

# Pre-autoimmune thyroid abnormalities in the biobreeding diabetes-prone (BB-DP) rat: a possible relation with the intrathyroid accumulation of dendritic cells and the initiation of the thyroid autoimmune response

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## Abstract

Thyroid autoimmune reactions start with an accumulation of mainly dendritic cells in the thyroid. There is increasing evidence that, apart from being antigen-presenting cells, they are also able to control the growth and hormone synthesis of neighbouring endocrine cells. The questions thus arise: are dendritic cells accumulating in the pre-autoimmune thyroid in response to an altered proliferative or metabolic activity of thyrocytes, and do cytokines, monocyte chemoattractants, or both, have a role in their accumulation? We have investigated these questions in thyrocytes of the biobreeding diabetes-prone (BB-DP) rat in relation to the start of the intrathyroid accumulation of dendritic cells – that is, at about 9 weeks of age.

BB-DP rats and Wistar rats (controls) were studied from 3 to 20 weeks of age. Hyperplastic goitre development was studied by assessing the thyroid weight and by measuring the number of thyrocyte nuclei per 0.01 mm<sup>2</sup> thyroid section. In addition, the *in situ* expression of interleukin-6 (IL-6), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), monocyte-chemotactic protein-1 (MCP-1), and intercellular adhesion molecule-1 (ICAM-1) were studied by immunohistochemistry. The *in vitro* proliferative capacity of BB-DP and Wistar thyrocytes was measured by tritiated-thymidine ([<sup>3</sup>H]TdR) and bromodeoxyuridine (BrdU) incorporation into reconstituted, TSH- and non-TSH-stimulated, cultured thyroid follicles. Further *in vitro* studies consisted of measurement of the production of thyroxine (T<sub>4</sub>), triiodothyronine (T<sub>3</sub>), thyroglobulin, IL-6, TNF- $\alpha$  and MCP-1 by the thyroid follicles.

BB-DP rats developed a small hyperplastic goitre between the ages of 9 and 12 weeks. The *in vitro* proliferative rate of thyrocytes isolated from hyperplastic BB-DP thyroids was significantly lower than that of Wistar thyrocytes. This phenomenon also occurred in

follicles isolated from BB-DP rats before hyperplastic goitre development, which produced significantly less T<sub>4</sub>, but more T<sub>3</sub>, than did Wistar follicles of the same age. At the time of and after hyperplastic goitre development, BB-DP follicles exhibited altered metabolic behaviour and produced significantly more T<sub>4</sub>, but equal amounts of T<sub>3</sub> compared with both Wistar follicles of the same age and follicles of younger BB-DP rats (both under basal conditions and TSH-stimulated). *In vitro* IL-6 production by these BB-DP thyroid follicles was also increased. There was no noteworthy difference in production of thyroglobulin and MCP-1 between BB-DP and Wistar follicles at any age. TNF- $\alpha$  was not produced by BB-DP or Wistar thyroid follicles.

Immunohistochemistry revealed the expression of IL-6 by both BB-DP and Wistar thyroid follicle cells at all times of sampling. MCP-1 and TNF- $\alpha$  were expressed only when infiltrates were present in BB-DP thyroids (restricted to leucocytes, ages >18 weeks). Modest ICAM-1 expression was restricted to large blood vessels in both BB-DP and Wistar thyroids; in the case of infiltrates (BB-DP rat) alone, high ICAM-1 expression was found on blood vessels and leucocytes in these infiltrations.

At the time of intrathyroidal dendritic cells accumulation, BB-DP rats develop a small hyperplastic goitre. At that time there is also *in vitro* evidence for a shift to a higher production of thyroxine and IL-6 from thyrocyte follicles. The *in vitro* proliferation rate of BB-DP thyrocytes is, however, abnormally low (both in the pre- and hyperplastic period). Similar pre-autoimmune thyroid growth abnormalities have been described in another animal model of thyroid autoimmune disease, the obese strain chicken.

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## Introduction

The animal models of spontaneously developing autoimmune thyroid disease (AITD) are the biobreeding diabetes-prone (BB-DP) rat, the non-obese diabetic (NOD) mouse and the obese strain (OS) chicken. The first immunological sign of AITD in these three animal models is an accumulation of antigen-presenting cells (APCs), mainly dendritic cells, in the thyroid (Voorbij *et al.* 1990, Many *et al.* 1995, Hala *et al.* 1996). This accumulation of APCs precedes the triggering of autoreactive T and B lymphocytes in the thyroid-draining lymph nodes and the subsequent influx of T and B lymphocytes into the thyroid (focal thyroiditis; Voorbij *et al.* 1990, Many *et al.* 1995). High numbers of APCs can also be detected in human forms of AITD, Hashimoto's and Graves' goitres (Kabel *et al.* 1988). In Graves' goitres, dendritic cells often accumulate in the absence of a noteworthy lymphocytic infiltration (Kabel *et al.* 1988). The question thus arises: 'Why do dendritic cells start to accumulate in thyroid glands that later become the target of an autoimmune attack?'

For a further exploration of this question it is important to note that endocrine tissues normally contain dendritic cells and macrophages. Although dendritic cells are present in low numbers in the normal thyroid (Kabel *et al.* 1988, Mooij *et al.* 1993a), networks of relatively large numbers can be found in the anterior pituitary (Allaerts *et al.* 1996, 1997) and in the gonads (Brännström & Norman 1993, Hoek *et al.* 1996). In the anterior pituitary, dendritic cells form a subgroup of the network of folliculo-stellate cells (Allaerts *et al.* 1996). Apart from being excellent accessory cells in T cell stimulation, these dendritic cells are also capable of regulating the hormonal responses of neighbouring endocrine cells (Allaerts *et al.* 1994, 1996, 1997). The cytokine, interleukin-6 (IL-6), is believed to have an important role in this regulation (Allaerts *et al.* 1994, 1997). With regard to the presence of dendritic cells and macrophages in the ovary and testis, these classical APCs have a role not only in the regulation of ovulation (Brännström & Norman 1993), but also in the growth and steroid production of granulosa, theca, luteal and Leydig cells (Gaytan *et al.* 1995, Hoek *et al.* 1996, Katabuchi *et al.* 1996). Recently, we have obtained preliminary evidence that dendritic cells are also involved in the regulation of the growth of thyrocytes: Wistar rat splenic dendritic cells – which are excellent APCs – were capable of downregulating the uptake of tritiated thymidine ( $[^3\text{H}]\text{TdR}$ ) and bromodeoxyuridine (BrdU) of Wistar rat thyrocytes kept in follicle culture (manuscript in preparation). Interestingly, dendritic cells accumulate in the thyroid during goitrous conditions, for example in simple human goitres (Kabel *et al.* 1988) and in iodine-deficient goitres both in humans (Wilders-Truschnig *et al.* 1989) and in the Wistar rat (Mooij *et al.* 1993a).

This accumulating evidence that dendritic cells have a role in the regulation of growth and hormone production of endocrine cells (Brännström & Norman 1993, Allaerts *et al.* 1994, 1996, 1997, Gaytan *et al.* 1995, Hoek *et al.* 1996, Katabuchi *et al.* 1996), is highly suggestive that the accumulation of dendritic cells in thyroid glands, which later become the targets of an autoimmune reaction, may be a consequence of an abnormal proliferative rate or metabolic activity of pre-autoimmune thyrocytes. Interestingly, many papers have, indeed, reported pre-autoimmune abnormalities in the growth and function of thyrocytes of the OS chicken (reviewed by Sundick 1989).

In this study, we have investigated whether there are pre-autoimmune abnormalities in the proliferative rate and in the metabolic activity of BB-DP thyrocytes compared with Wistar thyrocytes. We studied hyperplastic goitre development (thyroid weight increase and the number of thyrocyte nuclei per area of histological thyroid section), uptake of  $[^3\text{H}]\text{TdR}$  in isolated and *in vitro* reconstituted thyroid follicles (verified by BrdU labelling), and the production of thyroxine ( $\text{T}_4$ ), tri-iodothyronine ( $\text{T}_3$ ), and thyroglobulin by isolated and *in vitro* reconstituted thyroid follicles.

Adhesion molecules on thyroid endothelial cells and locally produced cytokines/monocyte-chemokines in pre-autoimmune stages are probably instrumental in the enhanced attraction of dendritic cells (for which monocytes are among the important precursors, Mooij *et al.* 1994, Peters *et al.* 1996) into the BB-DP thyroid. Because of limited availability of antibodies, from all the various cytokines and growth factors involved in monocyte extravasation and the transition of monocytes to dendritic cells, we were able to study only intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1), IL-6 and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ). ICAM-1 is one of the important endothelial adhesion molecules, MCP-1 is an important monocyte chemoattractant, and IL-6 and TNF- $\alpha$  are, alongside granulocyte monocyte colony stimulating factor (GM-CSF) and IL-4, important cytokines for monocyte to dendritic cell transition (Peters *et al.* 1996). The IL-6, TNF- $\alpha$  and MCP-1 production by BB-DP thyrocytes, and the ICAM-1 expression in the BB-DP thyroid were studied *in situ* (immunohistochemistry) as well as *in vitro* (thyroid follicle cultures).

Data were analysed taking into account the time at which intrathyroid accumulation of dendritic cells begins in the BB-DP rat, which is at about 9 weeks of age (Voorbij *et al.* 1990, Mooij *et al.* 1993b).

## Materials and Methods

### Animals

BB-DP rats were bred for more than 20 generations in the Experimental Animal Centre of the Erasmus University

Rotterdam, The Netherlands. The strain originates from the BB/WOR strain via the BB/Organon strain. All rats were kept under controlled conditions of light (12 h light : 12 h darkness cycle) during the experiments. Between 12 and 20 weeks of age, 90% of our BB-DP rats develop  $\alpha$ -colloid antibodies detectable in serum, and during the same period 70–80% of our rats become diabetic (glucosuria). Normal pellets (Am-II, Hope Farms BV, Woerden, The Netherlands; 0.35 mg iodine/kg) and tap water were available to all rats *ad libitum*. Control Wistar rats were purchased from Harlan, Zeist, The Netherlands.

#### *Histology of the thyroid, quantification of histomorphological thyroid parameters and detection of cytokines in situ*

BB-DP and Wistar rats were asphyxiated with carbon dioxide. Thyroid glands were excised, weighed and thereafter either immediately fixed overnight in a 10% (v/v) formalin solution (for haematoxylin–eosin staining) or embedded in OCT (Tissue-Tek, Miles, Elkhart, IN, USA), snap-frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until required for immunohistochemistry.

The formalin-fixed tissues were processed routinely and embedded in paraffin. Serial sections (6  $\mu\text{m}$ ) of blocks containing six separate thyroids were cut and routinely stained with haematoxylin–eosin. These sections were used to measure the thyrocyte nuclear areas and the number of thyrocyte nuclei per unit surface section area. For this purpose, a microscope (Axioscope, Zeiss, Weesp, The Netherlands) connected to a VIDAS-RT image-analysis system (Kontron Elektronik GmbH/Zeiss) was used; magnifications were  $\times 400$  and  $\times 200$  respectively.

Immunostaining for cytokines was performed as described earlier (Eertwegh van den *et al.* 1991) with minor modifications. In short, cryostat sections (8  $\mu\text{m}$ ) were cut, mounted on glass slides and kept overnight under high humidity at room temperature. Tissue sections were fixed for 10 min in acetone containing 0.02% (v/v)  $\text{H}_2\text{O}_2$  (Merck, Munich, Germany) to block endogenous peroxidase activity. Slides were dried and incubated with the primary antibody diluted in PBS (Merck) containing 0.2% (v/v) BSA (Organon Technika, Eppenheim, Germany) for 1 h at room temperature. The following polyclonal rabbit antibodies were used: anti-human IL-6 (1 : 10; Genzyme, Diagnostics, Cambridge, UK), anti-mouse TNF- $\alpha$  (1 : 10; Genzyme) and anti-mouse MCP-1 (1 : 50; a kind gift from Dr R M Strieter; Paine *et al.* 1993). These antibodies crossreact with rat cytokines (Diamond & Pesek 1991, Paine *et al.* 1993). For the detection of MCP-1, mouse anti-rat MCP-1 was also used (undiluted culture supernatant; a kind gift from Dr K Takahashi; Sakanashi *et al.* 1994). Immunostaining with anti-rat ICAM-1 (1:10; Cedarlane, Hornby, Ontario, Canada) was performed omitting the overnight step under high humidity. After incubation, slides were rinsed three

times with PBS–0.2% BSA and subsequently further incubated with peroxidase-conjugated secondary antibodies (in PBS containing 1% normal rat serum) and developed as described by Eertwegh van den *et al.* (1991).

#### *Isolation and in vitro culture of thyroid follicles*

Thyroid follicles were isolated according to slightly modified methods described in detail elsewhere (Denef *et al.* 1980, Spinel-Comez *et al.* 1990). The isolated follicles were resuspended under adherent conditions and cultured in 5–10 ml Ham's F12 medium (Gibco, BRL-Life Technologies, Breda, The Netherlands), supplemented with a five-hormone mixture (Ambesi-Impombato *et al.* 1980) and 1% fetal calf serum (FCS). The viability of the isolated follicles was routinely assessed by the trypan blue exclusion test.

After reconstitution for 24 h, the follicles were further cultured ( $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$ , water-saturated atmosphere) in 24-well plates (Costar Europe Ltd, Badhoevedorp, The Netherlands) in the presence of 0.1  $\mu\text{M}$  KI (Merck) with or without various concentrations of bovine (b) thyroid-stimulating hormone (TSH; Sigma Chemical Co., Axel, The Netherlands) or recombinant human (rh) TSH (Sigma; see Results). After 24 h the culture supernatants were aspirated, centrifuged at 500  $g$  for 5 min and stored at  $-20^{\circ}\text{C}$  until required for thyroid hormone, thyroglobulin or cytokine determinations.

#### *The measurement of in vitro thyroid hormone, thyroglobulin and cytokine production*

The  $\text{T}_4$  and  $\text{T}_3$  contents of the supernatants were measured by conventional radioimmunoassays (Dr T J Visser, Dr R Docter, Mr H van Toor, Department of Internal Medicine, University Hospital Dijkzigt, Rotterdam, The Netherlands).  $\text{T}_3$  and  $\text{T}_4$  standards were diluted in Ham's F12 culture fluid plus supplements. Each individual supernatant sample was assayed in duplicate.

Determination of the thyroglobulin concentrations in the follicle supernatants was performed using a newly developed sandwich ELISA. Microtitre plates (Falcon, Becton Dickinson Labware, New Jersey, USA) were coated overnight at  $4^{\circ}\text{C}$  with a specific polyclonal anti-serum against rat thyroglobulin in PBS (4.0  $\mu\text{g}$  protein A purified immunoglobulins per well). Non-specific binding was prevented by preincubating the plates with PBS–0.5% BSA–0.05% (v/v) Tween 20 (Merck) for 1 h. Rat thyroglobulin (31–2000 ng/ml), which was used as a standard, and the culture supernatants were then diluted in Ham's F12 medium (plus supplements) and incubated for 1.5 h. Finally, the plates were incubated during 1.5 h with a mixture of two monoclonal (mouse) antibodies against rat thyroglobulin (1 : 2000 in PBS; clones 2F2 and 2H11), followed by incubation for 1 h with a polyclonal anti-mouse serum conjugated with peroxidase (1 : 1000

in PBS; DAKO, Glostrup, Denmark). Bound enzyme activity was measured with 0.05 M  $\text{KNaPO}_4$  (Merck) buffer (pH 5.6) containing 6 mg/ml ortho-phenyldiamine dihydrochloride (Sigma) and 0.015%  $\text{H}_2\text{O}_2$  (Merck) as substrate. This reaction was stopped with 2 M HCl (Merck) and the optical density was read at 492 nm. All incubation steps were performed at room temperature and between these steps the wells were washed at least three times with 0.9% (w/v) NaCl (Merck)–0.1% Tween 20 in aqua dest. Rat thyroglobulin and antibodies against this thyroglobulin were developed and kindly provided by Dr J J M de Vijlder, Academic Medical Centre, Department of Pediatrics, Amsterdam, The Netherlands.

IL-6 was detected in the B9 bioassay developed by Aarden *et al.* (1987). The murine fibroblast cell line, WEHI 164-13, was used to assay TNF bioactivity according to Espevik & Nissen-Meyer (1986). Both techniques have been described in detail before. Thyroid follicle culture supernatants were serially diluted and assayed in triplicate.

Rat MCP-1 in the culture supernatants of the follicles was determined with a commercial available ELISA kit (Immunosource, Zoersal-Halle, Belgium) according to techniques advised by the manufacturer.

#### *The measurement of in vitro thyrocyte proliferation*

Thyroid follicles were labelled with [ $^3\text{H}$ ]TdR during the last 5 h of culture. In brief, the follicles were washed and put on fresh Ham's F12 medium (plus supplements and different concentrations of bTSH) in 96-well plates (Costar). Subsequently, 1  $\mu\text{Ci}$ /well methyl- $^3\text{H}$ ]TdR (Amersham International, Amersham, England) was added. After the period of labelling at 37 °C in a 5%  $\text{CO}_2$  water-saturated incubator, the follicles were harvested on filter paper and radioactivity was counted in a liquid scintillation analyser (LKB Betaplate; Wallac, Turku, Finland). In some experiments, follicles (obtained from both rat strains) were co-cultured with 5  $\mu\text{g}/\text{ml}$  aphidicolin (an inhibitor of DNA polymerase; Sigma; Okugawa *et al.* 1995) or exposed to 2000 rad X-irradiation. These treatments were applied to prevent replication of DNA, and thus to ensure that radioactivity measured in the samples that did not undergo these treatments reflected the S-phase of the cell cycle (Maurer 1981). Each individual experiment was performed in duplicate.

To validate further the [ $^3\text{H}$ ]TdR incorporation assay, data were also confirmed with BrdU labelling. For that purpose, the follicles were incubated at 37 °C in a 5%  $\text{CO}_2$  water-saturated incubator in the presence of 10  $\mu\text{M}$  BrdU (BrdU-kit I; Boehringer) during the last 5 h of culture. Thereafter, the samples were thoroughly washed in PBS, cytocentrifuged onto glass slides, air-dried and fixed in pre-cooled 70% (v/v) ethanol (Merck) for 30 min at –20 °C. The BrdU incorporation was visualized

immunocytochemically according to the instructions of the manufacturer. The percentage of BrdU-positive (BrdU $^+$ ) follicles was evaluated by counting at least 500 follicles per sample. For negative controls, the primary incubation step was buffer solution without anti-BrdU antibody.

#### *Statistical analysis*

The results are presented as means  $\pm$  s.d. or s.e.m. (see Figures). Statistical analyses of the quantitative histomorphological measurements were performed using the Student's *t*-test (unpaired, two-tailed, InStat computer program). Statistical analyses of the production of hormones, thyroglobulin and cytokines and the proliferative rate of the *in vitro* cultured thyroid follicles were performed using Wilcoxon's rank sum test (two-tailed, InStat computer program).

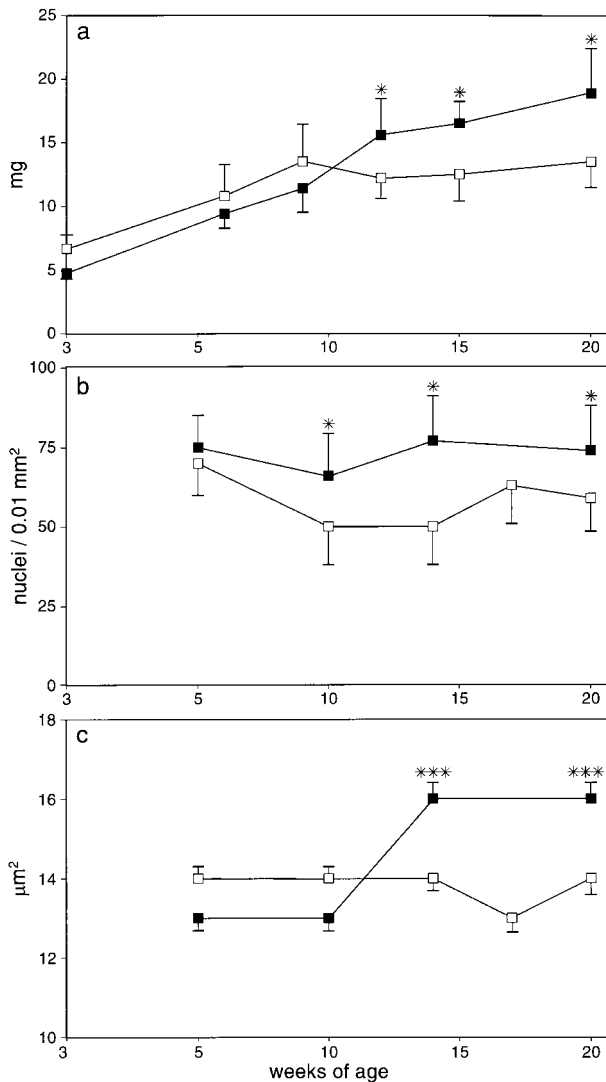
## **Results**

#### *Thyroid weight and number of thyrocyte nuclei per surface area of a thyroid section*

Figure 1a shows that, between the ages of 9–12 weeks, the thyroid weight of BB-DP rats became statistically greater than that of Wistar rats, and that the BB-DP rat thyroid continued to grow during our experimental period of 20 weeks, whereas the Wistar thyroid weight had reached a plateau from 9 weeks of age. From the age of 10 weeks onwards, morphological signs of thyroid hyperplasia in the BB-DP rat thyroid were evident: the number of thyrocyte nuclei per 0.01  $\text{mm}^2$  thyroid section surface area was significantly greater in BB-DP rats than in Wistar rats (Fig. 1b). Lymphocytic infiltrations were observed in our BB-DP colony only from the age of 18 weeks, therefore lymphocytic accumulation did not contribute to goitre formation in this pre-autoimmune phase.

#### *In vitro thyrocyte proliferation*

During the 24 h of culture after the isolation procedure, fragmented follicles of both BB-DP and Wistar rats became sphere-like structures again, and contained 30–50 thyrocytes per follicle (judged by inverted microscopy and haematoxylin–eosin staining). There was no difference in the morphology between BB and Wistar follicles. Because our culture conditions were low in FCS, the basal–apical polarity was normal. The reconstituted follicles were washed and seeded in 24-well plates at a density of 1000 follicles/well. At this stage, 0.1  $\mu\text{M}$  KI and bTSH in concentrations ranging from  $1 \times 10^{-5}$  to  $5 \times 10^2$  mU/ml were added to the follicles. Cultures were also performed in the absence of bTSH.



**Figure 1** Thyroid weight (a), number of nuclei per section area (b, hyperplasia) and the nuclear areas (c, 'hypermetabolism') of BB-DP rats (■) and Wistar rats (□) at various ages. Means ( $n \geq 30$ )  $\pm$  S.D. are shown. \* $P < 0.05$ , \*\*\* $P < 0.001$  compared with age-matched Wistar rats (Student's *t*-test).

During the period of follicle reconstitution, there was scarcely any spontaneous and inducible uptake of [ $^3$ H]TdR (<100 c.p.m.). Preliminary experiments established a time-dependent effect of the bTSH-induced [ $^3$ H]TdR uptake in thyroid follicles from BB-DP and Wistar rats after reconstitution time. Maximum bTSH-induced [ $^3$ H]TdR uptake was obtained in cultures of 24 h duration. Exposure to bTSH increased the [ $^3$ H]TdR uptake in a dose-dependent manner in thyroid follicles from both strains, although optimal concentrations differed between the two strains (Fig. 2). Figure 2 also shows that the *in vitro* proliferative responses of BB-DP and Wistar

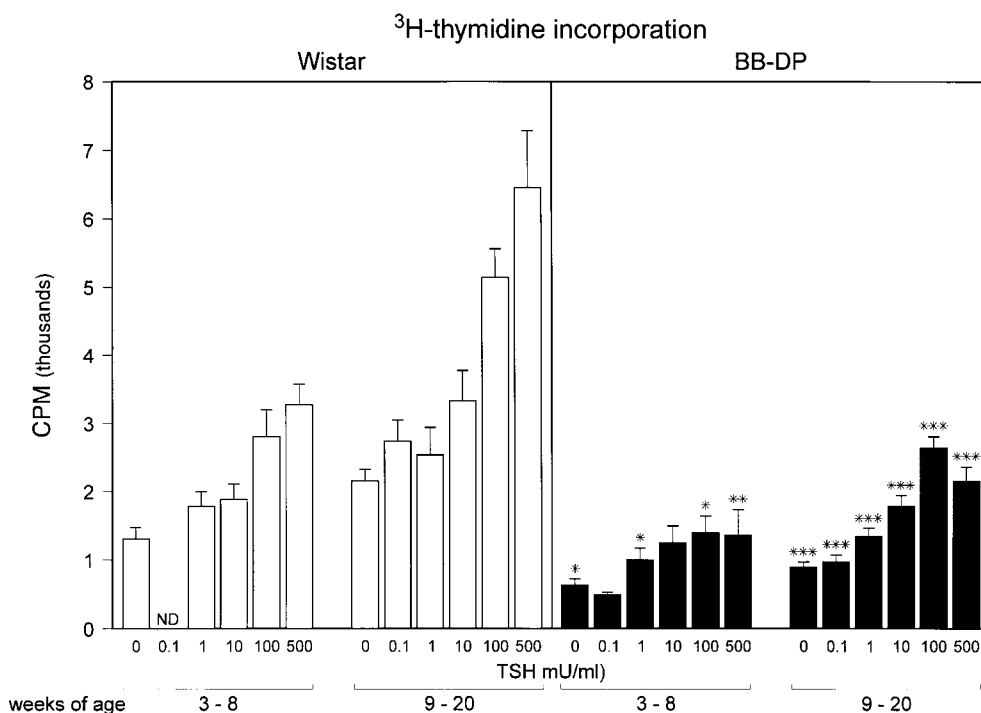
follicles differed: BB-DP rat follicles showed a clearly lower [ $^3$ H]TdR uptake than that in Wistar rat follicles. This lower uptake was found regardless of the times of sampling: BB-DP thyroid follicles isolated before or during hyperplastic goitre development both showed a decreased uptake. In view of the discrepancy between our *in vivo* and *in vitro* data, the specificity of the [ $^3$ H]TdR uptake with regard to DNA replication was demonstrated with aphidicolin and X-irradiation treatment. Both antiproliferative treatments reduced >80% (five separate experiments) of the total [ $^3$ H]-radioactivity in Wistar and BB-DP follicles when added to the cultures. This reduction could not be ascribed to cytotoxicity of aphidicolin or X-irradiation: none of the follicles showed an increased trypan blue uptake or altered morphology, and furthermore, production of  $T_3$  remained unchanged (data not shown).

The data from BrdU labelling were compatible with those from [ $^3$ H]TdR uptake. The percentages of BrdU $^+$  follicles (with and without bTSH) were again significantly greater in Wistar rats than in BB-DP follicles of young rats (3–8 weeks of age): without bTSH stimulation there were  $21 \pm 2\%$  and  $13 \pm 2\%$  positive follicles respectively ( $P < 0.05$ ;  $n=6$ ), and after bTSH stimulation there were  $43 \pm 4\%$  and  $27 \pm 3\%$  ( $P < 0.01$ ;  $n=6$ ). There was also no age-dependency with regard to this difference in BrdU uptake (pre- and hyperplastic periods). During the hyperplastic period (9–20 weeks of age), BrdU uptake in Wistar and BB-DP rats respectively was: non-bTSH stimulated,  $26 \pm 3\%$  and  $16 \pm 2\%$  ( $P < 0.05$ ;  $n=8$ ); bTSH stimulated,  $61 \pm 4\%$  and  $39 \pm 3\%$  ( $P < 0.005$ ;  $n=8$ ). After bTSH stimulation, Wistar follicles often demonstrated 10–20 BrdU $^+$  nuclei per follicle, whereas BB-DP follicles never exceeded 5–10 BrdU $^+$  nuclei per follicle.

To study further whether the discrepancy between our *in vivo* (goitre) and *in vitro* (low proliferation) data could be attributable to a different response of BB-DP and Wistar thyroid follicles towards other growth factors contaminating the bTSH preparation, we performed additional experiments with rhTSH with a purity greater than 99%. We obtained data similar to those with bTSH, namely a lower [ $^3$ H]TdR uptake in BB-DP thyroid follicles than in Wistar follicles.

#### *Production of thyroid hormones and thyroglobulin by in vitro cultured rat thyroid follicles*

For both the Wistar and the BB-DP follicles, 100 mU/ml bTSH appeared to be the optimal concentration for the measurement both of hormone secretion and of cytokine production. Addition of 6-propyl-2-thiouracil (Sigma; 2 mM) during the culture period inhibited basal and bTSH-induced hormonal levels in the culture supernatants (data not shown), indicating that the hormones assayed in the supernatant were mainly produced by the cells during the culture period rather than released via non-specific mechanisms.



**Figure 2** Uptake of [ $^3\text{H}$ ]TdR by cultured BB-DP (■) and Wistar (□) rat thyroid follicles (1000/well) in the absence or presence of various dosages of bTSH. Follicles were isolated from two age groups: 3–8 weeks and 9–20 weeks of age. Means ( $n \geq 6$ )  $\pm$  S.E.M. are shown. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with Wistar (age-matched groups; Wilcoxon's rank sum test).

**Table 1** *In vitro* production of  $T_3$ ,  $T_4$ , IL-6 and thyroglobulin (Tg) by BB-DP and Wistar rat thyroid follicles. Values are means  $\pm$  S.E.M.

	3–8 weeks of age				9–20 weeks of age			
	BB-DP rat	<i>n</i>	Wistar rat	<i>n</i>	BB-DP rat	<i>n</i>	Wistar rat	<i>n</i>
<b>Unstimulated</b>								
$T_3$ (fmol/2000 follicles)	188 $\pm$ 15**	10	96 $\pm$ 16	10	199 $\pm$ 20	27	231 $\pm$ 24	14
$T_4$ (fmol/2000 follicles)	890 $\pm$ 87*	10	1378 $\pm$ 171	10	1518 $\pm$ 209*	9	854 $\pm$ 113	8
Ratio $T_3/T_4$	0.22 $\pm$ 0.02***	10	0.08 $\pm$ 0.01	10	0.21 $\pm$ 0.05*	9	0.33 $\pm$ 0.06	7
Tg (ng/2000 follicles)	2638 $\pm$ 229	3	2884 $\pm$ 390	5	2935 $\pm$ 640	6	3128 $\pm$ 352	6
IL-6 (units/2000 follicles)	75 $\pm$ 19	8	40 $\pm$ 15	4	104 $\pm$ 41	3	63 $\pm$ 15	6
<b>TSH (100 mU/ml)-stimulated</b>								
$T_3$ (fmol/2000 follicles)	268 $\pm$ 28*	10	163 $\pm$ 26	10	335 $\pm$ 35	27	312 $\pm$ 31	14
$T_4$ (fmol/2000 follicles)	1262 $\pm$ 154	10	1771 $\pm$ 239	10	2130 $\pm$ 244**	9	968 $\pm$ 142	8
Ratio $T_3/T_4$	0.22 $\pm$ 0.02**	10	0.10 $\pm$ 0.02	10	0.26 $\pm$ 0.05	9	0.37 $\pm$ 0.08	7
Tg (ng/2000 follicles)	2675 $\pm$ 62*	3	3708 $\pm$ 291	5	3425 $\pm$ 626	6	3349 $\pm$ 416	6
IL-6 (units/2000 follicles)	232 $\pm$ 53	7	154 $\pm$ 53	4	477 $\pm$ 99*	3	190 $\pm$ 35	6

*n*, Number of experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with age group-matched Wistar rats (Wilcoxon's rank sum test).

BB-DP follicles isolated at young age (3–8 weeks of age; pre-hyperplastic stage) showed a significantly lower basal  $T_4$  secretion compared with age-matched Wistar follicles (Table 1). The  $T_3$  output, however, was significantly greater in the BB-DP follicles (both basal and bTSH-stimulated, Table 1), resulting in a relatively high  $T_3/T_4$  ratio in very young BB-DP rats compared with Wistar

rats. The bTSH-stimulated production of thyroglobulin from BB-DP follicles was less than that in Wistar follicles (Table 1).

BB-DP follicles isolated from rats of 9 weeks of age and older (hyperplastic stage) demonstrated *in vitro* a clear increase in  $T_4$  production compared with BB-DP follicles isolated from younger rats (Table 1). This increased

production after 9 weeks of age was also significantly enhanced (both basal and bTSH-stimulated) compared with that in Wistar follicles of the same age group. Production of T<sub>3</sub> and thyroglobulin did not differ between follicles of BB-DP and Wistar rats.

Morphological signs of hypermetabolism of the thyrocytes at the time of *in vitro* enhanced T<sub>4</sub> production were also evident in the BB-DP hyperplastic goitres. Pathologists often use the size of the nuclear area of thyrocytes as a parameter of the metabolic state of the cell, and we also measured this parameter in thyroid sections (Klencki *et al.* 1994, Paschke *et al.* 1995): the nuclear area of thyrocytes was significantly greater in BB-DP rats of 14 and 20 weeks of age than in Wistar rats (Fig. 1c).

#### *Production and expression of IL-6, MCP-1, TNF- $\alpha$ and ICAM-1*

On cryostat sections, thyrocytes of BB-DP rats in the period before hyperplastic goitre development (before 9 weeks of age) and of Wistar rats were seen to be stained with anti-IL-6 antibodies. The immunohistochemical technique used did not allow a reliable quantification of this cytokine. No staining was observed with antibodies against MCP-1 and TNF- $\alpha$ . ICAM-1 expression was modest and restricted to some large venules in sections of thyroid glands of both the BB-DP and Wistar rat.

In the period during hyperplastic goitre development (from 9 weeks of age onwards), thyrocytes of BB-DP and Wistar rats were again positively stained for IL-6. TNF- $\alpha$  and MCP-1 were detected at this time, but only when infiltrates of lymphoid cells were present in the thyroid glands of the BB-DP rats (aged 18 weeks and over). In these cases, cytokine expression was restricted to the areas of leucocytic infiltration, and in these areas a high expression of ICAM-1 was also observed on blood vessels and leucocytes. Thyrocytes were negative.

IL-6 was clearly detectable in the culture supernatants of rat thyroid follicles. Before 9 weeks of age, there was no difference with regard to IL-6 production between BB-DP and Wistar thyroid follicles (Table 1). However, BB-DP follicles isolated after 9 weeks of age produced significantly more IL-6 than Wistar follicles, when stimulated with bTSH (Table 1).

Release of MCP-1 was highly variably, ranging from 0 to 580 pg/2000 follicles, and no difference was found between BB-DP rat and Wistar rat thyroid follicles. Furthermore, MCP-1 release was not upregulated by bTSH.

TNF- $\alpha$  was not produced by BB-DP or Wistar thyroid follicles at any age.

#### **Discussion**

Our data show that BB-DP rats develop a small goitre with histomorphological signs of hyperplasia and hyperfunction

between the age of 9–12 weeks. Our *in vitro* findings of a lower proliferation rate of 'hyperplastic' BB-DP thyrocytes kept in follicle culture, compared with that in Wistar thyrocytes, are thus puzzling. There are a number of possible explanations for the discrepancy between the *in vivo* and *in vitro* data. First, the inclusion of FCS in our culture system may have exposed the thyrocytes to uncharacterized growth factors (or growth inhibitors); our experiments therefore cannot exclude differences in sensitivity for such factors between the two different strains of rats. Secondly, the [<sup>3</sup>H]TdR uptake into *in vitro* cultured thyrocytes may not reflect true cell proliferation (Maurer 1981). However, our experiments using aphidicolin, X-irradiation and BrdU labelling indicate that our data represent valid measurement of such proliferation. If valid, these data underline once more the danger of extrapolating *in vitro* data to the *in vivo* situation. *In vivo* goitre development is, indeed, far more complex than *in vitro* thyrocyte proliferation. First, goitre development is influenced by growth factors produced by cells other than thyrocytes – that is, by stromal cells normally present in the vicinity of the thyrocytes, such as fibroblasts and endothelial cells (Bechtner *et al.* 1993). Such cells were virtually absent from our *in vitro* follicle culture system. It is known also that the addition of matrix factors to thyroid follicle cultures greatly alters the proliferative rate of the thyrocytes (Toda *et al.* 1995). Secondly, goitre development is not only the outcome of thyrocyte proliferation, but also influenced by the rate of apoptosis of thyrocytes. Therefore, further experiments, such as *in vivo* BrdU labelling and the use of apoptosis markers, are needed, to shed more light on the discrepancy we have described here.

Regardless of the need to explore that discrepancy further, there is one striking similarity of our *in vitro* observations with those made in the OS chicken: a decreased *in vitro* growth rate of thyrocytes in the pre-autoimmune stage (Truden *et al.* 1983). It has been suggested that this abnormal, intrinsic, low proliferative rate of OS chicken thyrocytes reflected an inborn derangement of function (Truden *et al.* 1983) – that is, a defect in the handling of iodine (fewer I atoms were found to be built into the chicken thyroglobulin; Sundick 1989). Other pre-autoimmune thyroid abnormalities found in the OS chicken included TSH-independent hyperfunction, such as a high oxidative metabolism, a high iodine uptake and an incomplete suppression of this uptake (Sundick 1989). These signs of hypermetabolism were suggested to be mechanisms compensating for the iodine-handling defect.

It is tempting to speculate that the hyperplastic goitre development and the relatively high follicular T<sub>4</sub> output of our BB-DP rats of 9 weeks and older also represent compensatory mechanisms for the postulated mild defect in the handling of iodine. Evidence for such a mild defect (a low iodine/thyroglobulin ratio, and an abnormal, incomplete Wolff–Chaikoff effect) has been reported by Li & Boyages (1994). We have observed that young BB-DP

rats are extremely sensitive to perchlorate discharge and respond to low iodine diets with an extremely low plasma concentration of  $T_4$  (Mooij *et al.* 1992, Mooij 1993). However, the existence of a mild defect in iodine organification in BB-DP rats has also been disputed (Rajatanivan *et al.* 1983), and clearly more investigations are needed in the very young BB-DP rat (less than 9 weeks old) to establish or refute such metabolic abnormality and its compensations. In patients with Hashimoto's thyroiditis, however, organification defects have been well described (Takeuchi *et al.* 1970).

Whatever the reasons may be for the small goitre development in the BB-DP rat, we believe that the dendritic cells accumulate in such hyperplastic thyroids to serve a function: the regulation of the abnormal growth rate and metabolism of the thyrocytes (see Introduction).

What factors and cytokines are attracting these dendritic cells to the BB-DP rat thyroid? Histologically, we could not find a difference in ICAM-1 expression between BB-DP and Wistar thyroid endothelial cells in the pre-thyroiditis phase. However, after the development of focal thyroiditis in the BB-DP rat, ICAM-1 became clearly expressed on the endothelial cells in the thyroiditis areas, suggesting a role of this adhesion molecule late in the autoimmune process.

MCP-1 was produced *in vitro* by thyroid follicles, but in variable quantities, and there was no difference between BB-DP and Wistar thyroid follicles. Low levels of MCP-1 have also been observed in primary cultures of human thyrocytes isolated from patients with Graves' disease. Only after stimulation with cytokines such as IL-1- $\alpha$ , TNF- $\alpha$  and, to a lesser extent, IFN- $\gamma$ , were significant amounts of MCP-1 detected (Kasai *et al.* 1996).

BB-DP thyroid follicles isolated in the hyperplastic goitre phase did, however, produce more IL-6 than Wistar thyrocytes. Contaminating leucocytes could not have been the source of this IL-6, as such contaminating cells were not present in our culture system. *In vitro* IL-6 production by thyrocytes has been reported before. It is stimulated by TSH (see also this report), IL-1- $\alpha$  and epidermal growth factor (Hirano *et al.* 1990, Bartalena *et al.* 1995). IL-6 has a wide variety of activities: it is involved in the regulation of the immune response by an enhancement of T cell proliferation, a stimulation of B cells to produce antibodies and an enhancement of the activity of GM-CSF (Hirano *et al.* 1990). It also has a role in the transition of monocytes to dendritic cells (Mooij *et al.* 1994, Peters *et al.* 1996), hence a role of this cytokine in the intrathyroid accumulation of dendritic cells in BB-DP rats is likely. IL-6 also has a role in the stimulation of growth of thyrocytes (Nishiyama *et al.* 1993, Shimomura *et al.* 1994), and it is thus intriguing that the BB-DP thyrocytes, although having a low *in vitro* proliferative rate, produced more IL-6 (see above).

With regard to TNF- $\alpha$ , we were unable to detect, either *in situ* or *in vitro* (culture of isolated follicles), a noteworthy production of TNF- $\alpha$  by thyrocytes at a

stage at which lymphocytes had not yet infiltrated the thyroid.

With regard to yet other factors that play a part in accumulation of dendritic cells, namely autoantigens, we found no noteworthy difference between the production of thyroglobulin in BB-DP and Wistar rat thyroid follicles kept in culture. This might indicate that the expression of this autoantigen does not play a major part in the increased accumulation of the APCs. However, we cannot exclude the possibility that the degree of iodination of thyroglobulin is important (Sundick 1989), and that other autoantigens, such as thyroperoxidase, are expressed at a higher level in our BB-DP thyrocyte cultures.

In conclusion, our report describes hyperplastic goitre development in the BB-DP rat at a stage when the thyroid autoimmune reaction is initiated. At the same time there was also an increased production of  $T_4$  and IL-6 by the thyrocytes. It is conceivable that these thyroid abnormalities create a micromilieu that favours the attraction of APCs and the initiation of the thyroid autoimmune response.

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