunately used in earlier studies (11)), in particular because we (29) and other (30, 31) had become aware that patients and animals with goitrous conditions with a definite non-autoimmune pathogenesis, viz iodine deficiency, could also be positive for “Feulgen and thymidine uptake-stimulating Igs”. Indeed, one could wonder what was the cause or consequence (“chicken or egg?”) in the association “goitre formation–immune stimulation–production of thyroid reactive antibodies”.

After initial enthusiasm, the concept of TSH-R-ab inducing goitre formation was challenged in the second half of the 1980s with the following arguments.

Firstly it was doubted whether the CBA and FRTL-5 assay systems used to detect Ig-induced thyrocyte proliferation were indices for such proliferation. In particular, Dumont et al. (5, 6) viewed the Feulgen histochemical staining of the guinea pig explants to measure at best nuclear activation, whereas the activation of G-6-PD was not seen as a (required) step in the pre-replicative chain of events leading to mitosis. With regard to assays using the uptake of [3H]thymidine in thyrocytes, these assays should be checked by unambiguous demonstrations of a mitogenic response, because false positives and negatives are possible. Reliable measurements for mitogenesis in thyrocyte culture systems would only be increases in cell number, in total DNA or in the frequency of mitoses, especially after treatment with a microtubule inhibitor (5). Obviously, the ideal subject for such assay systems would be the human thyroid in vivo (5).

Other criticisms focused on the use of not fully purified Igs, and argued that contaminating growth factors, i.e. EGF or TSH, could be responsible for the observed in vitro growth effects (7, 32). The discussion was hampered further by the controversy of TSH indeed being a growth factor for thyrocytes (33), let alone of TSH receptor agonists. A new series of experiments carried out in the late 1980s took full account of these criticisms, and the above-listed points of doubt could be fully refuted with regard to the thyrorophic effects of Graves’ Igs: protein A-purified Graves’ IgGs were capable of increasing cell numbers and frequency of mitoses both in vitro (in FRTL-5 cells (34)) as well as in vivo (in human thyroid explants in nude mice (35)). The report in this issue of European Journal of Endocrinology published by Kashima et al. (2) confirms the trophic effect of injected Graves’ IgG on the intact thyroid of the mouse, using c-myec and PCNA expression as parameters for proliferation.

After a general acceptance of Graves’ IgG as a trophic factor for thyrocytes both in vivo and in vitro, it was questioned whether the growth-stimulating effects of the IgGs were separate from the thyroid hormone-
stimulating effects of the IgGs (7). In the prevailing view, 2nd messenger systems other than cAMP could not or hardly be involved, because c-AMP was considered the most important 2nd messenger in TSH-induced thyrocyte proliferation (6, 36). Moreover, the autoantibody reaction to the TSH receptor is of restricted heterogeneity (37), and in view of this restricted number of epitopes for TSH-R-ab and the idea at the time that TSH-R-ab bound to a rather small part of the TSH receptor, i.e. the TSH binding place (38), it was too provocative to visualize the autoantibody response to the TSH receptor in Graves’ disease as rather heterogeneous, triggering more than one 2nd messenger system. Graves’ IgGs were considered to be not at all able to activate 2nd messenger systems other than cAMP in dog, rat or human cell culture systems (39).

In line with this concept, various reports using FRTL-5 cells and fetal thyroid cultures were published that definitely showed a growth effect of Graves’ IgGs, but with a—sometimes strict—parallelism between growth and cAMP accumulation-stimulating activities of Graves’ IgGs (40–42). Opposing this view were reports showing that polyclonal and monoclonal antibody preparations reacting with the TSH receptor could be devoid of cAMP—stimulating capability yet able to stimulate [3H]thymidine uptake or Feulgen staining (43–45). One of these reports (45) was a double-blind study using IgGs of well-defined Graves’ patients collected in Denmark; the IgGs were tested in a TSH receptor competition assay, a cAMP assay and the Feulgen–CBA. This study had a complex outcome: thyroid volume was correlated to Feulgen-stimulating Ig activity but also to cAMP-stimulating activity, yet Feulgen-stimulating Ig activity did not correlate to cAMP-stimulating Ig activity in the same Ig preparations; and Feulgen-stimulating IgGs were present in the serum of patients with very large goitres, while largely absent in those with small or no goitres. The message taken from this report was that the TSH-R-ab formed a heterogeneous population and that stimulating effects—amongst which was thyrocyte proliferation—could be reached via the cAMP pathway but also via other intracellular pathways. Additional Ig stimulation of these latter pathways may lead to large goitre growth (45).

In the past few years a new series of experiments has been published which might shed more light in the darkness. First of all, Dumont’s group (46) published a new report showing that Graves’ IgGs were capable of stimulating the PIP2 cascade when an assay system other than dog, rat or human thyrocytes was used, i.e. TSH-receptor-transfected Cos cells. However, a role of TSH-R-ab in the activation of the PIP2 cascade in the human thyroid in vivo was considered to be unlikely by the authors, because only very potent TSH receptor IgGs were able to induce such activation in the Cos cells and because the authors were of the opinion that in normal human and rat thyrocytes these effects could not be induced (46). The report of Atwa et al. (4) in the next issue of European Journal of Endocrinology refutes this assumption by showing a stimulation of the PLA2 and PLC system in cultured human thyrocytes with purified IgG concentrations normally also used for c-AMP stimulation (0.1–1 mg/ml culture fluid). The report of Atwa et al. (4) confirms an earlier series of studies published by Di Cerbo et al. (47, 48) and by Kosugi et al. (49, 50) regarding rat thyroid cells. These latter reports show that IgGs from Graves’ patients are able to cause a significant increase in arachidonic acid release (a product of the PLA2 cascade) and of inositol phosphate (the latter in rat TSH-receptor-transfected Cos cells). The reports of Di Cerbo et al. (47, 48) additionally show that a subpopulation of Graves’ IgGs stimulates arachidonic acid release in FRTL-5 cells without having an effect on the adenyl cyclase cascade: such IgGs are able to stimulate [3H]thymidine incorporation in FRTL-5 cells. Graves’ patients with this variety of TSH-R-ab have small goitres; patients with the largest goitres were found to possess IgGs that were capable of stimulating both the cAMP as well as the arachidonic acid pathway. The reports of Kosugi et al. (49, 50) additionally show that when alanine at position 623 in the third cytoplasmic loop of the rat TSH receptor was changed, the PIP2 signal transduction exerted by the Graves’ IgG was interrupted, while the cAMP signal was not or hardly affected.

In conclusion, the recent observations strongly suggest that TSH-R-ab form a heterogeneous polyclonal population of antibodies, binding to different portions of or with different affinities to the TSH receptor, and stimulating distinct 2nd messenger pathways. Different mixtures of these TSH-R-ab variants will lead to differences in thyrocyte hormone production and thyrocyte proliferation.

A word of caution is, however necessary: again, it must be realized that data were obtained using in vitro assay systems that are highly artificial, making use of transfected cells or rat tumour cells. The confusing results obtained in the past highlight the danger of attempting to extrapolate to the organized tissue effects found in such cell culture systems in which thyrocytes are isolated from the normal structure of the organ (51). There is, however, a report (52) on heterogeneity of stimulating Graves’ IgGs making use of integrated human thyroid tissue; this report shows that thyroid follicle count and [3H]thymidine incorporation can be stimulated independently from iodine retention and cell nuclear volume in human thyroid explants in nude mice after injection of TSH-R-ab. According to the authors, their data demonstrate that Ig-induced thyroid function and growth are independent phenomena (52) in an experimental situation that comes close to the in vivo patient situation.

A second word of caution: it is probably too simplistic to consider the TSH-R-ab as the sole factors in Graves’ goitrogenesis. This notion is highlighted by the report of
Kashima et al. (2) in this issue of *European Journal of Endocrinology*, where the authors suggest that there is a direct effect of infiltrated and activated leucocytes in Graves’ goitres on the control of thyrocyte proliferation. In this commentary it is felt that this concept might turn out to be of value in explaining the confusing and puzzling presence of thyroid-reactive antibodies in the sera of patients with goitrous conditions such as sporadic and iodine-deficient goitres.

What is the present state of affairs regarding thyroid growth-stimulating and other thyroid reactive antibodies in patients with sporadic or iodine-deficient goitre? Again the literature gives contradictory results. While some investigators were able to confirm the presence of thyroid-stimulating antibody activity in sera of patients with sporadic or iodine-deficient goitre (30, 31, 53–57) with various frequencies and using various bioassay systems) others were unable to confirm these data (58, 59). Because the positive findings mostly showed a low titre/potency of the thyroid-stimulating Igs in these goitrous conditions, the sensitivity of the assay systems employed may have played a role in the discrepant findings (10). An analogy may exist with regard to antibodies to thyroglobulin: only when ultra sensitive assays are used can antibodies to thyroglobulin be detected at higher frequency in non-toxic nodular goitre (60).

Another factor relevant for the explanation of the discrepant results may be patient selection. What is indeed the definition of the clinical entity “sporadic non-toxic goitre”? In our previous studies we have selected patients with euthyroid (normal TSH) diffuse, euthyroid nodular or toxic (TSH low) nodular goitres, irrespective of TPO or thyroglobulin antibody positivity (11, 23, 61). In fact, around 10–30% of such thyroid antibody positivity was found in our sporadic goitre patients, and it can be argued that such thyroid antibody-positive patients represent cases of mild forms of euthyroid Hashimoto’s disease. The BB rat kept on a normal iodine diet has the same thyroid phenotypical picture, i.e., euthyroidism throughout life, focal thyroiditis and anticolloid antibody positivity (62, 63). This point of a possible inclusion of patients with Hashimoto goitre when studying “non-autoimmune” goitres has also been considered in the report of Aust et al. (1) in this issue of *European Journal of Endocrinology*. In Japan, a cohort of 108 patients with non-toxic diffuse goitre without antithyroid antibodies was followed for 5–14 years; five developed Graves’ disease (64). Hence, diffuse non-toxic goitre in a small proportion of patients may also represent an early form of Graves’ disease.

Despite this difficulty in disease definition, reports are now accumulating on a stimulation of the cell-mediated immune (CMI) system in sporadic non-toxic goitres. The report of Aust et al. (1) in this issue of *European Journal of Endocrinology* on the presence of activated T cells in NTG and TA is an example of that. Recently, de Groot and collaborators reported TSH-R- and TPO-specific T cells in colloid “non-autoimmune” goitres (65, 66). A higher influx of activated dendritic (antigen-presenting) macrophages has been reported in sporadic goitre by Kabel et al. (67). Also, iodine-deficient goitres (both in humans as well as in animal models) contain higher numbers of dendritic macrophages (68, 69) and lymphocytes (31, 68). Antithyroglobulin antibodies have been reported to be produced in higher frequencies in iodine deficiency (69, 70). Although both the activation of the CMI system and the production of low titres of thyroid reactive antibodies might be consequences of preceding thyrocyte abnormalities in growth, decay and function in non-toxic goitres (60, 65, 66), immune cell activation in sporadic and endemic goitres might nevertheless play a secondary role in regulating thyrocyte proliferation and function. Indeed, various cytokines produced by activated leucocytes (2, 71) are capable of regulating the growth and function of thyrocytes. We recently showed that dendritic macrophages are also capable of down-regulating TSH-induced thyrocyte proliferation (BrDU incorporation) and function (T3 production) (to be published). Similar endocrine regulatory roles have been described for (dendritic) macrophages in the anterior pituitary (72), the ovary (73) and the testis (74).

In summary, the state of affairs is as follows:

(i) There is general agreement that IgG fractions prepared from Graves’ sera are capable of stimulating thyrocyte proliferation both in vitro as well as in vivo, and are likely to be involved in Graves’ goitrogenesis (see this issue).

(ii) There is general agreement that IgG fractions prepared from Graves’ sera are capable of stimulating 2nd messenger systems other than cAMP. There is at present more than one report that such IgG fractions are as potent in the stimulation of PLA2 and PLC pathways as they are in the stimulation of the adenylate cyclase system (see this issue).

There are recent reports (47–50) of existing Graves’ IgG fractions only stimulating the PLA2 pathway or the cAMP pathway; both can be found in patients with Graves’ goitre and both are capable of inducing in vitro thyrocyte proliferation. When both varieties of distinct 2nd messenger stimulating IgGs are found together in the serum of Graves’ patients, these patients show the largest goitres and the most severe expression of hyperthyroidism (or ophthalmopathy).

(iii) There is general agreement that the antibody response in Graves’ patients to the TSH-R is polyclonal and heterogeneous, and directed to various, partly overlapping, non-linear (conformational) epitopes in the TSH-binding as well as in the non-TSH-binding domain (75–77).
In view of the heterogeneity of the TSH-R-ab with regard to 2nd messenger stimulation, and in view of the involvement of different (parts of the) intracellular loops of the TSH-R in the signal transduction to these various 2nd messenger systems (78, 79), the likelihood is increasing that TSH-R ab varieties are against distinct isomers of the TSH-R coupled to distinct G-proteins (76).

(iv) There is accumulating evidence for an immuno-endocrine regulatory circuit on the level of the thyroid. This immuno-endocrine regulatory circuit seems to be activated in goitrous conditions and is most likely involved in the control of thyrocyte proliferation, function and integrity. This immuno-endocrine regulatory circuit predominantly involves interactions between thyrocytes and (products of) activated, infiltrated lymphocytes and (dendritic) macrophages. Whether thyroid antibodies produced in low titre (as an epiphenomenon?) contribute to this regulation needs further clarification. Exchange of serum samples between laboratories should be a cornerstone in such further research (8, 10): a drawback in this approach is that, due to the severe criticism and the difficulty in maintaining the special equipment, CBAs are not (or practically not) performed anymore.

In my view, the past and future studies on “autoimmunity and goitre formation” have opened and will open new avenues for the understanding (and hopefully treatment) of prevalent disabling thyroid abnormalities.

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