THE THYROID, IODINE AND AUTOIMMUNITY

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THE THYROID, IODINE AND AUTOIMMUNITY

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When you know a thing, to hold that you know it; and when you do not know a thing, to allow that you do not know it – this is knowledge.

(Confucius 551-479 BC)

CONTENTS

Chapter 1

GENERAL INTRODUCTION

Chapter 1.1

THYROID HORMONE SYNTHESIS AND RELEASE. REGULATION BY DIETARY IODINE

Iodine is an essential trace element and part of the thyroid hormones. It is thus obvious that the metabolism of iodine, the thyroid function and the thyroid hormone production are highly interconnected.

The thyroid hormones, chemically known as the iodothyronines, are synthesized in the follicular cells of the thyroid gland[1,2]. The main secretory product of the thyroid gland is thyroxine (T_4), which has little intrinsic bioactivity and is generally regarded as a prohormone[3,4]. Less than 20% of the bioactive hormone triiodothyronine (T_3) is produced by the thyroid gland, while the majority of T_3 is generated outside the thyroid (in the brain, liver and kidney) by monodeiodination of the phenolic ring of T_4[5,6,7,8,9,10] (see also later). The hormonal effects of the inactive prohormone T_4 are therefore *in vivo* largely due to its conversion to T_3.

The synthesis and release of the thyroid hormones by thyrocytes is regulated by Thyrotropin or Thyroid Stimulating Hormone (TSH), which is secreted by the thyrotrophic cells of the anterior pituitary gland. The synthesis of TSH by these pituitary cells is regulated by Thyrotropin Releasing Hormone (TRH), synthesized in the hypothalamus. A negative feedback loop exists, by which the T_3 and T_4 concentrations regulate the synthesis and secretion of both TSH and TRH (see figure 1).

The sequence of events that lead to the synthesis and finally the secretion of the iodothyronines T_3 and T_4 into the circulation are: 1) an active concentration of iodine as iodide by the thyrocytes in the thyroid gland, 2) the synthesis of thyroglobulin (Tg) by the thyrocytes, 3) iodination of tyrosyl residues of Tg mainly at the apical cell membrane of the thyrocytes, 4) coupling of mono- and diiodotyrosine residues with formation of iodothyronines also mainly at the apical cell membrane, and 5) resorption and subsequent proteolytic digestion of the iodothyronine-rich Tg by the thyrocytes and the release and secretion of the iodothyronines[1,2] (see figure 1).

1.1.1 Concentration of iodide by the thyroid gland; iodide transport

The dietary iodine is quickly resorbed and spread through the body. Two third is cleared by the kidney and one third by the thyroid[11].

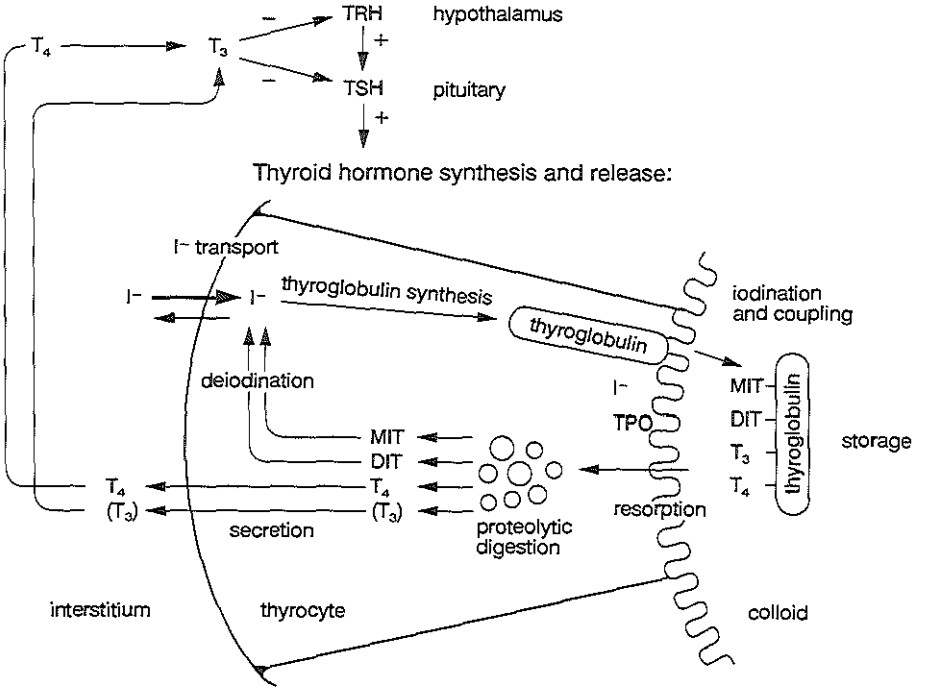

Figure 1. The sequence of events leading to thyroid hormone synthesis and release. Regulation via TRH and TSH

The thyrocytes internalize the iodine as iodide via an active transport process using an iodide pump[12], which is located in the basolateral membrane of the thyrocytes[13]. This iodide transport is dependent on a Na^+/K^+-ATPase, which exchanges 3 Na^+ ions for 2 K^+ ions[14]. The iodide is cotransported with Na^+ via a specific carrier protein[15]. In TSH stimulated thyrocytes the activity of the iodide pump can reach such activation that the iodide concentration in the cell is 500 times higher than the plasma iodide concentration. The active iodide transport can be blocked by the anion perchlorate (CLO_4^-) through a competitive binding process. CLO_4^- is concentrated in the thyrocytes and decreases the iodide influx, while increasing the iodide efflux.

Once internalized, the iodide is transported, or it diffuses to the apical pole of the thyrocyte. Mainly in the extracellular follicular lumen iodide is coupled to tyrosine

residues in the storage protein Tg, but intracellular locations of the iodination system have also been proposed[16]. The coupling process is catalyzed by the enzyme thyroid peroxidase (TPO) in the presence of H_2O_2, which is generated by a NADPH-dependent transmembrane enzyme complex (see later).

1.1.2 Thyroglobulin

After translation of the mRNA for the Tg molecule in the rough endoplasmic reticulum (rER) several post-transnational modifications like glycosylation[17], sulfatation[18] and phosphorylation[19,20] occur before the large protein Tg, transported in vesicles, encounters the apical membrane of the thyrocyte. The complete Tg molecule is a glycoprotein of 660 kD, consisting of two identical subunits of approximately 2800 aminoacids each[21]. At the apical thyrocyte membrane the tyrosine residues of Tg are iodinated, which process is catalyzed by TPO. Before the tyrosyl residues of the Tg molecule can be iodinated, Tg, iodide, TPO and H_2O_2 must be present at the same time and at the same location (the apical thyrocyte membrane).

1.1.3 Thyroid peroxidase and the related myeloperoxidase

TPO is a glycosylated transmembrane-bound enzyme with its COOH-terminal end projecting into the cytoplasm of the thyrocyte and the active site projecting into the follicular lumen[22]. Human TPO and human myeloperoxidase (MPO), the latter enzyme present in mononuclear phagocytes, have diverged from a common ancestral gene, since their DNAs and their amino acid sequences show a 46% and 44% homology, respectively[23]. MPO plays a major role in the H_2O_2 dependent anti-microbial system during the respiratory burst of activated mononuclear phagocytes. It has been demonstrated that the enzyme TPO is oxidized by H_2O_2. However, the precise nature of the transmembranous H_2O_2-generating system in the thyroid is still unknown. It has been shown that the production of H_2O_2 is NADPH-dependent[24,25] and that it transfers electrons across the membrane in order to generate H_2O_2 from $2H^+$ and O_2.

A similar NADPH oxidase is present in mononuclear phagocytes which is activated

by certain stimuli such as binding of bacteria to the cytoplasm membrane[26]. During the respiratory burst the NADPH oxidase produces superoxide and subsequently H_2O_2 and $OH^{\bullet}$. H_2O_2 and the radical products are capable of destroying the bacteria. Granules of the mononuclear phagocytes release MPO, which in the presence of H_2O_2 and halide ions (such as Cl^- and I^-) produce further active radicals such as hypochlorous acid, ClOH, or hypoiodous acid, IOH (in which the iodonium ion is I^+).

1.1.4 Iodination of tyrosyl residues of thyroglobulin and coupling of monoiodotyrosine and diiodotyrosine residues

The enzyme TPO is oxidized by H_2O_2, resulting in the formation of Compound I, which subsequently oxidizes iodide. This leads to the formation of the iodonium ion (I^+)[27] that reacts with H_2O to form hypoiodite (IO^-) probably via the formation of hypoiodous acid (IOH)[28]. These reaction products, that are still bound to the enzyme TPO, react with the tyrosyl residues of Tg to form monoiodotyrosine (MIT) and diiodotyrosine (DIT) residues. Compound I is reduced to the native form concomitantly with the iodination of Tg. The coupling of one MIT and one DIT or two DIT residues is catalyzed by the enzyme TPO, which leads to the formation of T_3 and T_4, respectively, bound to Tg[2]. *In vivo* coupling of MIT and DIT residues can lead to 1-2 molecules T_3 or T_4 bound to the Tg dimer[29]. The iodinated Tg molecule is stored in the follicular lumen (colloid).

The anti-thyroid drug methimazole (MMI), which is used to treat hyperthyroidism, inhibits TPO by competing with its active thioureylene group with iodide for the enzyme TPO[30]. It has been shown that the action of MMI is not restricted to the thyroid enzyme TPO, but that it also inhibits peroxidase systems of other cell types[31].

1.1.5 Resorption and lysosomal degradation of iodinated thyroglobulin

Upon TSH stimulation, the endocytosis of Tg is stimulated via a) macropinocytosis and the formation of pseudopods into the colloid, b) via fluid-phase micropinocytosis[32] and c) receptor mediated micropinicytosis[33]. The endocytosed iodinated Tg is transferred to the lysosomes of the thyrocytes for proteolytic cleavage and thyroid hormone release. Selective cleavage of Tg by the endopeptidases

cathepsin D, B, and L is followed by a further degradation of the iodinated Tg molecule by exopeptidases[34]. The early degradation process leads to the release of T_3 and T_4 and further proteolysis results in the total degradation of Tg. The non-secreted iodotyrosines are deiodinated intracellularly by the enzyme iodotyrosine deiodase and the free iodide can be reused. T_3 and T_4 are secreted at the basolateral surface of the thyrocyte via an unknown mechanism, probably involving thyroid hormone carrier proteins[35].

1.1.6 Regulation by the dietary iodine of thyroid hormone synthesis and release

Excessive iodine diet

Besides the regulation by TSH, an autoregulation of the thyroid exists particularly in respect to the dietary iodine intake. A prominent and *acute* increase of the dietary iodine intake results in an acute but transient decrease of the iodide transport mechanism[36] and hence in a decrease in the iodination of Tg, leading to a decreased iodothyronine formation and thyroid hormone secretion. This phenomenon is known as the Wolff-Chaikoff effect[37]. When a high level of plasma iodide is maintained, the inhibitory effects of iodide disappears and iodothyronine formation is restored (escape from the Wolff-Chaikoff effect). The precise mechanisms causing this escape are still unknown. When the level of plasma iodide falls below a critical level (20-30μg/ml), iodothyronine formation is also restored[38].

The effects of a *chronic* excessive dietary iodine intake are largely unknown. Under most circumstances, overt abnormalities of thyroid function have not been observed in normal individuals receiving chronic iodide therapy but subtle changes do occur. A small, significant decrease in the serum concentrations of T_4 and T_3 with a small rise in serum TSH have been observed[39,40]. Changes are usually within the normal range for the various parameters evaluated.

Deficient iodine diet

During iodine deficiency the iodide extraction from the blood by the thyrocytes is more efficient due to an increased activity of the iodide pump. This increased activity

is partly due to a stimulatory effect of TSH[41] and partly to an autonomous control mechanism by the thyroid itself[42]. The iodide extraction from the blood is negatively correlated with the anorganic iodide plasma concentration, resulting in a constant iodide uptake by the thyrocytes per time period. However, complete compensation via the above described mechanisms is not reached when the dietary iodine intake is too low.

Besides an increased iodide clearance from the plasma, the iodide metabolism is changed during iodine deficiency. The intracellular iodide taken up by the thyrocytes or intracellular released during the degradation of MIT and DIT residues (see above) is very efficiently reused, resulting in a minimal iodide leakage. Furthermore, the iodide ions recently bound to tyrosyl residues in the Tg molecule are also the first ones to be used for the secretion of thyroid hormones ("last come - first served"). This aspect of the iodide metabolism is increased during iodine deficiency and a faster release of thyroid hormones occurs[43].

Additionally, the degree of iodination of the Tg molecule is lower during iodine deficiency, which leads to more MIT than DIT residues and thus to more T_3 than T_4. In this way an enhanced synthesis of the biologically active T_3 at the expense of the production of the prohormone T_4 occurs, thereby allowing an efficient usage of the small amounts of iodide available with an optimal biological potency per Tg molecule[44].

The above described compensatory mechanisms of the thyroid are able to maintain euthyroidism during relatively mild iodine deficiency. Only during more severe forms of iodine deficiency these mechanisms will fail and hypothyroidism will be the result.

Chapter 1.2

THE ACTION OF THYROID HORMONES AND THYROID HORMONE METABOLISM

1.2.1 The action of thyroid hormones

Thyroid hormones play a key role in the regulation of metabolic processes involved in temperature adaptation and calorigenesis[45]. Furthermore, a lack of thyroid hormones in fetuses causes growth retardation and a hampered neurological and mental development. In humans, over 99% of the circulating T_4 and T_3 is bound to plasma proteins: 75% to thyroxine-binding globulin (TBG), 15% to thyroxine-binding prealbumin (TBPA) and 10% to albumin[46,47,48]. Although thyroid hormones are hydrophobic molecules, they do not enter cells by simple diffusion. Cellular uptake of thyroid hormones is an active process, mediated by specific, sodium and energy dependent carrier systems located in the cell membrane[49,50,51]. Since only nonprotein-bound thyroid hormone is taken up by the cells, the free iodothyronine concentration in serum determines hormone transfer to the cells. Furthermore, transfer of T_3 from the cytoplasm to the nucleus may also be a specific and energy-dependent process, determining the hormone levels at the nuclear T_3 receptor site[52].

By binding to nuclear receptors, T_3 exerts its effect at the transcriptional level[52,53,54,55,56]. It functions as a signalling substance by regulating the transcription of certain T_3-responsive genes[57] (most notably the c-erbA proto-oncogenes[58]). Cellular responses to T_3 hence depend - amongst other factors - on the bioavailability and concentration of T_3 within the nucleus and cytoplasm, which is regulated by different mechanisms.

Apart from the exchange of T_4 and T_3 between tissues and plasma, the bioavailability of T_3 also depends on the intracellular thyroid hormone metabolism. The major pathways in this intracellular thyroid hormone metabolism are: 1) removal of iodine substituents by deiodination (conversion of T_4 into T_3 or rT_3), 2) conjugation of the phenolic hydroxyl group of the iodothyronines with glucuronic acid or sulfate, 3) oxidative deamination and decarboxylation of the alanine side chain and 4) ether link cleavage[59,60]. Except for the conversion of T_4 into T_3 all other pathways lead to inactivation of thyroid hormone.

1.2.2 Deiodination

The bioinactive prohormone T_4 can be transformed into the bioactive thyroid hormone T_3 through elimination of an iodide from the phenolic outer ring. In contrast to this activation step, an inactivation through deiodination can also occur, through deiodination of the inner phenolic ring. This leads to the formation of reverse T_3 (rT_3), a compound without hormonal activity. Like T_4, T_3 is inactivated by inner ring deiodination, which yields 3,3'-T_2 (see figure 2, 3,5-T_2 and 3',5'-T_2 are minor metabolites). Further stepwise deiodination results via T_1 intermediates in the formation of thyronine (T_0), the noniodinated backbone molecule of thyroid hormones.

The deiodination reactions are catalyzed by at least 3 different iodothyronine-deiodinating enzymes.

The type I iodothyronine deiodinase is most abundant in the endoplasmic reticulum of liver, the plasma membrane of kidney and membrane fractions of the thyroid. It is capable of deiodinating the inner ring as well as the outer ring of iodothyronines and is largely responsible for the production of plasma T_3 as well as clearance of plasma rT_3 (see figure 2).

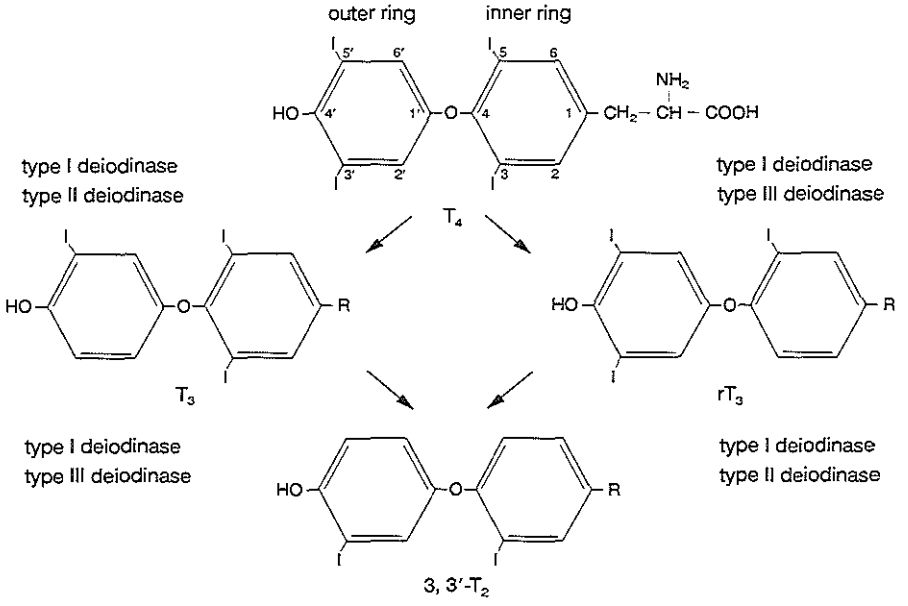

Figure 2. Deiodination of thyroxine (T_4).

The type II iodothyronine deiodinase acts only on the outer ring and is present in pituitary, brown adipose tissue and the central nervous system. This type II enzyme is important for the local supply of T_3 (see figure 2).

The type III enzyme is an inner ring deiodinase and catalyzes conversion of T_4 to rT_3 and of T_3 to 3,3'-T_2 and is mainly found in brain and skin (see figure 2).

1.2.3 Conjugation, oxidative deamination and decarboxylation, and ether-link cleavage

The second important pathway in the metabolism of thyroid hormones is *conjugation* of the phenolic hydroxyl group with glucuronic acid or sulfate (see figure 3). This reaction increases the water-solubility of lipophilic substances and facilitates their excretion in bile and urine. Furthermore, type I deiodination of T_3 is enhanced after sulfatation[61].

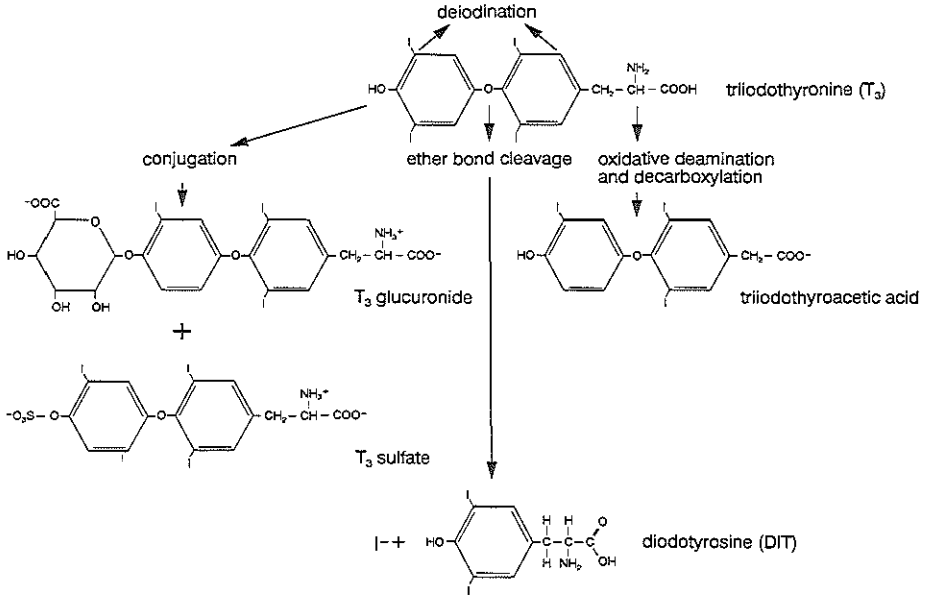

Figure 3. Metabolic pathways of triiodothyronine (T_3).

Transformation of the alanine side chain of an iodothyronine and subsequent conversion by *oxidative deamination and decarboxylation* leading to the corresponding iodothyroacetic acid derivative is a relatively minor pathway in the normal iodothyronine pathway and occurs in the liver, kidney and brain (see figure 3). Iodothyroacetic acids are metabolized via the same deiodination and conjugation pathways as the normal iodothyronines. Although the iodothyroacetic acid derivatives can be found in tissues and in the circulation, the reason for oxidative deamination and decarboxylation of iodothyronines still remains unclear.

Cleavage of the diphenyl ether link between the two phenolic ring structures of the thyronine molecules requires H_2O_2 and occurs in the granular fraction (which contains MPO) of activated phagocytes during the respiratory burst. Activated leukocytes use T_4 as substrate and DIT and iodide are released (see figure 3). This is a minor pathway by which iodothyronines are metabolized.

Chapter 1.3

THE REGULATION OF THYROID GROWTH. EFFECTS OF DIETARY IODINE

The last few decades many studies have been focussed on the mechanisms of the regulation of thyroid growth under physiological as well as pathological conditions. However, many controversies and discrepancies with regard to the nature and action of growth factors have arisen due to the use of different investigative *in vitro* and *in vivo* models and different end-point measurements. These models and end-point measurements include a whole variety ranging from biochemical techniques (for instance the preparation and use of cell fractions for binding studies and growth factor receptor assays) and cellular biological techniques (for instance the measurement of ^{3}H-thymide incorporation into DNA of primary cell cultures and long-term established cell lines of human or animal origin) to *in vivo* approaches (for instance the histological effects seen in thyroid tissue transplants in nude mice).

The regulation of thyroid cell growth was once a classical example of the concept one hormone - one cell type - one intracellular secondary messenger with its pleiotypic effects. Nowadays it is considered to be the result of a complex network of cross-linked regulatory steps in which extracellular and intracellular signal molecules act on their receptors to turn other pathways on or off. Such regulatory networks differ from one cell type to another and, for a given cell type, from species to species. Taken this into account, many apparent discrepancies in the literature can be explained.

1.3.1 The growth effects of thyrotropin or TSH

The main and best known regulatory network involves thyrotropin (TSH) stimulation of plasma membrane adenylate cyclase (see later). By activating cyclic AMP-dependent protein kinases, the main functions of the thyroid gland, namely the iodination of Tg and iodothyronine formation, i.e. the synthesis of thyroid hormones, and the uptake of Tg and its hydrolysis, i.e. the secretion of thyroid hormones are enhanced. Also the proliferation of thyrocytes is stimulated by TSH, although depending on the *in vivo* or *in vitro* system used.

Extracellular signals such as hormones and growth factors bind selectively to specific receptors and are transformed through intracellular systems into second messengers. When these transducing systems are activated by the binding of hormone to receptor, there is an increased production of intracellular second

messengers that act largely by stimulating protein phosphorylation. Most normal cells have at least two classes of transducing systems via membrane receptors. The transducing systems activated by TSH in the normal thyroid gland are the adenylate cyclase - protein kinase A system and the phosphoinositide (PI) turnover protein kinase C calcium system (phospholipase C system)[62,63,64,65,66]. TSH stimulates the adenylate cyclase and the phospholipase C system by first binding to the TSH receptor on the cell surface. In both transducing systems the TSH signal is transmitted through guanyl nucleotide regulatory proteins (G proteins) that link the TSH receptor to the catalytic unit of the enzymes adenylate cyclase and phospholipase C. The binding of TSH to its receptor alters the conformation of the G proteins in such a way that GTP is able to bind to the G proteins. In the GTP-bound state the G proteins are able to stimulate the activity of adenylate cyclase or phospholipase C. Once the G proteins have activated their effector molecules, the intrinsic GTPase activity converts GTP to GDP resulting in an inactive G protein that is then able to be reactivated by interaction with the hormone-receptor complex.

Adenylate cyclase converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) that activates protein kinase A by binding to the regulatory subunit, releasing the active subunit. Protein kinase A subsequently phophorylates cellular proteins.

The activated enzyme phospholipase C hydrolyses phosphatidylinositol 4,5-biphosphate (PIP_2) and generates two intracellular signalling molecules: inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 causes the release by endoplasmic reticulum of stored Ca^{2+} which results in a rise of the intracellular Ca^{2+} concentration. An increased intracellular Ca^{2+} concentration leads to cell proliferation in some cell types by yet unknown mechanisms. DAG activates the protein kinase C, which (like protein kinase A) phosphorylates cellular proteins.

Both receptor-transducer systems rapidly converge on several events such as the phosphorylation of tyrosine residues of proteins and both systems are capable to give rises in c-fos and c-myc protooncogene mRNA. These early events are assumed to be necessary for growth stimulation, but a causative relationship with late commitment to DNA replication remains unclear[67].

1.3.2 The effects of other growth factors such as Epidermal Growth Factor and Insulin-like Growth Factors

Epidermal growth factor (EGF) is a well known growth factor and plays a role in the regulation of thyroid growth and fuction[68,69,70,71,72,73]. EGF exerts its effects via protein tyrosine kinase activity. The EGF receptor is like the TSH receptor located on the basolateral cell surface of the thyrocyte[74,75] and TSH synergizes with EGF in growth stimulation by mechanisms involving upregulation of the EGF receptor by TSH[76].

Insulin-like growth fators (IGF-I, IGF-II) and insulin have been shown to be involved in thyroid growth[77,78]. Insuline has a mitogenic action on the thyroid, besides the stimulation of metabolism[79]. A simultaneous stimulation of human thyrocytes with TSH and IGFs resulted in a more than additive effect on thyrocyte proliferation. However, the effect of these growth factors depend on the model system used.

TSH, EGF and IGFs are thus important thyrocyte growth stimulators. The precise second messenger systems triggered by these growth factors and their interrelationships are not completely unraveled. Further investigations are necessary to elucidate the complex interactions of growth regulation in human thyrocytes.

1.3.3 The effects of iodine on thyroid growth

Iodine deficiency leads in the majority of individuals to thyroid growth (endemic goitre). Iodine depletion has been considered by some to be directly responsible for thyrocyte growth by a direct antimitotic effect of iodine on thyrocytes[80]. The proliferative effect of EGF on thyroid follicles in suspension culture inversely depends on the iodine supplementation of these follicles, indicating that thyroid growth is mainly inversely correlated with the iodine content of the thyroid gland. In other words mainly during iodine deficiency thyroid growth is observed. However, goitre formation during excessive dietary iodine intake has also been described[81].

Endemic goitre, of sometimes enormous size, due to iodine deficiency is according to most investigators not solely due to the rises in TSH observed in these patients. One should keep in mind that TSH and EGF act synergistically on thyroid growth. Other paracrine growth factors like IGF and cytokines may also be involved in this growth regulation.

An intimate role of the immune system in regulating iodine-deficient[82,83,84] (and iodine excess[81]) goitre formation has been suggested by us and others before. This thesis is particularly meant to study the relationship between dietary iodine intake, thyroid metabolism, and thyroid autoimmunity.

Chapter 1.4

THYROID AUTOIMMUNE RESPONSES

In healthy individuals some autoreactivity towards thyroid cells and other body components is nowadays considered to be a normal process controlled by several suppressor mechanisms. Malfunction of these suppressor mechanisms may result in autoimmune *disease*. A considerable proportion of the thyroid *diseases* in industrialized countries are due to aberrant immune reactions towards self-antigens of the thyroid.

1.4.1 Tolerance to thyroid autoantigens. Defects in thyroid autoimmune disease[85]

As in normal immune reactions, T lymphocytes and B lymphocytes play an important role in thyroid autoimmunity.

$CD8^+$ T lymphocytes, which recognize the self-antigens only when presented on MHC class-I molecules, are primarily cytotoxic (Tc) for thyrocytes.

$CD4^+$ T lymphocytes recognize the self-antigens only in the context of MHC class-II molecules and these lymphocytes can be divided into two subsets: The T helper 1 (Th_1) subset produces cytokines such as interferon-gamma (IFN-γ), which is capable of activating macrophages to become auto-aggressive, and Interleukin-2 (IL-2) which stimulates the proliferation of $CD8^+$ cytotoxic T cells. The T helper 2 (Th_2) subset secretes cytokines such as IL-4 and IL-5; these cytokines assist the B lymphocytes in the production of autoreactive antibodies of IgG isotype.

Normally autoreactivity towards thyroid antigens is controlled and self-tolerance is acquired by mechanisms that delete or inactivate auto-reactive T and B cell clones.

Deletion of large numbers of autoreactive T cytotoxic, Th_1 and Th_2 cells occurs within the thymus during the differentiation of prothymocytes to mature T cells[86]. T cells with high affinity towards self-determinants presented on MHC class-I and MHC class-II positive cells of the thymic micro-environment are effectively eliminated via a process of programmed cell death (apoptosis)[87]. Self-reactive B cells can also be deleted as was first proposed by Burnett[88]. Premature contact of self antigens by an immature B cell may prevent it from developing into a clone of antibody-forming cells. However, not all self reactive clones may be deleted which is indicated by the presence of Tg-reactive T cells in the peripheral blood of normal rats[89].

A second mechanism to maintain self-tolerance is the generation of active *immunosuppressor systems*[90]. These suppressor circuits are not only formed by $CD8^+$ T suppressor (Ts) cells, but also by $CD4^+$ T lymphocytes and by B lymphocytes. These may operate in (amongst others) an idiotype-anti-idiotypic network. In every healthy individual a balance exists between autoreactive effector and suppressor mechanisms in favor of the suppressor systems. It has been suggested that such a controlled network of autoreactive T lymphocytes is actually an advantage and facilitates the recognition of foreign invaders: foreign invaders are thought to share antigenic structures with evolutionary well conserved structures on our own cells[91]. With regard to thyroid self-antigens antigenic structures homologous to the TSH-receptor are present on Yersinia and Klebsiella bacteria.

In the animal models for spontaneous thyroid autoimmune disease (the OS-chicken, the BB-rat and the NOD-mouse) intrathymic clonal deletion and/or the induction of active suppressor systems is disturbed or even absent. This is due to maturation defects in the thymic micro-environment and defects at the post-thymic level[92,93,94,95,96].

When autoreactive T and B cells have escaped clonal deletion and when suppressor systems are insufficient a third control mechanism should come into operation, namely the process of *T cell anergy*[97,98]. When an autoantigen is presented to autoreactive effector T cells on *not-professional* antigen presenting cells, the T cells will become anergic. The induction of the anergic state is supposed to be due to the lack of provision of sufficient second signals, such as the induction of various adhesion molecules and the production of various cytokines, for T cell stimulation to occur. The recently discovered principle of T cell anergy suggests that the earlier found MHC class-II positivity of the thyrocytes in classical thyroid autoimmune disease may earlier be "suppressive" than being a stimulus for the thyroid autoimmune reaction[99]. The relatively late appearance of MHC class-II molecules on thyroid cells in the disease process after the appearance of thyroid autoantibodies in the circulation supports such a view[100].

The existing control over non-reactivity to thyroidal antigens can be broken when thyroid autoantigens are presented to the immune system on *professional* antigen-presenting cells capable of providing activating signals for T and B cells. Knight *et al.*[101] have shown that as few as 10^5 dendritic cells (the *professional* antigen presenting cells par excellence) pulsed with Tg were effective in inducing a thyroiditis after cell transfer to normal non-autoimmune BALB/c mice.

1.4.2 The thyroid autoimmune diseases: Hashimoto's goitre, primary hypothyroidism (atrophic myxedema) and Graves' disease

The major criteria of 'autoimmune disease' are defined as follows:

1. the presence of IgG autoantibodies and/or autoreactive T effector lymphocytes towards antigens of the affected organ or organ systems, this presence correlating to functional and/or morphological abnormalities of the affected organ or organ systems,
2. the possibility to induce these functional and/or morphological abnormalities in organs or organ systems by the transfer of the IgG autoantibodies and/or the autoreactive T lymphocytes.

According to these criteria only three thyroid diseases can presently be considered as autoimmune thyroid diseases; namely Hashimoto's goitre, primary hypothyroidism (atrophic myxedema) and Graves' disease.

Thyroid autoimmune reactions, however, do occur in several other thyroid abnormalities, such as sporadic and endemic goitre (see later), hyperthyroid nodular goitre (Plummer's disease), congenital hypothyroidism and endocrine ophthalmopathy. Further investigation is necessary to reveal whether these disorders can be included in the spectrum of autoimmune thyroid diseases.

The involvement of T lymphocytes in thyroid autoimmune responses is easily visualized in the histopathology of the various thyroid autoimmune reactions. Two types of lymphoid cell infiltrations can be discerned in thyroid glands affected by a thyroid autoimmune disease:

1. *Destructive lymphoid cell infiltrations*[102]. These infiltrations do not show a recognizable organization, and consist of a mixture of predominantly T cells and macrophages in destroyed and disrupted thyroid follicles. The function of the T cells in this type of infiltration is on the one hand cytotoxicity ($CD8^+$, T cytotoxic cells) and on the other hand the activation of the macrophages to become cytolytic (by $CD4^+$, Th_1 cells through the production of IFN-γ). The destructive lymphoid cell infiltrations are predominantly found in Hashimoto's goitre.
2. *Highly organized accumulations of lymphoid cells*[103]. These infiltrations are not destructive and are primarily found in Graves' disease and sporadic goitre adjacent to stimulated thyroid tissue, but can also be found in Hashimoto's goitre.

These lymphoid cell infiltrations represent an intrathyroidally developed lymphoid tissue with a similar architecture to that of mucosa-associated lymphoid tissue. Thyroid associated lymphoid tissue (TALT) is composed of T cell zones, B cell follicles and plasma cells in the periphery of the tissue. The plasma cells can also be observed in cord-like structures radiating from the lymphoid tissue in between the thyroid follicles. TALT is involved in thyroid autoantibody production[100]. The T cell zones of TALT consist of $CD4^+$ and $CD8^+$ lymphocytes in a ratio of 2-3:1. The function of these T lymphocytes is probably to regulate immune responsiveness and probably mainly involve Th_2 cells and other regulatory T and B lymphocytes.

Hashimoto's goitre

Upon histological examination Hashimoto's goitre forms a spectrum of inflammatory abnormalities ranging from mild lymphoid cell infiltration (juvenile form, difficult to distinguish from euthyroid sporadic goitre) via the most prevalent classical oxyphil variant (females of 30-40 years of age, dense lymphoid cell accumulations, areas of destructive thyroiditis, regrowth of follicles) to the fibrous form (older age, predominantly fibrosis, mainly infiltration with plasma cells).

Over 90% of patients with Hashimoto's disease show high levels of serum antibodies to thyroidal antigens. The mechanisms of destruction mediated by these antibodies are a) antibody-mediated direct cytotoxicity, b) antibody-dependent cell-mediated cytotoxicity (ADCC), and c) immune complex mediated reactions.

Although autoreactive T lymphocytes are more important in thyrocyte destruction, thyroid autoantibodies can nevertheless be considered as good markers of the destructive autoimmune process and are therefore diagnostically used.

The goitre of Hashimoto's disease is not only caused by lymphoid cell infiltrations but also by the regrowth of destroyed thyroid follicles. This regrowth is induced by a raised plasma TSH (compensation for the fall in thyroid hormones, subclinical hypothyroidism), or by thyroid growth stimulating antibodies (see later) that may further contribute to the regrowth and restimulation of the remaining thyrocytes. In a proportion of the Hashimoto's goitres the regeneration processes ultimately fail. The immune reaction finally leads to a total destruction of the gland with signs and symptoms of overt clinical hypothyroidism.

The first thyroid autoantigen discovered in relation to Hashimoto's goitre was Tg. Roitt and Doniach (1956) used a precipitation reaction in agar for the detection of these autoantibodies. In this assay only very high titres of Tg-autoantibodies could be detected. Thereafter, other detection methods have been developed such as the indirect immunofluorescence (IIF) assay, in which sections of thyroid tissue are exposed to the serum of patients. If autoantibodies towards thyroidal antigens are present in the serum, these antibodies bind to the thyroid antigens in the section. This can be visualized in fluorescence microscopy using a fluorescent-labeled animal antibody to the human antibodies (figure 4).

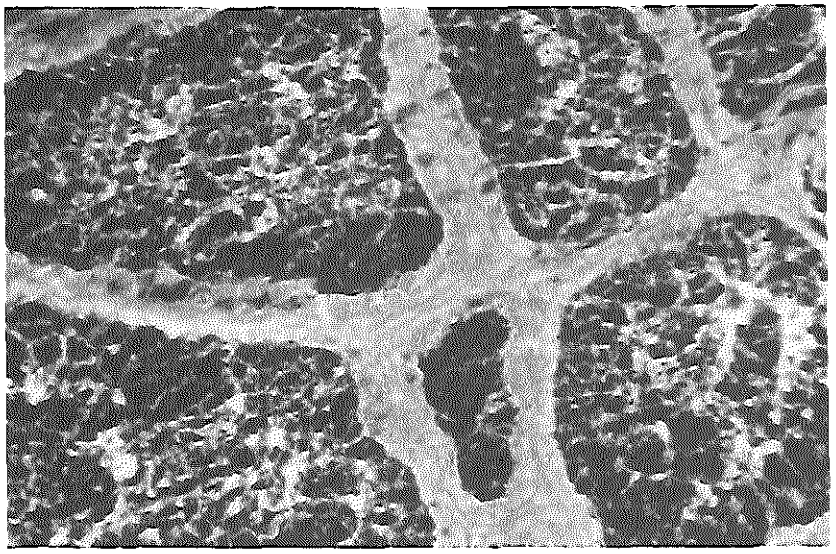

Figure 4. Anti-colloid antibodies visualized by indirect immunofluorescence using porcine thyroid sections (see text for more detail).

Thyroid autoantibodies discovered using IIF were directed towards colloidal components (mainly Tg) and towards the cytoplasmic microsomal fraction ('cytoplasmic' or 'microsomal' antibodies). Biochemical characterization revealed that the microsomal antigen was also expressed on the cell surface of living thyrocytes. This antigen later proved to be the enzyme TPO[104], which plays a key role in

oxidative reactions during the iodination of Tg (see before).

Although Tg and TPO are the most well-known thyroid self-antigens, other thyroidal antigens which can be the target of a thyroid autoimmune reaction (and to which autoantibodies have been found) are: the thyroid hormones[105], an undefined second colloid antigen (detected in IIF[106]), and a few poorly defined cell membrane proteins detected in Western blot analysis such a 64 kD cell membrane protein, occurring in thyrocytes and eye muscle cells[107].

Primary hypothyroidism or atrophic myxedema

A large number of patients with serum antibodies to TPO and Tg develop hypothyroidism and thyroid atrophy without a history of Hashimoto's goitre. This disorder is called primary hypothyroidism or atrophic myxedema. In general, patients suffering from this disorder have lower serum antibody titres to TPO and/or Tg in comparison to Hashimoto's goitre patients. The rapid development of the thyroid atrophy despite the excessively high plasma levels of TSH is presently explained by the additional existence of antibodies directed to the TSH receptor[108] that block the growth and hormone synthesis potential of the raised plasma TSH[109] (see below).

The disease "primary thyroid atrophy" mainly strikes at older age, but it can also affect younger women. If these younger women become pregnant, it is possible that they transfer the thyroid blocking antibodies to the neonates via the transplacental passage[110]. This may result in a transient or even permanent form of congenital hypothyroidism, the so-called familial congenital hypothyroidism (CHT). This neonatal disease constitutes approximately 1-5% of the CHT cases detected in infant screening programs in the USA and Western Europe[111].

Graves' disease

Besides antibodies directed to the TSH-receptor that block the action of TSH, there are TSH-receptor antibodies capable of stimulating thyroid cells and mimicking the action of TSH: upon binding of such antibodies to the TSH-receptor they often induce cAMP responses which result in the metabolic stimulation of the thyrocyte and

the production of thyroid hormones. The hyperthyroidism of Graves' disease is caused by these bioactive TSH-receptor antibodies which is indicated by the transplacental passage of such autoantibodies from Graves' mothers, resulting in a transient state of thyrotoxicosis in the newborn.

The Graves' autoantibodies capable of stimulating the hormone synthesis of thyrocytes are collectively called Thyroid stimulating Antibodies (TsAbs). The evidence that these TsAbs are directed to the TSH-receptor was presented in studies in which TsAbs positive Ig-fractions of Graves' patients exerted a dose-dependent inhibition of binding of radioactively labeled TSH to guinea pig and human thyroid cell membrane fractions[112]. The term Thyrotropin-Binding Inhibitory IgG (TBII) has been introduced for the IgGs exerting an effect in such TSH-binding assays. Presently, a kit is available to test for TBII on a routine clinical basis (Henning, Berlin). A new approach is the use of CHO-cells transfected with the TSH-receptor to perform a TBII-assay as well as a cAMP stimulation assay[113].

Although many thyroid-directed antibodies (Abs) can bind to the TSH-receptor (and are thus called TBII), only a few are also capable of actually stimulating the thyrocytes (TsAbs). In large groups of Graves' patients a limited correlation between the TBII assay, cAMP stimulation and thyroid hormone production was found. Some patient IgGs were positive in the TBII assay while having little or no activity in the cAMP assay. Since the TBII assay measures only the binding of the antibody to the TSH-receptor, it may give misleading results when a receptor antibody exhibits mainly blocking (antagonist) rather than stimulating (agonist) activity (figure 5, see also before with primary hypothyroidism). The discrepancy in the various assays could thus mean that the TSH-receptor antibody population of Graves' patients is heterogeneous and composed of a variable mixture of agonists, antagonists and perhaps non-agonist receptor antibodies. Conclusive evidence for the occurrence of functionally heterogeneous receptor antibodies within one individual patient has indeed been provided[114].

The basically polyclonal autoantibody response in Graves' disease is consonant with studies of individual patients revealing a changing relationship over time between the TBII, the TsAbs activity and the degree of thyrotoxicosis. In addition, the occasional emergence of hyperthyroidism in a patient who previously had well-documented primary hypothyroidism can also be explained[115].

Since the TSH-receptor antibodies of Graves' patients differ in respect to their functional effects, these antibodies will presumably also bind to different epitopes

within the TSH-receptor (see figure 5). Ongoing research in delineating the TSH-receptor epitopes may reveal to which epitopes the stimulating and the blocking antibodies are directed. The recent cloning of the TSH-receptor[116] is helpful in this research.

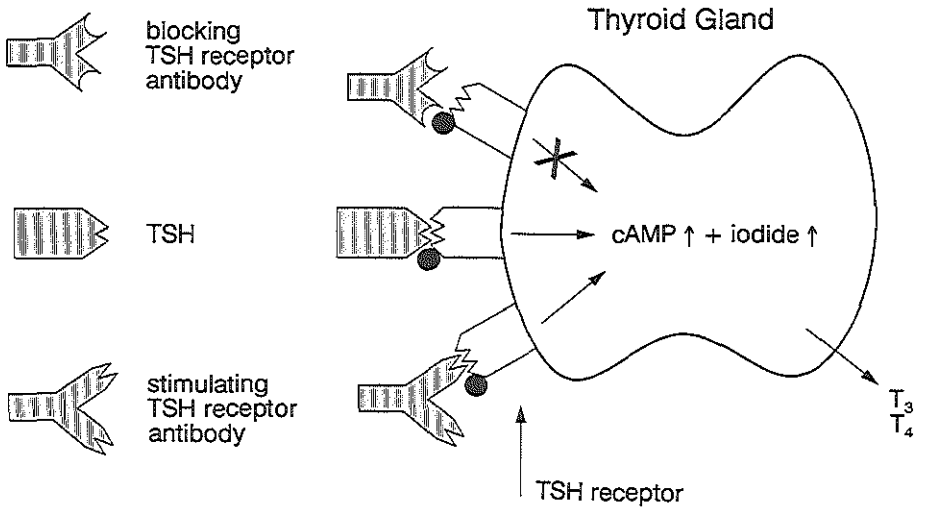

Figure 5. TSH-receptor antibodies (the so called TSH binding inhibiting immunoglobulins, TBII) can either have stimulating effects or blocking effects. The blocking antibodies probably bind to a different part of the TSH-receptor than the stimulating antibodies.

1.4.3 Thyroid growth stimulating antibodies and goitrous growth

Since 1980 it has also become clear that not only hormone production of thyrocytes can be stimulated or blocked by receptor antibodies, but also the proliferation of thyrocytes (goitre-induction). Deborah Doniach was the first to hypothesize that patients with euthyroid "colloid" goitres may possess circulating immunoglobulins (Igs) stimulating the growth of their thyrocytes. Such a pathogenic process would be analogous to that of Graves' disease where Igs stimulate thyroid hormone synthesis[117].

It lasted till 1980 before it proved possible to detect growth stimulating antibodies in euthyroid colloid goitre using a Cytochemical BioAssay (CBA) based on Feulgen densitometry; in this CBA system patient Igs stimulated the cells of guinea pig thyroid explants kept in organ culture to enter the S-phase. These *in vitro* findings suggested that the disease process of at least some of the patients with sporadic nontoxic goitre was autoimmune in nature and related to that of Graves' disease and Hashimoto goitre.

Since 1980 several investigators have provided additional evidence on the existence of Abs capable of stimulating the growth of thyrocytes *in vitro*. These Abs were found in large Graves' goitres, in Hashimoto's goitre and sporadic nontoxic goitre, but also in iodine deficient endemic goitre.

Description of the methods to detect thyroid growth stimulating antibodies

A CBA for detecting thyroid stimulating Abs was developed by Bitensky et al[118] and it is still the most sensitive method available. To measure growth stimulating Abs two other quantitative cytochemical techniques have been developed, one is based on nucleic acid cytophotometry (Feulgen-CBA) and the other on the measurement of glucose-6-phosphate dehydrogenase (G6PD) activity (G6PD-CBA).

The Feulgen-CBA involves the measurement of the amount of DNA in individual follicle-cell nuclei of a guinea-pig thyroid segment kept in organ culture and exposed to the putative growth stimulating Igs. The DNA measurement is performed by means of a microdensitometer or cytophotometer and the results are expressed by a computer as population histograms in which the nuclear DNA content is plotted against the cell number. The cell cycle forms the basis of the assessment and from the population histograms the percentage of cells in S-phase can easily be computed. The percentage of cells in S-phase reflects the proliferative state of the cultured thyroid segment and is thus a measure of proliferation induction. A detailed description of the methodology has been given elsewhere[119].

The other CBA for measuring DNA synthesis is the G6PD-CBA. G6PD is the first and rate controlling enzymatic step of the pentose-shunt pathway, which is the major source of ribose sugars needed for DNA synthesis[120]. It also provides much of the NADPH in the cytosol which is essential for many biosynthetic steps required for cellular growth. The total generation of NADPH from G6P and NADP by action of the

enzyme can be measured in the thyroid cells from the cultured guinea pig thyroid segments when the enzyme reaction is carried out in the presence of PMS. PMS transfers reducing equivalents directly from the generated NAPDH to the final acceptor neotetrazolium chloride which on reduction precipitates as a blue formazan. The amount of formazan is measured by microdensitometry. A detailed description of this methodology has been given elsewhere[121,122].

A good correlation exists between the outcomes of the Feulgen-CBA and those of the G6PD-CBA. Both CBA's detect TGAbs in about 90% of large Graves' goitres (> 40 gr) and in about 60-70% of euthyroid colloid goitres.

Because the CBA's are labour intensive more simplified methods for measuring TGAbs have been developed. Chiovato et al[123]., Schatz et al[124] and Wadeleux et al[125] have described methods in which ^{3}H-thymidine incorporation into reconstituted thyroid follicles in suspension culture was used as the growth parameter. These culture methods require less technical skill but are still labour intensive. A further drawback is that they are less sensitive in comparison to the CBA and only detect TGAbs in 70% of Graves' goitres and 10-35% of euthyroid colloid goitres.

The Fisher rat thyroid cell line-5 (FRTL-5)[126] is a continuous cell line in culture which can be used to measure cAMP stimulation induced by the classical TSAbs of Graves' disease patients. The assay system is nearly as effective as human thyroid-slice and cell systems. The most simple and direct method for detecting thyroid cell growth in FRTL-5 cells has been developed by Ealey et al[127,128] and is based on the enumeration of mitotic figures in the FRTL-5 cells. A mitotic arrest is induced during the endstages of a given culture by adding colchicine. The metaphase figures are visualized by staining and subsequently counted using an inverted light microscope.

Until shortly, FRTL-5 cell assays could only detect growth promoting antibodies in active Graves' disease, whereas Abs of sporadic euthyroid colloid goitre were negative. Wilders et al[83] have modified the mitosis arrest assay of Ealey et al[127,128] by cocultering Ig preparations of patients with a suboptimal dose of TSH to synergize the effects of the growth stimulating Abs. In this way they found 80% of sporadic nontoxic goitre patients positive for TGAbs. A similar assay approach has been used by Boyages et al[81].

Validation of the Feulgen cytochemical bioassay to detect thyroid growth stimulating antibodies in vitro

Criticism has been raised on the methods to detect TGAbs, their interpretations and the Ig-preparations used[129,130]. It has been argued that ^{3}H-thymidine incorporation into DNA may not reflect cell proliferation whereas the intensity of the Feulgen reaction product more likely measures the degree of unfolding of DNA than an increase in the quantity of DNA[129]. The modified FRTL-5 mitotic arrest assay developed by Wilders et al[83] avoids these technical pitfalls and directly measures cell proliferation by determining the percentage of cells in mitosis. To validate the Feulgen-CBA methodology a double blind study was carried out in which results obtained with a series of IgGs in the Feulgen-CBA were compared with those obtained in the "Wilders-modified" FRTL-5 mitotic arrest assay.

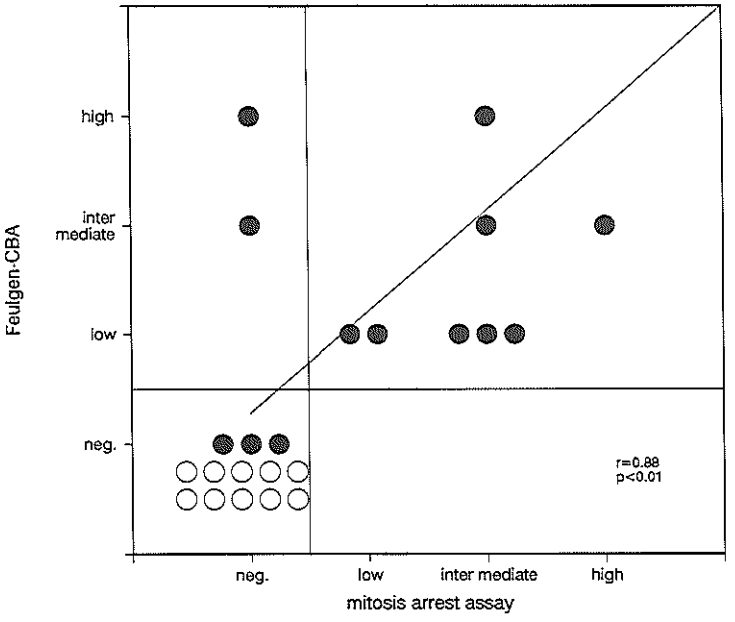

Figure 6. A comparison between the outcomes of the Feulgen-CBA and the FRTL-5 mitosis arrest assay (modification Wilders) stimulated with either 10 Ig-preparations of healthy individuals (open circles O) or 13 Ig-preparations of sporadic goitre patients (closed circles ●). Outcomes were scored "negative", or scored "low/intermediate/high positive" depending on the lowest amount of IgG needed to obtain a positive growth response in the assay (either > 5% of thyrocytes in S-phase or > 7% of FRTL-5 cells in mitosis).

Both assays gave almost identical results (see figure 6) thus showing the validity of the Feulgen-CBA to detect Abs stimulating mitogenesis *in vitro*. In fact the CBA proved to be more sensitive and detected TGAbs activity in three more sporadic goitre cases, while the assay needed 10 times less IgG to give a positive growth response.

The criticism that contaminations of the Ig preparations with growth factors other than TGAbs can be hold responsible for the observed growth effects[131] has now also been excluded in a series of experiments.

Firstly patients' TSH from the serum sample was removed before the preparation of Ig-fractions by pretreating the serum samples with a solid phase anti-TSH; this did not abolish the growth stimulating potential.

Secondly, protein-A sepharose column purification is used to prepare almost pure IgG fractions from patient serum; these fractions still contain TGAb activity. Furthermore, the growth promoting activity of the protein-A-sepharose purified IgG fractions can be neutralized by anti human IgG antibodies, whereas anti human EGF or anti human TSH have no effects[83].

These experiments provide ample evidence that it is IgG that is responsible for the observed *in vitro* growth effects. It has also been shown that a $(Fab)_2$ fragments prepared from a sporadic goitre IgG exerted the growth promoting activity in the Feulgen CBA in a fashion similar to that of the original IgG preparation[132].

Thyroid growth stimulating antibodies in sporadic nontoxic colloid goitre

To establish a role for TGAbs in the pathogenesis of sporadic colloid goitre, several studies have now been performed. At first we showed[133] a TGAb-positivity in 65% of patients using the Feulgen-CBA. Almost identical findings on the prevalence of TGAb in sporadic goitre were reported by McMullan and Smyth using the G6PD-CBA[122]. They found TGAb present in 68% of cases and they confirmed the earlier expressed view that the TGAb in sporadic goitre patients was less potent in comparison to the TGAb of goitrous Graves' disease patients.

Evidence for the presence of TGAb in sporadic goitre patients has also been given by Chiovatto et al[123] and Wadeleux et al[125], who found 10 and 35%, respectively, of sporadic goitre patients positive for TGAb using ^{3}H-thymidine incorporation assays on thyroid follicles in suspension culture.

Using the FRTL-5 assays, however, Igs of sporadic euthyroid goitre patients were

initially invariably negative for growth stimulation. To investigate this problem Wilders et al[83] modified the FRTL-5 mitosis arrest assay of Ealy et al[127,128] and tested the colloid goitre IgG preparations in the presence of a suboptimal dose of TSH. Stimulation of mitogenesis could now be detected in 9 out of 14 IgG preparations of patients with sporadic colloid goitre. Anti-human IgG abolished these growth promoting effects. Despite the TGAb activity, sporadic goitre patients do normally neither have TSH receptor Abs nor cAMP stimulating Abs in their serum. The discrepancy between the presence of growth stimulating effects and the absence of TSH receptor Abs and Igs stimulating cAMP strongly suggests that the TGAbs of sporadic goitre patients are not specific for the TSH binding site of the TSH receptor.

It has recently been shown by Wulffraat et al (to be published) that Abs directed against the IGF-1 receptor were present in 13 out of 14 patients with Graves' disease and in 10 out of 11 patients suffering from sporadic nontoxic goitre. This implies that apart from the TSH-receptor, other structures present on the thyrocytes, such as the IGF-1-receptor, may serve as targets for the TGAbs (figure 7).

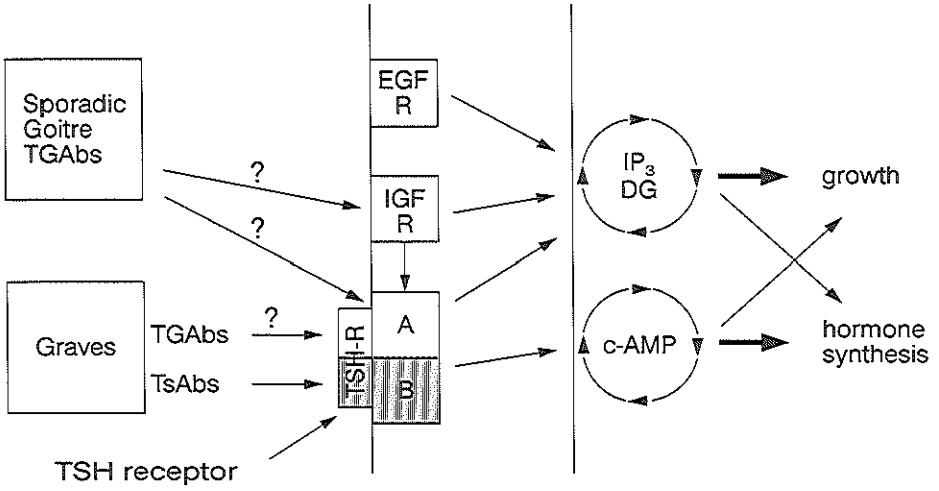

Figure 7. Thyroid stimulating antibodies (TsAbs) from Graves' patients bind to the TSH receptor, stimulate second messenger systems (cAMP, inositol triphosphate, IP_3 and diacylglycerol, DG) which results in thyroid growth and/or thyroid hormone synthesis. It is still not clear whether thyroid growth promoting antibodies (TGAbs) from Graves' patients or from sporadic goitre patients bind to special parts of the TSH receptor (separate from those involved in the stimulation of hormone synthesis) or to a growth receptor other than the TSH-receptor, for instance the Insuline-like Growth Factor receptor (IGF-R).

Thyroid growth stimulating antibodies in endemic goitre

Immunoglobulins capable of stimulating thyroid cell growth proved not only to be detectable in the serum of patients with sporadic goitre, but also in the serum of patients with iodine deficient endemic goitre.

Schatz et al[124] found 14% of endemic cases positive for TGAbs in the Giessen area in Germany, whereas Medeiros-Neto et al[84] reported a 50-60% positivity in cases from Brazil. In a recent study, Wilders et al[83] showed a growth promoting effect in 65 of 71 endemic goitre Ig preparations tested in their FRTL-5 mitotic arrest assay (91% positivity, Graz area). In her experiments the IgG preparations of 15 healthy controls living in an area of iodine sufficiency (Amsterdam) and of 18 Austrian subjects without an endemic goitre and without iodine deficiency did not stimulate FRTL-5 cell growth. The IgG samples from endemic goitre patients who reported a recent thyroid enlargement or who showed a postoperative recurrence of their goitre reached a maximal *in vitro* growth stimulation at relatively low concentrations of IgG. This indicates that such endemic goitre patients may have TGAbs with a higher growth promoting potency.

The mechanism by which the combination of a suboptimal concentration of TSH and endemic goitre TGAb synergize to promote the *in vitro* mitogenesis in FRTL-5 cells is still not understood, but it clearly shows that the TSH receptor needs to be stimulated before TGAb is able to fully induce FRTL-5 cell growth. It is tempting to speculate that such a combined action of TSH and TGAb will also play a role in endemic goitre formation *in vivo*. Already in 1968[134] it was reported that TSH has a stronger growth promoting action in *in vivo* conditions of iodine deficiency.

Chapter 1.5

THYROID AUTOIMMUNE RESPONSES AND DENDRITIC CELLS

Tolerance towards thyroid self antigens can be broken (see before). For the production of auto-antibodies and the expansion of autoreactive helper and cytotoxic T cells, an antigenic stimulation by so-called antigen-presenting accessory cells is required. Only a few antigen-presenting accessory cells are capable of inducing effective immune responses. These cells include MHC class-II positive B lymphocytes and macrophages, but the dendritic cell or veiled cell is amongst the accessory cells *the professional* antigen-presenting cell par excellence[135,136]. Dendritic cells expose a high density of MHC class-I and II molecules on their long cytoplasmic extensions or veils and actively seek contact with T lymphocytes. For the antigen-presentation and T cell stimulation the dendritic cell forms cell clusters with the lymphocytes and in this cluster formation various adhesion molecules are operative[137]. Apart from cluster formation in which a micro-environment is created suitable for antigen-presentation, the dendritic cell is capable of producing a series of cytokines which activate T and B lymphocytes[138]. The adhesion molecule interactions and the cytokine production provide enough second signal to make the dendritic cell the only cell capable of stimulating naive T cells to clonally expand[135].

Dendritic cells do not only occur in the T cell areas of spleen and lymph nodes as so-called interdigitating dendritic cells, but also in the skin as Langerhans cells and in the gut mucosa. It is now generally accepted that the dendritic cells of the skin and the gut mucosa travel via the lymph as veiled cells to the draining lymph nodes, while transporting antigens from the periphery to these lymphoid organs. The dendritic cells present the antigens to T cells in the lymph node and initiate the immune response[135].

Dendritic precursor cells originate from the bonemarrow. Such precursors probably circulate and reside within the blood monocyte pool. We and others have shown the maturation of purified blood monocytes into MHC class-II$^+$ cells with a dendritic morphology and function[139,140] (see also Chapter 6). It has also been established that the cytokine granulocyte-macrophage-colony-stimulating-factor (GM-CSF) induces the blood precursor cells to become fully active dendritic cells[141].

In autoimmune endocrine diseases such as Graves' disease and Hashimoto's goitre high numbers of class-II positive cells with a dendritic morphology and a phenotype of interdigitating dendritic cells can be found in the affected tissues[142,143]. An increase in the number of these cells, and a local clustering of these cells are the *very first signs* of a developing thyroid autoimmune

reaction[100,144] (figure 8). This local accumulation of the dendritic cells precedes the autoimmune reaction in the draining lymph nodes, the production of thyroid autoantibodies by these lymph nodes and the further signs and symptoms of thyroid autoimmune disease[100]. These morphological events indicate that the process of autoantigen presentation preceding the development of the thyroid autoimmune disease is similar to that of normal presentation of antigens; in other words, the morphological events strongly suggest that the dendritic cells which accumulate early in the endocrine tissue process endocrine autoantigens and travel with these to the draining lymph nodes to initiate the autoimmune reaction (figure 9). Dendritic or veiled cells have indeed been observed in lymph draining the thyroid and, additionally, Tg containing monocyte-like cells with a dendritic morphology have been observed in the marginal sinuses of draining lymph nodes[100].

Figure 8. Intrathyroidal dendritic cells forming clusters with each other during iodine deficiency. Rat thyroid sections were incubated with acid phosphatase and the monoclonal antibody OX-6 directed against MHC class-II antigens. Dendritic cells were identified as strong class-II positive cells with cytoplasmic processes and weak or absent acid phosphatase activity.

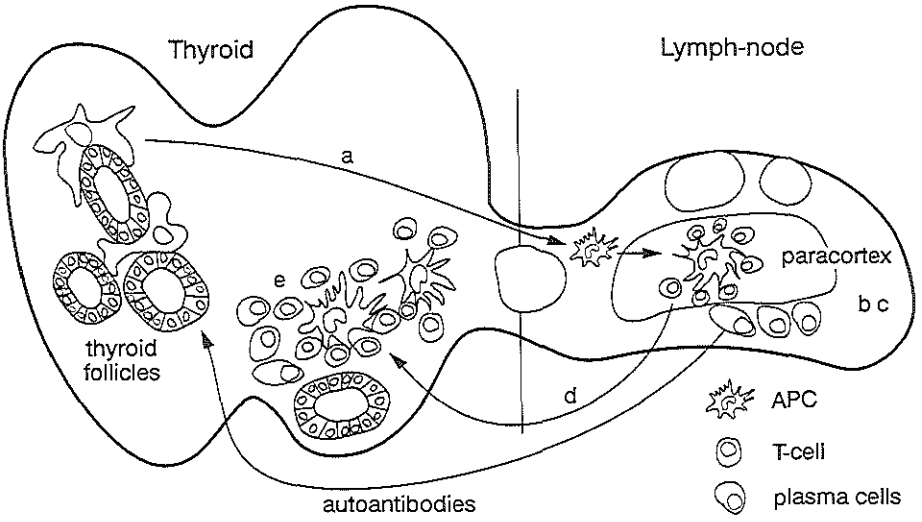

Figure 9. A schematic representation of the initiation of thyroid autoimmune reactivity. Intrathyroidal dendritic cells pick up thyroid antigens, travel to the draining lymph nodes (a) to initiate an immune response. Thyroid autoreactive T cells and B cells can be activated (b, c) and autoantibodies can be produced by plasma cells. The thyroid autoreactive T cells and antibodies exert their effects on the thyroid (d). The immune response can be continued in the thyroid-associated lymphoid tissue (TALT) in the thyroid itself (e).

Chapter 1.6

OUTLINE OF THE THESIS

Iodine is an essential trace element being part of the thyroid hormones. Therefore an iodine deficiency or excess of iodine leads to disturbances in thyroid physiology, thyroid growth and thyroid hormone production.

There are also strong indications that an iodine deficiency or an excess of iodine has effects on the thyroid autoimmune reactivity.

Iodine deficiency has been described to lead to:

a. a higher incidence of anti-Tg antibodies[145],
b. the generation of antibodies that affect the growth of thyrocytes *in vitro* (TGAbs) in individuals with endemic goitre[83,84] and endemic cretinism[146,147,148], and
c. intrathyroidal aggregates of epitheloid cells and dendritic cells in endemic goitres[149].

An excessive dietary iodine intake has also been described to lead to thyroid autoimmune reactivity:

a. in individuals with a preexisting thyroid abnormality, such as an iodine deficient goitre, an excessive dietary iodine intake results in a proportion of the individuals in the development of an attack of thyroiditis; those affected by the thyroiditis show anti-Tg and anti-microsomal antibodies in their serum[150,151,152],
b. in animals that spontaneously develop thyroid autoimmune disease, such as the Cornell C Strain of chicken, the Obese Strain of chicken and the BB rat, a high dietary iodine intake results in an increase in the incidence and severity of the thyroiditis[153,154].

The mechanisms underlying the thyroid autoimmune phenomena induced by an excessive or iodine deficient dietary intake are only partly understood.

In order to study these phenomena further and in more detail, we developed two animal models in which 3-weeks-old Wistar rats and 3-weeks-old BB rats (thyroid autoimmune prone) were kept on four dietary iodine regimens lasting for a period of 18 weeks, namely:

a. an enriched iodine diet (10 times the normal iodine intake; urinary iodine excretion 100 μg per day),
b. a normal iodine diet (urinary iodine excretion 7 μg per day)
c. a low iodine diet (2 days of 1% $KClO_4$ in the drinking water, followed by distilled drinking water and iodine deficient pellets; urinary iodine excretion undetectable), and
d. an extremely low iodine diet (continuously 1% $KClO_4$ in the drinkingwater and iodine deficient pellets, urinary iodine excretion undetectable).

These animal models have allowed us to study the effects of a low or a high dietary iodine intake in normal and thyroid autoimmune prone rats on thyroid performance (Chapters 2 and 3), on anti-colloid antibody production (Chapters 2, 4 and 5), the production of thyroid growth stimulating antibodies (TGAbs, Chapter 2.1.), and on the intrathyroidal influxes of dendritic cells, T lymphocytes, B lymphocytes and macrophages (Chapters 2 and 5).

In addition to these *in vivo* experiments, *in vitro* studies were performed on the effects of thyroid hormones and other iodinated compounds on the monocyte to veiled/dendritic cell transition (Chapter 6).

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Chapter 2.1

IODINE DEFICIENCY INDUCES THYROID AUTOIMMUNE REACTIVITY IN WISTAR RATS*

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SUMMARY

The last two decades it has become clear that iodine deficiency has a modulating effect on the thyroid autoimmune response in humans. Also in animals that spontaneously develop autoimmune thyroid disease evidence is accumulating that a low iodine intake can modulate thyroid autoimmune reactivity. However, it is still not clear what the effect of a low iodine intake on thyroid autoimmune reactivity is in normal non-autoimmune animals. To study the relationship of a dietary low iodine intake on the thyroid autoimmune reactivity in non-autoimmune animals, normal Wistar rats (female) were kept on an enriched iodine diet (EID; daily iodine intake of 100μg of iodine), a for our area normal (conventional) diet (COD; daily iodine intake of 7μg of iodine), a low iodine diet (LID; 2 days of 1% $KCLO_4$ followed by iodine deficient drinking water/pellets), or an extreme low iodine diet (LID^+; continuously 1% $KCLO_4$ in the drinking water, iodine deficient pellets).

The EID rats were euthyroid (T_3: around 8 nM/l, T_4: around 50 nM/l and TSH: around 2 ng/ml), had a normal thyroid weight (around 12.5 mg), and there were only minimal signs of a local thyroid immune reactivity: low numbers of intrathyroidal dendritic cells (DC, around 35 DC/mm^2), $CD4^+$ cells (around 2 cells/mm^2), and $CD8^+$ cells (around 2.5 cells/mm^2) were found in combination with a low anti-colloid antibody production (incidence of positive animals 12.5%).

The COD resulted in a normal thyroid function. The rats were euthyroid (range T_3: between 1.6 and 1.2 nM/l, T_4 around 50 nM/l and TSH around 2 ng/ml) and had a normal thyroid weight (around 12.5 mg). However, some signs of thyroid autoimmune reactivity could be found: number of intrathyroidal DC around 40/mm^2, around 3 $CD4^+$ cells/mm^2 and around 3 $CD8^+$ cells/mm^2 together with a 30% incidence of anti-colloid antibodies.

The LID and LID^+ not only induced goitre formation (thyroid weight 27.3 ± 4.2 mg (mean ± SD) after 12 weeks of LID and 38.4 ± 5.3 mg (mean ± SD) after 4 weeks of LID^+) and a low production of thyroxine from the thyroid (28 ± 3 nM/l, mean ± SD) after 12 weeks of LID and 14 ± 3 nM/l (mean ± SD) after 2 weeks of LID^+), they also induced various signs of thyroid autoimmune reactivity.

These constituted of:

a. influxes of DC into the thyroid (63.4 ± 3.8 DC/mm^2 (mean ± SD) after 12 weeks of LID and 64.4 ± 9.0 DC/mm^2 (mean ± SD) after 3 weeks of LID^+);

b. the formation of homotypic clusters of these DC;
c. influxes of intrathyroidal CD8 and CD4 positive T cells (10.3 ± 3.5 $CD4^+$ cells/mm^2 (mean $\pm$ SD) after 3 weeks of LID, 18.0 ± 4.7 $CD4^+$ cells/mm^2 (mean $\pm$ SD) after 4 weeks of LID^+ and 7.6 ± 4.1 $CD8^+$ cells/mm^2 (mean $\pm$ SD) after 6 weeks of LID, 25.7 ± 6.3 $CD8^+$ cells/mm^2 (mean $\pm$ SD) after 6 weeks of LID^+) and
d. increases in the production of anti-colloid antibodies (incidence of 60% positive animals after 18 weeks of LID and 73% after 10 weeks of LID^+).

These data collected in normal non-autoimmune female Wistar rats show that – as in humans – iodine deficiency precipitates thyroid autoimmune reactivity.

INTRODUCTION

The last two decades it has become clear that a high dietary iodine intake may aggrevate thyroid autoimmune reactivity in both humans as well as experimental animals suffering from thyroid (autoimmune) disease.

In experimental animals[1,2,3,4] and humans[5,6,7,8] with a preexisting thyroid abnormality, such as an iodine deficient goitre, a single administration of a high dose of iodine resulted in an attack of thyroiditis positive for anti-thyroglobulin and anti-microsomal antibodies. In the Cornell C Strain of chicken[9] and in the BB rat[10] it was shown that a high dietary intake of iodine led to an increase in the incidence and severity of the thyroiditis of these animals.

Evidence is also accumulating that iodine deficiency is able to precipitate thyroid autoimmune reactivity in humans:

a. a higher incidence of anti-thyroglobulin (Tg) antibodies has been described in areas of iodine deficiency[11];
b. the majority of patients suffering from diseases linked to iodine deficiency, such as endemic goitre[12,13] and endemic cretinism[14,15,16] show IgG's in their serum that affect the growth of thyrocytes *in vitro* (the so-called thyroid growth-stimulating immunoglobulins, TGIs). It must be noted however, that the character and meaning of these TGIs are still under debate[17,12]; some investigators have found these TGIs in the absence of TSH receptor

antibodies[18], whereas others consider the TGI effect as a classical TSH receptor stimulator effect of thyroid stimulating immunoglobulins (TSIs)[19];

c. thyroids of iodine deficient goitre patients contain aggregates of epitheloid cells and dendritic cells[20]. Dendritic cells are the antigen presenting cells par excellence[21] and play a major role in the initiation of immune responses. They have been described as the first cells arriving in thyroids before thyroid autoimmune reactivity develops[22,23]. Additionally, dendritic cells are capable of transferring autoimmune thyroiditis in an experimental animal model[24] and are thus considered to be responsible for the initiation of the thyroid autoimmune response. Others consider an abberant MHC class-II expression on thyrocytes as the primary event in thyroid autoimmune disease[25].

In animals, the effect of a low dietary iodine intake on thyroid autoimmune reactivity has only been studied in the existing models of autoimmune thyroid disease. To our knowledge, it has not been studied whether iodine deficiency precipitates thyroid autoimmune reactivity in non-autoimmune, normal, euthyroid animals. Such a study might be a better reflection of the effect of the dietary iodine intake on thyroid autoimmune reactivity in average human populations. Therefore we developed an animal model in which normal female Wistar rats were kept on four iodine regimens, namely an iodine enriched diet (EID), a for our area normal (conventional) diet (COD), a low iodine diet (LID), and an extremely low iodine diet (LID^+) for periods of up to 18 weeks starting from the third week of life. The extent of iodine deficiency/sufficiency was determined by the urinary iodine excretion. The severity of hypothyroidism was measured by the serum TSH and thyroid hormone levels. Goitre formation was determined by measuring the thyroid weight. Thyroid autoimmune reactivity was measured by counting the number of intrathyroidal infiltrated leucocytes (MHC class II positive dendritic cells, T cells and B cells) and measuring the incidence of anti-colloid and anti-cytoplasmic antibodies. The effects of the iodine diet on the serum levels of IgGs that stimulate the growth of thyrocytes *in vitro* (the socalled TGIs) will be discussed in another report.

MATERIALS AND METHODS

Animals and diets

Female Wistar rats were purchased from TNO Rijswijk, The Netherlands and kept at the central experimental animal housing facilities of the Erasmus University Rotterdam under standard conditions. Directly after weaning (at the age of 3 weeks) 5 consecutive cohorts of rats (n=30-60) were kept each on four dietary iodine regimens:

1. *An enriched iodine diet (EID)*; the rats received conventional pellets (Am-II, Hope Farms bv, Woerden, The Netherlands) ad libitum as well as an extra iodine supplementation of 6.5mg KI/l added to the drinking water. Consequently the iodine intake was about 100μg of iodine per day.
2. *A for our area normal (conventional) diet (COD)*; the rats received conventional pellets (Am-II, Hope Farms bv, ± 0.35 mg/kg iodine) and Rotterdam tap water ad libitum. The iodine intake in this group was about 7μg of iodine per day.
3. *A low iodine diet (LID)*; the rats received 1% $KCLO_4$ in the drinking water for a period of 2 days and thereafter distilled water and iodine deficient pellets (Modified Remmington diet, Hope Farms bv) ad libitum.
4. *An extreme low iodine diet (LID$^+$)*; the rats continuously received 1% $KCLO_4$ in their distilled drinking water and iodine deficient pellets (Modified Remmington diet, Hope Farms bv) ad libitum.

Wistar rats of the 5 cohorts were sacrificed by aortic exsanguination under ether anaesthesia after 3, 6, 9, 12 or 18 weeks of diet, in such a way that 5-18 animals per time group could be used for evaluation. Serum was prepared for T_3, T_4, TSH evaluation and anti-colloid antibody determination. T_3, T_4, and TSH were measured by conventional RIA's (Dr. W.M. Wiersinga, Mrs. M. Broenink, Academical Medical Centre Amsterdam, The Netherlands). For the rat TSH-RIA, the rat TSH reference preparation NIADDK-rTSH-RP-2 was used (176 times more potent than the NIADDK-rTSH-RP-1, kindly provided by Dr. Parlow from the UCLA Medical Centre, Torrance, USA. Thyroid glands were removed, weighed and stored at -80 °C until immunohistological examination.

Iodine measurement in rat urine

Rat urine was collected during 24 hours by keeping the rats in metabolic cages. Iodine excreted in the urine was measured using a modified method described by Benotti et al[26]. In brief, the urine samples were destructed by adding an equal volume of 30% chloric acid and incubating the samples at 200 °C for several hours. The iodine reacted as a cathalysing agent in the redox reaction between cerium ammonium sulphate and arsenic acid. During the reaction the yellow Ce^{4+} was converted into the colourless Ce^{3+} which was measured using an ELISA reader at 405 nm.

Immunohistological examination of thyroids

Immunohistological examination was performed according to Green et al.[27] with minor modifications. Of each frozen thyroid, one lobe was semiserially cut into 6 μm thin sections. The sections were airdried overnight and fixed in acetone at room temperature for 10 minutes. The sections were incubated with either a monoclonal antibody specific for MHC class II molecules, $CD4^+$ cells, $CD8^+$ cells or B cells (see table 1) for 60 minutes, washed with phosphate buffered saline (PBS) containing 0.2% bovine serum albumin (BSA, Sigma Chemical Co, USA) for 10 minutes, and further incubated with a rabbit anti-mouse Ig-HRP labeled conjugate (Dakopatts, Denmark) for 30 minutes, diluted 300 times in PBS containg 1% normal rat serum and 0.2% BSA. After washing the slides in PBS containing 0.2% BSA for 10 minutes and rinsing them with 0.1 M sodium acetate buffer, pH 6.0, the sections were developed with a metal-enhanced 3,3'-diaminobenzidine (DAB) solution containing 0.05% DAB (Sigma), 1% nickel sulphate (Merck), 0.068% imidazole (Sigma), and 0.8% sodium chloride in 0.1M acetate buffer pH 6.0 for 3-5 minutes. Hydrogen peroxide was added to a final concentration of 0.01%. After DAB development, the sections were washed briefly in 0.1M Tris-HCl buffer, pH 7.6, and immersed in a 0.5% solution of cobalt chloride in 0.1M Tris-HCl buffer, pH 7.2 at room temperature for 4 minutes. The slides were either counterstained with 0.1% nuclear fast red in 5% aluminium sulphate for 2 minutes or incubated with acid phosphatase at 37°C for 30 minutes and subsequently counterstained with hematoxylin. The slides were dehydrated and embedded in DePeX mounting

medium (Gurr, BDH Limited Poole, England).

For the quantification of intrathyroidal infiltrated cells, 4 sections of each thyroid, with intervals of at least 100μm, were reacted with the appropriate marker monoclonal antibody. Positive infiltrated cells were counted using light microscopy at a magnification of 400x. The surface area of the thyroid sections in which the infiltrated cells had been counted was measured using a camera attached to a Leitz Diaplan light microscope and a Videoplan image processing system (Kontron, Bild Analyse GmbH, Germany). The number of infiltrated DC (identified as strong MHC class-II$^+$ cells with cytoplasmic processes and weak or absent acid phosphatase activity), CD4$^+$ T cells and CD8$^+$ T cells were expressed per mm^2 surface area of a thyroid section. It is of importance to note that similar results were obtained when the number of DC and T cells were not expressed per mm^2 surface area of the section but were expressed per observed thyroid follicle in the plane of section or per surface area of thyrocyte parenchyma.

Table 1. Monoclonal antibodies used for the detection of leucocytes.

MoAb	Specificity	Reference
OX6 (Seralab, UK)	MHC class-II	28
W3/25 (Seralab, UK)	CD4 antigen	29
B115-4 (Holland Biotechnology, The Netherlands)	CD4 antigen	30
OX8 (Seralab, UK)	CD8 antigen	31
HIS 14 (F.G.M. Kroese)	B cells	32,33

Anti-thyroid antibody determination

6 μm thin frozen porcine thyroid sections were cut, airdried overnight and fixed in cold acetone (-20 °C) for 10 minutes. Porcine thyroid tissue was used, since it gave optimal results as compared to rat or human tissue. The sections were preincubated with normal rabbit serum (DAKO-immunoglobulins, Denmark), diluted 50 times in PBS with 1% BSA, for 10 minutes. Subsequently, the rat sera were applied (in duplicate, diluted 10 times in 0.9% NaCl) and the slides were incubated

at room temperature for 60 minutes. After washing in PBS, the sections were incubated with rabbit anti-rat immunoglobulins, fluorescein-isothiocyanate-(FITC)-labeled (DAKO immunoglobulins, Denmark) for 30 minutes (diluted 25 times in PBS with 1% BSA). After this second step and rewashing, the slides were embedded in aquamount (Gurr, BDH Limited Poole, England) and examined using a fluorescense microscope. Three control slides were included; one without incubation of rat serum, another incubated with a rat serum previously scored as negative and a third incubated with a rat serum previously scored as positive. The staining intensity of each serum was arbitrarily and blindly scored (under code, by two independent investigators) as negative, positive or strongly positive.

Statistical analysis

Differences inthyroid weight, serum T_3, T_4, and TSH, and number of intrathyroidal MHC class-II$^+$ DC, clusters of these cells and T cells were compared by Wilcoxon's rank sum test. The incidence of anti-colloid antibody production was tested by X^2 analysis.

RESULTS

Thyroid weight

The thyroid weight of animals kept on an *EID* and a *COD* increased slightly from 6.7 ± 1.1 mg to 13.3 ± 2.9 mg (mean $\pm$ SD) after 6 weeks of diet as could be aspected from the normal growth development and the thyroid/body weight index remained constant namely 0.07. The absolute thyroid weight after 6 weeks of diet remained at 13 to 14 milligrams for the further experimental period (figure 1A).

In animals kept on a *LID* however, a stronger increase in thyroid weight was found, that was statistically significantly different from the thyroid weights of animals kept on an EID or COD ($p<0.05$, Wilcoxon's rank sum test). After 6 weeks of diet the thyroid weight was 20.1 ± 2.8 mg (mean $\pm$ SD) and the goitre reached maximal values after 18 weeks of diet (26.3 ± 7.3 mg (mean $\pm$ SD), figure 1A).

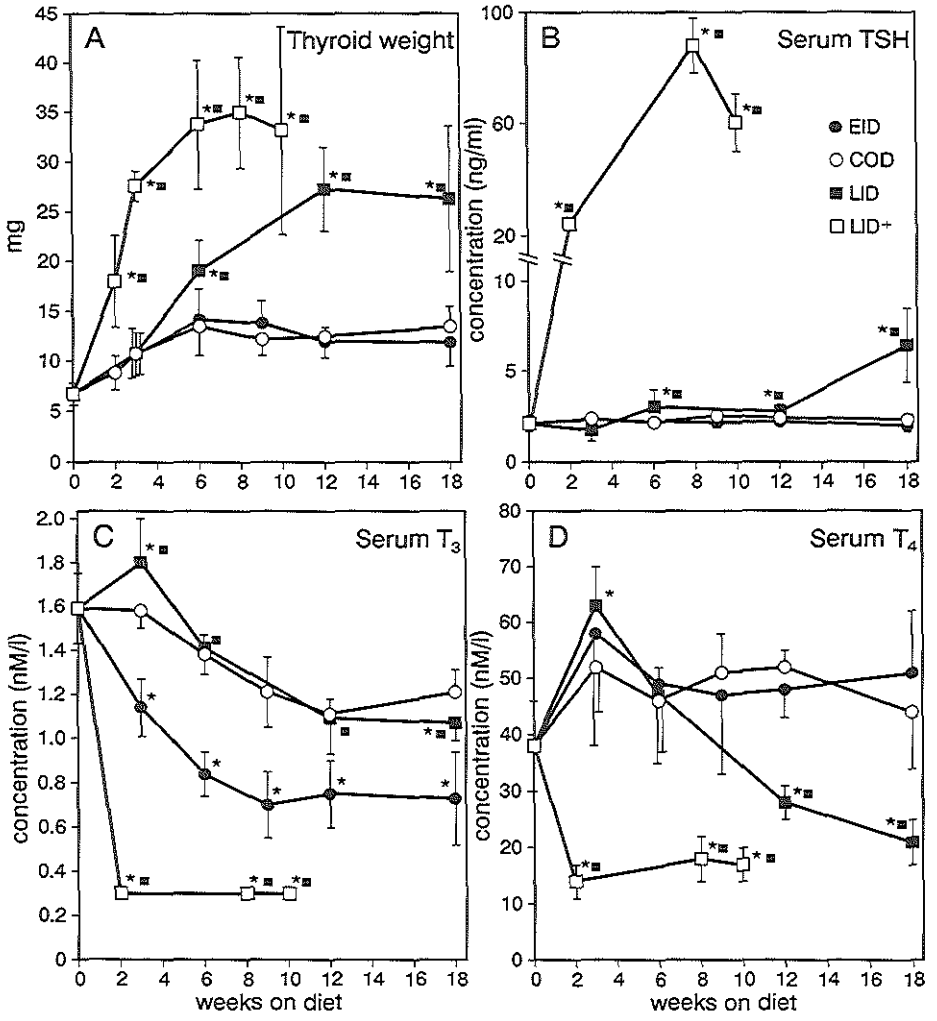

Figure 1. Thyroid weight and endocrine status. Female Wistar rats were kept on 4 dietary iodine regimens; an enriched iodine diet (EID), an conventional diet (COD), a low iodine diet (LID) and an extreme low iodine diet (LID^+) for a period of 18 weeks from 3 weeks of age onwards. Rats were sacrified and thyroids were removed and weighed to measure goitre development (A), serum was collected to measure serum TSH (B, please note the different scale for the upper and the lower part of the figure), serum T_3 (C), and serum T_4 (D) levels by RIA. The mean and standard deviation are given of 5-18 animals per group.

It is worthy to note that the increase in thyroid weight was neither in time, nor in extent related to increases in serum TSH levels in the LID group (figure 1A vs 1B, see also later).

Rats receiving a *LID*$^+$ showed a more pronounced goitre development. As soon as after 2 weeks of diet statistically significantly higher thyroid weights were found compared to rats kept on an EID, COD or LID ($p<0.05$, Wilcoxon's rank sum test). After 4 weeks of diet the thyroids reached a maximal weight of 38.4 ± 5.3 mg (mean $\pm$ SD), remaining constant thereafter (figure 1A).

Thyroid endocrine status and urinary iodine excretion

In animals kept on an *enriched iodine diet (EID)* the T_4 output from the thyroid was normal (figure 1D). Serum T_3 levels however were low (figure 1C). Similar low serum T_3 levels have been described by other investigators in rats treated with a high iodine dose (300-500 μg of iodine per day)[34,35]. Serum TSH levels in the EID rats were in the normal range (between 2.1 ± 0.4 (mean $\pm$ SD) and 2.5 ± 0.3 (mean $\pm$ SD)) during the entire experimental period (figure 1B). The urinary iodine excretion of the Wistar rats receiving an EID was high: an excretion of 50 μg I/day was measured after 1 week of diet and excretion reached a plateau after 3 weeks (6 weeks of life) of 100 μg I/day. These latter values reflected the estimated daily intake.

In animals receiving a *for our area normal (conventional) diet (COD)* serum T_4 levels started to increase during the first 3 weeks of diet to reach a constant level of 50 nM/l for the remaining experimental period (figure 1D). Serum T_3 levels were high (1.6 ± 0.2 nM/l (mean $\pm$ SD)) at the start of the experimentent. They gradually decreased after 3 weeks of diet from 1.6 ± 0.1 nM/l (mean $\pm$ SD) to 1.2 ± 0.1 nM/l (mean $\pm$ SD) at 12 weeks of diet and stayed at that level thereafter (figure 1C). Such relatively high serum T_4 and T_3 levels in prepubertal rats compared to adult rats have been described by others before[35] and these authors regarded the high thyroid hormone levels as necessary for optimal brain development in the prepubertal age. The need for the high serum thyroid hormone levels in the young rats was reflected by their urinary iodine excretion: the rats on a COD showed a relatively long period of low urinary iodine excretion; an output of 1.6 ± 0.3 μg I/day (mean $\pm$ SD) at the age of 3 weeks while it lasted till the age of 6 weeks that urinary

iodine excretion had reached 7.6±1.5 μg I/day (mean±SD), the latter value reflecting the daily iodine intake. This excretion level was maintained during the remaining experimental period. Serum TSH levels of the COD animals were in the normal range during the entire period of observation (figure 1B).

In animals kept on a *low iodine diet (LID)* the T_4 output of the thyroid gland gradually failed and the animals became subclinically hypothyroid after 12 weeks of diet. Serum T_4 levels were as low as 28.0±2.5 nM/l (mean±SD) and remained lower than the levels in EID and COD animals ($p<0.05$, see figure 1D). A very slight but statistically significantly rise in serum TSH levels could be observed after 6 weeks of a LID compared to the TSH levels found in EID and COD rats, but a more clear rise in TSH levels was not found until 18 weeks of LID (6.4±1.8 vs 2.0±0.4 and 2.3±0.3 ng/ml (mean±SD) compared to EID and COD animals respectively, figure 1B). The serum T_3 levels remained in the normal range (figure 1C). The low iodine intake was well reflected by the urinary iodine excretion. After 1 week of diet the excreted urinary iodine was under the lower limit of detection of our assay.

The animals kept on an *extreme low iodine diet (LID^+)* became severely hypothyroid. After 2 weeks of a LID^+ a decline in T_4 levels from 37.4±7.5 to 13.3±2.2 nM/l (mean±SD) was seen and levels remained low during the remaining experimental period (figure 1D). Also very low serum T_3 levels were found that were below the detection limit (≤0.03 nM/l) after 2 weeks of diet (figure 1C). The serum TSH levels of animals on a LID^+ were already high after 2 weeks of diet (24.3±2.1 ng/ml (mean±SD)) and reached maximal values after 8 weeks of diet (88±9.9 ng/ml (mean±SD)) slightly declining to 60.4±9.2 ng/ml (mean±SD) after 10 weeks (figure 1B). The urinary iodine excretion was extremely low: as early as after 1 week of diet iodine could not be detected in the excreted urine.

Observations were stopped after 10 weeks of LID^+ diet since then animals became severely ill due to their hypothyroidism.

Signs of immune reactivity in the thyroid

At the start of the experiment only a few DC were found in the thyroids of the animals. The DC were scattered throughout the thyroid and were localized in the interstitial connective tissue and never in between the thyrocytes. The DC were not only found as single cells, they also made contact with other DC and with mast

cells present in the interstitial tissue to form small cellular clusters (homotypic clusters, figure 2A). A few $CD4^+$ and $CD8^+$ T cells were also present and were, like the DC, found scattered throughout the thyroid in the interstitium. Sometimes however, the T cells could be observed between the thyrocytes being part of the thyroid follicle lining. The T cells were never grouped together or found clustered to other cells, but were present as single cells (figure 2B). B cells could not be detected in any of the young rat thyroids examined.

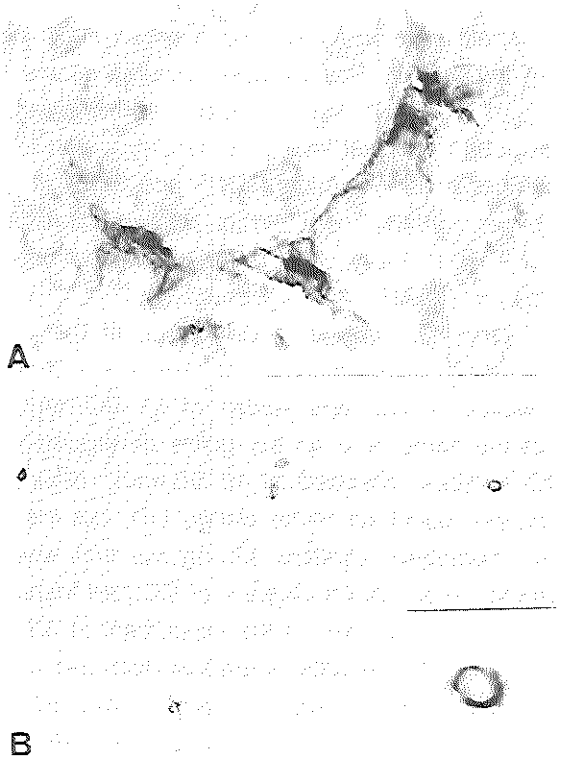

Figure 2. Intrathyroidal dendritic cells and T cells.

A. Intrathyroidal MHC class-II positive dendritic cells with cytoplasmic protrusions forming homotypic clusters. The thyroid section from a rat kept on a LID for 6 weeks was incubated with the monoclonal antibody OX-6 (Magn. 1000x).

B. Intrathyroidal single round CD8 positive T cells (Magn. 80x). The thyroid section from a rat kept on a LID^+ for 10 weeks was incubated with the monoclonal antibody OX8. Insert shows a higher magnification of a round T cell (Magn. 1500x).

In animals receiving an *EID* the intrathyroidal dendritic cells (DC) remained at a low constant level during the entire experimental period (range between 30 and 40 DC/mm^2, figure 3A). Also the number of intrathyroidal CD4$^+$ and CD8$^+$ T cells stayed low (range between 1.4±0.9 and 2.0±0.5 (mean±SD) for CD4$^+$ cells and between 1.7±1.6 and 2.8±1.0 (mean±SD) for CD8$^+$ cells, figure 3C, D).

It must be stressed that similar results were obtained when the number of DC and T cells were not expressed per mm^2 surface area of the section but were expressed per observed thyroid follicle in the plane of section or per surface area of thyrocyte parenchyma.

In the thyroids of rats kept on a *COD* the number of DC increased to around 45 DC/mm^2 in the first weeks. This increase occured at the same time as the increase in the serum T_4 level. After 9 weeks and thereafter the number of intrathyroidal DC dropped to a lower level of 35 DC/mm^2 and remained at this level (figure 3A). The number of intrathyroidal CD4$^+$ and CD8$^+$ T cells remained low during the entire experimental period in rats receiving a COD (range between 0.6±0.5 and 4.4±2.9 CD4$^+$ cells/mm^2 (mean±SD) and between 1.2±0.8 and 5.2±1.8 CD8$^+$ cells/mm^2 (mean±SD), figure 3C, D).

The *LID* caused a slightly stronger accumulation of DC and of T cells in the thyroid as compared to the EID and COD rats. There was a clear increase of DC numbers after 12 weeks of diet and values of 65 DC/mm^2 were found. This increase occured at the same time as the goitre development in these animals. After 18 weeks DC numbers dropped to 45 DC/mm^2 (figure 3A). Not only an increase in DC numbers could be found during LID, but the cells also became more active to form homotypic clusters. During the first weeks of the LID the number of intrathyroidal small cellular clusters of DC had increased from 0.6±0.8 clusters/mm^2 (mean±SD) at the start of the experiment to 3.9±1.4 clusters/mm^2 (mean±SD, 3 weeks of diet). Thereafter a gradual decrease was seen (figure 3B). The number of CD4$^+$ and CD8$^+$ T cells increased to 10.3 ±3.5 CD4$^+$ cells/mm^2 (mean±SD) and 7.6±4.1 CD8$^+$ cells/mm^2 (mean±SD) after 3 and 6 weeks of LID respectively. After a drop in numbers, relatively high values were again reached at 18 weeks of diet (6.6±2.5 CD4$^+$ cells/mm^2 (mean±SD) and 10.2±4.2 CD8$^+$ cells/mm^2 (mean±SD), figure 3C, D).

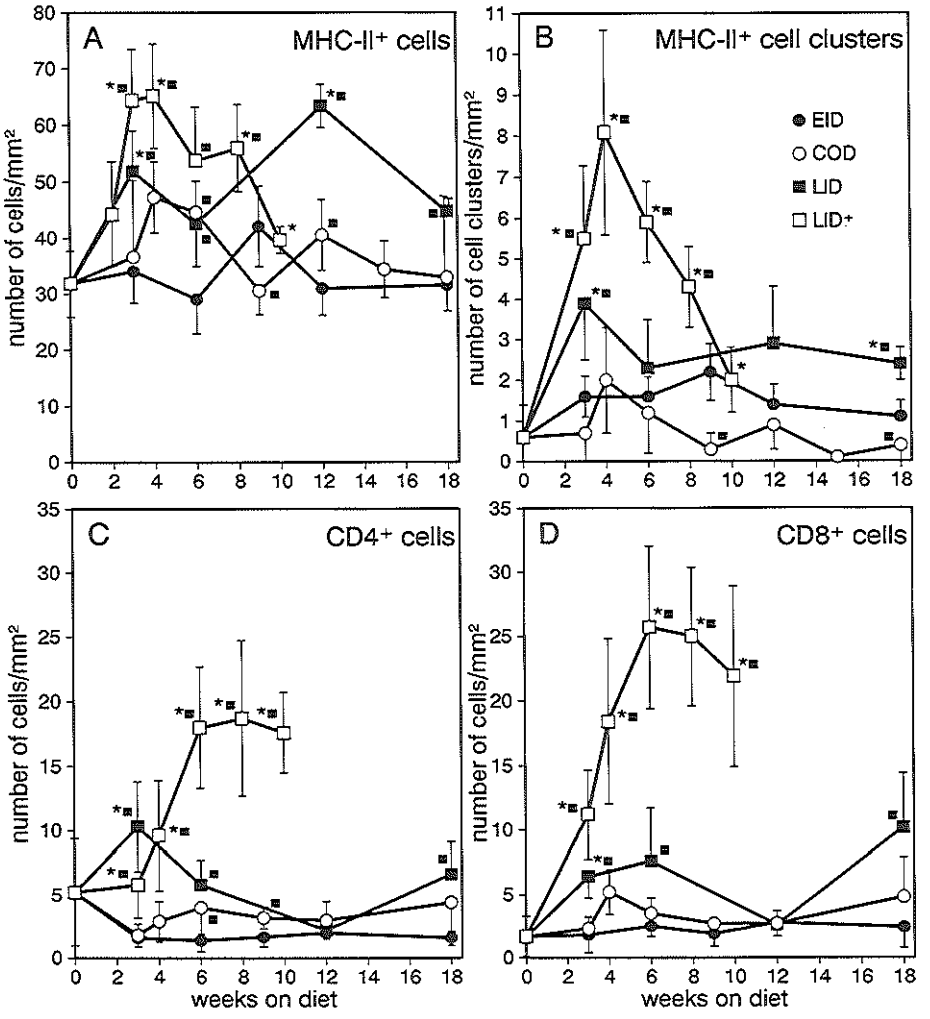

Figure 3. Immune reactivity in the thyroid. Thyroid lobes of groups of rats (n=5-12) kept on an enriched iodine diet (EID), a conventional diet (COD), a low iodine diet (LID) and an extreme low iodine diet (LID^+) were removed, frozen and sections of 6μm were incubated with several monoclonal antibodies to identify intrathyroidal leucocytes. The mean number and SEM of intrathyroidal dendritic cells (A), clusters of dendritic cells (B), intrathyroidal $CD4^+$ cells (C) and $CD8^+$ cells (D) are expressed per mm^2 counted in 4 semiserial cut sections for each thyroid.

The *LID*$^+$ caused a pronounced increase in cell infiltration of DC, CD4$^+$ and CD8$^+$ T lymphocytes and of homotypic clustering of DC. Values of around 65 DC/mm^2 were already seen after 3 weeks of the LID$^+$. After 6 weeks a decline could be observed and values of 40 DC/mm^2 were reached after 10 weeks of LID$^+$ (figure 3A). A pronounced peak of clusters of DC occured in the same period, reaching a maximal value of 8.1 ± 2.5 clusters/mm^2 (mean ± SD) after 4 weeks of LID$^+$ (figure 3B).

Not only an accumulation of DC was induced in the thyroid of LID$^+$ animals but also a rapid and clear increase in both the number of intrathyroidal CD4$^+$ and CD8$^+$ T cells (reaching maximal values of 18.7 ± 6.0 CD4$^+$ cells (mean ± SD) after 8 weeks of diet and 25.7 ± 6.3 CD8$^+$ cells (mean ± SD) after 6 weeks of diet, figure 3C, D). These T cells were as in the animals kept on other diets single cells present in the interstitium and in between the thyrocytes.

Anti-thyroid antibody production

At the start of the experiments the incidence of anti-colloid antibodies in the Wistar rats colony was 4% (n=24). Anti-cytoplasmic antibodies were not detected.

In rats receiving an *EID* the incidence of anti-colloid antibodies remained low and did not exceed an incidence of 12.5% from 6 weeks of diet onwards (n=8-16, figure 4). Anti-cytoplasmic antibodies were not detected during the entire observation period in rats receiving an EID.

Keeping the rats on a *COD* resulted in an incidence of anti-colloid antibodies of 30% (n=25) after 5 weeks and onwards (figure 4). This incidence was however, not statistically significanctly different from the incidence of anti-colloid antibodies in EID rats ($p>0.05$, X^2 analysis). Anti-cytoplasmic antibodies were sporadically found: in 1 out of 17 rats after 3 weeks of diet and in 1 out of 9 rats after 6 weeks of diet. The other rats were negative.

The *LID* resulted in a similar increase in anti-colloid antibody production as the COD, and incidences of 33% (n=18) were found after 5 weeks of diet. After 18 weeks however, the incidence was statistically significantly different from that of the EID rats and had reached 60% (n=10, $p<0.05$ compared to EID rats, X^2 analysis, figure 4). At that time the rats had become subclinically hypothyroid and had developed a goitre (see above). In rats on a LID anti-cytoplasmic antibodies were

not detected.

The *LID*$^+$ stimulated the production of anti-colloid antibodies in our Wistar colony even stronger than the LID. After 10 weeks of diet all the rats (n=7) showed anti-colloid antibodies in their serum (figure 4). At that time the rats were severely hypothyroid, showing clearly enlarged thyroids. Only a few rats showed anti-cytoplasmic antibodies in their serum: after 2 weeks of a LID$^+$ 1 out of 18 and after 6 weeks of diet 1 out of 7. The other rats were negative.

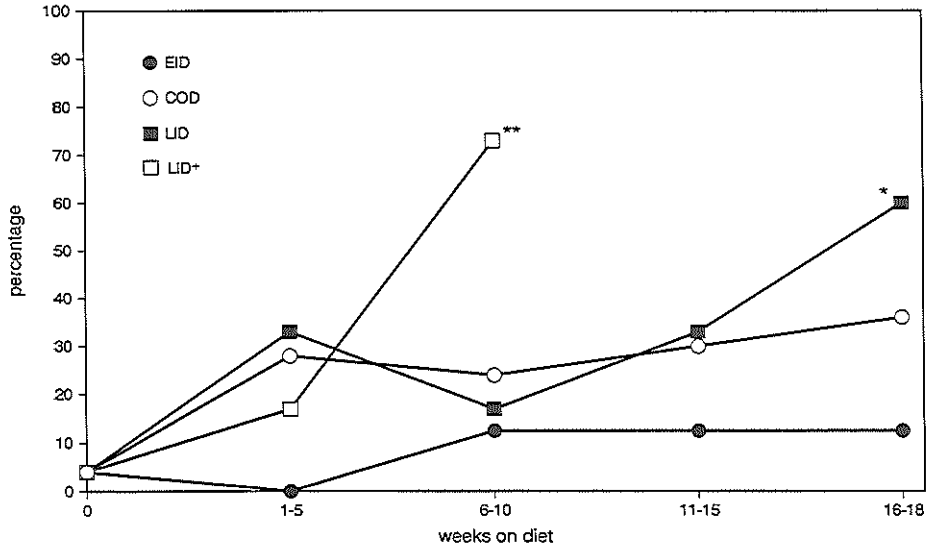

Figure 4. Incidence of anti-colloid antibody. The incidence of anti-colloid antibody production is given of various age groups of female Wistar rats (n=8-20 per age group) kept on an enriched iodine diet (EID), a conventional diet (COD), a low iodine diet (LID) and an extreme low iodine diet (LID$^+$) from 3 weeks of age onwards for a period of 18 weeks. * $p<0.05$, ** $p<0.001$ compared to EID, X^2 analysis.

DISCUSSION

This study in normal Wistar rats shows that the dietary iodine intake not only influences the thyroid weight and thyroid hormone profile, but also thyroid autoimmune reactivity.

An iodine-insufficient diet in our experimental animals resulted in:
a. an intrathyroidal accumulation of DC and T-cells;
b. a clustering of the thyroid infiltrated DC, and
c. an increased anti-colloid antibody production.

The Wistar rats suppleted with iodine showed a very low local thyroid immune response (few intrathyroidal dendritic cells and T-cells) and a very low production of anti-colloid antibodies.

The higher incidence of anti-colloid antibodies in the iodine-deficient goitrous Wistar rats is in accordance with the higher prevalences of anti-thyroglobulin antibodies reported in endemic goitre patients living in iodine-deficiency[11]. The direct pathogenic effects of anti-Tg antibodies are not clear. It must be noted that immunization with Tg can induce thyroiditis in experimental animals[36]; however, autoreactive T cells are considered more important in this phenomenon than thyroid autoantibodies. In a recent report not Tg, but TPO is thought to be of greater importance as an autoantigen in autoimmune thyroid disease[37]. In the thyroid autoimmune prone BB rat anti-cytoplasmic antibodies (anti-TPO antibodies) are indeed more frequent (10%) than in Wistar rat strains (3%, unpublished data). The here reported experiments also show that this low frequency of anti-cytoplasmic (anti-TPO) antibodies did not increase due to iodine deficiency or iodine excess. The anti-cytoplasmic antibodies are thus considered of minor importance in inducing thyroid autoimmune reactivity.

The observed increases in the number of intrathyroidal dendritic cells and homotypic clusters of these cells in the rat goitres find their human parallel in the reported increases in dendritic cell infiltration and clustering of these cells in thyroids of iodine-deficient endemic goitre patients[20].

Toxic effects of $KClO_4$ on thyrocytes, inducing thyroid antigen release and explaining the thyroid autoimmune effects of the iodine-deficient diets in our rats can practically be excluded. Firstly because $KClO_4$ was only given for the first two days of the diet in our LID group, while the increases in leucocyte infiltration and anti-colloid antibody production in this animal group were found only after 3-4 weeks and 12-18 weeks of LID respectively, and secondly because thyroid cell death could not be observed in our histological specimen of LID and LID^+ thyroids (on the contrary, an active high columnar epithelium was evident).

The mechanisms of the influxes of DC and lymphocytes into the Wistar thyroids

during iodine deficiency are speculative. DC are the antigen-presenting cells par excellence[38], and are the most potent inducers of experimental autoimmune thyroiditis after transfer[24]. They are the first cells to appear in the thyroid during the development of the BB rat thyroiditis[23]. It is likely that the macrophage-like dendritic cells accumulating early in the BB rat thyroid pick up thyroid antigens, travel with these via the lymph to the draining lymphnode to initiate the autoimmune response[38].

We hypothesize that the high thyroid metabolic activity and the enhanced proliferation rate of the thyrocytes induced by the iodine deficiency played a role in the attraction of the dendritic cells by an enhanced expression and release of thyroid autoantigens. This might have triggered the dendritic cells to accumulate and to start a thyroid autoimmune response. It has indeed been suggested that a high thyrocyte metabolism might be involved in initiating a thyroid autoimmune response in the OS chicken. An intrinsically high thyroid metabolism has been described in the OS-chicken as a primary genetic abnormality of the target organ that forms one of the basic conditions for susceptibility to thyroid autoimmune disease[39]. In our experiments the stimulation of the iodine-deficient Wistar thyrocytes by the relatively high levels of serum TSH might also have played an additional role. Indeed, the serum TSH levels of the LID^+ rats correlated well with the influxes of DC, the clustering of these cells and the incidence of anti-colloid antibodies. Chiovatto *et al*[40] observed that TSH enhances the expression of the antigen thyroid peroxidase[41] on the cell surface of the rat thyroid cell line FRTL-5, whereas TSH also induces a higher production of the autoantigen Tg from thyrocytes. In addition when serum levels of TSH were suppressed in BB/Wor rats by T4 treatment lower levels of Tg-antibodies and a decreased incidence of thyroiditis were found[42].

The here reported data also indicate that there is a marked contrast between Wistar rats on the one hand and BB rats and OS chickens on the other hand with regard to their response to deficient or sufficient iodine diets. In the OS-chicken and the BB/W rat, both well esthablished models of spontaneous autoimmune thyroid disease, a low iodine intake ameliorated the thyroid autoimmune reaction, whereas an increase in the incidence of thyroiditis was found during a high iodine intake[43,44].

The iodine intake in the studies on the BB rat and OS-chicken (5.000-10.000 μg I per day) are considerably higher in comparison to the iodine suppletion used in

the here reported Wistar study (100 μg I per day) which could explain the observed discrepancy. However, recent experiments performed by us (to be published) show that the relatively low excess of iodine of 100 μg I per day is sufficient to induce a marked and enhanced leucocytic infiltration in the thyroids of BB rats. This means that the discrepancy between Wistar rats and BB rats/OS chickens in their response towards high and low iodine diets must be due to specific differences between the animals, which also relates to their different susceptibility for spontaneous thyroid autoimmunity. It must be noted however - although the Wistar rat is not as severely genetically predisposed to thyroid autoimmune disease as the BB rat - the animal does show some susceptibility to thyroid autoimmune reactivity such as the presence of thyroid-specific T cells[45].

In several studies the thyroid autoimmune promoting effects of the high iodine diets in BB rats and OS chickens are generally attributed to the direct effects of iodine on the thyroid autoimmune response itself. Iodine increases the antigenicity of thyroglobulin[46,47] (although this is also disputed[48,49]) or directly stimulates the function of macrophages[50], T cells[51] and B cells[52]. To explain our results (the virtual absence of thyroid autoimmune reactivity in Wistar rats on a high iodine diet) one has to assume that either iodine has no such autoimune stimulating effects in the Wistar rat or that the autoimmune promoting effects of iodine are overruled by autoimmune suppressor mechanisms specific for the Wistar rat (tolerance induction). Since our histology showed that the thyrocytes of Wistar rats kept on an EID had a very low level of metabolic activity (the cells showed a flattened appearance with prominent colloid spaces, to be published), it could also be envisaged that the iodine intake of 100 μg per day during the iodine suppletion is the most optimal intake for the Wistar rat and that this intake allows the Wistar thyrocytes to function on a low metabolic level.

If the metabolic activity of thyrocytes is indeed coupled to their autoantigenicity (see discussion above) one could imagine that a dietary intake of 100 μg of iodine per day in Wistar rats is capable to keep the thyroid autoimmune reactivity at a very low level, despite the direct stimulating effects of the iodine on immune cells. Future studies need to unravel these possible mechanisms.

In conclusion, the here reported data show that an insufficient dietary iodine intake is capable to precipitate a thyroid autoimmune response in normal non-autoimmune Wistar rats, as in normal human populations.

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Chapter 2.2

THYROID GROWTH STIMULATING ACTIVITY IN AMMONIUM SULPHATE PRECIPITATES OF THE SERUM OF WISTAR RATS KEPT ON A LOW DIETARY IODINE REGIMEN. A PRELIMINARY REPORT

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INTRODUCTION

Patients suffering from diseases linked to iodine deficiency, such as endemic goitre[1,2] and endemic cretinism[3,4,5] often show IgG's in their serum that affect the growth of thyrocytes *in vitro*. The patients suffering from endemic goitre are often euthyroid and goitre development is not necessarily related to increases in TSH levels[6].

In Chapter 2.1 of this thesis we described that Wistar rats kept on a moderately low dietary iodine regimen developed a goitre 6 weeks after starting the diet (at 9 weeks of age) without any noteworthy increases in the serum TSH level. The observed thyroid growth in these rats could therefore not be due to TSH. Other growth factors must be involved in the iodine deficient goitre development.

At earlier occasions we suggested that immune factors, such as thyroid growth stimulating antibodies (TGAbs) might play a role in iodine deficient goitre formation. We therefore tested in a preliminary set of experiments crude immunoglobulin preparations of rats kept on several dietary iodine regimens on growth stimulating capacity in a mitotic arrest assay using the rat thyrocyte cell line FRTL-5 as indicator cells. Since it proved to be difficult to purify IgGs from rat sera via protein-A sepharose techniques, we first set out to study the effects of crude Ig preparations (ammonium sulphate precipitates) of the serum of the Wistar rats to establish any positivity in the mitotic arrest assay.

MATERIALS AND METHODS

Animals and diets

Female Wistar rats were purchased from TNO Rijswijk, The Netherlands and kept at the central experimental animal housing facilities of the Erasmus University Rotterdam under standard conditions. Directly after weaning (at the age of 3 weeks) groups of rats (n=5-18) were kept on several dietary iodine regimens:

1. *An enriched iodine diet (EID)*; the rats received conventional pellets (Am-II, Hope Farms bv, Woerden, The Netherlands) ad libitum as well as an extra iodine supplementation of 6.5mg KI/l added to the drinking water (the urinary

iodine excretion was about 100μg of iodine per day, for methods see ref[7]).

A for our area normal iodine diet (NID); the rats received conventional pellets (Am-II, Hope Farms bv, ± 0.35 mg/kg iodine) and Rotterdam tap water ad libitum (the urinary iodine excretion in this group was about 7μg of iodine per day).

A low iodine diet (LID); the rats received 1% $KCLO_4$ in the drinking water for a period of 2 days and thereafter distilled water and iodine deficient pellets (Modified Remmington diet, Hope Farms bv) ad libitum (urinary iodine excretion undetectable).

An extreme low iodine diet (LID$^+$); the rats continuously received 1% $KCLO_4$ in their distilled drinking water and iodine deficient pellets (Modified Remmington diet, Hope Farms bv) ad libitum (urinary iodine excretion undetectable).

Wistar rats were sacrificed by aortic exsanguination under ether anaesthesia at the start of the experiment and after 3, 4, 6, 8 and 9 weeks. Serum was collected and prepared for TGAb measurement. Each group consisted of 4-8 rats.

Preparation of the serum samples

Serum samples were diluted with an equal volume of phosphate buffered saline (PBS). Immunoglobulin (Ig) fractions were precipitated in 25% saturated ammoniumsulphate at 4°C and centrifuged at 1000x g. After several washing steps, the IgG fractions were dialyzed in PBS for 18 hours at 4°C. The protein concentration of each serum fraction was measured spectrofotometrically at 280 nm.

Measurement of thyroid growth stimulating activity

The growth stimulating activity of the serum samples was measured using the mitotic arrest assay described by Ealy et al[8] and modified by Wilders et al[1]. The rat thyroid cell line FRTL-5 used in this assay was cultured under standard conditions[9]. For the mitotic arrest assay $0.5.10^5$ cells/ml culture fluid were seeded in each well of 24-wells plates (Costar, Cambridge, MA), with round glass

coverslips on the bottom of each well. They were maintained in 6 hormone mixture (6H, see ref[1]) for 7 days and subsequently cultured in the same culture fluid without TSH (5 hormone mixture, 5H) for another 7 days. Every third or fourth day the culture fluid was replaced.

To test the serum samples for their growth potential, purified preparations were added in (when possible) four concentrations (dilutions in 1ml/well 5H, 0.2 mg/ml often proved to be the optimal concentration), plus a suboptimal dose of TSH, namely 10μU TSH/well. To serve as positive control, FRTL-5 cells were cultured in 6H, and as baseline control FRTL-5 cells were cultured in 5H plus the suboptimal dose of TSH (10μU/ml). After a culture period of 44 hours, Colcemid (Sigma), a mitotic spindle poison, was added to each well in a final concentration of 166μg/L to accumulate metaphases during the final 3 hours of culture. Culture fluid was removed and the glass coverslips were airdried for at least two hours. Thereafter, a May-Grünwald Giemsa staining of the cells on the coverslips was performed. Coverslips were removed and mounted upside down on microscope slides.

The mitosis index (MI) was determined by counting the percentage of cells in metaphase using light microscopy. Of each coverslip 500 cells were counted, with a minimum of 5 microscopic fields at a magnification of x400. The relative stimulation of each seperate test was calculated as the ratio between the MI of cells cultured in 5H with the purified serum samples plus 10μU/ml TSH and the MI of cells on 5H plus 10μU/ml TSH alone:

$$\text{relative stimulation} = \frac{\text{MI of cells with Ig preparation + 10}\mu\text{U/ml TSH}}{\text{MI of cells with 10}\mu\text{U/ml TSH}}$$

RESULTS

The growth stimulating activity of the ammonium sulphate-purified serum samples was considered to be positive - on the basis of the data in Wistar rats on a normal iodine diet - when one of the concentrations of serum samples tested resulted in a relative stimulation index (viz mitotic index in the presence of Ig preparation / mitotic index in the absence of Ig preparation) of > 3.

Serum samples of Wistar rats kept on a *normal iodine diet (NID)* did - by definition - not show any growth stimulating activity on the rat thyroid cell line FRTL-5 and relative stimulations ranged from 1 to 3 (4-6 rat Ig preparations tested in each group in 1-4 concentrations, figure 1).

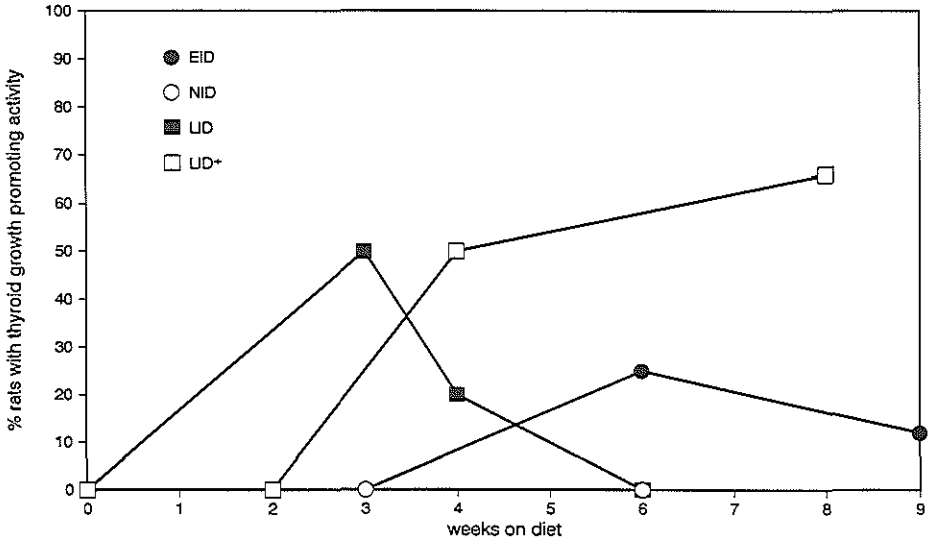

Figure 1. The percentage of rats kept on an enriched iodine diet (EID), a normal iodine diet (NID), a low iodine diet (LID) or an extreme low iodine diet (LID^+) with thyroid growth stimulating activity in their serum, measured in a mitotic arrest assay using the rat thyroid cell line FRTL-5 as indicator cells. The ammonium sulphate-purified serum samples were considered to be positive when relative stimulations of > 3 were found.

Also in rats kept on an *enriched iodine diet (EID)* growth stimulating activity was limited. After 6 weeks of diet 2 out of 8 (25%), and after 9 weeks of diet 1 out of 8 (12%) rat Ig preparations tested stimulated the growth rate of the FRTL-5 cells with a relative stimulation of 4. The other samples were negative (relative stimulation < 3, 6-8 rat Ig preparations tested in 1-5 concentrations).

However, when Wistar rats were kept on a *low iodine diet (LID)* or an *extreme low iodine diet (LID$^+$)*, a considerable proportion of serum samples of the rats had growth stimulating activity. 50% of the rats kept on a LID were positive after 3 weeks of diet (with a relative stimulation of 4, 4 rat Ig preparations tested, in 1-4 concentrations) which incidence declined to 20% after 4 weeks (with a relative stimulation of 6, 5 rat Ig preparations tested, in 2-4 concentrations) to drop to 0 after 6 weeks of diet (with relative stimulations of 1-3, 8 rat Ig preparations tested, in 1-2 concentrations).

The rats on a LID$^+$ also showed thyroid growth stimulating activity a few weeks after introducing the diet (viz at 4 weeks, with relative stimulations of 3.5-4.5, 6 rat Ig preparations tested, in 2-4 concentrations). These rats however remained positive till the end of the experiment: after 8 weeks of diet 66% of the rats tested still showed growth stimulating activity in their serum (relative stimulations of 4.5 and 5, 3 rat Ig preparations tested in 4 concentrations, figure 1).

DISCUSSION

This preliminary study shows that thyroid growth stimulating activity was indeed found in a considerable proportion of ammonium sulphate precipitates of the serum samples of Wistar rats kept on a moderately low (LID) or extreme low iodine diet (LID$^+$). This activity was not detectable in the ammonium sulphate precipitates of the serum samples of Wistar rats kept on a normal iodine diet (NID) and only in a limited number of the precipitates of rats kept on an enriched iodine diet (EID).

Rats kept on a LID showed thyroid growth stimulating activity in their serum fractions relatively early after introducing the diet. During this time serum TSH and thyroid growth were normal (see Chapter 2.1). Goitre development was only observed after 6 weeks of diet (when the incidence of thyroid growth stimulating activity of the serum samples already declined) and at which time serum TSH levels were still within the normal range.

It is possible that the observed thyroid growth stimulating activity of the serum samples in the early phases of the experiment is responsible for or modifies/regulates the goitre development observed somewhat later in these LID rats. The thyroid growth stimulating activity cannot be ascribed to TSH since serum TSH levels were within the normal range. Whether the growth stimulating activity

represents an immunoglobulin effect (for instance an immunoglobulin directed against the TSH receptor or against the IGF-1 receptor) or whether an other growth factor present in the ammonium sulphate precipitates is responsible for the thyroid growth stimulating activity needs further investigation.

Experiments are planned based on a further purification of Ig fractions from the ammonium sulphate precipitates or a neutralization of the thyroid growth stimulating activity via a treatment with α-Ig antibodies or with antibodies to IGF-1, EGF or other growth factors.

In rats kept on a LID^+, thyroid growth stimulating activity was observed in the serum fractions 4 and 8 weeks after introducing the diet. During this time the rats showed a pronounced goitre development (see Chapter 2.1). During this period the rats were hypothyroid and showed highly elevated serum TSH levels. It is however not possible that the observed thyroid growth stimulating activity is due to TSH in the ammonium sulphate-purified serum fractions, since the suboptimal dose of TSH (10μU/ml) added to the FRTL-5 cells in the mitotic arrest assay is already much higher than the TSH present in the serum of the hypothyroid rats.

Our data nevertheless indicate that circulating thyroid growth factors are present in iodine deficient Wistar rats. The stimulation of thyroid autoimmune phenomena in iodine deficient Wistar rats (see Chapter 2.1) suggests that these circulating thyroid growth stimulating factors might likely be thyroid growth stimulating antibodies (TGAbs).

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Chapter 3.1

THYROID AUTOIMMUNE PRONE BB RATS ARE CAPABLE OF COMPENSATING FOR MODERATELY LOW IODINE REGIMENS, RESTORING FULL EUTHYROIDISM*

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ABSTRACT

Female Wistar rats and female autoimmune-prone BB rats were kept on a low iodine diet (LID) for a period of 18 weeks from 3 weeks of age onwards. The LID consisted of 1% $KCLO_4$ in the drinkingwater for 2 days, followed by distilled water and iodine deficient pellets for the remaining period. Rats kept on a for our area normal iodine diet (NID: normal pellets and Rotterdam tap water) served as controls.

In the Wistar rats, the LID resulted in a relatively slow goitre formation (thyroid weights at 18 weeks of diet: 13.5 ± 2.0 (mean ± SD, NID) vs 26.3 ± 7.3 mg (mean ± SD, LID) $p < 0.05$), and a gradual development of a mild hypothyroidism (at 18 weeks of diet NID vs LID values: $T_3 = 1.21 \pm 0.1$ (mean ± SD) vs 1.07 ± 0.1 nmol/l (mean ± SD), $p < 0.05$; $T_4 = 44 \pm 10$ (mean ± SD) vs 21 ± 4 nmol/l (mean ± SD), $p < 0.05$; TSH = 2.3 ± 0.3 (mean ± SD) vs 6.4 ± 2.1 ng/ml (mean ± SD), $p < 0.05$).

In the BB rats the LID resulted in a relatively rapid goitre formation, and maximal thyroidal weight (31.2 ± 8 mg, mean ± SD) was already reached after 6 weeks of diet. Also and in contrast to the Wistar rats, the BB rats only developed a transient subclinical hypothyroidism from 3 till 6 weeks of LID (NID vs LID values after 6 weeks of diet: $T_3 = 1.21 \pm 0.24$ (mean ± SD) vs 1.15 ± 0.14 nmol/l (mean ± SD); $T_4 = 44.8 \pm 8.8$ (mean ± SD) vs 18.1 ± 2.4 nmol/l (mean ± SD), $p < 0.05$; TSH = 1.59 ± 0.8 (mean ± SD) vs 10.7 ± 3.6 ng/ml (mean ± SD), $p < 0.05$). Euthyroidism was fully restored after 9 weeks of the low iodine diet and NID vs LID value were: $T_3 = 1.05 \pm 0.18$ (mean ± SD) vs 1.28 ± 0.1 nmol/l (mean ± SD); $T_4 = 56.5 \pm 8.6$ (mean ± SD) vs 43.8 ± 6.5 nmol/l (mean ± SD) and TSH = 1.35 ± 0.4 (mean ± SD) vs 3.0 ± 1.1 ng/ml (mean ± SD).

Our data show that the autoimmune-prone BB rats of our colony are capable to compensate for mild iodine deficiency by a rapid goitre formation, this in contrast to the Wistar rats that became gradually hypothyroid. Hypothetical explanations for the compensating mechanisms are: 1) an autonomous high intrinsic metabolism and growth rate of BB-thyrocytes such has been described in another thyroid autoimmune animal model, namely the OS chicken and 2) an enhanced production of thyroid growth stimulating antibodies as has been described to be present in our BB rat strain.

INTRODUCTION

Over the past few years we have used two experimental animal strains - viz. the

Wistar rat and the BB rat - to study the effects of the dietary iodine intake on thyroid autoimmune reactivity. The development of thyroid autoimmune reactivity was studied in relation to thyroid performance (serum thyroid hormone levels, TSH levels) and goitre development[1,2,3,4]. During our studies we observed that the thyroid autoimmune prone BB rats were capable to compensate for a moderately low dietary iodine intake in contrast to normal Wistar rats. After a short transient period of subclinical hypothyroidism the BB rats were able to increase their thyroidal T_4 output, restoring full euthyroidism. This report describes the experimental details of this phenomenon and discusses possible explanations.

MATERIALS AND METHODS

Animals and diets

Female Wistar rats were purchased from TNO Rijswijk, The Netherlands and kept at the central experimental animal housing facilities of the Erasmus University Rotterdam under standard conditions.

BB rats were initially provided by Dr. H.A.M. Verheul and Mr. E. Cremers from Organon BV, The Netherlands; presently the strain is bred and kept at the central experimental animal housing facilities of the Erasmus University Rotterdam.

BB rats which became diabetic (at the age of approximately 10 weeks; daily controlled by urinary glucose levels using Gluketur-Test sticks, Boehringer, Germany) were treated subcutaneously with 2IU protamine zinc insulin (daily injections of isofane protamine bovine insulin, Insulinum N.P.H., Organon Oss bv, The Netherlands).

Directly after weaning (at the age of 3 weeks) groups of female Wistar and BB rats (n=5-18) were kept on either a normal or a low iodine diet:

1. *The normal iodine diet (NID)*; the rats received normal pellets (Am-II, Hope Farms bv, Woerden, The Netherlands, ± 0.35 mg/kg iodine) and Rotterdam tap water ad libitum. The daily iodine intake in this group was about 7μg of iodine, verified by urinary iodine excretion (for details of assay see ref[5]).
2. *The low iodine diet (LID)*; rats received 1% $KCLO_4$ in the drinking water for a period of 2 days and thereafter distilled water and iodine deficient pellets (Modified Remmington diet, Hope Farms bv) ad libitum. After 1 week of diet the excreted

urinary iodine during 24 hours was under the detection limit of our assay (for details of assay see ref[5]).

Various groups of these Wistar and BB rats were sacrificed by aortic exsanguination under ether anaesthesia after 3, 6, 9, 12 or 18 weeks of diet. Serum was prepared for T_3, T_4 and TSH evaluation (measured by RIA, Dr. W.M. Wiersinga, Mrs. M. Broenink, Academical Medical Centre, Amsterdam, The Netherlands). The rat TSH reference preparation NIADDK-rTSH-RP-2 was kindly provided by Dr. Parlow from the UCLA Medical Centre, Torrance, and was 176 times more potent than the NIADDK-rTSH-RP-1. Thyroid glands were removed and weighed.

Statistical analysis

Differences in serum T_3, T_4, and TSH were compared by Wilcoxon's rank sum test. The correlation between thyroid weight and serum TSH levels were calculated using linear regression analysis.

RESULTS

Thyroid weight development

Normal iodine diet (NID)

The thyroid weight of female Wistar rats kept on a NID increased gradually from 6.7 ± 1.1 mg (mean $\pm$ SD) to 13.3 ± 2.9 mg (mean $\pm$ SD) after 6 weeks of diet (9 weeks of age), remaining constant thereafter (figure 1A). This thyroid weight development was in line with the body weight development of the Wistar rats and the thyroid/body weight index remained constant namely: 0.07.

The thyroids of the autoimmune-prone BB rats kept on a NID, grew till the end of the experiment and thyroid weights increased from 4.8 ± 0.5 mg (mean $\pm$ SD) at the start of the experiment (3 weeks of age) to reach 18.9 ± 3.5 mg (mean $\pm$ SD) at 21 weeks of age (figure 1B).

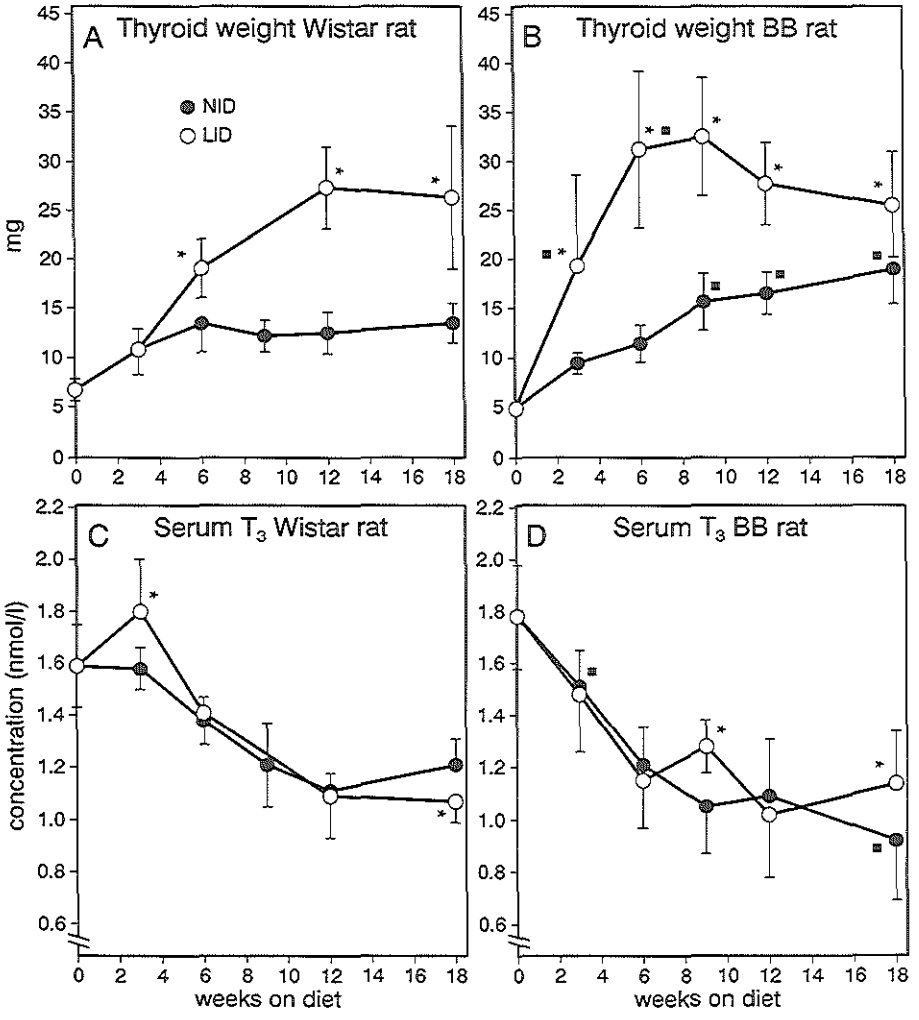

Figure 1. Thyroid weight and serum T_3 levels.
Wistar rats and BB rats were kept on a normal iodine diet (NID) or a low iodine diet (LID) for a period of 18 weeks from 3 weeks of age onwards. Rats were sacrificed, thyroids were removed and weighed to measure goitre development (A, Wistar rat and B, BB rat), serum was collected to measure serum T_3 by RIA (C, Wistar rat and D, BB rat). The mean and standard error are given of 5-18 rats per group.

In fact the thyroid weights of the BB rats on a normal iodine diet became higher than the thyroid weights of the Wistar rats on a normal iodine diet and a statistical significant difference was reached between the two strains at 12 weeks of age (9 weeks of diet, $p<0.05$, Wilcoxon's rank sum test). These results of spontaneous BB goitre development (in relation to the Wistar rat) are in agreement with earlier findings reported by us[6].

Low iodine diet (LID).

In the Wistar rats, a LID resulted in a clear weight increase of the thyroid that was statistically significantly different from the thyroid weights of Wistar rats kept on a NID: after 6 weeks of diet the Wistar thyroid weight was already 19.1 ± 3.0 mg (mean ± SD) and the goitre reached maximal values after 18 weeks of diet, viz. 26.3 ± 7.1 mg (mean ± SD) (figure 1A).

In the BB rats the LID caused an even more rapid and more pronounced goitre development than in the Wistar rats. Already after 3 weeks of diet the thyroid weight was statistically significantly higher than the thyroid weight of the BB rats on a normal iodine diet and also higher than that of the Wistar rats on LID, viz. 19.3 ± 9.3 vs 9.4 ± 1.1 and vs 10.8 ± 2.1 mg (mean ± SD) respectively (both $p<0.05$). Maximal goitre development was observed after 9 weeks of LID (32.5 ± 6.0 mg, mean ± SD) whereafter thyroidal weight slightly decreased to 27.7 ± 4.2 mg (mean ± SD) after 12 weeks of LID and to 25.5 ± 5.4 mg (mean ± SD) after 18 weeks of LID (figure 1B).

Serum thyroid hormone and TSH levels

Rats kept on a normal iodine diet

In Wistar rats receiving a normal diet serum T_3 levels were high at the start of the experiment (1.6 ± 0.2 nmol/l, mean ± SD) and decreased from 6 weeks of age onwards to gradually reach 1.2 ± 0.1 nmol/l (mean ± SD) at 15 weeks of age, remaining at that level thereafter (figure 1C). Serum T_4 levels increased during the first 3 weeks of the experiment to a level of 50 nmol/l, remaining constant thereafter for the further experimental period (figure 2A).

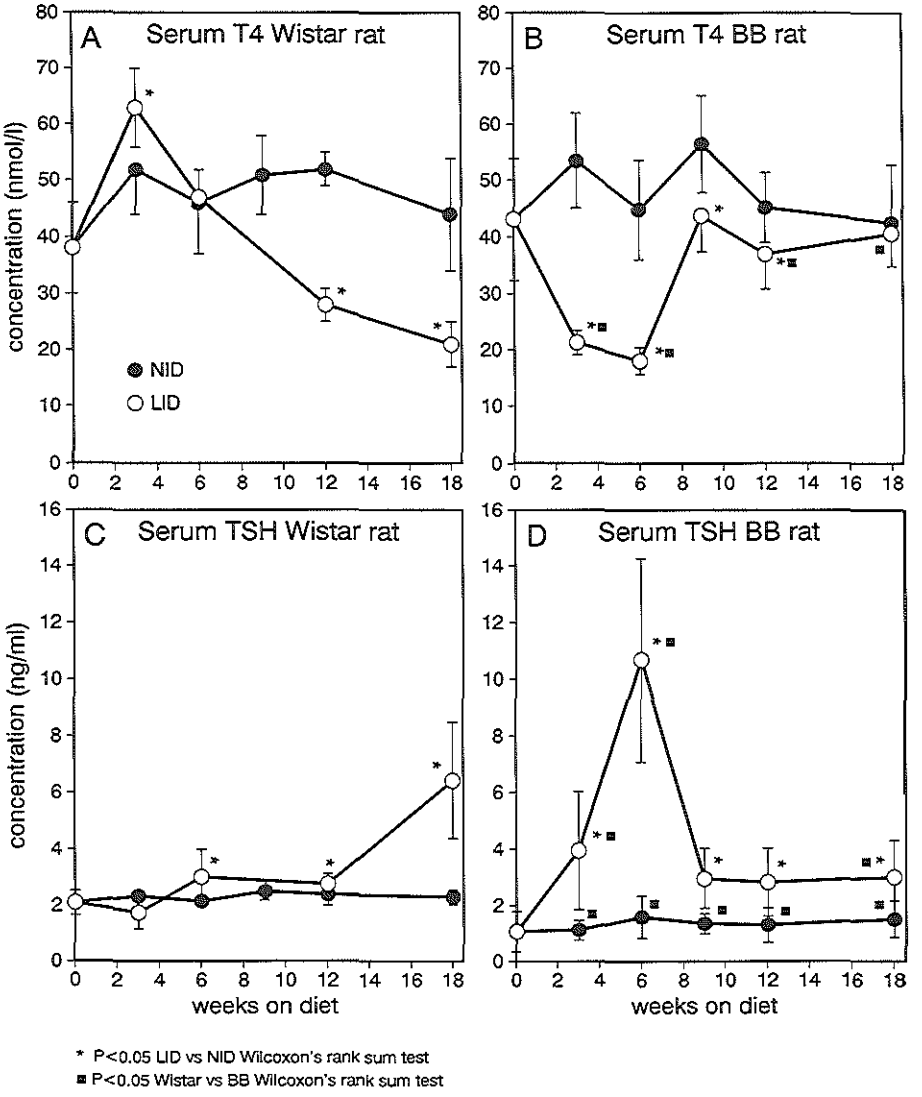

Figure 2. Serum T_4 and serum TSH levels. Wistar rats and BB rats were kept on a conventional (COD) or low iodine diet (LID) for a period of 18 weeks from 3 weeks of age onwards. Rats were sacrificed, serum was collected to measure serum T_4 (A, Wistar rat and B, BB rat) and TSH (C, Wistar rat and D, BB rat). The mean and standard error are given of 5-14 rats per group.

Such relatively high serum T_3 (and T_4) levels in prepubertal rats compared to adult rats have been described before[7] , and these high thyroid hormone levels were regarded by the reporting authors as necessary for optimal brain development in these young rats.

Comparable levels of serum T_3 and serum T_4 were found in the BB rats kept on a normal iodine diet (figure 1D, 2B). Serum T_3 levels were as in the Wistar rats high to begin with (1.8 ± 0.2 nmol/l, mean $\pm$ SD) and gradually declined to 0.9 ± 0.2 nmol/l (mean $\pm$ SD) at 21 weeks of age. Serum T_4 levels in the BB rat were around 50 nmol/l during the entire experimental period.

Serum TSH levels were relatively low in both Wistar and BB rats kept on normal diets as compared to data reported in the literature, but both were within the normal range[3]. The TSH levels of BB rats were lower in comparison to those of the Wistar rats ($p<0.05$, figure 2C, D).

Rats kept on a low iodine diet.

In Wistar rats the T_4 output of the thyroid gradually failed during the LID and the animals became subclinically hypothyroid after 12 weeks of diet. Serum T_4 levels were as low as 28.0 ± 2.5 nmol/l (mean $\pm$ SD) and remained after 12 weeks of diet statistically significantly lower than the levels of the Wistar rats on a normal iodine diet ($p<0.05$, figure 1D). Despite these low serum T_4 levels, serum T_3 levels were normal till 12 weeks of diet. Nevertheless, after 18 weeks compensation started to fail and a slight but statistically significant decrease in serum T_3 was seen when compared to the serum T_3 level of Wistar rats on a normal iodine diet (figure 1C).

The fall in serum T_4 (and later T_3) levels in iodine deficient Wistar rats was accompanied by a significant rise in serum TSH level. A small rise in serum TSH levels could already be detected after 6 weeks of LID, but a more pronounced rise was found at 18 weeks of LID (the latter 6.4 ± 1.8 ng/ml, mean $\pm$ SD, LID vs 2.3 ± 0.3 ng/ml mean $\pm$ SD, NID figure 2C).

It is worthy to note that the increase in thyroid weight during the first 12 weeks of the diet was not related to a meaningful increase in serum TSH levels.

In BB rats, a LID resulted in a completely different thyroid hormone output when compared to the Wistar rats output during a LID. A clear, but short transient subclinical hypothyroidal state was observed after introducing the low iodine diet. This subclinical

hypothyroidal state only lasted for a period of upto 9 weeks of LID. From 9 weeks of LID onwards full euthyroidism was restored. In detail: serum T_4 levels started to drop after 3 weeks of LID (from 53.5 ± 8.5, mean ± SD to 21.3 ± 2.2 nmol/l, mean ± SD) and reached a minimal value of 18.1 ± 2.4 nmol/l (mean ± SD) after 6 weeks of LID (figure 2B). At the same period a steep and clear increase in serum TSH levels was found: after 3 weeks of LID serum TSH rose from 1.1 ± 0.7 (mean ± SD) to 4.0 ± 2.1 nmol/l (mean ± SD) and after 6 weeks of LID a maximal value of 10.7 ± 3.6 nmol/l (mean ± SD) was reached (figure 2D). It must be recalled that during this period of subclinical hypothyroidism a rapid goitre development was observed in the BB rat; in fact a positive correlation existed between thyroid weight development, rises in serum TSH levels and falls in T_4 production in this period ($r=0.72$, $p<0.01$ and $r=-0.70$, $p<0.01$ respectively). Serum T_3 levels in the BB rat remained in the normal range during the entire experimental period (figure 1D).

DISCUSSION

This study shows that BB rats are capable to compensate for moderately low dietary iodine intakes. After an initial transient period of biochemical hypothyroidism, the BB thyroid was capable to increase its T_4 output, thereby restoring normal serum T_4 levels. The initial sharp drop in the serum T_4 level in the BB rat was accompanied by an increase in the serum TSH level and a rapid goitre development. This goitre development was earlier in time and more pronounced than the goitre development of Wistar rats kept on a the same low dietary iodine regimen. In these latter Wistar rats the iodine deficiency gradually led to thyroid insufficiency resulting in a state of biochemical hypothyroidism at the end of the experimental period. When BB rats are kept on a severe iodine deficient diet (continuously $KClO_4$ in the drinking water and iodine deficient pellets) there is again a rapid goitre development; however, thyroid metabolism fails completely and biochemical hypothyroidism is reached at 2 weeks of severe iodine deficient diet (data not shown).

The mechanisms underlying the capability of the BB rats to compensate for moderately low iodine intakes are unknown and remain speculative. It is tempting to speculate that the mechanisms are related to the thyroid autoimmune proneness of the animals.

An intrinsically high metabolism of thyrocytes has been described to be one of the prerequisites for the development of thyroid autoimmunity in the OS chicken[8], and there are indications that the thyrocyte metabolism, the iodine turnover and the growth rate are also intrinsically high in BB rats:

- firstly histological parameters indicate that BB thyrocytes are more active than normal Wistar thyrocytes (the nuclear volume of BB thyrocytes is larger and the thyrocytes show a high columnar appearance, data to be published),
- secondly, Li et al have reported[9] that (iodine treated) BB rats have a higher radioactive iodine uptake and release than Wistar rats, and
- thirdly, prelimenary data of our own group show that basic thyrocyte proliferation and the proliferative response to TSH is higher and much more pronounced in cultured BB rat thyroid follicles in comparison to these parameters in cultured Wistar rat thyroid follicles (data not shown).

If there is such an intrinsically high and autonomous metabolism and proliferation rate of BB thyrocytes, this autonomously high proliferative and metabolic activity might explain the rapid thyroid growth and the early rapid phase of low T_4 output (viz. rapid development of failure due to high iodine turnover) in BB rats during iodine deficiency. It might also form the basis for the development of the thyroid autoimmune reactivity in the BB rat if one envisages that the high metabolic and proliferative activity of the BB thyrocytes induces an enhanced expression of autoantigens and/or a significant release of antigenic degradation products, thereby triggering the animal's immune system (see similar mechanisms in the OS chicken, ref[10]).

However, there might also be an other explanation for the observed compensation mechanisms of the BB rats to moderate iodine deficiency. This explanation is a direct consequence of the thyroid autoimmune response of the animals. The BB rat is capable of producing thyroid growth stimulating immunoglobulins (TGIs)[2] and the presence of these TGIs might induce the rapid thyroid growth, possibly in combination with the raised TSH. Consequently there might be a higher T_4 output by the larger goitre. Research in our laboratory is ongoing to also study this latter hypothesis.

In conclusion, our experiments show that autoimmune prone BB rats are capable of compensating for mild iodine deficiency (in contrast to Wistar rats). The mechanisms for this compensation are speculative. Possibly analogous situations exists in human populations, where some individuals are capable to compensate for a low iodine intake by rapid goitre development. It is intruiging that these goitrous

individuals have been described to show various signs of thyroid autoimmunity viz. the presence of TGIs, increased numbers of intrathyroidal dendritic cells and aggregations of these cells and higher incidences of anti-thyroglobulin antibodies[11,12,13,14]. Again it is the question, whether these thyroid autoimmune phenomena form the basis for the goitre development[11,15] or whether they are mere markers of an intrinsically high autonomous thyroid growth rate and metabolism.

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Chapter 3.2

BB RAT THYROCYTES SHOW A HIGH PROLIFERATION RATE IN VITRO. A PRELIMINARY REPORT

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INTRODUCTION

The BB rat is a well known animal model for the study of autoimmune thyroid disease. The rat develops a focal thyroiditis, a goitre and shows a variety of thyroid reactive autoantibodies[1,2].

In Chapter 3.1 of this thesis we report that the BB rat is capable of compensating for a moderately low dietary iodine intake (in contrast to normal Wistar rats), restoring full euthyroidism by rapidly growing a large goitre. In the discussion of that Chapter we hypothesized that the BB thyrocytes might have an intrinsically high metabolism and growth rate. Such an intrinsically high thyroid metabolism has been described for the OS chicken, another animal model of autoimmune thyroid disease[3].

To verify this hypothesis we isolated thyroid follicles of autoimmune-prone BB rats and normal Wistar rats (10-15 weeks of age) and cultured these follicles in the absence or presence of TSH. The growth rate and growth rate response to TSH of the BB and Wistar rat thyroid follicles were measured using BrdU incorporation.

MATERIALS AND METHODS

Animals

Female Wistar rats were bred at TNO Rijswijk, The Netherlands and kept at the central experimental animal housing facilities of the Erasmus University Rotterdam under normal conditions. BB rats were kindly provided by Dr. H.A.M. Verheul and Mr. E. Cremers from Organon BV, The Netherlands and were further bred and kept at the central experimental animal housing facilities of the Erasmus University Rotterdam under normal conditions. BB rats which became diabetic (at the age of 10 weeks daily controlled by urinary glucose levels using Gluketur-Test sticks, Boehringer, Germany) were daily subcutaneously injected with 2IU protamine zinc insulin (isofane protamine bovine insulin, Insulinum N.P.H., Organon Oss bv, The Netherlands). All rats received commercial food (Am-II, Hope Farms bv, Woerden, The Netherlands) and tap water ad libitum. At the age of 10-15 weeks rats were sacrificed either under ether unaesthesia by aortic exsanguination or using CO_2 asphyxiation.

Isolation of thyroid follicles

Thyroid follicles were isolated as described before[4] with slight modifications. Thyroid lobes of 5 rats (10-15 weeks of age) were removed under sterile conditions and placed in Hanks medium without Ca^{2+} and Mg^{2+} (Gibco) at room temperature. The medium was removed and replaced by 5 ml Hanks (without Ca^{2+} and Mg^{2+}) supplemented with 5 mM $CaCL_2$, 475 U/ml collagenase (type II, Sigma) and 0.1 mg/ml DNAse (type I, grade 2, Boehringer, Germany). The lobes were mechanically dissected using small scissors and incubated for either 30 minutes (for BB rat thyroids) or 45 minutes (for Wistar rat thyroids) at 37°C in a 95% O_2, 5% CO_2 water-saturated atmosphere. After incubation the suspension was 10 times gently pipetted using a Pasteurs capillary pipette (short size, 150 mm, WU, Mainz, Germany). Thyroid fragments were left to sediment during 1 minute. The isolated follicles present in the supernatant were removed and kept at 4°C. Undigested fragments were incubated in 5 ml fresh collagenase/DNAse medium for either 15 minutes (for BB rat thyroids) or 20 minutes (for Wistar rat thyroids) at 37°C in a 95% O_2, 5% CO_2 water-saturated atmosphere. After incubation the suspension was again gently pipetted, thyroid follicles were removed and the remaining undigested fragments were incubated with 5 ml fresh collagenase/DNAse medium for the same period as described above. This procedure was repeated 3 to 4 times untill the complete thyroid was digested. The isolated follicles were centrifuged at 20 x g for 5 minutes and washed 3 times with 7 ml ice-cold Hanks (without Ca^{2+} and Mg^{2+}) supplemented with 5 mg/ml bovine serum albumin (BSA, Sigma) and 0.1 mg/ml DNAse. The final suspension was, when necessary, filtered through a 150 μm mesh nylon filter (Kabel Zaandam BV, The Netherlands). The isolated follicles were centrifuged at 500 x g for 5 minutes and resuspended in 1 ml Ham's F-12 medium (Flow, ICN Biomedicals BV, The Netherlands) supplemented with a 5 hormone mixture (5H) containing 10 μg/ml insulin (Sigma), 10 nM hydrocortison (Sigma), 5 μg/ml transferrin (Sigma), 10 ng/ml glycyl-L-histidyl-L-lysine-acetate (Sigma), 10 ng/m somatostatin (Sigma), and 0.5% heat inactivated (30 mins., 56 °C) fetal calf serum (FCS, Integro bv, Zaandam, The Netherlands), 100 U/ml penicillin G (Seromed, Biochrom, Berlin, Germany), 0.1 mg/ml streptomycin (Seromed, Biochrom, Berlin, Germany), and 2.5 μg/ml amphatracin B (Boehringer, Germany).

Viability of isolated follicles

The viability of isolated follicles was routinely assessed by the trypan blue exclusion test according to Phillips[5]. The counting was performed in a Bürker-Türk hemocytometer using light microscopy (magn. 100X). Directly after the isolation procedures 90-98% of the thyroid follicles were viable.

Cultering of thyroid follicles

The isolated thyroid follicles were seeded in 12 wells plates (Costar, 1-4.10^4 viable follicles/ml, 1 ml per well) in Ham's F-12 medium supplemented with 5H (see before), 0.5% FCS and antibiotics at 37°C in a 95% O_2, 5% CO_2 water-saturated atmosphere. The medium was refreshed at day 1 and 3. For stimulation experiments a concentration of 10 mU/ml of bovine TSH (Sigma) was added to the culture medium (in a preliminary study this concentration proved to be optimal) at day 1.

Growth of thyroid follicles

The growth of thyroid follicles was measured using BrdU incorporation according to a method that has been described before[6]. In brief, the follicles were harvested (1-4.10^4/ml), diluted 10 times in F-12 supplemented with 0.5% FCS and antibiotics, and incubated for 2 hours in the presence of BrdU (Sigma) in a final concentration of 10 μM at 37°C in a 95% O_2, 5% CO_2 water-saturated atmosphere. After the incubation period the follicles were washed 2 times (at 500 x g for 5 minutes) in phosphate buffered saline (PBS) supplemented with 1% glucose. The follicles were fixed for 30 minutes in 70% ethanol at 4°C and washed 2 times (at 500 x g for 5 minutes) in PBS. The incorporated BrdU was visualized by incubating the follicles for 60 minutes with a mouse monoclonal antibody directed against BrdU (Becton and Dickinson, USA) diluted 25 times in PBS containing 1% BSA at room temperature. Follicles incubated in PBS only containing 1% BSA for 60 minutes served as controls. The follicles were washed 2 times (at 500 x g for 5 minutes) in PBS containing 10% BSA and incubated for 30 minutes with a 25 times

diluted (in PBS with 1% BSA) rabbit anti-mouse-immunoglobulin monoclonal antibody labeled with FITC (fluorescein isothiocyanate, Dako, Denmark) at room temperature in the dark. The follicles were washed 2 times in PBS containing 10% BSA (at 500 x g for 5 minutes) and the final pellet was resuspended in 100 μl PBS with 10% BSA. 50 μl of the thyroid follicle suspensions at a concentration of 1.10^5/ml were cytocentrifuged (3 minutes, 1100 rpm) using a cytospin apparatus (Nordic Immunological Laboratories, The Netherlands) and cytocentrifuge preparations were embedded in aquamount (Gurr, BDH Limited Poole, England). The percentage positive labeled thyroid follicles was counted using a fluorescense microscope.

RESULTS

BB thyroid follicles isolated from 10-15 weeks old animals had a spontaneous higher growth rate as compared to Wistar thyrocytes when cultured in the absence of TSH, eg in the 5H mixture (see figure 1).

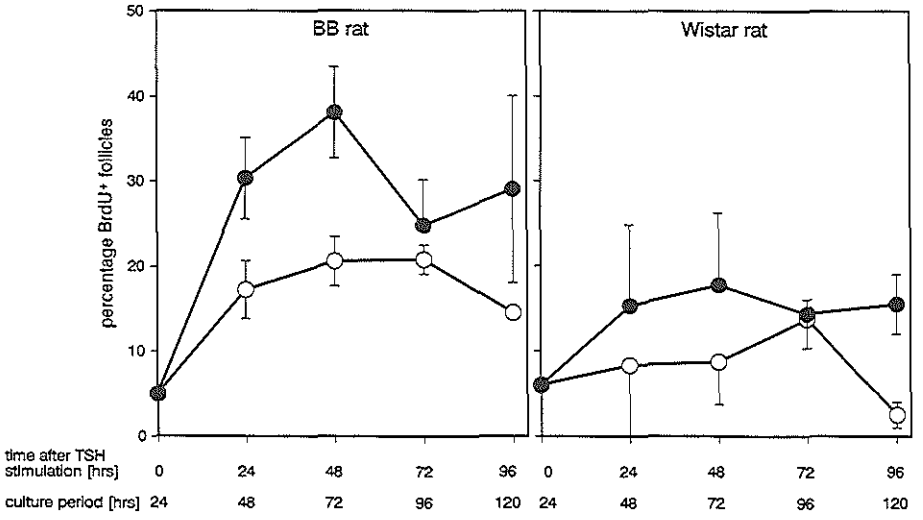

Figure 1. The growth rate measured using BrdU incorporation of thyroid follicles isolated from 5 (10-15 weeks old) BB rats (left panel) and 5 Wistar rats (right panel) cultured in the absence (open circles) or presence (closed circles) of 10 mU/ml TSH. The mean and standard deviation of 3 experiments are depicted.

A maximum of around 20% (the mean of 3 experiments) $BrdU^+$ BB rat thyroid follicles was observed after a culture period of 72 to 96 hours, whereas thyroid follicles of Wistar rats did not exceed a 14% positivity at 96 hours of culture (the mean of 3 experiments, figure 1).

Figure 1 also show the data of *in vitro* TSH stimulation, introduced at 24 hours of culture. The BB rat thyroid follicles clearly had a higher response to TSH in comparison to Wistar thyroid follicles ($38.5 \pm 5.4\%$ $BrdU^+$ follicles vs $17.7 \pm 8.5\%$, the mean of 3 experiments, 48 hours after TSH administration, optimal time; the optimal TSH dose was 10mU/ml for both rat thyrocyte cultures).

CONCLUSIONS

This preliminary study shows that BB rat thyroid follicles had a higher spontaneous growth rate in comparison to Wistar rat thyroid follicles when cultured in the absence of TSH. The response to an optimal dose of 10 mU/ml TSH was also in the BB rat thyroid follicle cultures increased compared to the Wistar rat thyroid follicle cultures. The slope of the curves suggests that this enhanced activity in the BB rat is an intrinsic phenomenon of the BB thyrocytes and not induced by a circulating factor outside the BB thyrocytes (in this case we had expected a strong BrdU incorporation in the beginning, declining during the culture period). Howerver, additional experiments are needed to clarify this issue.

We must also keep in mind that only a limited number of experiments were performed untill now (3 of each) and more experiments are needed to carry out a valid statistical analysis.

A high intrinsic proliferation rate of BB thyrocytes might indeed explain the rats' capability to rapidly grow a large goitre during iodine deficiency and to cope with mild iodine deficiency. It may also form the basis of the spontaneously developing autoimmune thyroiditis, since it has been shown for the OS chicken that genetically determined thyroid abnormalities exist prior to the development of the spontaneous thyroiditis in this animal (next to immunoregulatory disturbances)[3].

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Chapter 4

A HIGH IODINE INTAKE IN WISTAR RATS RESULTS IN THE DEVELOPMENT OF A THYROID-ASSOCIATED ECTOPIC THYMIC TISSUE AND IS ACCOMPANIED BY A LOW THYROID AUTOIMMUNE REACTIVITY*

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ABSTRACT

Evidence is accumulating that the dietary iodine intake is an important modulator of autoimmune thyroid reactions. To study this role of iodine intake further, female Wistar rats were kept on either an enriched iodine diet (EID, iodine intake: 100μg iodine/day), a normal iodine diet (NID, iodine intake 7μg iodine/day) or a low iodine diet (LID, 2 days of 1% $KCLO_4$ followed by iodine deficient drinking water/pellets) for periods up to 18 weeks.

The low iodine intake resulted - as could be expected - firstly in a gradual goitre development: thyroid weights: 27.3±4.2 mg (mean±SD, LID) vs 12.5±2.1 mg (mean±SD, NID) after 12 weeks of diet, and secondly in subclinical hypothyroidism after 18 weeks of diet: serum TSH: 6.4±2.1 ng/ml (mean±SD, LID) vs 2.3±0.3 ng/ml (mean±SD, NID) and serum T_4: 21.0±4.0 nM/l (mean±SD, LID) vs 44.0±10.0 nM/l (mean±SD, NID). Thyroid autoimmune reactions were also enhanced in LID animals and a higher incidence of anti-colloid antibodies, viz 60% positivity (vs 36%, NID) after 18 weeks of diet, and a higher thyroidal infiltration with lymphoid cells as compared to NID rats were found.

The enriched iodine intake resulted in a normal thyroid growth and in euthyroidism with borderline low serum T_3 levels. The excessive iodine diet however also resulted in a lowered thyroid autoimmune reactivity as compared to the NID and LID rats viz. in a lower incidence of anti-colloid antibody production (12.5%, EID vs 36%, NID and 60%, LID after 18 weeks) and lower numbers of intrathyroidal lymphoid cells, viz number of dendritic cells/mm^2: 34.1, EID vs 36.5, NID and 51.9, LID, number of $CD4^+$ cells/mm^2: 1.6, EID vs 1.8, NID and 10.3, LID and number of $CD8^+$ cells/mm^2: 1.8, EID vs 2.3, NID and 6.4, LID after 3 weeks of diet. Remarkable was also the development of a thyroid-associated ectopic thymic tissue in the rats on an enriched iodine diet (in 39% of the animals on EID vs 8% in NID rats and 0% in LID rats during the first 6 weeks, gradually declining to 16% in EID rats vs 8% in NID rats and 8% in LID rats from 9 to 12 weeks of diet). This thyroid-associated ectopic thymic tissue showed a similar marker pattern as normal rat thymus concerning TdT expression (positive cells in the cortex), CD4/CD8 positivity (double positive cells in the cortex, single positive cells in the medulla) and MHC class-II expression (in between the thymocytes).

It is hypothesized that this thyroid-associated ectopic thymic tissue is related to the low thyroid autoimmune response by playing a role in tolerance induction.

INTRODUCTION

The last two decades it has become clear that a high dietary iodine intake may aggrevate thyroid autoimmune reactivity in both humans as well as experimental animals suffering from thyroid (autoimmune) disease.

In humans with a preexisting thyroid abnormality, such as an iodine deficient goitre, a single administration of a high dose of iodine resulted in an attack of thyroiditis positive for anti-thyroglobulin and anti-microsomal antibodies[1,2,3]. A chronic excess of dietary iodine led in certain populations (Japan, China) to the development of a variant of endemic goitre. Patients with this goitre were for 60% positive for immunoglubulins that stimulate thyrocyte proliferation *in vitro*[4,5], the socalled thyroid growth stimulating immunoglobulins (TGI). It must be noted however, that the character and meaning of these TGIs are still under debate[6,7].

Uptill now experimental animal studies on the effect of the dietary iodine on thyroid autoimmune reactivity have predominantly been performed in animals genetically prone for autoimmune thyroid disease or in animals with a (mostly iodine deficient) hyperplastic goitre. In the Cornell C Strain of chicken and in the BB rat it was shown that a high dietary intake of iodine led to an increase in the incidence and severity of the thyroiditis[8,9] of these animals; a low iodine intake ameliorated the disease. A single high administration of iodine to iodine deficient hamsters with a hyperplastic goitre or to mice with a methimazole induced hyperplastic goitre resulted in an attack of transient thyroiditis[10,11,12], positive for thyroid autoantibodies.

Since it is not clear whether a high iodine intake also precipitates thyroid-directed autoimmune responses in normal, non-autoimmune animals we kept groups of normal female Wistar rats on an enriched, normal and low iodine diet for periods of up to 18 weeks from the third week of life onwards. Thyroid weight and thyroid hormone values were measured during the experimental period. Thyroid autoimmune reactivity was evaluated by measuring anti-colloid antibodies in the serum and by enumerating the number of thyroid infiltrated lymphocytes and dendritic cells.

MATERIALS AND METHODS

Animals and diets

Female Wistar rats were purchased from TNO Rijswijk, The Netherlands and kept at the central experimental animal housing facilities of the Erasmus University Rotterdam under standard conditions.

Directly after weaning (at the age of 3 weeks) groups of rats (n=5-18) were kept on three dietary iodine regimens:

1. *An enriched iodine diet (EID)*; the rats received normal pellets (Am-II, Hope Farms bv, Woerden, The Netherlands, ± 0.35 mg/kg iodine) as well as an extra iodine supplementation of 6.5mg KI/l added to the drinking water ad libitum.
2. *A for our area normal iodine diet (NID)*; the rats received normal pellets (Am-II, Hope Farms bv) and Rotterdam tap water ad libitum. These groups of rats served as controls.
3. *A low iodine diet (LID)*; the rats received 1% $KCLO_4$ in the drinking water for a period of 2 days and thereafter distilled water and iodine deficient pellets (Modified Remmington diet, Hope Farms bv) ad libitum.

Groups of above described Wistar rats were sacrificed by aortic exsanguination under ether anaesthesia at 3, 4, 6, 9, 12 or 18 weeks after starting the diet. Serum was prepared for T_3, T_4, TSH evaluation and anti-colloid antibody determination. T_3, T_4, and TSH were measured by RIA (Dr. W. Wiersinga, Margreet Broenink, Amsterdam, The Netherlands). The rat TSH reference preparation NIADDK-rTSH-RP-2 was kindly provided by Dr. Parlow from the UCLA Medical Centre, Torrance, USA, and was 176 times more potent than the NIADDK-rTSH-RP-1. Thyroid glands were removed, weighed and stored at -80 °C until immunohistological examination.

Iodine measurement in rat urine

Rat urine was collected during 24 hours by keeping the rats in metabolic cages. Urinary excreted iodine was measured using a modified method described

by Benotti et al[13]. In brief, the urine samples were destructed by adding an equal volume of 30% chloric acid and incubating the samples for several hours at 200 °C. The iodine reacted as a cathalysing agent in the redox reaction between cerium ammonium sulphate and arsenic acid. During the reaction the yellow Ce^{4+} was converted into the colourless Ce^{3+} which was measured using an ELISA reader at 405 nm.

Immunohistological examination

Immunohistological examination was performed according to Green et al.[14] with minor modifications. Of each frozen thyroid, one lobe was semiserially cut into 6 μm thin sections. The sections were airdried overnight and fixed in acetone at room temperature for 10 minutes. The sections were incubated with either a monoclonal antibody specific for MHC class II molecules, total T cells, $CD4^+$ T cells, $CD8^+$ T cells or B cells (see table 1) for 60 minutes, washed with phosphate buffered saline (PBS) containing 0.2% bovine serum albumin (BSA, Sigma Chemical Co, USA) for 10 minutes, and further incubated with a rabbit anti-mouse Ig-HRP labeled conjugate (Dakopatts, Denmark) for 30 minutes, diluted 300 times in PBS containg 1% normal rat serum and 0.2% BSA. After washing the slides in PBS containing 0.2% BSA (10 minutes) and rinsing them with 0.1 M sodium acetate buffer, pH 6.0, the sections were developed with a metal-enhanced 3,3'-diaminobenzidine (DAB) solution containing 0.05% DAB (Sigma), 1% nickel sulphate (Merck), 0.068% imidazole (Sigma), and 0.8% sodium chloride in 0.1M acetate buffer pH 6.0 for 3-5 minutes. Hydrogen peroxide was added to a final concentration of 0.01%. After DAB development, the sections were washed briefly in 0.1M Tris-HCl buffer, pH 7.6, and immersed in a 0.5% solution of cobalt chloride in 0.1M Tris-HCl buffer, pH 7.2 at room temperature for 4 minutes. The slides were either counterstained with 0.1% nuclear fast red in 5% aluminium sulphate for 2 minutes or incubated with acid phosphatase at 37°C for 30 minutes and thereafter counterstained with hematoxylin. The slides were dehydrated and embedded in DePeX mounting medium (Gurr, BDH Limited Poole, England).

For the immunohistological detection of terminal deoxynucleotidyl transferase (TdT) which is only found on immature lymphoid cells, such as cortical thymocytes, bone marrow precursor cells and malignant cells from acute lymphoblastic

leukemia patients[15,16,17], a method described by Gregoire et al was used[18]. In brief, thyroid sections were fixed in methanol at 4° C for 30 minutes. Thereafter the slides were washed 5 times with PBS at 4° C for 5 minutes and incubated in icecold PBS at room temperature for a period of 30 minutes. The slides were further incubated with a rabbit anti-TdT monoclonal antibody (Supertechs Inc. Bethesda, MD, USA) diluted 60 times in PBS containing 0.2% BSA at room temperature for 60 minutes. The slides were washed 3 times with PBS for 10 minutes and incubated with swine anti-rabbit Ig-HRP labeled (Supertechs Inc. Bethesda, MD, USA) diluted 100 times in PBS containing 0.2% BSA and 1% normal rat serum for 30 minutes. Thereafter slides were washed, developed and embedded as described above.

For the quantification of infiltrated leucocytes 4 sections of each thyroid with intervals of at least 100μm were reacted with the appropriate marker monoclonal antibody. Infiltrated $CD4^+$ and $CD8^+$ T cells were counted in each entire thyroid section. Infiltrated dendritic cells (DC, identified as strong MHC class-II positive cells with cytoplasmic processes, a reniform nucleus and weak or absent acid phosphatase activity) were counted in 10 microscopic fields of each thyroid section (total of 40 microscopic fields/thyroid) using light microscopy at a magnification of 400x. The surface area of the thyroid sections and of the microscopic fields in which the infiltrated cells had been counted was measured using a camera attached to a Leitz Diaplan light microscope and a Videoplan image processing system (Kontron, Bild Analyse GmbH, Germany) (surface area of 10 microscopic fields: 1.59 mm^2). The number of infiltrated dendritic cells, $CD4^+$ T cells and $CD8^+$ T cells were expressed per mm^2 surface area of a thyroid section. It is of importance to note that similar results were obtained when the number of dendritic cells and T cells were not expressed per mm^2 surface area of the section but for instance expressed per observed thyroid follicle in the plane of section or per surface area of thyrocyte parenchyma.

Table 1. Monoclonal antibodies (MoAbs) used for the detection of leucocytes

MoAb	Specificity	Reference
OX6 (Seralab, UK)	MHC class-II	19
W3/25 (Seralab, UK)	CD4 antigen	20
B115-4 (Holland Biotechnology, The Netherlands)	CD4 antigen	21
B115-5 (Holland Biotechnology)	CD5 antigen	21
OX8 (Seralab, UK)	CD8 antigen	22
HIS 14 (F.G.M. Kroese)	B cells	23,24
anti-human TdT (Supertechs, USA)	TdT	25

Anti-colloid antibody determination

6 μm thin frozen porcine thyroid sections were cut, airdried overnight and fixed in cold acetone (-20 °C) for 10 minutes. The sections were preincubated with normal rabbit serum (DAKO-immunoglobulins, Denmark) (diluted 50 times in PBS with 1% BSA) for 10 minutes. Subsequently, the rat sera were applied (in duplicate, diluted 10 times in 0.9% NaCl) and the slides were incubated at room temperature for 60 minutes. Porcine tissue was used , since it gave optimal results as compared to rat or human tissue. After washing in PBS, the sections were incubated with rabbit anti-rat immunoglobulins, fluorescein-isothiocyanate-(FITC)-labeled (DAKO immunoglobulins, Denmark) for 30 minutes (diluted 25 times in PBS with 1% BSA). After this second step and rewashing, the slides were embedded in aquamount (Gurr, BDH Limited Poole, England) and examined using a fluorescense microscope. Three control slides were included; one without incubation of rat serum, another incubated with a rat serum previously scored as negative and a third incubated with a rat serum previously scored as positive. The staining intensity of each serum was arbitrarily and blindly scored (under code, by two independent investigators) as negative, positive or strongly positive.

The indirect immunofluorencense technique to detect anti-colloid antibodies has the advantage that it also directly provides data on various other antigenic compartments: in the same simple, cheap and reliable test anti-colloid, anti-

cytoplasmic and anti-nuclear antibodies are simultaneously detected. The test can be performed in titration, however it is our experience that scoring the sera as negative, positive or strongly positive correlates well with titration or in other words, strongly positive sera in the dilution described above are of high titre.

Statistical analysis

Differences in serum T_3, T_4, and TSH, and number of intrathyroidal dendritic cells and T cells were compared by Wilcoxon's rank sum test. The incidence of anti-colloid antibody production and the incidence of thyroid associated ectopic thymic tissue was tested by X^2 analysis.

RESULTS

Thyroid endocrine performance

The *enriched iodine diet (EID)* had no observable thyroidal endocrine effects (figure 1A, B, C, D) and for our area normal thyroid weights and serum thyroid hormone levels were recorded (with borderline low serum T_3 levels in EID rats, figure 1C). The urinary iodine excretion rate in EID rats was however high: an excretion of 50 μg I/day was measured after 1 week of diet which reached a plateau of 100 μg I/day after 3 weeks (6 weeks of life), reflecting the estimated daily iodine intake. The urinary iodine excretion of rats on a NID was 7 μg of iodine per 24 hours.

The *low iodine diet (LID)* resulted, as could be expected, in a statistically significantly increased thyroid weight after 6 weeks of diet and a lowered thyroidal T_4 output after 12 weeks of diet ($p<0.05$, Wilcoxon's rank sum test, figure 1A and D). Serum T_3 levels stayed within the normal range (figure 1C) and the animals showed a normal body weight development (data not shown). Serum TSH levels started to increase after 12 weeks of diet ($p<0.05$, figure 1B). After 1 week of diet the urinary iodine excretion was already under the lower limit of detection of our assay, reflecting the low iodine intake.

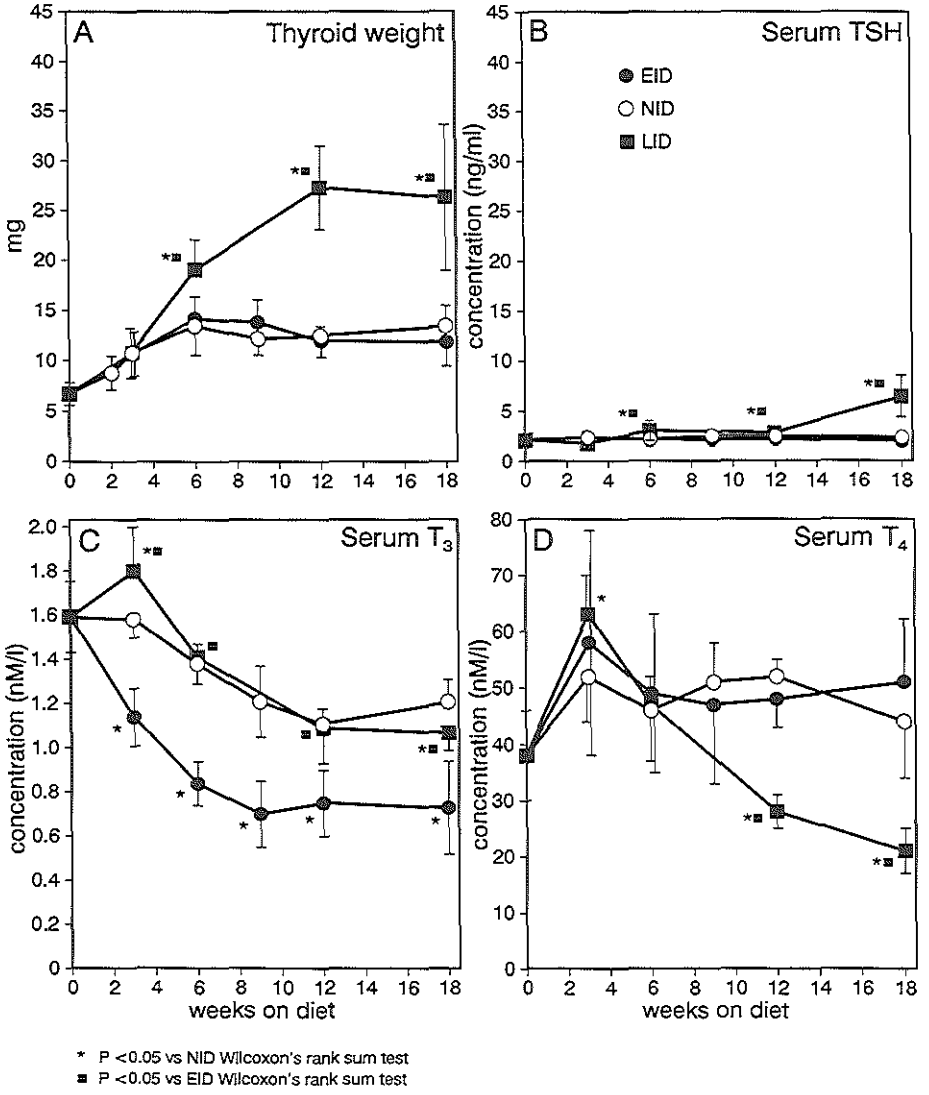


Figure 1. Thyroid weight and endocrine status. Female Wistar rats were kept on a normal iodine diet (NID), a low iodine diet (LID) or an enriched iodine diet (EID) for a period of 18 weeks from 3 weeks of age onwards. Rats were sacrified and thyroids were removed and weighed to measure goitre development (A), serum was collected to measure serum TSH (B), serum T_3 (C), and serum T_4 (D) levels by RIA. The mean and standard deviation are given of 5-18 animals per group.

Signs of thyroid autoimmune reactivity

Anti-colloid antibody production

At the start of the experiment (3 weeks of age) the incidence of anti-colloid antibodies was 4% (n=24). The anti-colloid antibodies showed a floccular staining pattern in the indirect immunofluorescence test and therefore most likely represent thyroglobulin-specific autoantibodies. Anti-cytoplasmic or anti-nuclear antibodies were not found in any of the groups of rats tested.

In rats receiving an *EID*, the incidence of anti-colloid antibodies did not increase in the period from 3 weeks to 8 weeks of life, and remained thereafter at a level not exceeding a 12.5% incidence (n=8-16, figure 2A).

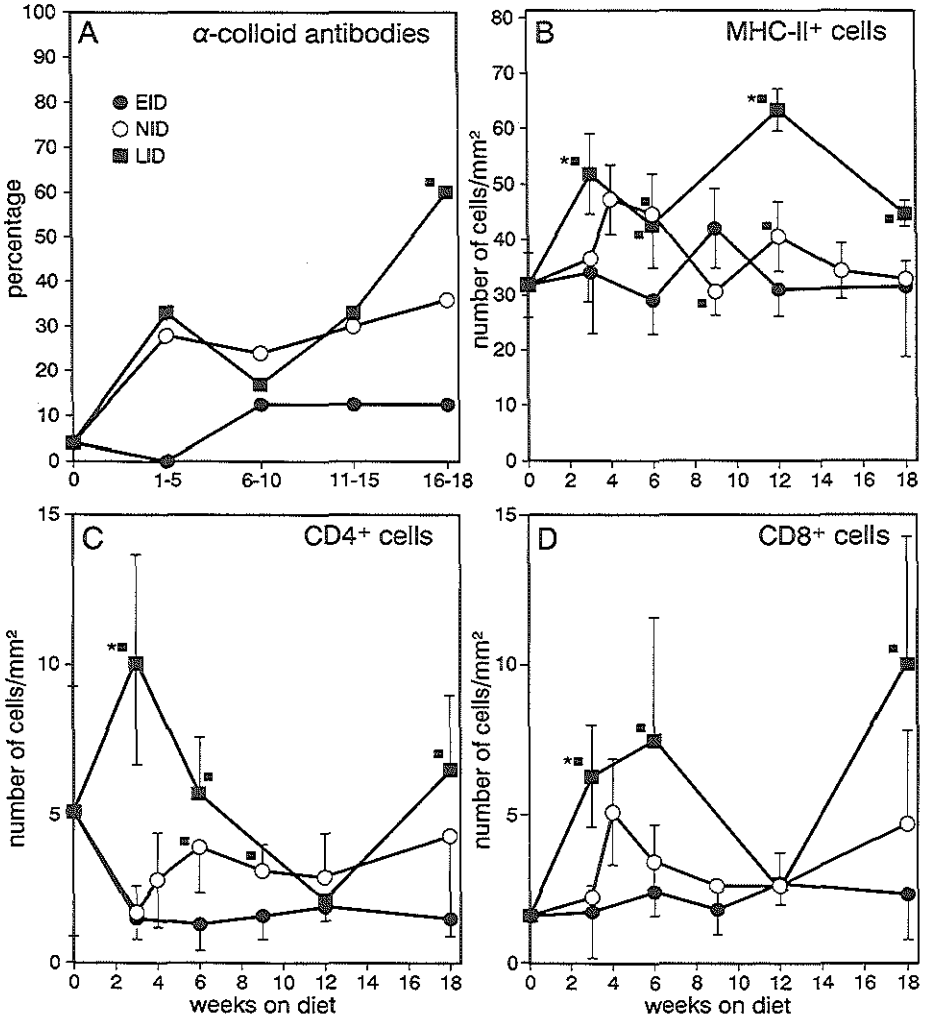
The rats on a *NID* however, showed from three weeks of life onwards an increase in the incidence of anti-colloid antibodies to reach 30% of positivity (n=25) after 5 weeks of diet. The incidence remained at that level thereafter (figure 2A).

The rats kept on a *LID* had up to 5 weeks of diet an incidence of anti-colloid antibodies similar to that of rats kept on a NID. However, the LID rats reached a higher level of 60% incidence of positivity at 18 weeks of diet; this latter value is statistically significantly higher than the incidence in rats kept on an EID at that time (figure 2A, $p<0.05$, X^2 analysis).

Intrathyroidal infiltrates of dendritic cells and T lymphocytes

During the *EID* a very low intrathyroidal infiltration of dendritic cells/mm^2 and T cells/mm^2 was observed after 3 weeks of diet; number of DC/mm^2: 34.1±5.3, mean±SD, EID vs 36.5±13.6, mean±SD, NID and 51.9±7.2, mean±SD, LID; number of CD4$^+$ T cells/mm^2: 1.6±0.7, mean±SD, EID vs 1.8±0.9, mean±SD, NID and 10.3±3.5 mean±SD, LID; number of CD8$^+$ T cells/mm^2: 1.8±0.9, mean±SD, EID vs 2.3±1.9, mean±SD, NID and 6.4±1.7, mean±SD, LID (figure 2B, C, D).

In fact a drop in the number of CD4$^+$ T cells (from around 5 to 2 cells/mm^2) was observed after 3 weeks of diet, numbers staying at that low level throughout the period of observation.



* P <0.05 vs NID Wilcoxon's rank sum test
▪ P <0.05 vs EID Wilcoxon's rank sum test

Figure 2. Thyroid autoimmune reactivity. The incidence of anti-colloid antibody production is given of various age groups of female Wistar rats (n=8-20 per age group) kept on a normal iodine diet (NID), a low iodine diet (LID) or an enriched iodine diet (EID) from 3 weeks of age onwards for a period of 18 weeks (A). ▪ $P<0.05$, compared to EID, X^2 analysis. The mean number and SD (n=5-12) of intrathyroidal dendritic cells (B), $CD4^+$ T cells (C) and $CD8^+$ T cells (D) are expressed per mm^2 counted in 4 semiserial cut sections for each thyroid. Statistical significances using the Wilcoxon's rank sum test are indicated with * and ▪.

With regard to the intrathyroidal infiltrates of dendritic cells and T cells, the normal iodine diet (*NID*) and more pronounced the *LID* resulted in a higher infiltration with these cells (see above). The latter diet showed two peaks of cell infiltration as compared to the normal and enriched iodine diet (figure 2B, C, D) namely at 3-6 weeks after starting the diet and at 12-18 weeks after starting the diet. The occurrence of these two peaks coincided with the higher prevalences of anti-colloid antibodies found in these groups of animals (figure 2A).

Incidence of thyroid associated ectopic thymic tissue

Remarkable in the *EID* rats was the occurence of a special lymphoid tissue in a high proportion of the animals in the early weeks of the diet (see table 2).

Table 2. Incidence of thyroid-associated ectopic thymic tissue

weeks on diet	EID	NID	LID
0 weeks	0/10 (0%)	0/10 (0%)	0/10 (0%)
3 weeks	8/14 (57%)*	2/14 (14%)	0/8 (0%)
6 weeks	7/14 (50%)*	1/14 (7%)	0/13 (0%)
9 weeks	3/14 (21%)	1/16 (6%)	-
12 weeks	1/8 (12.5%)	1/5 (20%)	1/9 (11%)
18 weeks	1/8 (12.5%)	0/5 (0%)	0/4 (0%)

Thyroid lobes of groups of rats (n=5-16) kept on an enriched iodine diet (EID), a normal iodine diet (NID) or a low iodine diet (LID) were removed, frozen and sections of 6μm were incubated with anti-MHC-II, anti-CD4 and CD8 and anti-TdT monoclonal antibodies. The number of rats with ectopic thymic tissue of each group is presented. The incidence of ectopic thymic tissue is given between brackets.

* $p<0.05$ versus NID and LID, X^2 analysis.

This observed lymphoid tissue was in contact with the thyroid and showed a marker pattern specific for thymus tissue. It occurred in 57% of the animals after 3 weeks, in 50% of the animals after 6 weeks, gradually declining to 12.5% of the animals after 12 weeks of excessive iodine diet, remaining at this level thereafter.

The cells of this ectopic thymic tissue reacted with monoclonal antibodies specific for MHC class-II molecules, some cells were double positive for CD4/CD8 and the tissue clearly showed a cortex and medulla (figure 3 A-F).

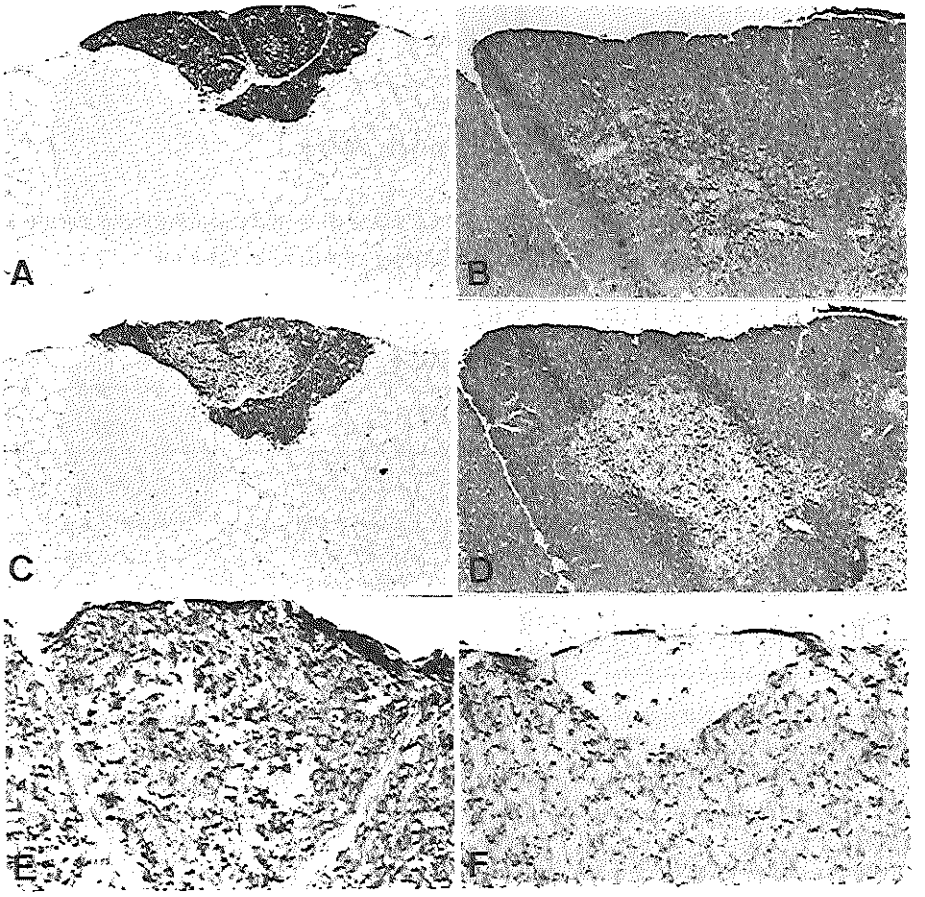


Figure 3. Thyroid associated ectopic thymic tissue. Frozen thyroid (A, C and E) and thymus (B, D and F) sections of rats kept on an EID of 6μm were incubated with several monoclonal antibodies directed against CD4 antigens (A and B, magn 200x), CD8 antigens (C and D, magn 200x) and MHC class-II molecules (E and F, magn 800x). Note the similarity of the thyroid associated ectopic thymic tissue with rat thymus.

To verify whether the observed lymphoid tissue was indeed thymic tissue, immuno-histochemistry was performed using a monoclonal antibody directed against the enzyme TdT, known to be present in immature cortical thymocytes. The staining pattern observed in the lymphoid tissue attached to the thyroid using the anti-TdT monoclonal antibody was exactly the same as in rat thymus (figure 4 A-F). Therefore it can be concluded that the observed lymphoid tissue was indeed thyroid-associated ectopic thymic tissue.

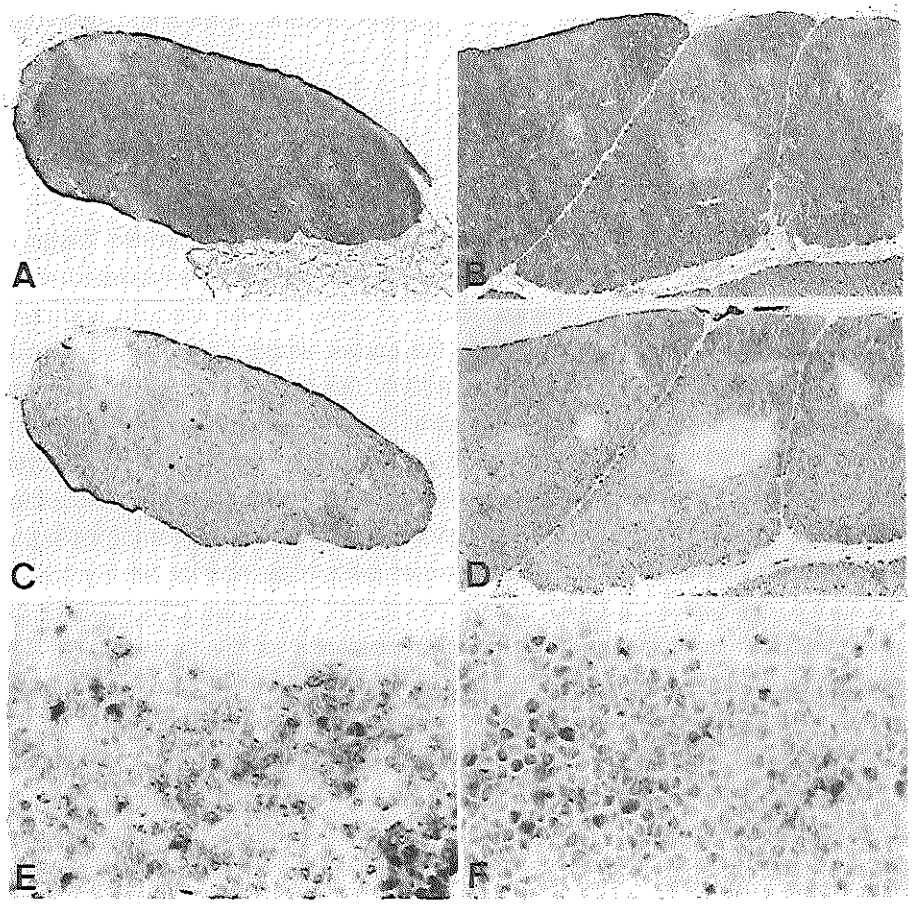


Figure 4. Thyroid associated ectopic thymic tissue. Frozen thyroid (A, C and E) and thymus (B, D and F) sections of rats kept on an EID of 6μm were routinely stained with heamatoxylin and eosin (A and B, magn 200x) or incubated with a monoclonal antibody specific for the enzyme TdT (C and D, magn 200x, E and F, magn 800x). Note the similarity of the thyroid associated ectopic thymic tissue with rat thymus.

Also in rats kept on a normal iodine diet (*NID*) this thyroid-associated ectopic thymic tissue could be observed, although only sporadically, incidences not exceeding a 20% level (table 2).

Only in a few rats on a *LID* this thyroid-associated ectopic thymic tissue could be observed (levels not exceeding 11% at 12 weeks of diet, table 2).

The occurrence of the thyroid-associated ectopic thymic tissue in EID rats is accompanied (see before) by the virtual absence of anti-colloid antibodies and low numbers of thyroid infiltrated MHC class-II positive dendritic cells and T cells (figure 2).

DISCUSSION

This study shows that a high dietary iodine intake in young female Wistar rats resulted in 57% of the animals in the development of a thyroid-associated ectopic lymphoid tissue. This ectopic lymphoid tissue showed a clear medulla and cortex, showed double CD4/CD8 positive cells in the cortex, single CD4 positive and CD8 positive cells in the medulla, showed TdT activity (an enzyme known to be present in immature T cells) in the cortex and MHC class-II expression in between the thymocytes. We therefore concluded on the basis of the histology that the thyroid-associated lymphoid tissue must be thymic tissue. The thyroid-associated ectopic thymic tissue found in rats on an excessive iodine diet was histomorphologically entirely normal, and showed the same marker pattern as normal rat thymus with regard to the expression of MHC class-II molecules, of double CD4/CD8 positive cells, single CD4 positive and CD8 positive cells and of the enzyme TdT. The presence of this tissue coincided with a very low infiltration into the thyroid of lymphoid cells and a minimal production of anti-colloid antibodies.

A for our area normal and a low dietary iodine intake lacked such effects, and a higher production of anti-colloid antibodies together with a limited but clear intrathyroidal infiltration of single dendritic cells and lymphocytes was present. It must be noted in this respect that the Wistar rats did not show any clear histological signs of thyroiditis (noticable lymphocytic infiltration in heamatoxylin-eosin staining and damage of thyroid follicles) although the rats did show some thyroid autoimmune reactions, such as anti-colloid antibody production (in 30% of the rats). It has also been reported that normal Wistar rats possess thyroid-specific

T cells in their circulation[26].

In humans with autoimmune thyroid disease and in the autoimmune-prone BB rat, areas of lymphoid tissue in the thyroid have been found before[27,28]. However, these areas of socalled "focal thyroiditis" are histologically clearly different in respect to marker pattern and structure from the thyroid-associated ectopic thymic tissue found in this study. The areas of "focal thyroiditis" consist of an organized lymphoid tissue that is composed of B cell follicles surrounded by a zone of T cells in which high endothelial venules, dendritic cells and macrophages are present. The thyroid-associated ectopic thymic tissue on the other hand consists of immature (double CD4/CD8 positive) and mature (single CD4 or CD8 positive) T cells arranged in a medulla and a cortex.

A similar thyroid-associated ectopic thymic tissue has been described as early as 1970 in the OS chicken[29,30] and it was then speculated by the authors that the tissue represented a breakdown of the thymus-thyroid barrier leading to the OS autoimmune thyroiditis. However, the incidence of the occurrence of the ectopic thymic tissue in the OS chicken was much lower (4 out of 64) than the incidence of thyroiditis (up to 90%) and its presence can thus not be considered as the cause for the development of the OS autoimmune thyroiditis. Furthermore many germinal centres were observed in the OS thyroid associated ectopic thymic tissue. Germinal centres do normally not occur in the normal thymus and the presence of these germinal centres may be taken as indicative for a gross abnormality of the thyroid-associated ectopic thymic tissue in the OS chicken. The thymus of the OS chickens itself is also abnormal (thymic nurse cell deficiency[31]).

The mechanisms underlying the development of the thyroid-associated ectopic thymic tissue triggered by the high dietary iodine intake remain speculative. Recently, Many et al[32] observed the development of thyroid-associated ectopic thymic tissue in NOD mice. These autors speculated that the development of the tissue was due to a form of dysembryogenesis, since the thyroid and the thymus are embryonically related, both being derived from the third endodermal pouch and ectodermal cleft[33]. It is indeed possible that fragments of the thymic "anlage" stay behind, close to the ductus thyroglossus to later develop into mature thymic tissue during a high dietary iodine intake. Vladutiu et al[34] also described ectopic thymic tissue in rat and mouse thyroid, showing the close embryological evolution of the two organs.

This report is special in that we showed that the development of ectopic thymic

tissue is influenced by an environmental factor such as the dietary iodine intake. Iodine has been described as having direct stimulating effects on various lymphoid cells[35,36,37]; whether it has also effects on thymic tissue is unknown.

The thymus plays a prominent role in the shaping of the T cell repertoire and the generation of tolerance induction. It has been described that intrathymic transplantation of pancreatic islets prevents autoimmune diabetes in BB rats[38,39], and in NOD mice[40]. Since the Wistar rats with the thyroid-associated ectopic thymic tissue had low thyroid (auto)immune reactivity, we like to hypothesize that the occurrence of the thyroid-associated ectopic thymic tissue represents tolerance induction towards thyroidal antigens and hence linked to the observed low thyroid autoimmune reactivity.

Our observations of the suppressive effects of a high iodine diet on thyroid autoimmune reactions in normal Wistar rats are in contrast to the observations of others on the stimulating effects of a high iodine diet in animals/individuals with spontaneously developing forms of autoimmune thyroiditis (such as the Obese Strain of chicken and the BB rat). In these thyroid autoimmune animals an excess of iodine induced a higher incidence and more severe forms of thyroiditis[8,9].

There are two hypothetical explanations for this discrepancy:

- Firstly, there might be a direct effect of iodine on the dysregulated immune system of the autoimmune prone animals. In normal, non-autoimmune animals, autoreactive T and B cells are present in the periphery, but these cells are suppressed by several control mechanisms. In BB rats and OS chickens however, several abnormalities in immunoregulation exist, by which eventually the thyrocyte becomes the victim of an autoimmune-mediated assault by these autoreactive cells. It has for instance been described that the BB rat becomes lymphopenic mainly because of a lack of RT6 positive regulator (suppressor) T cells[41,42], which are normally generated in the thymus. In the Obese Strain of chicken hyperreactive T cells, hyperreactive macrophages and a high density of Il-2 receptor expression were found[31]. Since iodine has direct stimulatory effects on lymphoid cells (see above), it is possible that a high dietary iodine intake results in a complete dysregulation of an already malfunctioning immune system in autoimmune prone animals, this in contrast to the situation in non-autoimmune prone animals where in fact a suppression of the autoimmune reactivity was observed (see this report).

- Secondly, iodine may be toxic for the thyrocytes of the autoimmune prone animals.

Not only abnormalities in the control mechanisms of the immune system are important for the development of autoimmune thyroid disease in thyroid autoimmune animal strains. There are genetically determined abnormalities in the thyroid cells themselves that are essential before fully blown thyroiditis can develop in BB rats and OS chickens. An autonomous high thyrocyte function (iodine uptake) has been described in the OS strain of chicken[43,44,45]. Our histomorphological observations (high thyrocyte epithelium) and observations of Li et al.[46] show that also the metabolic activity of BB thyrocytes is high before thyroid autoimmunity develops. It is therefore also possible that a high dietary iodine intake is toxic for BB and OS thyrocytes, leading to thyrocyte destruction, release of autoantigens and an enhanced stimulation of thyroid autoimmunity. A similar toxic action of iodine has been observed by Many et al in thyroid hyperplastic mice[11,12], resulting in thyrocyte destruction, inflammation of the thyroid and aggrevation of autoimmune thyroiditis[47].

In the here described Wistar rats, the high iodine intake is apparently not toxic for the thyrocytes and might indeed be more optimal for the function of the cells, resulting in a low thyrocyte metabolism and a lower release of antigenic degradation products and autoantigens. Histomorphology indeed shows a low thyrocyte metabolism in Wistar rats on a high iodine diet, viz a flat thyrocyte epithelium (data not shown).

In conclusion, this study shows that in female Wistar rats a high iodine intake results in the development of a thyroid-associated ectopic thymic tissue. This development is accompanied by a low thyroid autoimmune reactivity. Further investigations on this thyroid-associated ectopic thymic tissue might lead to a better understanding of the mechanisms of tolerance induction and the role of dietary iodine in thyroid autoimmune diseases.

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Chapter 5

AN EXCESS OF DIETARY IODINE ACCELERATES THE DEVELOPMENT OF A THYROID-ASSOCIATED LYMPHOID TISSUE IN AUTOIMMUNE PRONE BB RATS*

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ABSTRACT

It has been described before that dietary iodine enhances the severity and incidence of thyroiditis in autoimmune BB rats and OS chickens. It has however not been studied in detail which lymphoid cells are involved in the iodine induced focal thyroiditis and what the consequences are for the anti-colloid antibody production. We therefore performed a study in which female BB rats were kept on either an enriched iodine diet (EID; iodine intake: 100μg iodine/day) or a normal iodine diet (NID; iodine intake 7μg iodine/day) for a period of 18 weeks starting at 3 weeks of age. The intrathyroidal lymphoid tissue was immunohistologically studied using monoclonal antibodies specific for MHC class-II antigens, various subsets of T cells, B cells and various subsets of macrophages. Immunohistological data were compared to thyroid hormone status and anti-colloid antibody production.

Our data confirm that a high dietary iodine intake results in an accelerated development of the focal lymphoid cell infiltrates in the thyroid of the BB rat. After 12-18 weeks of an EID 50% of the BB rats developed these infiltrates. Our data additionally show that a) the process started with increases in the number of infiltrating MHC class-II positive dendritic cells (from 51.8 ± 2.3 cells/mm^2, mean $\pm$ SD, at the start of the experiment tot 79.8 ± 9.0 cells/mm^2, mean $\pm$ SD, at 9 weeks of diet) and a clustering of these cells with T cells, B cells and some macrophages and b) that the focal infiltrates were highly organized and consisted of central B cell follicle-like structures (positive for the monoclonal antibodies HIS-14 and OX-33) surrounded by rims and areas of T cells (B115-5 positive) that were mainly CD4$^+$ (B115-4). Dendritic cells (MHC class-II positive, OX-41 and OX-42 positive) and macrophages positive for ED1, ED7, ED8 and ED9 were mainly found in the T cell areas. Only minor signs of thyrocyte destruction were observed at the edges of the organized focal lymphoid cell accumulations (consisting of macrophages and CD4$^+$ T cells) and the BB rats remained euthyroid.

Since the architecture of the focal lymphoid cell infiltrates is very similar to mucosa associated tissue and secondary lymphoid organs (spleen and lymph node) and since only minor signs of thyrocyte destruction were observed, we prefer the term "thyroid-associated lymphoid tissue". The presence of the thyroid-associated lymphoid tissue in the BB rats was positively correlated to the incidence of anti-colloid antibodies in the serum of the BB rats.

To explain the accelerated development of the thyroid-associated lymphoid

tissue in BB rats on an enriched iodine diet, it is speculated that the dietary iodine might have direct effects on cells of the immune system or on cells forming the microenvironment of lymphoid tissue (reticulum cells). An effect of highly iodinated thyroglubulin on the accelerated development of thyroid-associated lymphoid tissue is also possible.

INTRODUCTION

The last few decades it has become clear that the dietary iodine intake can be a modulator of the autoimmune reactivity towards thyroidal antigens:

a. A high dietary iodine intake in individuals with a preexisting thyroid abnormality, such as an iodine deficient goiter, results in a proportion of the individuals in the development of an attack of thyroiditis; those affected show anti-thyroglobulin and anti-microsomal antibodies in their serum[1,2,3].
b. In animals that spontaneously develop thyroid autoimmune disease, such as the Cornell C Strain of chicken, the Obese Strain of chicken and the BB rat, a high dietary iodine intake results in an increase in the incidence and severity of the thyroiditis[4,5]. The OS chicken rapidly becomes severely hypothyroid under circumstances of high dietary iodine intake; the BB rat however, only shows minor signs of thyrocyte destruction[6] and the rats remain euthyroid during and after the dietary regimen[5].

It has not been studied in detail which subtypes of lymphocytes and which subtypes of mononuclear phagocytes contribute to the iodine induced thyroiditis and what the precise consequences of the diet are for the incidence of anti-colloid antibodies. We therefore performed a study in which BB rats were either kept on a high iodine diet or a for-our-area-normal iodine diet for a period of 18 weeks. The thyroid was studied immunohistologically at various time intervals after introducing the diet using monoclonal antibodies specific for MHC class-II antigens, various T cell markers, B cell markers, and markers for monocyte/macrophage subsets. Immunohistological infiltration data were compared to the thyroid performance (thyroid weight, T_3, T_4 and TSH levels) and the incidence of anti-colloid antibodies in the serum of the various groups of animals.

MATERIALS AND METHODS

Animals and diets

BB rats were kindly provided by Dr. H.A.M. Verheul and Mr. E. Cremer from Organon BV, The Netherlands. The rats were further bred and housed in the central animal facilities of the Erasmus University of Rotterdam under standard conditions. Rats that became diabetic (recorded by urinary glucose levels using Gluketur-Test sticks, Boehringer, Germany) were daily treated with subcutaneous injections of 2 IU Protamine Zinc Insulin (isofane protamine bovine insulin, Insulinum N.P.H., Organon Oss bv, The Netherlands).

Directly after weaning (at the age of 3 weeks) groups of rats (n=8) were kept on two dietary iodine regimens:

1. *A for-our-area-normal iodine diet (NID)*; the rats received normal pellets (Am-II, Hope Farms bv, Woerden, The Netherlands, ± 0.35 mg/kg iodine) and Rotterdam tap water ad libitum. The daily iodine intake in this group was about 7μg of iodine, measured by the urinary iodine excretion (see for details of technique ref[7]). These groups of rats served as controls.
2. *An enriched iodine diet (EID)*; the rats received normal pellets (Am-II, Hope Farms bv) ad libitum and an extra iodine supplementation of 6.5mg KI/l added to the drinking water. Consequently the daily iodine intake was about 100μg of iodine, measured by the urinary iodine excretion (see for details of technique ref[7]).

Groups of BB rats were sacrificed by aortic exsanguination under ether anaesthesia at 3, 6, 9, 12 or 18 weeks. Serum was prepared for T_3, T_4, TSH evaluation and anti-colloid antibody determination. T_3, T_4, and TSH were measured by RIA (Dr. W. Wiersinga, Margreet Broenink, Amsterdam, The Netherlands). The rat TSH reference preparation NIADDK-rTSH-RP-2 was kindly provided by Dr. Parlow from the UCLA Medical Center, Torrance, USA, and was 176 times more potent than the NIADDK-rTSH-RP-1. Thyroid glands were removed, weighed and stored at -80 °C until immunohistological examination.

Immunohistological examination

Immunohistological examination was performed according to Green et al.[8] with minor modifications. Of each frozen thyroid, one lobe was semiserially cut into 6 μm thin sections. The sections were airdried overnight and fixed in acetone at room temperature for 10 minutes. The sections were incubated with either a monoclonal antibody specific for MHC class II molecules, dendritic cells (DC), macrophages, $CD5^+$ cells, $CD4^+$ cells, $CD8^+$ cells or B cells (see table 1) for 60 minutes, washed with phosphate buffered saline (PBS) containing 0.2% bovine serum albumin (BSA, Sigma Chemical Co, USA) for 10 minutes, and further incubated with a rabbit anti-mouse Ig-HRP labeled conjugate (Dakopatts, Denmark) for 30 minutes, diluted 300 times in PBS containing 1% normal rat serum and 0.2% BSA.

After washing the slides in PBS containing 0.2% BSA for 10 minutes and rinsing them with 0.1 M sodium acetate buffer, pH 6.0, the sections were developed with a metal-enhanced 3,3'-diaminobenzidine (DAB) solution containing 0.05% DAB (Sigma), 1% nickel sulphate (Merck), 0.068% imidazole (Sigma), and 0.8% sodium chloride in 0.1M acetate buffer pH 6.0 for 3-5 minutes. Hydrogen peroxide was added to a final concentration of 0.01%. After DAB development, the sections were washed briefly in 0.1M Tris-HCl buffer, pH 7.6, and immersed in a 0.5% solution of cobalt chloride in 0.1M Tris-HCl buffer, pH 7.2 at room temperature for 4 minutes. The slides were either counterstained with 0.1% nuclear fast red in 5% aluminum sulphate for 2 minutes or incubated with acid phosphatase at 37°C for 30 minutes and subsequently counterstained with hematoxylin. The slides were dehydrated and embedded in DePeX mounting medium (Gurr, BDH Limited Poole, England).

Since the infiltrates in the thyroids of the BB rats consisted predominantly of dendritic cells, T cells and B cells, we have counted the number of these cells in the BB thyroid sections. The number of infiltrated dendritic cells (identified as strong MHC class-II, OX-6, positive cells with cytoplasmic protrusions, a reniform nucleus and weak or absent acid phosphatase activity), $CD4^+$ T cells (B115-4 positive round cells), $CD8^+$ T cells (OX-8 positive round cells) and B cells (OX-33 or His-14 positive cells) were counted in 4 complete sections of each thyroid, with section intervals of at least 100μm and using light microscopy at a magnification of 400x (this sampling procedure covered almost the entire thyroid). The surface area of the thyroid sections in which the infiltrated cells had been counted was measured

using a camera attached to a Leitz Diaplan light microscope and a Videoplan image processing system (Kontron, Bild Analyse GmbH, Germany).

Table 1. Monoclonal antibodies (MoAbs) used for the detection of intrathyroidal leucocytes

MoAb	Specificity	Source	Reference
OX-6	MHC class-II	Seralab, UK	9
B115-5	CD5 antigen	Holland Biotech, The Netherlands	10
B115-4	CD4 antigen	Holland Biotech	10
OX-8	CD8 antigen	Seralab, UK	11
HIS-14	Leucocyte Common Antigen (LCA) on B cells	FGM Kroese	12,13
OX-33	LCA on B cells	Serotec, UK	14
ED1	mainly monocytes, (some macrophages, dendritic cells)	CD Dijkstra	15
ED2	tissue macrophages	CD Dijkstra	15
ED3	macrophage subset, particularly in phagocytosing compartments	CD Dijkstra	15
ED7	granulocytes monocytes, macrophages	CD Dijkstra	16
ED8	granulocytes, monocytes, macrophages	CD Dijkstra	16
ED9	granulocytes, monocytes, spleen red pulpa macrophages	CD Dijkstra	16
OX-41	macrophages, granulocytes, dendritic cells	Serotec, UK	17
OX-42	macrophages, granulocytes, dendritic cells	Serotec, UK	17

The number of infiltrated cells was expressed per mm^2 surface area of a thyroid section. It is of importance to note that similar results were obtained when the number of DC, T cells and B cells were not expressed per mm^2 surface area of the section but were expressed per observed thyroid follicle in the plane of section or per surface area of thyrocyte parenchyma.

In some experimental animals a dense, packed lymphoid cell infiltration into the interfollicular area of the thyroid occurred (see figure 2-4; these infiltrates had in fact an architecture of peripheral lymphoid tissue). The dense packing of the lymphoid cells hampered a detailed counting of the cells. A semiquantification of the number of lymphoid cells in such areas of organized lymphoid tissue was made and scored as - (no cells), ± (1-10 cells), + (10-25 cells), ++ (25-50 cells), +++ (>50 cells) per focal infiltrate (see table 3).

Anti-colloid antibody determination

6 μm thin frozen porcine thyroid sections were cut, airdried overnight and fixed in cold acetone (-20 °C) for 10 minutes. Porcine thyroid tissue was used, since it gave optimal results as compared to rat or human tissue. The sections were preincubated with normal rabbit serum (DAKO-immunoglobulins, Denmark), diluted 50 times in PBS with 1% BSA, for 10 minutes. Subsequently, the rat sera were applied (in duplicate, diluted 10 times in 0.9% NaCl) and the slides were incubated at room temperature for 60 minutes. After washing in PBS, the sections were incubated with rabbit anti-rat immunoglobulins, fluorescein-isothiocyanate-(FITC)-labeled (DAKO immunoglobulins, Denmark) for 30 minutes (diluted 25 times in PBS with 1% BSA). After this second step and rewashing, the slides were embedded in aquamount (Gurr, BDH Limited Poole, England) and examined using a fluorescence microscope. Three control slides were included; one without incubation of rat serum, another incubated with a rat serum previously scored as negative and a third incubated with a rat serum previously scored as positive. The staining intensity of each serum was arbitrarily and blindly scored (under code, by two independent investigators) as negative or positive.

Statistical analysis

Differences in thyroid weight, serum T_3, T_4, and TSH, and number of single infiltrated intrathyroidal dendritic cells, small clusters of these cells and T cells were compared by Wilcoxon's rank sum test. The incidence of anti-colloid antibody production was tested by X^2 analysis.

RESULTS

Thyroid weight

The thyroid weights of BB rats kept on an *enriched iodine diet (EID)* were not statistically significantly different from the thyroid weights of such rats kept on a *for-our-area-normal iodine diet (NID)* and gradually increased from 4.8±0.5 mg (mean±SD) at the start of the experiment to 18.9±3.5 mg (mean±SD) after 18 weeks (figure 1A); this weight increase could be expected from the normal development of the body weight of the rats (in fact thyroid/body weight index remained constant, namely: 0.08). The thyroid weights of the BB rats were higher than the thyroid weights of age and sex matched groups of Wistar rats, indicating that normal BB rats developed a small goiter in relation to Wistar rats. This observation has earlier been reported[18,19].

Thyroid hormone and TSH levels

In BB rats receiving a *NID* serum T_4 levels slightly increased the first 3 weeks of the experiment from around 40 nM/l to around 50 nM/l and remained around this level during the entire experimental period (figure 1D). Serum T_3 levels were high (1.8±0.2 nM/l, mean±SD) at the start of the experiment and gradually decreased to 0.9±0.2 nM/l (mean±SD) at 18 weeks of diet (figure 1C). Such relatively high serum T_3 levels in prepubertal rats compared to adult rats have been described by others before[20] and these high thyroid hormone levels were regarded as necessary for optimal brain development in these young rats. Serum TSH levels were low during the entire observation period ranging between 1.1±0.7

ng/ml (mean ± SD) and 1.9 ± 1.2 ng/ml (mean ± SD, figure 1B), both values were however within the normal range.

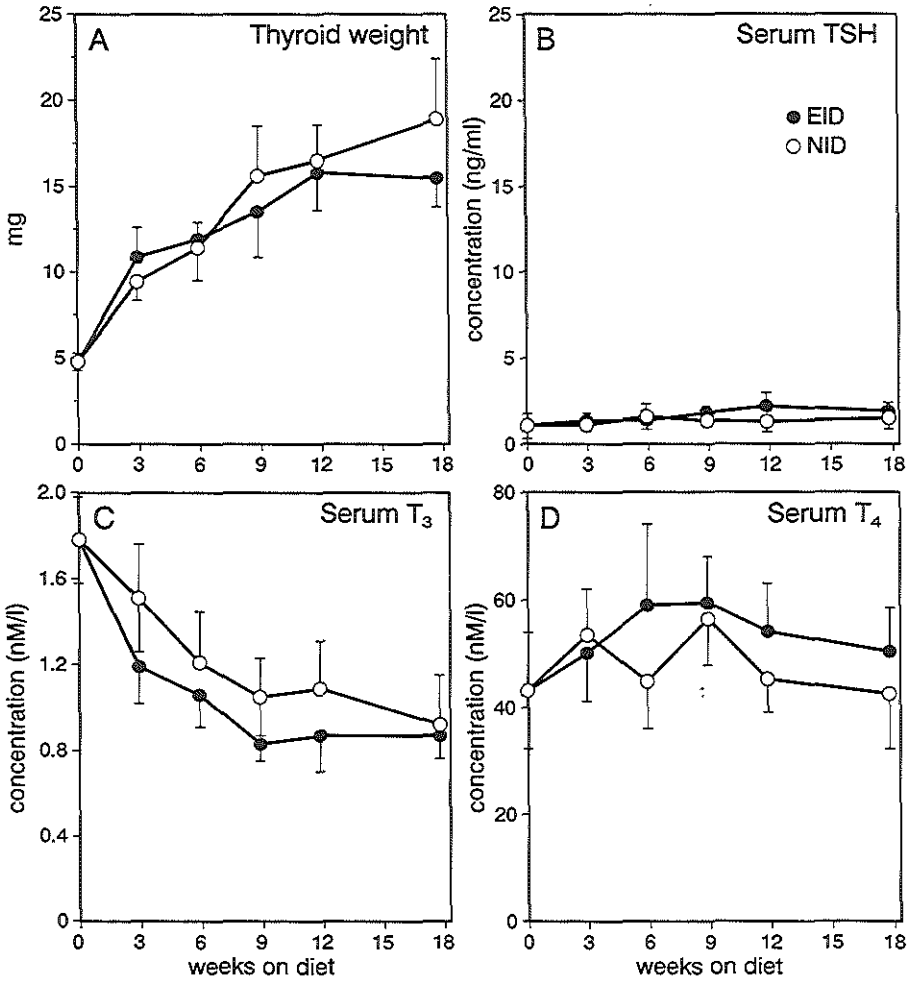

Figure 1. Thyroid weight and serum TSH and thyroid hormone levels. BB rats were kept on a normal iodine diet (NID) or an enriched iodine diet (EID) for a period of 18 weeks from 3 weeks of age onwards. Rats were sacrificed, thyroids were removed and weighed to measure goiter development (A) and serum was collected to measure serum TSH (B), T_3 (C) and T_4 (D) by RIA. The mean and standard error are given of 5-18 rats per group.

In BB rats kept on an *EID* the serum T_4 and T_3 levels were the same as in rats kept on a NID (see figure 1C and D). Serum TSH levels in the EID rats were in the normal range (between 1.4 ± 0.4 (mean $\pm$ SD) and 2.2 ± 0.8 ng/ml, mean $\pm$ SD) during the entire experimental period (figure 1B).

The development and architecture of focal infiltrates of lymphoid cells in the thyroids of BB rats kept on an enriched iodine diet (EID)

In 50% of the BB rats, areas of extensive, focal accumulation of lymphoid cells were found to develop 12 to 18 weeks after introducing the enriched iodine diet (viz. at 15-21 weeks of age, table 2). In BB rats that were shorter than 12 weeks on an enriched iodine diet (<15 weeks of age) such focal accumulations could not be detected.

Table 2. Incidence of focal infiltrates of lymphoid cells in the thyroid of BB rats

weeks on diet	NID	EID
0	0/5	-
3	0/5	0/5
6	0/5	0/5
9	0/5	0/5
12	0/5	2/5
18	0/5	3/5

The typical architecture of these focal accumulations was as follows: All the lymphoid cells in the focal infiltrates showed varying degrees of MHC class-II positivity, indicating their activation (table 3 and figures 3B and 4B). The majority of the lymphoid cells were B cells that were positive for the monoclonal antibodies His-14 and OX-33. These B cells were found grouped together. The groups of B cells (reminiscent of early B cell follicles) were surrounded by rims and areas of T cells (figures 2A and B, 3C and D, 4C and D) of which many were $CD4^+$ cells and only a few $CD8^+$.

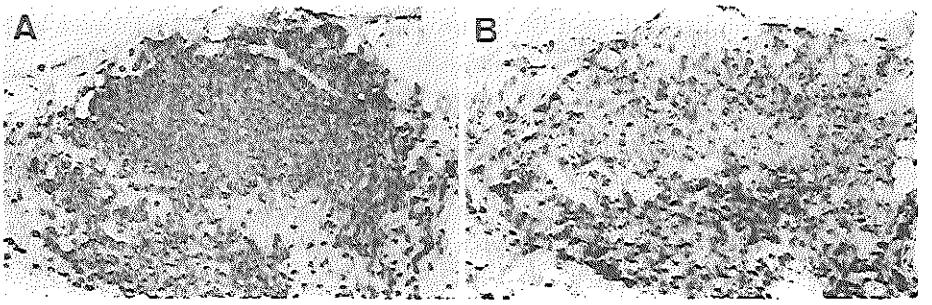

Figure 2. An area of "thyroid-associated lymphoid tissue" in a thyroid of a BB rat 18 weeks on a diet of excessive iodine. Note the distinguishable areas of B cells (A, His-14 positive cells) and T cells (B, B115-5 positive cells). Magn. x160.

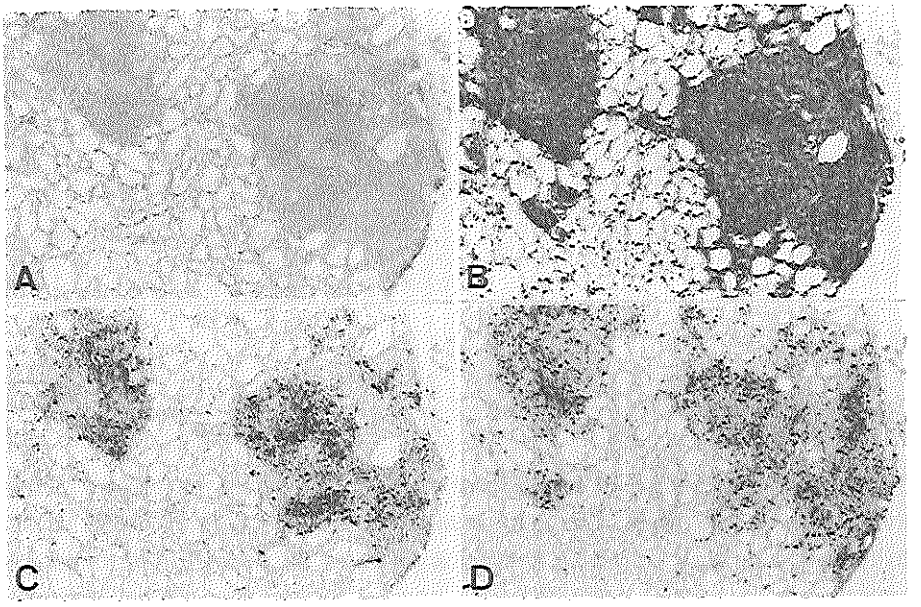

Figure 3. Two areas of "thyroid-associated lymphoid tissue in a thyroid of a BB rats 12 weeks on a diet of excessive iodine. A: control (hematoxylin, eosin staining), B: MHC class-II positive cells (OX-6 positive cells), C: B cells (OX-33 positive cells), D: T cells (B115-5 positive cells). Magn. x50. Note the sharp demarcations of the lymphoid tissue in the thyroid, the MHC class-II positivity of this tissue and the beginning of distinguishable areas of B cells and T cells.

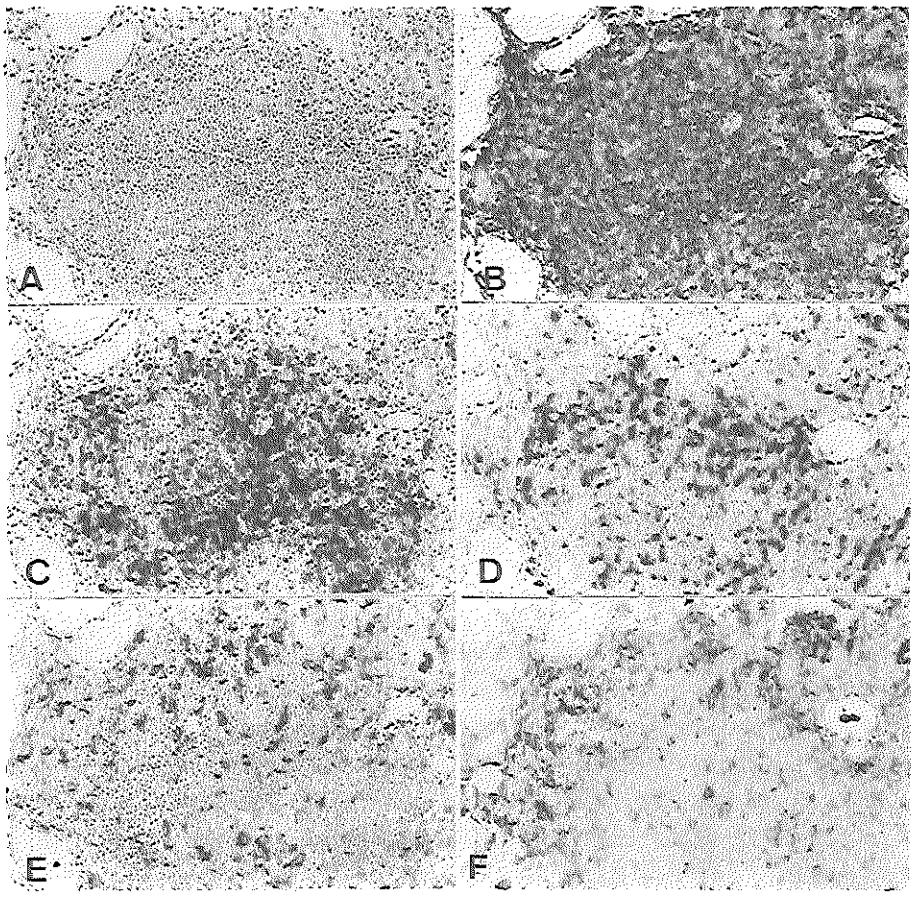

Figure 4. An area of "thyroid-associated lymphoid tissue" in a thyroid of a BB rat 12 weeks on a diet of excessive iodine. A: control (hematoxylin, eosin staining), B: MHC class-II positive cells (OX-6 positive cells), C: B cells (OX-33 positive cells), D: T cells (B115-5 positive cells), E: macrophages and dendritic cells (OX-42 positive cells) and F: macrophages (ED7 positive cells). Magn. x160. Note the MHC class-II positivity of the lymphoid tissue, the beginning of distinguishable areas of B cells and T cells, the accumulation of macrophages and dendritic cells particularly in the lymphoid tissue, and the accumulation of ED7 positive macrophages at the edges of the lymphoid tissue close to the thyroid follicles, sometimes filling a thyroid follicle.

Table 3. Locations of dendritic cells, T cells, B cells and subsets of macrophages in the focal infiltrates of lymphoid cells in the thyroid of BB rats kept on an EID

MoAb	Specificity	B cell area	T cell area	Surrounding areas of minor destruction
OX-6	MHC class-II	+++	+++	++
His-14	B cells	+++	-	-
OX-33	B cells	+++	-	-
B115-5	CD5 antigen	+	+++	+
B115-4	CD4 antigen	±	+++	+
OX-8	CD8 antigen	-	±	±
ED1	mono, mφ, DC	±	++	+
ED2	tissue mφ	-	-	-
ED3	mφ	-	-	-
ED7	granulo, mono, mφ	±	+	+
ED8	granulo, mono, mφ	±	+	+
ED9	granulo, mono, mφ	±	+	+
OX-41	granulo, mφ, DC	-	+	+
OX-42	granulo, mφ, DC	±	+	+

Mono: monocytes, mφ: macrophages, DC: dendritic cells, granulo: granulocytes.
- no cells stained, ± 1-10 cells stained, + 10-25 cells stained, ++ 25-50 cells stained, +++ >50 cells stained per area of focal infiltrate.

Dendritic cells and macrophages (ED1, ED7, ED8, ED9, OX-41 and OX-42 positive) were also abundantly present in these areas of focal lymphoid cell infiltration. These dendritic cells and macrophages were mainly found in the T cell areas (figure 4E and F), but a few could also be observed inside the B cell follicle structures (apart from OX-41 that was not found in the B cell areas, table 3). Only half of the ED7, ED8, ED9 and OX-42 positive cells were positive for acid phosphatase.

Lymphoid tissue with a similar architecture has earlier been reported by us in the BB rats of our colony that where on a normal diet[21]. However, these BB rats that developed such intrathyroidal lymphoid tissue were much older (24 weeks of age and older).

At the edges of the focal lymphoid cell infiltrates of the BB rats on EID minor signs of thyroid follicle destruction were observed. At these sites some T cells and macrophages positive for ED7, ED8, ED9 and OX-42 were present, sometimes completely filling a thyroid follicle (figure 4F). The majority (75%) of these macrophages were positive for acid phosphatase.

The thyroid epithelium that was adjacent to the areas of focal lymphoid cell

infiltrates a more cuboidal appearance than the thyrocytes remote from these areas. The adjacent thyrocytes sometimes also showed a weak MHC class-II positivity.

The goiter development of BB rats in comparison to Wistar rats (see before) had nothing to do with the development of the focal lymphoid cell accumulations, since BB rats that did not develop these accumulations also had a weight increase of their thyroids as compared to Wistar rats.

The number of single dendritic cells, T cells and B cells in the thyroids of BB rats prior to the development of the focal infiltrates of lymphoid cells

Since we earlier reported[21] that the development of the lymphoid tissue in older BB rats on a normal iodine diet started with the accumulation of dendritic cells in the thyroid and a clustering of such cells with T lymphocytes, and since the focal lymphoid cell accumulations found in BB rats kept on an enriched iodine diet were predominantly composed of dendritic cells, T cells and B cells, we investigated the number and infiltration pattern of these lymphoid cells in the interfollicular area of the thyroids of EID-BB rats prior to the time of the development of the focal lymphoid cell infiltrates (viz at 3, 6 and 9 weeks after introducing the diet).

In the interfollicular connective tissue area of the 3 weeks old BB rats - before starting the diet - the number of dendritic cells was already considerable (51.8 ± 2.3 DC/mm^2, mean $\pm$ SD, figure 5A). In BB rats kept on an EID the number of interfollicular dendritic cells started to increase after introducing the diet to reach levels of 71.2 ± 3.7 DC/mm^2 (mean $\pm$ SD) at 3 weeks, 75.8 ± 11.4 DC/mm^2 (mean $\pm$ SD) at 6 weeks and 79.8 ± 9.0 DC/mm^2 (mean $\pm$ SD) at 9 weeks of diet (statistically significantly different from NID values, $p < 0.05$, Wilcoxon's rank sum test). Thereafter the number of interfollicular dendritic cells declined to normal values in those rats not developing the focal infiltrates of lymphoid cells (to 49.5 ± 10.6 DC/mm^2, mean $\pm$ SD, 18 weeks after introducing the diet). However, in the rats that did develop these infiltrates, numbers of dendritic cells increased to uncountable numbers (figure 5A).

It is worthy to note that DC are capable of forming clusters with themselves and with lymphocytes (this process is adhesion molecule dependent[22], and essential for the initial phases of antigen presentation[23]).

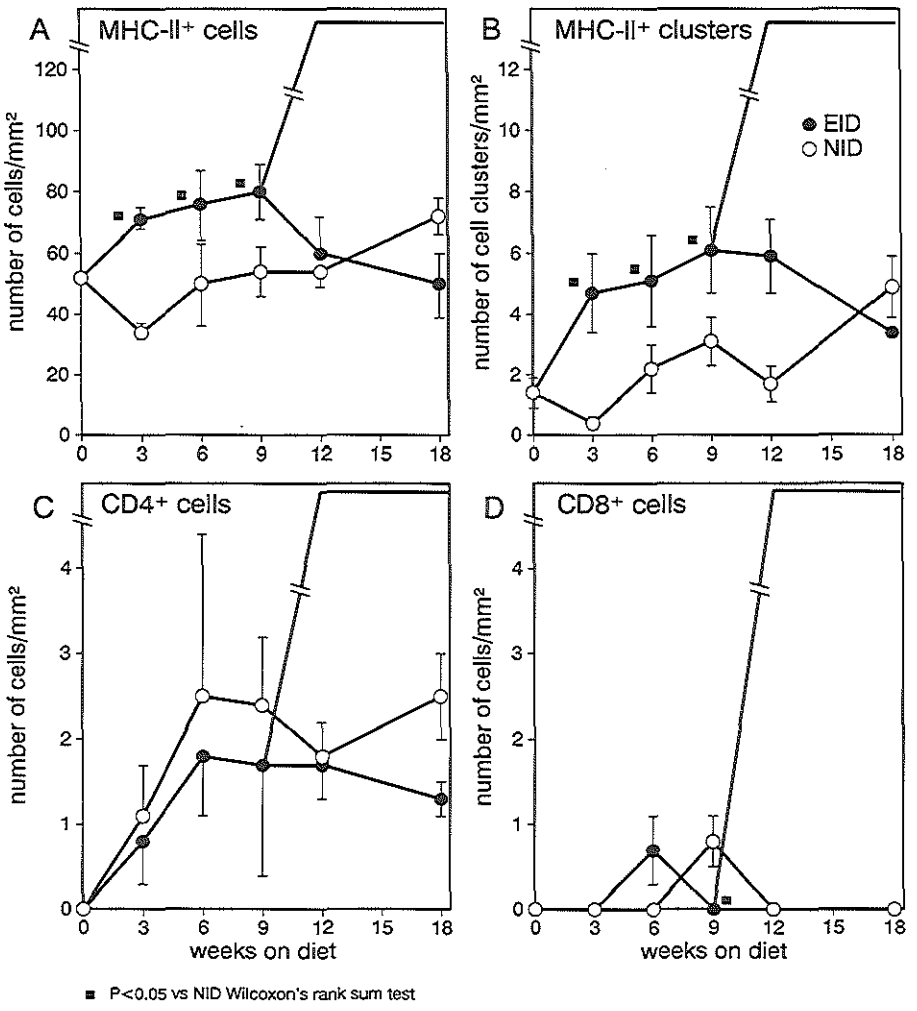

Figure 5. Number of lymphoid cells in the interfollicular area of the thyroid.
BB rats were kept on a normal iodine diet (NID) or an enriched iodine diet (EID) for a period of 18 weeks from 3 weeks of age onwards (5 rats per age group). Rats were sacrificed, thyroids were removed and immunohistologically examined. The number of MHC class-II$^+$ dendritic cells (A), MHC class-II$^+$ cell clusters (B), CD4$^+$ cells (C) and CD8$^+$ cells (D) were counted in 4 thyroid sections for each thyroid. Data are expressed as mean ± SD per surface area (mm^2). The // interrupted lines represent the uncountable numbers of lymphoid cells in the "thyroid-associated lymphoid tissue".

Also in the thyroid specimen of the here investigated BB rats, cluster formation of the interfollicular dendritic cells was evident (figure 5B). The number of interfollicular clusters of 3-6 lymphoid cells was higher in EID BB rats than in NID BB rats ($P<0.05$, Wilcoxon's rank sum test).

After 3 weeks of an EID 4.7 ± 1.3 (mean $\pm$ SD) clusters/mm^2 (vs 0.4 ± 0.2, mean $\pm$ SD, clusters/mm^2, NID) were found which increased to 6.1 ± 1.4 (mean $\pm$ SD) clusters/mm^2 (vs 3.1 ± 0.8, mean $\pm$ SD, clusters/mm^2, NID) after 9 weeks of diet (see figure 5B). In those rats not developing the focal lymphoid cell infiltrates, cluster formation declined to reach levels of 3 clusters/mm^2. In the rats that did develop the focal lymphoid cell infiltrates, the small lymphoid cell clusters were grouped into larger clusters as well as large areas of lymphoid cell accumulations with the architecture as described above. The larger lymphoid cell clusters were mainly composed of dendritic cells, CD4$^+$ T cells, B cells and a few ED1, ED7, ED8 and ED9 positive macrophages (see above).

The number of interfollicular CD4$^+$ T lymphocytes increased in the first 9 weeks after introducing the diet from nil to around 2 cells/mm^2 in both EID and NID BB rats. Numbers remained constant in animals that did not develop focal lymphoid cell infiltrates; however in those animals that did develop these infiltrates, CD4$^+$ T lymphocytes became uncountable (figure 5C).

The number of CD8$^+$ interfollicular T lymphocytes was and remained very low in both dietary groups, apart from the EID-BB rats that developed the focal lymphoid cell infiltrates (figure 5D).

B cells were absent from BB thyroids at the start of the experiment and 3, 6, and 9 weeks after introducing the diet. Only after 12 weeks of an EID when larger focal lymphoid cell infiltrates were detected, B cells could be identified and occurred only in these larger focal infiltrates together with dendritic cells, CD4 positive T cells and ED1, ED7, ED8 and ED9 positive macrophages.

Correlation between the presence of anti-colloid antibodies in the serum and the presence of the intrathyroidal focal lymphoid cell infiltrates in the BB rats kept on an enriched iodine diet

At the start of the experiment none of the rats were positive for anti-colloid antibodies ($n=8$). It is however well known that BB rats spontaneously develop

these antibodies, and indeed at 12 weeks of age (9 weeks of *NID*) 36% of the rats had become positive for anti-colloid antibodies, which incidence gradually increased to 58% at 15 weeks of age (12 weeks of NID), and to 93% at 21 weeks of age (18 weeks of NID, figure 6). We earlier reported that these anti-colloid antibodies are mainly produced outside the thyroid during the early phases of the autoimmune process (in the thyroid draining lymphnode) and not inside the thyroid[6].

The incidence of anti-colloid antibodies of rats kept on an enriched iodine diet was not statistically significantly different from the rats kept on a NID during the first 9 weeks of the experiment (figure 6). However, after 12 weeks of diet, the incidence of anti-colloid antibodies in rats kept on an EID developing intrathyroidal focal lymphoid cell infiltrates was 100%, whereas in those rats not developing these infiltrates, incidences of anti-colloid antibodies gradually declined to 0% after 18 weeks of diet (figure 6).

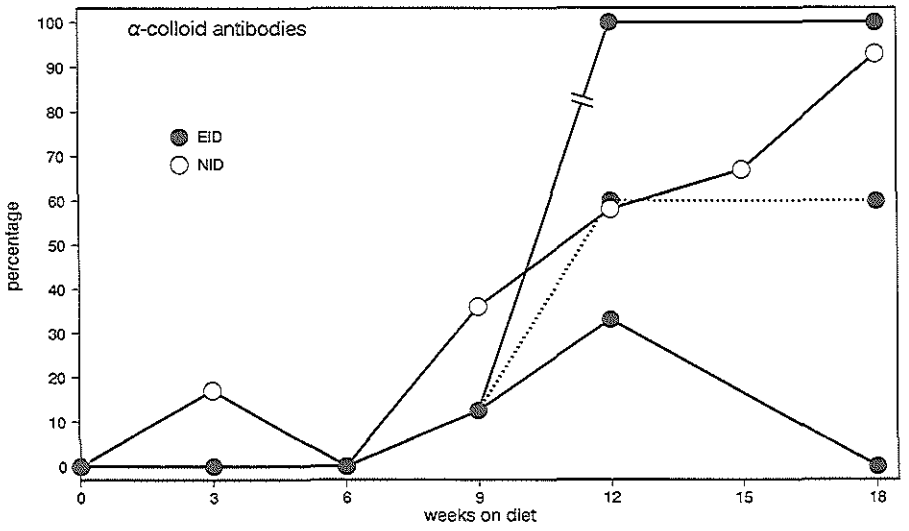

Figure 6. Incidence of anti-colloid antibody. The incidence of anti-colloid antibody production is given of various age groups of female BB rats (n=5-14) kept on a normal iodine diet (NID) or an enriched iodine diet (EID) from 3 weeks of age onwards for a period of 18 weeks. From 9 weeks of an EID and onwards the incidences of anti-colloid antibodies are given of the rats developing focal accumulations of lymphoid cells in the thyroid (represented by the // interrupted line), of rats not developing focal lymphoid cell accumulations (represented by the solid line) and of all the rats kept on an EID together (represented by the dashed line).

Table 4 shows the positive correlation between the development of intrathyroidal focal lymphoid cell infiltrates and the incidence of anti-colloid antibodies. It must be noted that the correlation could only be made for those animals from which the thyroid was immunohistochemically studied (5 of each group).

Table 4. Correlation between the presence of anti-colloid antibodies in the serum and the presence of the intrathyroidal focal lymphoid cell infiltrates in BB rats kept on an enriched iodine diet.

	rats without α-colloid Abs	rats with α-colloid Abs
rats without lymphoid cell infiltrates	4	1
rats with lymphoid cell infiltrates	0	5

DISCUSSION

At an earlier occasion we have reported that BB rats of our colony develop a highly organized lymphoid tissue in their thyroids starting at the age of 22-24 weeks, when kept on a for-our-area-normal iodine diet[6,21]. The process started with an increase in the number of interfollicular dendritic cells and a clustering of these cells, followed by a local trapping and accumulation in the clusters of T cells and B cells. At 24 weeks of BB rat age and older these aggregates of lymphoid cells start to show an organization and are composed of T cell areas with dendritic cells and high endothelial venules (HEVs), of B cell follicles adjacent to or in the center of the T cell areas and of plasma cells at the outer rims of the areas[24]. The structure of these lymphoid cell accumulations is hence very similar to the structure of mucosa associated lymphoid tissue (MALT) and of secondary lymphoid organs (lymphnodes, spleen).

The present study shows that a high dietary iodine intake of the BB rats (viz. 10 times the normal iodine intake) accelerates the development of this highly organized lymphoid tissue in the thyroids of the rats. The process again starts with an increase in the number and clustering of dendritic cells in the interfollicular area, and in 50% of the BB rats on an EID these clusters start to grow into the larger areas of lymphoid tissue at the age of 15 to 21 weeks.

Our observations support studies of the groups of Braverman and Sundick[1,2], who earlier showed that a high iodine intake in BB rats and OS chickens resulted in an increase of the incidence and severity of - what these authors called - focal "thyroiditis". In addition, the present report extends the findings of these authors by detailing the immunohistological composition of the areas of focal "thyroiditis".

Although many cells of the infiltrates exhibited macrophage characteristics, only minor signs of thyrocyte destruction were found. These small areas of destruction affected only few follicles which were located at the edges of the organized accumulations of lymphoid cells. We therefore consider the term "thyroiditis" as inappropriate, and prefer the term "thyroid-associated lymphoid tissue". Since the "thyroiditis" component was small, it is also not surprising that the BB rats on the enriched iodine diet remained euthyroid.

The majority of the cells in the areas of "thyroid-associated lymphoid tissue" were in fact B cells organized in structures reminiscent of early lymphoid follicles surrounded by rims and areas of T cells. The function of a B cell follicle is immunological memory formation and isotype switching. T cell areas in lymphoid tissue are normally involved in the generation of T helper 1, T helper 2 and T cytotoxic cells. We favor the idea that the organized lymphoid structures in the thyroid are responsible for the generation of thyroid autoreactive T cells and the production of thyroid autoantibodies. An indication for this function of the thyroid-associated lymphoid tissue is given by the fact that every rat which showed this intrathyroidal lymphoid tissue had anti-colloid antibodies in its serum.

The EID rats that did not develop thyroid-associated lymphoid tissue, showed a decline in the infiltration of dendritic cells and a decline in the cluster formation of these cells. Remarkably, these rats were almost all negative for anti-colloid antibodies. A similar suppressive effect of excess dietary iodine on the production of anti-colloid antibodies and the thyroidal infiltration with lymphoid cells has been found by us before in non-autoimmune Wistar rats[25]. It must also be noted in this respect that in earlier phases of the thyroid autoimmune process (at 6-18

weeks of age) anti-colloid antibody production mainly occurs in the thyroid draining lymphnode of the BB rat[6] and it seems therefore likely that the thyroid-associated lymphoid tissue developing in 50% of the BB rats kept on an enriched iodine diet is engaged in a further continuation of the thyroid autoimmune response, when the lymphnodal response subsides.

The mechanism by which the dietary iodine is capable of influencing the thyroid autoimmune reactivity and accelerating the development of the thyroid-associated lymphoid tissue in a proportion of the BB rats remains speculative.

A first possibility by which the dietary iodine might accelerate the development of the thyroid-associated lymphoid tissue is via a direct effect of iodine on dendritic cells/macrophages or T cells and B cells[26,27,28]. Iodine has been described as stimulating the myeloperoxidase activity of macrophages. It also increases the number of a circulating subset of T cells (involving both CD4 and CD8 positive T cells) and of MHC class-II positive T cells, and it induces a higher production of immunoglobulins from B cells/plasma cells. Recently we were able to demonstrate that highly iodinated proteins are capable of accelerating the maturation of dendritic cells from precursors in the blood[29,30]. Whether iodine has an effect on the stromal cells forming the microenvironment of lymphoid tissues (reticulum cells) needs further investigation.

A second possibility is that the enhanced antigenicity of highly iodinated thyroglobulin plays a role in accelerating the development of the thyroid-associated lymphoid tissue[31]. It has been shown that a high dietary iodine intake results in a high iodination grade of thyroglobulin and this highly iodinated thyroglobulin was proven to be (*in vitro*) more antigenic than thyroglobulin with a low iodination grade[32,33]. Together with a disturbed immune regulation in the BB rats caused by a lack of $RT6^+$ regulator (suppressor) T cells[34,35], and a disturbed immuno-endocrine feedback loop[36] the highly iodinated Tg might accelerate the development of thyroid-associated lymphoid tissue in the BB rat.

A third possibility for the accelerated intrathyroidal development of lymphoid tissue is that the high iodine diet induces the thyrocytes to produce more antigenic material. Li et al reported that iodine treated BB/W rats have a higher radioactive iodine release in comparison to non iodine treated BB/W rats or Wistar rats, indicating a leakage of autoantigen[37].

Toxic effects of iodine on metabolically active thyrocytes resulting in lymphoid cell infiltration have also been described[38,39]. However, the histological signs

of thyrocyte destruction were minor in our study and were only observed at the edges of the areas of the thyroid-associated lymphoid tissue. Toxicity of iodine with subsequent thyrocyte destruction and the release of antigenic material, thereby activating the thyroid autoimmune reactivity do not seem to play a prominent role in the here described study.

ACKNOWLEDGEMENTS

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Chapter 6

THE EFFECT OF THYROID HORMONES AND OTHER IODINATED COMPOUNDS ON THE TRANSITION OF MONOCYTES INTO VEILED/DENDRITIC CELLS. A ROLE OF GM-CSF, TNFα AND IL-6*

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ABSTRACT

We earlier reported that a pretreatment of blood monocytes with metrizamide (30 min, 37°C, non-adhering conditions) resulted in an enhanced maturation of monocytes into veiled/dendritic cells (DC) after an overnight culture (37°C, non-adhering conditions[1]). Since metrizamide is an iodinated compound structurally related to the thyroid hormones T_3 and T_4, we investigated the effect of these hormones on the maturation of monocytes into veiled/dendritic cells, as well as that of metrizamide, rT_3 and iodinated thyroglobulin (Tg). Since cytokines are essential for the differentiation and survival of blood dendritic cells in vitro[2] we also studied the effect of administration of α-GM-CSF, α-TNFα, α-IL-6, α-M-CSF, and α-IL-1β on the dendritic cell transition. The production of GM-CSF, IL-6 and IL-1β by the stimulated monocytes was measured in a limited series of experiments.

A stimulation of the blood monocytes with T_3 and T_4 enhanced their capability to mature into cytological and functional characteristic veiled/dendritic cells (cells with actively moving veiled and dendritic cytoplasmic protrusions, strongly positive for MHC class-II antigens with weak or absent acid phosphatase acivity, and good stimulators in the MLR). Veiled/dendritic cell transition induced by T_3 and T_4 was dependent on GM-CSF, TNFα and IL-6 produced in the culture since α-GM-CSF, α-TNFα and α-IL-6 had blocking effects, α-M-CSF and α-IL-1β had no effects. Contaminating T cells and B cells were not necessary for the transition and it is therefore likely that GM-CSF, TNFα and IL-6 were produced by the monocytes themselves. There was indeed a considerable production of cytokines in the culture system. Since the production of these cytokines was not increased in the thyroid hormone stimulated monocyte cultures, it is likely that the thyroid hormones influence monocyte to veiled/dendritic cell transition on the cytokine receptor level or on a post cytokine receptor level.

A stimulation of the blood monocytes with an optimal concentration of metrizamide (14.5%), rT_3 (2.10^{-10}M) and highly iodinated Tg (2.10^{-11}M) also resulted in an increased transition of monocytes to cytological and functional characteristic veiled/dendritic cells, but to a lesser extent in comparison to the thyroid hormones (T_3: 31±6%, T_4: 25±5% vs rT_3: 22±8% and 0.37% I Tg: 20±4% veiled/dendritic cells). Remarkably, α-GM-CSF, α-TNFα and α-Il-6 administration to the culture system had also blocking effects on the transition from monocytes to veiled/dendritic cells induced by the iodinated compounds, but again

an enhanced production of these cytokines under the influence of the iodinated compounds was not found. The mechanisms by which such compounds act on the monocyte to veiled/dendritic cell transition can only be speculated on.

Our data nevertheless show that:

a. exposure to thyroid hormones stimulates monocytes to mature into antigen-presenting veiled/dendritic cells; this observation is in line with earlier observations that thyroid hormones are necessary for optimal immune function, and
b. that iodinated compounds like metrizamide, rT_3 and iodinated Tg are also capable to enhance the transition of monocytes into veiled/dendritic cells; these latter observations are in line with earlier observations of a direct effect of iodine on cells of the immune system, such as T cells, B cells and macrophages.

INTRODUCTION

Dendritic cells (also called veiled cells when free in body fluids[3]) are excellent antigen presenting cells[4,5]. The cells play a crucial role in the initiation of normal as well as of autoimmune responses[6,7]. Knight et al[8] showed that autoimmune thyroiditis could be induced in Balb/c mice by the transfer of as few as 10^5 dendritic cells pulsed with thyroglobulin (Tg). Furthermore, the development of autoimmune lesions, such as arthritis[9,10,11], thyroiditis[12,13] or insulitis[14] starts with an increase in the number of dendritic cells and a local clustering of these cells with other lymphoid cells before autoantibodies can be detected in the circulation.

The precursor of the dendritic cells has not yet been fully characterized. We[1] and others[15,16] have reported that cells with a veiled or dendritic morphology, MHC class-II positivity and weak to absent acid phosphatase positivity, can be maturated from cells belonging to the blood monocyte pool: it was possible to induce 30-40% of veiled/dendritic cells from elutriator purified blood monocyte fractions (over 98% nonspecific esterase positive cells) after an exposure of these cells to 14.5% metrizamide for 30 minutes followed by an overnight culture under nonadhering conditions[1]. These monocyte-derived veiled/dendritic cells had an increased capability to act as stimulator cells in a MLR when compared to the

control, non-pulsed monocyte populations[1,17]. The veiled/dendritic cells were also less phagocytic and expressed less CD14. Since metrizamide is an iodinated compound structurally related to the thyroid hormones T_3 and T_4 we speculated that thyroid hormones - and maybe iodinated compounds in general - play a role in enhancing the transition of monocytic cells to veiled/dendritic cells.

The here reported study describes the effect of the thyroid hormones T_3 and T_4 and of a few iodinated compounds besides metrizamide, such as reverse T_3 (rT_3, the hormonally inactive form of T_3) and highly- low- and non-iodinated thyroglobulin (Tg), on the maturation of human peripheral blood monocyte fractions into functionally active veiled/dendritic cells. Since cytokines, and in particular granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor α (TNFα), have been described as essential for the differentiation and survival of blood dendritic cells in vitro[2,18,19], we also measured in a series of experiments the effect of the administration of α-GM-CSF, α-TNFα, α-interleukin-6 (α-IL-6), α-macrophage colony-stimulating factor (α-M-CSF) and α-IL-1β, on the monocyte to veiled/dendritic cell transition. The production of GM-CSF, IL-6 and IL-1β by the stimulated monocytes was measured in a limited series of experiments.

MATERIALS AND METHODS

The isolation of human peripheral blood monocytes

Peripheral blood monocytes from healthy volunteers were isolated by Ficoll-Isopaque (density 1.077 g/ml, Pharmacia, Uppsala, Sweden) density gradient centrifugation, followed by Percoll[20] (density 1.063 g/ml, Pharmacia) gradient centrifugation. In brief, heparinized blood was diluted with an equal volume phosphate buffered saline (PBS), pH 7.4, placed on Ficoll Isopaque and centrifuged for 15 minutes at 1000 g. The cells were collected from the interface and washed twice with PBS (5 minutes, 500 g). The cells were resuspended in RPMI 1640 (with 25 mM HEPES and L-glutamin, GIBCO, USA) containing antibiotics (100 U/ml penicillin G and 0.1 mg/ml streptomycin, Seromed, Biochrom, Berlin, Germany) and counted in a Bürker-Türk hemocytometer using light microscopy (magn 250x). 10-20.10^6 cells were placed on 2 ml Percoll and centrifuged for 40 minutes at 400 g. The collected cells from the interface were washed twice (5 minutes, 500 g) in

RPMI 1640 supplemented with antibiotics. The percentage of monocytes was determined by positive staining with non specific esterase (NSE) according to Mullink et al[21]. The cell suspension contained 60-80% NSE positive monocytes, the contaminating cells mainly consisted of $CD3^+$ T cells.

In some experiments a) elutriator-purified monocytes were used (purity >98% of NSE positive monocytes, for technical details see ref[1]) or b) Percoll purified monocyte preparations freed from contaminating T and B cells using a MACS (Magnetic Cell Separation system, Miltenyi Biotec GmbH, Germany) with mouse anti-human CD3 (Becton and Dickinson, USA) and CD19 (Coulter, England) monoclonal antibodies coated onto biotin labeled magnetic beads (Becton and Dickinson) via streptavidin (Zymed Laboratories Inc, USA) and a goat anti-mouse biotin labeled conjugate (Dakopatts, Denmark) yielding monocyte suspensions with a purity >95% NSE positive monocytes, this to verify whether contaminating T cells or B cells were necessary to provide stimuli for the monocyte-to-veiled/dendritic cell transition.

Incubation of human peripheral blood monocyte suspensions with hormones or iodinated compounds to obtain veiled or dendritic cells

From the purified monocyte suspensions a cell suspension containing 1.10^6 cells/ml was made in serum free culture fluid (ISCOVE's Modified Dulbecco's Medium, Gibco) supplemented with SF-1 (Costar, USA) and antibiotics.

A total of $3\text{-}4.10^5$ NSE^+ monocytes were incubated with culture fluid alone or were stimulated under nonadhering conditions (polypropylene tubes Falcon, Becton & Dickinson, USA) (37°C, 5% CO_2, 100% humidity) for 30 minutes with the following stimuli:

a. with metrizamide (14.5% w/v, Serva, Heidelberg, Germany) according to Kabel et al.[1],
b. with T_4, T_3, rT_3 (Sigma) and
c. with highly-iodinated Tg (0.37% I), low-iodinated Tg (0.2% I) and non-iodinated Tg (0% I), kindly provided by JJM de Vijlder (Academical Medical Centre, Amsterdam, The Netherlands).

The concentrations of the thyroid hormones and the iodinated compounds ranged from 2.10^{-18}M to 2.10^{-9}M.

After exposure to the hormones or iodinated compounds, the cells were washed 3 times in RPMI 1640 (5 minutes, 500 g) and further cultered in supplemented ISCOVE's for a period of 16 hours (overnight) under nonadhering conditions (polypropylene tubes, 37°C, 5% CO_2, 100% humidity).

To determine the influence of endogenous produced cytokines on the monocyte to veiled/dendritic cell transition, several concentrations of the monoclonal antibody α-GM-CSF (Genzyme Corporation, Boston, USA), α-TNFα (Genzyme), α-IL-6 (kindly provided by Dr. W.A. Buurman, University of Limburg, Maastricht, The Netherlands), α-M-CSF (Genzyme) and α-IL-1β (Glaxo, Geneva, Switzerland) were added 2 hours after starting the overnight culture period, the antibody remained present in the culture fluid during the further culture period (in a series of prelimenary experiments this time schedule proved to be the most optimal to reach the effects described in the results section).

Determination of the percentage of monocyte derived veiled or dendritic cells

After the 16 hour overnight culture period, cells were centrifuged (5 minutes, 500 g), the supernatant was removed and stored at -20°C untill the measurement of produced GM-CSF. The cells were resuspended and the morphology of the living cells was studied using light microscopy at a magnification of x400. Large cells, with actively moving, veiled cytoplasmic processes and/or dendritic extensions were considered as veiled/dendritic cells (figure 1).

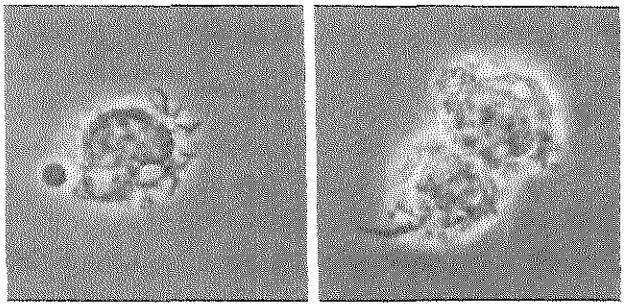

Figure 1. Typical morphology of T_3-induced living veiled/dendritic cells in suspension using phase contrast microscopy. Note the irregular cytoplasmic protrusions. Magn x800.

The number of living veiled/dendritic cells counted directly in suspension were verified using cytocentrifuge preparations: 50 μl of the cellsuspensions at a concentration of $0.5.10^6$/ml were cytocentrifuged (3 minutes, 800 rpm) using a Cytospin apparatus (Nordic Immunological Laboratories, The Netherlands). Cytocentrifuge preparations were airdried for at least 1 hour and fixed in 100% acetone for 10 minutes. Thereafter, indirect immunoperoxidase staining was performed using the monoclonal antibody HLA-DR in a dilution of 1:50 (specific for MHC class-II antigens, Becton and Dickinson, USA). After washing the slides a rabbit-anti-mouse IgG peroxidase labeled conjugate (1:50, Dako Imunoglobulins, Denmark) in 1% normal human pool serum was applied to the slides. The cytocentrifuge preparations were developed with a 3,3'-diaminobenzidine (DAB) solution containing 0.05% DAB (Sigma) for 3-5 minutes. Hydrogen peroxide was added to a final concentration of 0.01%. After DAB development, the cytospin preparations were incubated with acid phosphatase (with naphtol AS-BI phophate as substrate and hexazotized pararosanilin as diazoniumsalt) at 37°C for 30 minutes, counterstained with hematoxylin, dehydrated and embedded in DePeX mounting medium (Gurr, BDH Limited Poole, England). Cells with long cytoplasmic protrusions, a reniform nucleus, strong MHC class-II positivity but absent or weak acid phosphatase reactivity were considered to be the veiled/dendritic cells (this criterium is used by us before in fixed histological preparations[22,23,24,25,26]).

Purification of the veiled/dendritic cell suspensions

In a few experiments veiled/dendritic cells were enriched in the suspensions using the following technique (based on the characteristic of veiled/dendritic cells not to spontaneously adhere to surfaces, but to actively move around in the culture fluid[3]): after the overnight culture period under nonadhering conditions, the cell suspension containing monocytes and induced veiled/dendritic cells was washed (5 minutes, 500 g in polypropylene tubes) and cells were resuspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS, Integro BV, Zaandam, The Netherlands). The cell suspension was placed in a Boyden chamber system seperated by a polycarbonate microfilter (5μm, Nuclepore, polyvinylpyrrolidone free, Costar Corp, USA) in which the top chamber (polypropylene) contained the

washed monocyte/dendritic cell suspension in RPMI 1640, 10% FCS and the bottom chamber RPMI 1640, 10% FCS and 10 nM of the chemoattractant fMLP (n-formyl-methionyl-leucyl-phenylalanine, Sigma).

Cells in the top chamber were allowed to adhere to the polycarbonate filter and to migrate through the filter towards the fMLP gradient in the bottom chamber during a 2.5 hour incubation period (37°C, 5% CO_2, 100% humidity). Subsequently the cells were collected from the top chamber and the bottom chamber. The percentage of cells with a veiled/dendritic morphology were counted in both cell suspensions as described above under light microscopy (magn 400x).

Mixed leucocyte reaction (MLR)

To verify whether the monocyte derived cells with a veiled or dendritic morphology were functionally active veiled/dendritic cells capable of stimulating T cells to proliferate, an allogeneic mixed leucocyte reaction (MLR) was performed (details in ref[1]) with the veiled/dendritic cells as stimulator cells.

To obtain the responder population, a T lymphocyte isolation was performed according to standard procedures with subsequent Ficoll-isopaque, Percoll density gradient centrifugation (the pellet containing T and B lymphocytes and NK cells) and nylon wool adherence[27] (Leuko-Pak, fenwall Laboratories, Il, USA). The nonadhering cells were >90% positive for the marker CD3.

The mixed lymphocyte cultures were performed in triplicate in 96 wells flatbottomed microtitre plates (Falcon). As stimulator cells (x-irradiated, 2000 RAD) were used:

a. non cultered, freshly isolated Percoll monocytes,
b. Percoll-purified monocytes cultured overnight at 37°C under nonadhering conditions,
c. Percoll-purified monocytes cultured overnight at 37°C under nonadhering conditions after a pulse with T_3, T_4, rT_3 or the iodinated Tgs (containing raised numbers of veiled/dendritic cells), in some instances these populations were enriched for veiled/dendritic cells by the Boyden chamber method (see above) and
d. blood veiled/dendritic cell populations obtained according to the well established separation method described by Knight et al[28] (control).

Three.10^4 of stimulator cells were cultured with 1.5.10^5 allogeneic T lymphocytes in a total volume of 200 μl RPMI 1640 supplemented with 10% human A^+ serum and antibiotics for 5 days at 37°C, 5% CO_2. Three.10^4 x-irradiated monocytes/dendritic cells alone or 1.5.10^5 T lymphocytes in the presence of 10-50 μg/ml phytoheamagglutinin (PHA, Wellcome Diagnostics, UK) served as controls. Stimulator and responder populations were of various healthy donors.

During the last 16 hours 0.5 μCi ^{3}H-thymidine was added to each well, the cells were harvested on filter paper and ^{3}H-thymidine incorporation was measured in a liquid scintillation analyzer (Packard tricarb 2500 TR).

Cytokine production

The cytokine production was measured in a limited series of experiments in stored supernatants of various monocyte cultures after the over night culture period. Frozen samples were thawed and GM-CSF, IL-6 and IL-1β were measured using an enzyme amplified sensitivity immuno assay system (EASIA, MEDGENIX, Diagnostics, Belgium) following the instructions of the manufacturer.

Statistical analysis

Differences in monocyte to veiled/dendritic cell transition were analyzed using the Wilcoxon's rank sum test. Differences in the stimulating capability of monocyte or veiled/dendritic cell populations in the MLR and the cytokine production by these cell populations were tested using a paired binomial test.

RESULTS

The enhancing effect of thyroid hormones and other iodinated compounds on the transition of monocytes to veiled/dendritic cells

Incubation of Percoll-purified monocytes in the defined serum free culture fluid

alone resulted after an overnight culture period (non-adhering conditions, 37°C, 5% CO_2) in 13.2±2.7% (mean±SD) cells with a veiled or dendritic morphology (figure 2, represented by the hatched area). The incubated cells showed a viability of >90% (trypan blue exclusion).

An exposure of the monocytes to the thyroid hormones T_3 and T_4 prior to the overnight culture enhanced the capability of the blood monocytes to mature into veiled/dendritic cells in a dose-dependent manner (figure 2). A stimulation of the blood monocytes with optimal T_3 or T_4 concentrations (2.10^{-10} M) resulted in 30.5±5.7% (mean±SD) veiled/dendritic cells or 24.5±4.8% (mean±SD) veiled/dendritic cells respectively. Thyronine (T_0, the non-iodinated backbone molecule of thyroid hormones) was unable to enhance the transition of monocytes into veiled/dendritic cells (figure 2): at best (at a T_0 concentration of 2.10^{-10} M) 13.5±3.4% (mean±SD) dendritic/veiled cells were found; this value is not statistically significantly different from the spontaneous maturation (during incubation with serum free culture fluid, represented by the hatched area in figure 2).

In two series of experiments Percoll-purified monocyte suspensions that had been freed of contaminating T cells and B cells (using a MACS, yielding over 95% NSE^+ monocytes), or elutriator purified monocytes (yielding over 98% NSE^+ monocytes) were used. The thyroid hormone T_3 also gave in these experiments a clear enhanced monocyte to veiled/dendritic cell transition and 41% (MACS seperation) and 29±10% (n=10, elutriator purification) veiled/dendritic cells were found respectively, 2.10^{-11}M being the optimal T_3 concentration (data not shown). These experiments using almost pure monocyte suspensions show that T cells and B cells contaminating the monocyte population or products of these T or B cells are not necessary for the transition of monocytes to veiled/dendritic cells.

A pretreatment of the monocytes with metrizamide prior to the overnight culture, increased - as expected - the monocyte to veiled/dendritic cell transition, and 26.8±2.9% (mean±SD) dendritic/veiled cells were found, as has been reported before[1] (although in cultures containing 10% fetal calf serum). Reverse T_3 (rT_3) and both iodinated forms of thyroglobulin (Tg) were also capable of enhancing the monocyte to dendritic cell transition, although to a somewhat lesser extent as compared to the thyroid hormones and metrizamide (figure 2).

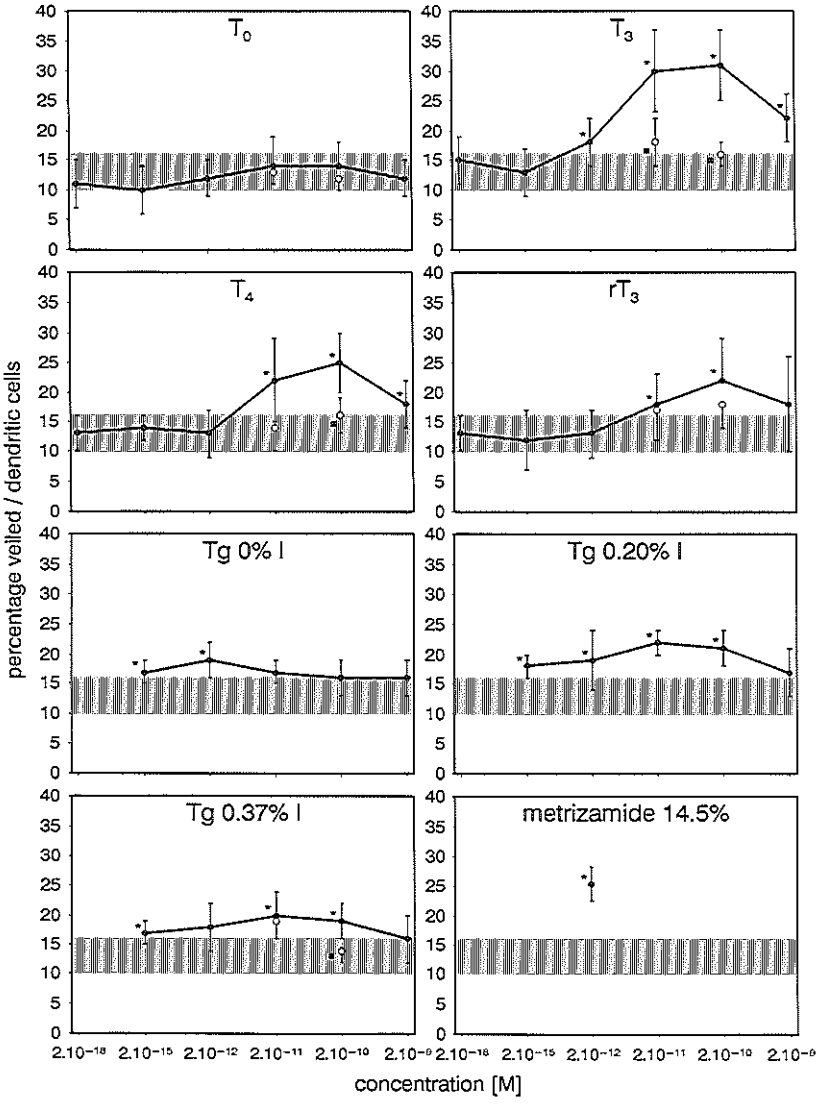

Figure 2. The monocyte into veiled/dendritic cell transition induced by an exposure to thyroid hormones or other iodinated compounds (mean ± SD, n=4-20). The hatched area represents the mean ± SD (n=20) of the spontaneous monocyte to dendritic cell transition (monocytes exposed to culture fluid alone). The open circles give the mean ± SD (n=3-6) of the monocyte to veiled/dendritic cell transition after α-GM-CSF treatment. * $p<0.05$ induced vs spontaneous transition (hatched area), Wilcoxon's rank sum test. ▪ $p<0.05$ induced transition vs induced transition after α-GM-CSF treatment, Wilcoxon's rank sum test.

At a concentration of 2.10^{-10}M of rT_3 (optimal concentration) 22±7% (mean±SD) veiled/dendritic cells were found. With regard to the iodinated forms of Tg at a concentration of 2.10^{-11}M Tg 0.2%I (optimal concentration) 22±2% (mean±SD) veiled/dendritic cells were found and at a concentration of 2.10^{-11} M Tg 0.37%I (optimal concentration) 20±4% (mean±SD) veiled/dendritic cells were found. Non-iodinated Tg was practically not capable of stimulating the transition of monocyte to veiled/dendritic cells and low values of 19±3% and 17±2% veiled/dendritic cells (mean±SD) were maximally found (figure 2, note however the much lower concentrations, namely at 2.10^{-15} and 2.10^{-12} M Tg where this transition was induced; at these concentrations also the iodinated Tg gave similar values and one may wonder whether this transition is induced for instance by the ingestion of the large Tg molecule).

Immunocytochemistry

To verify the character of the veiled/dendritic cells counted directly in the wet cell suspensions, immunocytochemical studies were performed on fixed cytocentrifuge preparations of the cell populations after overnight culture. Cells with cytoplasmic protrusions, strong MHC class-II positivity and weak or absent acid phosphatase activity were considered as veiled/dendritic cells in the fixed and stained preparations.

A strong correlation was found ($r=0.87$, $p<0.01$) between the percentage of veiled cells counted directly in suspension (cells with actively moving veils or dendritic cytoplasmic protrusions in the wet preparations) and the percentage of MHC class-II positive, acid phosphatase negative dendritic cells counted in the cytocentrifuge preparations (figure 3).

The mixed leucocyte reaction (MLR)

To verify the functional capacity of the induced veiled/dendritic cells, MLRs were carried out.

With regard to the control cultures, PHA was able to stimulate T lymphocytes to proliferate depending on the concentration used and $114.10^3 \pm 20.10^3$ cpm could

be reached in our assay at 50μg/ml of PHA. Irradiated monocyte suspensions and suspensions containing veiled/dendritic cells alone (other control cultures) were unable to proliferate and the cells did virtually not incorporate ^{3}H-thymidin (100-300 cpm). Purified T lymphocytes incubated with culture fluid alone (without stimulator cells) also incorporated ^{3}H-thymidin at a very low level and <150 cpm were measured.

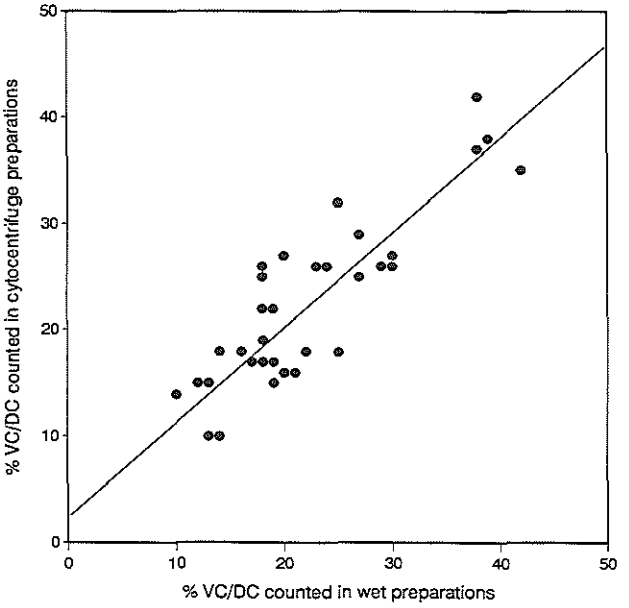

Figure 3. Correlation between percentage of living veiled/dendritic cells (VC/DC) counted directly in wet preparations (x-axis) and fixed veiled/dendritic cells (VC/DC) counted in cytocentrifuge preparations using immunocytochemistry (y-axis). Slope = 0.85, r = 0.87, $p<0.01$.

In figure 4 the results of the MLRs are depicted as proliferation indices (PI) of the tests which were calculated as follows:

cpm of incorporated ^{3}H-thymidin in T lymphocyte suspensions stimulated with cell suspensions containing monocytes and veiled/dendritic cells as stimulator cells

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cpm of incorporated ^{3}H-thymidin in the same T lymphocyte suspensions but without any stimulating cell suspension.

Figure 4 gives the results of a series of experiments using various donor-stimulator combinations.

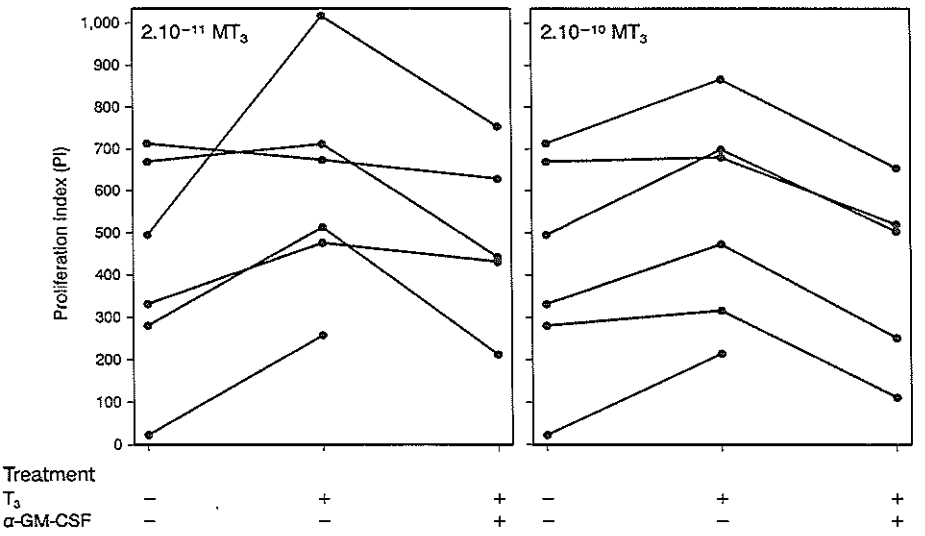
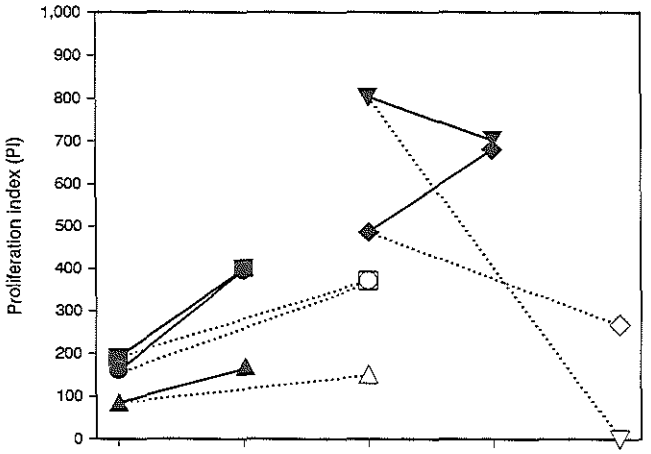


Figure 4. Mixed leucocyte reaction depicted as proliferation indeces (PI) of T cells stimulated with cultured monocytes, T cells with T_3-pulsed monocytes and T cells with T_3-pulsed monocytes treated with α-GM-CSF. The results are given of monocytes pulsed with 2.10^{-11} M T_3 (A) and with 2.10^{-10} M T_3 (B).

First it must be noted that we found a high range of PIs depending on the donor-stimulator combination used. Figure 4 further shows that monocyte suspensions exposed to T_3 prior to the overnight culture and contaning the highest percentages of veiled/dendritic cells (viz around 30%, see figure 2) were more potent stimulator populations in the MLR in comparison to cell suspensions that had not been exposed to any compound prior to the overnight culture (in 11 out of 12 cases, $p<0.003$, paired binomial test); the latter preparations only contained $13 \pm 3\%$ (mean $\pm$ SD) veiled/dendritic cells (see figure 2, hatched area). It must be noted however that this increase in MLR stimulator capacity is in fact minor and not proportional to the increases in the number of veiled/dendritic cells.

With regard to pretreatment of the monocytes with thyroxine, rT_3 and highly iodinated Tg it must be noted that this procedure yielded higher numbers of veiled/dendritic cells in comparison to non-pulsed monocyte populations and that such pretreated cell suspensions were more effective than the non-pulsed monocyte suspensions in stimulating allogeneic T cell proliferation in the MLR. However, statistical significant differences in the MLRs could not be reached (data not shown), probably because the increases in the number of veiled/dendritic cells are too low to give significant increases in the apparently insensitive MLR (see before).

In a series of other MLR experiments (figure 5) veiled/dendritic cell suspensions maturated via a pulse with T_3 and containing in this series of tests relatively high percentages of veiled/dendritic cells (viz 46±5%, mean±SD) were again potent stimulators in the MLR.



Stimulator population:					
mono's pulsed with 2.10^{-11} MT_3	–	+	–	–	–
DC purified according to Knight	–	–	+	–	–
DC purified using Boyden Chamber:	–	–	–	+	+
- non migrating cells	–	–	–	+	–
- migrating cells	–	–	–	–	+

Figure 5. Mixed leucocyte reaction depicted as proliferation indeces of T cells stimulated with cultured monocytes, T cells with 2.10^{-11} M T_3-pulsed monocytes, T cells with dendritic cells purified according to the method of Knight et al. and T cells with T_3-pulsed monocytes further purified using the Boyden chamber technique (yielding migrating cells and non-migrating cells).

In fact they were as potent MLR stimulators as veiled/dendritic cell suspensions obtained via the well established purification method of Knight et al (based on density purification); the latter "Knight" cell suspensions also contained around 40% veiled/dendritic cells in this experiment.

The cell suspensions enriched for veiled/dendritic cells with the purification technique based on adherence and chemotaxis in the Boyden chamber (non-adhered and non-migrated cells in the top chamber) contained 56% of veiled/dendritic cells and these cell suspensions belonged to the most potent stimulators in the MLR (figure 5). The cells chemotactically attracted and migrated through the membrane in this purification method (into the bottom chamber) only contained 15% of cells with a veiled/dendritic morphology and these cell populations only weakly stimulated T cells in the MLR (see figure 5).

Effects of α-cytokine administration on the transition of monocytes to veiled/dendritic cells

The enhanced monocyte to veiled/dendritic cell transition induced by the pulse with the thyroid hormones T_3 and T_4 was dependent on the endogenous production of GM-CSF, TNFα and IL-6 in the culture; figure 2 and 6 show that a treatment of the pulsed cells with 5 μg/ml (optimal concentration) α-GM-CSF, α-TNFα (optimal effects at dilution 1:10) and α-IL-6 (optimal effects at dilution 1:2500) during overnight culture (from 2-16 hours) prevented the transition of monocytes to dendritic/veiled cells. α-G-CSF (data not shown), α-M-CSF and α-Il-1β did not have such effects.

With regard to functional effects, in the MLR, α-cytokine treated T_3-pulsed monocyte suspensions (containing fewer veiled/dendritic cells, viz around 15%) were considerably less effective as stimulator cell populations (in 10 out of 10 cases, $p<0.001$ paired binomial test, figure 4).

α-GM-CSF, α-TNFα and α-IL-6 treatment also had effects on the metrizamide induced monocyte to veiled/dendritic cell transition (figure 6). These blocking effects were however more variable than in the case of T_3 induced maturation.

In the case of other induction pulses, namely rT_3 and Tg 0.37% I less consistent data were obtained (figure 2), probably due to the fact that these induction pulses were less strong as compared to T_3 and metrizamide.

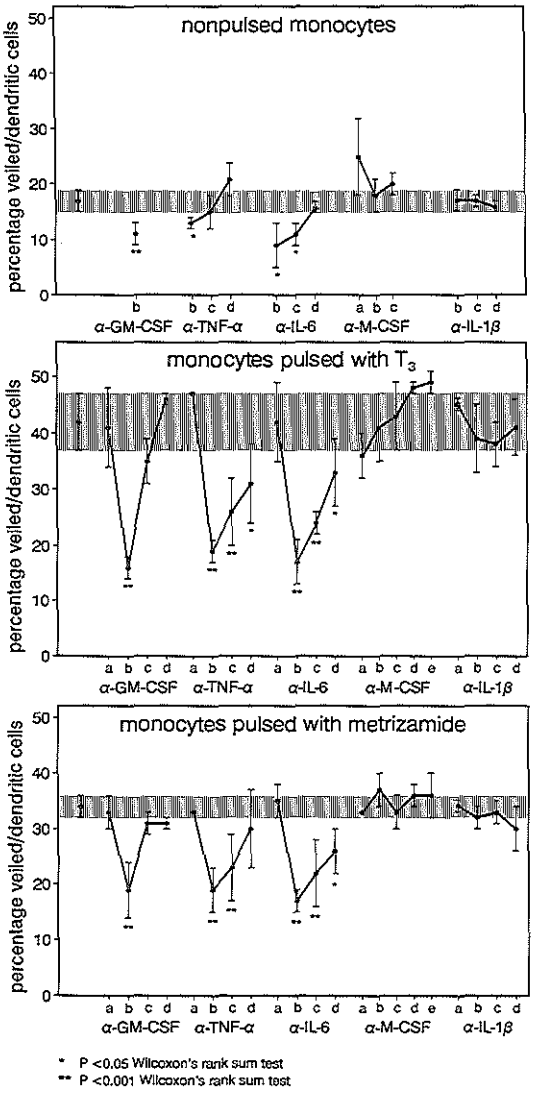


Figure 6. Inhibition of the veiled/dendritic cell transition (hatched area) of nonpulsed monocytes, T_3 pulsed monocytes and metrizamide pulsed moncytes by α-GM-CSF (a: 10μg/ml, b: 5μg/ml, c: 1μg/ml, d: 0.5μg/ml), α-TNFα (a: 1:5, b: 1:10, c: 1:50, d: 1:100), α-IL-6 (a: 1:500, b: 1:2500, c: 1:10000, d: 1:25000), α-M-CSF (a: 50 μg/ml, b: 5 μg/ml, c: 0.5 μg/ml, d: 0.05μg/ml, e: 0.05 μg/ml) and α-IL-1β (a: 1:500, b: 1:2500, c: 1:10000, d: 1:25000).

The production of GM-CSF, IL-6 and IL-1β by the pulsed monocytes

The GM-CSF, IL-6 and IL-1β production in the monocyte cultures was only tested in a limited series of experiments. The cytokine production of monocytes incubated in culture fluid alone was clear but varied considerably depending on the donor (n=5-10) and the time of collection; GM-CSF values were found between <1 and 1800 pg/ml/1.10^6 cells, IL-6 values between 200 and 500 ng/ml/1.10^6 cells and IL-1β values between 2 and 20 ng/ml/1.10^6 cells after an overnight culture period. Exposure of the monocytes to thyronine (T_0), T_3, T_4, Tg or metrizamide for half an hour did neither have quantitative important nor statistical significant effects on the cytokine production.

DISCUSSION

This study firstly shows that a pretreatment of blood monocytes for 30 minutes with thyroid hormones, and in particular with the bioactive thyroid hormone T_3, enhances the capability of the monocytes to mature into cytologically and functionally active veiled or dendritic cells when further cultured overnight under nonadhering conditions. The dendritic cell maturation during the culture was dependent on the endogenous production of GM-CSF, TNFα and IL-6 since α-GM-CSF, α-TNFα and α-IL-6 had blocking effects on the transition. Indeed, GM-CSF, IL-6 and IL-1β were produced in the cultures and these cytokines are most likely produced by the monocytes themselves since a removal of the few contaminating T cells and B cells did not affect the monocyte to veiled/dendritic cell transition.

The percentage of veiled/dendritic cells with long, actively moving cytoplasmic protrusions counted directly in the wet preparations correlated well with the percentage of "histologically classical" dendritic cells in fixed and stained cytocentrifuge preparations, viz cells with a dendritic morphology that were strongly MHC class-II positive and acid phosphatase negative. Also cell suspensions with the highest percentages of T_3-induced-veiled/dendritic cells, including the Boyden chamber purified cell fractions containing over 40% of T_3 induced veiled/dendritic cells, were the most potent stimulators in the MLR (comparable to effects of stimulator populations purified accordng to the classical "Knight" procedure). It must be admitted however that the increases in stimulator capacity in the MLR

were often minor and not proportional to the increases in the number of veiled/dendritic cells. We nevertheless feel justified to conclude that the cells with a veiled morphology in the wet preparations and induced by the action of the thyroid hormone must be considered as morphological and functional typical antigen-presenting accessory cells of veiled/dendritic type[22,23,24,25,26].

Remarkable are also the relatively low thyroid hormone concentrations at which the optimal effects were noticable, e.g. at 2.10^{-10}M. In other systems (liver) often concentrations in the micromolar to nanomolar range are optimal[29]. The thyroid hormone T_3 functions as a signalling substance by regulating the transcription of certain T_3-responsive genes[30]. Cellular responses to this hormone depend on the concentration of T_3 within the cell. This concentration is regulated by different mechanisms such as the intracellular synthesis from T_4 and the transport of T_3 through the cell membrane. Intracellular T_3 can only mediate its function through binding to nuclear T_3 receptors, which receptors are encoded for by the c-erbA proto-oncogene family. The T_3 receptors act as transcription factors and their activity not only depends on the availability of thyroid hormone, but also on the concentration of the receptor within the nucleus. Only after dimerization with certain T_3 receptor auxiliary proteins (or TRAPs), including retinoid receptors, a functional hormone-occupied T_3 receptor monomer can regulate transcription of certain genes by binding to specific DNA elements the so-called T_3-responsive elements (TRE). The retinoic acid receptors are hence also able to regulate T_3 regulated transcription.

It is therefore clear that further experiments are needed to unravel the exact mechanisms of the T_3-induced enhancement of monocyte to veiled/dendritic cell transition, in particular the transport mechanisms of thyroid hormone into monocytes, the role of the deiodinase activity of monocytes (conversion of T_4 into bioactive T_3), the concentrations of T_3 receptor isoforms and of TRAP isoforms in monocyte nuclei and the effect of the monocytic T_3 receptors on the transcription of genes that are involved in the here described GM-CSF, IL-6 and TNFα effects. T_3 apparently does not have an effect on the transcription of genes regulating the production of cytokines, since the production of GM-CSF, IL-6 and IL-1β was not increased in the cultures. T_3 might have an effect on genes regulating cytokine receptor expression or post cytokine receptor effects.

Veiled/dendritic cells are excellent antigen presenting cells and play a crucial role in the initiation of immune responses. Our observations that particularly T_3

stimulates antigen-presenting function is in agreement with earlier observations of Balàzs et al[31] who showed that T_3 supplements enhanced PHA stimulation of human lymphocytes in vivo and in vitro and of Chatterjee et al[32] who found an increased blastogenic response of lymphocytes from thymus, peripheral blood and mesenteric lymphnodes to pokeweed mitogen (PWM) after in vivo T_3 and T_4 administration. Additionally, the observations of Fabris et al[33] that hypothyroidism induces immunodeficiency, which is corrected by thyroid hormone supplements demonstrate that thyroid hormonal activity is necessary for optimal immune function.

Our study also shows that iodinated compounds such as metrizamide, rT_3 and iodinated Tg are capable to enhance the monocyte to veiled/dendritic cell transition. It is clear that such capability can not be transmitted via the T_3 receptor. Our observation is nevertheless in agreement with earlier observation that iodinated compounds can have direct stimulating effects on cells of the immune system, such as the T cells[34], the B cells[35] and the macrophages[36].

When monocytes were exposed to the highly iodinated compound metrizamide (containing 3 iodine atoms per molecule) high numbers of veiled/dendritic cells were induced (comparable to the numbers found after the exposure to T_3). These veiled/dendritic cells were capable of significantly stimulating the MLR (earlier experiments, see ref[1]) and GM-CSF, TNFα and IL-6 played a role in the transition, since the α-cytokines had blocking effects.

The transition induced by rT_3 and iodinated Tg was however not as pronounced as the transition that was induced by metrizamide and T_3; also in the MLR, significant stimulation could not be observed (probably because the increases in MLR are minor and not proportional to the increases in number of veiled/dendritic cells). One can only speculate about the mechanisms that are involved in the enhancement of the monocyte to dendritic/veiled cell transition under for instance the influence of metrizamide or rT_3. It is possible that iodonium groups (I^+) may be irritants for monocytes and it is tempting to speculate that these active redox groups are capable of interfering with myeloperoxidase and the H_2O_2 generating system in the monocytes, thereby triggering the monocyte to maturate into a cell with a less active H_2O_2 generating system, the dendritic cell. In support of such a view we found in a few experiments the peroxidase interfering drug methimazole also capable of enhancing the monocyte to veiled/dendritic cell transition (data not published). How α-cytokines exert their effects in such an

irritative action clearly needs further study.

With regard to a direct effect of the iodinated compounds on cells of the immune system, it has been described that a high dietary iodine intake may accelerate autoimmune thyroid disease, particularly in individuals or animals with a preexisting thyroid abnormality[37,38,39,40,41]. An enhanced antigenicity of highly iodinated thyroglobulin has been suggested to play a role in such acceleration of the thyroid autoimmune response[42]. It was for instance shown that a high dietary iodine intake resulted in vivo in a high iodination grade of thyroglobulin and highly iodinated thyroglobulin was proven to be (in vitro) more antigenic than thyroglobulin with a low iodination grade[43,44]. Since dendritic cells play a crucial role in the initiation of thyroid autoimmune responses[6,7,8] and since a high iodine diet (viz highly iodinated Tg) accelerates the development of focal thyroiditis in the BB rat[41], it is tempting to speculate that an enhanced iodine-induced monocyte-veiled/dendritic cell transition may play at least an additional role in such an accelerated development of thyroiditis.

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Chapter 7

CONCLUSIONS AND GENERAL DISCUSSION

Chapter 7.1

GENERAL CONCLUSIONS OF THE EXPERIMENTS DESCRIBED IN THIS THESIS

Our study firstly shows that dietary iodine deficiency may lead, apart from goitre formation and hypothyroidism, to a triggering of the thyroid autoimmune reactivity: an accumulation of dendritic cells was found in the thyroids of normal female Wistar rats, followed by intrathyroidal increases in T cells, and higher incidences of anti-colloid antibodies and thyroid growth stimulating antibodies (TGAbs) in the serum of the animals (see table 1). These observations in the Wistar rat are in agreement with observations made in human populations, in which a low dietary iodine intake led to goitre formation[1], hypothyroidism[2,3,4], increased numbers of intrathyroidal aggregations of dendritic cells, epitheloid cells and giant cells[1], an increased incidence of anti-thyroglobulin (Tg) antibodies[5] and a high incidence of TGAbs[6,7].

Not every individual, however, shows such reactivity to iodine deficiency and the response is variable[8,9,10,11,12]. Indeed in our hands females of another rat strain, eg the autoimmune prone BB rat, showed a different response to iodine deficiency in comparison to the normal Wistar rat. To our surprise, the BB rat was capable of compensating a relatively mild low dietary iodine intake by a rapid and increased goitre formation, and after 9 weeks of iodine deficient diet full euthyroidism was restored (see table 1). In Chapters 3.1 and 3.2 we suggest that the basis for this rapid goitre formation may be attributed to a higher intrinsic growth rate of the BB thyrocytes or to the effects of TGAbs or other growth factors in the circulation of the animal. During iodine deficiency, the BB goitres showed higher numbers of intrathyroidal dendritic cells and T cells as compared to BB rats kept on a normal iodine diet. It should also be noted that numbers of such cells in BB rats on a normal iodine diet are already higher than in Wistar rats on a normal iodine diet (see table 1). The fact that this rat strain with a clear genetically determined proneness for thyroid autoimmunity is capable of coping with mild iodine deficiency may be helpful in explaining the heterogeneous response to iodine deficiency observed in random human populations. There might be individuals with a "BB likeness (autoimmune-proneness)" who, upon a moderately low dietary iodine intake, remain euthyroid, however, at the expense of rapid goitre formation with various signs and symptoms of involvement of the immune system.

Table 1. The effects of several iodine regimens on thyroid endocrine reactions, thyroid autoimmune reactions and the immune system.

	Thyroid endocrine reactions		Thyroid autoimmune reactions		Reaction of the immune system in general	
	Wistar rat (normal)	BB rat (AI-prone)	Wistar rat (normal)	BB rat (AI-prone)	Wistar rat (normal)	BB rat (AI-prone)
Iodine deficiency						
Mild	goitre + hypothyroid +	goitre + + euthyroid	Abs ↑ (TGAb,colloid) DC ↑, Tcells ↑	Abs ↑ (colloid) DC ↑↑, Tcells ↑	=	=
Severe	goitre + + hypothyroid + +	goitre + + hypothyroid + +	Abs ↑↑ (TGAb,colloid) DC ↑, Tcells ↑↑	Abs ↑↑ (colloid) ND	↓↓	↓↓
Iodine excess						
Mild chronic	euthyroid	euthyroid, tendency to become hypo-thyroid	Abs ↓↓ (colloid) DC ↓, Tcells ↓ ectopic thymus (upto 57%)	50% of animals: Abs ↑↑ (colloid) DC ↑↑, Tcells ↑↑ TALT	=	=
Severe acute on hyperplastic goitre	hyperthyroidism, followed by hypothyroidism (transient outburst of thyroiditis)	outburst of thyroiditis, followed by thyroid AID (hypothyrodism)	DC ↑↑, mϕ ↑↑ (transient)	DC ↑↑, mϕ ↑↑ (permanent, induction of thyroid AID)	ND	ND

Reference point normal Wistar rat of our colony on a normal iodine diet (urinary iodine excretion 7 μg per day).
↑: increase, ↓: decrease, =: no difference, ND: not done, AI: autoimmune, Abs: antibodies, TGAb: thyroid growth stimulating antibodies, DC: dendritic cells, AID: autoimmune disease, TALT: thyroid-associated lymphoid tissue.

Iodine excess also resulted in a complex modulation of thyroid autoimmune responsiveness: there was a depression of thyroid autoimmune reactivity in the normal Wistar rat, but an acceleration of thyroid autoimmune reactivity in the autoimmune-prone BB rat.

The Wistar rat developed in the early phases of the experiment a thyroid-associated ectopic thymic tissue (of which we speculated in Chapter 4 that it is related to tolerance induction), together with a low intrathyroidal infiltration with lymphoid cells and a low production of anti-colloid antibodies (table 1). There were no disturbances in thyroid function. This relatively high dietary iodine intake therefore seems to be the most optimal iodine intake for female Wistar rats of our colony.

The same relatively high dietary iodine intake in the BB rat, however, resulted in an acceleration of the intrathyroidal development of the thyroid-associated lymphoid tissue (TALT) that normally occurs in 50% of BB rats older than 18-22 weeks of age[13,14]. The architecture of TALT suggests that it plays a prominent role in the production of autoantibodies and autoreactive T cells and therefore in the continuation of the thyroid autoimmune process (Chapter 5 and table 1).

Our observations on the high dietary iodine intake in rats are again in agreement with observations in human populations. In areas with a relatively high iodine intake (for instance in the United States and Japan), the incidence of classical Hashimoto's thyroiditis is higher in comparison to areas with a normal or relatively low iodine intake[15,16,17,18,19]. The observations in the autoimmune-prone BB rat - in contrast to the normal Wistar rat - suggest that the individuals already genetically prone to thyroid autoimmunity easily develop thyroid autoimmune disease under the influence of a high iodine diet, whereas those without such proneness may even be protected by the high iodine intake (table 1).

It has been nicely shown by the group of Wick et al[20], using the Obese strain (OS) of chicken, that the genetic predisposition to thyroid autoimmune disease and the factors involved in such an abnormality are complex and multifactorial:

a. Firstly, the basis for the genetic predisposition to thyroid autoimmune disease is formed by a preexisting abnormality of the thyroid. For the OS chicken[21] there is ample evidence that this is an intrinsic higher iodine uptake by the thyrocytes. For the BB rat Li et al[22] have also described such a higher thyrocyte iodine uptake. We observed a higher growth rate and a higher

responsiveness to TSH of BB thyrocyte follicles in suspension culture (that might be intrinsic) when comparised to Wistar thyrocyte follicles (Chapter 2.2).

b. Secondly, there are clear and often extensive abnormalities in the regulation of the immune response. These abnormalities play an important role in the actually enhanced production of autoreactive antibodies and T cells. In the Obese strain of chicken, hyperreactive T cells, with a higher expression of IL-2 receptors and a higher production of IL-2 have been observed[23,24], as well as hyperreactive macrophages[20]. Furthermore, a thymic nurse cell deficiency in the thymus of OS chickens may be responsible for a disturbed T cell development in which autoreactive T cells may not be deleted within the thymus[20].

   In the BB rat, a lymphopenia has been described, due to loss of $RT6^+$ T regulator (suppressor) cells[25,26]. Additionally, thymic epithelial cells devoid of MHC class-II antigens have been observed in the BB rat, which can explain the presence of autoreactive T cells in the periphery[27].

   A malfunctioning immuno-endocrine feedback loop plays an additional role in the disregulation of immune responses in such animals. In the OS chicken decreased basal glucocorticoid levels have been found, together with a decreased level of glucocorticoids in response to immunological stimuli[20]. Also in BB rats a decreased response of glucocorticoids to endotoxin was found, probably due to a deficient production of sufficient IL-1 by macrophages[28].

c. Thirdly, for the above described thyroidal and immunological abnormalities to fully develop into overt thyroiditis it is necessary that the thyroid gland becomes susceptible to the excessively produced autoantibodies and autoreactive T cells. It is not yet known what this - in the OS chicken genetically determined - susceptibility is. Possibilities that have been suggested are an abnormal expression of MHC class I or II molecules or an abnormal susceptibility of the thyrocytes to cytokines[20].

In the OS chicken iodine - as well as the sex hormones - were clear *modulators* of the spontaneously occurring thyroid autoimmune response. The iodine and the sex hormones were, however, of minor importance (and thus modulators) in comparison to the major factors of genetically determined target organ abnormality and susceptibility and the genetically determined immune disregulation. Our data in fact support such a view, clearly stating only a modulating role of the dietary iodine

intake in Wistar rats and in an animal model of thyroid autoimmune disease, the BB rat.

With regard to the differences between the Wistar rat and the BB rat it is likely that the outcome of the dietary iodine intake is different, depending on the different setpoints in regulation of the immune system, and differences in target organ (thyroid) metabolism and target organ susceptibility.

To explain at least part of the effects of the dietary iodine intake on thyroid autoimmune reactivity in both Wistar and BB rats our study shows that iodinated compounds such as iodinated Tg, metrizamide[29] and rT_3 have direct effects on the immune system, eg on the maturation of dendritic cells from blood cell precursors (probably the monocytes, see Chapter 6). Such a direct effect has been described before by other investigators in the case of B cells[30], T cells[31] and macrophages[32]. This phenomenon may play a role in the accelerated development of the thyroid-associated lymphoid tissue in the BB rats kept on a high dietary iodine regimen (Chapter 5), as well as the development of the thyroid-associated thymic tissue in the Wistar rats on a high dietary iodine regimen (Chapter 4).

Hypothyroidism in rats (induced by an extremely low dietary iodine regimen) had a clear immune suppressive effect. Furthermore, lowered thymic, splenic and thyroid draining lymph nodal weights were found (table 1, data not shown). This is in agreement with the observations of Fabris[33] who reported that hypothyroidism in the rat induces immunodeficiency, which is restored by thyroid hormone supplements.

Other investigators also stated that thyroid hormones are necessary for optimal immune function. Balàzs et al[34] showed that T_3 supplements enhanced PHA stimulation of human lymphocytes *in vitro* and *in vivo*. In addition, Chatterjee et al[35] found an increased blastogenic response of lymphocytes from thymus, peripheral blood and mesenteric lymph nodes to pokeweed mitogen after T_3 or T_4 administration *in vivo*.

Our findings that thyroid hormones are necessary for the transition of monocytes into veiled/dendritic cells are in agreement with these observations (Chapter 6).

In conclusion, both a low dietary iodine intake as well as a high dietary iodine intake have influences on the immune system and may lead to the development of thyroid autoimmune reactions. Other factors, such as genetically determined target

organ (thyroid) abnormalities and susceptibilities of the thyroid and a genetically determined disregulation of the immune network determine the actual consequences of the iodine intake. In this way reactivity ranges from tolerance (the Wistar rat with a high dietary iodine intake) to thyroid autoimmune reactivity (the Wistar rat on a low iodine diet) that may be helpful to cope with iodine deficiency (the BB rat on a low iodine diet) and overt thyroid autoimmune disease (the BB rat with a high dietary iodine intake).

Chapter 7.2

CRITICICAL REMARKS ON THE TECHNIQUES USED TO COME TO OUR GENERAL CONCLUSIONS

7.2.1 Related to the rat strains used

In our experiments we only used two rat strains, and one has therefore to be careful to draw general conclusions. In our studies we took the BB rat as the prototype of an autoimmune-prone individual and the Wistar rat as the prototype of a normal non-autoimmune individual. The Wistar rat is indeed the ancestral strain of the BB rat and by selectively breeding diabetic Wistar rats, a new rat strain emerged that spontaneously developed an insulitis and a "thyroiditis", namely the BB rat. However, we must keep in mind that the observed results of the dietary iodine regimens in the Wistar rat and the BB rat may be specific characteristics of each particular rat strain not directly or indirectly related to thyroid autoimmunity. It is therefore necessary in the future to perform the same dietary studies in other "normal" rat strains such as the AUG[36], PVG or the Brown Norway rat and "autoimmune rat and mouse strains like the Buffalo rat strain[37] and the non obese diabetic (NOD) mouse[38].

7.2.2 Related to the time schedule of iodine administration and the age of the rats

The time schedule of iodine administration and histological examination of the thyroid might also be critical in determining the outcome of the results of the different iodine regimens. In our study with Wistar and BB rats we used very young animals and the diet was introduced directly after weaning (3 weeks of age): the thyroid was studied histologically every 3 weeks.

The group of Braverman et al[39] also found that a high dietary iodine intake in young BB/W rats resulted in the acceleration of thyroiditis (we confirmed their observations by finding an acceleration of the development of TALT in the BB rats, Chapter 5). However, when Braverman et al introduced their diets in adult BB/W rats, they did not observe such acceleration. We never performed any dietary iodine studies in older animals.

Using Wistar rats of 3 weeks of age we observed a rapid development of a

thyroid-associated ectopic thymic tissue. This tissue was not present anymore at 12 weeks of age (9 weeks after starting the high iodine diet). The group of Braverman did not observe this thyroid-associated ectopic thymic tissue in their Wistar group on diet. We like to believe that this discrepancy is because they started to investigate the thyroids at a Wistar age of 11-12 weeks (at 8 weeks of diet).

In their hands a low dietary iodine intake resulted in BB/W rats in a decrease of the spontaneously developed "thyroiditis". We were not able to confirm these observations, in fact a slight increase in thyroid autoimmunity was observed (data not shown).

Differences in the BB rat strains used might also play a role. The BB/W strain used by the group of Braverman already spontaneously developed their TALT from 9 weeks of age and onwards, whereas in the BB rat strain of our colony characteristic TALT developed not earlier than at 18-22 weeks of age and onwards.

Using the very young Wistar rats we also observed an increase in thyroid autoimmune reactivity, viz higher numbers of intrathyroidal dendritic cells and T cells and an increased anti-colloid antibody production and thyroid growth stimulating antibodies, when these Wistar rats were kept on a low dietary iodine regimen (chapter 2 and addendum 1).

Braverman et al[39] did not observe such an effect of low dietary iodine regimens in Wistar rats. However, they did not study the thyroid immunohistologically and in fact their experiments need to be prolonged in order to be able to observe the effect of the moderately low iodine intake on anti-colloid antibody production.

7.2.3 Related to the preexisting thyroidal state

The thyroidal state at the beginning of the experiment, and in relation to that, the acuteness of the high dietary iodine administration and the dose of iodine administered will also have clear influences on thyroid autoimmune reactivity. At the start of our experiments, all rats were euthyroid, had a normal thyroid weight and the thyrocytes were at a metabolically basal level (although there are probably differences in the latter respect between Wistar rats and BB rats, Chapter 3.2). We chronically administered a moderately high iodine dose (10 μgI/day) during 18 weeks. During this time no histological signs of thyrocyte destruction were

observed in both Wistar and BB rats.

It has been described, however, that an acute high dose of iodine administered i.p. during 8 days to iodine deficient C3H mice[40] or hamsters[41] showing an iodine deficient hyperplastic goitre (with metabolically active thyrocytes), does result in goitre involution, together with thyrocyte destruction. During the iodine-induced goitre involution and thyrocyte destruction, a transient increase in the numbers of intrathyroidal macrophages and dendritic cells was observed. When autoimmune prone NOD mice were used for the experiments, the numbers of intrathyroidal macrophages and dendritic cells became permanently increased[38]. These experiments are again in agreement with observations made in humans: individuals with an iodine deficient goitre are highly susceptible for thyroid autoimmunity after a high dose of iodine supplementation[42,43,44].

The experiments also highlight the differences between the outcomes of our experiments (chronic iodine diets) versus experiments using acute high iodine administrations.

Chapter 7.3

THE POSSIBLE MECHANISMS BY WHICH IODINE MODULATES THYROID AUTOIMMUNE REACTIVITY

7.3.1 Toxicity for thyrocytes

One of the mechanisms by which iodine may precipitate thyroid autoimmune reactivity is demonstrated in the earlier mentioned experiments of Mahmoud et al[40] and Follis et al[41]. These investigators administered a high dose of iodine to iodine deficient mice and hamsters with a hyperplastic goitre. The thyrocytes of the hyperplastic goitres are metabolically highly active and are damaged by the iodine. The investigators argued that excessive amounts of the iodide ion in the hyperplastic thyrocytes were rapidly oxidized by TPO thereby producing excessive amounts of either hypoiodous acid or oxigen radicals, reactive elements reported as intermediates in TPO catalyzed oxidation of iodide[45] (see Chapter 1). These oxidative elements damage thyroid cell membranes by the oxidation of membrane lipids or proteins[46,47]. This mechanism probably only plays a role in acute and high iodine supplementation in iodine deficient individuals with a hyperplastic goitre. However, there is some indirect evidence that oxidative damage is an early event in the development of the spontaneous thyroiditis of the OS chicken as well; treating the OS chickens from hatching with anti-oxidants reduced the intrathyroidal lymphocytic infiltration and anti-Tg antibody synthesis. The drugs were ineffective after disease initiation[48].

In our experiments using a chronic high iodine diet only minor signs of thyrocyte destruction could be observed in the BB rat thyroids after an excessive iodine intake (this might, however, be due to the autoimmune process, see Chapter 5). In the Wistar rats the thyroid autoimmune reactivity was even reduced after an excessive iodine intake. In our studies we used animals with a normal thyroidal state. It is therefore unlikely that oxidative damage of thyrocytes plays a major role in the acceleration of thyroid autoimmune phenomena (quicker TALT development, development of ectopic thymic tissue) observed in our studies.

7.3.2 Immunogenicity of highly iodinated thyroglobulin

The creation of immunogenic epitopes in the Tg molecule by the ingestion of

excess iodide can explain the acceleration of the iodine-induced thyroid autoimmune phenomena observed in our studies. It has been demonstrated that highly iodinated Tg is a better immunogen than Tg of low iodine content, both *in vivo*[49] and *in vitro*[50]. Champion et al showed that immunization of mice with highly iodinated Tg and adjuvant, but not poorly iodinated Tg, induced autoimmune thyroiditis[51,52].

Although there is thus some evidence emphasizing the importance of highly iodinated Tg as an immunogen, it must be noted that real autoimmune thyroiditis could only be elicited with this immunogen in genetically susceptible rats[53].

Also in OS chickens and in patients suffering from Hashimoto's thyroiditis it is not clear whether highly iodinated Tg is a major causal factor for the development of autoimmune thyroid reactivity.

In OS chickens it is evident that

a. small amounts of iodine within the thyroid are sufficient to cause lymphocytic infiltration[54],
b. intrathyroidal Tg of OS chickens has a lower than normal iodine content (although it still has many iodine atoms per molecule of Tg)[55] and
c. antibodies to Tg in OS sera react equally well to highly-iodinated Tg and Tg of low iodine content[49].

Patients suffering from Hashimoto's thyroiditis have

a. poorly iodinated Tg[56],
b. antibodies to various thyroidal antigens next to Tg and even occasionally antibodies to such antigens in the absence of antibodies to Tg.

If antigens play a role in the development of ectopic lymphoid tissue (both thymus and TALT), it is possible that this is related to an increase in the antigenicity of iodinated Tg.

7.3.3 Direct stimulating effects of iodine on cells of the immune system

Direct stimulating effects of iodine on immune cells, such as macrophages[57], T cells[58] and B cells[59], have been described to play a role in the development

of thyroid autoimmune reactivity. In Chapter 6 we found an enhancing effect of metrizamide, rT_3 and iodinated Tg on the transition from monocyte to veiled/dendritic cell. This phenomenon may play a role in the iodine effects on the thyroid autoimmune processes observed in the BB rat and the Wistar rat and in the higher antigenicity of highly iodinated Tg.

One should also keep in mind that it is possible that iodine may have direct stimulating effects on cells forming the microenvironment (epithelial and reticulum cells) of lymphoid organs since such cells are probably more important in the development of thyroid-associated ectopic thymic tissue in the Wistar rat and TALT in the BB rat. Further investigations are necessary to resolve this.

7.3.4 Increased release of antigenic degradation products during iodine deficiency

Our study also shows that cells belonging to the dendritic cell family are normally present in low numbers in the thyroid and increase in number during iodine deficiency in both the Wistar rat and the BB rat[60]. Recent data from our group and from others indicate that dendritic cells are also normally present in other endocrine tissues (the pituitary, the ovary), and that such cells are capable of protecting endocrine cells from overstimulation[61,62,63].

One might therefore hypothesize that the above described intrathyroidal accumulation of dendritic cells during iodine deficiency is involved in the protection of the stimulated thyrocytes (iodine deficiency) from overstimulation and does therefore not necessarily represent an autoaggressive infiltration in the Wistar rat. A possible explanation for the attraction of the dendritic cells into the thyroid is an increased release of antigenic degradation products from the metabolically highly active thyrocytes induced by the iodine deficiency. These degradation products could act as chemotactic and phlogistic agents for macrophages and dendritic cells resulting in their enhanced accumulation. Interestingly, the dendritic cell infiltration in the iodine-deficient Wistar rats was followed by the production of anti-colloid antibodies and an increased intrathyroidal infiltration with T cells. There are indications that these immune phenomena might also be involved in the regulation of the hyperfunctioning thyroid tissue. A "physiological" role of Tg autoantibodies in the clearance of waste Tg in situations of overproduction of this thyroidal product

has been described as early as 1959 by Grabar et al[64]. Furthermore, high titres of monoclonal anti-Tg antibodies have been reported to result in mice in the hypofunction of thyroid glands without inflammatory leukocyte inflammation[65]. Cytokines produced by thyroid infiltrating T cells (such as IFN-γ) are known to be capable of dampening the hormone production and growth of stimulated thyrocytes[66].

Chapter 7.4

CLINICAL RECOMMENDATIONS BASED ON THE EXPERIMENTS DESCRIBED IN THIS THESIS

The studies described in this thesis again clearly establish that for optimal thyroid function and growth every individual should strive for an optimal daily dietary iodine intake, in other words should not use too much or too little iodine intake. This optimal daily iodine intake can be assessed considering that the daily human thyroidal thyroid hormone production is around 120 nmol T_4 and around 10 nmol T_3[67]. To produce this amount of thyroid hormones, 4 x 120 + 3 x 10 = 510 nmol iodide is needed, which means an intake of around 60-70 μg of iodide per day. The amount of iodide necessary for normal thyroid hormone production without using the buffer capacity of the thyroid and preventing goitre formation can be estimated as follows: under normal circumstances the thyroid clears 1/3 of the anorganic iodide in the blood[68] (the daily iodine intake must therefore be around 3 x 60 = 180 μg); around 10-20 μg of iodide is daily excreted in the faeces as thyroid hormone or thyroid hormone metabolites (the daily iodine intake must therefore be 180 + 10 = 190 μg). However, around 50 μg of iodide can be reused after proteolytic degradation of iodinated Tg and MIT and DIT residues during thyroid hormone secretion (see figure 1 General Introduction, page 12). The estimated daily iodine intake is thus 190 - 50 = 140 μg of iodide. The mandatory daily iodine intake given by the World Health Organization (WHO) is at least 150 μg for adults and adolescents, 175 μg for pregnant women and 200 μg of iodide for nursing women.

An iodine intake below this level will result in goitre formation, sometimes hypothyroidism, and in the development of all sorts of thyroid autoimmune phenomena such as thyroid growth blocking antibodies[2,3,4], thyroid growth promoting antibodies[6,7], intrathyroidal accumulations of dendritic cells[1] and higher incidences of anti-Tg antibodies[5]. However, these autoimmune phenomena may not be necessarily harmful. During pregnancy a low iodine intake may lead to retarded brain and bone development of the fetus and cretinism can be the result. Our data, however, indicate that some individuals will be able to cope with some aspects of the low dietary iodine intake (the autoimmune-prone individuals?) such as the hypothyroidism at the expence of large goitre formation.

An iodine intake above the recommended level bears the risk to induce an acceleration of thyroid autoimmune phenomena in some individuals (the

autoimmune-prone individuals who are able to cope with moderately low iodine diets?). However, since the individual reaction patterns cannot yet be predicted, the best is to strive for a general optimal iodine intake of 150 μg per day.

Our data are also of relevance when treating individuals with iodine in a fall-out zone after a nuclear explosion or when treating endemic goitre patients with iodine. A single high iodine dose is often administered to these populations through iodine tablets or iodine injections, thereby saturating the thyroid with iodine (in the case of the fall-out to prevent a thyroidal accumulation of radioactive iodine).

Extrapolating our animal data and other literature data to these situations it is not surprising that some individuals will develop thyrocyte destruction and transient forms of thyroid autoimmunity, while others will even have more permanent forms of thyroid autoimmune reactivity (the "autoimmune prone" individuals?). However, one may argue that such thyroid damage caused by a transient or permanent thyroid autoimmune reaction elicited by the high iodine gift is preferable to late damage (tumour growth) caused by the radioactive iodine accumulating in the thyroid during a nuclear fall-out when iodine tablets are not taken. It is clear that in the case of iodine deficient endemic goitre one has to take special precautions to minimize the damage inflicted by reintroducing iodine. It is advisable to start with a relatively small iodine supplementation in the beginning, followed by a gradual increase of the daily iodide dose. Ideally it would be an advantage to be able to indicate those individuals that will react with a permanent thyroid autoimmune response (the "autoimmune-prone ones") before reintroducing the iodine in the diet.

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SUMMARY

Chapter 1: General introduction

Iodine is an essential trace element and part of the thyroid hormones. The thyroid function, the thyroid hormone production and the metabolism of iodine are hence highly interconnected.

Thyroid Stimulating Hormone (TSH or thyrotropin) is an important regulator of the synthesis and release of the thyroid hormones T_3 and T_4. TSH is secreted by the thyrotrophic cells of the anterior pituitary gland. The synthesis of TSH by these pituitary cells is regulated by Thyrotropin Releasing Hormone (TRH), synthesized in the hypothalamus. Negative feedback loops exist, via which the T_3 and T_4 concentrations in the circulation regulate the synthesis and secretion of both TSH and TRH.

Regulation of thyroid hormone synthesis and release by dietary iodine

An excessive dietary iodine intake

Besides the regulation by TSH, an autoregulation of the thyroid exists, particularly in respect to the dietary iodine intake. An *acute*, prominent increase of the dietary iodine intake results in an acute but transient decrease of the iodide transport mechanism over the thyrocyte membrane and hence in a decrease in the iodination of thyroglobulin (Tg, the main storage protein of iodine in the thyroid and the matrix for thyroid hormone synthesis). This leads to a decreased iodothyronine formation and thyroid hormone secretion. This phenomenon is known as the Wolff-Chaikoff effect. When the high levels of plasma iodide are maintained, the inhibitory effects of iodide disappears and iodothyronine formation is restored (the so-called "escape from the Wolff-Chaikoff effect"). The precise mechanisms causing this escape are still unknown. The long term effects of a *chronically* excessive dietary iodine intake are largely unknown. Clear abnormalities of thyroid function have not been observed in the majority of normal individuals receiving high dosages of iodine over longer periods, subtle changes do, however, occur. A small, significant decrease in the serum concentrations of T_4 and T_3 with a small rise in serum TSH have been observed, but such changes are usually within the normal range for the various parameters evaluated.

A deficient dietary iodine intake

During iodine deficiency, the degree of iodination of the Tg molecule is lower, which leads to more MIT than DIT residues in the Tg molecule and thus to the production of more T_3 than T_4. In this way an enhanced synthesis of the biological active T_3 at the expense of the production of the prohormone T_4 occurs, thereby allowing an efficient usage of the small amounts of iodine available with an optimal biological potency per Tg molecule. This compensatory mechanism of the thyroid is thought to be able to maintain euthyroidism during relatively mild iodine deficiency. Only during more severe forms of iodine deficiency these mechanisms will fail and hypothyroidism will be the result. Since thyroid hormones are essential for brain development of the fetus, iodine deficiency during pregnancy can lead to impaired brain development resulting in mental retardation and cretinism.

The role of iodine in thyroid autoimmunity

Besides having an effect on thyroid hormone synthesis and release, there are strong indications that abnormalities in the dietary iodine intake influence thyroid autoimmune responses.

Iodine deficiency in human populations has been described to lead to:

a. a higher incidence of anti-thyroglobulin (Tg) antibodies,
b. the generation of antibodies that affect the growth of thyrocytes *in vitro* (the so-called TGAbs) in individuals characterized by disturbances in thyroid growth (endemic goitre, thyroid atrophy) in such iodine-deficient regions and
c. the presence of aggregates of lymphoid cells (epithelioid cells and dendritic cells) in endemic goitres.

An excessive dietary iodine intake has also been described to lead to thyroid autoimmune reactivity:

a. In individuals with a preexisting thyroid abnormality, such as an iodine deficient goitre, an excessive dietary iodine intake results in a proportion of the individuals in the development of an attack of thyroiditis; those affected by the thyroiditis show anti-Tg and anti-microsomal antibodies in their serum, and

b. In animals that spontaneously develop thyroid autoimmune disease, such as the Cornell C Strain of chicken, the Obese Strain of chicken and the BB rat, a high dietary iodine intake results in an increase in the incidence and severity of the thyroiditis.

The existence of a certain degree of immune autoreactivity towards thyroid cells and other body components is nowadays considered to be normal in healthy individuals. This autoreactivity is controlled by various suppressor mechanisms (deletion of the majority of autoreactive T cells in the thymus, the existence of regulatory circuits of T and B lymphocytes, T cell and B cell anergy). Malfunction of these suppressor mechanisms, an otherwise hyperreactive immune system, target organ (thyroid) abnormalities (such as a higher iodine metabolism, resulting in a higher release of antigenic degradation products) and target organ susceptibilities to an autoimmune attack are thought to be factors disturbing the delicate mechanisms controlling the low levels of normal autoreactivity.

Outline of the thesis

The mechanisms underlying the thyroid autoimmune phenomena induced by an excessive or iodine deficient dietary intake are only partly understood. This thesis is particularly meant to study the relationship between the dietary iodine intake, the metabolism and growth of thyrocytes and the thyroid autoimmune response *in vivo* and *in vitro*.

Chapter 2.1: Iodine deficiency induces thyroid autoimmune reactivity in wistar rats

The effects of iodine deficiency on thyroid performance and thyroid autoimmune reactivity in normal Wistar rats are described in Chapter 2.1.

Moderate and severe iodine deficiency led to goitre development, hypothyroidism and an increased thyroid autoimmune reactivity: higher numbers of intrathyroidal dendritic cells, clusters of these cells, higher numbers of intrathyroidal $CD4^+$ and $CD8^+$ cells and a higher incidence of anti-colloid antibodies in the serum of the

animals were observed.

Chapter 2.2: Thyroid growth stimulating activity in ammonium sulphate precipitates of the serum of Wistar rats kept on a low dietary iodine regimen. A preliminary report

The thyroid growth stimulating activity of ammonium sulphate precipitates of the iodine-deficient Wistar rat sera is described in Chapter 2.2. Sera of moderate and severe iodine deficient rats showed thyroid growth stimulating activity, in contrast to the sera of normal rats or rats with a high dietary iodine intake. Whether this growth stimulating activity is caused by an immunoglobulin and not by other growth factors (such as EGF and IGF) present in the ammonium sulphate precipitates, is presently under investigation.

Conclusion: iodine deficiency leads to thyroid autoimmune reactivity in normal Wistar rats.

Chapter 3.1: Thyroid autoimmune prone BB rats are capable of compensating for moderately low iodine regimens, restoring full euthyroidism

The effect of a moderate low iodine diet on thyroid performance in the autoimmune-prone BB rat as compared to the normal Wistar rat is described in Chapter 3.1. Moderately iodine deficient Wistar rats gradually developed goitres, reaching their maximal weight (27 mg) after 12 weeks of diet. The Wistar rats became subclinically hypothyroid after 18 weeks of diet (decreased serum T_3 and T_4 and increased serum TSH levels). The moderately iodine deficient BB rats, on the other hand, developed more rapidly goitres, reaching maximal weights (31 mg) after 6 weeks of diet. The BB rats showed a transient period of hypothyroidism (during the first 9 weeks of diet). Full euthyroidism was restored after 12 weeks of diet. Conclusion: the autoimmune-prone BB rat is better capable of compensating for a moderately low dietary iodine intake as compared to the Wistar rat. It is likely that the autoimmune background of the animal is linked to this capability (target organ abnormalities, such as a higher iodine metabolism, a higher thyrocyte proliferation rate or TGAbs).

Chapter 3.2: BB rat thyrocytes show a high proliferation rate *in vitro*. A preliminary report

In Chapter 3.2 the basal proliferation rate and the proliferation rate in response to TSH of BB rat thyroid follicles and Wistar rat thyroid follicles are described. BB rat thyroid follicles cultured *in vitro* showed a higher proliferation rate and a higher response to TSH in comparison to Wistar rat thyroid follicles. This phenomenon (that might be an inborn phenomenon of the BB rat thyrocytes, or induced by TGAbs) may explain the rapid goitre development of BB rats during moderate iodine deficiency and the capability to compensate for this moderate iodine deficiency (described in Chapter 3.1). Further investigations are necessary the confirm and clarify these preliminary experiments.

Chapter 4: A high iodine intake in Wistar rats results in the development of a thyroid-associated ectopic thymic tissue and is accompanied by a low thyroid autoimmune reactivity

The effect of a high dietary iodine intake on thyroid performance and thyroid autoimmune reactivity in normal Wistar rats is described in Chapter 4.

The high dietary iodine intake had no effect on thyroid performance: the animals did not develop a goitre and remained euthyroid. Remarkable was in 50-57% of these animals the occurrence of a thyroid-associated ectopic thymic tissue during the first 6 weeks of the experiments. Concomitant with this ectopic thymic tissue was the low level of thyroid autoimmune reactivity in animals with a high dietary iodine intake: lower numbers of intrathyroidal $CD4^+$ and $CD8^+$ cells and a lower incidence of anti-colloid antibodies were found in comparison to Wistar rats on a normal or iodine deficient diet. It is suggested that this ectopic thymic tissue is involved in the low thyroid autoimmune reactivity via mechanisms of thymus related tolerance induction.

Chapter 5: An excess of dietary iodine accelerates the development of a thyroid-associated lymphoid tissue in autoimmune-prone BB rats

In Chapter 5 the effects of a high dietary iodine intake in the autoimmune-prone

BB rat on thyroid performance and thyroid autoimmune reactivity are described.

The high dietary iodine intake lacked - as in the normal Wistar rat - an effect on thyroid performance. The effect of the excessive dietary iodine intake on thyroid autoimmune reactivity was however remarkable and completely different from that in the normal Wistar rat (described in Chapter 4). The high iodine intake led to an acceleration of the development of lymphoid cell infiltrates in the thyroid, that normally arise in our BB rat colony at the age of 21 weeks and older (these infiltrates could be observed from 15 weeks of age onwards). Chapter 5 gives a detailed description of the histology of these lymphoid infiltrates. In fact they consisted of T cell areas with interdigitating cells and B cell areas, showing the organized structure of the lymphoid cell infiltrates, comparable to the lymphoid structures seen in mucosas such as the gut mucosa (mucosa-associated lymphoid tissue, MALT). At the edges of the lymphoid tissue in the thyroid of the BB rats on an excessive iodine diet, minor signs of destruction were observed, in which area macrophage-like cells were found. The presence of this thyroid-associated lymphoid tissue (TALT) was correlated with the presence of anti-colloid antibodies in the serum. It is speculated that the TALT is involved in the production of these antibodies and in the continuation of the thyroid autoimmune reactivity.

Chapter 6: The effect of thyroid hormones and other iodinated compounds on the transition of monocytes into veiled/dendritic cells, a role of GM-CSF, TNFα and IL-6

The effect of the thyroid hormones T_3 and T_4 and a few other iodinated "hormonally inactive" compounds, such as rT_3, metrizamide and iodinated Tg, on the maturation of human peripheral blood monocytes into functionally active dendritic cells is described in Chapter 6.

A pretreatment of blood monocytes for 30 minutes with the thyroid hormones, and in particular with the active thyroid hormone T_3, enhances the capability of the monocytes to mature into immunocytologically typical and immunologically active (MLR) veiled or dendritic cells, when further cultured overnight under nonadhering conditions. The dendritic cell maturation during the culture was dependent on the endogenous production of various cytokines, since α-GM-CSF, α-TNFα and α-IL-6 had blocking effects on the transition. Since there is no increased cytokine production

induced by T_3, the bioactive thyroid hormone T_3 probably excerts its effects via binding to the nuclear T_3 receptor at the cytokine receptor level (up regulation receptor expression) or expression at the post cytokine receptor level.

The iodinated compounds metrizamide, rT_3 and iodinated Tg were also capable to enhance the monocyte to veiled/dendritic cell transition, but to a lesser extent than T_3. A possible mechanism involved in the enhanced monocyte to dendritic cell transition induced by these hormonally inactive iodinated compounds is an interaction of the iodinated compounds with the H_2O_2 generating system of the monocytes. In what way such interaction leads to a higher expression of cytokine receptors or have effects at the post cytokine receptor level is completely unknown. Since dendritic cells play a crucial role in the initiation of thyroid autoimmune responses and since a high iodine diet (viz highly iodinated Tg) accelerates the development of the thyroid autoimmune reaction (TALT, see before) in the BB rat, it is speculated that an enhanced iodine-induced monocyte-veiled/dendritic cell transition may play at least an additional role in such an accelerated development.

Chapter 7: Conclusions and general discussion

In Chapter 7 general conclusions as well as some critical remarks on the studies are given. The experiments described in this thesis show that the dietary iodine intake has clear effects on thyroid autoimmune reactivity. Both stimulation as well as suppression of thyroid autoimmune reactions have been observed, depending on the diet (chronic too low or too high) and the genetically determined susceptibility of the animal for thyroid autoimmune disease. Together with data from the literature it is clear that the dietary iodine intake must hence be considered as a modulator of thyroid autoimmune reactivity. Other factors such as genetically determined disturbances in the regulation of immune responses, intrinsic thyroid metabolic abnormalities (such as an intrinsic higher iodine uptake and metabolism, maybe resulting in a higher release of antigenic degradation products) and an abnormal susceptibility of thyrocytes to an autoimmune attack are probably of major importance.

The possible mechanisms by which the dietary iodine modulates thyroid autoimmune reactivity are:

a. an increased immunogenicity of highly iodinated thyroglobulin,

b. direct stimulating effects of iodine on cells of the immune system and
c. stimulation of the immune system via an increased release of degradation products of the thyrocytes during iodine deficiency.

The data in this thesis again highlight the notion that a daily optimal intake of iodine (around 150μg) will preserve optimal thyroid performance in a given population. The data in this thesis however do also show that individual differences (depending on the genetic background and susceptibility to thyroid autoimmune disease) can be expected in response to a given dietary iodine regimen.

SAMENVATTING

Hoofdstuk 1: Algemene introduktie

Jodium is een essentieel sporeelement en maakt deel uit van de schildklier hormonen T_3 en T_4. De schildklier funktie, de schildklier hormoon produktie en jodium metabolisme zijn daarom sterk met elkaar verbonden.

De synthese en afgifte van de schildklier hormonen wordt gereguleerd door "Thyroid Stimulating Hormone" (TSH of thyrotropine). TSH wordt uitgescheiden door de thyrotrofe cellen in de hypofyse. De synthese van TSH door deze hypofyse cellen wordt gereguleerd door "Thyrotropin Releasing Hormone" (TRH), gesynthetiseerd door de hypothalamus. De concentraties van de schildklier hormonen T_3 en T_4 in de circulatie reguleren de synthese en secretie van zowel TRH als TSH via negatieve terugkoppelings mechanismen.

Regulatie van schildklier hormoon synthese en secretie door jodium

Een te hoge jodium inname

Naast de regulatie door TSH bestaat er een autoregulatie van de schildklier met betrekking tot de jodium inname. Een *acute* overmaat aan jodium leidt tot een acute maar tijdelijke afname van het jodide transport mechanisme over de schildklier celmembraan en dus in een afname van de jodering van thyreoglobuline (Tg, het belangrijkste opslag eiwit van jodium in de schildklier en de matrix voor schildklier hormoon synthese). Dit leidt tot een afname van de vorming van jodothyronines en dus een afname van de synthese en secretie van schildklier hormoon. Dit fenomeen is bekend als het Wolff-Chaikoff effect. Wanneer de plasma jodide concentratie hoog blijft, verdwijnen de remmende effecten van het jodium en de schildklier hormoon synthese is genormaliseerd (ontsnappen aan het Wolff-Chaikoff effect). De mechanismen die leiden tot deze "ontsnapping" zijn nog niet bekend.

De effecten op lange termijn van een *chronische* overmaat aan jodium zijn nog voor een groot gedeelte onduidelijk. Ernstige abnormaliteiten van de schildklier funktie zijn niet waargenomen in de meeste individuen die hoge doseringen jodium gedurende een langere periode binnenkrijgen. Geringe veranderingen komen echter voor. Een lichte daling van de serum T_3 en T_4 concentraties met een

geringe stijging van de TSH spiegel zijn waargenomen, hoewel deze veranderingen meestal binnen de grenzen van de normaal waarden vallen.

Een te lage jodium inname

Tijdens jodium deficiëntie is er een lagere jodering van het thyreobloguline (Tg) molekuul, wat leidt tot de vorming van meer MIT dan DIT residuen en dus tot de produktie van meer T_3 dan T_4. Op deze manier wordt een optimaal gebruik gemaakt van het beperkte aanbod van jodium en een verhoogde produktie van het aktieve schildklier hormoon T_3 ten koste van het inaktieve schildklierhormoon T_4 vindt plaats. Gedurende relatieve milde jodium deficiëntie zal op deze manier euthyreoidie gehandhaafd blijven, echter tijdens ernstige jodiumdeficiëntie zal de compensatie niet voldoende zijn en is hypothyreoidie het gevolg.

Daar schildklier hormoon essentieel is voor de ontwikkeling van de hersenen van de foetus, kan hypothyreoidie tijdens de zwangerschap leiden tot een slechte hersen ontwikkeling en mentale redardatie en cretinisme zijn het gevolg.

De rol van jodium in schildklier autoimmuniteit

Een tekort of een overmaat aan jodium beïnvloeden niet alleen de funktie van de schildklier, schildklier hormoon synthese en secretie, er zijn ook sterke aanwijzingen dat jodium effecten heeft op schildklier autoimmuun responsen.

Het is beschreven dat een tekort aan jodium in humane populaties leidt tot:

a. een hogere incidentie van anti-Tg antilichamen,
b. de ontwikkeling van antilichamen die de groei van thyreocyten *in vitro* beïnvloeden (de zogenaamde schildklier groeistimulerende immuunglobulinen), in individuen gekarakteriseerd door afwijkingen in schildkliergroei (endemisch struma en schildklier atrofie) en
c. de aanwezigheid van aggregaten van lymfoïde cellen (epitheloïde cellen en dendritische cellen) in endemisch struma's.

Het is ook beschreven dat een overmaat aan jodium leidt tot schildklier autoimmuun reaktiviteit:

a. In een deel van individuen met een schildklier abnormaliteit, zoals een jodium deficiënt struma, leidt een te hoge jodium inname tot een aanval van thyreoiditis. De patiënten vertonen anti-Tg en anti-TPO antilichamen in de circulatie.
b. Een te hoge jodium inname in diermodellen die spontaan schildklier autoimmuun ziekten ontwikkelen, zoals de Obese Strain (OS) van de kip en de BB rat, leidt tot een toename van de incidentie en een verergering van de thyreoiditis.

Een geringe mate van autoimmuunreaktiviteit gericht tegen schildklier cellen en andere lichaamseigen componenten in gezonde individuen wordt tegenwoordig als normaal beschouwd. Deze geringe autoreaktiviteit wordt normaal gesproken gecontroleerd door verscheidene suppressor mechanismen (deletie van de meeste autoreaktieve T cellen in de thymus, regulatie door netwerken van T en B lymfocyten, T cel en B cel anergie). Wanneer deze suppressor mechanismen niet goed funktioneren, het immuunsysteem daardoor hyperaktief is of wanneer het doelwit orgaan (in dit geval de schildklier) zelf niet goed funktioneert (verhoogde jodium metabolisme wat mogelijk leidt tot een hogere expressie en uitscheiding van autoantigenen) of overgevoelig is voor de autoimmuun reaktiviteit, worden de mechanismen die de autoreaktiviteit normaliter controleren verstoord. De schildklier autoimmuun reaktiviteit kan dan uiteindelijk leiden tot schildklier autoimmuun ziekte.

Doel van het onderzoek

De mechanismen die ten grondslag liggen aan de schildklier autoimmuun fenomenen geïnduceerd door een te hoge of een te lage jodium inname zijn nog niet geheel duidelijk.

Dit onderzoek houdt zich bezig met de verdere bestudering van de relatie tussen jodium inname, het metabolisme en de groei van de schildklier cellen en de schildklier autoimmuun respons *in vivo* en *in vitro*.

Hoofdstuk 2.1: Jodium deficiëntie induceert schildklier autoimmuun reaktiviteit in de Wistar rat

In hoofdstuk 2.1 wordt het effect van jodium deficiëntie op schildklier groei en funktie en op schildklier autoimmuun reaktiviteit beschreven in de normale Wistar rat. Milde en extreme jodium deficiëntie leidde tot een struma vorming, hypothyreoidie en tot schildklier autoimmuun reaktiviteit: een verhoogd aantal intrathyreoidale dendritische cellen, clusters van deze cellen, een verhoogd aantal intrathyreoidale $CD4^+$ en $CD8^+$ cellen en een verhoogde incidentie van anti-colloïd antistoffen in het serum van de dieren werd waargenomen.

Hoofdstuk 2.2: Schildkliergroei stimulerend vermogen van ammonium sulfaat precipitaten van het serum van Wistar ratten met een lage jodium inname. Een preliminaire studie

Het schildkliergroei stimulerend vermogen van ammonium sulfaat precipitaten van de jodium deficiënte Wistar ratte sera wordt in hoofdstuk 2.2. beschreven. De sera van milde en extreem jodium deficiënte ratten vertoonden schildkliergroei stimulerende aktiviteit, die van ratten met een normale of een hoge jodiuminname niet of nauwelijks. Of deze groei stimulerende aktiviteit ook veroorzaakt wordt door een immuunglobuline en niet door andere groeifaktoren (zoals EGF of IGF), aanwezig in de ammonium sulfaat precipitaten, wordt op het ogenblik bestudeerd.

Conclusie: jodium deficiëntie leidt tot schildklier autoimmuun reaktiviteit in normale Wistar ratten.

Hoofdstuk 3.1: Schildklier autoimmune BB ratten zijn in staat milde jodium deficiëntie te compenseren, waardoor euthyreoidie volledig wordt hersteld

Het effect van een milde jodium deficiëntie op de schildklier funktie van de autoimmune BB rat in vergelijking tot de Wistar rat wordt beschreven in hoofdstuk 3.1. Milde jodium deficiënte Wistar ratten ontwikkelden geleidelijk struma's die hun maximale gewicht (27 mg) na 12 weken bereikten. De Wistar ratten werden uiteindelijk (na 18 weken dieet) subklinisch hypothyreoot (verlaagde serum T_4 en

T_3 en een verhoogde TSH spiegel). De milde jodium deficiënte BB ratten daarentegen ontwikkelden snel struma's, die hun maximale gewicht (31 mg) na 6 weken bereikten. De BB ratten vertoonden een tijdelijke periode van hypothyreoidie (gedurende de eerste 9 weken van het experiment). De euthyreote status was na 12 weken dieet volledig hersteld. Conclusie: de autoimmune BB rat is beter in staat de milde jodium deficiëntie te compenseren in vergelijking tot de Wistar rat. Waarschijnlijk heeft de autoimmune achtergrond van de BB rat (een intrinsiek hoger jodium metabolisme, een snellere groei van de schildklier cellen of schildklier groei stimulerende immuunglobulinen) te maken met de mogelijkheid te kunnen compenseren voor milde jodium deficiëntie.

Hoofdstuk 3.2: BB ratte thyrocyten vertonen een hoge proliferatie *in vitro*. Een preliminaire studie

In hoofdstuk 3.2 wordt de basale proliferatie en de proliferatieve respons op TSH van BB ratte schildklier follikels en Wistar ratte schildklier follikels beschreven. BB ratte schildklier follikels gekweekt *in vitro*, hadden een hogere basale proliferatie en een hogere proliferatieve respons op TSH dan Wistar ratte schildklier follikels. Dit fenomeen (dat een aangeboren fenomeen van de BB schildkliercellen zou kunnen zijn of geïnduceerd door schildklier groei stimulerende antistoffen) zou de snelle struma vorming van de BB rat tijdens milde jodium deficiëntie en de mogelijkheid om deze milde jodium deficiëntie te compenseren (beschreven in hoofdstuk 3.1) kunnen verklaren. Verder onderzoek is echter nodig om deze preliminaire gegevens te verifiëren en te verhelderen.

Hoofdstuk 4: Een hoge jodium inname in de Wistar rat leidt tot de ontwikkeling van een schildklier-geassocieerd ectopisch thymus weefsel, vergezeld van een lage schildklier autoimmuun reaktiviteit

Het effect van een chronisch te hoge jodium inname op de schildklier funktie en op de schildklier autoimmuun reaktiviteit in normale Wistar ratten wordt beschreven in hoofdstuk 4. Een chronisch te hoge jodium inname had geen effect op de schildklier funktie: de dieren bleven euthyreoot en er was geen struma

ontwikkeling. Opvallend was in 50-57% van deze dieren de aanwezigheid van een schildklier-geassocieerd ectopisch thymus weefsel gedurende de eerste 6 weken van het experiment. Dit ectopisch thymus weefsel was vergezeld van een lage schildklier autoimmuun reaktiviteit: een lagere incidentie van anti-colloid antistoffen en een lager aantal intrathyreoidale $CD4^+$ en $CD8^+$ cellen werd waargenomen in vergelijking tot normale en milde jodium deficiënte Wistar ratten. Er wordt gesuggereerd dat dit ectopisch thymus weefsel betrokken zou kunnen zijn bij de waargenomen lage schildklier autoimmuun reaktiviteit via thymus gerelateerde tolerantie inductie.

Hoofdstuk 5: Een te hoge jodium inname versnelt de ontwikkeling van een schildklier-geassocieerd lymfoïd weefsel in de autoimmune BB rat

In hoofdstuk 5 wordt het effect van een chronisch te hoge jodium inname op de schildklier funktie en schildklier autoimmuun reaktiviteit in de autoimmune BB rat beschreven. Net als in de Wistar rat had een chronisch te hoge jodium inname in de BB rat geen effect op de schildklier funktie. De BB ratten bleven euthyreoot en hadden een normaal schildklier gewicht. Het effect van een hoge jodium inname op de schildklier autoimmuun reaktiviteit was in de autoimmune BB rat daarentegen aanzienlijk en compleet verschillend van dat in de normale Wistar rat (beschreven in hoofdstuk 4). Een chronisch te hoge jodium inname leidde tot een versnelling van de ontwikkeling van lymfoïde celinfiltraten in de schildklier, die normaal op een leeftijd van 21 weken of ouder in onze BB ratte stam voorkomen (nu werden deze infiltraten op een leeftijd van 15-21 weken waargenomen). In hoofdstuk 5 wordt een nauwkeurige en gedetailleerde beschrijving van de histologische opbouw van de lymfoïde celinfiltraten gegeven. Ze bestonden uit T cel gebieden met interdigiterende cellen en B cel gebieden, wat de georganiseerde opbouw van de lymfoïde infiltraten weergeeft, vergelijkbaar met lymfoïde strukturen in de slijmvliezen van bijvoorbeeld de darm ("mucosa-associated lymphoid tissue, MALT"). Aan de randen van het lymfoïde weefsel in de schildklieren van de BB ratten met een te hoge jodium inname werd geringe destructie van schildklier follikels waargenomen. Hier werden macrofaag achtige cellen gevonden. De aanwezigheid van het schildklier-geassocieerde lymfoïde weefsel ("thyroid-associated lymphoid tissue, TALT") was gecorreleerd aan de aanwezigheid van anti-colloïd antistoffen in het

serum van de ratten. TALT zou een rol kunnen spelen in de produktie van deze antilichamen en in de continuatie van de schildklier autoimuun reaktiviteit.

Hoofdstuk 6: Het effect van schildklier hormonen en andere gejodeerde stoffen op de uitrijping van monocyten tot sluier of dendritische cellen. Een rol voor GM-CSF, TNFα en IL-6

Het effect van de schildklier hormonen T_3 en T_4 en een aantal andere gejodeerde, hormonaal inaktieve stoffen (zoals rT_3, gejodeerd thyreoglobuline en metrizamide) op de uitrijping van perifere bloed monocyten tot funktionele dendritische cellen wordt beschreven in hoofdstuk 6. Een voorbehandeling van bloed monocyten met de schildklier hormonen, voornamelijk het bioaktieve T_3, verhogen de mogelijkheid van monocyten uit te rijpen tot immunocytologisch typische en immunologisch aktieve (in de MLR) dendritische cellen. De uitrijping is waarschijnlijk afhankelijk van verscheidene cytokinen, omdat α-GM-CSF, α-TNFα en α-IL-6 blokkerende effecten hadden. Omdat er geen verhoogde cytokine produktie wordt geïnduceerd door T_3, heeft het bioaktieve schildklier hormoon T_3 waarschijnlijk, via binding aan de T_3 kernreceptor, effecten op de cytokine receptor expressie (op regulatie) of effecten op het post cytokine receptor nivo.

De gejodeerde stoffen metrizamide, rT_3 en gejodeerd Tg waren ook in staat de uitrijping van monocyten tot dendritische cellen te verhogen, echter in mindere mate dan T_3. Een mogelijk mechanisme dat een rol zouden kunnen spelen bij de verhoogde monocyt uitrijping geïnduceerd door de hormonaal inaktieve gejodeerde stoffen is een interaktie van de gejodeerde stoffen met het H_2O_2 systeem van de monocyt. Hoe dergelijke interakties zouden kunnen leiden tot een hogere expressie van de cytokine receptoren of effecten kunnen hebben op het post cytokine receptor nivo, is geheel onbekend.

Daar dendritische cellen een cruciale rol spelen bij de initiatie van schildklier autoimmuun responsen en omdat een te hoge jodium inname (leidend tot een hoog gejodeerd Tg molekuul) de ontwikkeling van de schildklier autoimmuun reaktie (TALT, zie eerder) in de BB rat versnelt, zou een verhoogde uitrijping van monocyten tot dendritische cellen onder invloed van gejodeerde stoffen en schildklier hormonen een rol kunnen spelen bij deze versnelde ontwikkeling van schildklier autoimmuun reaktiviteit.

Hoofdstuk 7: Conclusies en algemene discussie

In hoofdstuk 7 worden conclusies van de studies met enige kritische kanttekeningen gegeven. De experimenten beschreven in dit proefschrift laten zien dat de jodium inname duidelijke effecten op de schildklier autoimmuun reaktiviteit heeft. Zowel stimulatie als onderdrukking van schildklier autoimmuun reakties is waargenomen, afhankelijk van de jodium inname (chronisch te veel of te weinig) en de genetisch bepaalde gevoeligheid van de dieren voor schildklier autoimmuun ziekte. Samen met literatuur gegevens wordt het duidelijk dat de jodium inname via het dieet beschouwd moet worden als een modulator van schildklier autoimmuun reaktiviteit. Andere faktoren, zoals genetisch bepaalde afwijkingen in de regulatie van immuun responsen, intrinsieke metabolische schildklier afwijkingen (zoals een hogere jodium opname en metabolisme waardoor mogelijk een hogere uitscheiding van antigene afval stoffen plaats vindt) en een abnormale gevoeligheid van de schildklier cellen voor een autoimmologische aanval spelen waarschijnlijk een veel belangrijkere rol in de ontwikkeling van schildklier autoimmuun reaktiviteit.

De mogelijke mechanismen via welke jodium de schildklier autoimmuun reaktiviteit kan moduleren zijn:

a. een verhoogde immunogeniciteit van hoog gejodeerd Tg,
b. direkt stimulerende effecten van jodium op cellen van het immuun systeem en
c. stimulatie van het immuunsysteem via een verhoogde uitscheiding van afval produkten van schildklier cellen tijdens jodium deficiëntie.

De data in dit proefschrift benadrukken nog eens dat een dagelijkse optimale jodium inname (ongeveer 150 μg) de optimale schildklier funktie in een gegeven populatie handhaaft. Echter, individueel verschillende reakties op een bepaalde jodium inname (afhankelijk van de genetische achtergrond en gevoeligheid voor schildklier autoimmuun ziekte) kunnen worden verwacht.

ABBREVIATIONS

Abs	:	Antibodies
ADCC	:	Antibody-Dependent Cell-mediated Cytotoxicity
ATP	:	Adenosin Triphosphate
BB	:	Bio Breeding
cAMP	:	cyclic Adenosin Monophosphate
CBA	:	Cytochemical Bioassay
CHT	:	Congenital Hypothyroidism
COD	:	Conventional Diet
DAG	:	Diacylglycerol
DC	:	Dendritic Cell
EGF	:	Epidermal Growth Factor
EID	:	Enriched Iodine Diet
FCS	:	Foetal Calf Serum
GM-CSF	:	Granulocyte Macrophage Colony Stimulating Factor
G6PD	:	Glucose-6-Phosphate Dehydrogenase
IFN-γ	:	Interferon-γ
Ig	:	Immunoglobulin
IGF	:	Insulin-like Growth Factor
IgG	:	Immunoglobulin G
IIF	:	Indirect Immuno Fluorecense
IL-1	:	Interleukin-1
IP_3	:	Inositol 1,4,5-triphosphate
LID	:	Moderate Low Iodine Diet
LID^+	:	Extreme Low Iodine Diet
M-CSF	:	Macrophage Colony Stimulating Factor
MHC	:	Major Histocompatibility Complex
MMI	:	Methimazole
MPO	:	Myelo Peroxidase
NID	:	Normal Iodine Diet (= COD)
OS	:	Obese Strain
PBS	:	Phosphate Buffered Saline
PI	:	Phosphoinositide
PIP_2	:	Phosphatidylinositol 4,5-biphosphate
rT_3	:	reverse T_3
T_0	:	Thyronine
T_3	:	Triiodothyronine
T_4	:	Thyroxine
TALT	:	Thyroid-Associated Lymhoid Tissue
TBG	:	Thyroxin Binding Globulin
TBII	:	Thyrotropin-Binding Inhibitory Immunoglobulin
TBPA	:	Thyroxin Binding Prealbumin
Tc cell	:	T cytotoxic cell
Tg	:	Thyroglobulin
TGAbs	:	Thyroid Growth-stimulating Antibodies
TGI	:	Thyroid Growth-stimulating Immunoglobulin
Th_1 cell	:	T helper 1 cell
Th_2 cell	:	T helper 2 cell
TPO	:	Thyroid Peroxidase
TRH	:	Thyrotropin Releasing Hormone
TsAbs	:	Thyroid-stimulating Antibodies
Ts cell	:	T suppressor cell
TSH	:	Thyroid Stimulating Hormone or Thyrotropin

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CURRICULUM VITAE

1 december 1964 : Geboren te Amsterdam

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Stages:
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  Afdeling Oncologie, Vrije Universiteit Amsterdam
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Extra cursussen:
- Theoretisch gedeelte oncologie
- Theoretisch gedeelte immunologie
- Medische parasitologie
- Biochemische technieken
- Veiligheids cursus radioaktiviteit
- Scriptie virale immunopathologie

1988-1993 : Promotieonderzoek: Afdeling Immunologie, Erasmus Universiteit, Rotterdam
Promotoren: prof. dr. H.A. Drexhage en prof. dr. R. Benner
Project:
- De Schildklier, Jodium en Autoimmuniteit

Cursussen:
- Immunologie voor gevorderden (Advanced Immunology, Male et al 1987)
- Medische immunologie (Essentials of Clinical Immunology, Chapel and Heany 1984 2e druk)
- Erasmus Summer Programme on Endocrinology / Immunology
- Inleiding tot principes van medisch wetenschappelijk onderzoek (proefopzet en biostatistiek)
- Proefdierkunde (ex art 9 Wet op de dierproeven)
- The Oxford Examination in English as a foreign Language
- Wetenschapsfilosofie en Logica

Onderwijs:
- Praktikumassistent Immunologie
- Praktikumassistent Histologie

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