The thymus expresses proinsulin, among many other tissue-specific antigens, and the inheritance of genetically determined low thymic proinsulin expression has been associated with impaired proinsulin-specific autoreactive T-cell tolerance and type 1 diabetes susceptibility. The cellular and molecular biology of proinsulin expression in the thymus remains unknown, and contradictory reports exist regarding the identity of proinsulin-producing cells. Using knock-in mice expressing β-galactosidase (β-Gal) under the control of an endogenous insulin promoter, we found that thymic proinsulin and β-Gal transcripts were detectable at high levels in purified thymic epithelial cells. Immunohistochemical analysis of β-Gal activity showed that most proinsulin expression can be accounted for by rare medullary epithelial cells of the Hassall’s corpuscles. Moreover, flow cytometry analyses of β-Gal-positive cells showed that only 1–3% of all epithelial cells express proinsulin, and this technique will now provide us with a method for isolating the proinsulin-producing cells in mouse thymus. Diabetes 53:354–359, 2004

T-cell reactivity when compared with control animals (8). This corroborates our hypothesis (5) that genetically determined low thymic proinsulin expression levels predispose to human type 1 diabetes through less efficient insulin-specific autoreactive T-cell selection. In addition, it has recently been shown that NOD mice with drastically reduced insulin expression in the thymus present an accelerated type 1 diabetes development (9).

The thymus is a central lymphoid organ responsible for the generation of the T-cell repertoire, during which those T-cells that recognize self-antigens must be eliminated during T-cell development in the thymus by negative selection through clonal deletion (10–13). The importance of negative selection for tolerance to tissue-specific autoantigens such as insulin had, until recently, been in doubt. Such antigens were traditionally thought to be unavailable for presentation in the thymus, and tolerance to them was believed to occur by extrathymic peripheral mechanisms such as anergy, ignorance, or deletion (14–16). Growing evidence suggests that this may not be the case, and an increasing number of proteins previously considered to be strictly tissue-specific can be detected in the thymus. These include hormones, transcription factors, and secreted proteins. Many of the ectopically expressed antigens (proinsulin, thyroglobulin, myelin basic protein, and retinal S-antigen) are associated with organ-specific autoimmune disease (type 1 diabetes, thyroiditis, multiple sclerosis, and uveitis, respectively) (17–19). However, the expression of these self-antigens is limited to a very small subset of thymic cells, and studying how these specific cells result in the deletion of a wide variety of autoreactive T-cells has not been straightforward.

The thymus is anatomically divided into subcapsular, cortical, and medullary compartments. Thymic stroma contains a variety of professional antigen-presenting cells, including bone marrow–derived dendritic cells (DCs), macrophages, B-cells, endoderm-derived cortical epithelial cells, and medullary epithelial cells (mTECs) (20). A striking morphologic feature of the medulla is the presence of Hassall’s corpuscles (HCs), which consist of concentric whorls of stratified keratinizing epithelium and share antigenic properties with ectodermic epithelium (21). Well developed in human thymus, they are small and sparse in murine thymus. The function of HC remains unknown, although recent studies in human thymus have demonstrated that they are the only regions in the medulla to contain apoptotic T-cells (22). In addition to this, the mTECs that make up the corpuscles have been shown to express a leukocyte and hematopoietic attractant, stromal-derived factor-1, and CD30-l, a membrane-associated glycoprotein related to tumor necrosis factor that is in-

From the Endocrine Genetics Laboratory, McGill University Health Center (Montreal Children’s Hospital Research Institute), Montreal, Quebec, Canada. Address correspondence and reprint requests to Constantin Polychronakos, Endocrine Genetics Laboratory, McGill University Health Center (Montreal Children’s Hospital) Research Institute, 2300 Tupper, Office C244, Montreal, PQ, Canada H3H 1P3. E-mail: constantin.polychronakos@mcgill.ca. Received for publication 4 July 2003 and accepted in revised form 22 October 2003. A.A.C. and M.P. contributed equally to this work. β-Gal, β-galactosidase; DC, dendritic cell; mTEC, medullary epithelial cell; HC, Hassall’s corpuscle; TEC, thymic epithelial cell. © 2004 by the American Diabetes Association.
volved in T-cell signaling (23, 24). Moreover, it has also been shown that CD30-deficient mice contain elevated numbers of thymocytes and show a gross defect in negative but not positive selection (25). Together, these results suggest that the HCs are central to negative T-cell selection.

In mice, conflicting reports exist regarding the identity of proinsulin-producing cells in the thymus (19, 26). To provide a definite answer, we used knock-in mice expressing β-galactosidase (β-Gal) under the control of endogenous Ins2 promoter. We show that most of insulin promoter–directed transcription can be accounted for by epithelial cells in HC, which can be isolated by flow cytometry using a fluorescent β-Gal substrate.

**RESEARCH DESIGN AND METHODS**

Ins1-KO and Ins2-KO mice were a gift of Dr. J. Jami (Institut Cochin, Paris, France) and were generated as described by Duvallé et al. (27). Ins2-KO mice have their Ins2 gene replaced by a functional LacZ gene that is under the regulation of the endogenous Ins2 gene promoter, whereas Ins1 was disrupted by introducing a neomycin cassette against the same genetic background. All mice were bred at our animal facility under conditions specified by the Canadian Council of Animal Care.

**RNA preparation and cDNA synthesis.** Pancreatic and thymic RNA from 2- to 4-week-old mice was isolated by Trizol (Gibco, Rockville, MD) and treated with DNase (Gibco). RNA was isolated from enriched thymic cell populations with RNaseasy Mini Kit (Qiagen, Mississauga, Ontario, Canada) with typical yields of RNA between 50 and 400 ng. An equal amount of each RNA (2.5 µg of tissue-extracted and 50–100 ng of single cells or more as with the case of DCs) was reverse-transcribed using random primers and Superscript II Reverse-Transcriptase (Gibco). Parallel samples in which reverse transcriptase was omitted were always included to confirm the absence of DNA contamination.

**Quantitative and semiquantitative RT-PCR.** Insulin expression in the thymus- and pancreas-extracted tissues was analyzed by quantitative RT-PCR. An equal amount of each cDNA sample (2 µl from thymic and 2 µl from 1:300 diluted pancreatic cDNA) was then added to serial dilutions of the insulin competitor (56, 28, 14, 7, 3.5, 1.7, or 0 µmol/tube) or with the cyclophilin competitor (2.5, 1, 0.5, 0.25, 0.1, or 0 × 10−6 copies/tube) for loading control. The insulin competitor is an internally deleted cloned insulin sequence amplified with the same primers but 45 bp shorter, constructed as described by Forster (28). For cyclophilin analysis, we used the PCR Kit for cyclophilin (Quantum Biological Products; 1:300 dilutions for DCs) was then directly FACs sorted with CD11c alone, DEC205 (Serotech, NLC-145) alone, or CD11c+ MHC-II (MRC Pharmingen, Mississauga, Ontario, Canada) with essentially identical results. Thymic epithelial cells (TECs) were isolated according to Klein et al. (30). Briefly, 4- to 5-week-old mice were digested for 30–40 min with Collagenase/Dispase (Sigma; 2 mg/ml each) and DNase (Roche, Mannheim, Germany; 25 µg/ml) in PBS-1% FCS. EDTA was added to a final concentration of 10 mM/ml for the last 5 min. Cells were then separated on a discontinuous Percoll (Amersham Biosciences, Uppsala, Sweden) density gradient. The TEC-enriched fraction of the two upper phases were harvested, washed, and stained with CD11c followed by phycoerythrin-conjugated rabbit anti-rat IgG antibody (Serotech). After 30 min at 4°C, cells were washed with anti-CD45-FITC (Pharmingen) to distinguish epithelial cells from G8.8-positive narrow-derived cells. TECs were sorted according to the phenotype of CD45+ and G8.8+. Blocking with the anti-FcR mAb 2.4G2 (Pharmingen) preceded the staining for DCs, whereas blocking with 1% horse serum was done for the TEC isolation. The average cell yield per thymus was 30,000 TECs.

**β-Gal activity assay/FACS.** Approximately 106 cells from the Percoll gradient isolation (epithelial cells) or from the DC isolation (low-density fraction) were labeled with G8.8 or CD11c antibodies and resuspended in 1 ml of RPMI (10% FCS) with 10 µl of endogenous β-Gal–like activity inhibitor from the C57/6FG kit (ImaGene Green Kit; Molecular Probes, Eugene, OR) for 30 min at 37°C. After this, 0.5 ml of prewarmed C12FDG (15 µmol/l) substrate was added to the cells and incubated for 20 min at 4°C, room temperature, or 37°C. Results from the incubations at all three temperatures were very similar, but the background was significantly lower at 4°C and the results shown were obtained at this temperature. This method was used to compare the percentage of cells positive for both G8.8 (or CD11c) and C12FDG between Ins2-KO mice (β-Gal expressed under the Ins2 promoter) and Ins1-KO mice (no β-Gal transgene). The difference in double-positive cells between the two genotypes was taken to represent cells that truly express transgenic β-Gal. The number, as a percentage of all G8.8+ cells, was much higher (10- to 30-fold higher) in Ins2-KO mice than in Ins1-KO in all four experiments regardless of whether the background, in the absence of C12FDG, was subtracted from each. In CD11c+ cells, no significant difference from background was seen.

**RESULTS**

Proinsulin/β-Gal expression in the thymus and pancreas. In mice, two unlinked, nonallelic, Ins1 and Ins2 genes encode insulin. Ins1 is expressed at two to three times lower than Ins2 in the pancreas and thymus (8, 31, 32). Figure 1A shows proinsulin expression in the thymus of 2- to 4-week-old mice. Proinsulin is expressed two to three times less in the Ins2-KO mice when compared with the Ins1-KO mice, confirming previous results (8). For both mice, the concentration of proinsulin was normalized with cyclophilin (Fig. 1B). As expected, β-Gal...
expression was detected in thymus and pancreas of Ins2-KO but not Ins1-KO mice (Fig. 1C). These results show that proinsulin is abundantly expressed in the thymus of Ins1-KO mice and bioactive β-Gal in the thymus of Ins2-KO mice. These mice were used for the analyses of proinsulin/β-Gal expression in fractionated thymic stromal cells.

**Proinsulin/β-Gal expression in thymic stromal cells.**

To examine proinsulin/β-Gal gene expression in thymic stromal cells, we enriched distinct cell populations to high purity by a combination of density fractionation and cell sorting. DCs were isolated from thymi of 2- to 4-week-old mice using the standard protocol (29) that includes enrichment by adherence to culture dishes after overnight incubation before cell sorting with anti-CD11c antibody. Alternatively, for avoiding possible phenotypic changes during the overnight incubation, possibly comparable to DC maturation in vivo (33), the overnight incubation step was omitted, and the carefully removed low-density cell fraction from the BSA gradient was sorted immediately using an anti-CD11c or anti-DEC205 antibody (Fig. 2A). Both techniques yielded CD11c-positive cells within the expected range of 30,000–70,000 DCs per mouse thymus (29). Without overnight incubation, sorting by CD11c and I-A^b^ (MHC II) antibodies yielded similar results (Fig. 2B). Virtually the entire population of CD11c^+^ cells were also positive for I-A^b^.

Epithelial cells were isolated as described by Klein et al. (30) and enriched by cell sorting using G8.8 and CD45 antibodies. The G8.8^+^ population consists of 40–60% TECs, with the remainder of cells being mostly thymocytes (30,34). Marrow-derived cells were eliminated by selecting for G8.8^−^/CD45^−^/lo providing exclusively medullary and cortical epithelial cells (19,30) (Fig. 2C and D). Such isolations yielded very similar results to Derbinski et al. (19), as we consistently isolated ~30,000 epithelial cells/thymus. G8.8^−^/CD45^−^ and G8.8^−^/CD45^lo^ cell fractions were also isolated (Fig. 2D).

By RT-PCR, strong proinsulin and β-Gal bands were detected in G8.8^−^/CD45^−^/lo TECs from Ins1-KO and Ins2-KO mice, respectively, whereas only a very faint band was detected in the CD11c^+^, DEC205^+^, or CD11c^+^/I-A^b^ DCs from the same mice, isolated without overnight incubation (Fig. 3B, lanes 1–5, and C, lanes 1–4). Even with a two- to threefold greater number of DCs, as compared with TECs, and with a substantially larger amount of RNA used for RT-PCR, controlled for by cyclophilin, only very weak proinsulin and β-Gal bands could be detected within the DC extracts (Fig. 3B, lanes 1, 2, and 4, and C, lanes 1 and 2). Reducing the cDNA quantity in the DCs only reduced the intensity of the proinsulin and β-Gal expression.
bands, ruling out saturation of cDNA as a possible inhibitor of the PCR (Fig. 3B, lanes 1 and 2). Similarly and as expected, the intensity of the proinsulin band increased with epithelial enrichment of the G8.8+ fraction by double sorting (G8.8+/CD45lo; Fig. 3A, lanes 2 and 3). β-Gal was also detectable in G8.8+ and G8.8+/CD45lo cells from Ins2-KO mice (Fig. 3, lanes 6 and 7; note that loading was not normalized for this result). No proinsulin/β-Gal expression was detected in G8.8+/CD45hi cells (Fig. 3D) and from DCs with overnight incubation (Fig. 3A, lanes 1 and 5). Proinsulin was also strongly detected in G8.8+/CD45- epithelial cells even when poor RNA yields did not allow for the detection of cyclophilin (Fig. 3C, lane 4). All of these data demonstrate that in the thymus, proinsulin mRNA transcripts are predominantly detected in TEC.

Proinsulin/β-Gal is produced by rare TECs of the HC.

To be more specific about which population of cells is responsible for insulin gene transcription in the thymus, we localized β-Gal activity in histologic sections. Figure 4 shows histochemistry results of the β-Gal activity on pancreatic and thymic frozen sections of Ins1-KO and Ins2-KO mice. These results show that β-Gal activity is specifically detected in pancreatic islets of Ins2-KO mice but not in Ins1-KO mice (Fig. 4A and B). In the thymus, β-Gal activity is detected in Ins2-KO mice but not in Ins1-KO and was localized in the mTECs forming the concentric structure that resembles HC. Approximately 1 in every 10 HC were positive for β-Gal activity, some of which contained as many as four X-gal–stained cells (Fig. 4C–F). Because our sections represent only a slice of a three-dimensional HC, the true proportion must be higher, and it is indeed possible that every HC contains these cells. Immunohistochemistry co-localized all of the β-Gal–positive cells with G8.8 (Fig. 5B–E) but never with CD11c (DC; Fig. 5A). These data demonstrate that proinsulin-producing cells in the mouse thymus are mTECs of the HC.

Flow cytometry analysis of β-Gal/proinsulin-producing rare TEC. We further localized β-Gal activity in purified populations of thymic stromal cells by flow cytometry using fluorescent β-Gal substrate (C12FDG) and cell type–specific antibodies. The results consistently revealed a population of between 1 and 3% G8.8+/β-Gal–positive cells within the purified G8.8+ thymic epithelial cell extract (TEC) from Ins2-KO mice (Fig. 6B). We could not detect a similar β-Gal–positive population in G8.8+ cells of control Ins1-KO mice (Fig. 6A). In a separate experiment, we incubated sorted G8.8+ cells with C12FDG, under similar conditions as above and observed them by fluorescence microscopy. The results showed a similar proportion of β-Gal–positive cells in the Ins2-KO preparation, consistent with the FACS data (Fig. 6C). The same experiments were done for sorted CD11c+ DCs, and no population of β-Gal–positive DCs was observed in Ins2-KO mice that was above the background levels obtained with DCs from Ins1-KO mice (data not shown).

**DISCUSSION**

Our results show that in mouse thymus, proinsulin expression is limited to a very rare fraction of mTECs forming the HC. Previous studies that attempted to identify which cells

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**FIG. 4. Histochemistry of β-Gal activity on pancreatic and thymic frozen sections.** A and B: Pancreatic β-Gal activity detected in β-cells of Ins2-KO mice but not in Ins1-KO. C and D: Thymic β-Gal activity detected in mTECs (arrows) of HC of Ins2-KO mice but not in Ins1-KO mice. E and F: Zoom of HC cells positive for β-Gal activity. Magnification ×40.

**FIG. 5. Immunohistochemistry of β-Gal activity and thymic stromal cells.** A: Co-localization of β-Gal activity and CD11c (DC). B–E: Co-localization of β-Gal activity and G8.8 (TEC). No staining was observed in Ins1-KO sections (data not shown). Magnification ×40.
are responsible for this proinsulin expression, by RT-PCR, gave contradictory results with expression being detected in DCs (26) or TECs (19) but not both. To resolve this discrepancy and to best characterize the identity of these cells, we used β-Gal as a marker of transcriptional activity of the endogenous Ins2 promoter. Semiquantitative RT-PCR shows that proinsulin/β-Gal is very strongly and easily detected in TECs but only very faintly detected in DCs that are sorted without overnight incubation. Throsby et al. (26) also detected a very faint band in DC extracts, and they required using a radiolabeled probe to see it adequately. This band may be the result of some epithelial cell contamination in the DC extracts, or it might represent expression at much lower levels or much fewer cells among the DCs.

Immunohistolocalization experiments revealed that these thymic cells were confined to the thymic medulla and were part of the HC. The expression of insulin and other ectopic or promiscuous tissue-specific self-antigens by nTECs has been documented extensively by Derbinski et al. (19). The authors, however, did not identify the exact location of the nTECs responsible for the production of insulin or other antigens and their spatial relationship to the rest of the stroma. Our immunohistochemistry of β-Gal activity showed that proinsulin production is a unique property of rare nTECs of the HC.

It has been reported that self-antigen–expressing cells in the thymus, including proinsulin-producing cells, were frequently found in a cluster of two to four positive cells when analyzed ex vivo (35). This may reflect in vivo clustering, as suggested by our observation of clusters of more than one β-gal–positive cell. Rodewald et al. (36) demonstrated that the thymus medulla consists of epithelial cell islets, each derived from a single progenitor. It is not known how this clonality relates to the epigenetic differences that distinguish the rare cells involved in the thymic expression of tissue-restricted antigens from the rest of the TECs. It also is not known whether such expression is the property of a large number of cells, each specializing in a particular antigen, or of a small number of cells that have developed the promiscuous transcriptional machinery to express many diversely regulated antigens.

Our ability to isolate the insulin-producing cells by scaling up the flow cytometry depicted in Fig. 6B will enable us, in studies currently under way, to provide an answer to these questions.

Thymocyte-negative selection occurs mainly in the medulla (37,38), and the HC cells have been associated with apoptotic thymocytes (22). DCs and macrophages have been frequently observed around HC (23,38), and the DCs were shown to be responsible for phagocytosis of apoptotic thymocytes (23). In human thymus, Pugliese et al. (39) found that insulin can be detected in a number of thymic and secondary lymphoid DCs by double immunostaining. Our findings are compatible with the presence of a small number of proinsulin-expressing DCs, but it also is conceivable that these DCs do not express insulin at the transcriptional level but merely take it up for presentation. For tolerizing both CD4+ and CD8+ T-cells, presentation of the self-antigen by major histocompatibility complex class I and II molecules is necessary. It is known that nTECs can present endogenously produced self-antigens by both classes, whereas DCs do not seem to present endogenously produced antigen on class II (40).

If proinsulin expression in thymus medulla is important in negative selection, then how can contact of all or most thymocytes with the few, scattered insulin-producing cells be ensured? This may be the result of chemoattractant properties (29) that drive each thymocyte to scan many HCs. Alternatively or in parallel, other antigen-presenting cells, particularly DCs, may also contribute by taking up secreted antigen for presentation.

We have previously shown that thymic insulin expression levels modulate insulin-specific autoreactive T-cell tolerance even in non–diabetes-prone mice (8). Against the NOD background, the Ins2 knockout mice (with extremely low insulin expression in the thymus but normal levels in the pancreas) develop diabetes with higher incidence and earlier than the wild type (20), confirmed by our own

**FIG. 6.** β-Gal activity in sorted thymic G8.8+ cells. A and B: Percoll-purified TECs were incubated with C12FDG substrate (see RESEARCH DESIGN AND METHODS). A percentage of 1–3% more G8.8β-Gal–positive epithelial cells was consistently observed in the Ins2-KO sorts when compared with the Ins1-KO sorts. Percentages shown are normalized for the percentage of G8.8+ cells in each extraction. No similar increase over the Ins1-KO control was seen in BSA-purified CD11c+ cells (DC). C: G8.8+ TECs were incubated with C12FDG substrate and viewed by fluorescence microscopy. An increased number of β-Gal–positive cells in the Ins2-KO sample as compared with the Ins1-KO sample was observed (data not shown), confirming the FACS data. Magnification ×10.
preliminary observations and those of the group of Dr. G. Eisenbarth; Moriyama et al., personal communication).

We believe that the isolation of proinsulin-producing cells, using the techniques that we have developed here, will allow detailed study of the cellular and molecular biology of proinsulin production, central tolerance mechanisms, and the possibility of designing novel forms of gene or cellular therapy.

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AA. CHENTOUFI, M. PALUMBO, AND C. POLYCHRONAKOS

350