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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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SUMMARY

Evidence is accumulating that 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 an enriched iodine diet (EID, iodine intake 100 μg iodine/day) for a period of up to 18 weeks. Control rats were either on a normal iodine diet (NID, iodine intake 7 μg iodine/day) or a low iodine diet (LID, 2 days of 1% $KClO_4$ followed by iodine-deficient drinking water/pellets). During the first 6 weeks of the EID rats developed a thyroid-associated ectopic thymic tissue (50–57% of the animals on EID versus 7–14% of NID rats and 0% of LID rats). This thyroid-associated ectopic thymic tissue showed a similar histology (cortex and medulla) and a similar marker pattern as normal rat thymus concerning TdT expression (positive cells in the cortex) and CD4/CD8 positivity (double-positive cells in the cortex, single-positive cells in the medulla).The excessive iodine diet also resulted in a lowered thyroid autoimmune reactivity as compared to the NID and LID, viz. (1) in a lower incidence of anti-colloid antibodies in serum (12·5% positivity in EID rats versus 36% in NID and 60% in LID rats at 18 weeks) and (2) lower numbers of intrathyroidal lymphoid cells, viz. lower numbers of dendritic cells and lower numbers of CD4 and CD8 positive lymphocytes. It is hypothesized that the development of the thyroid-associated ectopic thymic tissue in the EID rats is related to their low thyroid autoimmune responsiveness; the tissue might play a role in tolerance induction to thyroidal autoantigens.

INTRODUCTION

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

In humans with a pre-existing 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.[1–3] 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 60% positive for immunoglobulins that stimulate thyrocyte proliferation *in vitro*,[4,5] the so-called thyroid growth stimulating immunoglobulins (TGI). It must be noted however, that the character and function of these TGI are still under debate.[6,7]

Up until now experimental animal studies on the effect of the dietary iodine on thyroid autoimmune reactivity have predominantly been performed in animals genetically prone to 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 thyroiditis;[8,9] 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,[10–12] positive for thyroid autoantibodies.

As 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 iodine diet for periods of up to 18 weeks from 3 weeks of age. Control rats were kept on either a normal or low iodine diet. 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 via immunohistochemical techniques.

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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 3 weeks of age) groups of rats ($n = 5$–18) were kept on three dietary iodine regimens:

(1) *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 6·5 mg KI/l added to the drinking water *ad libitum*. To verify the dietary regimen iodine was measured in urine (for details of the technique see Mooij *et al.*[13]): 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, reflecting the estimated daily iodine intake.

(2) *Normal iodine diet (for our area) (NID).* The rats received normal pellets (Am-II, Hope Farms bv) and Rotterdam tap-water *ad libitum*. These groups of rats served as controls. The urinary iodine excretion of rats on a NID was 7 μg I/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*. After 1 week of diet the urinary iodine excretion was already under the lower limit of detection of our assay, verifying the low iodine intake of the animals.

Groups of the above-described Wistar rats were killed 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, thyroid-stimulating hormone (TSH) evaluation and anti-colloid antibody determination. T_3, T_4 and TSH were measured by radioimmunoassay (RIA) (Dr W. Wiersinga, Margreet Broenink, Amsterdam, The Netherlands). The rat TSH reference preparation NIADDK-rTSH-RP-2 was kindly provided by Dr A. Parlow from the UCLA Medical Centre (Torrance, CA) and was 176 times more potent than the NIADDK-rTSH-RP-1. Thyroid glands were removed, weighed and stored at −80° until immunohistological examination.

Immunohistological examination

Immunohistological examination was performed according to Green *et al.*[14] with minor modifications. From each frozen thyroid, one lobe was semiserially cut into 6-μm thin sections. The sections were air dried overnight and fixed in acetone at room temperature for 10 min. The sections were incubated with either a monoclonal antibody specific for major histocompatibility complex (MHC) class II molecules, total T cells, $CD4^+$ T cells, $CD8^+$ T cells or B cells (see Table 1) for 60 min, washed with phosphate-buffered saline (PBS) containing 0·2% bovine serum albumin (BSA; Sigma Chemical Co, St Louis, MO) for 10 min, and further incubated with a rabbit anti-mouse Ig horseradish peroxidase (HRP)-labelled conjugate (Dakopatts, Glostrup, Denmark) for 30 min, diluted 300 times in PBS containing 1% normal rat serum and 0·2% BSA. After washing the slides in PBS containing 0·2% BSA (10 min) 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, Darmstadt, Germany), 0·068% imidazole (Sigma) and 0·8% sodium chloride in 0·1 M acetate buffer pH 6·0 for 3–5 min. Hydrogen peroxide was added to a final concentration of 0·01%. After DAB development, the sections were washed briefly in 0·1 M Tris-HCl buffer, pH 7·6, and immersed in a 0·5% solution of cobalt chloride in 0·1 M Tris-HCl buffer, pH 7·2 at room temperature for 4 min. The slides were either counterstained with 0·1% nuclear fast red in 5% aluminum sulphate for 2 min or incubated with acid phosphatase at 37° for 30 min and thereafter counterstained with haematoxylin. The slides were dehydrated and embedded in DePeX mounting medium (Gurr, BDH Ltd, Poole, U.K.).

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

mAb	Specificity	Reference
OX6 (Sera Lab, Crawley Down, U.K.)	MHC class II	15
W3/25 (Sera Lab)	CD4 antigen	16
B115-4 (Holland Biotechnology, The Netherlands)	CD4 antigen	17
B115-5 (Holland Biotechnology)	CD5 antigen	17
OX8 (Sera Lab)	CD8 antigen	18
HIS 14 (F. G. M. Kroese)	B cells	19,20
Anti-human TdT (Supertechs Inc., Bethesda, MD)	TdT	21

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 leukaemia patients,[22–24] a method described by Gregoire *et al.* was used.[25] In brief, thyroid sections were fixed in methanol at 4° for 30 min. Thereafter the slides were washed five times with PBS at 4° for 5 min and incubated in ice-cold PBS at room temperature for a period of 30 min. The slides were further incubated with a rabbit anti-TdT mAb (Supertechs Inc. Bethesda, MD) diluted 60 times in PBS containing 0·2% BSA at room temperature for 60 min. The slides were washed three times with PBS for 10 min and incubated with swine anti-rabbit Ig HRP-labelled (Supertechs Inc.) diluted 100 times in PBS containing 0·2% BSA and 1% normal rat serum for 30 min. Thereafter slides were washed, developed and embedded as described above.

For the quantification of infiltrated leucocytes four sections of each thyroid with intervals of at least 100 μm were reacted with the appropriate marker mAb. 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 400×. 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.

Anti-colloid antibody determination

Six-micrometre thin frozen porcine thyroid sections were cut, air dried overnight and fixed in cold acetone (−20°) for 10 min. The sections were preincubated with normal rabbit serum (Dako, Copenhagen, Denmark) (diluted 50 times in PBS with 1% BSA) for 10 min. 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 min. Porcine tissue was used, as it gave optimal results compared to rat or human tissue. After washing in PBS, the sections were incubated with rabbit anti-rat immunoglobulins, fluorescein isothiocyanate-(FITC)-labelled (Dako) for 30 min (diluted 25 times in PBS with 1% BSA). After this second step and rewashing, the slides were embedded in aquamount (Gurr, BDH Ltd, Poole, U.K.) 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, positive or strongly positive.

The indirect immunofluorescence 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 χ^2 analysis.

RESULTS

The development of thyroid-associated ectopic thymic tissue in EID rats

The development of a special lymphoid tissue was remarkable in a high proportion of the EID rats during the early weeks of the diet (Table 2). This lymphoid tissue was in contact with the thyroid and showed a marker pattern specific for thymus. It occurred in about half of the animals after 3 and 6 weeks of excessive iodine diet, declining to a 12·5% incidence after 12 weeks, remaining at this level thereafter. The tissue clearly showed a cortex and medulla as in thymus and cortical lymphocytes were double positive for CD4 and CD8 (Fig. 1). To verify whether the observed lymphoid tissue was indeed thymic tissue, immunohistochemistry 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 (Fig. 2). Therefore it can be concluded that the observed lymphoid tissue is indeed thyroid-associated ectopic thymic tissue.

Table 2. Incidence of thyroid-associated ectopic thymic tissue

Weeks on diet	EID	NID	LID
0	0/10 (0%)	0/10 (0%)	0/10 (0%)
3	8/14 (57%)*	2/14 (14%)	0/8 (0%)
6	7/14 (50%)*	1/14 (7%)	0/13 (0%)
9	3/14 (21%)	1/16 (6%)	—
12	1/8 (12·5%)	1/5 (20%)	1/9 (11%)
18	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 6 μm were incubated with anti-MHC class II, anti-CD4 and CD8 and anti-TdT mAb. The number of rats with ectopic thymic tissue of each group is presented. The incidence of ectopic thymic tissue is given in parentheses.

* $P<0·05$ versus NID and LID, χ^2 analysis.

In rats kept on the NID thyroid-associated ectopic thymic tissue could only occasionally be observed, and incidences did not exceed the 20% level (Table 2).

In the 34 rats on the LID thyroid-associated ectopic thymic tissue could only be detected in one animal (Table 2).

Thyroid autoimmune reactivity in EID rats

The occurrence of thyroid-associated ectopic thymic tissue in EID rats was accompanied by the virtual absence of anti-colloid antibodies later in the circulation and a low infiltration of MHC class II positive dendritic cells and T cells into the thyroid (Fig. 3).

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 the EID, the incidence of anti-colloid antibodies did not increase much and remained at a level not exceeding 12·5% incidence ($n=8$–16, Fig. 3A).

As reported in detail earlier[13] rats on the NID and LID developed higher incidences of serum anti-colloid antibodies, particularly at later stages of the dietary regimens and positivity reached a 30% and a 60% incidence respectively (see also Fig. 3A).

During the EID there was also a steady and low infiltration into the thyroid of MHC class II positive dendritic cells (Fig. 3B) and $CD4^+$ and $CD8^+$ T cells (Fig. 3C, 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.

With regard to such intrathyroidal infiltrations of dendritic cells and T cells during the NID and a LID we reported previously[13] that such diets, especially the LID resulted in a higher infiltration with these cells (see also Fig. 3B–D).

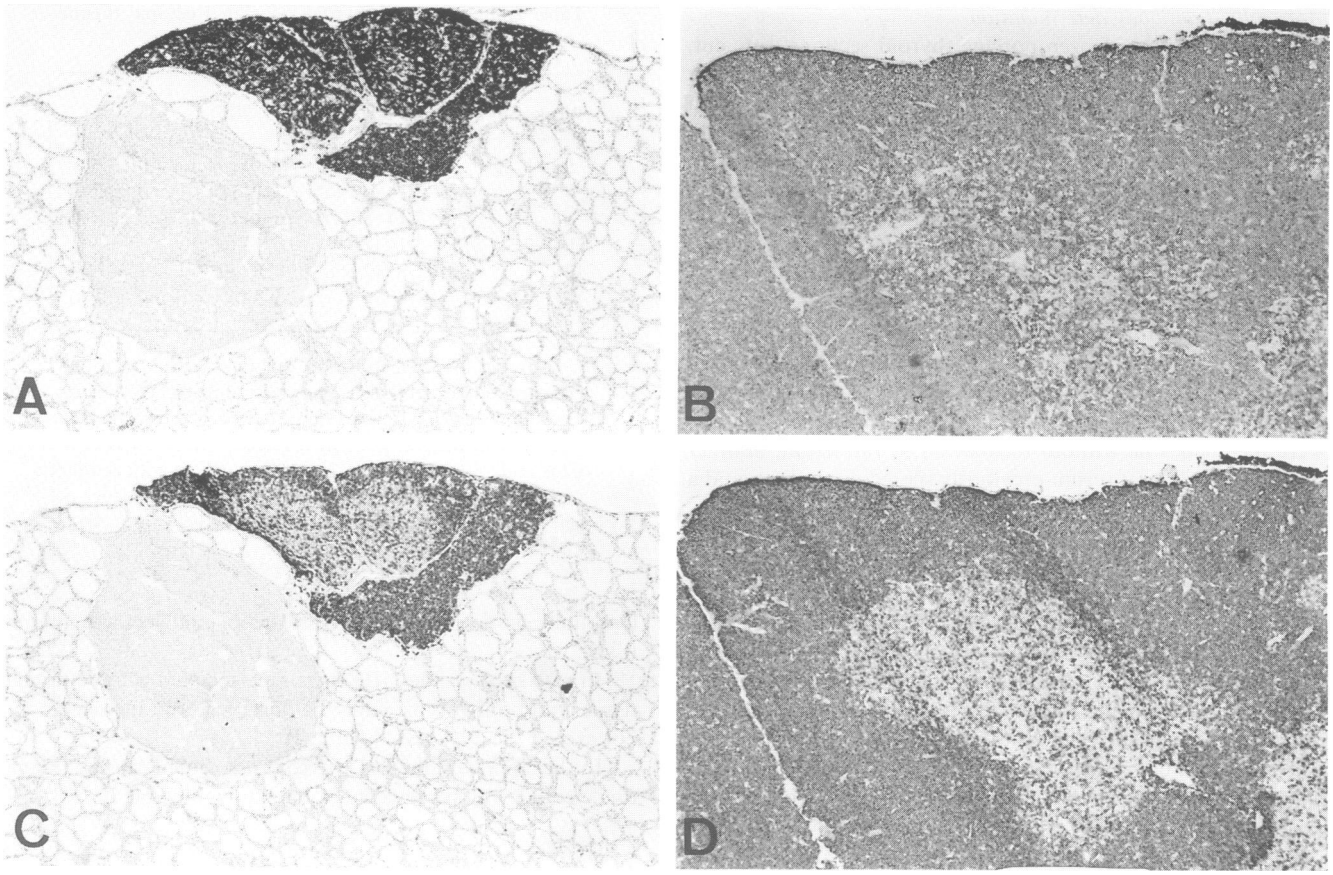

Figure 1. Thyroid-associated ectopic thymic tissue. Frozen thyroid (A and C) and thymus (B and D) sections of rats kept on an EID of 6 μm were incubated with monoclonal antibodies directed against CD4 antigens (A and B, magnification $\times$ 200) and CD8 antigens (C and D, magnification $\times$ 200). Note the similarity of the thyroid-associated ectopic thymic tissue with rat thymus, and the clear cortex and medulla in Fig. 3C and D.

The thyroid endocrine performance of the various diet groups has also been published in detail previously.[13] In short, the EID had no observable thyroidal endocrine effects and thyroid weights and serum thyroid hormone levels were within the limits of normality. The 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. Serum T_3 levels stayed within the normal range and the LID animals showed a normal body weight development. Serum TSH levels started to increase after 12 weeks of diet.

DISCUSSION

This study shows that a high dietary iodine intake in young female Wistar rats resulted in around 50% of the animals in the development of a thyroid-associated ectopic lymphoid tissue. On the basis of immunohistology we identified this tissue as ectopic thymus tissue. It showed: (1) a clear medulla and cortex; (2) double CD4/CD8 positive lymphocytes in the cortex; (3) single CD4 positive and CD8 positive lymphocytes in the medulla; and (4) TdT activity in the cortex (an enzyme known to be present in immature T cells). The thyroid-associated ectopic thymic tissue was histomorphologically entirely normal, and showed the same marker pattern as normal rat thymus. The development of this tissue in EID rats coincided with a very low infiltration into the thyroid of lymphoid cells and a minimal production of anti-colloid antibodies.

In humans with autoimmune thyroid disease and in the autoimmune-prone BB rat, areas of lymphoid tissue in the thyroid have been found before. However, these areas of so-called 'focal thyroiditis' are histologically clearly different in respect to marker pattern and structure from the thyroid-associated ectopic thymic tissue found in this study:[26,27] 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 presence of ectopic thymic tissue in or around the thyroid has been reported before.[28–31] The ectopic tissue has been observed as early as 1970 in the obese strain (OS) chicken[28,29] and it was then speculated that it represented a breakdown of the thymus–thyroid barrier leading to the OS autoimmune thyroiditis. However, the incidence of ectopic thymic tissue in the OS chicken was much lower (four out of 64) than the incidence of thyroiditis (up to 90%) and it is therefore doubtful that its presence can be considered as the cause for the development of the OS autoimmune thyroiditis. There were also many germinal centres in the OS thyroid-associated ectopic thymic tissue. Germinal centres do not usually occur in the normal thymus and the presence of these germinal centres may be taken as an

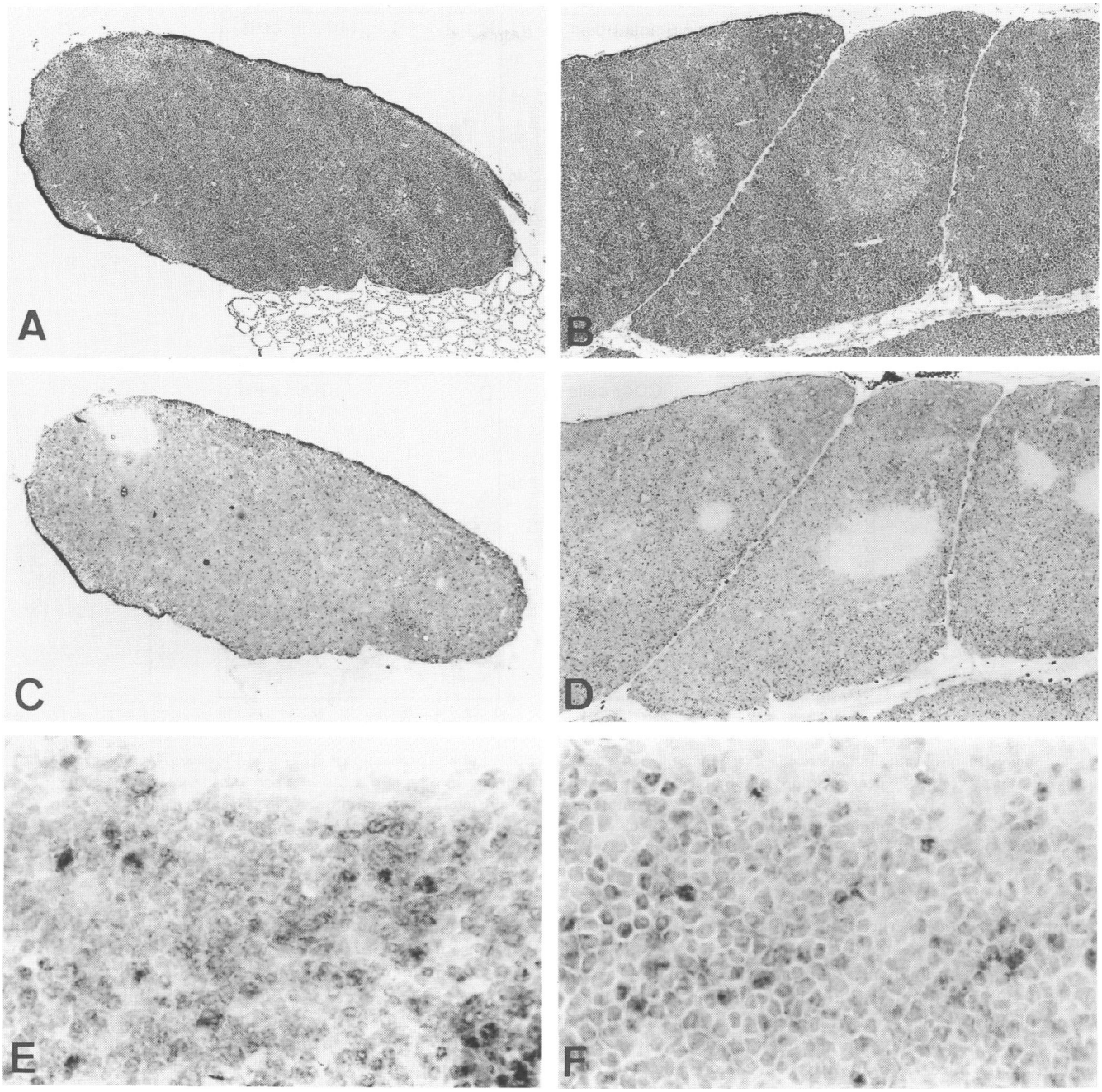

Figure 2. Thyroid-associated ectopic thymic tissue. Frozen thyroid (A, C and E) and thymus (B, D and F) sections of rats kept on an EID of 6 μm were routinely stained with haematoxylin and eosin (A and B, magnification $\times 200$) or incubated with a monoclonal antibody specific for the enzyme TdT (C and D, magnification $\times 200$; E and F, magnification $\times 800$). Note the similarity between the reactivity of the thyroid-associated ectopic thymic tissue and that of the thymus.

indication of abnormality of the OS thyroid-associated ectopic thymic tissue. The OS thymus itself is also abnormal (thymic nurse cell deficiency[30].)

The mechanisms underlying the development of thyroid-associated ectopic thymic tissue remain speculative. Recently, Many *et al.*[31] observed the development of thyroid-associated ectopic thymic tissue in NOD mice. These authors speculated that the development of the tissue was due to a form of dysembryogenesis, as the thyroid and thymus are embryonically related, both being derived from the third endodermal pouch and ectodermal cleft.[32] It is indeed possible that fragments of the thymic 'anlage' stay behind, close to the ductus thyroglossus to develop later into mature thymic tissue during a high dietary iodine intake. Vladutiu *et al.*[33] 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 have shown that the development of ectopic thymic tissue is influenced by an environmental factor such as dietary iodine intake. Iodine has been described as having direct stimulating effects on various

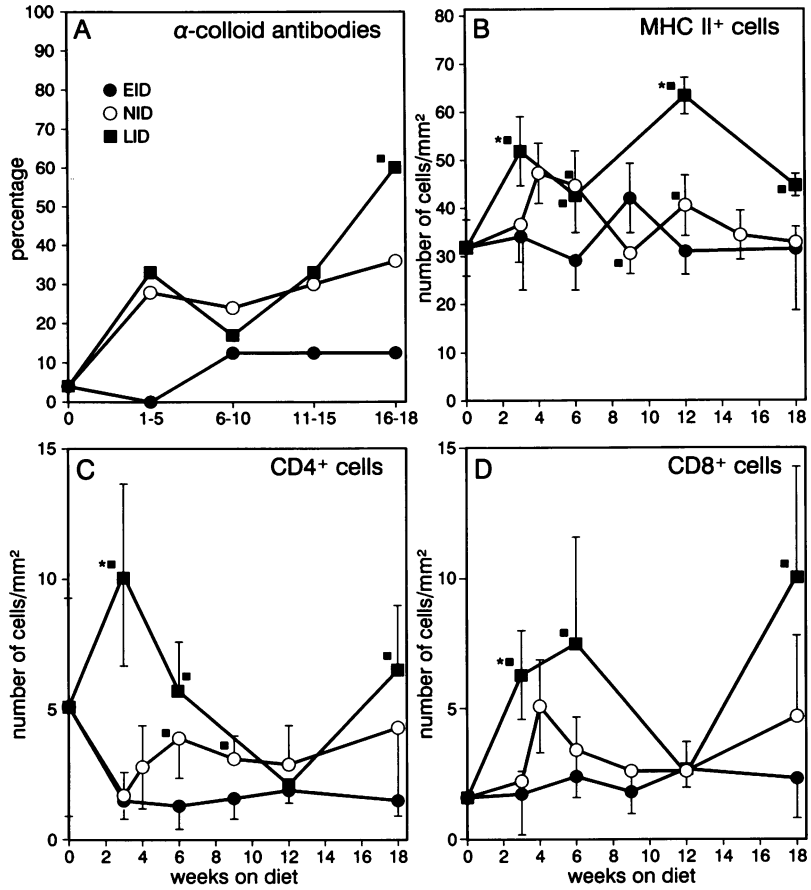

Figure 3. Thyroid autoimmune reactivity. (A) The incidence of anti-colloid antibody production is given of various age groups of female Wistar rats ($n = 8$–20 per age group) kept on the NID, LID or EID from 3 weeks of age onwards for a period of 18 weeks. ■ $P < 0{\cdot}05$, compared to EID, χ^2 analysis. Thyroid lobes of groups of rats ($n = 5$–12) were removed, frozen and sections of 6 μm were incubated with several monoclonal antibodies to identify intrathyroidal leucocytes. The mean ± SD of intrathyroidal MHC class II$^+$ dendritic cells (B), CD4$^+$ T cells (C) and CD8$^+$ T cells (D) are expressed per mm^2 counted in four semiserial cut sections for each thyroid. Statistical significances using the Wilcoxon's rank sum test: *$P < 0{\cdot}05$ versus NID; ■ $P < 0{\cdot}05$ versus EID.

lymphoid cells;[34-36] whether it also affects the development of thymic tissue is unknown.

The thymus normally 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,[37,38] and in NOD mice.[39] As an EID also led to a low thyroid autoimmune reactivity, in the Wistar rats we hypothesize that the development of the thyroid-associated ectopic thymic tissue represents a sign of tolerance induction towards thyroidal autoantigens.

Our observations on the 'immunosuppressive' effect of a high iodine diet on thyroid autoimmune reactivity in normal Wistar rats are in contrast to the observations of others showing immunostimulating effects of a high iodine diet in animals/individuals with an underlying thyroid (autoimmune) disease: in OS chickens and BB rats an excess of dietary iodine induced a higher incidence and more severe forms of thyroiditis.[8,9]

There are two hypothetical explanations for this discrepancy:

(1) Firstly, there might be a stimulating effect of iodine on the immune system of the autoimmune prone OS chickens and BB rats, as these animals have a dysregulated immune system. In normal, non-autoimmune animals, autoreactive T and B cells are present in the periphery, but these cells are suppressed by various immunoregulatory mechanisms. In BB rats and OS chickens however, there are abnormalities in this immunoregulation. It has, for instance, been described that the BB rat becomes lymphopenic mainly because of a lack of RT6 positive regulator (suppressor) T cells,[40,41] which are normally generated in the thymus. In the OS of chicken hyperreactive T cells, hyperreactive macrophages and a high density of interleukin-2 (IL-2) receptor expression were found.[30] As iodine has direct stimulatory effects on lymphoid cells, it is possible that a high dietary iodine intake results in a complete dysregulation of an already malfunctioning immune system in autoimmune prone animals/individuals.

(2) Secondly, iodine may be toxic especially for thyrocytes of autoimmune prone OS chickens and BB rats. Abnormalities in the regulatory mechanisms of the immune system are not the only factors important in the development of thyroid disease in autoimmune-prone animal strains. Genetically determined abnormalities in the thyroid cells themselves are also essential for the development of fully blown thyroiditis in OS chickens and BB rats. In the OS strain of chicken an autonomous high thyrocyte function (iodine uptake) has been described,[42-44] and

the metabolic activity of BB thyrocytes has also been reported as high before thyroid autoimmunity develops. It is therefore possible that the high iodine diet is toxic for OS and BB 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 the thyroids of hyperplastic mice,[11,12] resulting in thyrocyte destruction, inflammation of the thyroid and an attack of autoimmune thyroiditis.[45]

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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