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INFLUENCE OF THE CHONDROITIN 6-SULFATE OLIGOSACCHARIDE UNIT ON THE IMMUNOPATHOGENICITY OF HUMAN THYROGLOBULIN IN CBA/J(*H-2*^{k}) MICE

MULTIPLE EFFECTS ON ANTIGEN PROCESSING AND T CELL RESPONSES

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INDEX

1. SUMMARY

The site of type D (chondroitin 6-sulfate) oligosaccharide unit addition to human thyroglobulin (hTg) was localized. Furthermore, hTg and its fractions endowed with chondroitin 6-sulfate oligosaccaride units (hTg-CS) and devoid of it (hTg-CS$^-$), were compared, with respect to their ability to induce experimental autoimmune thyroiditis (EAT) in CBA/J(*H-2*^{k}) mice, by subcutaneous administration, in the presence of complete adjuvant.

HTg was chromatographically separated into hTg-CS and hTg-CS$^-$ molecules, on the base of their uronic acid content. In an ample number of hTg preparations, the fraction of hTg-CS in total hTg ranged from 32.0 to 71.6 percent. By exploiting the electrophoretic mobility shift and metachromasia conferred by chondrotin-6-sulfate upon the products of limited proteolysis of hTg, chondroitin 6-sulfate was first restricted to a carboxy-terminal region, starting at residue 2513. 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 being LTAGXGLRE (residues 2725-2733, X being Ser2729 linked to the oligosaccharide chain). In an *in vitro* assay of enzymatic iodination, hTg-CS produced higher yields of 3,5,5'-triiodothyronine (T3) (171%) and 3,5,3',5'-tetraiodothyronine (T4) (134%), than hTg-CS$^-$. Unfractionated hTg behaved as hTg-CS. Thus, chondroitin 6-sulfate addition to a subset of hTg molecules enhanced the overall level of T4 and, particularly, T3 formation. Furthermore, the chondroitin 6-sulfate oligosaccharide unit of hTg-CS protected peptide bond Gly2713-Lys2714 from proteolysis, during the limited digestion of hTg-CS with trypsin.

Although immunization with all forms of hTg was accompanied by thyroid cell damage, as judged from the increase of T4 in blood, a higher degree of mononuclear infiltration of the thyroid was associated with unfractionated hTg, in comparison both with hTg-CS and with

hTg-CS$^-$. Thus, it appears that both hTg subfractions contributed to the immunopathogenic potency of unfractionated hTg, as neither one reproduced fully the histological picture associated with the latter. Significant differences were observed also upon restimulation *in vitro* of splenic lymphocytes obtained from mice immunized *in vivo* with the different forms of hTg. Restimulation *in vitro* with hTg-CS of splenocytes from mice immunized with the same antigen was followed by low-level, dose-dependent proliferation and IFN-γ production, whereas cross-stimulation with hTg-CS$^-$ of the same cells was followed by proliferative and secretory responses of even lower degree. On the other hand, restimulation *in vitro* with hTg-CS$^-$ of splenocytes primed *in vivo* with the same antigen was followed by higher-level, dose-dependent increases of IFN-γ production, accompanied by proliferative responses of low degree and inversely related with the antigen dose, while cross-stimulation with hTg-CS of the same cells was followed by dose-dependent increases, both of proliferation and IFN-γ production, of the highest level observed in this study. Similar results were obtained when splenocytes, primed *in vivo* with hTg-CS$^-$, were restimulated with purified glycopeptide hTg-CSgp, containing the chondroitin 6-sulfate unit, but not with its non-glycosylated, synthetic homologue. These data indicate that hTg-CS$^-$ was more effective than hTg-CS in priming autoreactive T lymphocytes, recognizing thyroiditogenic epitopes shared between murine and human Tg, whereas hTg-CS was a stronger inducer of proliferation of antigen-sensitized T cells. Moreover, different molecular signals, including structural determinant(s) associated with the chondroitin 6-sulfate chain, were required, in addition to epitope recognition, for the activation of T cell proliferation, together with IFN-γ production.

These findings provide insights into the molecular mechanism of regulation of the hormonogenic efficiency and of the T4/T3 ratio in hTg, and may bear important implications in the processing and presentation of hTg as an autoantigen, and in the mechanisms of activation of Th-1-mediated and cytotoxic lymphocyte responses involved in EAT.

2. INTRODUCTION

2.1. Generalities on autoimmune thyroid disease (AITD)

Autoimmune thyroid disease (AITD) is quite common, affecting 2% of females and 0.2% of males (Jacobson et al., 1997). Chronic autoimmune thyroiditis is the most frequent cause of acquired hypothyroidism with goitre in iodine-sufficient areas. It may present with or without goitre, and may progress from euthyroidism or, rarely, hyperthyroidism, to hypothyroidism with goitre (Hashimoto's thyroiditis, HT) or atrophic thyroiditis (primary mixoedema). Graves' disease (GD) is characterized by goitre and thyrotoxicosis, although hypothyroidism can develop in the course of the disease. Infiltrative ophthalmopathy and dermopathy are associated in 50 and 10% of cases, respectively. Both conditions are characterized by thyroid infiltration with T lymphocytes, proliferative responses of T lymphocytes against thyroid autoantigens *in vitro*, and production of autoantibodies against human thyroglobulin (hTg), thyroperoxidase (hTPO), and thyroid-stimulating hormone receptor.

2.2. Genetic factors in AITD

AITD develops as a result of complex interactions between multiple susceptibility genes, and environmental and endogenous factors (Weetman et al., 2003). Epidemiological evidences for a genetic predisposition include familial clustering, and the higher prevalence in females and concordance rate in monozygotic versus dizygotic twins (Brix et al., 2000; Brix

et al., 2001). Family-based studies indicated the linkage of GD to HLA genes (extended haplotype *DRB1*0304-DQB1*02-DQA1*0501*, region 6p21) (Heward et al., 1998) and CTLA-4 (2q33), (Vaydia et al., 1999) confirming earlier associations. The role played by polymorphic *HLA-DR* and *HLA-DQ* genes in the development of autoimmune thyroiditis in response to hTg was demonstrated in murine EAT, by introducing the permissive transgenes *HLA-DRA /DRB1*0301* (*DR3*) and *DQA1*0301/DQB1*0302* (*DQ8*) into class II-deficient *H-2Ab*^{0} mice (Wan et al., 2002). Microsatellite-based, whole-genome linkage studies indicated additional susceptibility loci, such as *AITD-1* (6p), *hTg* (8q), and region 10q for HT and GD, loci *GD-1* (14q), *GD-2* (20q) and region 7q for GD, and locus *HT-2* (12q) for HT (Tomer et al., 2003). The association and linkage of the *hTg* gene with AITD was confirmed by direct analysis of chromosomal region 8q24 (Tomer et al., 2002). Thus, some AITD susceptibility genes may be immune-modifying genes, which increase the general susceptibility to autoimmunity (e.g. HLA, CTLA-4), while others may be thyroid-specific (e.g., *hTg*) (Tomer et al., 2002).

2.3. Experimental autoimmune thyroiditis (EAT)

Experimental autoimmune thyroiditis (EAT), a model of Hashimoto's thyroiditis, can be induced in mice with haplotype *H-2*^{s} and *H-2*^{k} by immunization with mouse Tg (mTg) or human Tg (hTg) in complete Freund's adjuvant (CFA) or lipolysaccharide (Rose et al., 1971). EAT is characterized by infiltration of thyroid by mononuclear cells, production of specific antibody and *in vitro* cell proliferative responses against Tg. EAT is a T cell-mediated disease that can be transferred from mice with immunized 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 (Tange 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).

2.4. Genetic regulation of susceptibility to EAT

The development of EAT is under the influence of H-2 molecules of 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 (Biesel 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).

2.5. 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 homomlogous 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 mojetes, 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 2748 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 omodimers, having a molecular mass of 330,000.

Post-translational modifications of hTg: glycosilation

Carbohydrate 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 effiient 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).

2.6. Immunopathogenic epitopes of Tg and EAT

The recognition of a certain number of conserved epitopes of mTg and hTg by $H\text{-}2^k$- and $H\text{-}2^s$-restricted T lymphocytes seems at the base of the development of murine EAT (Simon et al., 1986). Their current inventory is shown in Table 1. The epitopes recognized by autoreactive T cells are essentially linear in nature, as their reactivity was independent of polypeptide fragment size, and insensitive to thermal denaturation and reduction (Shimoio et al., 1988). By using two mTg-autoreactive T cell hybridomas, reacting selectively with T4-

containing tryptic peptides of hTg, peptide 2551–2559, with T4 at position 2553, was identified among a set of synthetic peptides, centered on the four hormonogenic sites of hTg (Champion et al., 1991). Such a strategy was adopted because iodinated mTg was capable of inducing EAT and activating selectively autoreactive T cells in CBA/J(*H-2A*^{k}) mice, whereas iodine-poor mTg only triggered the production of anti-mTg autoantibodies (Champion et al., 1987).

However, peptide 2549–2560, with T4 at position 2553, termed T4(2553), stimulated proliferative responses *in vitro* and the adoptive transfer of EAT by T cells, primed *in vivo* either with the same peptide or with mTg, but was incapable of direct EAT induction (Hutchings et al., 1992). Homologous peptides with tyrosine or deiodinated T4 at position 2553 also failed to elicit proliferative T cell responses (Dawe et al., 1996). In contrast, others reported that in CBA/J(*H-2A*^{k}) mice, synthetic hTg peptide 2549–2560, with thyronine (T0) at position 2553, was able to induce proliferative responses and activate the transfer of EAT by peptide-primed or mTg-primed lymphonode cells (LNCs), although with lower efficiency than peptide 2549–2560 with T4 2553 (Kong et al., 1995). On the other hand, peptide 1–12, containing T4 at position 5, this being the main hormonogenic site in Tg, elicited only mild thyroiditis and weakly stimulated mTg-primed, but not peptide-primed, LNCs to proliferate and adoptively transfer EAT (Kong et al., 1995; Wan et al., 1997). The observation that the peripheral blood T cells of patients with chronic autoimmune thyroiditis were equally responsive to hTg with variable iodine contents seemed to limit the immunopathogenic role of Tg iodination (Shimojo et al., 1988). However, it was later reported that T cells from patients with chronic thyroiditis and normal controls proliferated in response to minimally iodinated, but not iodine-free hTg (Rasooly et al., 1998). A different, non-iodinated peptide of hTg, spanning residues 1672–1711, named F40D, induced EAT in CBA/J mice. It was partly homologous with a tryptic pTg peptide, which activated a cytotoxic T cell hybridoma derived

from the LNCs of pTg- or hTg-primed mice (Texier et al., 1992) Two peptides of rat Tg (rTg), identified by using predictive algorithms, induced strong secondary proliferative responses by peptide-specific LNCs. Rat Tg peptide 2496–2512 (TgP1, p2495) caused extensive EAT in C3H(*H-2*^{k}) and SJL(*H-2*^{s}) mice (Chronopoulou et al., 1992). The nonamer 2497–2505 was the minimal T-cell epitope in TgP1, and could be presented within the context of the non-isotypic H-2E^k and H-2A^s molecules. Instead, epitope 2500–2508, which was restricted to the H-2A^k and H-2A^s molecules, induced only weak EAT and did not elicit proliferative responses in either strain of mice (Rao et al., 1994). The rTg peptide 2696–2713 (TgP2, p2695) caused only focal EAT by direct induction in *H-2A*^{s} mice, and extensive EAT by the adoptive transfer of peptide-specific LNCs to naive syngeneic recipients (Carayanniotis et al., 1994). A major immunopathogenic T-cell epitope was localized in the rTg peptide 2696–2707, while its human homologue, with the substitutions Glu2703Ser and Thr2704Ser, caused only focal thyroiditis by the adoptive transfer of LNCs, and did not stimulate secondary T-cell responses *in vitro* (Rao et al., 1997). Another hTg peptide (2730–2743), selected because of its partial homology with sequence 118–131 of hTPO, activated only the adoptive transfer of EAT by mTg-primed LNCs (Hoshioka et al., 1993). All the epitopes described were non-dominant, as none was able to prime in vivo LNCs that could proliferate or transfer EAT to syngeneic hosts, upon stimulation with intact Tg *in vitro*. Only peptides 2496–2512 and 1672–1711 caused extensive EAT by direct induction, whereas most of them activated in vitro the transfer of EAT by peptide-specific LNCs. Peptides 2496–2512 and 2696–2713 could not stimulate in vitro hTg-primed LNCs, either. However, when SJL (*H-2*^{s}) mice were immunized with maximally iodinated mTg (I-Tg, containing more than 60 iodine atoms per Tg monomer), they developed more severe EAT, and the behaviour of peptide 2496–2512 changed to dominant, as though iodination enhanced the presentation of a cryptic epitope, which, in turn, activated resting autoreactive T cells. I-Tg-primed LNCs

proliferated strongly in vitro against peptide 2496–2512, and so did a peptide-specific T cell hybridoma, activated with I-Tg-pulsed splenocytes (Dai et al., 2002). On the basis of previous observations (Duthoit et al., 2000; Dunn et al., 1983), it is possible that mTg fragmentation and the exposure of cryptic epitopes had occurred already during the incorporation of non-physiological iodine levels into mTg in vitro. All of the above peptides induced peptide-specific antibody responses, but only peptides 2549–2560 withT4(2553) and 2496–2512 induced the formation of anti-hTg antibodies (Kong et al., 1995; Wan et al., 1997; Rasooly et al., 1998; Texier et al., 1992; Chronopoulou and Carayanniotis, 1992). A recent search, conducted by using an algorithm for A^k-binding motifs in mTg, identified five additional immunogenic, non-iodinated and non-dominant epitopes, which induced the proliferation *in vitro* of peptide-primed, but not mTg-primed, LNCs (Table 1). Four of them (peptides 306–320, 1579–1591, 1826–1835 and 2596–2608) induced very mild or mild EAT directly, and all induced EAT of low to moderate grade, by the adoptive transfer of peptide-primed LNCs. Peptides 2102–2116 and 2596–2608 elicited peptide-specific antibodies that did not react with intact mTg (Verginis et al., 2002). Finally, one (or more) non-dominant T-cell epitope(s) was (were) found in peptide 2340–2359 of hTg, which contained several E^k-binding motifs. This elicited mild to moderate EAT by direct subcutaneous challenge in complete Freund's adjuvant in AKR*/J* mice, and specific proliferative and secretory responses by peptide-primed, but not hTg-primed, LNCs in vitro. Peptide-primed LNCs did not respond to intact hTg, either (Karras et al., 2003). Notably, this peptide coincided with a peptide (2339–2358, TgP15) recognized by Tg-reactive B cells in GD patients (Thrasyvoulides et al., 2001) (see Table 1 and Fig. 1). More recently, hTg p2340 has been found able to induce EAT in *HLA-DR3* transgenic mice, suggesting that it could be presented by DR3 molecules in patients with Hashimoto's thyroiditis and participate in the development of the disease (Karras et al., 2005). The EAT-causing epitopes of Tg are listed in Table 1, while Figure 1 illustrates their

location along the polypeptide chain of Tg, also in relation with its repetitive organization and with the epitopes recognized by human anti-hTg autoantibodies. Seven out of 13 epitopes were located within the AChE-homologous domain, and six of these were comprised within a fragment having a mass of 40,000, and starting at residue 2384, which was produced during the iodination or the oxidation of hTg *in vitro* and *in vivo*, and was recognized by the autoantibodies in the sera of AITD patients (Duthoit et al., 2000; Duthoit et al., 2001). Peptides 2102–2116 and 2696–2713 were also comprised within two cross-reacting tryptic fragments of hTg, with masses of 23,000 and 15,000, starting at residues 2089 and 2657, respectively, and bearing HT-related B cell epitopes (Saboori et al., 1995; Saboori et al., 1999). Of particular interest was the coexistence of GD-related B cell epitopes and EAT-causing T cell epitopes in the 20-mer peptide 2339–2358 (Thrasyvoulides et al., 2001; Verginis et al., 2002). The remaining EAT-causing peptides lay in cysteine-rich regions, namely repeats 1A.4, 3A.1 and 3B.1. The immunopathogenic, non-dominant character of these epitopes is compatible with the hypothesis, originally put forth for Tg type-1 motifs, that the cysteine-rich repeats of Tg may inhibit lysosomal proteases (Molina et al., 1996) and thus contain cryptic epitopes, which are not normally processed and presented to the immune system, and whose exposure may be responsible for the breaking of peripheral tolerance to hTg. Notwithstanding numerous studies, the diversity of immunogenic and pathogenic epitopes of hTg, and their location, are still open to investigation. All the EAT-related Tg epitopes characterized so far, mostly on the base of predictive algorithms, were non-dominant. The detection of immunodominant epitopes may be precluded by the limitations of algorithm-based approaches (Verginis et al., 2002). The binding of peptide 2496–2512 to nonisotypic H-2A^s and H-2E^k products illustrates the fact that the requirements for the presentation of peptides on MHC molecules may exceed binding motifs, by incorporating

processing constraints (Rao et al., 1994). It can be expected that other determinants of pathogenic importance will be identified in Tg by more direct methods.

Table 1 – Immunopathogenic Tg peptides causing EAT in mice

Position	Historical name	Murine strain	H-2 haplotype	MHC restriction	Tg	EAT induction		T cell responses	Abs	Ref.
						Direct	Adoptive			
1-12 [(a)]	T4(5)	CBA	k	ND	hTg	±	±	±	+	Kong et al., 1995 Wan et al., 1997
306-320 [(b)]	p306	CBA	k	A^k	mTg	+	+	+	–	Verginis et al., 2002
1579-1591 [(b)]	p1579	CBA	k	A^k	mTg	±	+	+	–	Karras et al., 2003
1672-1711 [(a)]	F40D	CBA	k	ND	hTg	+	ND	ND	+	Karras et al., 2003
1826-1835 [(b)]	p1826	CBA	k	A^k	mTg	±	+	+	–	Verginis et al., 2002
2102-2116 [(b)]	p2102	CBA	k	A^k	mTg	–	±	+	+	Karras et al., 2003
2340-2359 [(c)]	p2340	AKR	k	E^k	hTg	+	ND	+	+	Karras et al., 2003

2496-2512 [d]	TgP1, p2495 2495-2511 [a]	B10.BR, C3H, SJL	k, s	E^k, A^s	rTg	+	ND	+	+ [e]	Chronopoulou Carayannniotis 1994
2497-2505 [d]	2496-2504 [a]	C3H, SJL	k, s	E^k, A^s	rTg	+	ND	+	+	Rao et al. 1994
2500-2508 [d]	2499-2507 [a]	C3H, SJL	k, s	A^k, A^s	rTg	±	ND	–	–	Rao et al. 1994
2549-2560 [a]	T4(2553)	CBA	k	A^k	hTg	–	+ [f, g]	+	+	Kong et al., 1995 Wan et al., 1997
2596-2608 [b]	p2596	CBA	k	A^k	mTg	±	+	+	+	Verginis et al., 2002
2696-2713 [d]	TgP2, p2695 2695-2713 [a]	SJL	s	A^s	rTg	±	+	+	+	Carayanniotis et al., 1994
2696-2707 [d]	2695-2706 [a]	SJL	s	A^s	rTg	ND	+	+	+	Rao and Carayanniotis, 1997
2730-2743 [a]	2730-2743	CBA	k	ND	hTg	–	+ [f]	+	ND	Hoshioka et al. 1993

ND = not determined

[a] Amino acid residues numbered as in the cDNA-derived sequence of hTg, according to Malthiéry and Lissitzky, 1987

[b] Amino acid residues numbered as in the cDNA-derived sequence of mTg (Kim et al., 1998)

(c) Amino acid residues numbered as in the cDNA-derived sequence of hTg, as modified by van de Graaf et al., 1997. Peptide 2340-2359 coincides with peptide 2339-2358 (TgP15) (Figure 1) whose extremities were numbered according to Malthiéry and Lissitzky, 1987

(d) Amino acid residues numbered as in the cDNA-derived sequence of rTg (Hishinuma et al., 2000). The extremities of these rTg peptides were originally numbered as in the cDNA-derived sequence of hTg, according to Malthiéry and Lissitzky, 1987

(e) Antibodies cross-reacting with Tgs of various animal species

(f) By transfer of LNC primed *in vivo* and restimulated *in vitro* with the peptide

(g) By transfer of LNC primed *in vivo* with mTg and restimulated *in vitro* with the peptide

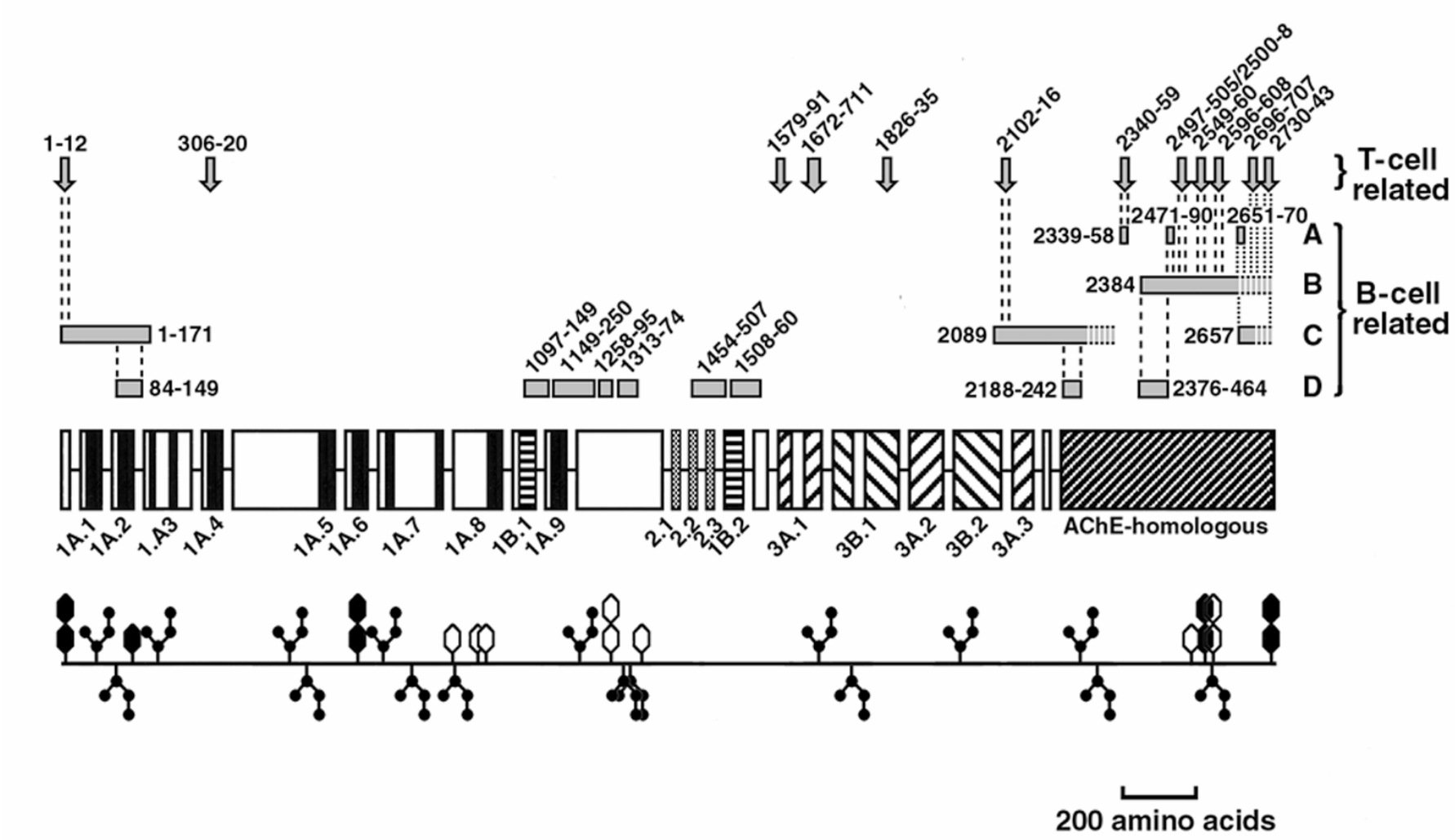

1-12
306-20
1579-91
1672-711
1826-35
2102-16
2340-59
2497-505/2500-8
2549-60
2596-608
2696-707
2730-43
T-cell related
2471-90
2651-70
2339-58
A
2384
B
B-cell related
1-171
1097-149
1149-250
1258-95
1313-74
1454-507
1508-60
2089
2657
C
84-149
2188-242
2376-464
D
1A.1
1A.2
1.A3
1A.4
1A.5
1A.6
1A.7
1A.8
1B.1
1A.9
2.1
2.2
2.3
1B.2
3A.1
3B.1
3A.2
3B.2
3A.3
AChE-homologous
200 amino acids

Figure 1. Diagrammatic representation of hTg, showing the epitope-bearing peptides recognized by human anti-hTg autoantibodies and the immunopathogenic peptides, causing EAT in mice. Tandem repeats of hTg are according to Malthiéry and Lissitzky, 1987, and Molina et al., 1996a. Repeated motifs and other parts of hTg are represented as boxes filled as follows: black, type-1A repeats; horizontal hatch, type-1B repeats; grey, type-2 repeats; upward right cross-hatch, type-3A repeats; downward right cross-hatch, type-3B repeats; upward right narrow cross-hatch, AChE-homologous domain; white, unrelated sequences interrupting individual repeats and spacers separating tandem repeats. Spacer 1 indicates the region between type-1 and type-2 repeats, encompassing residues 1191-1435. Spacer 2 indicates the region between type-1B.2 and type-3 repeats, encompassing residues 1546-1582. Spaces have been introduced for clarity between contiguous motifs and bear no relationship to gene introns;  , glycosylation site;  , hormonogenic acceptor and  , donor tyrosine (filled symbols represent those demonstrated in hTg, empty symbols those found in other animal species). T cell- and B cell-related epitope-bearing peptides are identified by the amino acid numbers at their extremities, as in Table 1, and are represented as grey boxes on top of the hTg diagram, whose lenght is proportional to the polypeptide length. Peptides whose C termini are based on presumptive estimates are identified by their amino-terminal residue number only, and are represented by grey boxes with broken C-terminal extremities. Legend: a) chemically synthesized peptides; b) fragments produced by oxidative cleavage; c) peptides obtained by chemical or enzymatic proteolysis; d) recombinantly expressed peptides. Overlapping regions of different epitope-bearing peptides are connected by vertical broken lines (dotted lines for peptides with presumptive C termini). Notice that the B-cell epitope-bearing peptide 2339-58 and the T-cell epitope-bearing peptide 2340-59, whose extremities were numbered as in the hTg sequences reported by Malthiéry and Lissitzky, 1987 and van de Graaf et al., 1997, respectively, actually coincide.

2.7. Chondroitin 6-sulfate oligosaccharide units and immune responses

A number of studies were focused on the role of chondroitin sulfate oligosaccharide units in the modulation of immune responses. A small proportion of invariant chain molecules (Ii), associated with class II MHC molecules at the surface of antigen-presenting cells (APC), is modified with the addition of chondroitin sulfate oligosaccharide units (Ii-CS). Ii-CS dramatically enhances the ability of APC to stimulate mitogenic and allogeneic T cell response, through the interaction with CD44 (Naujokas et al., 1993). CD44, a transmembrane glycoprotein, widely expressed on leukocytes, fibroblasts, endothelial and epithelial cells, is the principal cell surface receptor for hyaluronan (HA), but recognizes also chondroitin 4- and 6-sulfate, although with lower affinity (Aruffo, 1990; Sy, 1991). Its co-stimulatory role in T cell activation is supported by a number of studies (Sommer, 1995; Yashiro, 1998).

Serglycine, a family of secretory granule proteoglycans, modified with chondroitin-4 and -6 sulfate and expressed in lymphoid and myeloid cells, bind specifically CD44 and their addition to cytotoxic T lymphocytes (CTL) clones promotes the release of cytokines and proteases from secretory granules (Toyama-Sorimachi et al., 1997). CD44 also interacts with aggrecan, a major component of bovine and rat cartilage. Such binding is dependent on chondroitin sulfate-A and chondroitin sulfate-C lateral chains of aggrecan. The interaction of aggrecan with CD44 determines oligomerization of CD44 molecules and the activation of intracellular signaling (Fujimoto et al., 2001). CD44 also binds versican, a CS-bearing proteoglican, expressed in fibroblasts, keratinocytes and arterial smooth muscle cells in kidney, skin, brain, and other tissues. The interaction occurs through the chondroitin sulfate-B and -C side chains of versican (Kawashima et al., 2000).

3. AIM OF THE STUDY

Tg is not only the molecular site of thyroid hormone formation, but also a major autoantigen, involved in the pathogenesis of thyroid autoimmunity. Numerous post-translational modifications contribute to the molecular microheterogeneity of hTg. Iodine addition and hormone formation at specific sites have the most obvious effects on thyroid function, but effects of glycosylation on the hormone-forming efficiency at specific sites and on the immunogenicity of Tg have also been clearly documented Mallet et al., 1995; Fenouillet et al., 1986). HTg is modified with the addition of several kinds of oligosaccharide units among which N-linked type A (high-mannose) and type B (complex) units have been characterized best, as to their composition and localization (Arima et al., 1972; Arima and Spiro, 1972; Yang et al., 1996). Among O-linked oligosaccharide chains, type D units were reported to be composed of a repeating disaccharide of the chondroitin 6-sulfate type, attached to the polpeptide chain through a galactosyl-serine linkage region (Spiro, 1977; Spiro and Bhoyroo, 1988; Schneider et al., 1988). However, the number and localization of type D oligosaccharide units in the hTg molecule was not established. Several studies have suggested the influence of oligosaccharide chains in the processing and/or presentation of glycoproteic antigens (Glant et al., 1998; Vlad et al., 2002; Hanisch et al., 2003; Anderton, 2004), and the involvement of chondroitin 6-sulfate oligosaccharide chains in the modulation of cellular immune responses (Naujokas et al., 1993; Toyama-Sorimachi et al., 1997; Fujimoto et al., 2001). Prompted by these observations, we set up to determine: 1) the number and localization of chondroitin 6-sulfate oligosaccharide chain(s) in hTg, and 2) the influence of post-translational addition of chondroitin 6-suoftae unit(s) on the immunopathogenicity of hTg in a murine model of experimental autoimmune thyroiditis.

4. MATERIALS AND METHODS

4.1. Material

Thermolysin from *Bacillus thermo-proteolyticus rokko* (EC 3.4.24.4) and L-1-tosylamide-2-phenylethylchloromethyl- (TosPheCH2Cl-) treated trypsin from bovine pancreas (EC 3.4.21.4), endoproteinase Glu-C from *Staphylococcus areus* (EC 3.4.21.19), lactoperoxidase from bovine milk (EC 1.11.1.7), and glucose oxidase from *Aspergillus niger* (EC 1.1.3.4) were purchased from Sigma-Aldrich (Milan, Italy), protease-free chondroitinase ABC from *Proteus vulgaris* (EC 4.2.2.4) from Boehringer Mannheim (Milan, Italy), aminopeptidase M from porcine kidney (EC 3.4.11.2) and pronase from *Streptomyces griseus* from Calbiochem (San Diego, CA, USA). Sephacryl S-300 HR, HiTrap™ Q Sepharose HR, DEAE-Sepharose Fast Flow, and Sephadex G50 fine were obtained from GE Healthcare, BioGel P-2 and electrophoresis products from Bio-Rad Laboratories (Milan, Italy), Immobilon P from Millipore (Vimodrone, Italy), HPLC-grade solvents from Carlo Erba (Milan, Italy), bicinchoninic acid (BCA) Protein Assay Reagent from Pierce (Rockford, IL, USA), diethyl-methyl-dibenzo-thiacarbocyanine (DMTCC, Stains All™) from ICN Biomedicals (Milano, Italy), solid-phase kits for radioimmunoassays of total T4 and T3 from Medical Systems (Rio Torbido, Italy), other analytical grade chemicals from Sigma-Aldrich.

4.2. Purification of hTg and its chondroitin 6-sulfate-containing (hTg-CS) and chondroitin 6-sulfate-devoid (hTg-CS-) subfractions by ion-exchange chromatography on Hi-Trap™ Q-Sepharose HR (Q-IEC)

HTg was prepared from thyroid tissue obtained from informed euthyroid patients hemilaryngectomized for non-thyroidal disease, and patients undergoing subtotal thyroidectomy for non-familial, simple or multinodular goiter. HTg was prepared either by extraction or by homogenization with an Omnimixer homogenizer, in 0.05 M sodium phosphate, pH 7.2 (1 ml/g tissue), at 4 °C for 6 hours, followed by fractional precipitation with 1.4-1.8 ammonium sulphate, 0.05 M Tris·HCl, pH 7.2, and gel filtration on Sephacryl S-300 in 0.13 M NaCl, 0.05 M Tris·HCl, pH 7.4 (buffer A). In order to increase the immunogenicity of hTg, poor-iodine hTg from goiter was iodinated enzymatically *in vitro*, using 2 μg/mL of bovine lactoperoxidase and 4 x 10^{-5} M KI, plus 0.23 μg/mL of glucose oxidase from *Aspergillus niger* and 1 mM D-glucose, as the H_2O_2 generating system, over 90 min at 25 °C. Under these conditions, the amount of iodine incorporated into hTg was regularly 0.5% (percent of hTg mass, corresponding to 25 moles of iodine atoms/mole of hTg). Subsequently, hTg was dialyzed against phosphate-buffered saline (PBS), containing 136 mM NaCl, 6 mM Na_2HPO_4, 1,5 mM KH_2PO_4,, 3 mM KCl). Protein concentration was estimated by measuring the absorbance at 280 nm, using an extinction coefficient of 10 cm^{-1} for a 1% solution. The iodine content was measured by a non-incinerative method, using L-thyroxine as the standard (Palumbo et al., 1982).

HTg molecules containing type D (chondroitin 6-sulfate) oligosaccharide units (hTg-CS) were separated from the residual hTg molecules, devoid of them (hTg-CS^-), by ion-exchange chromatography on trimethylamino-substituted Q-Sepharose (Q-IEC), using 5-mL HiTrap™ Q-Sepharose HR columns (GE Healthcare), 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 the 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. 1-mL fractions were analyzed, or stored at -80 °C until use. Areas under peaks were calculated using the NIH Image software.

In order to minimize contamination of hTg preparations with bacterial products, namely endotoxins, both the head of the gel filtration column and anion-exchange column were provided with polyethersulphone ultrafiltration membrane Biomax, with 10 KDaltons pores (Pellicon XL, Millipore). The concentration of bacterial endotoxin in hTg preparations to be used both for mice immunization and stimulating lymphocytes proliferation, measured by *Limulus amebocytes lysate* test, resulted <0.01 Endotoxin Units (EU)/mL. Unfractionated hTg, $hTgCS^-$ and hTgCS have been concentrated by centrifugation in Centriprep-30 to a final concentration of 2.0 g/L and stored at -80°C until use.

4.3. Compositional analysis of the fractions of the Q-IEC of hTg

Uronic acids were assayed in Q-IEC fractions by the *meta*-hydroxy-biphenyl mehod (Blumenkrantz et al., 1973), using D-glucuronic acid (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 speed vacuum concentrator, and redissolved in 0.2 mL of dH_2O in borosilicate Pyrex tubes. Samples were processed by the addition of 1.2 mL of 0.0125 M $Na_2B_4O_7$•10 H_2O in H_2SO_4, followed, after heating in boiling water bath for 5 min and cooling on ice, by 0.02 mL of 0.15% *m*-hydroxy-biphenyl 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, except that *m*-hydroxy-biphenyl in the 0.5% NaOH reagent was omitted. Total neutral hexoses were assayed in duplicate samples of 20-300 μg of hTg, by the anthrone method (Spiro, 1966), 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 (Warren, 1963), 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 (Palumbo et al., 1982).

4.4. Enzymatic *in vitro* iodination of hTg and analysis of the iodine, T3 and T4 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/L, was performed in 0.02 M imidazole/HCl, pH 7.0, using 2 μg/mL of bovine milk lactoperoxidase, 4 x 10^{-5} M KI, 1 mM D-glucose and 0.19 μg/mL of glucose oxidase from *Aspergillus niger*. Iodination was stopped with 0.05 M 2-mercapto-1-methylimidazole (MMI). Comparative iodination of hTg, hTg-CS and hTg-CS^- was performed with 0.65 mg of each protein, under identical conditions, except that 7.5 x 10^{-5} M KI, and 0.21 μg/mL of glucose oxidase were used. Aliquots of 0.1 mg of hTg were withdrawn at intervals of 5, 15, 30, 50, 70 and 90 min, supplemented with MMI, dialyzed against 0.01 M NH_4HCO_3, 0.005 M NaCl, and assayed in duplicate for protein content, using the BCA Protein Assay Reagent (Bio-Rad Laboratories) and bovine serum albumin as the standard, and iodine content, as already described. For the assay of T4 and T3, 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, pH 8.0, 0.05 M MMI, 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 prolonged for 24 h at 37 °C, after which T4 and T3 were measured by solid-phase RIA (Medical Systems).

4.5. Limited proteolysis of hTg

HTg, at the concentration of 1 g/l 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 TosPheCH_2Cl-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 x 10^{-5} M antipain, 2 x 10^{-6} M aprotinin, 5 x 10^{-4} M benzamidine, 4 x 10^{-5} M leupeptin, 1 x 10^{-4} M tosyl-lysil-chloromethylketone, 2 x 10^{-5} M phenyl-methyl-sulfonyl fluoride, 5 x 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% bromophenol blue, and the samples were heated in a boiling water bath for 1.5 min and immediately subjected to SDS-PAGE.

4.6. Enzymatic digestion of chondroitin 6-sulfate oligosaccharide units of hTg 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 Na acetate, 0.1 M Tris/HCl, pH 8.0, and 200 mU/mL of chondroitinase ABC from *Proteus vulgaris*, which degrades chondroitin 4-sulfate and 6-sulfate oligosaccharide chains into sulfated disaccharides, by hydrolizing the glycosidic bonds between glucuronic acid and N-acetylgalactosamine sulfate in the repeating disaccharide units. The protease inhibitors already indicated with regard to limited proteolysis were added, when not already present. Samples were incubated at 37 °C for 4 h, and then subjected to precipitation in methanol/chloroform/water (Wessel and Flügge, 1984) and immediately analyzed by SDS-PAGE.

4.7. Separation and identification of the products of limited proteolysis of hTg

Analytical PAGE of the digestion products in Tris-glycine-SDS was performed, using 4-17% total acrylamide, 2.7% *N,N'*-methylene-bis-acrylamide gradient gels. Molecular mass standards were: myosin (205,000), β-galactosidase (116,000), phosphorylase *b* (94,000), bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,000). Two replicas of each gel were made. One was stained with 0.1% Coomassie Brilliant Blue (CBB) R-250 in 25%(v/v) 2-propanol, 10%(v/v) acetic acid, destained in 25% (v/v) methanol, 10% acetic acid. The other was fixed in 25% (v/v) 2-propanol, 10% acetic acid, thoroughly rinsed in double-distilled H_2O, stained with 0.005% diethyl-methyl-dibenzo-thiacarbocyanine (DMTCC) in 50% formamide for 48 h, and finally destained in 5% glycerol (v/v) in tap water (Dahlberg et al., 1969). 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 (Gentile and Salvatore, 1993). Proteolytic fragments to be identified *ex novo* were separated by reducing or non-reducing SDS-PAGE, in 4-17% acrylamide gradient gels, and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore) by semi-dry blotting in 25 mM Tris, 10 mM 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% CBB R-250 in 50% methanol, and destained in 50% methanol, 10% acetic acid. Bands were subjected to NH_2-terminal peptide sequencing at the Protein Structure Laboratory, University of California, Davis. Peptide sequences were aligned with the cDNA-derived sequence of hTg (Malthiéry and Lissitzky, 1987).

4.8. Isolation and sequencing of glycopeptide hTg-CSgp

Fifty mg of hTg-CS were denatured and reduced in 15 mL of 0.3 M Tris/HCl, pH 8.0, 6 M guanidine/HCl, 1 mM EDTA, 10 mM 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 phosphate, pH 7.8, and digested with endoproteinase Glu-C (protease V8) from *Staphilococcus 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 HR 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 optical absorbance at 280 nm, and uronic acid content. A single uronic acid-containing peak was subjected to size exclusion chromatography on a 1.5-by-100-cm column of BioGel P-2 (Bio-Rad Laboratories), in 0.01 M NH_4HCO_3. An uronic acid-containing peak, eluted in the void volume, was liophylized and further purified by gel chromatography on a 0.5-by-40-cm column of Sephadex G-50 fine (GE Healthcare), in 0.01 M NH_4HCO_3. One-mL fractions were monitored for peptide-related optical absorbance at 220 nm and uronic acid content, and a single peptide- and uronic acid-containing peak was liophylized. An aliquot of it was subjected to NH_2-terminal peptide microsequencing. Purity was checked by PAGE in Tris-tricine-SDS, in a 16.5% T, 6% C gel, containing 6.0 M urea (Schägger and von Jagow, 1987), followed by semi-dry transfer to Immobilon P (Millipore), as already described, for 25 min. The membrane was stained with DMTCC in 50% formamide, and destained in tap water.

4.9. Synthesis of a non-glycosilated peptide homologue of hTgCSgp

A chondroitin 6-sulfate-devoid peptide homologue of hTg chondroitin 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.

4.10. Isolation and characterization of tryptic fragment h4bis$_{TR}$ by HPLC and ElectroSpray Mass Spectrometry

The fragments obtained from the limited digestion of 20 mg of hTg with trypsin for 20 min, under the conditions described above, were freed from trypsin immediately after digestion, by passage through a 10-mL column of DEAE-Sepharose Fast Flow (GE Healthcare), 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-by-100-cm column of BioGel P-2 (Bio-Rad Laboratories), 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 x 10 mm, 5 μm), equilibrated in 0.1% (v/v) trifluoroacetic acid in H_2O (solvent A), containing 2% of 0.07% trifluoroacetic acid in acetonitrile (solvent B). After 2 min at 35% of solvent B, the elution was conducted 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 non-reducing conditions. Corresponding peaks from repeated runs were pooled for further mass spectrometric analysis.

ES mass spectra of peptide h4bis$_{TR}$ were recorded with a PLATFORM mass spectrometer (Fisons, Manchester, UK), equipped with an electrospray ion source. Samples from the HPLC separation (10 μL, 20 pmol) were injected into the ion source at a flow rate of

10 μL/min; the spectra were scanned at the speed of 10 sec/scan. Mass calibration was carried out using the multiple charged ions from a separate introduction of horse heart myoglobin (average relative molecular mass of 16950.5). 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 in the spectra were associated with the corresponding peptides, on the basis of the expected molecular masses, using a dedicated software.

4.11. Experimental animals

All the experiments were conducted using female, 10-12 week-old, CBA/J(*H-2*^{k}) mice genetically susceptible to EAT induction, purchased from Charles River.

4.12. Induction of EAT

On day 0, groups of 4-6 mice, were immunized s.c. with 100 μg of hTg, hTgCS^{-}, hTgCS (2 mg/mL) in PBS, emulsified at 1:1 ratio in Complete Freund Adiuvant (CFA), containing 3.5 mg/mL *Mycobacterium tuberculosis* (H37-RA, Difco Laboratories, Detroit, Michigan). Ten 10 days later, mice were boosted s.c. with 50 μg of the same antigens in Incomplete Freund Adiuvant (IFA). Six weeks after the first immunization, mice were killed under anesthesia. Blood from tale veins, spleen and thyroid were immediately obtained. Serum was separated and stored at -20°C, thyroid lobes were fixed in 10% formalin and lymphocytes were used for proliferation assays.

4.13. Histological scoring of EAT

EAT was assessed by histological examination of the thyroid, using a modification of the criteria adopted by Dai et al., 2005. Mononuclear cell infiltration index was scored as follows: 0 = no infiltration; 1 = interstitial accumulation of cells between two or three follicles; 2 = one or two foci of cells at least the size of one follicle; 3 = extensive infiltration, 10-40% of total area; 4 = extensive infiltration, 40-80% of total area, and 5 = extensive infiltration > 80% of total area. Each mouse was assigned the average infiltration index observed from both thyroid lobes (at least 10 sections per lobe were read and the average infiltration index per lobe was scored).

4.14. Assay of T4 and T3 in serum

T4 and T3 concentrations in the serum of experimental animals were determined on 10-100-µL serum samples, by using solid-phase radio-immunometric assay kits (Medical Systems), with reference curves ranging from 0.3 to 7.7 pmoles for T4, and 30 to 920 fmoles for T3.

4.15. Lymphocyte proliferation assays

Spleens were aseptically collected (soon after mice killing) in 60-mm Petri dishes in 5 mL of RPMI medium, containing Na pyruvate, L-glutamine, 2x penicillin-streptomycin. Splenocytes have been obtained through mechanical disruption of the spleen with the upper extremity of a disposable syringe plunge and transferred to sterile 50 mL Falcon tubes, resuspended by gentle pipetting, after the addition of another 5 mL of medium, diluted 1/100 with PBS and counted with a Burker cell. After centrifugation, medium was aspirated and cells were resuspended in complete medium containing 2% fetal bovine serum (FBS), so to obtain a cell density of $5x10^6$ cells/mL. 100 μL aliquots of cell suspension ($5x10^5$ cells/100μL/well) plus 80 μL of complete medium 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 absence or in presence of 20 μL of varying concentrations of antigen (0-30 μg). 48 hours after initiation of culture cell, supernatants were removed from single well and frozen at-80° for cytokine assay. The remaining cells were cultured for additional 16 hours, pulsed with 1μCi/well of [H^3] thymidine, harvested on glass-fiber filters and counted in Betaplate liquid scintillation counter. All cultures 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.

4.16. Detection of specific antibodies by ELISA

The levels of hTg, hTgCS$^-$, and hTgCS-specific total IgG, and IgG1, IgG2a and IgG3 were determined by ELISA, using serum from individual mouse. The wells of 96-well plates were coated with 100μL of a 10μg/mL solution of hTg, hTg, hTgCS$^-$, and hTgCS in 0.1 M

Na_2HPO_4, pH 9.6, and incubated overnight. After washing, the reactive sites were blocked with PBS-10%FCS for 30 minutes. For total IgG measurements, 100 μL of serum diluted 1/6000 with 0.2% Tween 20 in PBS-10%FCS were added, and plates were incubated for 2 hours at room temperature. After 4 washes, goat anti-mouse biotin-conjugated Abs diluted 1/3000 were added for 1 hour. After 6 washes 1/500 ExtrAvidin peroxidase was added for 30 minutes. The reaction was developed using *o*-phenylenediaminedihydrochloride (Sigma Fast OPD) as the peroxidase substrate, and the absorbance at 450 was after 30 min in an ELISA plates reader. IgG1 and IgG2a concentrations were determined in a similar manner, using goat biotin-conjugated, specific antibodies for IgG1 and IgG2a. Optimal dilutions of serum (1/6000 and 1/5000 for IgG1 and IgG2a, respectively) and subclass-specific antibodies (1/20000 and 1/500 for IgG1 and IgG2a, respectively) were determined in preliminary titrations.

4.17. Cytokine assays

Cytokine production was determined in culture supernatants. Assays of INFγ, IL-4 and IL-10 were performed by ELISA, using cytokine-specific capture and detection antibodies, according to the manufacturer's instructions (Peprotech). Standard curves were generated for each cytokine, by using known amounts of murine rINFγ, rIL-4 and rIL-10.

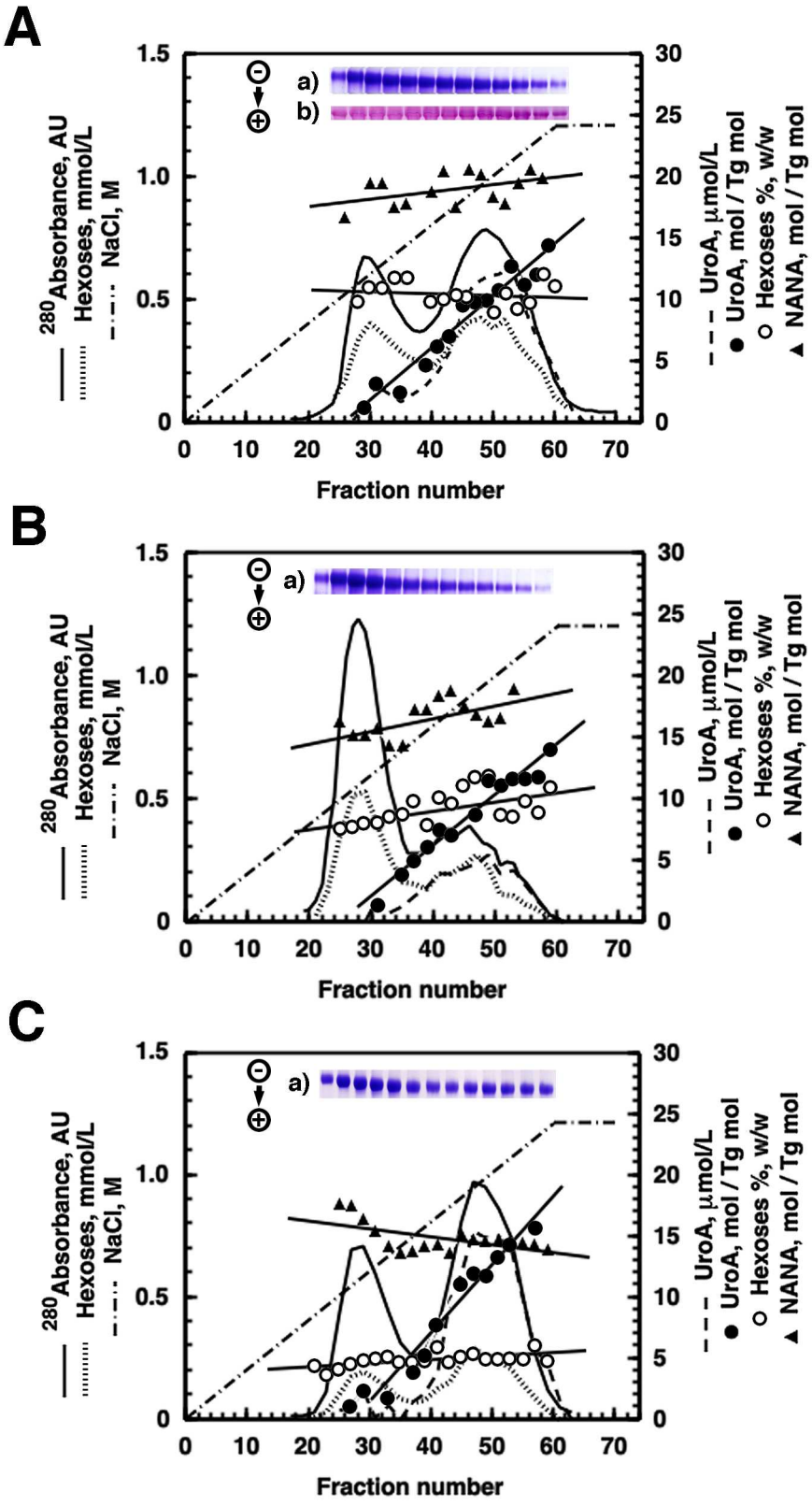
4.18. Statistical analysis

Data are expressed as mean ± SD, unless otherwise specified. Two-tailed Student's, Kruskal-Wallis and Mann-Whitney tests were employed for statistical analysis. A value of $p < 0.05$ was considered to be statistically significant.

5. RESULTS

5.1. A relevant fraction of hTg is regularly composed by hTg molecules provided with uronic acid-containing, type D (chondroitin 6-sulfate) oligosaccharide units (hTg-CS)

Type D (chondroitin 6-sulfate) oligosaccharide unit-containing hTg molecules were separated from residual hTg molecules, using ion-exchange chromatography on trimethylamino-substituted HiTrap™ Q-Sepharose HR (GE Healthcare). The Q-IEC of 40 hTg preparations, mostly from simple and multinodular goiters but also from normal thyroids, using a NaCl gradient from 0 to 1.2 mol/L 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 (at the column head) of 0.49 ± 0.01 M and 0.80 ± 0.01 M, respectively (Figure 2). In 18 chromatograms, the area of the Q2 peak varied from 32.0 to 71.6% of total (mean, 52.8%; median, 51.7%). The hTg subpopulations thus separated were stable, and separation was satisfactory for most practical purposes, as shown by pooling and rechromatography of the two peaks (Figure 3, panel A). We traced chondroitin 6-sulfate oligosaccharide units in hTg fractions, by assaying their uronic acid content, using a *meta*-hydroxybiphenyl-based method. Uronic acids were largely restricted in the Q2 peak, which thus coincided with a fraction of hTg, marked by the presence of uronic acid-containing, type D (chondroitin 6-sulfate) oligosaccharide units (henceforth referred to as hTg-CS). Negligible amounts of uronic acids in the Q1 peak possibly reflected the presence of hTg molecules with very short, incomplete type D oligosaccharide chains (Figure 2 and Table 2). The neutral hexose content stayed constant or increased slightly, along the elution profile of several chromatograms, while the sialic acid content showed no variation or moderate changes in either sense.


A
B
C
a)
b)
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Hexoses, mmol/L
NaCl, M
UroA, µmol/L
UroA, mol / Tg mol
Hexoses %, w/w
NANA, mol / Tg mol
Fraction number

Figure 2 - Fractionation by ion-exchange chromatography on HiTrap™ Q-Sepharose HR (Q-IEC) of selected hTg preparations, and compositional analysis of the fractions.

Twenty-mg aliquots of hTg preparation O. (*panel A*), Ma. (*panel B*), and D. (*panel C*), in 0.025 M Tris/HCl, 0.065 M NaCl, pH 7.4, were loaded onto a HiTrap™ Q-Sepharose HR 5-mL 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 (dashed-dotted line). One-mL fractions were monitored for protein content, by measuring the optical absorbance at 280 nm (continuous line), and carbohydrate content. Legend: neutral hexoses, mmol/L (hatched line) and % on a weight basis (❍); uronic acid, μmol/L (dashed line) and mol/Tg mol (●); N-acetylneuraminic acid (NANA), mol/Tg mol (▲). The analysis of a fraction every other three by PAGE under native conditions, in a 4-9% total acrylamide gradient gel stained with CBB R-250, is shown on top of *panels A* and *B*. The analysis by SDS-PAGE, in a 4-9% total acrylamide gradient gel stained with DMTCC, also shown on top of *panel A*, evidences the metachromasia, on going from the Q1 peak to the Q2 peak, along the elution profile of the Q-IEC.

TABLE II – **Compositional analysis of selected hTg preparations subjected to Q-IEC on HyTrap™ Q-Sepharose HR**

hTg preparation [a)]	hTg-CS, % of hTg	Uronic Acid in Q2 peak			Iodine, % of hTg mass
		mmol/L [b)]	mmol/hTg mol [b)]	range [c)], mmol/hTg mol	
O. [d]	48.0	11.8	10.0	3.8-14.3	0.50
Ma.	38.9	3.9	7.0	3.9-14.0	0.12
T.	48.3	7.2	7.7	3.7-14.0	0.02
C.	51.2	7.5	7.3	2.8-14.2	0.45
D.	66.3	14.8	11.7	3.9-15.7	0.16
Mi.	71.6	20.9	15.6	3.1-23.8	0.16
Mi. Nod. [e)]	63.1	16.8	12.0	3.6-24.0	0.08
Mi. Nod. Q2 peak in urea [f)]	68.0	20.2	13.6	3.2-14.8	-

[a)] hTg preparations were named after the patients (last name initial). All preparations were from non-familial, simple or multinodular goiters, except preparation O.;

[b)] Values measured in the fractions with maximal protein concentration in the Q2 peak;

[c)] Indicates the uronic acid concentration range across the span of peak Q2, starting from the valley between peaks Q1 and Q2;

[d)] hTg preparation from normal thyroid tissue of an euthyroid patient, hemilaryngectomized for a non-thyroidal disease;

[e)] Preparation obtained from a large 2 x 3-cm colloid nodule, surrounded by apparently normal thyroid tissue, of patient Mi.;

[f)] Pooled fractions of the Q2 peak from the Q-IEC of hTg preparation Mi. Nod. were dissociated in 2.5 M Urea, 0.04 M Tris/HCl, pH 9.0 for 18 h at 20 °C, and then subjected again to Q-IEC, using a gradient from 0 to 1.2 M NaCl in 2.5 M urea, 0.055 M Tris/HCl, pH 7.4.

Instead, the number of moles of uronic acid per hTg mole regularly exhibited a linear increase across the fractions of the Q2 peak, spanning the range between 3 and 14 moles, in several hTg preparations in which hTg-CS was between 39 and 51% of total, but reaching values of 24, in preparations more enriched with hTg-CS (Table II). In particular, preparations Mi. and Mi. Nod. had elution profiles, in which shoulders in the trailing edge of the Q2 peak indicated the presence of a secondary component, eluting at 0.9 M NaCl (Figure 3, panel B). We hypothesized that the complexity and extended span of uronic acid content of these Q2 peaks may reflect the coexistence of heterodimeric hTg-CS, with a single chondroitin 6-sulfate-bearing subunit, and homodimeric hTg-CS. Pooled fractions of the Q2 peak from the Q-IEC of hTg Mi. Nod. were dissociated in 2.5 M urea, 0.04 M Tris/HCl, pH 9.0, at 20 °C over night, as previously reported (Veneziani et al., 1998), and subjected again to Q-IEC, in the presence of 2.5 M urea. As shown in figure 3, panel B, the hTg monomers produced by the dissociation of hTg-CS in urea were separated into peaks Q1 and Q2, eluting at NaCl concentrations of 0.48 and 0.81 mol/L, respectively. Once again, uronic acids were restricted largely in the Q2 peak, and their concentration showed a linear increase from 3.2 to 14.8 moles/hTg mole across the peak span, as opposed to the increase from 3.6 to 24.0 moles/mole of undissociated hTg-CS (Table II). The Q2 peak of monomeric hTg-CS was superimposed on the primary, leading component of the Q2 peak of native hTg-CS, with no secondary shoulder at the trailing edge. The Q2/Q1 ratio of hTg-CS dissociated in urea was 2/1. These data demonstrate that the native hTg-CS pool of hTg Mi. Nod. included both an earlier-eluting, heterodimeric subfraction, composed of chondroitinated and non-chondroitinated monomers in the 1/1 ratio, and a later-eluting, homodimeric component, composed only of chondroitinated subunits, and representing about one-third of total hTg-CS. Thus, hTg-CS regularly represented a relevant and, sometimes, predominant fraction of hTg. It was microheterogeneous, conceivably because of the variable number of repeating disaccharide

units per oligosaccharide chain, which is typical of glycosaminoglycans (Malsch et al., 1996). In hTg preparations with the highest fractions of hTg-CS, heterodimeric hTg-CS molecules coexisted with homodimeric ones. Panels A and B of figure 2 also show the PAGE, under native conditions, of the Q-IEC fractions of hTg preparations O. and M., evidencing the anodal shift, on going from the Q1 to the Q2 peak, due to the added negative charge of glucuronic acid residues and sulfate groups of the chondroitin 6-sulfate unit(s).

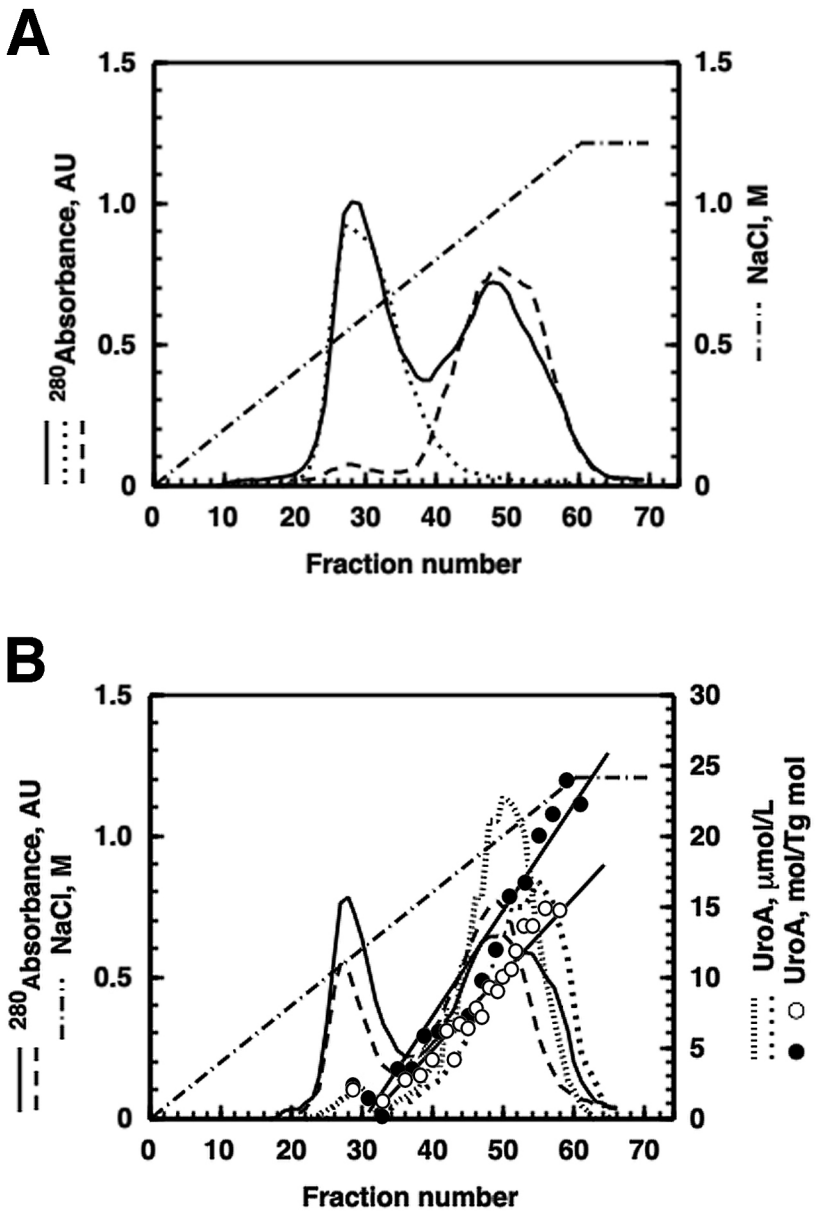


Figure 3 - Fractionation of selected hTg preparations by Q-IEC, followed by rechromatography of subfractions under various conditions.

Panel A: following the Q-IEC of 20 mg of hTg preparation I., as specified under Experimental Procedures and in the legend to Fig. 1, the fractions of the Q1 peak and the Q2 peak were reunited in separate pools, with the exclusion of three fractions at the valley between peaks, dialyzed against buffer A, and each pool was subjected to rechromatography, under identical conditions. The optical absorbance at 280 nm of the fractions of the Q-IEC (continuous line), and of the rechromatography of the Q1 peak (dotted line) and Q2 peak (dashed line) was monitored. *Panel B*: following the Q-IEC of 20 mg of hTg preparation Mi. Nod., 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 over night, as previously reported (30), and subjected to Q-IEC, using a NaCl gradient from 0 to 1.2 mol/L in 2.5 M urea, 0.055 M Tris/HCl, pH 7.4. Legend: optical absorbance at 280 nm of the fractions of primary Q-IEC (continuous line), and Q2 peak rechromatography (dashed line); uronic acid, μmol/L of primary Q-IEC (dense-hatched line) and rechromatography (loose-hatched line); uronic acid, mol/Tg mol of primary Q-IEC (●) and rechromatography (❍).

5.2. hTg-CS has a higher efficiency of T4 and, especially, T3 formation than hTg-CS$^-$, and enhances the overall hormonogenic efficiency of hTg

The proximity of Ser2729 to the preferential site of T3 formation (Ser2746) (Fassler et al., 1988; Lamas et al., 1989) prompted us to investigate the possible influence of the chondroitin 6-sulfate oligosaccharide unit(s) on the hormonogenic function of hTg-CS, in comparison with hTg-CS$^-$, and unfractionated hTg. To this aim, iodine-poor hTg, hTg-CS and hTg-CS$^-$ from goiters (with no more than 0.09% of iodine, on a weight basis) were iodinated enzymatically *in vitro*, using 2 μg/mL of bovine lactoperoxidase and 7.5 x 10^{-5} M KI, 0.21 μg/mL of glucose oxidase from *Aspergillus niger* and 1 mM D-glucose, over 90 min at 25 °C (Lamas et al., 1986). Figure 4 shows the mean amounts of iodine incorporated into hTg, expressed as percent of the hTg mass, during 4 time-course experiments, and the levels of T3 and T4 formed, as a function of iodine bound. Similar amounts of iodine were incorporated at plateau in hTg and hTg-CS, and slightly higher amounts in hTg-CS$^-$ (Figure 4, panel A). However, the number of T3 mmoles (panel B) and T4 moles (panel C) synthesized per hTg-CS mole were in the ratios of 1.70 and 1.34, respectively, with those synthesized per hTg-CS$^-$ mole. Instead, hTg and hTg-CS did no differ, as for the efficiency of formation of T3 and T4. These data indicated that hTg-CS had a higher efficiency of T4 and, especially, T3 formation than hTg-CS$^-$, and that the entire population of unfractionated hTg molecules benefited from this property of hTg-CS. This conclusion was supported by the observation that no significant variations were observed in the yields of T3 and T4, among the different fractions of various preparations of hTg, which were first iodinated as a whole, and then subjected to Q-IEC. These included hTg from normal subjects, physiologically iodinated *in vivo* (Figure 5, panel A), and hTg from patients with goiter, which had undergone low-level iodination *in vivo* (Figure 5, panel B), or enzymatic iodination *in vitro* (Figure 5, panel C).

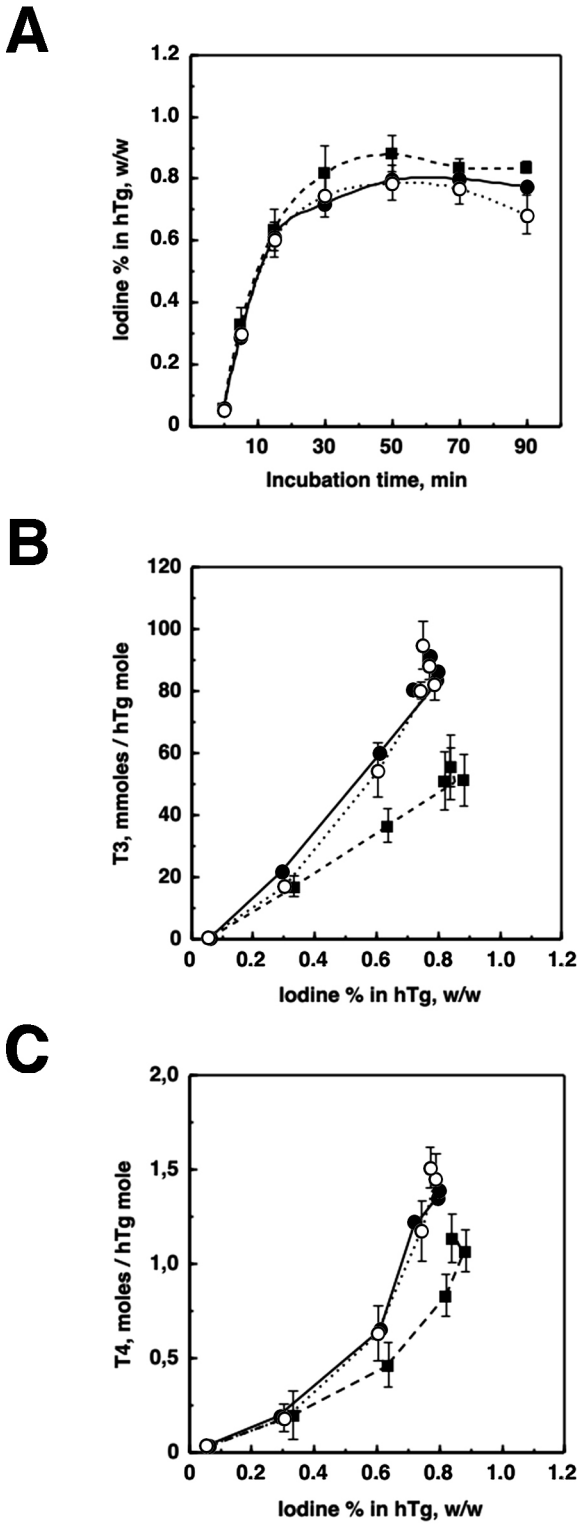

A
1.2
1.0
0.8
0.6
0.4
0.2
0
Iodine % in hTg, w/w
10
30
50
70
90
Incubation time, min
B
120
100
80
60
40
20
0
T3, mmoles / hTg mole
0
0.2
0.4
0.6
0.8
1.0
1.2
Iodine % in hTg, w/w
C
2,0
1,5
1,0
0,5
0
T4, moles / hTg mole
0
0.2
0.4
0.6
0.8
1.0
1.2
Iodine % in hTg, w/w

Figure 4 - Enzymatic *in vitro* iodination and analysis of the hormone-forming efficiency of hTg, hTg-CS and hTg-CS$^-$.

Iodine- and hormone-poor hTg (●), and subfractions hTg-CS$^-$ (■) and hTg-CS (❍), prepared by Q-IEC, at the concentration of 0.45 g/L in 0.02 M imidazole/HCl, pH 7.0, were iodinated enzymatically *in vitro*, using 2 μg/mL of bovine lactoperoxidase, 7.5 x 10^{-5} M KI, 0.21 μg/mL of glucose oxidase from *Aspergillus niger* and 1 mM D-glucose, over 90 min at 25 °C. At times indicated, aliquots were removed, dialyzed against 0.01 M NH_4HCO_3, 5 mM NaCl, and the iodine content was assayed and expressed as percent of the protein content on a weight basis (*Panel A*). After digestion with Pronase and aminopeptidase M, as described in Experimental Procedures, T3 (*Panel B*) and T4 (*Panel C*) were also assayed by radioimmunoassay. Points represent mean ± S.E.M. values of 4 experiments. For the sake of clarity, S.E.M. are indicated only for hTg-CS and hTg-CS$^-$.

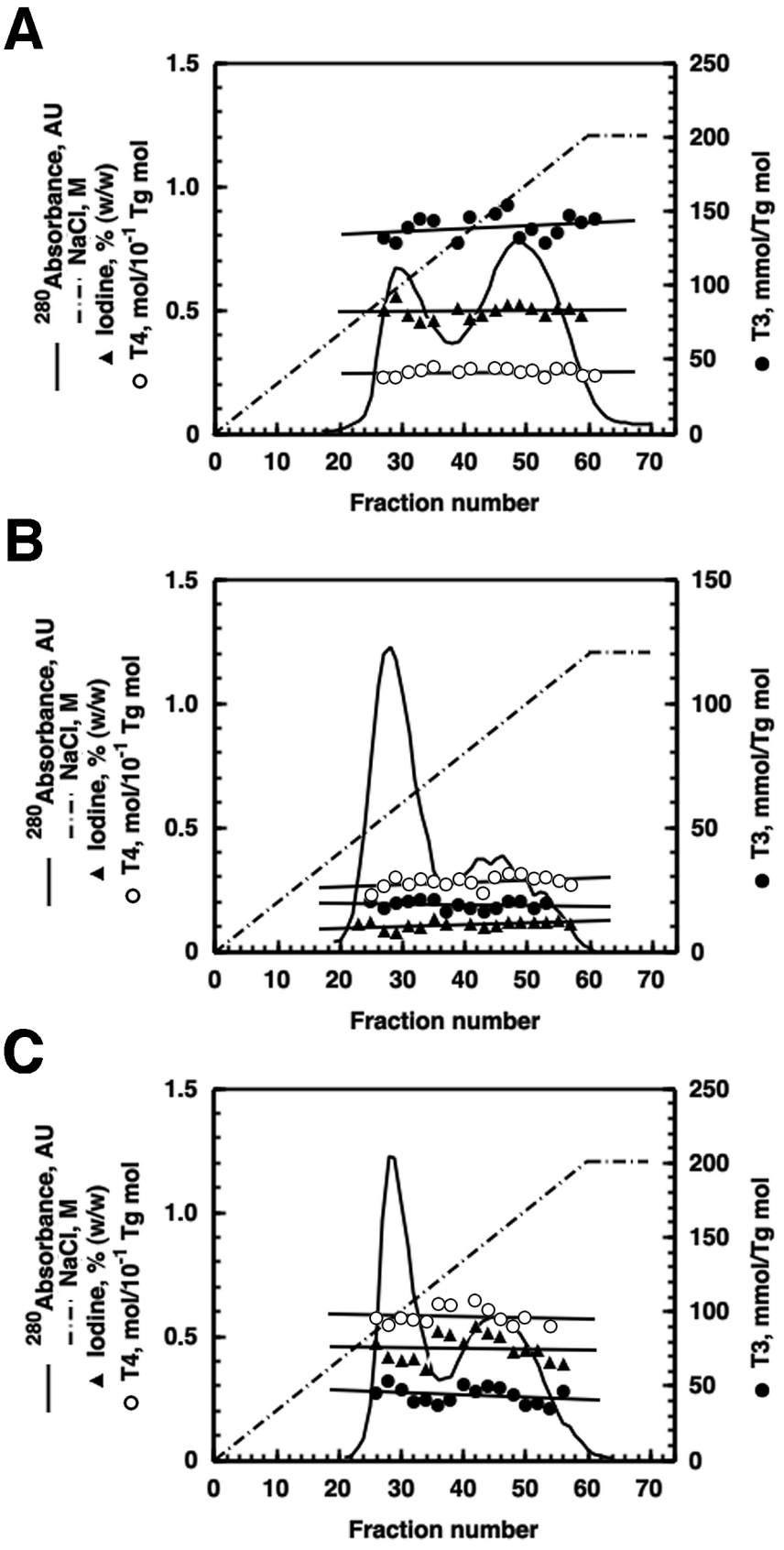

A
B
C
280Absorbance, AU
NaCl, M
Iodine, % (w/w)
T4, mol/10-1 Tg mol
T3, mmol/Tg mol
Fraction number
0
0.5
1.0
1.5
50
100
150
200
250
10
20
30
40
60
70

Figure 5 - Iodine and hormone content of fractions of the Q-IEC of selected hTg preparations, iodinated both physiologically and *in vitro*.

The three hTg preparations shown were subjected to Q-IEC, and the fractions obtained were analyzed for their iodine (▲), T3 (●) and T4 (❍) content, as described under Experimental Procedures. *Panel A*, physiologically iodinated hTg preparation O., from normal thyroid tissue, containing 0.50% iodine (w/w). *Panel B*, low-iodine, goitrous hTg preparation Ma., containing 0.12% iodine (w/w). *Panel C*, low-iodine, goitrous hTg preparation P., containing 0.09% iodine (w/w), was iodinated enzymatically *in vitro* to the iodine content of 0.45% (w/w), and subsequently fractionated by Q-IEC.

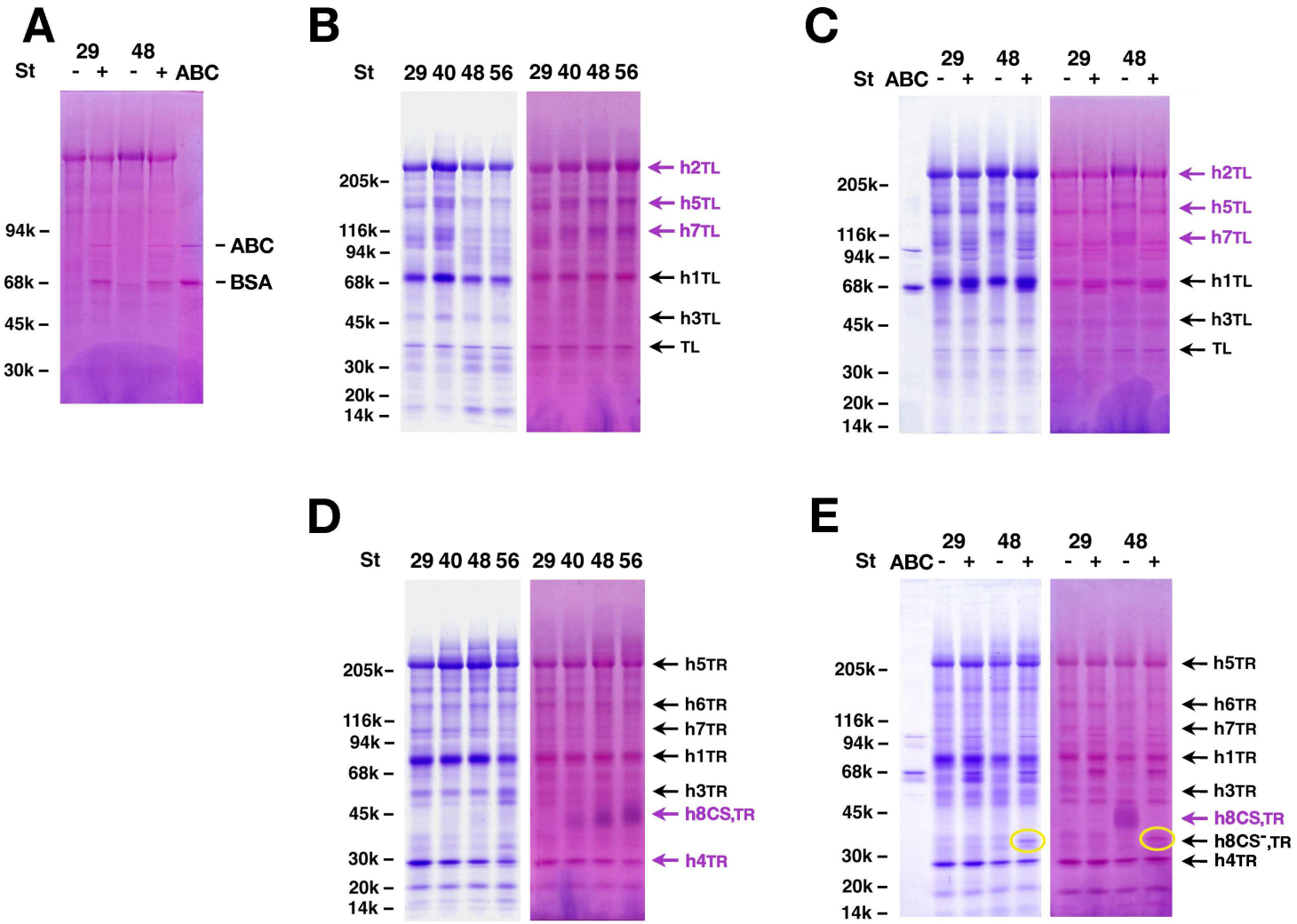
5.3. Limited proteolysis indicates that the chondroitin 6-sulfate oligosaccharide unit(s) of hTg-CS are restricted in the carboxy-terminal domain, downstream Thr2513

We were able to differentiate hTg-CS from hTg-CS^- in SDS-PAGE gels, by staining with diethyl-methyl-dibenzo-thiacarbocyanine (DMTCC) (Stains All™, ICN). This dye was reported to stain mucopolysaccharides metachromatically and, particularly, chondroitin sulfate purple, while staining proteins red (Bader et al., 1972). In fact, fractions in the Q1 (hTg-CS) peak were stained pink red with DMTCC, whereas those in the Q2 (hTg-CS^-) peak were stained purple (Figure 2, panel A). Side-by-side comparison of the two peak fractions revealed also a subtle cathodal shift in hTg-CS. Both metachromasia and the mobility shift were abolished by digestion with 200 mU/mL of chondroitinase ABC, at 37 °C for 4 h, prior to SDS-PAGE (Figure 6, panel A). In order 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 (Gentile and Salvatore, 1993), and were identified on the base of their mobilities. Figure 6, panel B shows the SDS-PAGE of the digestion products of fractions 29, 40, 48, and 56 of hTg preparation 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, on 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 exhibit lower than average mobilities in SDS-PAGE (Pitt-Rivers and Ambesi-Impiombato, 1968). 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, while

no changes were brought about in fraction 29 (Figure 6, 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 carboxy-terminal side of hTg, and shared the region downstream Leu1831 (Figure 7, panel A).

On the other hand, a diffuse band ($h8CS_{TR}$), with an average apparent mass of 41000, appeared among the tryptic fragments of hTg, on going from fraction 29 to fraction 40. It was stained purple with DMTCC, but was inapparent in the gel stained with CBB R-250. Its intensity of staining and apparent mass increased, on going from fraction 40 to 56 (Figure 6, panel D), in keeping with the increase of the uronic acid content revealed by the analysis of the Q-IEC fractions along the Q2 peak (Figure 2 and Table II). Upon digestion of the tryptic fragments of fraction 48 with chondroitinase ABC, band $h8CS_{TR}$ was replaced by a well-focused band ($h8CS^{-}_{TR}$), with an apparent mass of 36000, which was stained blue with CBB R-250, and red with DMTCC. No changes were caused by chondroitinase ABC in fraction 29 (Figure 6, panel E). Band $h8CS^{-}_{TR}$ was prepared by limited tryptic digestion of 0.5 mg of hTg-CS, followed by chondroitinase digestion, reducing SDS-PAGE and transfer to PVDF. NH_2-terminal peptide microsequencing revealed a single sequence (TSSKTA), corresponding to hTg residues 2513-2518 (Malthiéry and Lissitzky, 1987) (Table III). The flow-diagram of the proteolysis of hTg with trypsin shows the carboxy-terminal location of peptide $h8\text{-}CS_{TR}$, in keeping with the results with thermolysin (Figure 7, panel B). Because the NH_2-terminal sequence of peptide $h8_{TR}$ was identical with one of two sequences previously found in band $h4_{TR}$ (Gentile and Salvatore, 1993), we determined also the sequence of band $h4_{TR}$, obtained from hTg-CS. As expected, two sequences were found, one starting at the residue 1 of hTg ($h4_{TR}$), and another at residue 2513 ($h4bis_{TR}$) (Gentile and Salvatore, 1993). Thus, of two

peptides, both starting at residue 2513, one (h8-CS_{TR}) contained chondroitin 6-sulfate oligosaccharide unit(s), while the other (h4bis_{TR}) did not. Inspection of figure 6, panel D reveals that the increase of staining intensity of band h8-CS_{TR}, on 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}, co-existed with homodimeric hTg-CS, yielding only peptide h8-CS_{TR}, in the late part of the Q2 peak (Figure 3, panel B).


A
St
29
48
- + - + ABC
94k –
68k –
45k –
30k –
– ABC
– BSA
B
St
29 40 48 56
29 40 48 56
205k –
116k –
94k –
68k –
45k –
30k –
20k –
14k –
h2TL
h5TL
h7TL
h1TL
h3TL
TL
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St ABC
29
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29
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- + - + - + - +
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14k –
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h1TL
h3TL
TL
D
St
29 40 48 56
29 40 48 56
205k –
116k –
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45k –
30k –
20k –
14k –
h5TR
h6TR
h7TR
h1TR
h3TR
h8CS,TR
h4TR
E
St ABC
29
48
29
48
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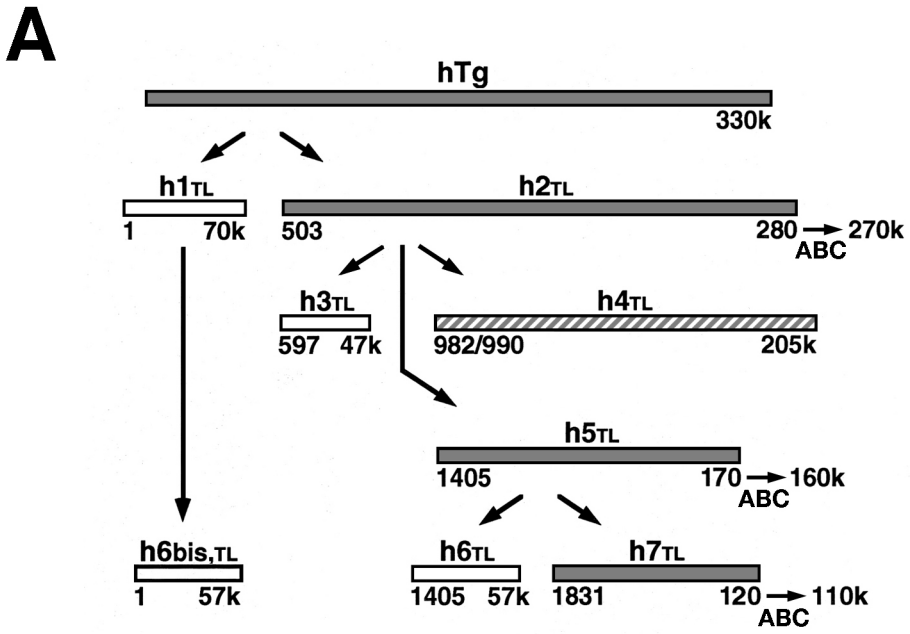
205k –
116k –
94k –
68k –
45k –
30k –
20k –
14k –
h5TR
h6TR
h7TR
h1TR
h3TR
h8CS,TR
h8CS⁻,TR
h4TR

Figure 6 - SDS-PAGE under reducing conditions of selected fractions from the Q-IEC of hTg, with and without limited proteolysis with thermolysin or trypsin, and/or digestion with chondroitinase ABC.

Panel A, fractions corresponding to the Q1 peak (#29) and Q2 peak (#48) of the Q-IEC of hTg preparation O., analyzed before (-) and after (+) digestion with 200 mU/mL of chondroitinase ABC (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 30 °C for 80 min, analyzed in replicate 4-17% total acrylamide gels, stained with CBB R-250 (*left*) and DMTCC (*right*). Fragments produced are marked at right, in accordance with ref. 21. Fragments exhibiting cathodal shifts and metachromasia, on going from the Q1 to the Q2 peak, are marked in purple. *Panel C*, results of further digestion with chondroitinase ABC of the proteolysis products 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 30 °C for 40 min. *Panel E*, further digestion with chondroitinase ABC of the proteolysis products of fractions #29 and #48 with trypsin. Band $h8CS^{-}_{TR}$ is circled. In all panels, molecular mass standards are marked at the left side of the gels.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h1TL
1
70k
h2TL
503
280
270k
ABC
h3TL
597
47k
h4TL
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205k
h5TL
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1405
57k
h7TL
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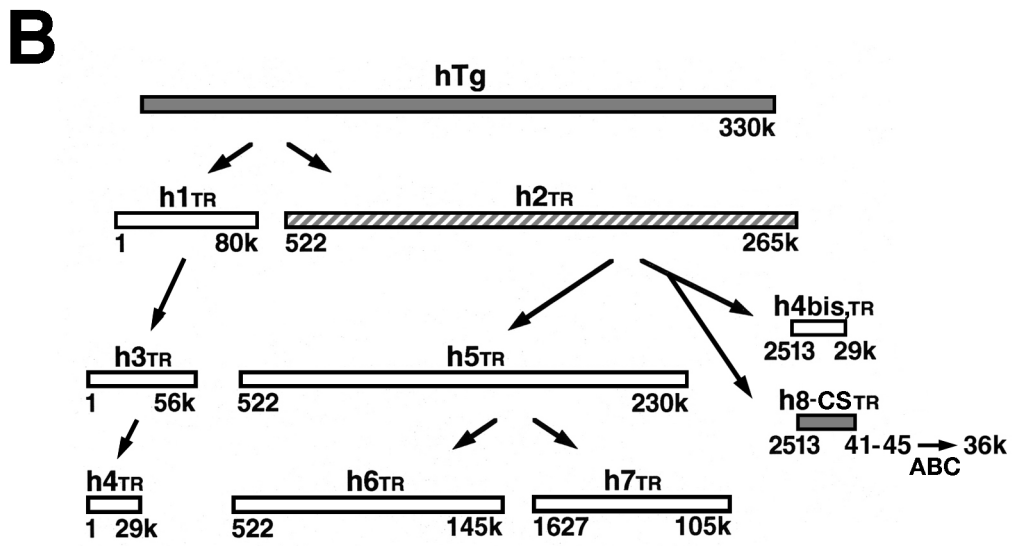
120
110k
ABC


B
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330k
h1TR
1
80k
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522
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h4bis,TR
2513
29k
h3TR
1
56k
h5TR
522
230k
h8-CSTR
2513
41-45
36k
ABC
h4TR
1
29k
h6TR
522
145k
h7TR
1627
105k

Figure 7 - Flow-diagrams of the limited proteolysis of hTg with thermolysin (*Panel A*) and trypsin (*Panel B*) (modified from Gentile and Salvatore, 1993).

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 Gentile and Salvatore, 1993. Fragments of hTg-CS, which exhibited metachromasia upon staining with DMTCC and cathodal shifts, with respect to their counterparts from hTg-CS$^-$, were drawn 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 employed, the corresponding peptides were not visible any more in the gels shown in Fig. 6.

TABLE III – **NH_2-terminal microsequence analysis of tryptic peptides of hTg-CS and hTg**

The phenylthiohydantoin derivatives detected by HPLC analysis for subsequent cycles of Edman degradation are shown. Amino acid residues are indicated using the single-letter code, while the numbers that follow the residues are the yields in picomoles.

Cycle	hTg-CS						hTg			
	$h4_{TR}$ [a]				$h8\text{-}CS^-_{TR}$ [b]		$h4_{TR\text{-}NR}$ [c]			
	$h4_{TR}$		h4bis,$_{TR}$				Sequence 1		Sequence 2	
1	N	9	T	8	T		T	70	T	-
2	I	13	S	3	S		S	35	A	12
3	F	21	X	-	S		S	29	F	6
4	E	11	K	6	K		K	19	Y	5
5	X	-	T	5	T		T	26	Q	6
6	Q	8	A	7	A		A	39	A	-
7							F	19	L	8
8							Y	9	Q	5
9							Q	17	N	5
10							A	20	S	8
11							L	14	L	-
12							Q	12	-	-

[a] $h4_{TR}$ was obtained by digestion of hTg-CS with trypsin, at the enzyme/substrate weight ratio of 1/100, at 30 °C for 40 min, followed by SDS-PAGE of the fragments in a 4-17% total acrylamide gel, under reducing conditions, and electrophoretic transfer to PVDF;

[b] $h8\text{-}CS^-_{TR}$ was obtained from hTg-CS as indicated for $h4_{TR}$, with the difference that proteolysis with trypsin was followed by digestion with 200 mU/mL of chondroitinase ABC of *Proteus vulgaris*, in 0.1 M Na acetate, 0.1 M Tris/HCl, pH 8.0, at 37 °C for 4 h;

[c] $h4_{TR\text{-}NR}$ was obtained by digestion of unfractionated hTg with trypsin, at the enzyme/substrate weight ratio of 1/100, at 30 °C for 20 min, followed by SDS-PAGE of the fragments in a non-reducing 4-16.5% acrylamide gel, and transfer to PVDF.

5.4. A single chondroitin 6-sulfate oligosaccharide unit is linked to Ser2729 of hTg-CS

Fifty mg of hTg-CS were reduced and carboxymethylated with iodoacetamide, as described in Experimental Procedures, and hydrolyzed with endoproteinase Glu-C from *Staphylococcus aureus*, at the enzyme/substrate weight ratio of 1/100, in 0.05 M 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 HR 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 of the protein was discarded in the flow-through, while a unique uronic-acid containing peak, having negligible absorbance at 280 nm, was eluted in the late portion of the gradient (Figure 7, panel A). This was subjected to size exclusion chromatography on BioGel P-2 (size exclusion limit of 1800 relative mass units), in 0.01 M NH_4HCO_3, which yielded an uronic acid-containing peak in the void volume. Its further purification by size exclusion chromatography on Sephadex G-50 fine, in 0.01 M NH_4HCO_3, monitored at 220 nm, is shown in panel B of figure 8. Of two peaks resolved, only one contained uronic acid. NH_2-terminal peptide microsequencing of this material revealed a single, homogeneous nonapeptide with the LTAGXGLRE sequence, corresponding to residues 2725-2733 of the cDNA-derived sequence of hTg (Malthiéry and Lissitzky, 1987), X being Ser2729 linked with the chondroitin 6-sulfate oligosaccharide unit. This glycopeptide will be henceforth referred to as hTg-CSgp. The comparison between the sequence surrounding Ser2729 and a *consensus* for the recognition of core protein serine residues by UDP-D-xylose:proteoglycan core protein β-D-xylosyltransferase, deriving from the alignment of 51 chondroitin 6-sulfate attachment sites from 19 proteoglycan core proteins (Brinkmann et al., 1997), revealed a 90% concordance, provided that two insertions of 3 and 2 residues were allowed (Figure 9). Electrophoresis of hTg-CSgp in a 16% polyacrylamide gel in Tris-tricine-SDS, followed by transfer to a PVDF membrane, stained with DMTCC, revealed an intensely metachromatic band, with the typical,

diffuse migration of glycosaminoglycans (Dahlberg et al., 1969; Naujokas et al., 1993), and an apparent molecular mass of 13,000-20,000 (Figure 8, panel C).

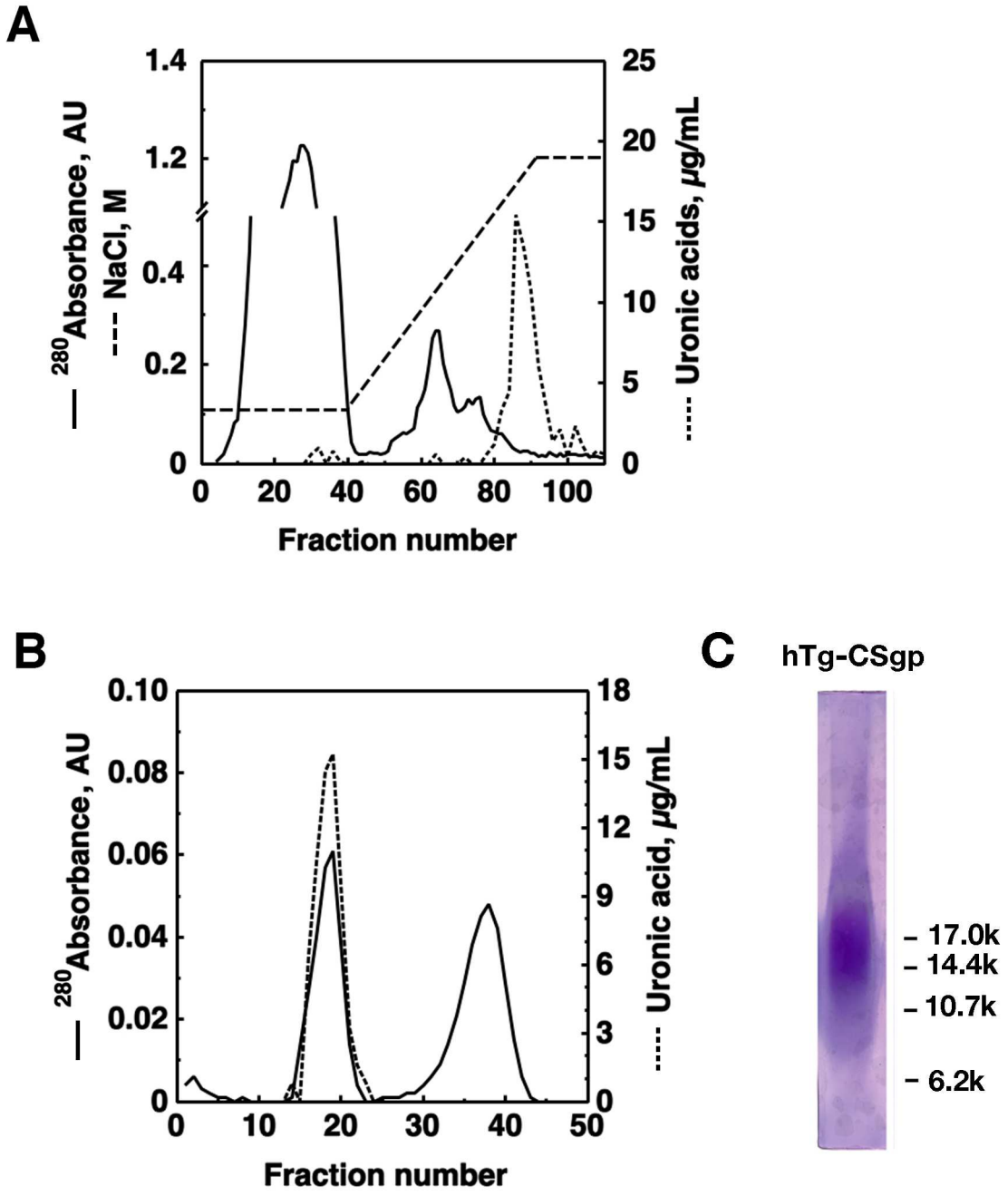

Figure 8 - Isolation and purification of the chondroitin 6-sulfate-containing glycopeptide hTg-CSgp.

Panel A, the products of digestion of 50 mg of carboxymethylated hTg-CS with endoproteinase Glu-C were loaded onto a 5-mL HiTrap™ Q-Sepharose HR column in 0.025 M Tris/HCl, 0.1 M NaCl, 2.0 M urea, pH 7.4, and eluted with a gradient from 0.1 to 1.2 M NaCl; one-mL fractions were monitored for protein content, by measuring the optical absorbance at 280 nm, and uronic acid content. *Panel B*, size-exclusion chromatography on Sephadex G-50, in 0.01 M NH_4HCO_3, of the uronic acid-containing peak eluted in the void volume of the size-exclusion gel chromatography on BioGel P-2 (not shown) of the uronic acid-containing fractions of the Q-IEC shown in *panel A*; one-mL fractions were monitored for peptide content, by measuring the optical absorbance at 220 nm (continuous line), and uronic acid content (dotted line). *Panel C*, Analysis of the purity of glycopeptide hTg-CSgp, by electrophoresis in a 16% polyacrylamide gel in Tris-tricine-SDS (Schägger and von Jagow, 1987), and electrophoretic transfer to a PVDF membrane, stained with DMTCC. Molecular mass standards (myoglobin cleavage products and glucagon from Sigma Marker Kit MW-SDS-17S).

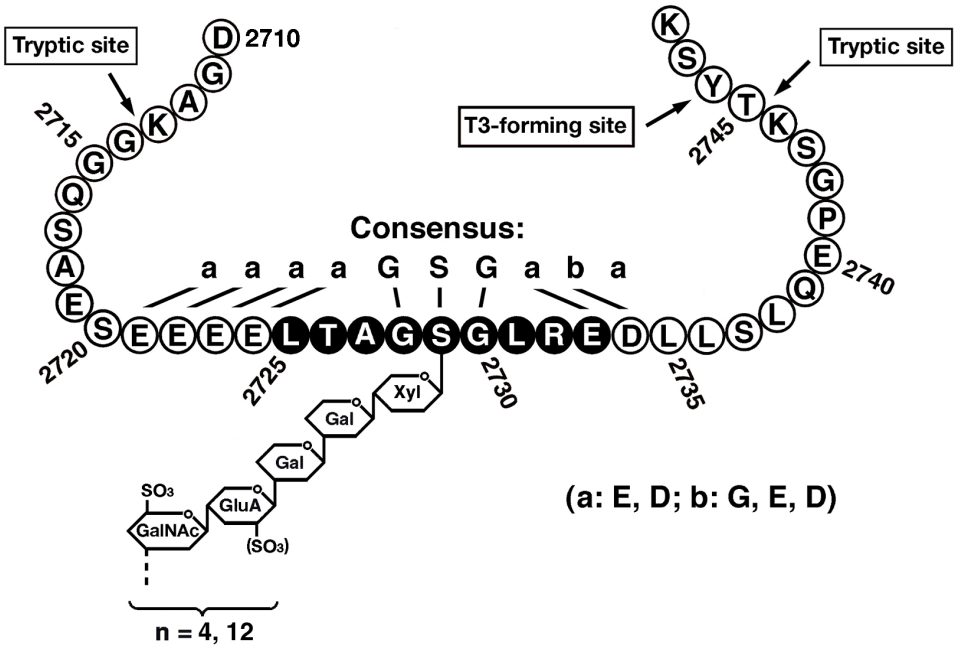

Figure 9. Diagrammatic representation of the localization and structure of the type D (chondroitin 6-sulfate) oligosaccharide unit of hTg. The cDNA-deduced amino acid sequence of hTg, from residue 2710 to residue 2748, is represented in single-letter code (Malthiéry and Lissitzky, 1987). Black circles with white lettering mark the sequence found for the purified glycopeptide hTg-CSgp (with the exclusion of Ser2729, which appeared as a blank). A diagram of the chondroitin 6-sulfate oligosaccharide unit attached to Ser2729 is shown. Legend: Xyl, xylose; Gal, galactose; GluA, glucuronic acid; GalNAc, N-acetylgalactosamine. 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. The two sites of tryptic cleavage between residues 2713-2714 and 2744-2745, and the preferential site of T3 formation at Tyr2746 (Fassler et al., 1988; Lamas et al., 1989), are also evidenced.

5.5. The chondroitin 6-sulfate oligosaccharide unit of hTg-CS protects peptide bond Lys2713-Gly2714 from proteolysis

Next, we seeked explanation for the difference between the apparent molecular masses of peptides h8CS$^-_{TR}$ (36,000) and h4bis$_{TR}$ (29,000), by determining the carboxyl terminus of the latter. Although, in the experiments described above, peptide h4bis$_{TR}$ co-migrated with h4$_{TR}$ in reducing SDS-PAGE, previous observations indicated that the homologous peptide b11$_{TR}$ of bovine Tg was obtained free of co-migrating species, under non-reducing conditions, as it was the only fragment not bound to the others by disulfide bridges (Veneziani et al., 1998). In fact, non-reducing SDS-PAGE of the tryptic fragments of hTg yielded only two bands, instead of several bands seen under reducing conditions (Figure 10, panel B). NH_2-terminal microsequencing of band h4$_{TR-NR}$, transferred to PVDF, revealed the same sequence as peptide h4bis$_{TR}$, starting at residue 2513, together with traces of a sequence starting at residue 2517 (Table III). Therefore, the products of limited digestion of hTg with trypsin, at the enzyme/substrate weight ratio of 1/100, at 30 °C for 20 min, were subjected to RP-HPLC in the absence of reduction, using a Vydac C-4 column (250 x 10 mm, 5 μm) and an acetonitrile gradient (Figure 10, panel A). Non-reducing SDS-PAGE of the resulting peaks revealed band h4$_{TR-NR}$ in peak 2. Upon reduction, this band exhibited the same apparent mass as peptide h4bis$_{TR}$ (Figure 10, panel B). The mass of peptide h4bis$_{TR}$ was determined by ES/MS. The results (Table IV) defined a peptide with ragged amino- and carboxy-terminal ends (residues 2511-2513 and 2712-2713, respectively) and a high-mannose oligosaccharide unit, composed of 2 N-acetylglucosamine and 8 or 9 mannose residues, linked to Asn2562, in agreement with previous data (Rawitch et al., 1968; Yang et al., 1996). ES/MS analysis of HPLC peak 1 also revealed a mass value corresponding to hTg peptide 2714-2744 (Table IV).

Thus, peptide h4bis$_{TR}$ was truncated at Lys2713, whilst peptide bond Lys2713-Gly2714 was protected from proteolysis in peptide h8-CS$_{TR}$, which included Ser2729, with its bound chondroitin 6-sulfate unit. A tryptic site also lay between Lys2744 and Thr2745 (Figure 9).

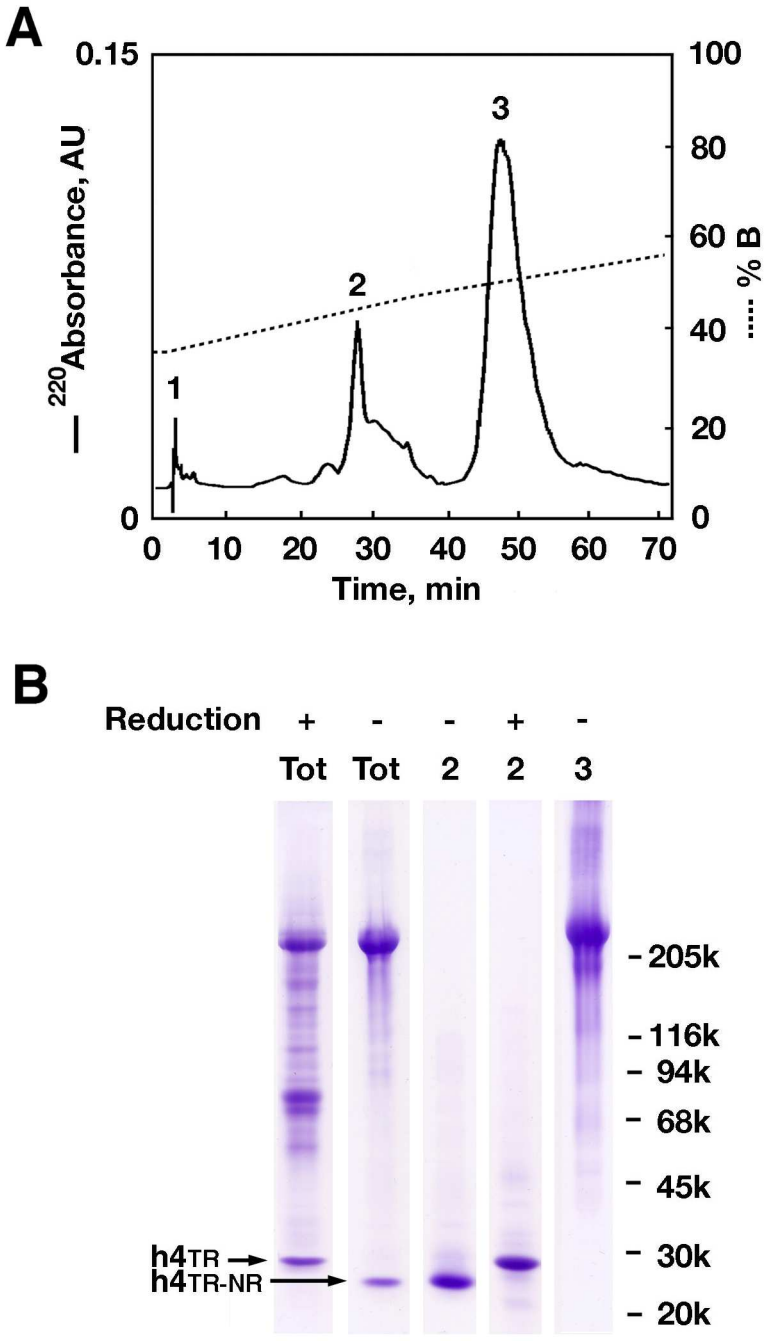

Figure 10 - Isolation of the carboxy-terminal tryptic fragment h4bis$_{TR}$ of hTg.

Panel A, reverse-phase HPLC of the non-reduced products of limited tryptic digestion of hTg. One-mg aliquots of the digestion products of hTg with trypsin, at the enzyme/substrate weight ratio of 1/100, at ph 7.8 and 30 °C for 20 min, were fractionated with a Vydac C-4 column (250 x 10 mm, 5 μm) equilibrated in 0.1% (v/v) trifluoroacetic acid in water (solvent A), containing 2% of 0.07% TFA in acetonitrile (solvent B). The column was developed with the gradient of solvent B indicated by the dotted line (see under Experimental Procedures for details). One-mL fractions were collected, and monitored for peptide content, by measuring the optical absorbance at 220 nm (continuous line), and uronic acid content (dotted line). The main peaks are numbered. *Panel B*, Analysis in SDS-PAGE of the mixture of tryptic digestion products of hTg (Tot), and of peaks 2 and 3 of the HPLC shown in *panel A*, in a 4-17% total acrylamide gradient gel, under reducing and non-reducing conditions, as indicated. Bands h4$_{TR}$ and h4$_{TR-NR}$ are indicated. Molecular mass standards are marked at right.

TABLE IV – **Analysis by ES/MS of selected peaks of the RP-HPLC on a Vydac C-4 column of the products of digestion of hTg with trypsin**

hTg was digested with trypsin, at the enzyme/substrate weight ratio of 1/100, at 30 °C for 20 min. One-mg aliquots of the fragment mixture were fractionated, in the absence of reduction, by reverse-phase HPLC, using a Vydac C-4 column (250 x 10 mm, 5 μm). and corresponding peaks from repeated runs were pooled and dried under nitrogen for subsequent analysis by ES/MS, as described under Experimental Procedures.

HPLC peak [a)]	Measured mass [b)] (mean ± SD)	Theoretical mass [c)]	Peptide [d)]	Post-translational modifications [e)]
1	3204.0 ± 0.3	3204.1	2714-2744	-
2	24296.9 ± 1.5	24296.3	2513-2712	GlcNAc2-Man8
	24422.2 ± 4.9	24424.4	2513-2713	GlcNAc2-Man8
	24458.6 ± 1.7	24458.4	2513-2712	GlcNAc2-Man9
	24587.7 ± 1.9	24586.6	2513-2713	GlcNAc2-Man9
	24510.2 ± 1.1	24509.5	2511-2712	GlcNAc2-Man8
	24671.3 ± 4.9	24671.7	2511-2712	GlcNAc2-Man9
	24797.5 ± 4.0	24799.8	2511-2713	GlcNAc2-Man9

[a)] Numbers refer to the peaks of the chromatogram shown in Fig. 10, panel A;

[b)] Average relative molecular masses (mean ± S.D.) obtained by integrating the multiple peaks corresponding to each molecular species, differing only in the total number of charges, measured by ES/MS;

[c)] Numbers indicate the amino acid residues at the extremities of each peptide, numbered according to Malthiéry and Lissitzky, 1987;

[d)] Masses calculated on the basis of the cDNA-derived sequence of human Tg (Malthiéry and Lissitzky, 1987), taking into account the post-translational modifications indicated;

[e)] Composition of the N-linked high-mannose oligosaccharide unit linked to Asn2562 (Rawitch et al., 1968; Yang et al., 1996).

5.6. Immunization of CBA/J(*H-2*^{k}) mice with either hTg-CS or hTg-CS$^-$ did not reproduce fully the histological picture associated with immunization with unfractionated hTg

Next, we compared the ability of hTg, hTg-CS and hTg-CS$^-$ to induce EAT in CBA/J(H-2^k) mice. Groups of 4-6 animals were immunized with 100 μg of each antigen, in 50 μL of PBS, emulsionated with 50 μL of CFA and administered subcutaneously in the dorsal region. A second dose of 50 μg of antigen, in incomplete Freund's adjuvant, was administered 10 days later, by the same route. Experimental animals were sacrificed 4 weeks later, by excess anesthesia, and the thyroids were evaluated histologically, as described in detail in Materials and Methods. Typical aspects of thyroid infiltration with mononuclear cells obtained following the immunization with the three antigens are shown in Figure 11, 12 and 13, respectively. Histological scores are reported in Table V. Thyroiditis developed in all experimental groups of mice; however, the most severe scores were observed in mice immunized with unfractionated hTg. Instead, the immunization with both hTg-CS and hTg-CS$^-$ resulted associated with lower scores of EAT. Thus, it appears that both hTg subfractions contributed to the immunopathogenic potency of unfractionated hTg. No thyroid infiltration was seen in control mice.

Table V – **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 Dai et al., 2005: 0 = no infiltration; 1 = interstitial accumulation of cells between two or three follicles; 2 = one or two foci of cells at least the size of one follicle; 3 = extensive infiltration, 10-40% of total area; 4 = extensive infiltration, 40-80% of total area, and 5 = extensive infiltration > 80% of total area. Each mouse was assigned the average infiltration index observed from both thyroid lobes (at least 10 sections per lobe were evaluated and the average infiltration index per lobe was scored). Data are referred to three experiments (n = 17).

Immunizing agent	**EAT Score**				
	0	1	2	3	4
	% of animals				
Unfractionated hTg	35	35	24	6	0
hTg-C6S	41	35	24	0	0
hTg-C6S$^-$	53	29	12	6	0

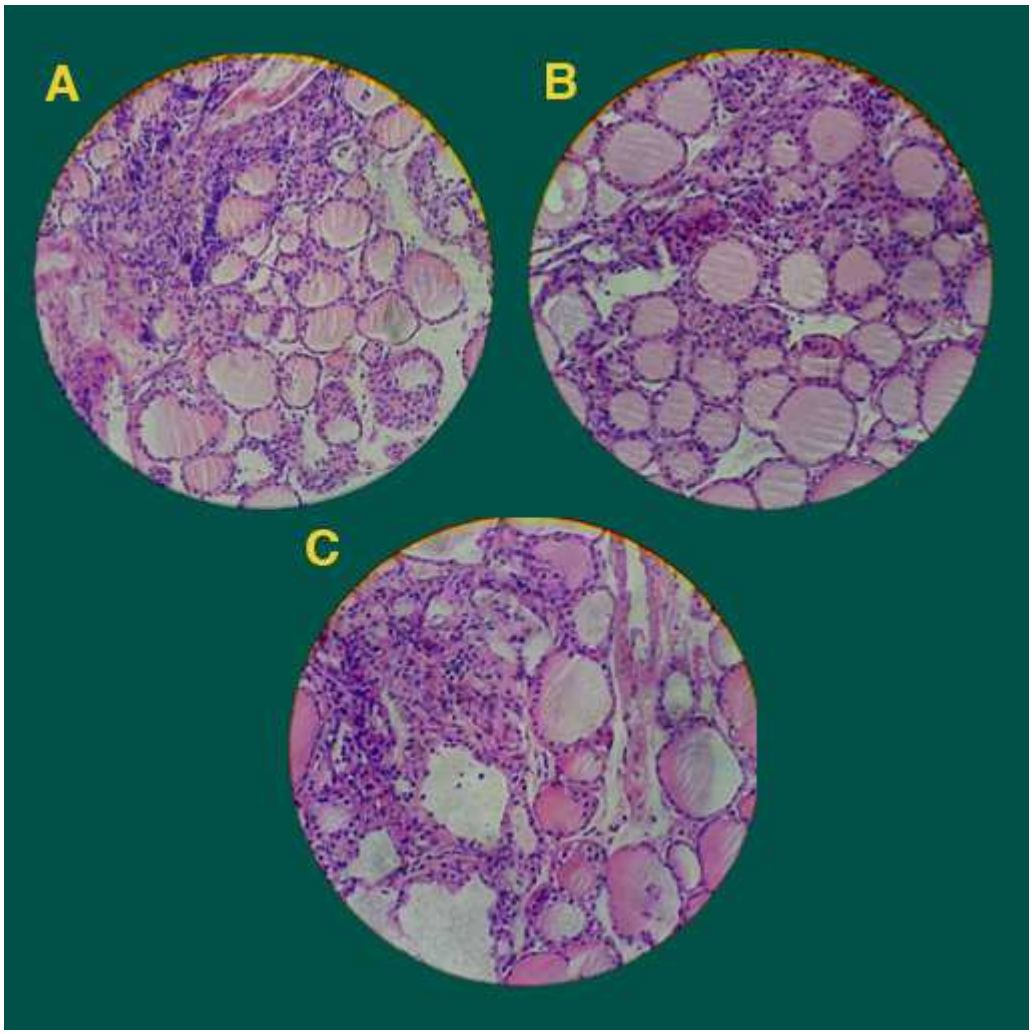

Figure 11 – Typical histological pictures of mononuclear infiltration of the thyroid.

The thyroid sections shown were prepared from CBA/J(H-2k) mice immunized with hTg (A), hTg-CS (B), and hTg-CS$^-$ (C)

5.7. Immunization with hTg, hTg-CS and hTg-CS$^-$ is associated with thyroid cell damage

Significant tissue damage in all groups of immunized animals accompanied the immunization, as judged by the increased levels of T4 in serum, compared to control mice (2.4 ± 0.8 mg/L), without any significant difference between mice immunized with hTg, hTgCS, and hTgCS$^-$ (Figure 12). Instead, serum levels of T3 were not increased in immunized mice, in comparison with control mice (87.3 ± 15.1 ng/L). These findings could reflect the limited extent of intrathyroidal deiodination of T4 to T3 in mice.

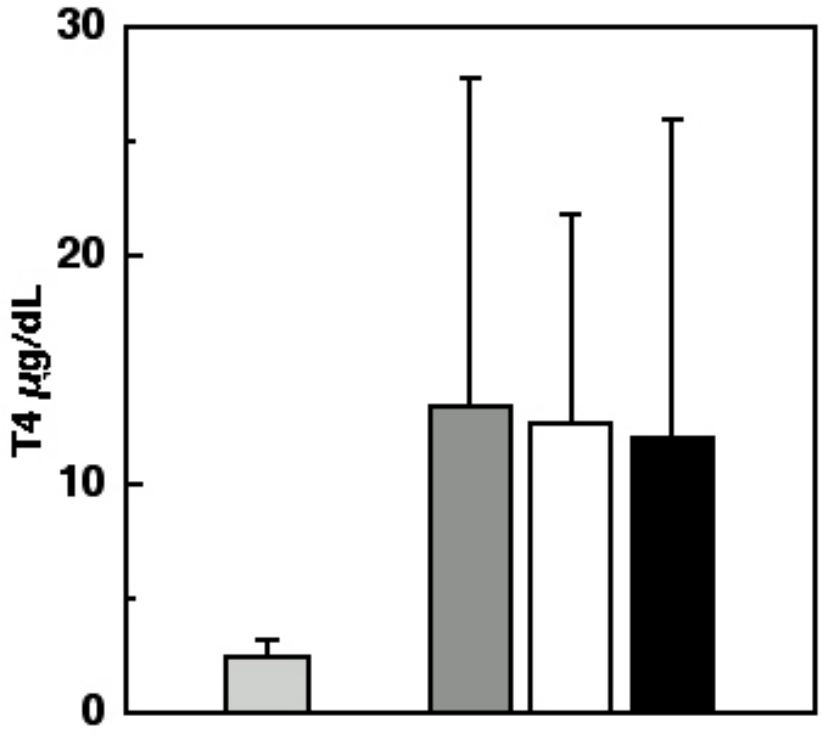

Figure 12 – Serum T4 levels in CBA/J(H-2^k) mice immunized with: ■ hTg; □ hTg-CS$^-$; ■ hTg-CS, and ■ control animals.

5.8. Proliferative responses and IFN-γ production by splenic lymphocytes *in vitro* are maximal after immunization of mice with hTg-CS$^-$ and restimulation with hTg-CS

The T cell responses to hTg, hTgCS, hTgCS$^-$ and to glycopeptide hTgC6Sgp were tested on splenic lymphocytes taken at the time of sacrifice from mice immunized with hTg, htg-CS and hTg-CS$^-$, and cultured for 72 h in the absence or presence of different concentrations of antigens. Panel A of Figure 13 shows the results of the restimulation *in vitro* of the splenic lymphocytes of each one of the three experimental groups with the same antigen respectively used for immunization. Results are expressed in terms of stimulation indices (SI). Panels B and C, on the other hand, show the results of the cross-stimulation of the splenic lymphocytes from the three experimental groups with hTg-CS$^-$ and hTg-CS, respectively. Panels D-F show the results of the assays of INFγ production measured in the supernatants of the cell cultures, whose stimulation indices are shown in panels A-C, respectively. An inspection of panels A and D reveals that the restimulation with hTg-CS of splenocytes from mice immunized with the same antigen was followed by low-level, dose-dependent proliferative responses, accompanied by proportional IFN-γ production, whereas restimulation with hTg-CS$^-$ of splenocytes primed *in vivo* with the same antigen was followed by higher-level, dose-dependent increases of IFN-γ production, accompanied by proliferative responses inversely related with the antigen dose. Finally, a mixed response characterized the restimulation with hTg of hTg-primed splenocytes in which a dose-dependent production of IFN-γ and a non-monotonic trend of cell proliferation, peaking at the dose of 15μg/mL, were observed. Altogether, these data showed a dissociation between T cell proliferation and IFN-γ production. The former was more pronounced in hTg-primed splenocytes restimulated with the same antigen (with the exclusion of the dose of 5μg/mL), while the latter reached the maximum levels in the culture supernatants of mice immunized and restimulated with hTgCS$^-$

. On the other hand, the cross-stimulation *in vitro* with hTg-CS$^-$ of splenocytes primed *in vivo* with hTg or hTg-CS was followed by a similar pattern of proliferative and secretory responses, although lower in level, as observed upon restimulation with the respective immunizing agents (panels B and E). Instead, hTg-CS$^-$-primed splenocytes exhibited strong, dose-dependent responses to the cross-stimulation with hTg-CS, both in terms of proliferation and IFN-γ production; similar, albeit milder responses were observed in mice immunized with hTg and restimulated with hTg-CS (panels C and F). On the whole, maximal and concordant T cell proliferation and INF-γ production were recorded following cross-stimulation with hTgCS of splenocytes from mice immunized with hTgCS$^-$. Moreover, a dissociation between INF-γ production and T cell proliferation was seen when hTgCS$^-$-primed lymphocytes were restimulated *in vitro* with the same antigen.

Furthermore, Figure 14 shows the results observed upon restimulation *in vitro* of the splenic lymphocytes obtained from mice immunized *in vivo* with the different forms of hTg, cultured in the presence of purified hTgC6Sgp (panels A and C) or its synthetic, non-glycosilated homologue with the LTAGSGLRE sequence (panels B and D). Restimulation with hTgC6Sgp of hTg-CS$^-$-primed lymphocytes was accompanied by dose-dependent proliferative responses and proportional INF-γ production. At variance, synthetic peptide LTAGSGLRE, bearing the same peptide sequence as hTgC6Sgp, but devoid of carbohydrate chains, was not able to elicit either proliferative responses or cytokine production. Glycopeptide hTgC6Sgp also induced proliferation, but not INF-γ production, of splenocytes from control mice, possibly owing to aspecific mitogenic properties.

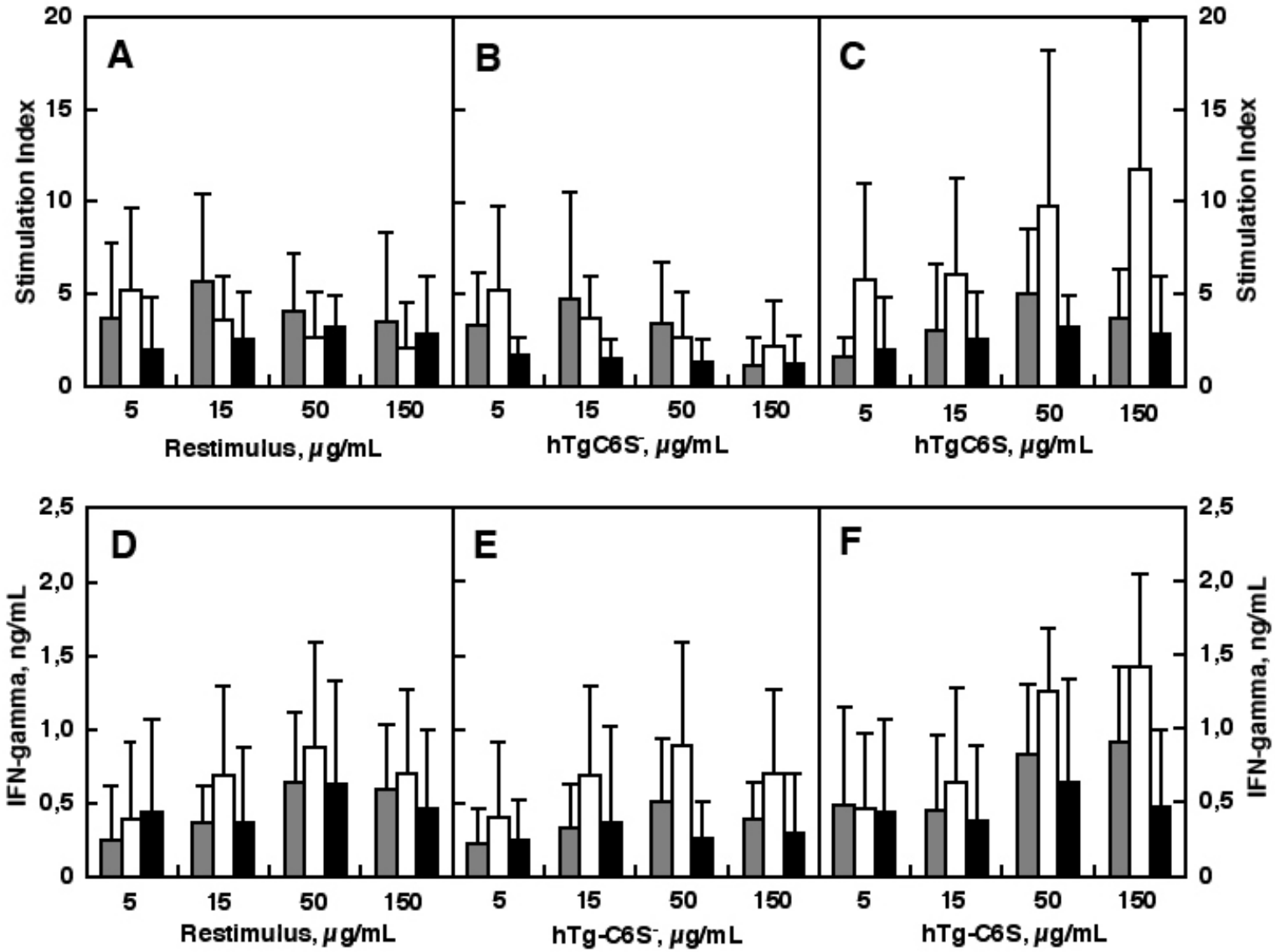
A
B
C
D
E
F
Stimulation Index
IFN-gamma, ng/mL
0
5
10
15
20
0,5
1,0
1,5
2,0
2,5
5
15
50
150
Restimulus, μg/mL
hTgC6S⁻, μg/mL
hTgC6S, μg/mL
hTg-C6S⁻, μg/mL
hTg-C6S, μg/mL

Figure 13 (previous page) – Proliferative responses (panels A-C) and IFN-γ production (panels D-F) by splenic lymphocytes from CBA/J(H-2k) mice immunized and restimulated *in vitro* with the various forms of hTg. The responses are shown of mice immunized with: ■ hTg; □ hTg-CS$^-$; ■ hTg-CS and restimulated with the same antigen used for immunization (A, D), hTg-CS$^-$ (B, E) or hTg-CS (C, F).

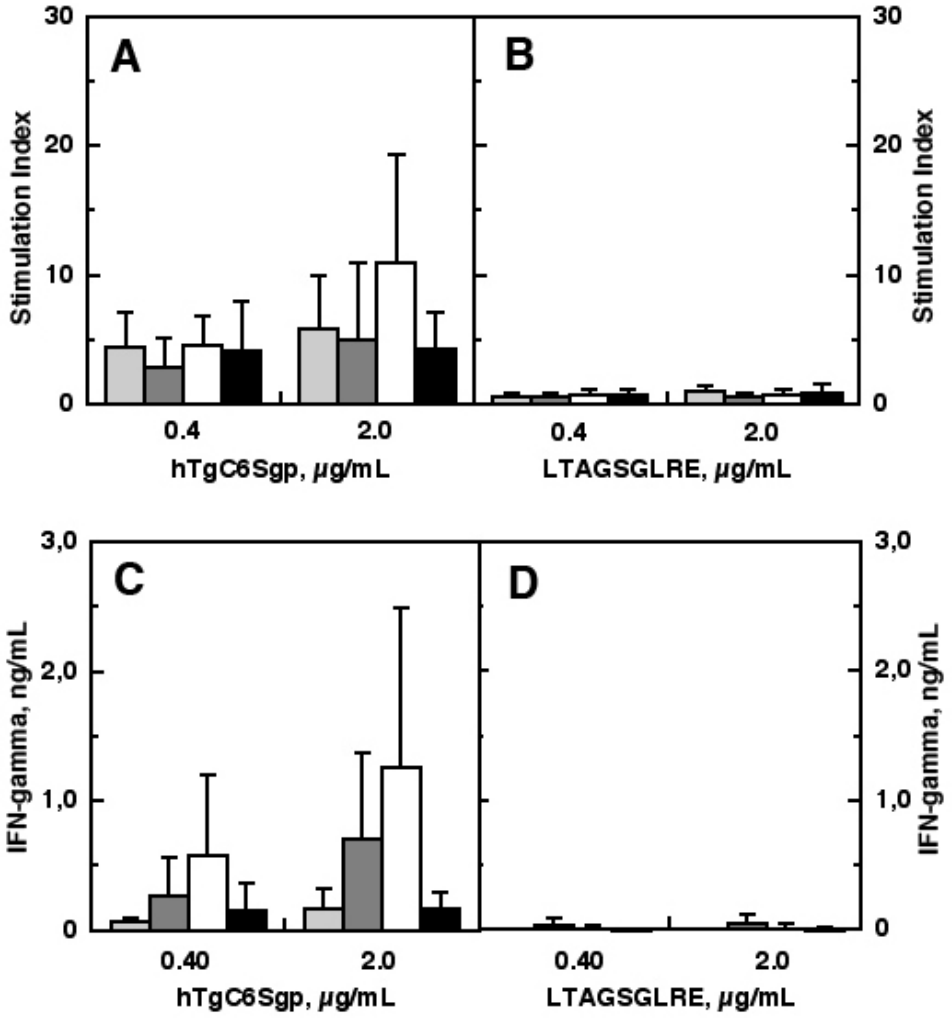

Figure 14. Proliferative responses (panels A, B) and IFN-γ poduction (panels C, D) by splenic lymphocytes of CBA/J(H-2^k) mice immunized with: ■ hTg; □ hTg-C6S$^-$; ■ hTg-

C6S and restimulated *in vitro* with the hTgC6Sgp glycopeptide (A, C), or the synthetic, non-glycosylated homologue 2725-2733 LTAGSGLRE (B, D).

5.9. IgG2a antibody responses of immunized mice parallel thyroid histological disease scores

We also investigated the effects of hTg, hTgCS and hTgCS- on the production of antibodies of the IgG class (Figure 15) and for IgG1 and IgG2a subclasses (Figure 16). Immunization with hTg, hTgCS and hTgCS$^-$ caused the production of comparable serum levels of total IgG and IgG1. Instead, the levels of IgG2a against all three antigens were maximal in the animals immunized with unfractionated hTg. Mice immunized with hTgCS produced levels of IgG2a almost as high, while the lowest levels of IgG2a were found following hTgCS$^-$ immunization. These findings seem to indicate that the presence of the chondroitin 6-sulfate was essential for the development of Th-1-polarized responses to hTg, as judged from IgG2a production (Snapper et al., 1993).

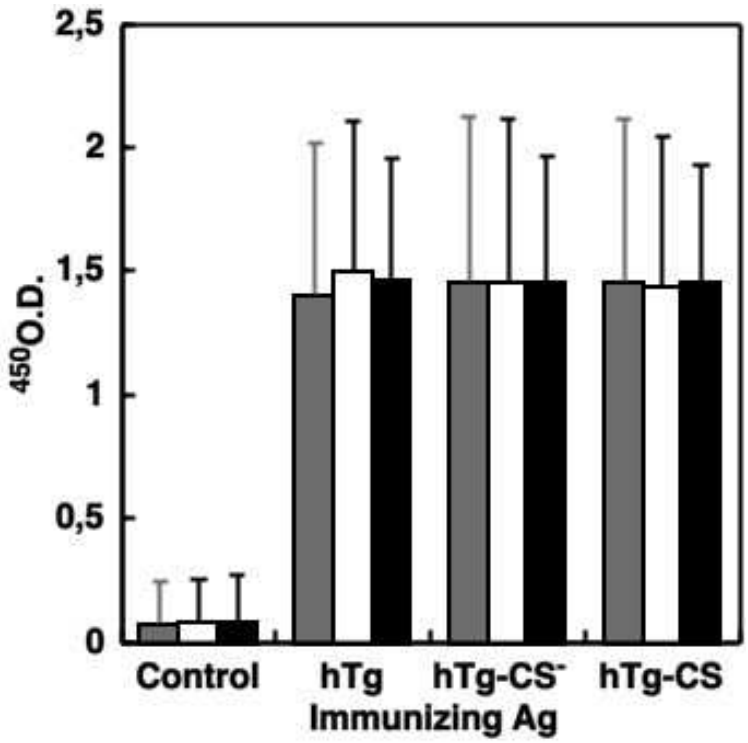

Figure 15 – Concentrations of IgG against: ■ hTg; □ hTg-CS^{-}; ■ hTg-CS in the sera of CBA/J(H-2^{k}) mice immunized with each one of the three antigens.

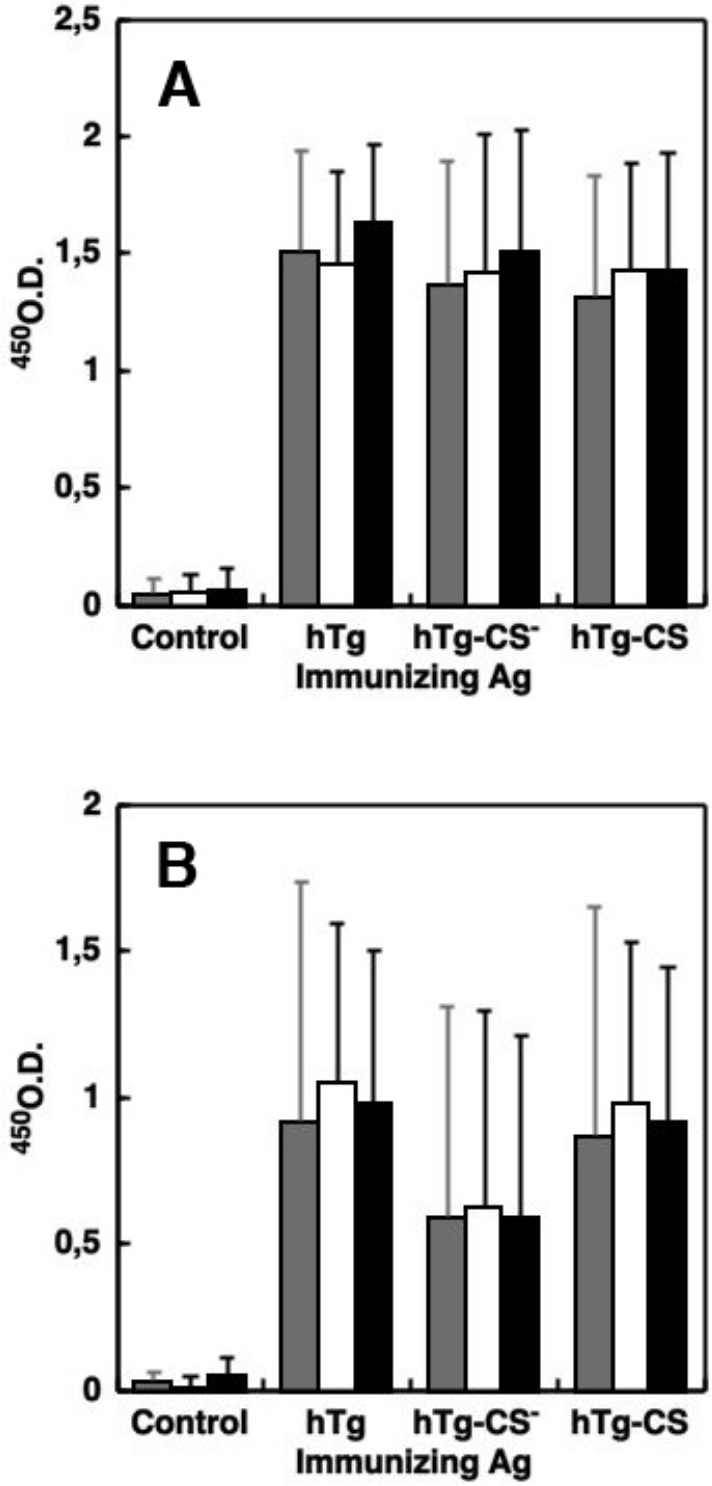

Figure 16 – Concentrations of IgG1 (A) and IgG2a (B) against: ■ hTg; □ hTg-CS$^-$; ■ hTg-CS in the sera of CBA/J(H-2^k) mice immunized with each one of the three antigens.

6. DISCUSSION

The present study documents that type D (chondroitin 6-sulfate) oligosaccharide chains are a main source of microheterogeneity of hTg, being regularly found in a significant, and sometimes predominant fraction of hTg molecules. We developed an ion-exchange chromatographic method, which permitted us to separate chondroitin 6-sulfate-containing hTg molecules (hTg-CS) from residual hTg molecules (hTg-CS^-). By exploiting the changes of electrophoretic mobility and staining properties imparted by chondroitin 6-sulfate chains upon the fragments deriving from the limited proteolysis of hTg, we were able to restrict the search for chondroitin 6-sulfate-containing regions of hTg to a carboxy-terminal peptide, starting at Thr2515. Subsequent purification, from the products of digestion of hTg-CS with endoproteinase Glu-C, of a homogeneous, glucuronic acid-containing nonapeptide, whose sequence corresponded to residues 2725-2733 and bore substantial homology with a *consensus* sequence for the recognition of core protein serine residues by UDP-D-xylose:proteoglycan core protein β-D-xylosyltransferase, permitted us to establish Ser2729 as the sole site of chondroitin 6-sulfate addition in hTg. The presence of two insertions in this peptide, with respect to the *consensus* sequence, is in keeping with the observation that proteins which are not modified quantitatively with chondroitin sulfate, such as aggrecan and type IX collagen, have recognition sequences for xylosyltransferase that match the *consensus* sequence less well than those of proteins, such as bikunin, which are modified quantitatively (Brinkmann et al., 1997). Spiro reported previously the presence of O-linked oligosaccharide units in hTg, containing from 7 to 11 moles of glucuronic acid and N-acetylgalactosamine, 1 mole of xylose, 2 moles of galactose and up to 14 moles of sulfate per mole of serine residues

(Spiro, 1977). She suggested that hTg may contain one chondroitin sulfate unit per protein monomer. Subsequently, Schneider *et al.* showed that the repeating disaccharide units were of the chondroitin 6-sulfate kind (Schneider *et al*., 1988). However, none of them conclusively established the number of type D oligosaccharide chains, nor their localization in hTg. Our data demonstrate that hTg-CS molecules contain a single chondroitin 6-sulfate unit per polypeptide chain, which is usually made of a variable number of repeating disaccharide units. By taking into account that, in hTg preparations with the highest content of hTg-CS, heterodimeric hTg-CS molecules, containing a single chondroitin 6-sulfate-bearing protein subunit, coexisted with homodimeric hTg-CS molecules, containing up to 24 moles of glucuronic acid per hTg mole, the maximum number of repeating disaccharide units in chondroitin 6-sulfate chains of hTg is not in excess of 12.

We also show that hTg-CS has a higher efficiency of hormone, particularly T3, formation than hTg-CS^-, and that all molecules in unfractionated hTg benefit fom this property of hTg-CS. The relatively greater advantage conferred by chondroitin 6-sulfate addition upon T3, with respect to T4 formation, and the proximity of Ser2729 to the site of preferential T3 formation at Tyr2746, suggest that the effects of the chondroitin 6-sulfate oligosaccharide chain in hormonogenesis may entail intermolecular interactions between the carboxy-terminal domains of both hTg-CS and hTg-CS^- molecules. Thus, chondroitin 6-sulfate addition seems to constitute an ergonomic mechanism, by which post-translational modification of a fraction of hTg molecules influences the overall hormone-forming efficiency of hTg. The regulation of the T4/T3 ratio in thyroid secretion is crucial for achieving and maintaining physiological concentrations of active thyroid hormone (T3) in blood, and is finely tuned by multiple mechanisms, all of which are under the control of TSH. A prominent mechanism is the intrathyroidal conversion of T4 to T3, especially in severe iodine deficiency, through the action of a thyroid iodothyronine 5'-deiodinase (Ishii, 1983).

Enhanced T3 release from Tg under hyperstimulation by TSH results also from increased proteolysis at the COOH-terminus of Tg by cathepsin B (Dunn, 1991). However, the T4/T3 ratio is regulated first at the biosynthetic level in Tg, which decreases in experimental animals given TSH (Fassler, 1988). In several animal species studied, Tyr5 is the main site of T4 formation in Tg (Lejeune, 1983; Rawitch et al., 1983, 1984; Dunn et al., 1987; Fassler et al., 1988), while Tyr2746 represents the site of preferential T3 formation (Dunn et al., 1987; Fassler, 1988; Lamas, 1989; Marriq, 1983). In rabbit and guinea pig Tg, TSH stimulated T3 formation at tyrosine 2746, and decreased T4 synthesis at tyrosine 5. The diminished T4 formation at the amino terminus was mediated by the TSH-stimulated maturation of N-linked oligosaccharide chains linked to Asn91 from the high-mannose to the complex type (Mallet, 1995). Should the addition of chondroitin 6-sulfate chain to hTg be also controlled by TSH, a common mechanism, mediated by TSH-dependent modifications of the composition and number of N-linked and O-linked oligosaccharide chains in Tg, could account for the changes in hormonogenic efficiency at both Tg termini, affecting the overall ratio of T4 over T3 formation. For hormone release to occur, Tg must be internalized into thyroid follicular cells by fluid-phase pinocytosis, followed by degradation of hTg in lysosomes. A part of Tg, which is internalized via the endocytic receptor megalin, bypasses lysosomes and is transcytosed across cells and released from basolateral membranes into the bloodstream (Marinò, 1999, 2000). A heparin-binding region of rat Tg, and its homologous region of hTg, encompassing residues 2469-2483, are involved in Tg binding to megalin (Marinò, 1999a), which is facilitated by accessory interactions with heparin-like molecules (heparan-sulfate proteoglycans) on the surface of thyroid cells (Marinò, 1999b). Experiments are in progress to establish whether chondroitin 6-sulfate units interfere with hTg binding to megalin. In FRTL-5 cells, Tg molecules with a low hormone content are favored over those with a high hormone content for megalin-mediated transcytosis, which, therefore, appears to promote lysosomal

degradation of hormone-rich Tg, by diverting hormone-poor Tg from lysosomes (Lisi, 2003). Conceivably, chondroitin 6-sulfate addition to hTg, in addition to improving the yield of active hormone, may also prevent the non-productive internalization of hTg by transcytosis.

Furthermore, we have identified two sites, namely Lys2713-Gly2714 and Lys2744-Thr2745, in the vicinity of the site of chondroitin 6-sulfate addition, which were amenable to cleavage, during the limited proteolysis of hTg with trypsin. The data that we present document that the former, at least, was protected from proteolysis in hTg-CS, but not in hTg-CS^- molecules. The influence of the C6S oligosaccharide chain on the proteolytic susceptibility of the extreme carboxy-terminal region of hTg could have important implications in modifying the processing and presentation of hTg by antigen-presenting cells (APCs) and in the ability of hTg to function as an autoantigen, particularly because the site of chondroitin 6-sulfate addition to hTg is localized 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 (Gentile, 2004). On the other hand, the effects of N-linked and O-linked glycosylation on the activity of proteolytic enzymes involved in antigen processing, as well as on the affinity of epitope binding to MHC or T-cell receptor molecules, have been reviewed (Anderton, 2004). To determine whether the addition of oligosaccharide unit C6S to hTg would modify its immunopathogenicity, we used intact hTg, hTgCS and $hTgCS^-$ to induce EAT in CBA/J susceptible mice. By using this animal model, we provided evidence that the presence of C6S oligosaccharide unit modulates the immunopathogenic properties of hTg. In fact, although, both subunit are able to provoke thyroid damage, as judged from serum FT_4 increases, $hTgCS^-$ immunization was accompanied by a less severe thyroidal mononuclear cell infiltration compared to hTgCS. However, neither one fully reproduced the histological picture associated with unfractionated hTg. Apparently, the two isoforms possess distinct

molecular pathogenic determinants necessary to obtain the full development of the organ disease. This initial conclusion has been confirmed by restimulation experiments of splenic lympocytes. Restimulation with hTg-CS of splenocytes from mice immunized with the same antigen was followed by low-level, dose-dependent proliferative responses, and IFN-γ production, whereas restimulation with hTg-CS$^-$ of splenocytes primed *in vivo* with the same antigen was followed by higher-level, dose-dependent increases of IFN-γ production, accompanied by proliferative responses inversely related with the antigen dose. Thus, an evident dissociation between INFγ production and T cell proliferation has been recorded after restimulation with hTgCS$^-$ of splenic lymphocytes from mice immunized with the same antigen. To this respect, it has been reported that myelin basic protein (MBP)-reactive T cells may express variable pattern of cytokine production and proliferation in a model of experimental autoimmune encephalomyelitis, depending from dose and type of antigen. In particular, encephalitogenic peptide MBP (68-88) stimulated considerable amounts of INFγ by MBP-reactive T cells in the absence of cell proliferation (Sun et al., 1995), when stimulated with suboptimal dose of antigen. Other peptides may have capacity to induce IL-4 production, but not proliferation of the responder T cells (Evavold and Allen, 1991; Allen et al., 1994). Further studies demonstrated that activation of T helper cells to a threshold required for INFγ or IL-2 production or proliferation is not required to achieve induction of T helper cell effector functions (Brown et al., 1997). On the other hand, a model of Th1 cell activation has been already described, which requires two types of signals: one for cytokine production and a separate one other for proliferation (Sommer et al., 1995). These findings further illustrate the complexity of T-cell-mediated autoimmune diseases; a same T cell population may express distinct immune function, depending on the degree they are activated, by the quality and quantity of antigens available.

Interestigly, we also observed that *in vitro* cross-stimulation with hTg-CS$^-$ of splenocytes from mice immunized with hTg-CS was followed by a similar pattern of proliferative and secretory responses, although lower in level, as observed upon restimulation with hTg-CS$^-$. Instead, cross-stimulation with hTg-CS of splenocytes primed *in vivo* with hTg-CS$^-$ was followed by maximal., dose-dependent increases both of proliferative responses and IFN-γ production. These data indicate that hTg-CS$^-$ is more effective than hTg-CS in the priming of autoreactive T lymphocytes, recognizing shared autoepitopes between murine and human Tg, whereas hTg-CS is a stronger inducer of proliferation of antigen-sensitized T cell clones. In keeping with these conclusions, are the similar results obtained when splenocytes primed *in vivo* with hTg-CS$^-$, were restimulated with purified glycopeptide hTg-CSgp, containing the chondroitin 6-sulfate unit, but not with its synthetic, non-glycosylated homologue peptide. It is clear that a diversity of molecular signals, including structural determinant(s) associated with the chondroitin 6-sulfate chain, are required for the concomitant activation of T cell proliferation and IFN-γ production, possibly via the interaction with specialized T cell subsets. Among the implications of our results the possibility of using an immunizing protocol in which mice primed with hTgCS$^-$ are cross-stimulated with hTgCS appear very promising as it could lead to a more severe thyroiditis compared to that induced with unfractionated hTg, usually mild and focal., and therefore more resembling to human thyroiditis. This model could represent an appropriate tool in the hands of the researchers to better understand the cellular and molecular mechanisms of chronic autoimmune thyroiditis.

The observation that hTgCS$^-$ is more effective than hTgCS in priming autoreactive T cells is in agreement with the role of oligosaccharide chains on the activity of proteolytic enzymes involved in antigen processing, as well as on the affinity of epitope binding to MHC or T-cell receptor molecules. (Anderton, 2004). O-linked mono- and disaccharides in tumor-

associated glycoprotein MUC1 were not removed during processing by dendritic cells (Vlad, 2002). They 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, 2003), or by preventing epitope recognition by a peptide-specific T cell hybridoma. However, they could also enrich the epitope repertory, as some of the disaccharide-substituted peptides, presented by dendritic cells, were able to prime and reactivate specific T cell clones (Vlad, 2002). N-linked oligosaccharide chains also inhibited the generation of a self epitope from glutamate receptor subunit 3, by limiting extracellular cleavage by granzyme B (Gahring, 2001), and the endosomal generation of cytotoxic lymphocyte-specific epitopes from influenza A nucleoprotein (Wood, 1998). As for glycosaminoglycan chains, a keratan sulfate chain was reported to mask an arthritogenic T cell epitope in the G1 domain of aggrecan, a high density proteolycan of human adult cartilage, 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 in the development of T cell responses (Glant, 1998). The chondroitin 6-sulfate chain of hTg is expected to inhibit the processing of hTg peptide 2730-2743, among several peptides known for their immunopathogenic properties in CBA mice. Although incapable of direct EAT induction, this peptide, bearing partial homology with sequence 118-131 of human thyroperoxidase, stimulated *in vitro* strong proliferative responses and the adoptive transfer of EAT by splenic lymphocytes of CBA mice immunized with mouse Tg (Hoshioka, 1993). Other epitopes may be abrogated by the chondroitin 6-sulfate chain of hTg, whose generation requires cleavage at or in the vicinity of Ser2729, within a range which includes, on the amino side, the Lys2713-Gly2714 peptide bond.

The probability that the chondroitin 6-sulfate unit influence the immunopathogenic properties of hTg is augmented by the documented effects of chondroitin sulfate

oligosaccharide units on cellular immune responses. A small percentage (2-5%) of invariant chain (Ii) molecules associated with class II MHC molecules are modified with the addition of a single chondroitin sulfate chain at Ser 291. In this form, they remain associated with class II molecules at the surface of APCs (Sant, 1985; Miller, 1988), 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. Such an effect occurs through interaction of Ii-CS with CD44 on responding T cells, as it can be inhibited both by anti-CD44 antibodies, and by a soluble form of CD44 (CD44Rg), which binds Ii-CS directly (Naujokas, 1993). Treatment of spleen cells with xyloside, which inhibits glycosaminoglycan addition, interferes with their antigen-presenting capabilities (Rosamond, 1987). It was suggested that Ii-CS may allow stimulation of memory T cells, which express high levels of CD44 (Butterfield, 1989), by APC types that either are lacking other types of co-stimulatory molecules, or only express them after an initial encounter with T cells (Naujokas, 1993). CD44, a broadly distributed transmembrane glycoprotein, is the principal cell surface receptor for hyaluronan (HA), but recognizes also chondroitin 4- and 6-sulfate, although with lower affinity (Aruffo, 1990; Sy, 1991). Among various carbohydrates, it can be modified by chondroitination. Its co-stimulatory role in T cell activation is supported by a number of studies (Sommer, 1995; Yashiro, 1998). Moreover, monoclonal antibodies directed against CD44 at the surface of cytotoxic T lymphocytes were able to trigger cytolytic activity in a TCR-independent manner (Seth, 1991). Serglycins, small proteoglycans stored in secretory granules of hematopoietic cells, activate the CD3-dependent release of cytokines and proteases from CD44-positive CTL clones (Toyama-Sorimachi, 1995), by interacting with CD44 through their chondroitin 4-sulfate and 6-sulfate side chains (Toyama-Sorimachi, 1997). 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 oligomerization

of CD44 molecules and activation of intracellular signaling (Fujimoto, 2001). Expression of CD44 in an active form, which is capable of hyaluronan binding and primary adhesion, can be induced by antigen stimulation of the TCR (DeGrendele, 1997a), and is required for extravasation of activated T lymphocytes and monocytes to sites of inflammation (DeGrendele, 1997b; Stoop, 2002; Weiss, 1998). Increased CD44 expression ensues also B cell activation (Camp, 1991). The number of circulating T cells showing functional activation of CD44 expression is increased in patients with systemic lupus erythematosus and arthritis (Estess, 1998), and administration of anti-CD44 antibodies inhibits inflammation in murine models of inflammatory bowel disease and of collagen- and proteoglycan-induced arthritis (Pure, 2001). For these reasons, CD44 has raised interest as a marker of disease activity or a target for therapeutic intervention in autoimmune diseases. Different effects of the CD44 stimulation in different cell types depend on the CD44 isoforms involved, which vary by the insertion of variant exon products between exon 5 and 6 of the CD44 standard isoform (CD44s) (Ponta, 2003). Both CD44v6 and CD44v7 are transiently up-regulated during lymphocyte activation, the former being mainly expressed on T cells, and the latter on a fraction of $CD4^+$ cells, B cells and monocytes. Antibodies to CD44s and CD44v7 inhibit antigenic and mitogenic T and B cell responses, while antibodies to CD44v6 selectively inhibit T cell responses (Seiter, 2000). Both mitigate delayed-type hypersensitivity reactions, although by different mechanisms. Blockade of the former prevented activation of $CD8^+$ cells and secretion of IL-2 and IFN-γ from $CD4^+$ and $CD8^+$ cells, whereas blockade of the latter apparently interfered with the delivery of signals from monocytes, resulting in decereased IL-12 secretion by B cells, and increased IL-10 secretion by B and CD4+ cells (Seiter, 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, 2005).

Finally, the close clinical and temporal association between Graves' hyperthyroidism and thyroid-associated ophthalmopathy (TAO) led to the hypothesis that the latter is the result of an autoimmune response directed against one or more orbital autoantigens that are also present within the thyroid. Much effort has been directed at identifying target cells and mapping critical epitope(s) on autoantigens they display. Candidate antigens, besides the TSH receptor, have included Tg (reviewed in ref. Prabhakar, 2003). Some Authors detected Tg in orbital tissues in patients with TAO, and hypothesized that transfer of Tg to orbital tissues might occur via thyroid-orbit connections evidenced by radioisotope-based lymphography (Marinò, 2001). Tg was found to be predominantly localized in fibroadipose tissue (Lisi, 2002). The same Authors proposed that the ability of hTg to bind to GAGs, including chondroitin sulfate B and C, may mediate its localization in orbital tissues, where it may function as the target of immune responses originally directed towards the thyroid (Marinò, 2003). Metachromatic GAGs accumulate in thyroid-associated ophthalmopathy and dermopathy (Prabhakar, 2003). Edematous connective perimysial tissues of patients with TAO are composed predominantly of hyaluronan and chondroitin sulfate (Kahaly, 1998). Accumulation of GAGs, together with adipose tissue expansion, is also apparent in the fatty connective tissue of the posterior orbit (Hufnagel, 1984). UDP-glucose dehydrogenase, the enzyme which catalyzes the conversion of UDP-glucose to UDP-glucuronate, was induced by proinflammatory cytokines in orbital fibroblasts (Spicer, 1998). In our opinion, the presence of an integral GAG chain in a subpopulation of hTg molecules may represent a more direct mechanism, by which autoaggressive responses towards hTg may spread to connective tissue antigen with shared GAG chains, particularly in the event that the synthesis of both be quantitatively and/or qualitatively disregulated by pathogenic stimuli. Proving such an hypothesis will require the fine structural characterization of the chondroitin 6-sulfate chains

of hTg and of the GAGs of orbital connective tissues, and the demonstration of cross-reacting autoantibodies and/or T cell clones in the tissues and/or blood of patients with TAO.

In conclusion, in dozens of hTg preparations examined, mostly from goiters, hTg-CS was constantly found, its abundance varying broadly from 31 to 72%. The presence of a single chondroitin 6-sulfate chain linked to Ser2729 of hTg influenced significantly the hormone-forming efficiency of hTg, the proteolytic accessibility of its carboxy-terminal domain, and its immunopathogenic capacity in CBA/J(*H-2*^{k}) mice, by a complex mechanism.

Further work will be aiming to determine:

1) the physiological limits of hTg-CS abundance in hTg;
2) the regulation of the synthesis of chondroitin 6-sulfate chains of hTg, with particular regard to the role of TSH, and the possible adaptive role of changes in hTg-CS abundance in the presence of iodine deficiency or inherited defects of hormone synthesis and/or secretion;
3) the possible correlations between variations of the hTg-CS/hTg-CS^{-} ratio and thyroid function. It is our opinion that a systematic investigation could shed light on the pathogenesis of thyroid disease, particularly AITD;
4) the effects of the immunization of CBA/J(*H-2*^{k}) mice, using hTgCS^{-} for priming, and hTgCS for boosting, in comparison with the use of unfractionated hTg for both; such a protocol may lead to a fuller-blown form of EAT and a better model of AITD;
5) the effects of the immunization of CBA/J(*H-2*^{k}) mice with hTg peptide 2714-2744, containing peptide bonds which are protected from proteolysis in hTgCS, and, perhaps, epitopes whose processing by APCs is inhibited in hTg-CS;
6) the effects of the stimulation with hTgCSgp of autoreactive T cell clones from CBA/J(*H-2*^{k}) mice immunized with the various forms of hTg, using such techniques as

gene microarrays, 2-D proteome analysis and differential mRNA display, and paying particular attention to the activation of signal transduction cascades.

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8. REFERENCES

- Allen PM. Peptides in positive and negative selection: a delicate balance. *Cell* 1994; **76**:593-596.
- Anderton SM. Post-translational modifications of self antigens: implications for autoimmunity. *Curr. Opin. Immunol.* 2004; **16**:753-8
- Arima T, Spiro MJ and Spiro RG. Studies on the carbohydrate units of thyroglobulin. Evaluation of their microheterogeneity in the human and calf proteins. *J. Biol. Chem.* 1972; **247**: 1825-1835
- Arima T and Spiro RG. Studies on the carbohydrate units of thyroglobulin. Structure of the mannose-N-acetylglucosamine unit (unit A) of the human and calf proteins. *J. Biol. Chem.* 1972; **247**, 1836-1848
- Aruffo A, Stamenkovic I, Melnick M, Underhill CB, Seed B. CD44 is the principal cell surface receptor for hyaluronate. *Cell* 1990; **61**:1303-13.
- Asuncion M, Ingrassia R, Escribano J, Martin U, Mendez F, Di Lauro R, Lamas L. Efficient thyroid hormon formation by in vitro iodination of a segment of rat thyroglobulin fused to Staphylococcal protein A. *Febs Lett* 1992; **297**: 266-270
- Baas F, van Ommen GJB, Bikker H, Arnberg AC and de Vijlder JJM. The human thyroglobulin gene is over 300 kb long and contains introns of up to 64 kb. *Nucl. Acids Res.* 1986; **14**: 5171-5186
- Bader, J., P., Ray, D. A., and Steck, T. L. Electrophoretic determinations of hyaluronate produced by cells in culture. *Biochim. Biophys. Acta* 1972; **264**, 73-84
- Beisel KW, David CS, Giraldo AA, Kong YM and Rose NR. Regulation of experimental autoimmune thyroiditis: mapping of susceptibility to the *I-A* subregion of the mouse *H-2*. *Immunogenetics* 1982; **15**: 427-430

- Blumenkrantz N and Asboe-Hansen G. New method for the quantitative determination of uronic acids. *Anal. Biochem.* 1973; **54**, 484-489
- Brown DR, Swier K, Moskowitz NH, Naujokas MF, Locksley RM, Reiner SL. T helper subset differentiation in the absence of invariant chain *J Exp Med* 1997; **185**: 31-41
- Braley-Mullen H, Sharp GC, Medling B, Tang H. Spontaneous thyroiditis in NOD.H-2h4 mice. *J. Autoimmun.* 1999; **12**: 157-65
- Braley-Mullen H, Yu S. Early requirement for B cells for development of spontaneous autoimmune thyroiditis in NOD.H-2h4 mice. *J. Immunol.* 2000; **165**: 7262-9
- Brinkmann T, Weilke C, Kleesiek K. Recognition of acceptor proteins by UDP-D-xylose proteoglycan core protein beta-D-xylosyltransferase. *J Biol Chem* 1997; **272**:11171-5.
- Brix TH, Kyvik KO, Christensen , Hegedüs L. Evidence for a major role of heredity in Graves' disease. A population-based study of two Danish cohorts. *J. Clin. Endocrinol. Metab.* 2001; **86**: 930-4
- Brix TH, Kyvik KO, Hegedüs L. A population-based study of chronic autoimmune hypothyroidism in Danish twins. *J. Clin. Endocrinol. Metab.* 2000; **85**: 536-9
- Carayanniotis G, Chronopoulou E and Rao V.P. Distinct genetic pattern of mouse susceptibility to thyroiditis induced by a novel thyroglobulin peptide. *Immunogenetics* 1994; **39**: 21-28
- Carayanniotis G, Masters SR and Noelle RJ. Suppression of murine thyroiditis via blockade of the CD40-CD40L interaction. *Immunology* 1997; **90**: 421-426.
- Champion BR, Page KR, Parish N, Rayner DC, Dawe K, Biswas-Hughes G, Cooke A, Geysen M and Roitt IM. Identification of a thyroxine-containing self-epitope of

thyroglobulin which triggers thyroid autoreactive T cells. *J. Exp. Med.* 1991; **174**: 363-370

- Champion BR, Rayner DC, Byfield PGH, Page KR, Chan CTJ and Roitt IM. Critical role of iodination for T cell recognition of thyroglobulin in experimental murine thyroid autoimmunity. *J. Immunol.* 1987; **139**: 3665-3670
- Charreire J. Immune mechanisms in autoimmune thyroiditis. *Adv. Immunol.* 1989; **46**: 263-334
- Chronopoulou E and Carayanniotis G. Identification of a thyroiditogenic sequence within the thyroglobulin molecule. *J. Immunol.* 1992; **149**: 1039-1044
- Chronopoulou E and Carayanniotis G. H-2Ek expression influences thyroiditis induction by the thyroglobulin peptide (2495-2511). *Immunogenetics* 1993; **38**: 150-153
- Dahlberg AE, Dingman CW, and Peacock AC. Electrophoretic characterization of bacterial polyribosomes in agarose-acrylamide composite gels. *J. Mol. Biol* 1969*;* **41**, 139-147
- Dai Y, Carayanniotis KA, Eliades P, Lymberi P, Shepherd P, Kong YM, Carayanniotis G. Enhancing or suppressive effects of antibodies on processing of a pathogenic T cell epitope in thyroglobulin. *J. Immunol.* 1999; **162**: 6987-92
- Dai YD, Rao VP, Carayanniotis G. Enhanced iodination of thyroglobulin facilitates processing and presentation of a cryptic pathogenic peptide. *J Immunol.* 2002; **168**: 5907-11
- Dai YD, Eliades P, Carayanniotis KA, McCormick DJ, Kong YC, Magafa V, Cordopatis P, Lymberi P, Carayanniotis G Thyroxine-binding antibodies inhibit T cell recognition of a pathogenic thyroglobulin epitope. *J Immunol.* 2005; **174**:3105-10.
- Damotte D, Colomb E, Cailleau C, Brousse N, Charreire J, Carnaud C. Analysis of susceptibility of NOD mice to spontaneous and experimentally induced thyroiditis. *Eur. J. Immunol.* 1997; **27**: 2854-62

- Dawe KI, Hutchings PR, Geysen M, Champion BR, Cooke A and Roitt IM. Unique role of thyroxine in T cell recognition of a pathogenic peptide in experimental autoimmune thyroiditis. *Eur. J. Immunol.* 1996; **26**: 768-772
- DeGrendele HC, Estess P, Siegelmann MH. CD44 activation and associated primary adhesion is inducible via T cell receptor stimulation *J Immunol* 1997a **159**: 2549-2553
- DeGrendele HC, Estess P, Siegelman MH. Requirement for CD44 in activated T cell extravasation into an inflammatory site. *Science* 1997b; **278**:672-5.
- Dunn JT, Kim PS, Dunn AD, Heppner DG Jr, Moore RC. The role of iodination in the formation of hormone-rich peptides from thyroglobulin. *J. Biol. Chem.* 1983; **258**: 9093-9
- Dunn JT, Anderson PC, Fox JW, Fassler CA, Dunn AD, Hite LA, Moore RC. The sites of thyroid hormone formation in rabbit thyroglobulin.. *J. Biol. Chem.* 1987; **262**, 16948-16952
- Duthoit C, Estienne V, Delom F, Durand-Gorde J-M, Mallet B, Carayon P and Ruf J. Production of immunoreactive thyroglobulin C-terminal fragments during thyroid hormone synthesis. *Endocrinology* 2000; **141**: 2518-25
- Duthoit C, Estienne V, Giraud A, Durand-Gorde J-M, Rasmussen AK, Feldt-Rasmussen U, Carayon P and Ruf J. Hydrogen peroxide-induced production of a 40 kDa immunoreactive thyroglobulin fragment in human thyroid cells: the onset of thyroid autoimmunity? *Biochem. J.* 2001; **360**: 557-62
- Estess P, DeGrendele HC, Pascual V, Siegelmann MH. Functional activation of lymphocyte CD44 in peripheral blood is a marker of autoimmune disease activity. *J Clin Invest* 1998; **102:** 1173-1182
- Evavold BD, Allen PM. Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand. *Science* 1991; **252**:1308-1310

- Fassler, C. A., Dunn, J. T., Anderson, P. C., Fox, J. W., Dunn, A. D., Hite, L. A., Moore, R. C., and Kim, P. S. Thyrotropin alters the utilization of thyroglobulin's hormonogenic sites. *J. Biol. Chem.* 1988; **263**, 17366-17371
- Fenouillet, E., Fayet, G., Hovsepian, S., Bahraoui, E. M., and Ronin, C. Immunochemical evidence for a role of complex carbohydrate chains in thyroglobulin antigenicity. *J. Biol. Chem.* 1986; **261**, 15153-15158
- Flynn JC, Conaway DH, Cobbold S, Waldmann H and Kong YM. Depletion of $L3T4^+$ and $Lyt\text{-}2^+$ cells by rat monoclonal antibodies alters the development of adoptively transferred experimental autoimmune thyroiditis. *Cell. Immunol.* 1989; **122**: 377-90
- Franc JL, Venot N, Marriq C. Characterization of the two oligosaccharides present in the preferential hormonogenic domain of human thyroglobulin. *Biochem Biophys Res Comm.*1990; **166**: 937-944
- Fujimoto T, Kawashima H, Tanaka T, Hirose M, Toyama-Sorimachi N, Matsuzawa Y and Miyasaka M. CD44 binds a chondroitin sulfate proteoglycan, aggrecan. *Int. Immunol.* 2001; **13**: 359-366
- Gahring L, Carlson NG, Meyer EL, Rogers SW. Granzyme B proteolysis of a neuronal glutamate receptor generates an autoantigen and is modulated by glycosylation. *J Immunol* 2001; **166**:1433-8.
- Gentile F and Palumbo G. Trypsin-resistant regions of thyroglobulin: possible relationship with intermonomeric contact sites. *Biochem. Biophys. Res. Commun.* 1993; **196**: 1120-6
- Gentile F and Salvatore G. Preferential sites of proteolytic cleavage of bovine, human and rat thyroglobulin. The use of limited proteolysis to detect solvent-exposed regions of the primary structure. *Eur. J. Biochem.* 1993; **218**: 603-621
- Gentile F, Di Lauro R and Salvatore G. Biosynthesis and secretion of thyroid hormones. *Endocrinology*,1995 3rd edition (DeGroot, L. J., ed.) W. B. Saunders Company, Philadelphia, **Vol. 1**, pp. 517-542

- Gentile F, Ferranti P, Mamone G, Malorni A and Salvatore G. Identification of hormonogenic tyrosines in fragment 1218-1591 of bovine thyroglobulin by mass spectrometry. Hormonogenic acceptor Tyr-1291 and donor Tyr-1375. *J.Biol.Chem.* 1997a; **272**: 639-646
- Gentile F, Balì E and Pignalosa G. Sensitivity and applications of the non-denaturing staining of proteins on polyvinylidene difluoride membranes with Amido Black 10B in water followed by destaining in water. *Anal. Biochem.* 1997b; **245**: 260-262
- Gentile F., Conte M, Formisano S. Thyroglobulin as an autoantigen: what can we learn about immunopathogenicity from the correlation of antigenic properties with protein structure? *Immunology* 2004; **112**:13-25
- Glant TT, Buzas EI, Finnegan A, Negroiu G, Cs-Szabo G, Mikecz K. Critical roles of glycosaminoglycan side chains of cartilage proteoglycan (aggrecan) in antigen recognition and presentation. *J Immunol* 1998; **160**: 3812-9.
- Greenfield B, Wang W-C, Marquardt H, Piepkorn M, Wolff EA, Aruffo A and Bennett KL. Characterization of the heparan sulfate and chondroitin sulfate assembly sites in CD44. *J. Biol. Chem.* 1999; **274**: 2511-2517
- Hanisch F-G, Schwientek T, Von Bergwelt-Baildon MS, Schultze J, and Finn O. O-linked glycans control glycoprotein processing by antigen-presenting cells: a biochemical approach to the molecular aspects of MUC1 processing by dendritic cells. *Eur. J. Immunol.* 2003; **33**: 32-42-54
- Heward JM, Allahabadia A, Daykin J et al. Linkage disequilibrium between the human leukocyte antigen class II region of the major histocompatibility complex and Graves' disease: replication using a population case control and family-based study. *J. Clin. Endocrinol. Metab.* 1998; **83**: 3394-7
- Hishinuma A, Furudate S-I, Oh-Ishi M, Nagakubo N, Namatame T, Ieiri T. A novel missense mutation (G2320R) in thyroglobulin causes hypothyroidism in *rdw* rats. *Endocrinology* 2000; **141**: 4050-5

- Hoshioka A, Kohno Y, Katsuki T, Shimojo N, Maruyama N, Inagaki Y, Yokochi T, Tarutani O, Hosoya T and Niimi H. A common T cell epitope between human thyroglobulin and human thyroid peroxidase is related to murine experimental autoimmune thyroditis. *Immunol. Lett.* 1993; **37**: 235-41
- Hufnagel TG, Hickey WF, Cobbs VH, Jakobiec FA, Iwamoto T, Eagle RC. Immunohistochemical and ultrastructural studies on the exenterated orbital tissue of a patient with Graves'disease. *Ophthalmology* 1984; **91**:1411-1419
- Hutchings PR, Cooke A, Dawe K, Chamion BR, Geysen M, Valerio R and Roitt IM. A thyroxine-containing peptide can induce purine experimental autoimmune thyroiditis. *J. Exp. Med.* 1992; **175**: 869-872
- Hutchings PR, Verma S, Phillips JM, Harach SZ, Howlett S, and Cooke A. Both CD4+ and CD8+ cells are required for iodine accelerated thyroiditis in NOD mice. *Cell. Immunol.* 1999;**192**: 113-21
- Jacobson DL, Gange SJ, Rose NR and Graham NM. Epidemiology and estimated population burden of selected autoimmune diseases in the United States. *Clin Immunol Immunopathol* 1997; **84**: 223-43
- Kahaly G, Forester G, Hansen C. Glycosaminoglycans in thyroid eye disease *Thyroid* 1998; **8:** 429-432
- Karras E, Carayanniotis G and Lymberi P. Induction of murine thyroiditis by a non dominant E^k-restricted peptide of human thyroglobulin. *Immunology* 2003; **108**: 556-61
- Karras E, Yang H, Lymberi P, Christadoss P. Human thyroglobulin peptide p2340 induces autoimmune thyroiditis in HLA-DR3 transgenic mice. *J. Autoimm.* 2005; **24**: 291-296
- Kawashima H, Hirose M, Hirose J, Nagakubo D, Plaas AHK and Miyasaka M. Binding of a large chondroitin sulfate/dermatan sulfate proteoglycan, versican, to L-selectin, P-selectin, and CD44. *J. Biol. Chem.* 2000; **275**: 35448-35456

- Kim PS, Hossain SA, Park YN, Lee I, Yoo SE, Arvan P. A single amino acid change in the acetylcholinesterase-like domain of thyroglobulin causes congenital goiter with hypothyroidism in the cog/cog mouse: a model of human endoplasmic reticulum storage disease. *Proc. Natl. Acad. Sci. USA* 1998; **95**: 9909-9913
- Kong, Y. M., David, C. S., Giraldo, A. A., ElRehewy, M., and Rose, N. R. Regulation of autoimmune response to mouse thyroglobulin: influence of *H-2D*-end genes. *J. Immunol.* 1979; **123**: 15-18
- Kong YM, Lomo LC, Motte RW, Giraldo AA, Baisch J, Strauss G, Hämmerling GJ and David CS. HLA-DRB1 polymorphism determines susceptibility to autoimmune thyroiditis in transgenic mice: definitive association with HLA-DRB1*0301 (DR3) Gene. *J. Exp. Med.* 1996; **184**: 1167-1172
- Kong YM, McCormick DJ, Wan Q, Motte RW, Fuller BE, Giraldo AA and David CS. Primary hormonogenic sites as conserved autoepitopes on thyroglobulin in murine autoimmune thyroiditis. Secondary role of iodination. *J. Immunol.* 1995; **155**: 5847-5854
- Krejci E, Duval N, Chatonnet A, Vincens P and Massoulié P. Cholinesterase-like domains in enzymes and structural proteins: functional and evolutionary relationships and identification of a catalytically essential aspartic acid. *Proc. Natl. Acad. Sc.i USA* 1991; **88**: 6647-6651
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 1970; **227**, 680-685
- Lamas, L., Santisteban, P., Turmo, C., and Seguido, A. M. Evidence that level of in vitro iodination of thyroglobulin depends on the initial iodide concentration. *Endocrinology* 1986, **118**, 2131-2136
- Lamas L, Anderson PC, Fox JW and Dunn JT. Consensus sequences for early iodination and hormonogenesis in human thyroglobulin. *J. Biol. Chem.* 1989; **264**: 13541-13545

- Lejeune PJ, Marriq C, Rolland M and Lissitzky S. Amino acid sequence around a hormonogenic tyrosine residue in the N-terminal region of human thyroglobulin after in vivo and in vitro iodination. *Biochem. Biophys. Res. Commun.* 1983; **114**: 73-80
- Lisi S, Marinò M, Pinchera A, Mazzi B, Di Cosmo C, Sellari-Franceschini S, Chiovato L. Thyroglobulin in orbital tissues from patients with thyroid-associated ophthalmopathy: predominant localization in fibroadipose tissue. Thyroid 2002, **12**:351-360
- Mallet B, Lejeune PJ, Baudry N, Niccoli P, Carayon P, and Franc JL. N-glycans modulate in vivo and in vitro thyroid hormone synthesis. Study at the N-terminal domain of thyroglobulin. *J. Biol. Chem.* 1995; **270**, 29881-29888
- Malsch, R., Harenberg, J., Piazolo, L., Huhle, G., and Heene, D. L. (1996) *J. Chromatogr. B Biomed. Appl.* **685**, 223-231
- Malthiéry Y and Lissitzky S. Primary structure of human thyroglobulin deduced from the sequence of its 8448-base complementary DNA. *Eur. J. Biochem.* 1987; **165**: 491-498
- Marhaba R, Bourouba M, and Zoller M. CD44v6 promotes proliferation by persisting activation of MAP kinases. *Cell. Signal.* 2005; **17**: 961-973
- Marinò M, Zheng G, McCluskey RT. Megalin (gp330) is an endocytic receptor for thyroglobulin on cultured Fisher Rat Thyroid Cells. *J. Biol. Chem.* 1999a; **274**: 12898-12904
- Marinò M, Friedlander JA, McCluskey RT, Andrews D. Identification of a heparin-binding region of rat thyroglobulin involved in megalin binding. *J Biol Chem* 1999b; **274**, 30377-30386

- Marinò M, Lisi S, Pinchera A, Marcocci C, Menconi F, Morabito E, Macchia M, Sellari-Franceschini S, McCluskey RT, Chiovato L. Glycosaminoglycans provide a binding site for thyroglobulin in orbital tissues of patients with thyroid-associated ophthalmopathy. *Thyroid* 2003; **13**: 851-859
- Marriq C., Rolland M, Lissitzky S. Amino acid sequence of the unique 3,5,3'-triiodothyronine-containing sequence from porcine thyroglobulin. *Biochem. Biophys. Res. Commun.* 1983; **112**, 206-213
- Marriq C, Lejeune PJ, Venot N and Vinet L. Hormone formation in the isolated fragment 1-171 of human thyroglobulin involves the couple tyrosine 5 and tyrosine 130. *Mol. Cell. Endocrinol.* 1991: **81**: 155-164
- Matsuoka N, Unger P, Ben-Nun A, Graves P and Davies TF. Thyroglobulin-induced murine thyroiditis assessed by intrathyroidal T cell receptor sequencing. *J. Immunol.* 1994; **152**: 2562-68
- McKnight AJ, Mason DW and Barclay AN. Sequence of a rat MHC class II-associated invariant chain cDNA clone containing a 64 amino acid thyroglobulin-like domain. *Nucl. Acid. Res.* 1989; **17**: 3983-3984
- McMurray RW, Sharp GC, and Braley-Mullen H. Intrathyroidal cell phenotype in murine lymphocytic and granulomatous experimental autoimmune thyroiditis. *Autoimmunity* 1994; **18**: 93-102
- Miller J, Hatch JA, Simonis S, Cullen SE. Identification of the glycosaminoglycan attachment site of mouse invariant chain proteoglycan core protein by site-direct mutagenesis. *Proc Natl Acad Sci USA* 1988; **85:** 1359-1363

- Molina F, Bouanani M, Pau B and Granier C. Characterization of the type-1 repeat from thyroglobulin, a cysteine-rich module found in proteins from different families. *Eur. J. Biochem.* 1996a; **240**: 125-33
- Molina F, Pau B and Granier C. The type-1 repeats of thyroglobulin regulate thyroglobulin degradation and T3, T4 release in thyrocytes. *FEBS Lett.* 1996b; **391**: 229-31
- Nakashima M, Kong YM and Davies TF. The role of T cells expressing TcR Vβ13 in autoimmune thyroiditis induced by transfer of mouse thyroglobulin-activated lymphocytes: identification of two common CDR3 motifs. *Clin. Immunol. Immunopathol.* 1996; **80**: 204-10
- Naujokas MF, Morin M, Abderson MS, Peterson M, and Miller J. The chondroitin sulfate form of invariant chain can enhance stimulation of T cell responses through interaction with CD44. *Cell* 1993;**74**: 257-68
- Palumbo, G., Tecce, M. F., and Ambrosio, G. A non-incinerative rate-sensing methods for the determination of iodine in iodoproteins. *Anal. Biochem.* 1982; **123**: 183-189.
- Pitt-Rivers, R., and Ambesi-Impiombato, F. S. (1968) The binding of sodium dodecyl sulphate to various proteins. *Biochem. J.* **109**, 825-830
- Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. *Nat Rev Mol Cell Biol* 2003; **4**:33-45
- Prabhakar BS, Bahn RS, Smith TJ. Current perspective on the pathogenesis of Graves' disease and ophthalmopathy. *Endocrine Rev.* 2003; **24**: 802-835
- Pucci P and Sepe C. *Biomed. Environ. Mass Spectrom.* 1990; **17**, 287-291
- Pure E and Cuff CA. A crucial role for CD44 in inflammation. *Trends Mol Med*. 2001; **7**:213-21

- Rao VP, Balasa B and Carayanniotis G. Mapping of thyroglobulin epitopes: presentation pf a 9mer pathogenic peptide by different mouse MHC class II isotypes. *Immunogenetics* 1994; **40**: 352-359
- Rao VP and Carayanniotis G. Contrasting immunopathogenic properties of highly homologous peptides from rat and human thyroglobulin. *Immunology* 1997; **90**: 244-249
- Rachmilewitz J and Tykocinski ML. Differential effects of chondrotin sulfates A and B on monocyte and B cell activation: evidence for B-cell activation via a CD44-dependent pathway *Blood* 1998; **92**: 223-229
- Rasooly L, Rose NR, Saboori AM, Ladenson PW and Burek CL. Iodine is essential for human T cell recognition of human thyroglobulin. *Autoimmunity* 1998; **27**: 213-19
- Rawitch AB, Chernoff SB, Litwer MR, Rouse JB, Hamilton JW. Thyroglobulin structure-function. The amino acid sequence surrounding thyroxine. *J. Biol. Chem.* 1983; **258**, 2079-2082
- Rawitch, A. B., Liao, T. H., and Pierce, J. G. The amino acid sequence of a tryptic glycopeptide from human thyroglobulin. *Biochim. Biophys. Acta* 1968 **160**, 360-367
- Rawitch AB, Litwer MR, Gregg J, Dziadik Turner C, Rouse JB, d Hamilton JW. *Biochem. Biophys. Res. Commun.* 1984; **118**, 423-429
- Romball CG and Weigle O. Transfer of experimental autoimmune thyroiditis with T cell clones. *J. Immunol.* 1987; **138**: 1092-1098
- Rosamond S, Brown L, Gomez C, Braciale TJ, Schwartz BD. Xyloside inhibits synthesis of the class II-associated chondroitin sulfate proteoglycan and antigen presentation events. *J Immunol.* 1987; **139**:1946-51.

- Rose NR, Twarog FJ and Crowle AJ. Murine thyroiditis: importance of adjuvant and mouse strain for induction of thyroid lesions. *J. Immunol.* 1971; **106**: 698-704
- Saboori AM, Rose NR and Burek CL. Amino acid sequence of a tryptic peptide of human thyroglobulin reactive with sera of patients with thyroid disease. *Autoimmunity* 1995; **22**: 87-94
- Saboori AM, Rose NR, Yuhasz SC, Amzel LM and Burek CL. Peptides of human thyroglobulin reactive with sera of patients with autoimmune thyroid disease. *J. Immunol.* 1999; **163**: 6244-50
- Salamero J, Charreire J. Syngeneic sensitization of mouse lymphocytes on monolayers of thyroid epithelial cells. V. The primary syngeneic sensitization is under I-A subregion control. Eur J Immunol. 1983a; **13**: 948-51
- Salamero I and Charreire J. Syngeneic sensitization of mouse lymphocytes on monolayers of thyroid epithelial cells. Relation to *H-2* haplotypes. *Cell. Immunol.* 1983b; **78**: 387-391
- Schägger H, and von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 1987; **166**: 368-379.
- Sant AJ, Schwartz BD, Cullen SE. Cellular distribution of the Ia-associated chondroitin sulfate proteoglycan. *J Immunol* 1985; **135**:408-15.
- Schneider AB, McCurdy A, Chang T, Dudlak D and Magner J. Metabolic labeling of human thyroglobulin with (^{35}S)-sulfate: incorporation into chondroitin 6-sulfate and endoglycosidase-F-susceptible carbohydrate units. *Endocrinology* 1988; **122**: 2428-2435

- Seiter S, Schimdt DS, Zöller M. The CD44 variant isoforms CD44v6 and CD44v7 are expressed by distinct leukocytes subpopulations and exert non-overlapping functional activities. *Int Immunol* 2000; **12:** 37-49

- Seth A, Gote L, Nagarkatti M, Nagarkatti PM. T-cel-receptor-independent activation of cytolytic activaty T cytotoxic lymphocytes through mediated CD44 and gp90^{MEL14} *Proc Natl Acad Sci USA* 1991; **88**:7877-7881

- Shimojo N, Saito K, Kohno Y, Sasaki N, Tarutani O and N. Hironori. Antigenic determinants on thyroglobulin: comparison of the reactivities of different thyroglobulin preparations with serum antibodies and T cells of patients with chronic thyroiditis. *J. Clin. Endocrinol. Metab.* 1988; **66**: 689-695

- Simon LL, Justen JM, Giraldo AA, Krco CJ and Kong YCM. Activation of cytotoxic T cells and effector cells in experimental autoimmune thyroiditis by shared determinants of mouse and human thyroglobulins. *Clin. Immunol. Immunopathol.* 1986; **39**: 345-56

- Simon LL, Krco CJ, David CS and Kong Y. M. Characterization of the *in vitro* murine T cell proliferative responses to murine and human thyroglobulins in thyroiditis-susceptible snf resistant mice. *Cell. Immunol.* 1985; **94**: 243-253

- Snapper CM and Mond JJ. Toward a comprehensive view of immunoglobulin class switching. 1993 Immunol Today 14: 15

- Sommer F, Huber M, Rollinghoff M, Lohoff M. CD44 plays a co-stimulatory role in murine T cell activation: ligation of CD44 selectively co-stimulates IL-2 production, but not proliferation in TCR-stimulated murine Th1 cells. *Int Immunol* 1995; **7**:1779-86

- Sorimachi, K., and Ui, N. (1974) *J. Biochem. (Tokyo)* **76**, 39-45

- Spicer AP, Kaback LA, Smith TJ, Seldin MF. Molecular cloning and characterization of the human and mouse UDP-glucose dehydrogenase genes *J Biol Chem* 1998; **273**: 25117-25124

- Spiro RJ. Analysis of sugars found in glycoproteins. *Meth. Enzymol.* 1966; **8**, 3-25
- Spiro MJ. Presence of a glucuronic acid-containing carbohydrate unit in human thyroglobulin. *J. Biol. Chem.* 1977; **252**: 5424-5430
- Spiro RG and Bhoyroo VD. Occurrence of sulfate in the asparagine-linked complex carbohydrate units of thyroglobulin. Identification and localization of galactose 3-sulfate and N-acetylglucosamine 6-sulfate residues in the human and calf proteins. *J. Biol. Chem.* 1988; **263**: 14351-14358
- Stoop R, Gal I, Glant TT, McNeish JD, Mikecz K. Trafficking of CD44-deficient murine lymphocytes under normal and inflammatory conditions. *Eur J Immunol* 2002; **32**:2532-42.
- Sun D, Hu X-Z, Shah, R, Coleclough C. The pattern of cytokine gene expression induced in rat T cells specific for myelin basic protein depends on the type and quality of antigenic stimulus. *Cell. Immunol.* 1995;**166**: 1-8
- Sy MS, Guo YJ, Stamenkovic I. Distinct effects of two CD44 isoforms on tumor growth in vivo. *J Exp Med* 1991; **174**:859-66.
- Takagi Y, Omura T and Go M. Evolutionary origin of thyroglobulin by duplication of esterase gene. *FEBS Lett.* 1991; **282**: 17-22
- Tange H, Bedin C, Texier B and Charreire J. Autoantibody specific for a thyroglobulin epitope inducing experimental autoimmune thyroiditis or its anti-idiotype correlates with the disease. *Eur. J. Immunol.* 1990; **20**: 1535-9
- Texier B, Bédin C, Tang H, Camoin L, Laurent-Winter C and Charreire J. Characterization and sequencing of a 40-amino-acid peptide from human thyroglobulin inducing experimental autoimmune thyroiditis. *J. Immunol.* 1992; **148**: 3405-3411
- Thrasyvoulides A, Sakarellos-Daitsiotis M, Philippou G, Souvatzoglou A, Sakarellos C and Lymberi P. B-cell autoepitopes on the acetylcholinesterase-homologous region of

human thyroglobulin: association with Graves' disease and thyroid eye disease. *Eur. J. Endocrinol.* 2001; **145**: 119-27

- Tomer Y, Greenberg DA, Concepcion E, Ban Y and Davies TF. Thyroglobulin is a thyroid specific gene for the familial autoimmune thyroid diseases. *J. Clin. Endocrinol. Metab.* 2002; **87**: 404-7
- Tomer Y. Genetic dissection of familial autoimmune thyroid diseases using whole genome screening. *Autoimmun. Rev.* 2002; **1**: 198-204
- Tomer Y, Ban Y, Concepcion E, Barbesino G, Villanueva R, Greenberg DA and Davies TF. Common and Unique Susceptibility Loci in Graves and Hashimoto Diseases: Results of Whole-Genome Screening in a Data Set of 102 Multiplex Families. *Am. J. Hum. Genet.* 2003; **73**: 736-47
- Toyama-Sorimachi N, Sorimachi H, Tobita Y, Kitamura F, Yagita H, Suzuki K, Miyasaka M. A novel ligand for CD44 is serglycin, a hematopoietic cell lineage-specific proteoglycan. *J. Biol. Chem.* 1995; **270:**7437-7444
- Toyama-Sorimachi N, Kitamura F, Habuchi H, Tobita Y, Kimata K and Miyasaka M. Widespread expression of chondroitin sulfate-type serglycins with CD44 binding ability in hematopoietic cells. *J. Biol. Chem.* 1997; **272**: 26714-26719
- Vaidya B, Imrie H, Perros P et al. The cytotoxic T lymphocyte antigen-4 is a major Graves' disease locus. *Hum. Mol. Genet.* 1999; **8**: 1195-9
- van de Graaf SAR, Pauws E, de Vijlder JJM, Ris-Stalpers C. The revised 8307 base pair coding sequence of human thyroglobulin transiently expressed in eukaryotic cells. *Eur. J. Endocrinol.* 1997; **136**: 508-15
- Veenboer GJM and de Vijlder JJM. Molecular basis of the thyroglobulin synthesis defects in Dutch goats. *Endocrinology* 1993; **132:** 377-81

- Verginis P , Stanford MM and Carayanniotis G. Delineation of five thyroglobulin T cell epitopes with pathogenic potential in experimental autoimmune thyroiditis. *J. Immunol.* 2002; **169**: 5332-7
- Verma S, Hutchings P, Guo J, McLAchlan S, Rapoport B, e Cooke A. Role of MHC I Expression and CD8+ T cells in the evolution of iodine-induced thyroiditis in NOD-H2h4 and NOD mice. *Eur. J. Immunol.* 2000;**30**: 1191-202
- Vlad AM, Muller S, Cudic M, Paulsen H, Otvos L jr, Hanisch FG. Complex carbohydrates are not removed during processing of glycoproteins by dendritic cells: processing of tumor antigen MUC1 glycopeptides for presentation to major histocompatibility complex class II-restricted T cells. *J Exp Med* 2002; **196**:1435-46.
- Vladutiu AO. Experimental autoimmune thyroiditis in mice chronically treated from birth with anti-IgM antibodies. *Cell. Immunol.* 1989; **121**: 49-59
- Wan Q, Kita M, Flynn JC et al. Participation of Vb13+ and Vb1+ T cells in transfer thyroiditis after activation of mouse thyroglobulin-primed T cells by superantigen staphylococcal enterotoxin A. *Cell. Immunol.* 2001; **213**: 149-57
- Wan Q, Motte RW, McCormick DJ, Fuller BE, Giraldo AA, David CS and Kong YM. Primary hormonogenic sites as conserved autoepitopes on thyroglobulin in murine autoimmune thyroiditis: role of MHC class II. *Clin. Immunol. Immunopathol.* 1997; **85**: 187-94
- Wan Q, Shah R, McCormick DJ, Lomo LC, Giraldo AA, David DS and Kong YM. H2-E transgenic class II-negative mice can distinguish self from nonself in susceptibility to heterologous thyroglobulins in autoimmune thyroiditis. *Immunogen.* 1999; **50**: 22-30
- Wan Q, Shah R, Panos JC, Giraldo AA, David CS and Kong YM. HLA-DR and HLA-DQ polymorphism in human thyroglobulin-induced autoimmune thyroiditis: DR3 and DQ8 transgenic mice are susceptible. *Hum. Immunol.* 2002; **63**: 301-10
- Warren, L. Thiobarbituric acid assay for sialic acids. *Meth. Enzymol.* 1963; **6:** 463-464

- Weiss JM, Renkl AC, Sleeman J, Dittmar H, Termeer CC, Taxis S, Howells N, Schopf E, Ponta H, Herrlich P, Simon JC. CD44 variant isoforms are essential for the function of epidermal Langerhans cells and dendritic cells. *Cell Adhes Commun.* 1998; **6**:157-60
- Wessel, D., and Flügge, U. I. (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* **138**, 141-143
- Weetman AP. Autoimmune thyroid disease: propagation and progression. *Eur. J. Endocrinol.* 2003; 148: 1-9
- Weetman AP and McGregor AM. Autoimmune thyroid disease: further developments in our understanding. *Endocr. Rev.* 1994; **15**: 788-830
- Yang, S.-X., Pollock, G., and Rawitch, A. B. Glycosylation in human thyroglobulin: location of the N-linked oligosaccharide units and comparison with bovine thyroglobulin. *Arch. Biochem. Biophys.* 1996; **327**, 61-70
- Yashiro Y, Tai XG, Toyo-oka K, Park CS, Abe R, Hamaoka T, Kobayashi M, Neben S, Fujiwara H. A fundamental difference in the capacity to induce proliferation of naive T cells between CD28 and other co-stimulatory molecules. *Eur J Immunol* 1998; **28**:926-35
- Yu S, Sharp GC and Braley-Mullen H. Dual roles for IFN-gamma, but not for IL-4, in spontaneous autoimmune thyroiditis in NOD.H-2h4 mice. *J. Immunol.* 2002; **169**: 3999-4007
- Zaccone P, Hutchings P, Nicoletti, Penna G, Adorini L, and Cooke A. The involvement of IL-12 in murine experimentally induced autoimmune thyroid disease. *Eur. J. Immunol.* 1999; **29**: 1933-42

Curriculum Vitae

The author of this thesis was born on the 6th of May 1971 in Salerno, Italy. After graduating at high school (Liceo Classico) with the maximum score (60/60), she attended the Medical School at the Second University of Naples, where she graduated *cum laude* and special "academic mention" for the curriculum in 1996. In 1997 she obtained the license to practice as a Medical Doctor in Naples with the maximum mark (90/90). Between 1996 and 2001, she attended the School of Specialization in Endocrinology and Metabolic Diseases (Chair: Prof. Antonio Bellastella), at the Second University of Naples. In 2001, she obtained the Specialization in Endocrinology and Metabolic Diseases *cum laude*. From May 2002 she has been working at the Department of Cellular and Molecular Biology and Pathology "L. Califano" of University "Federico II" of Naples, under the guide of Prof. Fabrizio Gentile.

Publications

1. RECOMBINANT GROWTH HORMONE (GH) THERAPY IN GH-DEFICIENT ADULTS: A LONG-TERM CONTROLLED STUDY ON DAILY VERSUS THRICE WEEKLY INJECTIONS. Amato G, Mazziotti G, Di Somma C, Lalli E, De Felice G, **Conte M**, Rotondi M, Pietrosante M, Lombardi G, Bellastella A, Carella C, Colao A. *J Clin Endocrinol Metab.* 2000 85(10):3720-5.

2. ANTIPITUITARY ANTIBODIES IN ADULTS WITH APPARENTLY IDIOPATHIC GROWHT HORMONE DEFICIENCY AND IN ADULTS WITH AUTOIMMUNE ENDOCRINE DISEASES. De Bellis A, Bizzarro A, **Conte M,** Perrino S, Coronella C, Solimeno S, Sinisi AM, Stile LA, Pisano G, Bellastella A. *J Clin Endocrinol Metab.* 2003 88(2):650-654.

3. SERUM AND FOLLICULAR FLUID CYTOKINES IN POLYCYSTIC OVARY SYNDROME DURING STIMULATED CYCLES. Amato G, **Conte M**, Mazziotti G, Lalli E, Vitolo G, Tucker AT, Bellastella A, Carella C, Izzo A. *Obstet. Gynecol* 2003 101(6) 1177-1182.

4. RELATIONSHIP BETWEEN LONGITUDINAL BEHAVIOR OF SOME MARKERS OF EYE AUTOIMMUNITY AND CHANGES OF OCULAR FINDINGS IN PATIENTS WITH GRAVES' OPHTHALMOPATHY UNDERGOING CORTICOSTEROID THERAPY. De Bellis A, Bizzarro A, **Conte**

M, Coronella C, Solimeno S, Perrino S, Sansone D, Guaglione M, Wall JR, Bellastella A. *Clin Endocrinol* 2003 59: 388-395.

5. THYROGLOBULIN AS AN AUTOANTIGEN: WHAT CAN WE LEARN ABOUT IMMUNOPATHOGENICITY FROM THE CORRELATION OF ANTIGENIC PROPERTIES WITH PROTEIN STRUCTURE? F. Gentile, **M. Conte,** S. Formisano. *Immunology* 2004 112; 13-25

6. EXTRAOCULAR MUSCLE ANTIBODIES AND THE OCCURRENCE OF OPHTHALMOPATHY IN GRAVES' DISEASE A. De Bellis, S. Perrino, C. Coronella, D. Sansone, G. Ruocco, G. Tirelli, S. Di Martino, **M. Conte,** G. Bellastella, J.R. Wall, A. Bellastella, A. Bizzarro. *Clin Endocrinol* 2004 60: 694-698.

7. SERUM ANTIBODIES AGAINST COLLAGEN XIII: A FURTHER GOOD MARKER OF ACTIVE GRAVES' OPHTHALMOPATHY A. De Bellis, C. Coronella, D. Sansone, **M. Conte,** S. Iorio, S. Perrino, G. Bellastella, JR. Wall, A. Bellastella, A. Bizzarro. *Clin Endocrinol* 2005 62: 24-29

8. CHARACTERIZATION OF ANTIPITUITARY ANTIBODIES TARGETTING PITUITARY HORMONE-SECRETING CELLS IN ADULTS WITH ISOLATED APPARENTLY IDIOPATHIC GROWTH HORMONE DEFICIENCY AND IN ADULTS WITH AUTOIMMUNE ENDOCRINE DISEASES WITH OR WITHOUT GROWTH HORMONE DEFICIENCY A. De Bellis, A. Bizzarro, S. Perrino, C. Coronella, **M. Conte**, D. Pasquali, A.A. Sinisi, C. Betterle, A. Bellastella *Clinical Endocrinology* 2005 63: 45-49

9. EFFECTS OF TOTAL THYROIDECTOMY ALONE OR PLUS RADIOIODINE ABLATION ON THE COURSE OF GRAVES' OPHTHALMOPATHY: A TWO-YEAR LONGITUDINAL STUDY **M Conte**, A Bizzarro, G Ruocco, G Bellastella, A Savoia, LA Stile, O Fabozzi, JR. Wall, A Livrea, G Cennamo, A Bellastella, A De Bellis. *Under revision in Ophthalmology*

10. A SINGLE CHONDROITIN 6-SULFATE OLIGOSACCHARIDE UNIT AT SER2729 OF HUMAN THYROGLOBULIN ENHANCES HORMONE FORMING EFFICIENCY AND LIMITS PROTEOLYTIC ACCESSIBILITY AT THE CARBOXYL TERMINUS. POSSIBLE INSIGHTS INTO THYROID HOMEOSTASIS AND AUTOIMMUNITY **M. Conte** et al *J Biol Chem* submitted

11. INFLUENCE OF CHONDROITIN 6-SULFATE OLIGOSACCHARIDE UNIT ADDITION ON THE IMMUNOPATHOGENICITY OF HUMAN THYROGLOBULIN IN CBA/J(*H-2*^{k}) MICE. MULTIPLE EFFECTS ON ANTIGEN PROCESSING AND T CELL RESPONSES. **M. Conte** et al *manuscript in preparation*