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“The chondrotin 6-sulfate oligosaccharide chain is a major determinant of the immunopathogenicity of human thyroglobulin in CBA/J(H-2Ak) mice”

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“The chondrotin 6-sulfate oligosaccharide chain is a major determinant of the immunopathogenicity of human thyroglobulin in CBA/J(H-2Ak) mice”

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This dissertation is based upon the following publication:

Conte, M., Arcaro, A., D'Angelo, D., Gnata, A., Mamone, G., Ferranti, P., Formisano, S., and Gentile, F.
A single chondroitin 6-sulfate oligosaccharide unit at Ser2730 of human thyroglobulin enhances hormone formation and limits proteolytic accessibility at the carboxyl terminus. Potential insights into thyroid homeostasis and autoimmunity.
J. Biol. Chem. **281(31)**: 22200-22211, 2006 **IF: 7.368**

ABSTRACT

We investigated the influence of the chondroitin 6-sulfate oligosaccharide unit at Ser2750 of hTg on the ability of hTg to induce experimental autoimmune thyroiditis in CBA/J(H-2k) mice, by using purified preparations of chondroitin 6-sulfate-devoid hTg ($hTgCS_0$), chondroitin 6-sulfate-containing hTg (hTgCS), and of the chondroitin 6-sulfate-containing nonapeptide centred upon Ser2730. Immunization with hTgCS was associated with markedly more pronounced infiltration of thyroid wih mononuclear cells, in comparison with $hTgCS_0$, and larger secondary proliferative responses of splenocytes to both kinds of hTg *in vitro*. Cytokines secreted in the supernatants of splenocyte proliferations were predominantly of the TH1 type. Splenocytes from hTgCS-immunized mice secreted larger amounts of IFN-γ and IL-2 in response to both kinds of hTg, compared with splenocytes from $hTgCS_0$-immunized mice. The hTgCSgp glycopeptide elicited the production of significant levels of IFN-γ, GM-CSF, IL-6, TNF-α and IL-10, but not of IL-2 and IL-5, both in control and immunized groups. The effects of combined $hTgCS_0$ and hTgCSgp on IFN-γ and GM-CSF production were additive in hTgCS-immunized mice and synergistic in $hTgCS_0$-immunized mice. Synergistic effects were also noticed with IL-17 production in both groups. The presence of chondroitin sulfate did not influence the sensitization and response to hTg of IL-5-producing cells. In keeping with the latter results, comparable levels of anti-hTg antibodies of the IgG class and IgG1 and IgG2a subclasses were found in both immune groups. Thus, the chondroitin 6-sulfate oligosaccharide unit of hTg deeply affected the immunopathogenicity of hTg in murine EAT, by interfering at possibly more than one level in the differentiation process of TH1 cells in response to hTg.

1 BACKGROUND

Thyroglobulin

Thyroglobulin is a large homodimeric glycoprotein, with a Mr of 660,000. After being synthesized and assembled into dimers in the endoplasmic reticulum and glycosylated in the Golgi apparatus, it is condensed in secretory granules in the apical cytoplasm of thyroid follicular cells and secreted in a regulated way in the lumen of tyroid follicles. Thyroglobulin is the molecular site of biosynthesis of thyroid hormones, which are synthesized through the iodination and coupling of a small number of specific tyrosyl residues within the context of the polypeptide chains of thyroglobulin. Thyroglobulin is also a major autoantigen, involved in the pathogenesis of thyroid autoimmunity (Gentile et al. 2004).

Experimental autoimmune thyroiditis (EAT)

Experimental autoimmune thyroiditis (EAT), a murine model of Hashimoto's thyroiditis, can be induced in mice with haplotypes *H-2*^{s} and *H-2*^{k} by immunization with mouse Tg (mTg) or human Tg (hTg) in complete Freund's adjuvant (CFA) or lipopolysaccharide (Rose et al. 1971). EAT is characterized by the infiltration of thyroid by mononuclear cells, the production of specific antibodies and *in vitro* cell proliferative responses against Tg. EAT is a T cell-mediated disease, which can be transferred from mice immunized with mTg to syngeneic hosts, by injecting mTg-specific T-cell clones and cytotoxic T lymphocytes. CD4$^+$ cells play a dominant role in EAT (Flynn et al. 1989). The analysis of the T cell receptor Vβ gene repertoire of thyroid-infiltrating T cells, after immunization with Tg or adoptive transfer of mTg-primed T cells, showed the clonal expansion of Vβ13$^+$ T cells (Matsuoka et al. 1994; Nakashima et al. 1996). Also the characterization of mTg-primed T cells, expanded in vitro with staphylococcal enterotoxin A, demonstrated the involvement of Vβ13$^+$ and Vβ1$^+$ T cells in subsequent thyroiditis transfer (Wan et al. 2001). T helper type-1 responses are crucial for thyroiditis development, as underscored by the impaired induction of EAT in interleukin-12-deficient C57BL⁄6 mice (Zaccone et al. 1999) and by the association between the *IFN-γ* gene deletion and the marked reduction of anti-mTg IgG1 and IgG2b production, and thyroid infiltration with T, B and plasma cells in spontaneous autoimmune thyroiditis (SAT) (Yu et al. 2002). Anti-Tg antibodies may also contribute to the pathogenesis of murine autoimmune thyroiditis. A selective correlation was found between the activity of EAT and the levels of anti-idiotypic antibodies to a mAb (3B8G), recognizing a thyroiditogenic tryptic

fragment of pTg (Tang et al. 1990). Moreover, some murine mAbs facilitated the internalization of mTg by a murine B-cell hybridoma in vitro, and either suppressed or enhanced the presentation of the non-dominant pathogenic epitope 2549–2560 (Dai et al. 1999). In NOD-H-2^{h4} mice, the levels of mTg-specific IgG1 and IgG2b autoantibodies produced correlated closely with the severity of SAT (Braley-Mullen et al. 1999). Moreover, B cell-deficient NOD-H-2^{h4} mice developed minimal SAT, and B cell function could not be replaced by anti-mTg antibodies (Braley-Mullen et al. 2000).

Genetic regulation of susceptibility to EAT

The development of EAT is under the influence of H-2 molecules of the murine major hystocompatibility complex (Vladutiu 1989; Beisel et al. 1992). High susceptibility strains include C3H(*H-2*^{k}) and SJL(*H-2*^{s}), while BALB/C(*H-2*^{d}) and B10(*H-2*^{b}) are relatively resistant. Non-obese diabetic mice (NOD)(*H-2*^{g7}), a strain susceptible to diabetes, are also prone to develop SAT, even though at a very low rate with aging (Damotte et al. 1997). However, in NOD(*H2*^{h4}) transgenic mice, which express the *H-2A*^{k} allele, diabetes does not develop, while thyroiditis occurs at a much higher frequency and is accelerated and enhanced by the addition of iodide to drinking water (Braley-Mullen et al. 1999). In the context of the "high-responder" haplotype *H-2*^{k}, the region *H-2A* of the *H-2* complex is the major regulator of the susceptibility to EAT (Beisel et al. 1982), even though the regions *K* and *D* of the *H-2* complex also have influence (Kong et al. 1979). The primary response of mouse lymphocytes to epithelial syngeneic thyroid cells is under a similar control by the *H-2* region, and their proliferation seems to be triggered by the recognition of products of the *I-A* subregion (Salamero and Charreire 1983a and 1983b). Therefore, the recognition of a limited number of thyroiditogenic epitopes of mTg and hTg by HLA-restricted T lymphocytes seems pivotal in the development of EAT. Apparently, H-2 molecules are able to present T cell thyroiditogenic epitopes shared by mTg and hTg.

By using mice characterized by recombination within the *H-2* region, differences in the genetic influence on the induction of EAT by thyroiditogenic peptides of Tg have been observed, in comparison with EAT induction by whole Tg, which is under strong influence by H-2A^k products. The rat Tg peptide TgP1 causes EAT with a similar genetic pattern as entire Tg: B10.BR and C3H(*H-2*^{k}) and SJL(*H-2*^{s}) mice are susceptible, while BALB/C(*H-2*^{d}) are partially resistant and B10(*H-2*^{b}) are resistant. At variance from EAT induction by intact Tg, EAT induction by TgP1 (2495-2511), within the susceptible haplotype *H-2*^{k}, requires the expression of H-2E products, while the *H-2D* region does not seem to have influence. Such divergences probably reflect differences in antigen processing and presentation

between Tg and its peptides. The role of H-2E molecules in the presentation of peptides such as TgP1 could be masked, if most thyroiditogenic Tg epitopes were *H-2A*^{k}-restricted. Moreover, the non-immunodominant peptide TgP1 could be displaced by other peptides of Tg in the interaction with H2-E molecules, or, alternatively, it may not be generated by antigen presenting cells (APC), in the course of Tg proteolysis, whereas it may be generated and presented by intrathyroidal MHC class I- or class II-positive cells (Chronopoulou et al. 1993). Subsequently, it has been demonstrated that the 2496-2504 nonamer was the minimal T-cell epitope in TgP1 and could be presented within the context of the non-isotypic H-2E^{k} molecules of C3H(*H-2*^{k}) mice and H-2A^{s} molecules of SJL(*H-2*^{s}) mice (Rao et al. 1994). On the other hand, the genetic pattern of mouse susceptibility can vary, depending on the pathogenic epitope: rTg peptide 2696-2713 (TgP2) caused EAT in SJL(*H-2*^{s}) mice, but not in C3H or B10.BR(*H-2*^{k}), BALB/c(*H-2*^{d}), and B10(*H-2*^{b}) mice (Carayanniotis et al. 1994).
Last, the role of polymorphic genes *HLA-BR1* (*DR3*) in the development of EAT following Tg immunization has been addressed using *HLA-DR* and *HLA-DQ* transgenic mice: immunization of *HLA-BR1*0301(DR3)* transgenic mice with mTg or hTg resulted in severe thyroiditis, while transgenic mice expressing *HLA-BR1*1502(DR2)* gene were resistant to EAT (Kong et al. 1996). The introduction of the *H-2Ea*^{k} transgene into B10(*H2*^{b}) or MHC class II-negative *Ab*^{0} mice, both resistant to EAT, conferred upon them susceptibility to EAT induced by bovine, porcine or human Tg, but not murine Tg (Wan et al. 1999).

Structure and function of human thyroglobulin (hTg)

The Tg gene: structure and evolution

The hTg gene is located on chromosome 8 and it is one of the gratest genes known, encompassing 42 exons and spanning more than 300,000 base pairs (Baas et al. 1986). About two-thirds of Tg at its amino-terminal side consist of tandemly repeated cysteine-rich motifs of various kinds. This portion of the *hTg* gene probably derives from duplication of an ancestral unit made of four exons. Subsequent unequal crossing-over events, and the partial “exonization” of some introns and the loss of several others, seem to have resulted in the present structure. The carboxy-terminal third of Tg is homologous with acetylcholinesterase and appears to have been originated from the duplication of an ancestral gene, in common with a superfamily of esterases (Takagi et al. 1991; Krejci et al. 1991). Thus, Tg appears to be made of two mojeties, an amino-

terminal one and a carboxy-terminal one, with different evolutionary histories, both being able to support the biosynthesis of thyroid hormones.

Structure of hTg

The full-length sequence of the hTg mRNA has been determined from overlapping cDNA clones (Malthiéry and Lissitzky 1987). It consists of 8448 nucleotides, including an untranslated 5′-end, an open reading frame, and an untranslated 3′-end, and encodes a polypeptide of 2767 amino acids. The first 19 residues probably represent a hydrophobic signal sequence and are absent from the mature protein, which is composed of 2749 residues and has an expected Mr of 302,773. Two-thirds of the Tg sequence at its amino-terminal side consist of tandemly repeated motifs. The type-1 motif is 60-70-residue long, contains 6 cysteinyl residues, and is repeated 10 times. The type-2 motif consists of 14 to 17 amino acids, 2 of which are cysteines, and is repeated 3 times at the center of Tg. Five type-3 repeats, subdivided into three subtype-3a repeats, with 8 cysteines each, and 2 subtype-3b repeats, with 6 cysteines each, follow in alternating order. The type-1 motif is homologous with a cystein-rich motif of the invariant chain associated with the class II major histocompatibility antigen (McKnight et al., 1989). The sequence of 570 amino acids at the COOH-terminus of Tg shows a high degree of similarity (up to 60% in some regions) with those of the members of a superfamily of lipases and esterases, including the acetylcholinesterase of *Torpedo californica,* human serum cholinesterase, and others (Takagi et al. 1991; Krejci et al. 1991). Mature hTg is a mixture of both non-covalent and covalent homodimers, having a molecular mass of 330,000.

Post-translational modifications of hTg: glycosilation

Carbohydrates contribute about 10 per cent of the Tg mass. Two kinds of oligosaccharide units (A and B) are attached by glycosylamine linkages to asparagine residues of Tg. High-mannose A units contain a variable number (7-9) of mannose residues and 2 *N*-acetylglucosamine residues. Complex B units contain 3 mannose residues and a variable number of *N*- acetylglucosamine, galactose, fucose, and sialic acid residues (Arima et al. 1972). Human Tg also contains C and D oligosaccharide units. C units are linked to serine and threonine by O-glycosidic bonds and contain galactosamine; D units are chondroitin-6-sulfate-like oligosaccharides linked to serine and contain a repeating unit of glucuronic acid and galactosamine, plus xylose, galactose, and sulfate (Spiro et al. 1977). 90 per cent of the [^{35}S]-sulfate incorporated into human Tg is equally

distirbuted in: a) biantennary B units, containing galactose-3-sulfate, and tri- and tetra-antennary B units containing galactose-3-sulfate and *N*-acetylglucosamine-6-sulfate; b) chondroitin-6-sulfate-like D units (Spiro and Bhoyroo 1988; Schneider et al. 1988).

Post-translational modification of hTg: iodination, sulfation and hormonogenesis

Iodoamino acids in hTg include monoiodotyrosine (MIT), 3,5-diiodotyrosine (DIT), and the hormones 3,3′,5-triiodothyronine (T3) and 3,3′,5,5′-tetraiodothyronine (thyroxine, T4). Under normal conditions, the iodine level ranges from 10 to 40 moles of iodine atoms per mole of Tg. MIT, DIT and T4 appear to be in a precursor-product relationship, in the same order. For an iodine content of 0.5 per cent of the Tg weight (25 moles of iodine atoms per mole of Tg), 2.5-3 moles of T4 and less of than 1 mole of T3 are formed per mole of human Tg. T3 and T4 are formed in Tg via the iodination of specific tyrosyl residues and the subsequent transfer of an iodophenoxyl group from a "donor" iodothyrosine, which provides the outer ring, to an "acceptor" iodothyrosine, which provides the inner ring. The main T4-forming site of Tg has been located at tyrosine 5 (Lejeune et al. 1983). T4 formation in human Tg appears to involve tyrosine 5 as the acceptor site and tyrosine 130 as the donor site (Marriq et al. 1991). T4 is also formed at tyrosines 2553 and 2567 (Lamas et al. 1989). Dehydroalanine residues have been identified at several positions of Tg from various animal species, including Tyr1375 of bovine Tg (Gentile et al. 1997).
Several observations lead to the conclusion that the NH_2- and COOH-terminal regions of Tg represent autonomous hormone-forming domains. A mutation in amino acid 296 of Tg, resulting in premature termination and goitre in Dutch goats, determined the production of two NH_2-terminal fragments, with masses of 40,000 and 32,000. However, these were capable of efficient thyroid hormone production, in the presence of iodide supplementation (Veenboer and de Vijlder 1993). Thyroid hormones were also efficiently formed by the *in vitro* iodination of a carboxy-terminal fragment of rat Tg fused to Staphylococcal protein A (Asunciòn et al. 1992).

Chondroitin 6-sulfate oligosaccharide units

In a previous study (Conte et al. 2006), we characterized in detail the O-linked, type D chondroitin 6-sulfate (C6S) oligosaccharide unit of hTg. We developed an ion-exchange chromatography (IEC) method, which permitted us to

separate chondroitin 6-sulfate-containing hTg molecules (hTg-CS) from the residual hTg molecules (hTg-CS_0). By exploiting the changes of electrophoretic mobility and staining properties conferred upon the products of limited proteolysis of hTg by chondroitin 6-sulfate units, we first restricted the chondroitin 6-sulfate-containing regions of hTg to a carboxyl-terminal peptide, starting at Thr2514. The subsequent purification of a homogeneous, D-glucuronic acid-containing nonapeptide (hTg-CSgp), corresponding to hTg residues 2726-2734, permitted us to establish Ser2730 as the sole site of chondroitin 6-sulfate addition in hTg.
In an ample number of hTg preparations, the fraction of hTg-CS in total hTg ranged from 32.0 to 71.6%. The C6S unit was composed of a broadly varying number of D-glucuronic acid-N-acetyl-D-galactosamine disaccharide units. In some goiter hTg preparations with high contents of hTg-CS, hTg-CS heterodimers, in which only one hTg subunit contained a C6S chain, coexisted with hTg-CS homodimers. In the monomers deriving from the dissociation of hTg-CS in urea, the net number of disaccharide units per chondroitin 6-sulfate chain did not exceed 14. We also showed that hTg-CS has a higher efficiency of hormone formation than hTg-CS_0, and that the whole unfractionated hTg benefits from this property. Thus, chondroitin 6-sulfate addition represents an ergonomic mechanism, by which the post-translational modification of a fraction of molecules influences the overall function of hTg. The greater advantage in the formation of T_3, with respect to T_4, associated with the chondroitin 6-sulfate unit, and the proximity of the attachment site of the latter to the carboxyl terminus of hTg suggest that it may influence hormonogenesis by affecting the function of the site of preferential T3 formation at Tyr2747 (Van de Graaf et al. 2001; Fassler 1988; Lamas 1989).
Furthermore, we identified two sites, one between Lys2714 and Gly2715 and the other between Lys2745 and Thr2746, near the site of chondroitin 6-sulfate addition, which were susceptible to limited proteolysis with trypsin. Our data show that the former was protected from proteolysis in hTg-CS but not in hTg-CS_0. We reasoned that the addition of the C6S chain may exert modifying effects in the processing of hTg by antigen-presenting cells and in the ability of hTg to function as an autoantigen. These may go beyond the possible influences on the proteolytic accessibility and/or the recognition of specific T- and B-cell epitopes located in the vicinity of the chondroitin 6-sulfate addition site.

Effects of chondroitin sulfate proteoglycans

Chondroitin sulfate proteoglycans play important roles in cell adhesion, migration, differentiation and proliferation. Several reports indicate that some chondroitin sulfate proteoglycans affect the proliferation of a number of cell types. Versican 1 and 2 have opposite actions on the proliferation of NIH-3T3 cells:

versican 1 induces cell proliferation and inhibits apoptosis, while versican 2 inhibits cell proliferation and does not affect apoptosis (Sheng et al. 2005). TENB2, a chondroitin sulfate proteoglycan identified in prostate cancer, is involved in disease progression and in prostatic cell growth control (Glynne-Jones et al. 2001). The degradation of chondroitin sulfates A, B and C reduces the proliferation and invasion capacity of melanoma cells, while inducing caspase-3 activity and thus stimulating apoptosis (Denholm et al. 2001). Endothelial cells, treated with the same enzymes, behave similarly. Some Authors (Yang et al. 2004), by studying the chondroitin sulfate proteoglycan of melanoma cells (MCSP), demonstrated that it stimulates proliferation, migration and invasion by tumor cells, by enhancing FAK and ERK activation. By studying globular domains of proteoglycan molecules, specific regions were identified, modulating cellular responses, such as apoptosis or cell proliferation (Cattaruzza et al. 2004).

The action of chondroitin sulfate proteoglycans is often mediated by CD44, the most important chondroitin sulfate proteoglycan exposed on the leukocyte surface (Taylor and Gallo 2006). CD44 acts mainly as a receptor for hyaluronan, but can also bind chondroitin sulfates, heparan sulfates, fibronectin and osteopontin (Rudzki and Jothy 1997). CD44 is involved in inflammation, chemotaxis, cell proliferation and apoptosis (McVoy and Kew 2005; Castellone et al. 2004; Ayroldi et al. 1995). Generally, CD44 is involved in proliferative signalling, whereas it provides protection from apoptosis induced by dexamethasone (Ayroldi et al. 1995). CD44 is coded by a single gene, containing 20 exons; 10 of these exons (v1-v10) can be differentially included in the primary transcript, by alternative splicing. Splice variants have an important role in tumour progression, being up-regulated in several human tumours (Ponta et al. 2003). For example, CD44v6 is up-regulated in several papillary carcinomas of the thyroid, where it is able to modulate tumor invasiveness (Guarino et al. 2005).

Chondroitin sulfates are related to the differentiation degree of some tumours, such as Merkel skin cell carcinoma (Sames et al. 2001) and prostate cancer (Ricciardelli et al. 1999). Generally, high levels of proteoglycan chondroitin sulfates are predictive of a poor prognosis and malignant progression of the disease. It is conceivable that the hTg-CSgp glycopeptide may be involved in the progression of thyroid tumours and may induce cellular proliferation and inhibition of apoptosis, not only in thyroid cells, where it is expressed, but also in connective tissue and endothelial cells. The regulated growth of epithelial tissues depends on the controlled proliferation of epithelial cells and the adjacent connective tissue (Popp et al. 1996). Some peptides with regulatory functions are produced and released by epithelial cells themselves and act as essential parts of autocrine or paracrine mechanisms. Thyrotropin (TSH) promotes the growth and the differentiated functions of thyrocytes (Roger and Dumont 1984), e.g., thyroglobulin synthesis and iodide transport (Weiss et al. 1984; Santisteban et al. 1987).

Some functions exerted by TSH are mediated by molecules sythesized by thyroid epithelial cells. FRTL-5 cells stimulated with TSH release growth-promoting factors, such as insulin-like growth factor II (Maciel et al. 1988) and the secretory ectodomain of beta-amyloid precursor proteins (sAPP) (Graebert et al. 1995). A regulatory role in the growth of FRTL-5 cells was demonstrated for the latter (Popp et al. 1996). Moreover, it was proposed that sAPP operates as an autocrine growth factor, mediating the proliferative effect of TSH on neighboring thyroid epithelial cells (Pietrzik et al. 1998). The proposed functions of the APP family components were in part deduced from the domain structure of different APP forms, which include binding sites for collagen, laminin and glycosaminoglycans (Kibbey et al. 1993). A chondroitin sulfate modified form of APP, named appican, was also characterized (Pangalos et al. 1996). In thyroid cells, APP may act as a link between intracellular and extracellular matrix-dependent signals, which modulate the proliferative and differentiative status of thyroid cells. Yet, the role of the extracellular matrix in the control of thyrocyte growth and differentiation is poorly understood. On the other hand, a number of reports have demonstrated the role played by components of the extracellular matrix in the control of neural cell differentiation and neurite outgrowth. Some authors described both inhibitory and stimulatory effects of chondroitin sulfate proteoglycans on cell adhesion and neurite outhgrowth in vitro (Anderson et al. 1998). Glycosaminoglycans, specifically heparan sulfate, were essential in the action of glial-cell line-derived neurotrophic factor (GDNF) on axonogenesis in neurons (Barnett et al. 2002).

Thyroglobulin secreted by thyroid follicular cells is deposited in the lumen of thyroid follicles as a very concentrated colloidal solution, whose concentration is increased by the active extrusion of water (Nitsch and Wollman 1987; Chambard et al. 1987). In a sense, the colloid substance represents a semisolid extracellular matrix in direct contact with the apical functional specializations of thyroid epithelial cells. The presence, within the colloid substance, of a relevant proportion (from 30 to 70%) of hTg modified by the addition of a C6S oligosaccharide chain (hTg-CS) may prompt one to ask whether this hTg isoform may share with other proteoglycans of the extracellular matrix any signaling functions towards the epithelial cells with which it is in contact, including signaling related with cell polarity. It was shown that Tg is able to suppress the expression of thyroid-specific genes, such as the sodium/iodide symporter (NIS), thyroperoxidase (TPO), Tg itself, and the TSH receptor (TSH-R). Such effects were mediated by Tg binding to the apical asialoglycoprotein receptor (ASGPR), while the phosphorylation of serine/threonine residues of Tg not only suppressed such effects, but even promoted the transcription of thyroid-specific genes (Ulianich et al. 1999). These observations may provide grounds for a study of the effects of C6S addition to hTg on the modulation of the expression of thyroid-specific functions by hTg. It is possible that the addition and/or the elongation of C6S hTg chains be under the

regulatory control of TSH, as it occurs with N-linked oligosaccharide units (Di Jeso and Gentile 1992). If so, chondroitin 6-sulfate containing hTg (hTg-CS) might participate in the autocrine regulation of the differentiated phenotype of thyroid cells, under the control of TSH.

Involvement of C6S in Experimental Autoimmune Thyroiditis

C6S unit might influence the ability of hTg to induce experimental autoimmune thyroiditis (EAT) in genetically susceptible mice in several ways:
1. The influence of the C6S oligosaccharide chain on the proteolytic susceptibility of the extreme carboxy-terminal region of hTg could modify the processing and presentation of hTg by antigen-presenting cells (APCs), particularly because the site of C6S addition to hTg is localized within an epitope-rich region, harboring several T cell- and B cell-related epitopes (Gentile et al. 2004). O-linked mono- and disaccharides in tumor-associated glycoprotein MUC1 restricted the repertory of epitopes produced and/or presented in a site-specific manner, either by limiting the accessibility of specific cleavage sites to cathepsin L (Hanisch et al. 2003), or by preventing epitope recognition by a peptide-specific T cell hybridoma. N-linked oligosaccharide chains also inhibited the generation of a self epitope from glutamate receptor subunit 3 (Gahring et al. 2001), and of cytotoxic lymphocyte (CTL)-specific epitopes from influenza A nucleoprotein (Wood 1998). A keratan sulfate chain masked an arthritogenic T cell epitope in the G1 domain of aggrecan, whereas the enzymatic depletion of multiple chondroitin sulfate side chains generated clusters of chondroitin sulfate stubs, which activated specific B cells to function as APCs (Glant et al. 1998). Conceivably, the C6S oligosaccharide unit in hTg-CS may hamper the processing by APCs of the surrounding hTg region, within a range which includes the Lys2714-Gly2715 bond, at the amino side, and the Lys2745-Thr2746 bond, at the carboxy side.
2. Direct effects of chondroitin sulfate oligosaccharide units on cellular immune responses have been documented:

- Ii (invariant chain, CD74) is a non-polymorphic glycoprotein that participates in a number of immunological functions. The major functions of Ii are mediated through its association with MHC-II heterodimers (Hiltbolt 2002). In the ER, newly synthesized Ii self assembles into trimers. Three class II heterodimers are sequentially added to one Ii trimer to form a nine-chain complex. In the ER, Ii facilitates MHC-II heterodimer assembly and folding and occupies the MHC-II-binding groove, preventing premature binding of either peptides or unfolded polypeptides. The nonameric complex transits through the Golgi and is transported to late endosomes/lysosomes, where Ii is degraded, allowing MHC-II to be loaded with peptide. However, it has been

shown that a small percentage (2-5%) of Ii molecules associated with class II MHC molecules are modified with the addition of a single chondroitin sulfate chain at Ser 291. Chondroitin sulfate-containing invariant chain complexes (Ii-Cs) are transported rapidly from the *trans*-Golgi network to the cell surface, in spite of the presence of an intact endosomal localization signal. In this form, they remain associated with class II molecules at the surface of APCs (Sant et al. 1985; Miller et al. 1988), where they act as accessory molecules in antigen presentation, through interaction with CD44 (Naujokas 2003). Recently, it has been demonstrated that the presence of at least one chondroitin sulfate-containing subunit within the context of Ii/MHC-II nonamers is able to direct Ii to the cell surface (Arneson et al. 2007). Enhancing effects of Ii (CD74) on T cell mitogenic and allogenic responses occur through the interaction of Ii-CS (CD74) with CD44 on responding T cells, as they can be inhibited both by anti-CD44 antibodies, and by a soluble form of CD44 (CD44Rg) (Naujokas et al. 1993). Monoclonal antibodies against CD44 were able to trigger the cytotoxic activity of CTL in a TCR-independent manner (Seth et al. 1991). Serglycins, small proteoglycans in secretory granules of hematopoietic cells, activated the CD3-dependent release of cytokines and proteases from CD44-positive CTL clones (Toyama-Sorimachi et al. 1995), by interacting with CD44 through their chondroitin 4-sulfate and 6-sulfate side chains (Toyama-Sorimachi et al. 1997). Moreover, the binding of aggrecan to CD44, through its chondroitin 4- and 6-sulfate side chains, was able to trigger the oligomerization of CD44 molecules and the intracellular signaling (Fujimoto et al. 2001). Both CD44v6 and CD44v7 were transiently up-regulated during lymphocyte activation. Antibodies to CD44s and CD44v7 inhibited antigenic and mitogenic T and B cell responses, while antibodies to CD44v6 selectively inhibited T cell responses (Seiter et al. 2000). Finally, cross-linking of CD44v6, but not CD44s, promoted TCR/CD3-independent proliferation of T lymphoma cells, accompanied by IL-2 production and activation of MAP and SAP kinases (Marhaba et al. 2005).

- More recently, Ii-CS (called CD74) has been identified as the cell-surface receptor for Macrophage migration inhibitory factor (MIF) (Leng, 2003). MIF promotes pro-inflammatory cytokine production by macrophages, triggers proliferation of T cells, and induces the release of nitric oxide, matrix metalloproteases, COX-2, and prostaglandin E2. MIF binds to Ii by a high-affinity interaction and induces the serine phosphorylation of the CD74 intracytoplasmic domain in a CD44-dependent manner. CD44 might be necessary for many, if not all, of MIF's signal transduction properties. By using COS-7/M6 (deficient in both CD74 and CD44) cell lines engineered to stably express combinations of CD74, CD44, and CD44D67, some Authors found that CD74 alone imparts MIF binding to cells and that the presence of

CD44 did not confer additional binding activity over that of CD74 alone. There are not evidences for a specific interaction between MIF and CD44; however, MIF-mediated ERK1 and ERK2 activation required the expression of a full length CD44. MIF stimulation was associated with the PKA-dependent serine phosphorylation of CD74 and CD44. Investigations with siRNA and the kinase inhibitor PP2 indicate that MIF-induced ERK activation proceeded via the Src tyrosine kinase, which was previously shown to associate physically with the CD44 intracytoplasmic domain (Taher et al. 1996). Several of the biologic activities of MIF have been shown to proceed via ERK activation. These include arachidonic acid metabolism and prostaglandin production (via cytoplasmic phospholipase A2 and cyclooxygenase-2) (Mitchell et al. 1999; Sampey et al. 2001), regulation of p53 activity (Mitchell et al. 2002), and the activation of additional ERK effectors, such as the Ets family of transcription factors that regulateToll-like receptor expression (Roger et al. 2001).

- Finally, it has been demostrated that p41 fragment of invariant chain (containing an additional 64-amino acid sequence in the lumenal domain) is a potent inhibitor of cathepsin L. The additional segment (AS) is cysteine rich, and shares significant homology with the type-1 repetitive sequence of thyroglobulin (Bevec 1996). These data suggest that p41 may enhance Ag presentation, by reducing the proteolytic activity of the Ag-processing compartment, thus protecting a subset of antigenic epitopes from excessive degradation (Fineschi 1996).

2 AIM OF THE STUDY

The aim of this study was to determine whether and by which mechanisms the chondroitin 6-sulfate oligosaccharide unit of human thyroglobulin may modulate the immunopathogenicity of hTg in a murine model of experimental autoimmune thyroiditis (EAT). A significant fraction of hTg molecules is modified by the addition of a single chondroitin 6-sulfate oligosaccharide unit to Ser2730. In an ample number of hTg preparations, a chondroitin 6-sulfate-containing subfraction of hTg molecules was regularly detected, and its relative abundance varied broadly from less than 30% to over 70%. The presence of a chondroitin 6-sulfate oligosaccharide unit, thus, is a major source of molecular microheterogeneity in hTg. Among the most prominent biochemical effects of chondroitin 6-sulfate addition to Ser2730 in hTg, there are the enhancement of the T4-forming and, especially, the T3-forming efficiency of hTg, and the protection from proteolysis of some closely located sites of tryptic susceptibility. The regional location of the chondroitin 6-sulfate oligosaccharide unit of hTg coincides with a cluster containing some of the best characterized T cell-dependent epitopes in murine models of autoimmune thyroid disease, as well as of some of the most prevalent B cell-dependent epitopes in human autoimmune thyroid disease. Such circumstances suggest that chondroitin sulfate modification of hTg may influence the processing and presentation of hTg epitopes, as it occurs with oliosaccharide chains of many glycoproteins. Most importantly, in view of the well documented effects that chondroitin sulfate-containing molecules, as the invariant chain (Ii, CD74) associated with class II MHC molecules, serglycins, aggrecan, and CD44 exert on immune cell responses, via their chondroitin sulfate oligosaccharide units, it is conceivable that the chondroitin 6-sulfate oligosaccharide unit of hTg may also affect the intermolecular and intercellular interactions determining and controlling the development of immune responses to hTg.

Therefore, in order to investigate into further detail the molecular determinants of the ability of hTg to induce experimental autoimmune thyroiditis in a genetically susceptible strain of CBA/J(H-2k) mice, we have set to determine the possible differential effects of the presence of the chondroitin 6-sulfate oligosaccharide unit onto the ability of hTg to induce the distinctive histological features of EAT and antibody responses against Tg *in vivo*, as well as the characteristic T cell-mediated responses to hTg *in vitro*, by using purified preparations of chondroitin 6-sulfate-devoid hTg (hTgCS0) and chondroitin 6-sulfate-containing hTg (hTgCS), and of a homogeneously purified hTg nonameric glycopeptide (hTgCSgp), containing the chondroitin 6-sulfate unit linked to Ser2730.

3 MATERIALS AND METHODS

Purification of hTg

hTg was prepared as described (Gentile and Salvatore, 1993) from informed euthyroid patients, hemilaryngectomized for non-thyroidal disease, and patients undergoing thyroidectomy for non-familial, simple or multinodular goiter. Protein concentration was assayed by measuring the optical absorbance at 280 nm. Iodine content was assayed as described, using L-thyroxine as the standard (Palumbo et al. 1982).

Separation of hTg-CS from hTg-CS_0

HTg molecules containing type-D (chondroitin 6-sulfate) oligosaccharide units (hTg-CS) were separated from residual hTg molecules, devoid of type-D units (hTg-CS_0), by ion-exchange chromatography on trimethylamino-substituted Q-Sepharose (Q-IEC), using 5-mL HiTrap™ Q-Sepharose HP columns, equilibrated in 0.025 M Tris/HCl, pH 7.4 (buffer A). Up to 20 mg of hTg in buffer A, plus 0.05 M NaCl, were applied to a column. After washing with buffer A, a linear gradient from 0 to 100% of buffer B (1.2 M NaCl in buffer A) was developed in 24 min, at the flow rate of 2.5 mL/min. One-mL fractions were analyzed, or stored at -80 °C until use.The concentration of bacterial endotoxin in hTg, hTgCS and hTgCS_0 preparations to be used both for mice immunization and secondary splenocyte proliferation assays was measured by the Limulus amebocyte lysate test. Sample purity threshold for use with cell cultures was set at < 0.01 Endotoxin Units (EU)/mL.

Enzymatic *in vitro* iodination of hTg, hTg-CS_0 and hTg-CS' and analysis of the iodine, T3 and T4 content

HTg from goiters, with iodine contents lower than 0.5% on a weight basis were iodinated enzymatically *in* vitro, as previously described (Conte et al. 2006). Aliquots of samples were assayed for iodine content, as already described, and for protein content, using the BCA Protein Assay Reagent (Bio-Rad Laboratories) and bovine serum albumin as the standard.

Purification of the hTg-CSgp glycopeptide

The chondroitin 6-sulfate-containing glycopeptide of hTg (hTgCSgp) was purified as previously described (Conte et al. 2006). A 40-mg aliquot of hTgCS was denatured and reduced, in 15 mL of 0.3 M Tris/HCl, pH 8.0, 6.0 M

guanidine/HCl, 1 x 10^{-3} M EDTA, 0.01 M dithiothreitol, at 37 °C for 2 h. The reduced protein was carboxymethylated with a 5-fold molar excess of iodoacetamide, with respect to total –SH groups, at room temperature for 30 min in the dark. Alkylation was stopped with excess dithiothreitol. The sample was dialyzed against 0.05 M Na phosphate, pH 7.8, and digested with endoproteinase Glu-C (protease V8) from *Staphylococcus aureus*, at the enzyme:substrate weight ratio of 1:100, at 37 °C for 18 h. The sample was adjusted with concentrated solutions to 0.025 M TrisHCl, 0.1 M NaCl, 2.0 M urea, pH 7.4 (buffer C), and loaded onto a 5-mL HiTrap™ Q-Sepharose HP column, equilibrated in the same buffer. After washing with buffer C, a gradient was started, from 0 to 100% of buffer D (1.2 M NaCl in buffer C) in 55 min, followed by 100% buffer D for 10 min, at the flow rate of 1 mL/min. One-mL fractions were monitored for the optical absorbance at 280 nm and D-glucuronic acid content. A single D-glucuronic acid-containing peak was subjected to size exclusion chromatography on a 1.5-by-100-cm column of Bio-Gel P-2, in 0.01 M NH_4HCO_3. A D-glucuronic acid-containing peak, eluted in the void volume, was lyophilized and further purified by gel chromatography on a 0.5-by-40-cm column of Sephadex G-50 fine, in 0.01 M NH_4HCO_3. One-mL fractions were monitored for peptide content, by measuring the optical absorbance at 220 nm, and D-glucuronic acid content, and a single peptide- and D-glucuronic acid-containing peak was lyophilized.

Synthetic peptides

A chondroitin 6-sulfate-devoid nonapeptide homolog of hTg chondroitin 6-sulfate-containing glycopeptide hTg-CSgp, with the LTAGSGLRE sequence, to be used as a negative control for the effects specifically due to the glycosidic unit of peptide hTg-CSgp, was synthesized at AnaSpec®, Inc. (San Josè, California). The peptide was certified to be endotoxin-free.

A set of seven partially overlapping C-terminal dodecapeptides corresponding to hTg residues: 2702-2713 (C-peptide 1, H-ISSLKTSADGA-OH, Mr = 1212.2); 2709-2720 (C-peptide 2, H-SADGAKGGQSAE-OH, Mr = 1077.0); 2717-2728 (C-peptide 3, H-QSAESEEEELTA-OH, Mr = 1322.2); 2724-2735 (C-peptide 4, H-EELTAGSGLRED-OH, Mr = 1276.3); 2731-2742 (C-peptide 5, H-GLREDLLSLQEP-OH, Mr = 1369.5); 2738-2749(T3) (C-peptide 6, H-SLQEPGSKT-T3-SK-OH, containing T3 at residue 2747, Mr = 1324.3) were also synthesized at Anaspec®, Inc. (San Josè, California). The peptides were certified to be endotoxin-free.

Conjugation of hTg C-peptides with sulfo-NHS-LC-Biotin

Aliquots of 150 nmoles of hTg C-terminal peptides were dissolved in 0.1 mL of PBS, pH 7.2. Then, 45 μL of 20 mM sulfosuccinimidyl-6-(biotin-amido) hexanoate (EZ-Link® Sulfo-NHS-LC-Biotin, Pierce) were added to each sample (biotin:peptide molar ratio = 6:1), and mixtures were kept on ice for 2 h. Products were filtered on a 1.5-by-100-cm P2 column in 10 mM NH_4HCO_3, in order to remove unreacted biotin. 2-mL fractions were monitored at 220 nm and peaks were pooled and liophylized. Samples were assayed with the Lowry protein assay.

Experimental animals

All experiments were conducted using female, 4-week-old CBA/J(*H-2*^{k}) mice genetically susceptible to EAT induction, purchased from Charles River.

EAT induction

Experimental groups of six female, CBA/J(H-2k) mice were immunized at day 0 with 100 micrograms of $hTgCS_0$ or hTgCS in Freund's complete adjuvant (CFA), containing 3.5 μg/mL of *Mycobacterium tuberculosis*, in 50 μl of PBS, in the dorsal thoracic region. Control mice received PBS in CFA. On day 7, all animals were given a supplemental dose of 50 μg of the respective immunogen in 25 μl of PBS, emulsionated with the same volume of incomplete Freund's adjuvant (IFA). After 30 days, mice were sacrificed by excess anesthesia, and blood, spleens and thyroids immediately collected.

Evaluation of EAT

Thyroids were fixed, sliced and stained with May-Grünwald-Giemsa for histological examination, or embedded in paraffin for immunoistochemical characterization. Mononuclear infiltration of the thyroid was evaluated histologically, according to Verginis et al. (2002), in at least 30 sections per thyroid lobe per animal, and scored as follows: 0 = no infiltration; 1 = small interstitial accumulation between two or more follicles; 2 = one or two foci of inflammatory cells more than the size of one follicle; 3 = 10-40% of field area occupied by inflammatory cells; 4 = 40-80% of field area infiltrated.

Assay of T4 and T3 in mice sera

The concentrations of fT4 and fT3 in mice sera were determined on 10-100 μL-aliquots of sera, using solid-phase radio-immunometric assays (Medical Systems), with reference curves of 0.3-7.7 pmol for T4, and 30-920 fmol for T3.

Lymphocyte proliferation assays

Murine spleens were collected in 5 mL of RPMI 1640 medium, containing Na pyruvate, L-glutamine, penicillin and streptomycin. Splenocytes were obtained by mechanical disruption of spleens with the upper extremity of a disposable syringe plunger and transferred into sterile 50 mL Falcon tubes. In order to remove red cells and dead cells, spleen cells were layered onto a cushion of Lympholyte-M (Cedarlane) and centrifuged at 1000 *g* for 20 minutes. Live cells were carefully collected at the interface with Lympholyte-M, transferred to new tubes and resuspended by the addition of 5 mL of medium, diluted 1:100 with PBS and counted in a Burker's cell. After centrifugation, medium was aspirated and cells were resuspended in complete RPMI 1640 medium, containing 2% fetal bovine serum (FBS), so to obtain a cell density of 2.5 x 10^6 cells/mL. 200 µL aliquots of cell suspension (5 x 10^5 cells/100µL/well) were cultured for proliferation assays in flat-bottom 96-well plates and incubated at 37°C, in a 5% CO_2/90% air-humidified incubator in the absence or presence of graded doses (0-30 µg) of hTg-CS_0 or hTg-CS, or purified hTgCSgp glycopeptide, or its synthetic non-glycosylated peptide homolog (LTAGSGLRE). After 48 h of incubation, 50-µL aliquots of supernatants were removed from each well for cytokine assay. 1 µCi of [^{3}H]-thymidine was added to each well and incubation prolonged for 16 h, after which the cells were blotted onto glass-fiber filters and radioactivity counted in beta counter. All assays were performed in triplicate and results were expressed as Stimulation Index (S.I.), defined as follows: cpm in the presence of antigen/cpm in the absence of antigen.

Flow-cytometric analysis of CD74 and H2-I expression at the surface of isolated dendritic cells isolated from murine spleens

Spleen cell suspensions were prepared and incubated with microbeads conjugated with an anti-CD11c monoclonal antibody (MACS-anti-CD11c; Miltenyi Biotec). $CD11c^+$ cells were positively selected by high-speed magnetic cell sorting (AutoMACS, Miltenyi Biotec). The expression of invariant chain (CD74) at the surface of isolated dendritic cells was evaluated, using an anti-CD74 specific primary goat plyclonal antibody (clone C-16, Santa Cruz Biotechnology), recognizing the extracellular carboxy-terminal domain of human CD74, which was conjugated with R-phycoerythrin (RPE), using the LYNX Rapid Conjugation Kit (AbD Serotec). The expression of MHC II molecules at the DC surface was evaluated using a specific APC-conjugated goat anti-H2-A antibody (Pharmingen). Dendritic cells were incubated for 10 minutes in the presence of mouse serum, in order to block the Fc receptor. After washing, cells were further incubated with 10 µg/well of hTgCS_0 or hTgCS in 200 µL of RPMI 1640 medium, 10% fetal calf serum for 20 min at 4° C and washed two times in cold PBS. Data

were acquired, using a FACSCalibur flow cytometer (Becton Dickinson) and the results were analyzed usin the CellQuest Software (Becton Dickinson).

Assay of TH1/TH2 patterns of cytokine secretion

The concentrations of GMC-SF, INFγ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17 and TNF-α in the supernatants of splenocyte proliferative reactions were measured, using a multiparametric bead-based flow-cytometric Analyte Detection System (FlowCytomix Mouse Th1/Th2 10plex, Bender MedSystems®). Measurements were performed using a Dako flow cytometer.

Serum antibody assays

Total IgG, IgG1, IgG2a and IgG3 concentrations in mice sera were measured by ELISA assay in 96-well plates coated with 1 μg of hTg-CS_0, hTg-CS or hTg-CSgp as the capture antigen. IgG were assayed on 1:6000 dilutions of sera, using biotinylated goat anti-Ig γ chain $F(ab')_2$ secondary antibodies. IgG1, IgG2a and IgG3 were assayed on 1:20000, 1:500 and 1:500 dilutions of sera, respectively, using biotinylated rat monoclonal anti-mouse IgG1, IgG2a and IgG3 antibodies (LO-MG_1, LO-MG_{2a} and LO-MG_3 clones, Sigma). Detection was performed with streptavidine-peroxidase conjugates and orthophenylenediamine.

For the assay of antibodies against the purified hTgCSgp glycopeptide and the synthetic hTg carboxy-terminal peptides (hTg C-peptides), aliquots of 0.5 μg/well of biotinylated hTgCSgp glycopeptide and 120 pmol of biotinylated hTg C-peptides in 100 μL of PBS per well were incubated in Reacti-Bind Neutravidin Coated Plates, High-Binding Capacity, 8-well strips (Pierce) over night at 15 °C. After washing with PBS 0.05%, Tween-20, plates were incubated with 100 μL of 1:1000 dilutions of mice sera for IgG and 1:500 of mice sera for IgM detection, in blocking buffer for 1 h at room temperature. Secondary antibodies were peroxidase-conjugated goat anti-mouse IgG or anti-mouse IgM (Sigma). Assays were developed, using orthophenylenediamine as the substrate.

Statistical analysis

Data are expressed as mean ± SD, unless otherwise specified. The statistical significance between data obtained in experiments of splenocyte proliferation, cytokine assas and serum immunoglobulin concentrations was determined by *t* paired or two-tailed Mann-Withney test, as indicated. A value of $p < 0.05$ was considered to be statistically significant. Hystological evaluation of thyroid infiltration was analyzed by the non-parametric Wilcoxon test.

4 RESULTS AND DISCUSSION

Histological and biochemical evaluation of EAT

The use of the chondroitin 6-sulfate-devoid fraction and of the chondroitin 6-sulfate-containing fraction of human thyroglobulin (hTg), heretofore referred to as $hTgCS_0$ and hTgCS, respectively, for the subcutaneous immunization of CBA/J(H-2k) mice in adjuvant, had markedly different impacts on the severity of the EAT induced, as judged both from the changes in serum concentrations of the thyroid hormone T4 and its precursor T3, and from the histological disease score. Thyroid disease was more severe in mice immunized with hTgCS than in those immunized with $hTgCS_0$.

Figure 1 and Table 1 show the disease scores assigned to 48 animals, at the end of 3 immunization experiments, by the histological examination of 30 seriate sections per thyroid lobe, on the average, on the base of the extent of infiltration of thyroid tissue with mononuclear cells. Disease scores 2 and 3, corresponding, respectively, to one or more foci of infiltration, more than the size of a thyroid follicle, and 10-40% of field area occupied by mononuclear cells, were assigned only to mice immunized with hTgCS, whereas mice immunized with $hTgCS_0$ were either unaffected or showed very limited thyroid involvement, not exceeding score 1. A typical aspect of thyroid infiltration with mononuclear cells, which was scored 2 in our evaluation, is shown in Figure 2, aside a normally appearing thyroid section from a control mouse.

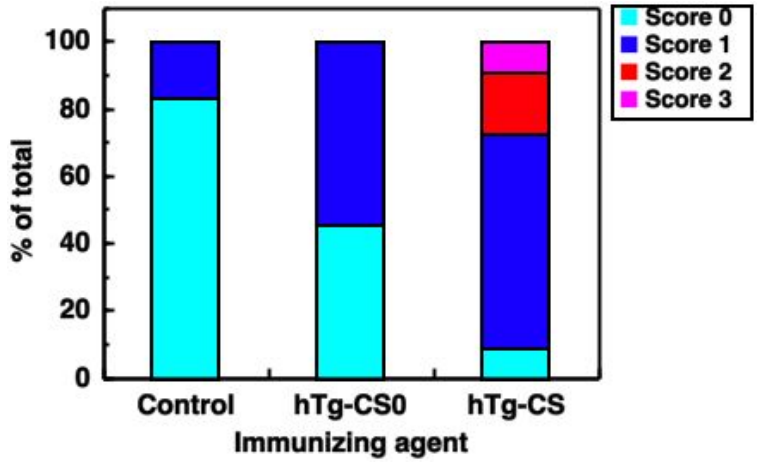

Fig. 1. Histological EAT score, indicating the severity of mononuclear infiltration of thyroids of CBA/J(H-2^k) mice immunized with the different forms of hTg. The EAT score was calculated, using a modification of the criteria adopted by Verginis et al., in at least 30 seriate

sections per thyroid lobe per animal, and scored as follows: 0 = no infiltration; 1 = small interstitial accumulation of infiltrating mononuclear cells between two or more follicles; 2 = one or two foci of infiltrating mononuclear cells more than the size of one follicle; 3 = 10-40% of field area occupied by infiltrating cells; 4 = 40-80% of field area infiltrated.

	Number of mice	Score 0	Score 1	Score 2	Score 3
Controls	10	90.0%	10.0%	0%	0%
$TgCS_0$	19	42.1%	57.9%	0%	0%
TgCS	19	15.8%	68.4%	10.5%	5.3%

Table 1. Number and percent of CBA/J(H-2^k) mice assigned disease scores from 0 to 4 in three different immunization experiments with $hTgCS_0$ and hTgCS. For score codes, refer to the legend to Figure 1.

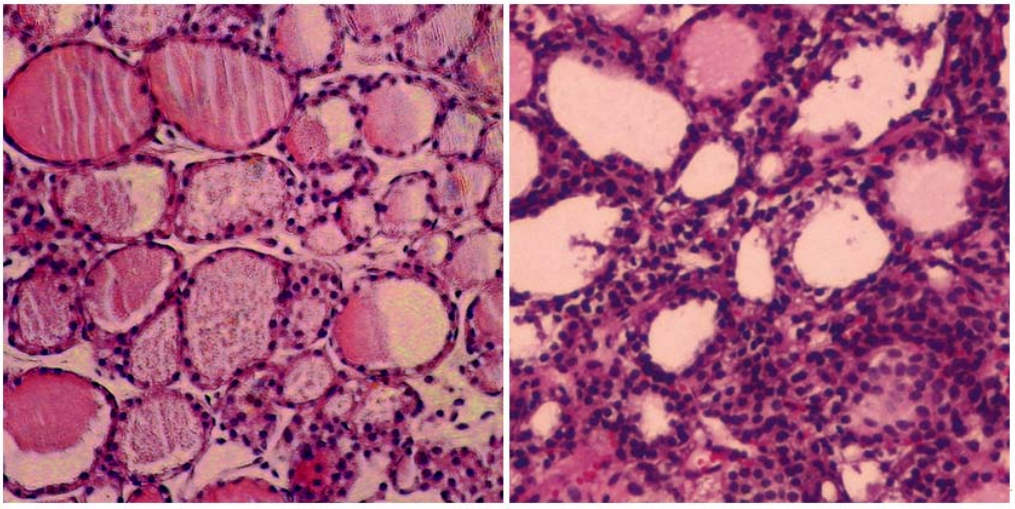

Fig. 2. Representative aspects of thyroid infiltration in EAT, compareed to a normally appearing thyroid section from a control animal. The thyroid section shown *at right* was obtained from a CBA/J(H-2k) mice immunized with hTgCS and was scored 2. A normally appearing thyroid section from a control animal is shown *at left*.

The increased severity of thyroid tissue involvement detected by histological examination was reflected in the differential increases in serum concentrations of T4 and T3, which were quite limited and did not reach statistical significance in mice immunized with $hTgCS_0$, whereas were large and significant in mice immunized with hTgCS.

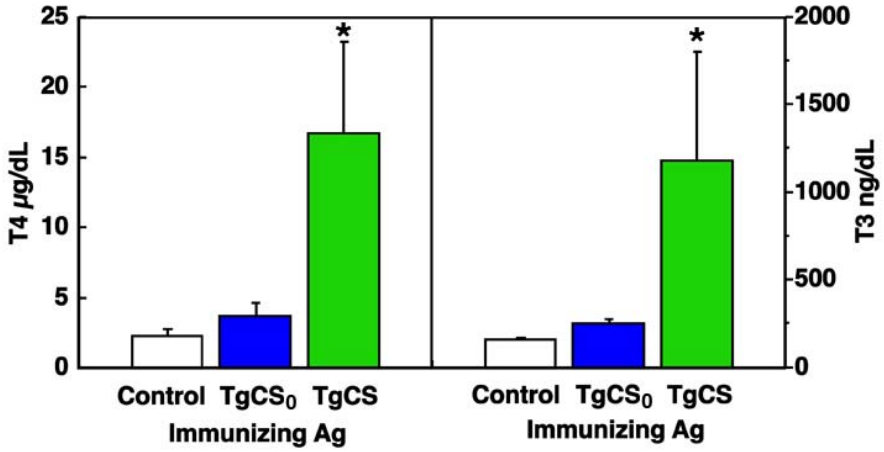

Fig. 3. Serum concentrations of T3 (3,5,5'-triiodothyronine) and T4 (thyroxine) in the sera of CBA/J(H-2^k) mice immunized with the different forms of hTg. Results are expressed as means ± S.D. * indicates the statistical significance between hTgCS and each of the control and $hTgCS_0$ condition (*p <0,05*).

It is known that murine EAT is characterized by low-grade, focal mononuclear infiltration of thyroid glands, even in genetically susceptible murine strains. Thyroid infiltration in experimental animals was even lower, when human Tg was used as the immunizing agent, instead of murine Tg, even though it is generally accepted that EAT results from the recognition by murine autoreactive T cells of epitopes that are conserved between the murine and human species. We have shown that hTg is regularly composed of a mixture of $hTgCS_0$ and hTgCS, in broadly variable proportions among different individuals. In the light of the data here reported, it appears that the induction of autoimmune thyroid disease in experimental animals depends prevailingly or solely on the hTgCS fraction of hTg. Because investigators of EAT have been using so far unfractionated hTg for their experiments, it appears that the variable severity of EAT observed in many studies was deeply influenced by the composition of the particular hTg preparations used, not only with regard to the percent abundance of hTgCS, but also to the relative proportion of heterodimeric and homodimeric hTgCS and to the average chain length of the chondroitin 6-sulfate chain linked to each hTg subunit.

Proliferative secondary responses of murine splenocytes to hTg

Proliferative responses both to hTgCS and $hTgCS_0$ were markedly higher in mice immunized with hTgCS, in comparison with mice immunized with $hTgCS_0$. In both groups, the responses to $hTgCS_0$ showed a tendency to inhibition at the highest doses, at variance with the responses to hTgCS.

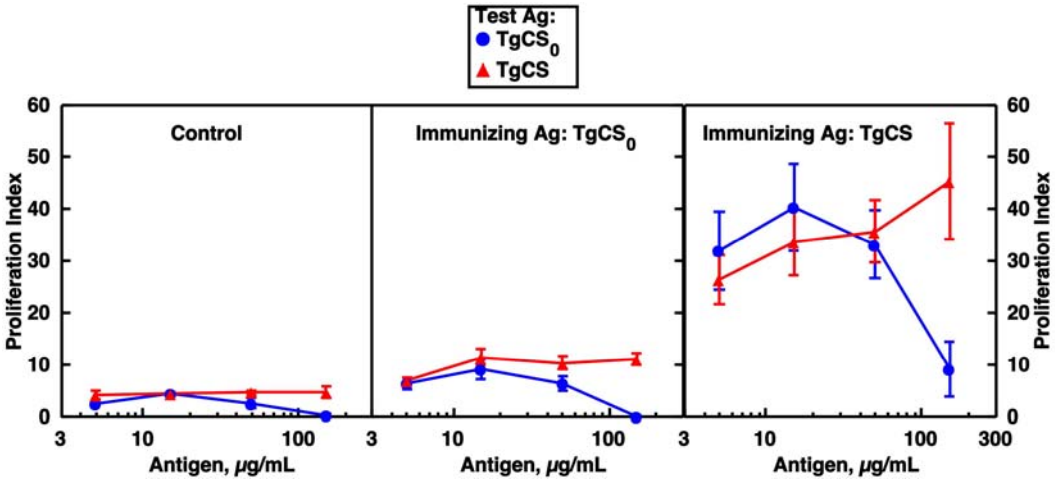

Fig. 4. Proliferative responses by splenic lymphocytes from CBA/J(H-2k) mice immunized and restimulated *in vitro* with the two forms of hTg. Mice were immunized at day 0 with 100 μg of hTgCS or $hTgCS_0$ in CFA, followed at day 8 by 50 μg of the same inìmmunnogen in IFA. On day 30, mice were sacrificed, spleens removed and splenocytes were restimulated with the indicated doses of the same antigen used for immunization. Results are expressed as means ± S.D.

Secondary proliferative responses of splenocytes to hTgCSgp and LTAGSGLRE

Secondary responses of murine splenocytes were investigated also by stimulating them with the purified hTg nonapeptide containing the site of chondroitin 6-sulfate addition to hTg at Ser2730 (referred to as hTgCSgp). The results were similar as those obtained with hTg, as the purified hTgCSgp glycopeptide – but not its non-chondroitinated, synthetic peptide homologue LTAGSGLRE - elicited much higher, dose-related levels of proliferation in animals immunized with hTgCS, in comparison with those immunized with $hTgCS_0$. Notably, the splenocytes of control animals also proliferated upon restimulation with hTgCSgp, but not with the LTAGSGLRE peptide.

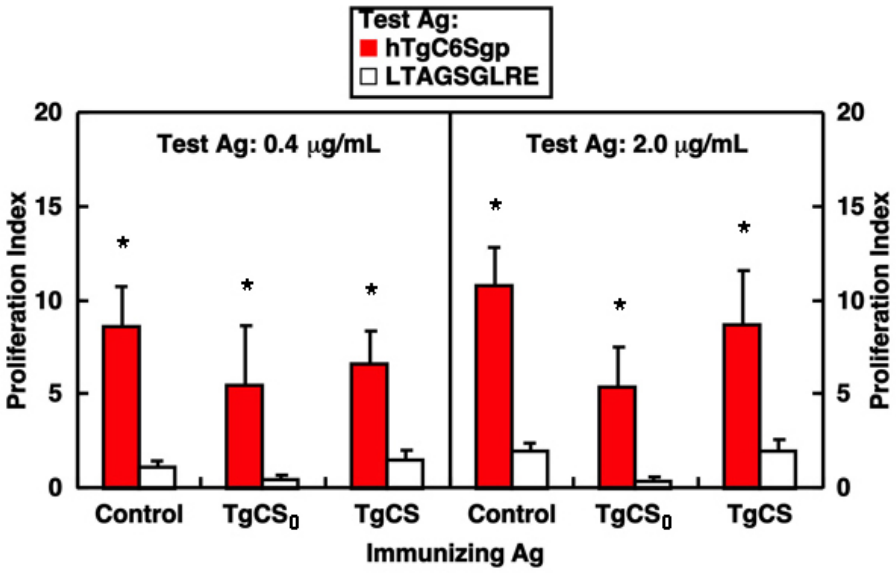

Fig. 5. Proliferative secondary responses of splenic lymphocytes of CBA/J(H-2k) mice to hTgCSgp and LTAGSGLRE. The splenocytes obtained at sacrifice from control and immunized CBA mice were stimulated *in vitro* with the purified chondroitin 6-sulfate-containing hTg peptide hTgCSgp and its non-glycosylated, synthetic homolog having the LTAGSGLRE sequence. Combined results of three esperiments are expressed as means ± S.D. * indicates the statistical significance between hTgCS and controls ($p < 0.05$).

Assay of cytokine production in secondary proliferative responses

Multiple cytokine concentrations were measured in the supernatants of the proliferation assays performed with the splenocytes from CBA/J(H-2k) mice immunized with $hTgCS_0$ and hTgCS, by the use of a multiparametric bead-based flow cytometric technique. The cytokines assayed were intended to dissect the polarization of the $CD4^+$ TH cell-dependent responses, as they included IFN-γ, GM-CSF, TNF-α, IL-2, IL-1β, IL-6, IL-4, IL-5, IL-17 and IL-10. Results are shown in Figure 6.

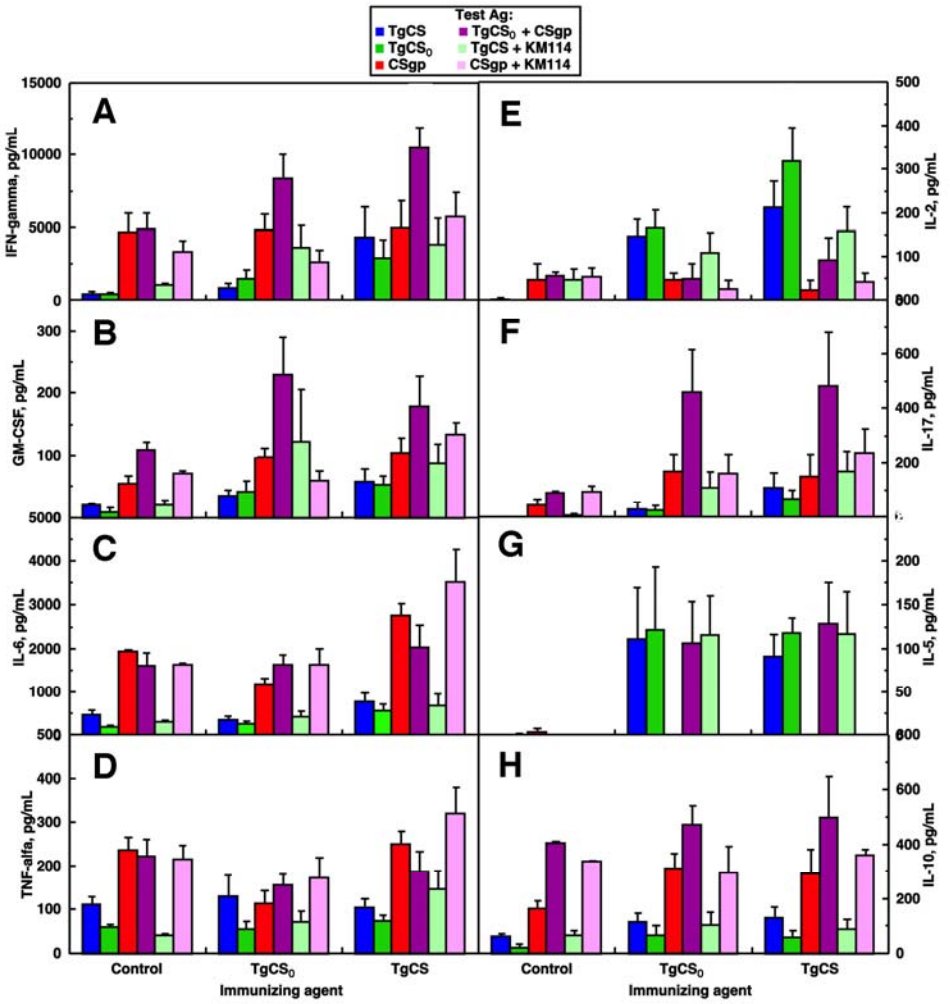

Fig. 6. Secondary proliferative responses of splenic lymphocytes from CBA/J(H-2k) mice to $hTgCS_0$, hTgCS, hTgCSgp or $hTgCS_0$ plus hTgCSgp, also in the presence of the KM114 anti-CD44 mAb. Results are means ± S.E.M. of five or six animals per experimental group. For the sake of clarity, responses to: a) PBS; b) the synthetic, non-glycosylated peptide homolog of hTgCSgp (having the LTAGSGLRE sequence) and c) the monoclonal anti-CD44 antibody KM114, which were all at baseline values in all conditions, are not shown. All ten cytokines were assayed by using a nultiparametric bead-based flow cytometric technique.

The following may be noticed in regard to IFN-γ, a typical indicator of TH1 polarization of the differentiation of $CD4^+$ TH cells (Figure 6, panel *A*): a) secretory responses to both $hTgCS_0$ and hTgCS attained higher levels in hTgCS-immune, with respect to $hTgCS_0$-immune mice, being absent or very low in

control animals; b) the addition of hTgCSgp evoked intense secretory responses of closely matching magnitude in all groups, including control animals; c) the synthetic, non-glycosylated peptide homolog of hTgCSgp, LTAGSGLRE, had no effect of its own, yielding responses close to baseline values in all cases, with all cytokines assayed (omitted in Figure 6 for the sake of clarity); d) the combined effects of $hTgCS_0$ and hTgCSgp were additive in hTgCS-immunized mice, and more than additive in $hTgCS_0$-immune mice, in which the isolated contribution by $hTgCS_0$ was lower than in hTgCS-immune mice. Parallel results were obtained with GM-CSF (Figure 6, panel *B*), another typical TH1-like cytokine, although the differences between the responses to hTg between the two experimental groups did non attain statistical significance; e) the monoclonal antibody KM114, reportedly inhibiting the binding of hyaluronic acid to CD44 (Katoh et al. 1995), did non have any significant effect on the action of hTgCS and of hTgCSgp.

The observed effects of hTgCSgp, also viewed in the light of the data reported above, concerning the proliferative responses of splenocytes, may actually represent the compound result of two distinct actions: 1) a prompt by the chondroitin 6-sulfate oligosaccharide unit of hTgCSgp to the proliferation of T cells, independent of their activation status, as it was apparent also in control splenocytes; 2) an aid to APCs in the sensitization of T cells to hTg, namely to antigenic determinants within its peptide mojety, as suggested by the fact that mice immunized with hTgCS showed improved secondary responses to both chondroitin sulfate-containing and chondroitin sulfate-devoid forms of hTg. This latter component was such that could be exerted by the chondroitin 6-sulfate oligosaccharide chain also upon secondary restimulation *in vitro* of splenocytes from $hTgCS_0$-immune mice, which had not been exposed to it *in vivo* at the time of immunization. In fact, upon restimulation, the response of $hTgCS_0$-immune mice to $hTgCS_0$ in combination with hTgCSgp, once cleared of the individual contribution by hTgCSgp, matched the response to $hTgCS_0$ (and hTgCS) observed in hTgCS-immune mice.

IL-2 secretion in response to $hTgCS_0$ and hTgCS was larger in mice immunized with the latter, in keeping with the supposed enhancing role of the chondroitin 6-sulfate oligosaccharide chain of hTg in the TH1 differentiation of hTg-reactive T cells. However, no significant IL-2 production accompanied the stimulation with hTgCSgp in any of the experimental groups. This finding contrasted with the similarity of the roles commonly attributed to IFN-γ and GM-CSF and IL-2 in the TH1 polarization of TH cell differentiation. In this regard, it has been reported that quantitative ratios of cytokine expression may fluctuate in response to changes in activation conditions, such as the quantity and the quality of specific or non-specific TCR ligands. In particular, truncated forms of a myelin basic protein (MBP) peptide induced the secretion of considerable amounts of IFN-γ from MBP-reactive T cells, without inducing IL-2 production or

proliferation. By contrast, the staphilococcal enterotoxin superantigen elicited both a proliferative response and Il-2 production, without inciting the secretion of IFN-γ or TNF-α (Sun et al. 1995). These reports, as well as our observations, illustrate the complexities of T cell activation and differentiation, particularly in relation with the role played by the modifications of autoantigens and different modes of interaction.

IL-6 and TNF-α invite common comments, as they seemingly obeyed a common response pattern: a) no significant differences in the responses to hTg between different groups were detected; b) hTgCSgp elicited as strong responses as observed with IFN-γ and GM-CSF, in all conditions in which it was present; c) no synergistic effects of the combined use of $hTgCS_0$ and hTgCSgp were observed in $hTgCS_0$-immune mice, and not even additive effects in hTgCS-immune mice; d) the administration of the KM114 anti-CD44 mAb in combination with the hTgCSgp glycopeptide was associated with increased IL-6 and TNF-α secretion in hTgCS-immune animals, at variance with the production of IFN-γ and GM-CSF. Given the predominant origin of IL-6 from stimulated antigen-presenting cells, the observations referred above seem to indicate that, aside T cells, the chondroitin 6-sulfate oligosaccharide chain of hTg was also able to exert its effects on some subpopulation(s) of APCs. This is in line with a number of observations (Rachmilewitz et al. 1998; Termeer et al. 2001). Proof of this may reside in the lack of changes detected, between different experimental groups, in the sensitization and responses of splenocytes to hTg, necessarily implying the contribution of T cells and, thus, better reflected by changes in IFN-γ, GM-CSF and IL-2. It may be argued that TNF-α secretion is normally also a hallmark of TH1 cell differentiation. It is possible, however, that, under the circumstances of our experiments, most of it derived from activated macrophages, DC or NK cells. This, in turn, may reflect a dissociation in the pattern of cytokine production by differentiating TH1 cells of a similar kind as the one that was already commented upon, between the secretion of IFN-γ and GM-CSF, on one hand, and IL-2, on the other hand.

Low-grade IL-17 production in response to hTg was detected only in hTgCS-immune mice, and low levels of IL-17 were also produced in response to hTgCSgp in immunized, but not in control animals. However, a synergistic effect between $hTgCS_0$ and hTgCSgp was apparent in immunized mice. IL-17 production is a hallmark of differentiation of TH17 cells, a specific subpopulation of TH cells, which is involved in the control of the inflammatory response, as well as in the development of autoimmunity. Even though factors promoting the development of the TH1 phenotype are able to suppress the differentiation of TH17 cells, there is ample evidence supporting the idea that both subpopulations of cells may cooperate in the induction of experimental autoimmune encephalitis (EAE) in

mice (Bettelli et al. 2007). Our data favour the involvement of IL-17-producing cells also in the development of EAT.

Significant IL-5 production was elicited by both forms hTg forms in both groups of immunized mice, but not in control animals, whereas hTgCSgp had no effect at all in any of them (Figure 6, panel *G*). It is quite well established that Tg is able to elicit, together with T cell responses, also well defined antibody responses, From our data, it was also quite clear that the chondroitin 6-sulfate oligosaccharide unit of hTg was not able to affect the differentiation of TH2 cells anyhow. By contrast, the levels of IL-4 produced were irrelevant in all groups, under all experimental conditions.

IL-10 was produced in all experimental groups in response to hTgCSgp and, to a lesser extent, both forms of hTg. The simultaneous production of cytokines characteristic of TH1 cells (IFN-γ and IL-2) and TH2 cells (Il-5 and IL-10) was previously observed with rat MBP-reactive T cells (Sun *et al.* 1995). It has also been reported that IL-10 has the ability of either enhancing or inhibiting the deveolpment of EAE (Sun *et al.* 1995). Like for T cells producing IL-10 in response to MBP, it remains to be established whether the ability of murine T cells to produce IL-10 in response to hTgCSgp or to $hTgCS_0$ in the presence of hTgCSgp is directly involved in their thyroiditogenic activity.

Finally, the results obtained with the KM114 anti-CD44 monoclonal antibody also deserve comment. The usage of this mAb as a presumptive competitor of hTgCSgp was based on the reports concerning, on one hand, the involvement of CD44 in the effects of the chondroitin sulfate-containing form of the invariant chain (CD74) on mitogenic and allogenic T cell responses (Naujokas *et al.* 1993) and, on the other hand, the inhibitory effect of the KM114 mAb in the binding of hyaluronic acid (HA) to CD44 (Zheng *et al.* 1995). The apparent lack of effect of the KM114 mAb in most cytokine secretory responses to hTgCS and hTgCSgp, with the only possible exception of IL-2 (Figure 6, panel *E*), does not rule out, in our opinion, the possible involvement of CD44 as a receptor for the chondroitin 6-sulfate oligosaccharide chain of hTg. As a matter of fact, the interactions of a number of mAbs with CD44 were described, which were very selective as to their epitopic specificities and functional consequences. Thus, KM114 may still be inhibiting the binding of HA, but not of hTgCSgp, to CD44 (Zheng *et al.* 1995). Only further experimentation in CD44-knockout mice may be able to prove or rule out the involvement of CD44 in the effects of the chondroitin 6-sulfate oligosaccharide unit of hTg.

Assay of surface expression of H-2 class II and CD74 antigens in isolated DCs from CBA/J(H-2k) mice immunized with $hTgCS_0$ and hTgCS

We assayed the surface expression of H-2 class II and CD74 (invariant chain) antigens in DCs isolated by immunomagnetic positive selection with anti-CD11c fluorescent antibodies from the spleens of mice immunized with $hTgCS_0$ and hTgCS, in response to incubation for 2 h with either immunogen. Similar levels of class II H-2 expression were detected in all experimental groups, and no changes were observed in association with the loading of DCs with the two forms of hTg. By contrast, a slight increase in CD74 expression was observed in DCs from hTgCS-immunized mice, in comparison with control and $hTgCS_0$-immunized mice. This experiment was undertaken in order to probe the hypothesis that loading DCs with chondroitin sulfate-bearing hTg may someway affect the intracellular trafficking of invariant chain (CD74), a fraction of which is also modified by the addition of chondroitin sulfate, and whose targeting to the cell surface is indeed directed by its chondroitin sulfate-substituted subunits (Arneson *et al.* 2007). Although the results indicate that feeding DCs with hTgCS did not alter the intracellular compartmentalization of CD74, they also suggest that some changes in environmental conditions, most probably the cytokine milieu in which the DCs from hTgCS-immunized mice matured, may have affected the level of CD74 expression at their surface. This, in turn, may favourably affect the ability of DCs to activate T cell responses, as previously reported (Naujokas *et al.* 1993). As the expression of CD74 in response to such pro-inflammatory stimuli as the IFNγ produced by NK, $CD4^+$ and $CD8^+$ cells is coordinately regulated with those of MHC class II antigens, HLA-DM and HLA-DQ, it is somewhat surprising that the effects seen here on the expression of CD74 were not paralleled by similar effects on H-2 class II antigen expression, which may indicate that regulative control mechanisms unique to CD74 are involved.

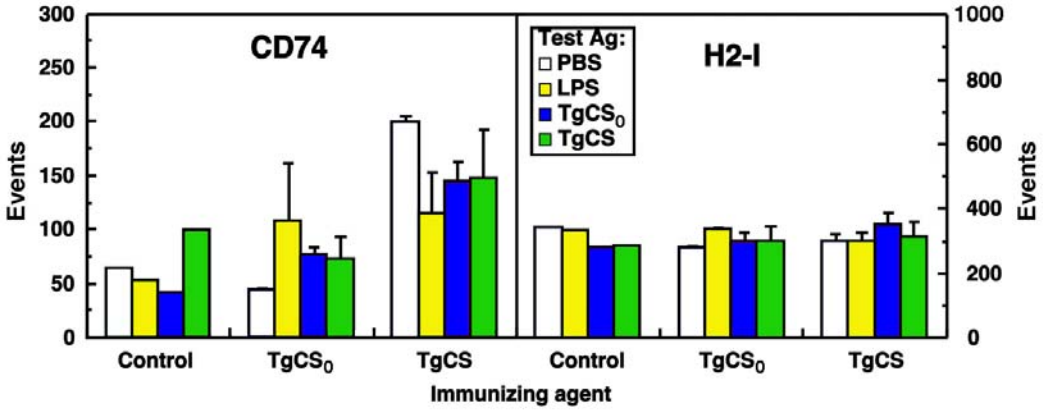

Fig. 7. Surface expression of H-2I and CD74 by DCs isolated from the spleens of CBA/J(H-2k) immunized with $hTgCS_0$ and hTgCS and incubated *in vitro* with both immunogens.

Freshly isolated DCs from the spleens of mice sacrificed at day 30 from immunization were incubated for 2 h with antigens, after which DCs were plated in 24-well plates at the concentration of 2 x 10^5 cells/well,,incubated with fluorescent antibodies to H2-I or CD74 for 20 min in the dark at 4° C and subjected to flow cytometric analysis. Stimulation with 1 μg of LPS was used as a positive control. Results are expressed as means ± S.E.M.

Production of antibodies against $hTgCS_0$ and hTgCS

Immunization with either form of hTg was accompanied by the production of significant amounts of IgG antibodies directed against $hTgCS_0$ and hTg-. Total IgG, IgG1 and IgG2a concentrations did not vary between experimental groups. No anti-hTg antibodies were detected in controls.

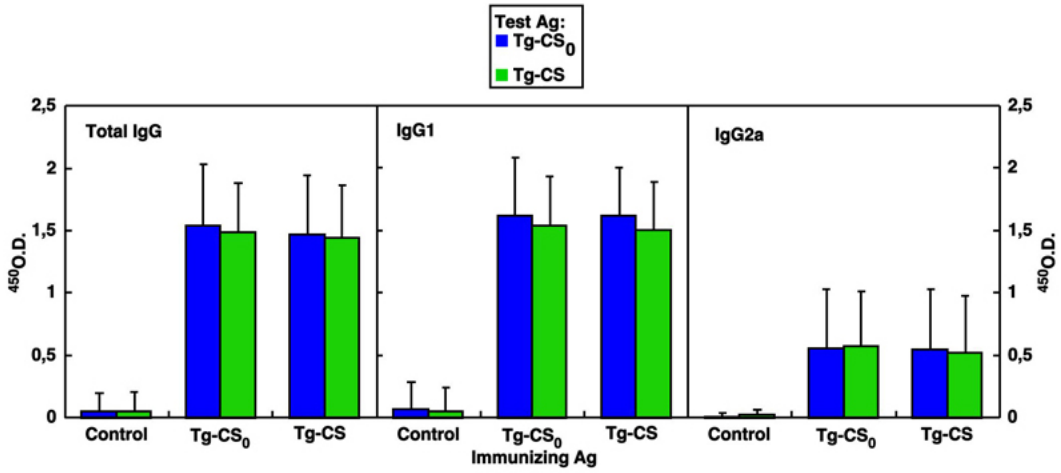

Fig. 8. Concentrations of antibodies of the IgG class and IgG1 and IgG2a subclasses directed against $hTgCS_0$ and hTgCS in the sera of CBA/J(H-2^k) mice immunized with both antigens. Antigens were used at the concentration of 1 μg/well. Results are expressed as means ± S.D. of the combined results from 3 (IgG) and 6 experiments (IgG1 and IgG2a).

These results outline the lack of significant effects of chondroitin 6-sulfate addition to hTg on its ability to elicit the production of antibodies, which was already suggested by the lack of effects of hTgCS and of the hTgCSgp glycopeptide in the production of type TH2 cytokines in secondary proliferative responses of splenocytes from mice immunized with both forms of hTg.

Next, we investigated whether there were antibodies recognizing the hTgCSgp glycopeptide in the sera of mice immunized with $hTgCS_0$ or hTgCS. Results are shown in Figure 10.

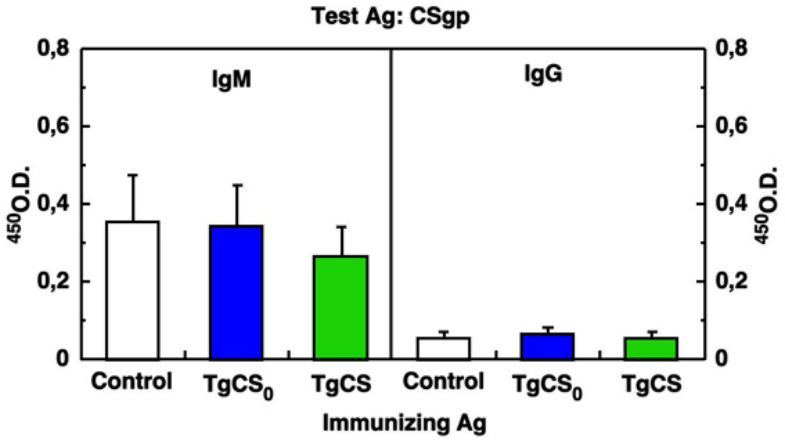

Fig. 9. Concentrations in the sera of CBA/J(H-2k) mice immunized with hTg and controls of IgM antibodies against the biotinylated, purified chondroitin 6-sulfate-containing glycopeptide hTgCSgp. Combined results of two immunization experiments are expressed as means ± S.E.M.

The differences between groups were not statistically significant. The presence of antibodies recognizing hTgCSgp in the sera of immunized animals did not seem to reflect a specific adaptive immune response to the glycosylated peptide, as no IgG and comparable levels of IgM antibodies were detected in control and immunized animals. This was not surprising, in the light of the diffusion and abundance of chondroitin sulfate-containing glycosaminoglycans and proteoglycans in the extracellular matrix of connective tissues throughout the mouse body. Low levels of IgM antibodies against this kind of polysaccharide antigens are usually produced by $CD5^+$ B1 cells, inhabiting the marginal zone of spleen and the serous membranes lining body cavities, within the context of type 2 thymus-independent responses to ubiquitous and abundant self antigens. Because these T-independent responses may still entail a limited isotype switch to IgG3 immunoglobulins, under circumstances in which help is provided by TH cells, upon non-clonotypic activation by certain stimuli, whose nature is still poorly defined, we also assayed the mice sera for anti-hTgCSgp antibodies of the IgG3 subclass, but detected none.

As the chondroitin 6-sulfate oligosaccharide unit of hTg did non appear to be involved in the differentiation of Th2 cells and of B cells producing antibodies against hTg, and did not seem to constitute or participate in any B cell-dependent epitope, either, we next investigated whether it may be affecting the processing and/or presentation of neighboring epitopes in the region of the polypeptide chain of hTg surrounding the site of chondroitin 6-sulfate addition to Ser2730.

Therefore, we designed a set of six dodecameric peptides, overlapping each other by four residues at both extremities and spanning the carboxy-terminal region of the hTg sequence, between residues 2702-2749, and had them chemically synthesized. The extreme carboxy-terminal peptide, containing the preferential site of T3 formation in Tg, was synthesized with T3 at position 2747, in view of the fact that significant T3 production is normally found at this hormonogenic site *in vivo*, even at low levels of iodine availability. The peptides were conjugated with biotin and used in ELISA assays of IgG concentrations in the sera of experimental mice. Results are shown in Figure 10. No assayable levels of IgG antibodies were found in control animals, even at low serum dilution. Significant levels of IgG antibodies were detected only against the carboxy-terminal peptide hTg2738-2749(T3), and – to a lower extent – the preceding peptide hTg2731-2742.

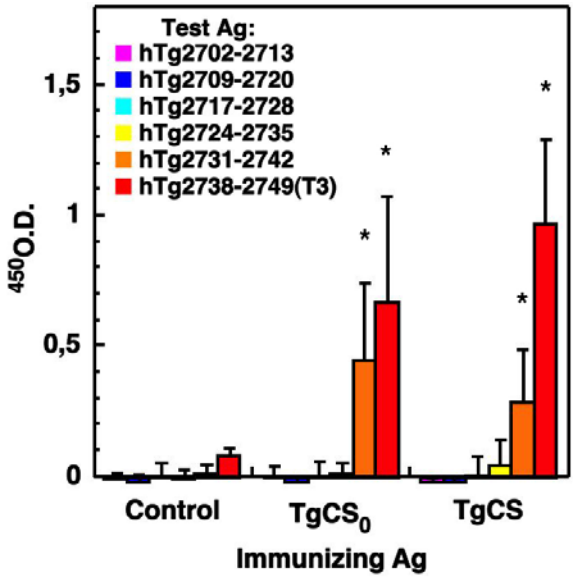

Fig. 10. Concentrations in the sera of CBA/J(H-2k) mice immunized with hTg and controls of IgG antibodies recognizing a set of chemically synthesized, overlapping dodecameric peptides, spanning the hTg sequence between amino acid residues 2702-2749. Combined results of two immunization experiments are expressed as means ± S.D. * indicates the statistical significance between mice immunized with either form of hTg and controls ($p < 0.05$).

The above findings, on one hand, let us rule out that the chondroitin 6-sulfate oligosaccharide unit of hTg may bear any impact on the availability of B cell-dependent epitopes in the neighboring sequence region to processing and presentation, at least within the sequence range that we explored. This may hold true, notwithstanding the general implications of the post-translational

modification with oligosaccharide chains in the antigenicity of proteins, which were recalled in the *Introduction* to the present work. As a matter of fact, the inventory and location of B cell-dependent epitopes of Tg from various animal species has been thoroughly investigated during the last three decades (reviewed in Gentile *et al.* 2004). The nearest epitope, with respect to the site of chondroitin 6-sulfate addition to hTg, that was documented in detail, by the use of a set of chemically synthesized, overlapping peptides, spanning altogether the entire cholinesterase-homologous domain of hTg (residues 2181-2749), was reported at residues 2651-2670 (Thrasyvoulides et al. 2001).
The data presented, however, do not rule out that effects of the chondroitin 6-sulfate addition on the amenability of hTg to the processing and presentation of B cell-dependent epitopes may be resented at other sites of the hTg structure, corresponding to more distant regions of the hTg sequence, which may come into contact or in the vicinity of the chondroitin 6-sulfate chain, as a consequence of the folding and dimerization of hTg polypeptide chains.

Concerning the immunogenicity of the T3-containing carboxyterminal domain of hTg, ours is the first documentation that immunization of EAT-susceptible mice with hTg can elicit the production of antibodies directed towards the extreme carboxy-terminal sequence of hTg, containing the site of preferential T3 formation at Tyr 2747. Other Authors previously reported the detection of antibodies in mice immunized with chemically synthesized peptides centered upon two major hormonogenic sites of Tg, corresponding to residues 1-12 and 2549-2560 of hTg (Kong et al. 1995; Wan et al. 1997). A number of evidences suggest that Tg antigenicity is influenced by its iodine and hormone content. CBA lymph node cells (LNC) primed in vivo with mouse Tg or with the T4-containing hTg peptide 2549-2560 were able to adoptively transfer EAT to naive syngeneic hosts, following in vitro activation with the peptide (Hutchings et al. 1992). Antigenicity appeared to be related as much to the formation of iodothyronine (an event naturally occurring in the course of iodination) as to iodination: in fact, hTg peptide 2549-2560 containing thyronine (T0) at position 2553 was also able, in CBA mice, to induce proliferative responses and activate adoptive transfer of EAT by LNCs (primed with mouse Tg or the peptide itself), after in vitro expansion, although with lower efficiency than the corresponding T4-containing peptide. Also peptides T4(1-12) and T0(1-12), containing T4 or T0 at position 5, respectively, stimulated weakly, in rank order of descending potency, mouse Tg-primed T cells in vitro (Kong et al. 1995). Conceivably, Tg normally present in low concentrations in the serum, which is almost entirely devoid of iodine and appears to be secreted from the basolateral pole of the follicular cells, without reaching the apical site of iodination (Ikekubo et al. 1981; Schneider et al. 1983), may have little immunogenic power, being a self-antigen participating in the process of clonal deletion during embryonic development (Savin et al. 1990), whereas the

more iodinated and hormone-bearing Tg, normally segregated within thyroid follicles and only released into the blood under abnormal conditions, such as thyroid cancer and acute or subacute thyroiditis, may activate specific T cells recognizing iodination-dependent antigenic determinants.

5 CONCLUSIONS

The data presented here indicate that the post-translational addition of the chondroitin 6-sulfate oligosaccharide chain at Ser2730 of hTg has a profound impact on the ability of hTg to induce experimental autimmune thyroiditis in genetically sysceptible CBA/J(H-2k) mice. It appears that chondroitin 6-sulfate-containing hTg molecules (hTgCS), which in previous studies were found to represent regularly a broadly variable percentage of whole hTg (Conte et al. 2006), may actually be the sole fraction of hTg actually capable of inducing EAT in mice. The variability in the percent abundance of hTgCS may explain the poor behavior of EAT as an animal model of thyroid autoimmune disease, and the often scarce content of hTgCS in whole hTg may help explain the somewhat unpredictable outcome of immunization and the focal distribution and limited extension often displayed by the experimentally induced disease. In view of the ease of separation of hTgCS from the rest of chondroitin 6-sulfate-devoid $hTgCS_0$, our observations may be of some value for investigators in the field.

The mechanism(s) by which the chondroitin 6-sulfate unit of hTg exerts its effect(s) on the immunopathogenicity of hTg appear to be complex. The results obtained with isolated DCs indicate that they may partly pertain to the cytokine milieu in which DC mature and attend to their antigen-presenting duties. Undoubtedly, the core effects brought about by the oligosaccharide unit appeared to affect T cells. Whichever the effects were on DCs, most probably they were indirect reflections of actions directed onto T cells. However, in the light of the effects reported for chondroitin sulfate on a variety of cells, including macrophages, DC and B cells, this point deserves further investigation, which is to include also to the role of NK cells.

The chondroitin 6-sulfate unit deeply affected the priming of murine T cells with hTg, with consequences both on thyroid infiltration and damage *in vivo*, and on secondary lymphocyte responses to hTg *in vitro*. It appeared capable of doing so both as an intrinsic component of hTgCS, and as an independent adjunct to $hTgCS_0$, within the experimental setting of proliferative reactions. Moreover, a relevant part of its effect(s) was brought about even in the absence of the main protein mojety of hTg, i.e., in the form of hTgCSgp, a glycopeptide with a very limited nonameric peptidic component, which had no effect of its own. The data reported here data raise a number of questions: 1) the first question pertains the mechanism(s) whereby the chondroitin 6-sulfate unit of hTgCS normally accesses the interaction with T cells: are they related with those which also mediate the transport of chondroitin sulfate-containing invariant chain (CD74) molecules to the surface of APCs? Do they lead to the immunological synapse?; 2) The second major questions relates to the mode of interaction of the chondroitin 6-sulfate unit with T cells: Are its actions on proliferation and secretion of resting T cells and of

hTg-reactive T cells related? Do they involve the same or different target molecules? Is the TCR involved in such interactions, even in a non-specific manner? Are non-clonotypic receptors at the surface of T cells involved, on their own or together with the TCR? Is CD44 involved, as suggested by prior observations? Which is the relationship between the activation state of T cells and their sensitivity to the effects of chondroitin 6-sulfate? Answering these questions will require the dissection of complex mechanisms at the interface between antigen-presenting cells and T cells, including the TCR-mediated activation of T cell responses and more than one co-stimulatory mechanism. However, the experimental system highlighted by these introductory results may turn out to be a privileged standpoint for enriching our understanding of the immunological synapse.

Human thyroglobulin emerges from this study as being seen from a novel perspective, that of an antigen which is bearing in itself sort of a "built-in adjuvant", which can make a potentially dangerous trigger of autoimmunity out of an otherwise innocuous self antigen. All this may be the consequence of some impact which the chondroitin 6-sulfate mojety of hTg seems to have onto T cell responses. As the chondroitin 6-sulfate addition to hTg appears to be a regulated physiological process, whose extent may vary in relation with factors as yet not well understood, it may be of the foremost importance to investigate in further detail which pathophysiological conditions may alter the balance between the chondroitin 6-sulfate-devoid and chondroitin 6-sulfate-containing fractions of hTg, and which relationship may exist between such alterations and autoimmune thyroid disease, not only in animal models, but also in humans.

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A Single Chondroitin 6-Sulfate Oligosaccharide Unit at Ser-2730 of Human Thyroglobulin Enhances Hormone Formation and Limits Proteolytic Accessibility at the Carboxyl Terminus

POTENTIAL INSIGHTS INTO THYROID HOMEOSTASIS AND AUTOIMMUNITY[*] [S]

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We localized the site of type D (chondroitin 6-sulfate) oligosaccharide unit addition to human thyroglobulin (hTg). hTg was chromatographically separated into chondroitin 6-sulfate-containing (hTg-CS) and chondroitin 6-sulfate-devoid (hTg-CS_0) molecules on the basis of their D-glucuronic acid content. In an ample number of hTg preparations, the fraction of hTg-CS in total hTg ranged from 32.0 to 71.6%. By exploiting the electrophoretic mobility shift and metachromasia conferred by chondroitin 6-sulfate upon the products of limited proteolysis of hTg, chondroitin 6-sulfate was first restricted to a carboxyl-terminal region, starting at residue 2514. A single chondroitin 6-sulfate-containing nonapeptide was isolated in pure form from the products of digestion of hTg with endoproteinase Glu-C, and its sequence was determined as LTAG*X*GLRE (residues 2726–2734, *X* being Ser^{2730} linked to the oligosaccharide chain). In an *in vitro* assay of enzymatic iodination, hTg-CS produced higher yields of 3,5,5′-triiodothyronine (T_3) (171%) and 3,5,3′,5′-tetraiodothyronine (T_4) (134%) than hTg-CS_0. Unfractionated hTg behaved as hTg-CS. Thus, chondroitin 6-sulfate addition to a subset of hTg molecules enhanced the overall level of T_4 and, in particular, T_3 formation. Furthermore, the chondroitin 6-sulfate oligosaccharide unit of hTg-CS protected peptide bond Lys^{2714}–Gly^{2715} from proteolysis, during the limited digestion of hTg-CS with trypsin. These findings provide insights into the molecular mechanism of regulation of the hormonogenic efficiency and of the T_4/T_3 ratio in hTg. The potential implications in the ability of hTg to function as an autoantigen and into the pathogenesis of thyroidal and extra-thyroidal manifestations of autoimmune thyroid disease are discussed.

Thyroglobulin (Tg),[2] the molecular site of thyroid hormone formation, is a large homodimeric glycoprotein with an M_r of 660,000. It is also a major antigen, involved in the pathogenesis of thyroid autoimmunity. In fact, experimental autoimmune thyroiditis (EAT) can be induced in genetically susceptible mice by immunization with human thyroglobulin (hTg) in complete adjuvant, and autoantibodies to hTg are found in the blood of humans affected with autoimmune thyroid disease (AITD), even though their pathogenic significance is unclear (1). Several post-translational modifications contribute to the molecular microheterogeneity of hTg, including iodination, glycosylation, phosphorylation, and sulfation (reviewed in Ref. 2). Iodine addition and hormone formation at specific sites have the most obvious implications for thyroid function, but the effects of glycosylation on the hormone-forming efficiency at specific sites and on the antigenicity of Tg have also been documented (3, 4). hTg is modified with the addition of several oligosaccharide units of different kinds, among which the *N*-linked type A (high-mannose) and type B (complex) units have been characterized best as to their composition and localization (5–7). Type C units are linked to serine and threonine by *O*-glycosidic bonds and contain D-galactosamine (5). Another *O*-linked type D oligosaccharide unit was described, which was composed of a repeating D-glucuronic acid-*N*-acetyl-D-galactosamine sulfate disaccharide attached to the polypeptide chain through a D-galactosyl-D-xylosyl-serine linkage region (8). Further study

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[2] The abbreviations used are: Tg, thyroglobulin; AITD, autoimmune thyroid disease; APC, antigen-presenting cell; DMTCC, 3,3′-diethyl-9-methyl-4,5,4′,5′-dibenzothiacarbocyanine; EAT, experimental autoimmune thyroiditis; GAG, glycosaminoglycan; h8-$deCS_{TR}$, peptide h8-CS_{TR} digested with chondroitinase ABC; hTg, human thyroglobulin; hTg-CS, type D (chondroitin 6-sulfate) oligosaccharide unit-containing hTg; hTg-CS_0, type D (chondroitin 6-sulfate) oligosaccharide unit-devoid hTg; hTg-CSgp, chondroitin 6-sulfate-containing glycopeptide 2726–2734 of hTg; Ii, invariant chain; Ii-CS, chondroitin sulfate-containing invariant chain; LPO, lactoperoxidase; MMI, 2-mercapto-1-methylimidazole; PVDF, polyvinylidene difluoride; Q-IEC, ion-exchange chromatography on trimethylamino-substituted Q-Sepharose; T_3, 3,5,5′-triiodothyronine; T_4, 3,5,3′,5′-tetraiodothyronine (thyroxine); TAO, thyroid-associated ophthalmopathy; TPO, thyroid peroxidase; ES-MS, electrospray mass spectrometry; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; HPLC, high pressure liquid chromatography; TSH, thyrotropin.

indicated that the repeating disaccharides were of the chondroitin 6-sulfate type (9, 10). However, the number and localization of type D oligosaccharide units in hTg were not determined.

A number of studies have documented the influence of oligosaccharide chains in the processing and/or presentation of glycoproteic antigens by antigen-presenting cells (APCs) (11–16), and the involvement of chondroitin 6-sulfate oligosaccharide chains in the modulation of cellular immune responses (17–20). Prompted by these observations, we began to determine in detail the number and localization of chondroitin 6-sulfate oligosaccharide unit(s) in hTg as a basis for further studies on their influence on the immunopathogenicity of hTg in a murine model of EAT. We found that the addition of chondroitin 6-sulfate unit(s) is a major source of molecular microheterogeneity of hTg. In dozens of hTg preparations examined, it was regularly found in a percentage of hTg molecules varying from 32.0 to 71.6% of total. Moreover, we determined that a single chondroitin 6-sulfate unit per hTg polypeptide chain is linked to Ser^{2730}. We also observed changes in the hormone-forming efficiency of hTg and in the proteolytic accessibility of the extreme carboxyl-terminal region of hTg, associated with the presence of the chondroitin 6-sulfate oligosaccharide chain. The former observation contributes to delineate a general mechanism, by which modifications in the composition and number of *N*-linked and *O*-linked oligosaccharide units determine changes in the hormone-forming efficiency of the main hormonogenic domains at both hTg extremities, whereas the latter finding supports the potential role of the chondroitin 6-sulfate oligosaccharide unit in modifying the susceptibility of hTg to processing by APCs and the repertoire of hTg epitopes thus generated.

EXPERIMENTAL PROCEDURES

Materials—Thermolysin from *Bacillus thermoproteolyticus rokko* (EC 3.4.24.4), L-1-tosylamide-2-phenylethylchloromethyl-treated trypsin from bovine pancreas (EC 3.4.21.4), endoproteinase Glu-C from *Staphylococcus aureus* (EC 3.4.21.19), lactoperoxidase (LPO) from bovine milk (EC 1.11.1.7), and glucose oxidase from *Aspergillus niger* (EC 1.1.3.4) were purchased from Sigma. Protease-free chondroitinase ABC from *Proteus vulgaris* (EC 4.2.2.4) was from Roche Applied Science. Aminopeptidase M from porcine kidney (EC 3.4.11.2) and Pronase from *Streptomyces griseus* were from Calbiochem. Sephacryl S-300 HR, HiTrap™ Q-Sepharose HP, DEAE-Sepharose Fast Flow, and Sephadex G-50 fine were obtained from GE Healthcare. Bio-Gel P-2 and electrophoresis products were from Bio-Rad. Immobilon P membranes and Centriprep 30 concentrators were from Millipore (Vimodrone, Italy). HPLC-grade solvents were from Carlo Erba (Milan, Italy). BCA protein assay reagent was from Pierce. 3,3′-Diethyl-9-methyl-4,5,4′,5′-dibenzothiacarbocyanine (DMTCC, Stains All™) was from ICN Biomedicals (Milan, Italy). Solid-phase radioimmunoassay kits for total 3,5,5′-triiodothyronine (T_3) and 3,5,3′,5′-tetraiodothyronine (thyroxine, T_4) were from Diagnostic Products Corp. (Los Angeles, CA). Marker kit MW-SDS-17S and other analytical grade chemicals were from Sigma.

Purification of hTg—hTg was prepared as described (21) from informed euthyroid patients hemilaryngectomized for non-thyroidal disease and patients undergoing thyroidectomy for nonfamilial, simple, or multinodular goiter. Protein concentration was assayed by measuring the optical absorbance at 280 nm, using an extinction coefficient of 10 cm^{-1} for a 1% solution. Iodine content was assayed as described, using L-thyroxine as the standard (22).

Ion-exchange Chromatography of hTg on HiTrap™ Q-Sepharose HP (Q-IEC)—hTg molecules containing type D (chondroitin 6-sulfate) oligosaccharide units (hTg-CS) were separated from residual hTg molecules, devoid of type D units (hTg-CS_0), by ion-exchange chromatography on trimethylamino-substituted Q-Sepharose (Q-IEC) using 5-ml HiTrap™ Q-Sepharose HP columns, equilibrated in 0.025 M Tris/HCl, pH 7.4 (buffer A). Up to 20 mg of hTg in buffer A, plus 0.05 M NaCl, were applied to a column. After washing with buffer A, a linear gradient from 0 to 100% of buffer B (1.2 M NaCl in buffer A) was developed in 24 min at the flow rate of 2.5 ml/min. One-ml fractions were analyzed or stored at −80 °C until use.

Compositional Analysis of Q-IEC Fractions of hTg—D-Glucuronic acid was assayed in Q-IEC fractions by the *meta*-hydroxybiphenyl method (23), using the same compound (0–5 μg) as the standard. Duplicate samples of 30–500 μg of hTg were dialyzed against 0.01 M NH_4HCO_3, dried in centrifugal evaporator, and redissolved in 0.2 ml of double-distilled H_2O in borosilicate Pyrex tubes. Samples were processed by the addition of 1.2 ml of 0.0125 M $Na_2B_4O_7 \cdot 10H_2O$ in H_2SO_4 and, after heating in boiling water bath for 5 min and cooling on ice, 0.02 ml of 0.15% *meta*-hydroxybiphenyl in 0.5% NaOH, after which the optical absorbance at 520 nm was read. Correction for aspecific color development was provided by a replicate set of samples, which were treated identically, with the exclusion of *meta*-hydroxybiphenyl in the 0.5% NaOH reagent. Total neutral hexoses were assayed in duplicate samples of 20–300 μg of hTg by the anthrone method (24), using D-galactose (0–20 μg) as the standard. Sialic acid was assayed in duplicate samples of 40–500 μg of hTg by the thiobarbituric acid method (25), using *N*-acetylneuraminic acid (0–15 μg) as the standard. Iodine was assayed in duplicate samples of 30–200 μg of hTg, using the method cited (22).

Enzymatic in Vitro Iodination of hTg and Analysis of the Iodine, T_3, and T_4 Content—hTg from goiters, with an iodine content not exceeding 0.09% on a weight basis, was iodinated enzymatically *in vitro*. Bulk iodination of 20 mg of unfractionated hTg, at the concentration of 0.45 g/liter, was performed in 0.02 M imidazole/HCl, pH 7.0, using 2 μg/ml of LPO from bovine milk, 4×10^{-5} M potassium iodide, 1×10^{-3} M D-glucose, and 0.19 μg/ml glucose oxidase from *A. niger*. Iodination was stopped with 0.05 M 2-mercapto-1-methylimidazole (MMI). Comparative iodination of unfractionated hTg and of hTg-CS_0 and hTg-CS subfractions of the same hTg preparation was performed with 0.65 mg of each protein, under identical conditions, except that 7.5×10^{-5} M potassium iodide, and 0.21 μg/ml glucose oxidase were used. Aliquots of 0.1 mg of hTg were withdrawn at 5, 15, 30, 50, 70, and 90 min, supplemented with MMI, dialyzed against 0.01 M NH_4HCO_3, 5×10^{-3} M NaCl, and assayed in duplicate for protein content, using the BCA protein assay reagent and bovine serum albumin as the standard, and for iodine content, as already described. For the assay of T_3 and T_4, 10–15

μg of hTg were hydrolyzed at 37 °C with Pronase, at the enzyme/substrate weight ratio of 1:1, in 0.2 ml of 0.1 M Tris/HCl, 0.05 M MMI, pH 8.0, to which 15 μl of toluene were added. After 24 h, aminopeptidase M was added, at the enzyme/substrate weight ratio of 1:10, and digestion was prolonged for 24 h at 37 °C, after which T_3 and T_4 were measured by solid-phase radioimmunoassays in antibody-coated tubes (26). In both assays, ^{125}I-labeled T_3 or T_4 competed with the respective hormone in the test samples for antibody sites. After the tubes were decanted and radioactivity was measured, the T_3 or T_4 concentration was obtained by interpolation from a calibration curve.

Limited Proteolysis of hTg—hTg, at the concentration of 1 g/liter in 0.05 M Tris/HCl, 0.1 M NaCl, pH 7.4, was digested with thermolysin, at the enzyme/substrate weight ratio of 1:50, at 30 °C for 80 min, or with L-1-tosylamide-2-phenylethylchloromethyl-treated trypsin (henceforth referred to as trypsin), at the enzyme/substrate weight ratio of 1:100, at 30 °C for 20 or 40 min. Proteolytic digestion was stopped with 3×10^{-5} M antipain, 2×10^{-6} M aprotinin, 5×10^{-4} M benzamidine, 4×10^{-5} M leupeptin, 1×10^{-4} M Nα-*p*-tosyl-L-lysine chloromethyl ketone, 2×10^{-5} M phenylmethylsulfonyl fluoride, 5×10^{-3} M EDTA and, in the case of trypsin, soybean trypsin inhibitor, at the inhibitor/enzyme weight ratio of 3:1. Thereafter, concentrated SDS-PAGE sample buffer was added to a final concentration of 0.01 M Tris/HCl, pH 6.8, 1% SDS, 5% 2-mercaptoethanol (v/v), 1.36 M glycerol, 0.0025% bromphenol blue, and the samples were heated in a boiling water bath for 1.5 min and immediately subjected to SDS-PAGE.

Enzymatic Digestion of Chondroitin 6-Sulfate Oligosaccharide Units of hTg, hTg Subfractions, and hTg Proteolytic Fragments with Chondroitinase ABC—hTg, or its proteolytic fragments in 0.1 M NaCl, 0.05 M Tris/HCl, pH 7.4, were supplemented with equal volumes of 0.1 M sodium acetate, 0.1 M Tris/HCl, pH 8.0. Pooled fractions from the Q2 peak of the Q-IEC of hTg were dialyzed against the same buffer. All samples were supplemented with 200 milliunits/ml of chondroitinase ABC from *P. vulgaris*, which degrades chondroitin 4-sulfate and 6-sulfate oligosaccharide chains into sulfated disaccharides by hydrolyzing the β-(1→4)-glycosidic bonds between *N*-acetyl-D-galactosamine 4- and 6-sulfate and D-glucuronic acid in the repeating disaccharide units. The protease inhibitors already indicated with regard to limited proteolysis were added, when not already present, and the samples were incubated at 37 °C for 4 h. Samples to be analyzed by SDS-PAGE were immediately precipitated in methanol/chloroform/water (27), although those to be used for enzymatic iodination *in vitro* or limited tryptic digestion, following chondroitinase ABC digestion, were subjected to Q rechromatography, as already described, concentrated in Centriprep 30 concentrators, and dialyzed against the appropriate buffer.

Separation and Identification of the Products of Limited Proteolysis of hTg—Analytical PAGE of the digestion products of hTg in Tris/glycine/SDS was performed using 4–17% total acrylamide, 2.7% *N,N′*-methylenebisacrylamide gradient gels. Molecular mass standards (Bio-Rad) were as follows: myosin (205,000 Da), β-galactosidase (116,000 Da), phosphorylase *b* (94,000 Da), bovine serum albumin (68,000 Da), ovalbumin (45,000 Da), carbonic anhydrase (30,000 Da), and soybean trypsin inhibitor (20,000 Da). Two replicas of each gel were prepared. One was stained with 0.1% Coomassie Brilliant Blue R-250 in 25% (v/v) 2-propanol, 10% (v/v) acetic acid and destained in 25% (v/v) methanol, 10% acetic acid. The other one was fixed in 25% 2-propanol, 10% acetic acid, thoroughly rinsed in double-distilled H_2O, stained with 0.005% DMTCC in 50% formamide for 48 h, and finally destained in 5% (v/v) glycerol in tap water (28). Bands were identified on the basis of their mobilities, according to the detailed characterization of their NH_2-terminal peptide sequences provided in a previous study (21). Proteolytic fragments to be identified *ex novo* were separated by reducing or nonreducing SDS-PAGE in 4–17% acrylamide gradient gels and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore) by semi-dry blotting in 0.025 M Tris, 0.01 M glycine, at the constant current of 0.8 mA/cm^2 for 1 h. Membranes were rinsed in double-distilled H_2O, stained with 0.1% Coomassie Brilliant Blue R-250 in 50% methanol, and destained in 50% methanol, 10% acetic acid. Bands were subjected to NH_2-terminal peptide sequencing at the Molecular Structure Facility, University of California, Davis. Peptide sequences were aligned with the cDNA-derived sequence of hTg (29).

Isolation and Sequencing of Glycopeptide hTg-CSgp—Forty seven mg of hTg-CS were denatured and reduced in 15 ml of 0.3 M Tris/HCl, pH 8.0, 6.0 M guanidine/HCl, 1×10^{-3} M EDTA, 0.01 M dithiothreitol at 37 °C for 2 h. The reduced protein was carboxymethylated with a 5-fold molar excess of iodoacetamide, with respect to total –SH groups, at room temperature for 30 min in the dark. Alkylation was stopped with excess dithiothreitol. The sample was dialyzed against 0.05 M sodium phosphate, pH 7.8, and digested with endoproteinase Glu-C (protease V8) from *S. aureus* at the enzyme/substrate weight ratio of 1:100 at 37 °C for 18 h. The sample was adjusted with concentrated solutions to 0.025 M Tris/HCl, 0.1 M NaCl, 2.0 M urea, pH 7.4 (buffer C), and loaded onto a 5-ml HiTrap™ Q-Sepharose HP column equilibrated in the same buffer. After washing with buffer C, a gradient was started from 0 to 100% of buffer D (1.2 M NaCl in buffer C) in 55 min, followed by 100% buffer D for 10 min at the flow rate of 1 ml/min. One-ml fractions were monitored for the optical absorbance at 280 nm and D-glucuronic acid content. A single D-glucuronic acid-containing peak was subjected to size-exclusion chromatography on a 1.5 × 100-cm column of Bio-Gel P-2 in 0.01 M NH_4HCO_3. A D-glucuronic acid-containing peak, eluted in the void volume, was lyophilized and further purified by gel chromatography on a 0.5 × 40-cm column of Sephadex G-50 fine in 0.01 M NH_4HCO_3. One-ml fractions were monitored for peptide content, by measuring the optical absorbance at 220 nm, and D-glucuronic acid content, and a single peptide- and D-glucuronic acid-containing peak was lyophilized. A solubilized aliquot was subjected to NH_2-terminal peptide microsequencing. Purity was checked by PAGE in Tris/Tricine/SDS in a 16.5% total acrylamide, 6% *N,N′*-methylenebisacrylamide gel, containing 6.0 M urea (30). Molecular mass standards (marker kit MW-SDS-17S, Sigma) were as follows: myoglobin fragments 1–153 (16,950 Da), 1–131 (14,440 Da), 56–153 (10,600 Da), 56–131 (8,160 Da), 1–55 (6,210 Da), glucagon (3,480 Da), and myoglobin fragment 132–153 (2,510 Da). SDS-PAGE was followed by semi-dry transfer to Immobilon P, as

described above, for 25 min. The membrane was stained with DMTCC in 50% formamide and destained in tap water (28).

Purification of Carboxyl-terminal Tryptic Fragments of hTg by HPLC—The fragments obtained from the limited digestion of 20 mg of goiter hTg (0.03% of iodine on a weight basis) with trypsin for 20 min, under the conditions described above, were freed from trypsin immediately after digestion by filtration through a 10-ml column of DEAE-Sepharose Fast Flow in 0.025 M Tris/HCl, pH 7.2. hTg fragments were eluted with 0.15 M NaCl in 0.025 M Tris/HCl, pH 7.2, and desalted by gel filtration on a 1.5 × 100-cm column of Bio-Gel P-2, equilibrated with 0.01 M NH_4HCO_3. One-mg aliquots of the fragments were fractionated by reverse-phase HPLC with a Vydac C-4 column (250 × 10 mm, 5 μm) equilibrated in 0.1% (v/v) trifluoroacetic acid in H_2O (solvent A) and containing 2% of 0.07% trifluoroacetic acid in acetonitrile (solvent B). After 2 min at 35% of solvent B, the fragments were eluted with a two-step linear gradient from 35 to 46% of solvent B over 35 min and from 46 to 55.5% over the following 35 min. The flow rate was 1 ml/min. Fractions were analyzed by SDS-PAGE in 4–17% total acrylamide gradient gels under reducing and nonreducing conditions. Corresponding peaks from repeated runs were pooled for further mass spectrometric analysis.

Characterization of Carboxyl-terminal Tryptic Fragments of hTg by Electrospray Mass Spectrometry—ES-MS of peptide $h4bis_{TR}$ were recorded on a Q-TOF Ultima hybrid mass spectrometer (Waters), equipped with an electrospray ion source and operating in positive ion mode. A nanoflow high pressure pump system model CapLC (Waters) was used to deliver a 1-μl/min flow rate of 10% acetonitrile to the mass spectrometer. The spectra were scanned at the speed of 10 s/scan. The source conditions were the following: capillary voltage, 3000 V; cone voltage, 100 V; extractor, 0 V; RF lens, 60. Raw data were processed using the MassLynx 3.5 software. Mass calibration was carried out using the multiple charged ions from a separate introduction of horse heart myoglobin (average molecular mass of 16,950.5 Da). The quantitative analysis was performed by integration of the multiple charged ions of the single species. Molecular masses are reported as average values. The mass signals recorded were associated with the corresponding peptides, on the basis of the expected molecular masses, using the Biolynx software (Waters).

RESULTS

A Relevant Fraction of hTg Is Regularly Composed of hTg Molecules Provided with Type D (Chondroitin 6-Sulfate) Oligosaccharide Units—Type D (chondroitin 6-sulfate) oligosaccharide unit-containing hTg molecules were separated from residual hTg molecules by using ion-exchange chromatography on trimethylamino-substituted HiTrap™ Q-Sepharose HP (GE Healthcare). The Q-IEC of 40 hTg preparations, mostly from nonfamilial, simple, and multinodular goiters but also from normal thyroids, using a NaCl gradient from 0 to 1.2 mol/liter in 0.05 M Tris/HCl, pH 7.4, regularly resulted in the elution of two peaks (henceforth referred to as peaks Q1 and Q2, in elution order) at NaCl concentrations of 0.45 ± 0.01 and 0.84 ± 0.03 M, respectively (Fig. 1). In 18 chromatograms, the area under the Q2 peak varied from 32 to 71.6% of total (mean, 52.8%; median, 51.7%), with no apparent relation with the iodine content (Table 1). The hTg subpopulations thus separated were stable, and separation was satisfactory for practical purposes, as shown by pooling and rechromatography of the two peaks (Fig. 2, *panel A*). We traced chondroitin 6-sulfate oligosaccharide units in hTg fractions by assaying their D-glucuronic acid content, using a *meta*-hydroxybiphenyl-based

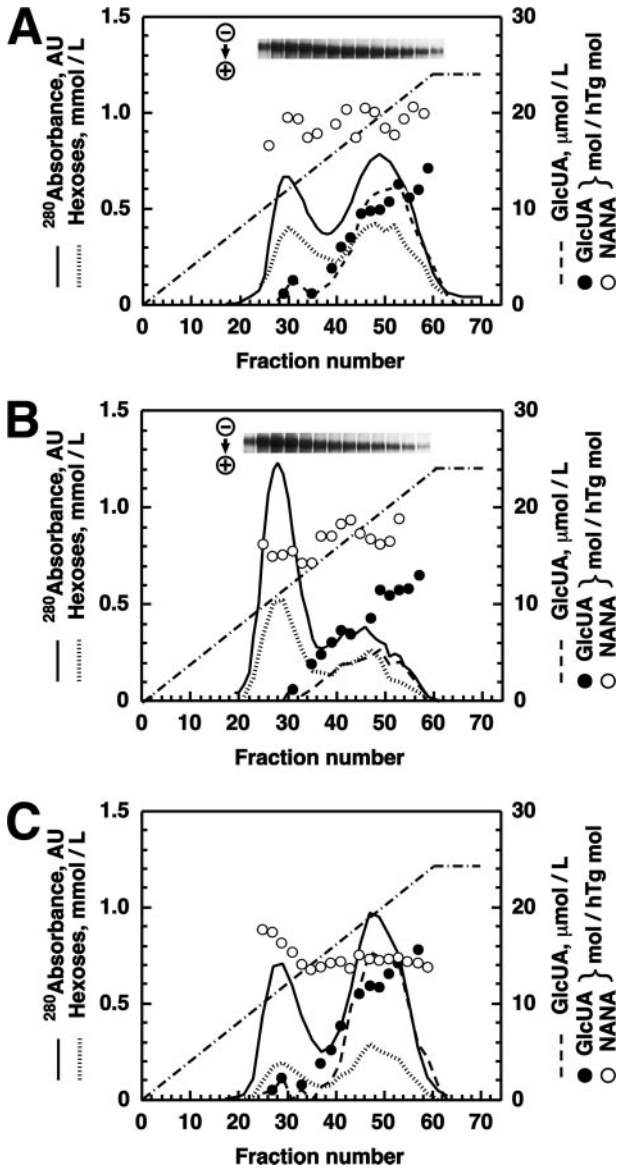

FIGURE 1. **Fractionation by ion-exchange chromatography on HiTrap™ Q-Sepharose HP (Q-IEC) of selected hTg preparations and compositional analysis of the resulting fractions.** Twenty-mg aliquots of hTg preparations O. (*panel A*), Ma. (*panel B*), and D. (*panel C*) in 0.025 M Tris/HCl, 0.05 M NaCl, pH 7.4, were loaded onto a 5-ml HiTrap™ Q-Sepharose HP column, equilibrated with 0.025 M Tris/HCl, pH 7.4 (buffer A). After washing with buffer A, a linear gradient from 0 to 100% of buffer B (1.2 M NaCl in buffer A), in 24 min, at the flow rate of 2.5 ml/min, was applied (the *dashed-dotted line* indicates the NaCl concentration at the column head, with reference to the left *y* axis; dead volume was 6.0 ml). One-ml fractions were monitored for protein content, by measuring the optical absorbance at 280 nm (*continuous line*), and carbohydrate content. The symbols used are as follows: neutral hexoses content, in mmol/liter (*hatched line*); D-glucuronic acid (*GlcUA*) content, in μmol/liter (*dashed line*), and mol/hTg mol (●); *N*-acetylneuraminic acid (*NANA*) content, in mol/hTg mol (○). The analysis of one fraction every other two by PAGE under native conditions, in a 4–9% total acrylamide gradient gel stained with Coomassie Brilliant Blue R-250, is shown on top of *panels A* and *B*.

TABLE 1
Compositional analysis of selected hTg preparations subjected to Q-IEC on HiTrap™ Q-Sepharose HP

hTg preparation[a]	hTg-CS, % of hTg	D-Glucuronic acid in Q2 peak			Total neutral hexoses range,[d] % (w/w)	*N*-Acetylneuraminic acid range,[d] mol/hTg mol	Iodine, % of hTg (w/w)
		μmol/liter[b]	mol/hTg mol[b]	Range,[c] mol/hTg mol			
O.	65.8	11.8	10.0	3.8–14.3	10.7–10.2	18.0–19.8	0.50
Ma.	38.9	3.9	7.0	3.9–13.2	8.0–10.4	15.1–18.2	0.12
T.	48.3	7.2	7.7	3.7–14.0	4.9–6.1	9.8–9.9	0.02
Ce.	51.2	7.5	7.3	2.8–14.2	7.0–8.5		0.09
D.	66.3	14.8	11.7	3.9–15.7	4.4–5.4	16.0–14.0	0.16
Mi.	71.6	20.9	15.6	3.1–23.8			0.16
Mi. N.[e]	63.1	16.8	12.0	3.6–24.0			0.08
Mi. N. Q2 peak in urea[f]	72.7	20.2	13.6	3.2–14.8			

[a] hTg preparations were named after the patients (using the last initial(s)). Preparation O. was from a euthyroid individual, hemilaryngectomized for a non-thyroidal disease; the other preparations were from nonfamilial, simple, or multinodular goiters.
[b] Values were measured in the fractions with the maximal protein concentration in the Q2 peak.
[c] Data indicate the D-glucuronic acid concentration range across the span of the Q2 peak, starting from the valley between the Q1 and the Q2 peaks.
[d] Data indicate the concentration range across the entire span of the Q-IEC, from the leading edge of the Q1 peak to the trailing edge of the Q2 peak.
[e] Preparation obtained from a large 2 × 3-cm colloid nodule, surrounded by apparently normal thyroid tissue, from patient Mi.
[f] Pooled fractions of the Q2 peak from the Q-IEC of hTg preparation Mi. N. were dissociated in 0.04 M Tris/HCl, 2.5 M urea, pH 9.0, for 18 h at 20 °C, as reported (31), and then subjected again to Q-IEC, using a gradient from 0 to 1.2 M NaCl in 0.055 M Tris/HCl, 2.5 M urea, pH 7.4.

method (23). D-Glucuronic acid was almost exclusively restricted in the Q2 peak, which coincided with a fraction of hTg, marked by the presence of D-glucuronic acid-containing type D (chondroitin 6-sulfate) oligosaccharide units (henceforth referred to as hTg-CS) (Fig. 1). Negligible amounts of D-glucuronic acid in the Q1 peak possibly reflected the presence of hTg molecules with very short, nascent type D oligosaccharide chains. The neutral hexose content remained constant or increased slightly along the elution profile of the chromatograms, while the sialic acid content showed no change or only moderate changes in either sense (Fig. 1 and Table 1). Instead, the number of moles of D-glucuronic acid per mol of hTg regularly exhibited a linear increase across the Q2 peak, ranging from a minimum of about 3 to a maximum of 13.2 mol in the hTg preparation with the lowest fraction of hTg-CS, and of 24.0 mol in some hTg-CS-rich goiter hTg preparations (Table 1). In particular, preparations Mi. and Mi. N. had elution profiles (preparations are named using last initial(s) of patients), in which a shoulder in the trailing edge of the Q2 peak indicated the presence of a secondary component, eluting at 0.96 M NaCl (Fig. 2, *panel B*). We hypothesized that the complexity and extended span of D-glucuronic acid content of these Q2 peaks might reflect the coexistence of heterodimeric hTg-CS, with a single chondroitin 6-sulfate-bearing subunit, and homodimeric hTg-CS. To prove this, pooled fractions of the Q2 peak from the Q-IEC of hTg Mi. N. were dissociated in 0.04 M Tris/HCl, 2.5 M urea, pH 9.0, at 20 °C overnight, as described previously (31), and subjected again to Q-IEC, using a NaCl gradient from 0 to 1.2 mol/liter in 2.5 M urea, 0.055 M Tris/HCl, pH 7.4. As shown in Fig. 2, *panel B*, the hTg monomers produced by the dissociation of hTg-CS in urea were separated again into peaks Q1 and Q2, eluting at NaCl concentrations of 0.44 and 0.86 mol/liter, respectively. Once again, D-glucuronic acid was restricted in the Q2 peak, and its concentration showed a linear increase from 3.2 to 14.8 mol/hTg mol across the peak span, as opposed to the increase from 3.6 to 24.0 mol/mol of undissociated hTg-CS (Table 1). The Q2 peak of dissociated hTg-CS was superimposed onto the leading component of the Q2 peak of native hTg-CS, with no secondary shoulder at the trailing edge. The Q2/Q1 ratio in hTg-CS dissociated in urea was 72.7 *versus* 27.3%. These data indicate that the hTg-CS fraction of hTg Mi. N. included both an earlier eluting subfraction of heterodimers, composed of nonchondroitinated (27.3%) and chondroitinated (27.3%) monomers in the 1:1 ratio, and a later eluting homodimeric subfraction, composed only of chondroitinated subunits, and representing the remaining 45.4% of hTg-CS. Thus, hTg-CS was microheterogeneous, both because of the variable number of repeating disaccharide units per chondroitin 6-sulfate chain, which is typical of glycosaminoglycans (GAGs) (32), and because of the coexistence of heterodimeric and homodimeric hTg-CS molecules in hTg-CS-rich goiter hTg preparations. When the pooled fractions of the Q2 peak from the Q-IEC of hTg preparation Ca. were digested with chondroitinase ABC and subjected to Q rechromatography, they eluted in a peak superimposed onto the Q1 peak of the initial Q-IEC of native hTg (Fig. 2, *panel C*). Fig. 1, *panels A* and *B*, also shows the native PAGE of the Q-IEC fractions of hTg preparations O. and Ma., evidencing the anodal shift, going from the Q1 to the Q2 peak, because of the added negative charge of D-glucuronic acid residues and sulfate groups of the chondroitin 6-sulfate unit(s).

hTg-CS Has a Higher Efficiency of T_3 and T_4 Formation than hTg-CS$_0$ and Enhances the Overall Hormonogenic Efficiency of hTg—We investigated the possible influence of the chondroitin 6-sulfate oligosaccharide unit(s) on the hormonogenic function of hTg-CS, in comparison with chondroitin 6-sulfate-devoid hTg (henceforth referred to as hTg-CS$_0$), and unfractionated hTg. Iodination of Tg at the apical pole of thyroid follicular cells occurs by the action of thyroid peroxidase (TPO) (reviewed in Ref. 2). Lactoperoxidase (LPO) is a common alternative for TPO in reconstituted systems of iodination and coupling *in vitro*. TPO and LPO are equally effective in the selective iodination of a subset of tyrosyl residues, which are able to undergo coupling to form T_3 and T_4. TPO and LPO also catalyze the coupling of iodotyrosines with similar efficiencies at pH 7.4, even though LPO is significantly less efficient at lower pH (33). Iodine-poor unfractionated hTg, hTg-CS$_0$, and hTg-CS from nonfamilial goiters (with no more than 0.09% of iodine, on a weight basis) were iodinated enzymatically *in vitro* by using 2 μg/ml bovine LPO, 7.5×10^{-5} M potassium iodide, 0.21 μg/ml glucose oxidase from *A. niger*, and 1×10^{-3} M D-glucose over 90 min at 25 °C (34). Fig. 3 shows the means ± S.E. of the amounts of iodine incorporated into hTg, expressed as percent of hTg on a weight basis, and of the levels of T_3 and T_4 formed as a func-

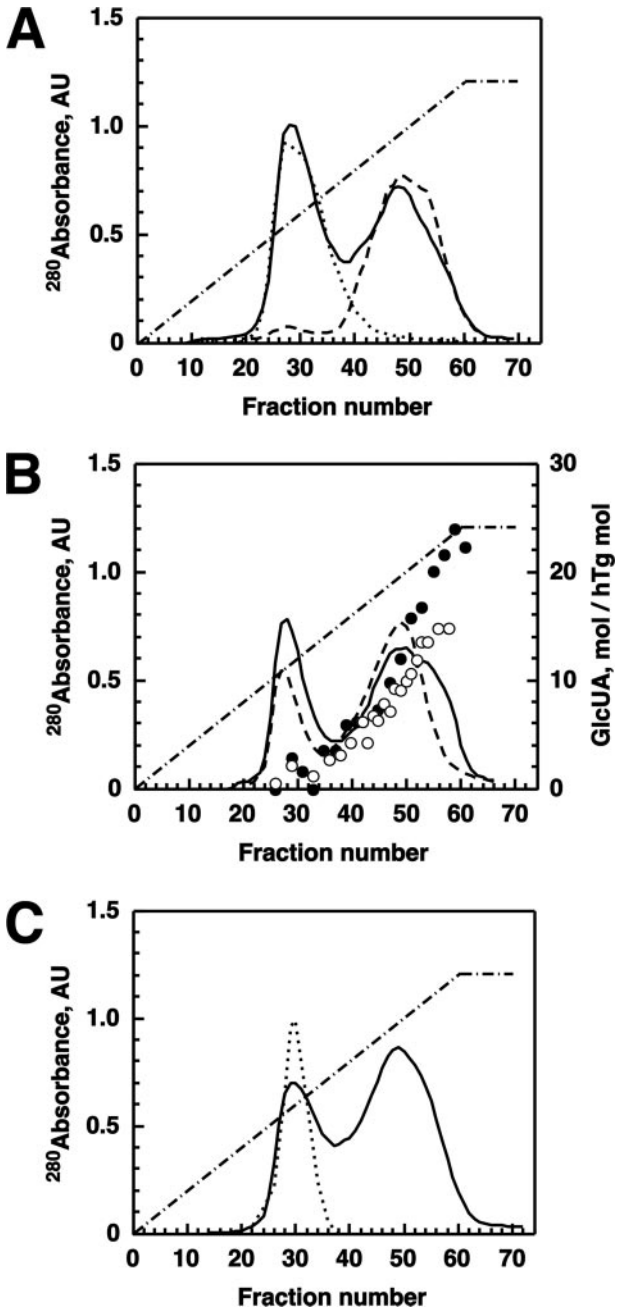

FIGURE 2. **Fractionation by Q-IEC of selected hTg preparations, followed by Q rechromatography of the peaks obtained therefrom, under various conditions.** *Panel A,* following the Q-IEC of 20 mg of hTg preparation I., as described in the legend to Fig. 1, the fractions of the Q1 and Q2 peaks obtained therefrom were pooled separately, with the exclusion of three fractions between peaks. Each pool was dialyzed against buffer A and subjected to Q rechromatography, under identical conditions as the initial Q-IEC. The optical absorbance at 280 nm of the fractions of the initial Q-IEC (*continuous line*) and of the Q rechromatography of the Q1 (*dotted line*) and Q2 peak (*dashed line*) was monitored. *Panel B,* following the Q-IEC of 20 mg of hTg preparation Mi. N., the fractions of the Q2 peak were pooled, dialyzed against buffer A, dissociated in 0.04 M Tris/HCl, 2.5 M urea, pH 9.0, at 20 °C overnight, as reported (31), and subjected to Q rechromatography by using a NaCl gradient from 0 to 1.2 mol/liter in 2.5 M urea, 0.055 M Tris/HCl, pH 7.4. The symbols used are as follows: optical absorbance at 280 nm of the fractions of the initial Q-IEC (*continuous line*) and of the Q rechromatography of the Q2 peak dissociated in urea (*dashed line*); D-glucuronic acid (GlcUA) content, in mol/hTg mol, of the fractions of the initial Q-IEC (●) and of the Q rechromatography of the Q2 peak dissociated in urea (○). *Panel C,* following the Q-IEC of 20 mg of hTg preparation Ca., the fractions of the Q2 peak were pooled. An aliquot of 6 mg of this material was taken to the volume of 1.5 ml with Centriprep 30 concentrators, dialyzed against 0.1 M Tris, 0.1 M sodium acetate, pH 8.0, digested with 200 milliunits/ml of chondroitinase ABC from *P. vulgaris* for 4 h at 37 °C, and finally subjected to Q rechromatography. The optical absorbance at 280 nm of the fractions of the initial Q-IEC (*continuous line*) and of the Q rechromatography of the Q2 peak digested with chondroitinase ABC (*dotted line*) was monitored.

tion of iodine bound during four experiments of time course of iodination *in vitro*. Each experiment was performed using unfractionated hTg, together with hTg-CS_0 and hTg-CS subfractions deriving from the same iodine-poor hTg preparation. The average fraction of hTg-CS in the hTg preparations used was 58.1%. Similar amounts of iodine were incorporated at plateau in hTg and hTg-CS, and slightly higher amounts in hTg-CS_0 (Fig. 3, *panel A*). However, the number of T_3 millimoles (*panel B*) and T_4 moles (*panel C*) synthesized per mol of hTg-CS were in the ratios of 1.70 and 1.34, respectively, with those synthesized per mol of hTg-CS_0. Instead, hTg and hTg-CS did not differ, as for the efficiency of formation of T_3 and T_4. These data indicated that hTg-CS had a higher efficiency of T_4 and, especially, T_3 formation than hTg-CS_0 and that the entire population of unfractionated hTg molecules benefited from this property of hTg-CS. A rationale for this may be that chondroitin 6-sulfate chains exerted an influence not only on the hTg-CS molecules they were linked to but also on the hTg-CS_0 molecules they interacted with or on the peroxidase. If so, one would not expect to see differences of hormone yields between hTg-CS and hTg-CS_0 when these were iodinated altogether in the pool of unfractionated hTg. Such a prediction was verified by the lack of significant variations in the amounts of T_3 and T_4 formed, between the Q-IEC fractions of hTg preparation O., physiologically iodinated *in vivo* (Fig. 4). Previous digestion of hTg-CS with 200 milliunits/ml of chondroitinase ABC from *P. vulgaris*, at pH 8.0 and 37 °C for 4 h, was not associated with reductions of the T_3 and T_4 yields, with respect to native hTg-CS, and of the differences of hormone-forming efficiency between hTg-CS and hTg-CS_0.

Chondroitin 6-Sulfate Oligosaccharide Unit(s) of hTg-CS Are Restricted in the Carboxyl-terminal Region, Downstream Thr^{2514}—We were able to differentiate hTg-CS from hTg-CS_0 in SDS-polyacrylamide gels by staining them with DMTCC (Stains All™, ICN). This dye was reported to stain GAGs metachromatically and, in particular, chondroitin sulfate purple, while staining proteins red (35). In fact, fractions in the Q1 (hTg-CS_0) peak were stained pinkish red with DMTCC, and those in the Q2 (hTg-CS) peak were stained purple. Side-by-side comparison of the two peak fractions also revealed a subtle cathodal shift in hTg-CS in the presence of SDS. Both metachromasia and the mobility shift were abolished by digestion with 200 milliunits/ml of chondroitinase ABC, at 37 °C for 4 h, prior to SDS-PAGE (Fig. 5, *panel A*). To identify the chondroitin 6-sulfate-containing region(s) of hTg, the Q-IEC fractions of hTg were subjected to limited digestion with thermolysin and trypsin, using DMTCC as a probe. The proteolytic fragments, separated by reducing SDS-PAGE, corresponded exactly to those characterized previously by NH_2-terminal peptide sequencing (21) and were identified on the basis of their mobilities. Fig. 5, *panel B,* shows the SDS-PAGE of the digestion products of fractions 29, 40, 48, and 56 of hTg preparation

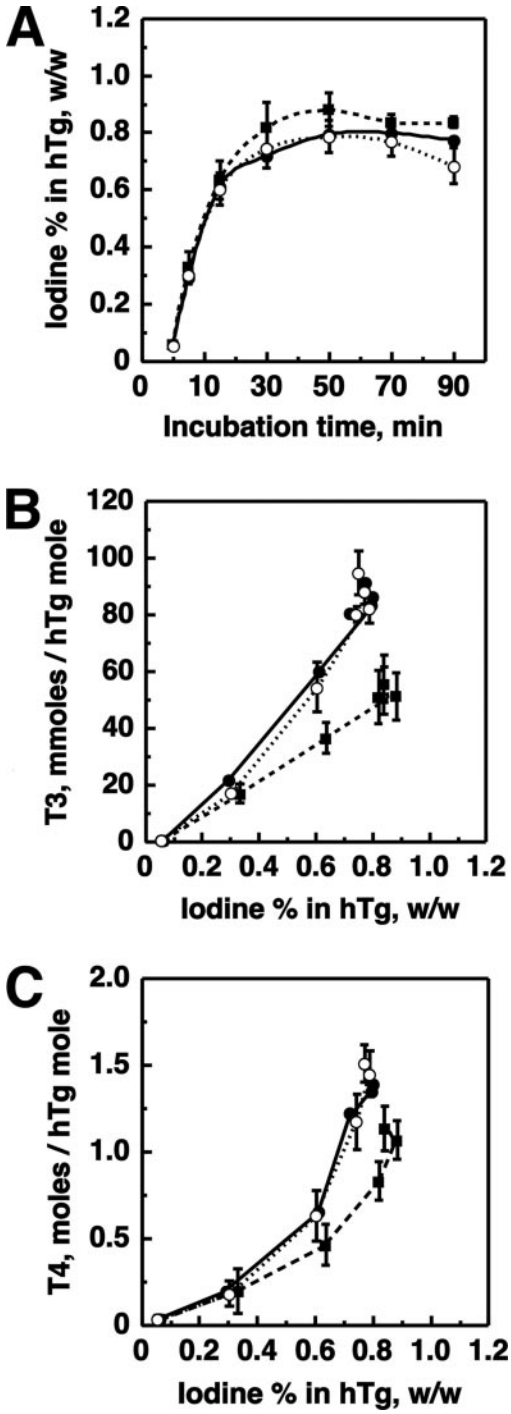

FIGURE 3. **Enzymatic *in vitro* iodination and analysis of the hormone-forming efficiency of hTg, hTg-CS_0, and hTg-CS.** Iodine- and hormone-poor hTg (●) and its subfractions hTg-CS_0 (■) and hTg-CS (○), prepared by Q-IEC, at the concentration of 0.45 g/liter in 0.02 M imidazole/HCl, pH 7.0, were iodinated enzymatically *in vitro*, using 2 μg/ml of bovine LPO, 7.5×10^{-5} M potassium iodide, 0.21 μg/ml of glucose oxidase from *A. niger* and 1×10^{-3} M D-glucose, over 90 min at 25 °C (34). Individual experiments were performed using unfractionated hTg, hTg-CS_0, and hTg-CS subfractions derived from the same iodine-poor hTg preparation. The average percent content of hTg-CS in the hTg preparations used was 58.1%. At the times indicated, aliquots were removed, dialyzed against 0.01 M NH_4HCO_3, 0.005 M NaCl, and the iodine content was assayed and expressed as percent of the protein on a weight basis (*panel A*). After digestion with Pronase and aminopeptidase M, as described under "Experimental Procedures," T_3 (*panel B*) and T_4 (*panel C*) were also assayed by radioimmunoassay. Points represent mean ± S.E. values of four experiments. For the sake of clarity, S.E. values are indicated only for hTg-CS_0 and hTg-CS.

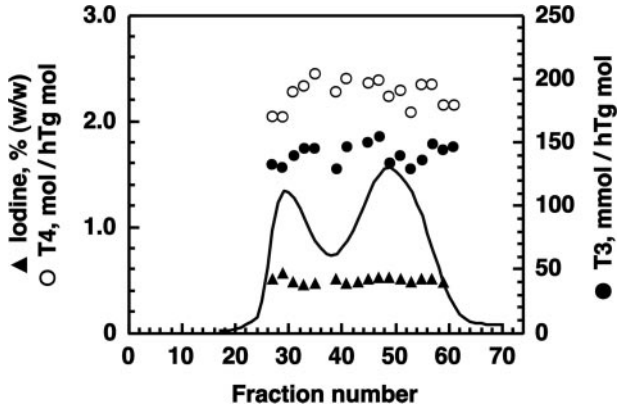

FIGURE 4. **Iodine and hormone content of the Q-IEC fractions of physiologically iodinated hTg preparation O., from normal thyroid tissue, containing 0.50% iodine (w/w).** The hTg preparation was subjected to Q-IEC, as described in the legend to Fig. 1, and the fractions obtained were analyzed for their iodine (▲), T_3 (●), and T_4 (○) content, as described under "Experimental Procedures." The *continuous line* indicates the optical absorbance at 280 nm, for which measuring units are not indicated as they were the same as in Fig. 1.

O. with thermolysin. Bands $h2_{TL}$, $h5_{TL}$, and $h7_{TL}$ exhibited metachromasia and a cathodal shift, whose extent was inversely related with the fragment apparent mass, going from fraction 29 in the Q1 peak to fractions 48 and 56 in the Q2 peak. This is in keeping with the notion that peptides with high negative net charges bind lower than average amounts of SDS, and they exhibit lower than average mobilities in SDS-PAGE (36). A mixed pattern was apparent in fraction 40, in the valley between the Q1 and Q2 peaks. Both changes were reverted by digestion of the proteolytic fragments of fraction 48 with chondroitinase ABC, prior to SDS-PAGE, although no changes were brought about in fraction 29 (Fig. 5, *panel C*). Inspection of the flow diagram of the limited proteolysis of hTg with thermolysin revealed that fragments $h2_{TL}$, $h5_{TL}$, and $h7_{TL}$ were all located at the carboxyl-terminal side of hTg and shared the region downstream from Leu^{1832} (Fig. 6, *panel A*).

On the other hand, a diffuse band (h8-CS_{TR}), with an average apparent mass of 41,000 Da, appeared among the tryptic fragments of hTg, going from fraction 29 to fraction 40. It was stained purple with DMTCC, but was not apparent in the gel stained with Coomassie Brilliant Blue R-250. Its intensity of staining and apparent relative mass increased going from fraction 40 to 56 (Fig. 5, *panel D*), in keeping with the increase of D-glucuronic acid content revealed by the analysis of the Q-IEC fractions across the Q2 peak (Fig. 1 and Table 1). Upon digestion of the tryptic fragments of fraction 48 with chondroitinase ABC, band h8-CS_{TR} was replaced by a well focused band, corresponding to dechondroitinated h8-CS_{TR} (h8-deCS_{TR}), with an apparent mass of 36,000 Da, which was stained blue with Coomassie Brilliant Blue R-250 and red with DMTCC. No changes were caused by chondroitinase ABC in fraction 29 (Fig. 5, *panel E*). The formation of band h8-deCS_{TR} was not prevented when the limited digestion of hTg-CS with trypsin was preceded, rather than followed, by digestion with chondroitinase ABC under identical conditions. Band h8-deCS_{TR} was prepared by limited tryptic digestion of 0.5 mg of hTg-CS, followed by chondroitinase ABC digestion, reducing SDS-PAGE and transfer to PVDF. NH_2-terminal peptide microsequencing of this

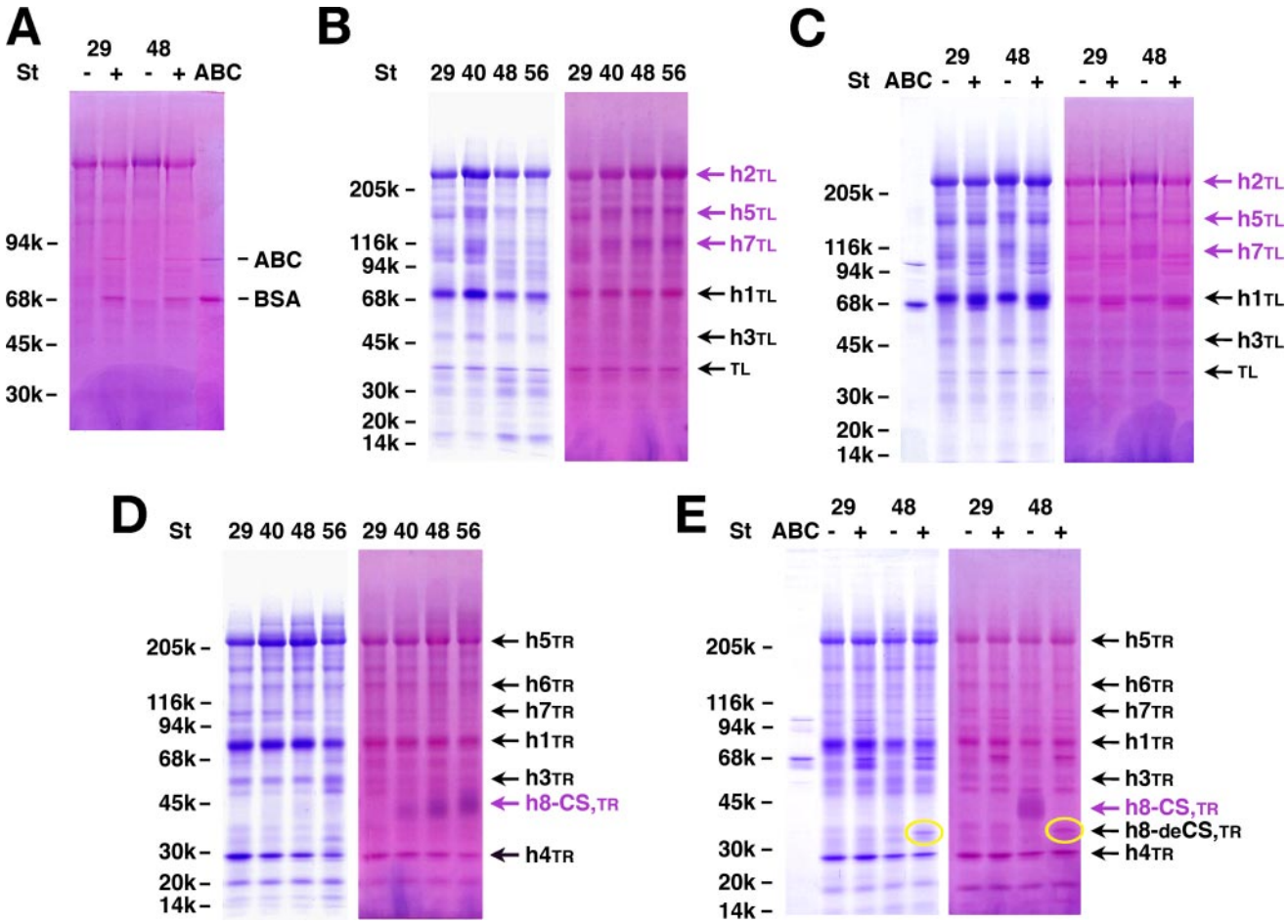

FIGURE 5. **SDS-PAGE under reducing conditions of selected Q-IEC fractions of hTg preparation O., either as such or subjected to limited proteolysis with thermolysin or trypsin and/or digestion with chondroitinase ABC.** *Panel A*, top fractions of the Q1 peak (fraction 29) and Q2 peak (fraction 48), analyzed before (−) and after (+) digestion with 200 milliunits/ml of chondroitinase ABC, in a 4–13% total acrylamide gradient gel stained with DMTCC. The positions of chondroitinase ABC (*ABC*) and carrier bovine serum albumin (*BSA*) are indicated. *Panel B*, digestion products of the fractions indicated with thermolysin (*TL*), at the TL/hTg ratio of 1:50, pH 7.8, and at 30 °C for 80 min, analyzed in replicate 4–17% total acrylamide gels, stained with Coomassie Brilliant Blue R-250 (*left*) and DMTCC (*right*). Fragments are marked at *right* in accordance with Ref. 21. Fragments exhibiting cathodal shifts and metachromasia, going from the Q1 to the Q2 peak, are shown in *purple*. *Panel C*, further digestion with chondroitinase ABC of the products of proteolysis of fractions 29 and 48 with thermolysin. *Panel D*, digestion products of the fractions indicated with trypsin (*TR*), at the TR/hTg ratio of 1:100, pH 7.8, and at 30 °C for 40 min. *Panel E*, digestion with chondroitinase ABC of the products of proteolysis of fractions 29 and 48 with trypsin. Dechondroitinated peptide h8-CS_{TR} (h8-deCS_{TR}) is *circled*. Relative molecular mass standards (*St*), as detailed under "Experimental Procedures," are marked at *left* of each gel.

band revealed a single sequence (TSSKTA), corresponding to hTg residues 2514–2519 (29) (see supplemental Table 1). Naturally, this was also the sequence of peptide h8-CS_{TR}. The flow diagram of limited proteolysis of hTg with trypsin shows the carboxyl-terminal location of peptide h8-CS_{TR}, in keeping with the results obtained with thermolysin (Fig. 6, *panel B*). Because the NH_2-terminal sequence of peptide h8-deCS_{TR} was identical with one of two sequences found previously in band $h4_{TR}$ (21), we also determined the sequence of band $h4_{TR}$ prepared from hTg-CS. As expected, two sequences were found, one starting at residue 1 of hTg ($h4_{TR}$) and the other one at residue 2514 ($h4bis_{TR}$) (supplemental Table 1). Thus, of two peptides, both starting at residue 2514, one (h8-CS_{TR}) contained chondroitin 6-sulfate oligosaccharide units and the other ($h4bis_{TR}$) did not. Inspection of Fig. 5, *panel D*, reveals that the increase of staining intensity of band h8-CS_{TR}, going from fraction 40 to 56, was paralleled by a decrease of intensity of band $h4_{TR}$, in keeping with the finding that heterodimeric hTg-CS, yielding peptides h8-CS_{TR} and $h4bis_{TR}$, and homodimeric hTg-CS, yielding only peptide h8-CS_{TR}, coexisted in the late portion of the Q2 peak (Fig. 2, *panel B*).

A Single Type D (Chondroitin 6-Sulfate) Oligosaccharide Unit Is Linked to Ser^{2730} of hTg-CS—Forty seven mg of hTg-CS were reduced and carboxymethylated, as reported under "Experimental Procedures," and hydrolyzed with endoproteinase Glu-C from *S. aureus*, at the enzyme/substrate weight ratio of 1:100, in 0.05 M sodium phosphate buffer, pH 7.8, at 37 °C for 18 h. Digestion products were subjected to Q-IEC on a 5-ml HiTrap™ Q-Sepharose HP column, using a gradient from 0.1 to 1.2 M NaCl in 0.025 M Tris/HCl, 2.0 M urea, pH 7.4, in 55 min, at the flow rate of 1 ml/min. Most protein was discarded in the flow-through, whereas a unique D-glucuronic acid-containing peak, having negligible absorbance at 280 nm, was eluted late in the gradient (supplemental Fig. 1, *panel A*). This peak was subjected to size-exclusion chromatography on Bio-Gel P-2 (size-exclusion limit of 1800 relative mass units) in 0.01 M NH_4HCO_3, which yielded a D-glucuronic acid-containing peak in the void volume. Its further purification by size-exclusion

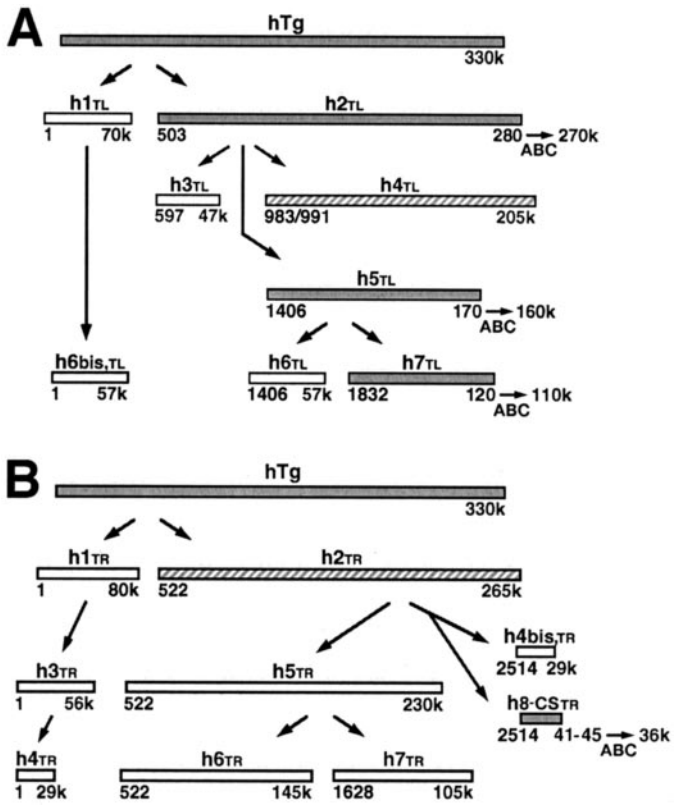

FIGURE 6. **Flow diagrams of the limited proteolysis of hTg with thermolysin (*panel A*) and trypsin (*panel B*) (21).** Each *bar* represents a peptide, with the peptide number *above* the bar, the apparent relative molecular mass below at *right,* and the amino-terminal residue number below at *left*, according to Ref. 29. Fragments of hTg-CS, which exhibited cathodal shifts and metachromasia upon staining with DMTCC, with respect to their counterparts deriving from hTg-CS_0, are shown in *gray*. Their apparent relative molecular masses, both before and after digestion with chondroitinase ABC (*ABC*), are indicated. *Cross-hatching* of bars $h4_{TL}$ and $h2_{TR}$ indicates that, under the digestion conditions used, the corresponding peptides were not detected in the gels shown in Fig. 5.

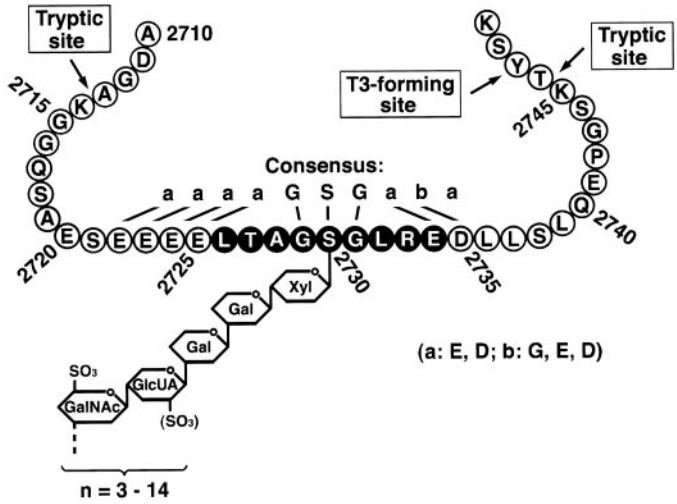

FIGURE 7. **Diagrammatic representation of the localization and structure of type D (chondroitin 6-sulfate) oligosaccharide unit of hTg.** The cDNA-deduced amino acid sequence of hTg, from residue 2710 to 2749, is represented in the single-letter code (29). *Black circles* with *white lettering* mark the sequence found for the purified glycopeptide hTg-CSgp (with the *proviso* that Ser^{2730} appeared as a *blank*). A consensus sequence for the recognition of core protein serine residues by UDP-D-xylose:proteoglycan core protein β-D-xylosyltransferase (37) is aligned with the hTg sequence, and concordances are indicated. A diagram of the chondroitin 6-sulfate oligosaccharide unit attached to Ser^{2730} is shown (8–10). The number of repeating β-D-glucuronic acid-*N*-acetyl-D-galactosamine 6-sulfate disaccharide units per chondroitin 6-sulfate chain found in the present study is indicated. The two sites of tryptic cleavage between residues 2714–2715 and 2745–2746 found in the present study and the preferential site of T_3 formation at Tyr^{2747} (29, 40, 41) are also marked.

chromatography on Sephadex G-50 fine, in 0.01 M NH_4HCO_3, monitored at 220 nm, is shown in supplemental Fig. 1, *panel B*. Of the two peaks resolved, only one peak contained D-glucuronic acid. NH_2-terminal peptide microsequencing of this material revealed a single, homogeneous nonapeptide with the LTAG*X*GLRE sequence, corresponding to residues 2726–2734 of the cDNA-derived sequence of hTg (29), *X* being Ser^{2730} linked with the chondroitin 6-sulfate oligosaccharide unit (supplemental Table 1). This glycopeptide will be henceforth referred to as hTg-CSgp. The comparison between the sequence around Ser^{2730} and a consensus for the recognition of serine residues by UDP-D-xylose:proteoglycan core protein β-D-xylosyltransferase, deriving from the alignment of 51 chondroitin 6-sulfate attachment sites of 19 proteoglycan core proteins (37), revealed a 90% concordance, provided that two insertions of 3 and 2 residues were allowed (Fig. 7). The purified aliquot of hTg-CSgp contained 41.1 μg of D-glucuronic acid and 25.2 μg of protein. On the basis of the M_r of 903, calculated for the peptide moiety, this indicated an average content of 8.4 mol of D-glucuronic acid per mol of glycopeptide. Electrophoresis of 18 μg of hTg-CSgp in a 16.5% polyacrylamide gel in Tris/Tricine/SDS, followed by transfer to a PVDF membrane and staining with DMTCC, revealed a homogeneous, metachromatic band with the diffuse migration, which is typical of GAGs (17, 32), and an apparent molecular mass of 13,000–20,000 Da, both indicating polydispersity (supplemental Fig. 1, *panel C*). hTg-CSgp was labile upon freezing and thawing.

The Chondroitin 6-Sulfate Oligosaccharide Unit of hTg-CS Protects Peptide Bond Lys^{2714}–Gly^{2715} from Proteolysis—Next, we sought an explanation for the difference between the apparent relative molecular masses of peptides h8-$deCS_{TR}$ (36,000 Da) and $h4bis_{TR}$ (29,000 Da), by determining the carboxyl terminus of the latter. In fact, only a minor part of the mass difference could be accounted for by the residual oligosaccharide (GlcUA-Gal-Gal-Xyl), which was left *in situ* after chondroitinase ABC digestion. Even though peptide $h4bis_{TR}$ comigrated with $h4_{TR}$, in reducing SDS-PAGE of the products of tryptic digestion of hTg described above, previous observations indicated that the homologous peptide $b11_{TR}$ of bovine Tg was free of comigrating species under nonreducing conditions, because it was the sole fragment not linked to other fragments by disulfide bonds (38). In fact, nonreducing SDS-PAGE of the tryptic fragments of hTg yielded only two bands instead of several bands seen with reduction (supplemental Fig. 2, *panel B*). NH_2-terminal microsequencing of band $h4_{TR\text{-}NR}$, transferred onto PVDF, revealed the same sequence as peptide $h4bis_{TR}$, starting at residue 2514, together with traces of a sequence starting at residue 2518 (supplemental Table 1). Thus, the products of limited tryptic digestion of goiter hTg (with an iodine content of 0.03%, on a weight basis), at the enzyme/substrate weight ratio of 1:100, at 30 °C for 20 min, were subjected to reverse-phase HPLC in the absence of reduction, using a Vydac C-4 column

(250 × 10 mm, 5 μm) and a gradient of acetonitrile in trifluoroacetic acid (supplemental Fig. 2, *panel A*). Nonreducing SDS-PAGE of peak 2 revealed band $h4_{TR\text{-}NR}$. Upon reduction, this band exhibited the same relative apparent mass as peptide $h4bis_{TR}$ (supplemental Fig. 2, *panel B*). The mass values associated with this band were determined by ES-MS (supplemental Table 2) and defined a peptide having ragged amino- and carboxyl-terminal ends (residues 2512–2514 and 2713–2714, respectively) and containing a high-mannose oligosaccharide unit, composed of 2 *N*-acetyl-D-glucosamine and 8 or 9 D-mannose residues, linked to Asn^{2563}, as reported previously (7, 29, 39). Furthermore, the ES-MS analysis of HPLC peak 1 revealed a mass value corresponding to hTg peptide 2715–2745 (supplemental Table 2). Thus, peptide $h4bis_{TR}$ was truncated at Lys^{2714}, whereas peptide bond Lys^{2714}–Gly^{2715} was protected from proteolysis in peptide $h8\text{-}CS_{TR}$, which extended through Ser^{2730}, with its bound chondroitin 6-sulfate unit. Another tryptic site was located between Lys^{2745} and Thr^{2746} (Fig. 7).

DISCUSSION

The present study shows that type D (chondroitin 6-sulfate) oligosaccharide units are a main source of molecular microheterogeneity of hTg, being regularly found in a significant and sometimes predominant fraction of it. We developed an IEC method, which permitted us to separate chondroitin 6-sulfate-containing hTg molecules (hTg-CS) from the residual hTg molecules ($hTg\text{-}CS_0$). By exploiting the changes of electrophoretic mobility and staining properties conferred upon the products of limited proteolysis of hTg by chondroitin 6-sulfate units, we first restricted the chondroitin 6-sulfate-containing regions of hTg to a carboxyl-terminal peptide, starting at Thr^{2514}. The subsequent purification of a homogeneous, D-glucuronic acid-containing nonapeptide (hTg-CSgp), corresponding to hTg residues 2726–2734, permitted us to establish Ser^{2730} as the sole site of chondroitin 6-sulfate addition in hTg. The surrounding sequence showed concordance with a consensus sequence for the recognition of core protein serine residues by UDP-D-xylose:proteoglycan core protein β-D-xylosyltransferase, with the exception of two insertions of three and two residues (Fig. 7), in keeping with the finding that recognition sequences of proteins, which are not modified quantitatively with chondroitin sulfate, match the consensus less well than those of proteins that are (37). The presence in hTg of *O*-linked oligosaccharide units, composed of 1 mol of D-xylose, 2 mol of D-galactose, 11 mol of a D-glucuronic acid-*N*-acetyl-D-galactosamine disaccharide, and up to 14 mol of sulfate per mol of serine was reported (8). Based on numbers of moles of D-glucuronic acid per mol of hTg varying from 4.8 to 8.7 in different hTgs, it was suggested that some hTg molecules might be devoid of type D units, although some might contain units of different sizes or multiple units (8). Subsequent studies showed that the repeating disaccharides were of the chondroitin 6-sulfate type (9, 10). Our data show that a relevant hTg fraction regularly contained a single chondroitin 6-sulfate unit per polypeptide chain, linked to Ser^{2730} and composed of a broadly varying number of repeating disaccharide units. In some goiter hTg preparations with high contents of hTg-CS, hTg-CS heterodimers, in which only one monomer contained a chondroitin 6-sulfate chain, coexisted with hTg-CS homodimers. In the monomers derived from the dissociation of hTg-CS in urea, the net number of disaccharide units per chondroitin 6-sulfate chain did not exceed 14.

We also show that hTg-CS had a higher efficiency of hormone formation than $hTg\text{-}CS_0$ and that the whole unfractionated hTg benefited from this property. Thus, chondroitin 6-sulfate addition represents an ergonomic mechanism, by which the post-translational modification of a fraction of molecules influences the overall function of hTg. The greater advantage in the formation of T_3, with respect to T_4, associated with the chondroitin 6-sulfate unit, and the proximity of the attachment site of the latter to the carboxyl terminus of hTg suggest that it may influence hormonogenesis by affecting the function of the site of preferential T_3 formation at Tyr^{2747} (29, 40, 41). Normal ranges of serum T_4 concentration (64–142 nmol/liter) and T_3 concentration (1.1–2.9 nmol/liter) have higher limits that exceed the lower ones only by a factor of 2.2 and 2.6, respectively (42), so that the increases in the rates of T_4 and T_3 formation in hTg-CS, in comparison with $hTg\text{-}CS_0$, observed in the present study may be physiologically significant. The regulation of the T_4/T_3 ratio in thyroid secretion is crucial for maintaining physiological concentrations of active hormone (T_3) in blood and is finely tuned by multiple mechanisms, under the control of TSH (2). The T_4/T_3 ratio is regulated first at the biosynthetic level in Tg and decreases in experimental animals given TSH. In rabbit and guinea pig Tg, TSH stimulates T_3 formation at tyrosine 2747 and decreases T_4 synthesis at tyrosine 5 (40). The diminished T_4 formation at the amino terminus of Tg is mediated by the TSH-stimulated maturation of the *N*-linked oligosaccharide chain linked to Asn^{91}, from the high-mannose to the complex type (3). The data that we present contribute to delineate a common mechanism, mediated by modifications of the composition and number of *N*-linked and *O*-linked oligosaccharide chains, which may be responsible for changes in the hormonogenic efficiency at both hTg termini, affecting the overall ratio of T_4 over T_3 formation in hTg. For hormone release to occur, Tg must be internalized into thyroid follicular cells and degraded in lysosomes. A part of Tg, internalized via the endocytic receptor megalin, bypasses lysosomes and is transcytosed across cells, to be secreted from the basolateral pole (43). Tg binding to megalin is facilitated by accessory interactions with heparan-sulfate proteoglycans on the surface of thyroid cells (44, 45). Experiments are in progress to determine whether chondroitin 6-sulfate units may interfere with the binding of hTg to megalin. Conceivably, the addition of chondroitin 6-sulfate to hTg, besides improving the yield of active hormone, may also prevent the nonproductive internalization of hTg by transcytosis.

Furthermore, we identified two sites, one between Lys^{2714} and Gly^{2715} and the other between $Lys^{2745}$and Thr^{2746}, near the site of chondroitin 6-sulfate addition, which were susceptible to limited proteolysis with trypsin. Our data show that the former was protected from proteolysis in hTg-CS but not in $hTg\text{-}CS_0$. Both the hormone-forming advantage of hTg-CS over $hTg\text{-}CS_0$ and the resistance of peptide bond 2714–2715 to proteolysis persisted after digestion of hTg-CS with chondroitinase ABC. Such findings indicate that the residual oligosaccharide that was left *in situ* was sufficient for the observed effects. This is not

surprising with respect to proteolysis, because *O*-linked mono- and disaccharides were able to protect specific cleavage sites from cathepsin L (12). Moreover, the production of proteolytic fragments by an oxidative cleavage mechanism was repeatedly observed in the course of Tg iodination both *in vitro* (46, 47) and *in vivo* (48). The protection from the occurrence of such cleavages in the carboxyl-terminal end of hTg, possibly afforded by the oligosaccharide chain at Ser^{2730}, even in its residual form after digestion with chondroitinase ABC, might contribute to improve the hormone yields. Verification of this hypothesis will be the aim of future work. It is worth noticing that the product of digestion of hTg-CS with chondroitinase ABC differed from native hTg-CS_0, because this enzyme hydrolyzes the β-(1→4)-glycosidic bonds between *N*-acetyl-D-galactosamine 6-sulfate and D-glucuronic acid, thus removing β-GlcUA-(1→3)-GalNAc-6S disaccharides, while leaving *in situ* a GlcUA-Gal-Gal-Xyl linkage oligosaccharide. Moreover, Hascall *et al.* (49) reported that the disaccharide closest to the linkage oligosaccharide in chondroitin 4-sulfate resisted digestion by chondroitinase ABC. Our data entail, even though some of the hTg molecules in the Q1 peak might contain short oligosaccharide chains of this kind, that all the hTg molecules from the Q2 peak digested with chondroitinase ABC contained residual stubs, in a heterodimeric or even in a homodimeric form.

The reported effects on proteolysis are in support of a possible modifying influence of the chondroitin 6-sulfate unit in the processing of hTg by APCs, and in the ability of hTg to function as an autoantigen, particularly because the chondroitin 6-sulfate unit was located within an epitope-rich region, harboring several T cell-related epitopes, capable of causing EAT in genetically susceptible mice, and B cell-related epitopes, recognized by circulating autoantibodies of patients with AITD (reviewed in Ref. 1). The effects of oligosaccharide chains on the activity of proteolytic enzymes involved in antigen processing and on the affinity of epitope binding to major histocompatibility complex or T cell receptor molecules have been reviewed (11). *O*-Linked mono- and disaccharides in tumor-associated glycoprotein MUC1 restricted the repertoire of the epitopes produced and/or presented in a site-specific manner, either by limiting the proteolytic accessibility of the protein (12) or by preventing epitope recognition by a peptide-specific T cell hybridoma (13). *N*-Linked oligosaccharide chains inhibited the generation of a self-epitope from glutamate receptor subunit 3 (14) and of cytotoxic lymphocyte-specific epitopes from influenza A nucleoprotein (15). Moreover, a keratan sulfate chain masked an arthritogenic T cell epitope in the G_1 domain of aggrecan, whereas the depletion of multiple chondroitin sulfate side chains generated clusters of chondroitin sulfate stubs, which activated specific B cells to function as APCs (16). We expect that the chondroitin 6-sulfate chain of hTg may inhibit the processing of hTg peptide 2731–2744 (amino acid numbering as per Ref. 29), known for its ability to stimulate *in vitro* strong proliferative responses and the adoptive transfer of EAT by splenic lymphocytes of CBA mice immunized with mouse Tg (50). Other epitopes might be abrogated by the chondroitin 6-sulfate unit, should their generation require cleavage in the vicinity of Ser^{2730}, within a range including the Lys^{2714}–Gly^{2715} peptide bond on the amino side.

In addition, significant effects of chondroitin sulfate oligosaccharide chains on cellular immune responses have been documented. A small percentage (2–5%) of invariant chain molecules, associated with class II major histocompatibility complex molecules, are modified with the addition of a single chondroitin sulfate chain at Ser^{291} (Ii-CS). In this form, they remain associated with class II molecules at the surface of APCs (51, 52), where they act as accessory molecules in antigen presentation, facilitating the interactions between APCs and T cells, and greatly enhancing class II-dependent allogeneic and mitogenic T cell responses (17). Such effects occur through interactions of Ii-CS with CD44 on responding T cells, as they can be inhibited both by anti-CD44 antibodies, and by a soluble form of CD44 (CD44Rg), which binds Ii-CS directly (17). Treatment of spleen cells with xyloside, which inhibits GAG addition (53), interferes with their antigen-presenting capabilities (54). Serglycins, small proteoglycans stored in secretory granules of hematopoietic cells, activate the CD3-dependent release of cytokines and proteases from CD44-positive cytotoxic lymphocytic clones (18) by interacting with CD44 through their chondroitin 4-sulfate and 6-sulfate side chains (19). Moreover, CD44 binding to aggrecan, a major proteoglycan of the cartilage matrix, through the chondroitin 4- and 6-sulfate side chains of the latter, can trigger the oligomerization of CD44 molecules and the activation of intracellular signaling (20). In keeping with these suggestions, preliminary results indicate that the chondroitin 6-sulfate oligosaccharide unit significantly affects the immunopathogenic capacity of hTg in CBA/J(H-$2A^k$) mice by a complex mechanism.[3]

Finally, the close clinical association between Graves hyperthyroidism and thyroid-associated ophthalmopathy (TAO) led to the hypothesis that the latter might be the result of an autoimmune response against orbital autoantigen(s) that are also present in the thyroid. Candidate antigens, besides the TSH receptor, include Tg (55). Some authors detected hTg in the orbital tissues of patients with TAO and hypothesized that it might be transferred there via thyroid-orbit connections evidenced by lymphography (56). hTg was found prevailingly in fibroadipose tissue (57). It was proposed that the ability of hTg to bind to GAGs, including chondroitin sulfate B and C, might mediate its localization in orbital tissues, where it might function as a target of immune responses directed against the thyroid (58). Metachromatic GAGs accumulate in TAO and thyroid-associated dermopathy (55). Edematous connective perimysial tissues of patients with TAO are composed mainly of hyaluronan and chondroitin sulfate (59). Accumulation of GAGs and adipose tissue expansion is apparent in the fatty connective tissue of the posterior orbit (60). In our opinion, the presence of an integral GAG chain, in a fraction of hTg molecules, may represent a mechanism by which autoaggressive responses against hTg may spread to connective tissue antigens with shared GAG chains, particularly in the event that the synthesis of both be quantitatively and/or qualitatively dysregulated. Proving this hypothesis will require the fine structural characterization of the chondroitin 6-sulfate chains of hTg and of the GAGs

[3] M. Conte, A. Arcaro, D. D'Angelo, A. Gnata, F. Fulciniti, S. Formisano, and F. Gentile, manuscript in preparation.

of orbital connective tissues from patients with TAO, and the demonstration of cross-reacting B and/or T cell clones.

In conclusion, a single chondroitin 6-sulfate chain linked to Ser^{2730} in a relevant fraction of hTg molecules influences both the hormone-forming efficiency and the proteolytic accessibility of the carboxyl-terminal region of hTg. These effects may bear consequences on thyroid homeostasis and autoimmunity. Further work will be aiming to determine the following: 1) What are the physiological limits of hTg-CS abundance in hTg? 2) How is the synthesis of the chondroitin 6-sulfate unit of hTg regulated, particularly regarding the role of TSH? May changes of hTg-CS abundance mediate thyroid adaptation to iodine deficiency or inherited defects of thyroid hormone synthesis or secretion? 3) Do any correlations exist between thyroid function and variations in the hTg-CS/hTg-CS_0 ratio or in the chondroitin 6-sulfate chain length, as analyzed by gel electrophoresis of the oligosaccharide units released from hTg by β-elimination, in comparison with appropriate standards? 4) How does the chondroitin 6-sulfate unit influence the processing of hTg by APCs and cellular immune responses to hTg *in vivo*? It is our opinion that a systematic investigation may shed light on the pathogenesis of thyroid diseases, particularly AITD.

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