Original Article

Isolation and Characterization of Proinsulin-Producing Medullary Thymic Epithelial Cell Clones

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Proinsulin, like many tissue-specific antigens, is expressed by rare (1–3%) cells of the thymus medullary stroma, presumably for the purpose of self-tolerance. Levels of this expression are associated with type 1 diabetes susceptibility in humans and in the NOD mouse. To further understand the mechanism of central tolerance induction by these rare cells, we have isolated and cultured two proinsulin-producing epithelial cell clones from murine thymus. These cells have a typical epithelial morphology and, by flow cytometry, a surface phenotype representative of mature thymic medullary epithelial cells (G8.8$^+$/UEA-1$^+$/DEC205$^-$/CD45$^-$/MHC II$^+$). By RT-PCR, they express predominantly *Ins2* as opposed to *Ins1*, as does whole thymus. Expression of the transcription factor *Aire*, implicated in enhancing promiscuous thymic expression of tissue-specific antigens, fell to very low levels after a few passages but increased 20-fold upon exposure to an agonistic anti-lymphotoxin B antibody, concurrent with 2.5-fold enhanced insulin expression. RNA of *Pdx-1*, *Glut-2*, and *Gck* was detectable by RT-PCR in whole thymus but not in the clones, suggesting thymic proinsulin expression is *Pdx-1* independent and that *Pdx-1*, *Glut-2*, and *Gck* are likely expressed in the thymus as antigens, not as regulatory molecules. *Diabetes* 55:2595–2601, 2006

The contribution of central tolerance to the prevention of autoimmune disease has acquired increased importance with the finding that many tissue-specific self-antigens are expressed in the thymic medulla (1,2). Proinsulin is one of these self-antigens, and it is also one of the major self-antigens involved in the T-cell–mediated destruction of the pancreatic β-cells that causes type 1 diabetes (3,4). Other antigens include glutamic acid decarboxylase (GAD65 and 67) and the tyrosine phosphatase I-A/2. However, proinsulin is the only type 1 diabetes autoantigen that is exclusively expressed by the β-cell and the only one that maps to a confirmed genetic susceptibility locus (4).

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DC, dendritic cell; FACS, fluorescence-activated cell sorted; FITC, fluorescein isothiocyanate; IFN-γ, γ-interferon; LTBR-Ab, lymphotoxin B receptor antibody; mTEC, medullary thymic epithelial cell; RPE, rhodophyta phycoerythrin; TSA, trichostatin A; UEA, ulex europaeus agglutinin.

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The importance of thymic proinsulin expression first became obvious from observations of tolerance induction to K^b and the SV40 large T-antigen when expressed in the thymus under the insulin 2 promoter, tolerance that was transferable by thymus transplantation (5–7). In addition, reducing proinsulin levels in thymus, while maintaining normal levels in pancreas, increased the number of proinsulin-specific autoreactive T-cells in mice (8,9), an effect also transferable by thymic transplantation (10). Against the NOD background, this genetic manipulation results in a significant acceleration of autoimmune diabetes in females and drastically increased frequency in males (11). These findings corroborate genetic evidence in humans, where a variable number of tandem repeat polymorphism just 5′ to the human proinsulin gene specifically modulates thymic proinsulin levels, and alleles resulting in reduced thymic expression predispose to type 1 diabetes (4,12).

The majority of thymic self-antigens are expressed by medullary thymic epithelial cells (mTECs) in both mice and humans (1,2). We and others have shown that proinsulin RNA can be detected very strongly in mTECs as opposed to other medullary thymic cell populations and also that this expression is limited to 1–3% of all thymic mTECs (1,13). In addition, we localized most of this proinsulin expression to what are believed to be the most mature of all thymic mTECs: those that make up the Hassall corpuscle–like structures in mice (13). Although this has yet to be confirmed using another experimental strategy, there is work demonstrating that self-antigens are produced in the Hassall corpuscles of human thymus (14,15), which was also recently shown to be a site of production of CD4+/CD25+ regulatory T-cells (16). Thymic dendritic cells (DCs), as well as peripheral DCs, have also been reported to be positive for proinsulin/insulin peptides (17), a finding that has not been reproduced in attempts by others (1,2,13,17). Interestingly, recent work demonstrated that although mTECs can produce self-antigens, their processing and presentation of the antigen is limited to CD8+ T-cells and that efficient presentation to CD4+ T-cells requires self-antigen uptake and presentation by DCs (18), which in the Hassall corpuscles, appears to be involved in the generation of T-regulatory cells (16,18).

The rarity of proinsulin-producing mTECs has precluded our detailed study aimed at answering important questions. Do such cells produce all the thymic self-antigens, only proinsulin, or a defined subset of self-antigens in addition to proinsulin? How are proinsulin and the other expressed antigens processed, and what is the role of this processing? Indeed, if a subset of other self-antigens is expressed by these cells, what is the transcriptional mechanism involved and is it related to the

reported chromosomal clustering of the genes encoding those antigens (2,19)? These findings have prompted the proposal that more than one lineage of mTECs exist, each of which produces a subset of all self-antigens localized in close proximity along the same chromosome. Additional questions include what is the transcriptional and translational regulation of proinsulin (and the other self-antigens produced by these cells), and how do these cells develop the capacity to express a single, many, or all of the tissue-specific antigens? Recently, the autoimmune regulator element (*Aire*) has been shown to play a very important role in the expression of proinsulin along with many other, but not all, thymic-expressed tissue-specific antigens (19,20). Mutations in mouse *Aire* abolish or drastically reduce ectopic thymic expression of tissue-specific antigens and give a phenotype similar to mutations in the human orthologue, which results in autoimmune polyendocrinopathy type 1 syndrome in which diabetes is a common feature (20).

Toward answering these questions, we developed a strategy to isolate and culture the proinsulin-producing cells from mouse thymus. Here, we report the generation and basic characterization of two insulin-positive clonal mTEC lines.

RESEARCH DESIGN AND METHODS

INS2KO (C57Bl6/129) mice were a gift from Dr. J. Jami (Institut Cochin, Paris, France) and were generated as described by Duvillié et al. (21) by replacing the *Ins2* gene with a functional *LacZ* copy, regulated by the endogenous *Ins2* promoter. The expression profile of both β-galactosidase and proinsulin (both *Ins1* and *Ins2*) in the thymus and pancreas of these mice has been previously described (8,13). These were crossed with mice homozygous for the Immortomouse transgene, a temperature-sensitive SV40 large T-antigen, which induces proliferation of thymic epithelial cells at 33°C (22–24). At 37°C, these cells revert to a more differentiated phenotype (22–24). Mice carrying this transgene against the C57Bl6 background were purchased from Charles River (Saint Constant, PQ, Canada) and have been previously described by Jat et al. (22). All mice were bred at our animal facility under conditions specified by the Canadian Council of Animal Care. Homozygous *Ins2*KO and Immortomouse animals were crossed to obtain F1 heterozygous Ins2KO/Immortomouse (INS2KO/Imm).

Isolation of thymic epithelial cells. We modified the previously described protocol (1,13) as follows: thymi from three 10-week-old INS2KO/Imm mice were individually extracted, finely minced, resuspended in RPMI (no phenol red, cat. no. 11835-030; Gibco, Rockville, MD), and stirred at room temperature for 10 min to release thymocytes. Following this, individual thymi were digested with collagenase/dispase (125 μg/ml; Sigma, Oakville, ON, Canada) and DNase I (125 μg/ml; Roche, Mannheim, Germany) for two rounds of ~45 min each at 37°C. Cells were then washed with PBS and resuspended in Dulbecco's modified Eagle's medium (Cat-M3861-20 with D-valine; US Biological, Swampscott, MA) to inhibit fibroblast growth and 100 μg/ml γ-interferon (IFN-γ; Roche), 10% FBS, 1× sodium pyruvate (Gibco), 1× nonessential amino acids (Gibco), 50 μmol/l β-mercaptoethanol, and 50 μg/ml gentamicin antibiotic (Gibco) and placed at 33°C. Media were changed every 3–4 days for the first 1–2 weeks, followed by passaging every 3–4 days at 3–4 weeks.

Enrichment by β-galactosidase activity assay (C_{12}FDG). After ~4 weeks in culture, two of three individual lines of extracted cells were treated with the fluorescent β-galactocidase substrate C_{12}FDG (ImaGene Green Kit; Molecular Probes, Eugene, OR) and green fluorescent cells were fluorescence-activated cell sorted (FACS; Vantage FACS; Becton Dickinson, Franklin Lakes, NJ) and recultured. Briefly, cells were immersed, while still in culture adhered to 10-cm plates, in RPMI media (standard RPMI with phenol red) with 10% FBS, and 100 μl endogenous β-gal–like activity inhibitor from the C_{12}FDG kit was added for 30 min at 37°C. Following this, prewarmed C_{12}FDG substrate in RPMI media without FBS was added to a final concentration of 15 μmol/l, and the cells were incubated at 37°C until green fluorescent cells could be observed by microscopy using the enhanced green fluorescent protein filter (Leica MZFLIII; Leica Microsystems, Wetzlar, Germany). Once green fluorescent cells were observed (~1 h), the reaction media was removed and cells were trypsinized washed and incubated with PBS (10 mmol/l EDTA, 1% BSA [fraction V]; Sigma) that included a stop reagent provided with the C_{12}FDG kit. Green fluorescent cells (~20–40% of the cell mix) were then FACS and placed in culture at 33°C with 100 μg/ml IFN-γ (required to maximize expression of the SV40-Tag transgene).

Limiting dilutions and β-galactosidase luminescence assays. After sorted cells were cultured for 1–2 weeks, sorted and nonsorted cells were cultured in flat-bottom 96-well plates at 33°C with IFN-γ, diluted to one cell per well (0.1 ml). Colonies with clear epithelial morphology were identified, trypsinized, and grown in 24-well plates. It should be noted that although we continuously cultured our cells in D-valine–containing media, a number of cells with fibroblast morphology were observed in those limiting dilutions. The selected colonies were than analyzed for β-galactosidase luminescence using the Galacto-Star Kit (Tropix; PE Biosystems, Bedford, MA). Cells from the 24-well plates were trypsinized, and an equivalent number of cells (~2×10^5) from each plate were lysed in 200 μl freshly made lysis buffer (0.5% NP40, 0.1 mol/l Tris HCl, pH 7.9, and 0.01 mol/l dithiothreitol). Galacton-Star substrate was added (1/50) to 100 μl of reaction buffer (100 μmol/l NaH_2PO_4, 1 μmol/l $MgCl_2$, and 5% sapphire enhancer; Tropix; PE Biosystems) in a nontranslucent 96-well assay plate (Corning, NY) followed by the addition of 20–30 μl cell extract to the wells. All reactions were done in triplicate. Luminescence readings were read at 30 and 60 min in an EG&G Berthold Microplate Luminometer, LB96V (Bad Wildbad, Germany). A total of 4 colonies with reproducibly higher than average luminescence were selected from one of the enriched cultures and 6 from the other, for a total of 10. As negative controls, we selected five colonies with background luminescence values, taken from the nonenriched plate. The difference in luminescence values between the positive colonies and the average colonies was two- to fourfold.

RNA preparation, cDNA synthesis, and RT-PCR. RNA was extracted using the RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada) and treated with Ambion DNase I (Austin, TX). RNA (~2 μg) was reverse transcribed using Random Primers (Gibco) and Superscript II Reverse Transcriptase (Gibco), and parallel samples in which reverse transcriptase was omitted were always included to confirm the absence of DNA contamination. Primer sequences, conditions, reagents, and amount of DNase I–treated RNA used for each of the PCRs are shown in Table 1. RT-PCR for insulin and cyclophilin was performed on RNA extracted from cells cultured at either 33 or 37°C, with or without IFN-γ.

FACS analysis. Antibodies used to characterize the surface-marker phenotype of our cells were rat IgG_{2a} G8.8 (anti-gp40, mouse homologue of human epithelial cellular adhesion molecule; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) with goat anti-rat IgG rhodophyta phycoerythrin (RPE) (Serotech, Raleigh, NC), rat anti-mouse DEC205 fluorescein isothiocyanate (FITC; Serotec), mouse anti-mouse I-A^b FITC or RPE (Pharmingen, Mississauga, ON, Canada), and mouse anti-mouse CD45.2 FITC (Pharmingen). Cells were washed with PBS and then incubated with PBS and 10 mmol/l EDTA for ~3 min. Cells were then resuspended (10^6 cells/ml) in PBS, 5 mmol/l EDTA, and 1% FCS (fraction V), and ~10^6 cells were incubated with the appropriate antibody. Generally, a 1:200 dilution was used for G8.8, whereas 1:10 to 1:20 dilutions were used for all other antibodies. Isotype controls were used at the same concentrations and included RPE-conjugated rat IgG_{2a} (Pharmingen) and FITC-conjugated mouse IgG_{2a} (Pharmingen). Fc blocking was done using rat anti-mouse CD16/CD32 (Pharmingen) for all reactions except when goat anti-rat IgG was used as a secondary antibody; horse serum was added to these samples instead. Similarly, 10^6 cells were incubated with a 1:10 dilution of fluorescein ulex europaeus agglutinin I (UEA-1 [an mTEC marker]; Vector Laboratories, Burlingame, CA). In some instances, cells were fixed with 1% formalin in PBS, 5 mmol/l EDTA, and 1% FCS (fraction V) if FACS analysis could not be done immediately.

Treatment to reactivate *Aire* expression. The mTEC clones were incubated with 100 nmol/l of trichostatin A (A.G. Scientific) for 24 h. After the incubation, rat anti-mouse lymphotoxin β-receptor antibody (IgG2a azide-free antibody; Serotec) was added to the cells at a concentration of 1 μg/ml for 6 h.

Real-time PCR was performed on 200 ng of cDNA, generated using random primers, both for *Aire* and insulin gene expression, and normalized to 18s rRNA gene expression using TaqMan Gene Expression assay kits for all genes (Applied Biosystems).

RESULTS

Isolation and culturing of thymic proinsulin-producing epithelial cells. As described in RESEARCH DESIGN AND METHODS, two of three plates of thymic cells cultured from INS2KO/Imm mice were subjected to an enrichment step using FACS with C_{12}FDG. In our previous study (13), using a shorter incubation time, we were able to identify and isolate the β-gal/proinsulin–producing cells to quite high purity (1–3% of the entire epithelial cell population). To

TABLE 1
Primers and PCR conditions

Gene	Sense primer	Antisense primer	Conditions	Product size (bp)
Ins1/2	GGCTTCTTCTACACACCCA	CAGTAGTTCTCCAGCTGGTA	1*	181
Ins1	CCAGCTATAATCAGAGACCA	GTGTAAAAAAAGCCACGCT	2†	197
Ins2	TCCGCTACAATCAAAAACCAT	GCTGGGTAGTGGTGGGTCTA	3	411
Pdx-1	TCGCTGGGATCACTGGAGCA	GGTTCCGCTGTGTAAGCACC	2	275
Glut-2	GAGCCAAGGACCCCGTCCTA	GTGAAGACCAGGACCACCCC	2	150
Gck	TGGATGACAGAGCCAGGATGG	ACTTCTGAGCCTTCTGGGGTG	3†	208
Aire	ACACTGCTGGCCCACTTTCTG	ACGGAATTCAGACCATGGCAG	2	298
Cd80	GAAACCCATCTGCAGACACTA	GACAACGATGACGACGACTG	4*	338
Cyclophilin	See ref. 10			

Conditions: 1: 94°C 2 min 30 s, 94°C 1 min, 60°C 1 min, 72°C 1 min 50 s 32×; 2: 95°C 10 min 1×, 97°C 30 s, 64°C 30 s, 72°C 30 s 5×, 95°C 30 s, 64°C 30 s, 72°C 30 s 45×, 72°C 6 min 1×; 3: 95°C 10 min 1×, 97°C 30 s, 62°C 30 s, 72°C 30 s 5×, 95°C 30 s, 62°C 30 s, 72°C 30 s 30×, 72°C 6 min 1×; and 4: 95°C 10 min 1×, 97°C 30 s, 61.5°C 30 s, 72°C 30 s 5×, 95°C 30 s, 61.5°C 30 s, 72°C 30 s 30×, 72°C 6 min. *0.1 units RedTaq (Sigma) was used for *Ins1/2*; regular Amplitaq was used for *CD80*. †60 ng of DNASE1-treated RNA used for PCR as opposed to 200 ng of treated RNA for all other PCRs and 15 μl as opposed to 25 μl final reaction volume used. Reagent concentrations in final reaction mix: 2 mmol/l $MgCl_2$, 1× PCR buffer, 0.2 mmol/l dNTPs, 0.1 units Amplitag Gold, and 1 μ of each primer. $MgCl_2$, 10× PCR buffer, and Amplitaq Gold are from Applied Biosystems (Foster City, CA) cat. no. N808-0249. All primers obtained from Alpha DNA (Montreal, Quebec, Canada).

maximize yield, at the expense of specificity, we extended the incubation time to 1 h, at which point 20–40% of the cells in culture were fluorescent green. This represents a two- to fourfold enrichment of proinsulin-producing cells in preparation for limiting dilution.

Over 1,000 limiting dilutions were prepared from both enriched and nonenriched mTEC cultures. Once growing in 96 well plates, ~25 epithelial colonies were chosen from each of the enriched cultures and ~10 were chosen from the nonenriched cultures. A total of 10 colonies from the enriched cells had β-gal luminescence values above the average signal for all colonies combined (two- to fourfold above the average). All 10 of these colonies, along with 5 colonies with average luminescence values from the nonenriched plate, were analyzed for proinsulin by RT-PCR. Two were found to be positive for proinsulin (PCR primers designed to detect both *Ins1* and *Ins2*); one positive colony was obtained from each of two enriched cultures indicating that they were separate colonies and not clonal (Fig. 1). Figure 2 shows RT-PCR proinsulin results of two negative colonies and one positive colony after 45 passages in triplicate with cyclophilin as a loading control. Similar results were obtained whether the cells were cultured in the presence of IFN-γ at 37°C for 3 days before RNA isolation or in the complete absence of IFN-γ at 37°C. Assuming a twofold enrichment in proinsulin-producing cells by C_{12}FDG-FACS, the percentage of proinsulin-producing cells in our cultures was 2 of 50 (~4%) and thus corresponds reasonably well with what was determined to be the actual percentage in vivo (~2%) (1,13). Of note is that the levels of proinsulin detected by RT-PCR in the cultured cells seems to be lower than what can be detected in the whole thymus when equal RNA amounts are used (data not shown). This is related to *Aire* expression (see below) and is probably attributable to loss of some endogenous stimulator. Identification of such factor(s) will be the subject of future research.

FACS analysis. Typical mTEC surface phenotype by FACS can be defined as $G8.8^+$ (25,26), $CD45^{-/lo}$ (1), $DEC205^-$ (27,28), UEA-1^+ (29,30), and MHC II^+ (24,30), with the UEA-1^+ phenotype believed to be restricted to more mature mTECs (31). No changes in surface phenotype were observed with changes in temperature (33 vs. 37°C), but I-A^b (MHC II) levels were markedly upregulated in the presence of IFN-γ as has been previously shown (24) (Fig. 3). The phenotype of two non–proinsulin-producing colonies and both proinsulin-producing colonies were found to conform to all these criteria (Fig. 3). Of note is that all colonies initially analyzed were $G8.8^+$, but at ~30 passages, the G8.8 positive phenotype was lost. This was assumed to be the result of cell culturing, no longer requiring adhesion molecules. Also to note is that previous studies have shown that MHC II expression is undetectable in the absence of IFN-γ (23,24), but FACS analysis with our colonies cultured without IFN-γ for >5 weeks still demonstrated a population of MHC II–positive cells (Fig. 3). Figure 4 is a phase contrast image of the proinsulin-positive and -negative colonies demonstrating a cuboidal, cobblestone epithelial morphology.

RT-PCR analysis for expression of *Ins1*, *Ins2*, and promiscuous expression markers. Unlike the murine pancreas, the murine thymus expresses very low levels of *Ins1* compared with *Ins2* (8,32). To determine whether

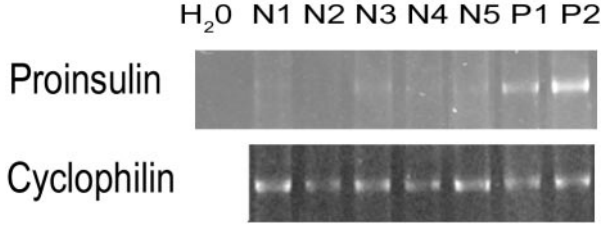

FIG. 1. RT-PCR for proinsulin (detecting both 1 and 2) in cell colonies grown at 33°C with IFN-γ. All colonies shown here had above average β-galactosidase luminescence values (two- to fourfold above background). N, negative for proinsulin; P, positive for proinsulin.

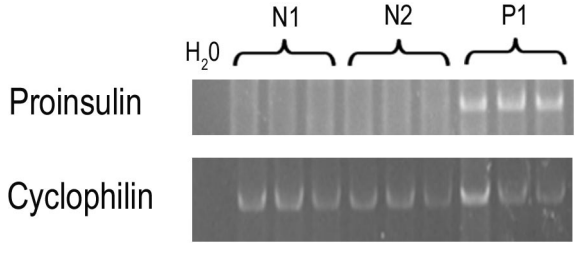

FIG. 2. RT-PCR for proinsulin (detecting both 1 and 2) in cell colonies grown at 33°C without IFN-γ and placed for 3 days at 37°C without IFN-γ before RNA was extracted. All reactions done in triplicate for both negatives and one of the positives; the other positive was also tested and gave the same results (data not shown). N, negative for proinsulin; P, positive for proinsulin.

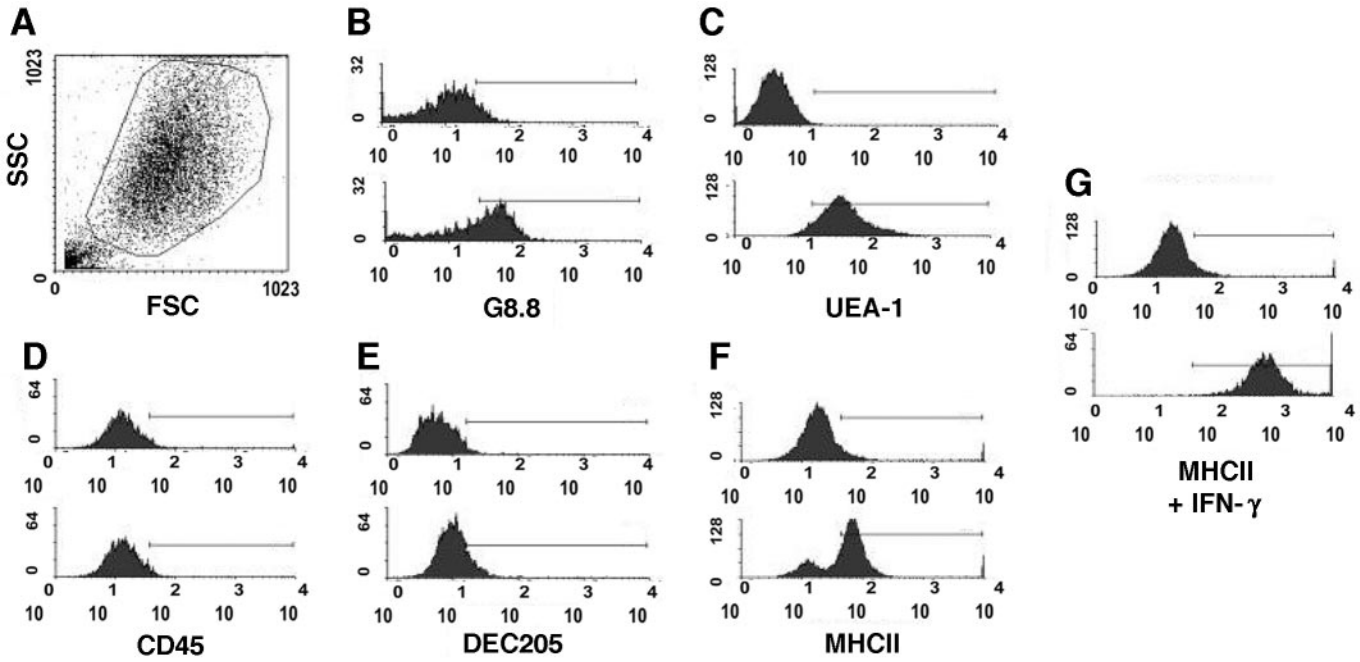

FIG. 3. FACS analysis results for one of the proinsulin-positive colonies. Identical results were obtained for the other proinsulin-positive clone and the two proinsulin-negative clones. *A*: A representative forward versus side-scatter plot indicating the gated population. The pattern was identical in all cell aliquots used in this experiment. *B*: G8.8 labeling. *C*: UEA-1 labeling. *D*: CD45 labeling. *E*: DEC205 labeling. *F*: MHC II labeling without IFN-γ. *G*: MHC II labeling with IFN-γ. There are two histograms for every analysis: the top one is the negative (UEA-1 labeling) or isotype control (all the rest), while the bottom one is with antibody or lectin (UEA-1). UEA-1 specificity was assayed by incubating 10^6 Jurkat cells with the same concentration of UEA-1 lectin used for the epithelial cells. No positive cells were observed (data not shown). No differences in signal were observed with cells incubated at 33 or 37°C with or without IFN-γ except for MHC II, which was upregulated significantly in the presence of IFN-γ, as shown.

this was also the case in our proinsulin-positive mTECs, we assayed for each individually by RT-PCR. As shown in Fig. 5*B* and *C*, *Ins2* is easily detectable in pancreas, thymus, and our proinsulin-positive mTECs. *Ins1*, in contrast, is easily detectable in pancreas but only gives a very faint band in thymus or the proinsulin-positive mTECs.

In addition to being positive for UEA-1 (31), more mature mTECs are also characterized by the expression of CD80 and *Aire*; both of which are expressed only by a subset of the UEA-1^+ mTECs (20,31,33). CD80 was detectable by RT-PCR in both the positive and negative colonies (Fig. 5*A*), but *Aire* was not. *Aire* was detected by RT-PCR when the cells were first placed in culture, but expression was lost quickly with passaging, a phenomenon that has been previously observed (34). *Aire* and CD80 have been associated with expression of tissue-restricted antigens in the murine thymus, including insulin (20,35). However, although *Aire* knockout reduces proinsulin expression significantly, some proinsulin is still detectable in *Aire*-deficient $CD80^{hi}$ mTECs (35). This explains how we could detect insulin in the absence of detectable *Aire* expression.

Induction of *Aire* expression and *Ins2* enhancement with anti-lymphotoxin B antibody. It has been previously reported that treatment with an agonistic antibody against the lymphotoxin B receptor (LTBR-Ab) in the presence of trichostatin A (TSA) restores *Aire* expression by mTECs in culture (34).

Indeed, after LTBR-Ab+TSA treatment, as described in RESEARCH DESIGN AND METHODS, *Aire* mRNA, measured by real-time RT-PCR, increased by 20-fold in the *Ins2*-expressing mTECs. This was accompanied by a 2.5-fold increase in *Ins2* mRNA (average of four separate experiments, $P = 0.01$) (Fig. 6).

Other β-cell–specific genes. In the search of other important proinsulin regulatory molecules, we also examined regulators known to be important in pancreatic β-cells. These were *Pdx-1*, a β-cell–specific transcription factor crucial for pancreas development and insulin expression, *Glut-2* (glucose transporter), and *Gck* (glucoki-

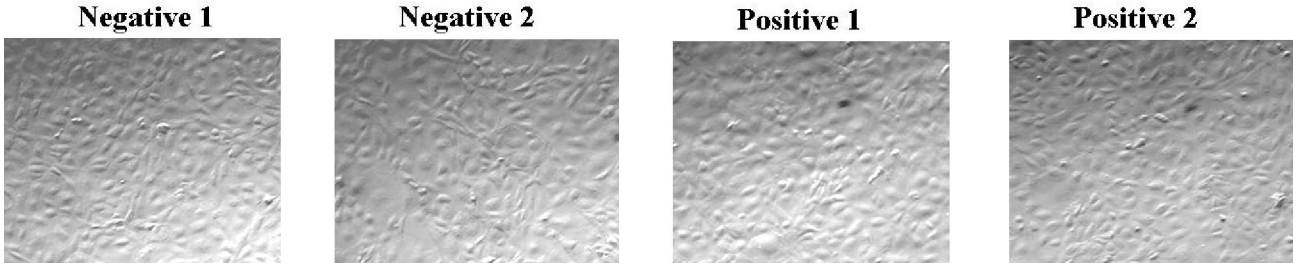

FIG. 4. Phase contrast microscopy of the proinsulin-negative and -positive mTECs demonstrating a cuboidal and "cobblestone" appearance characteristic of epithelial cells.

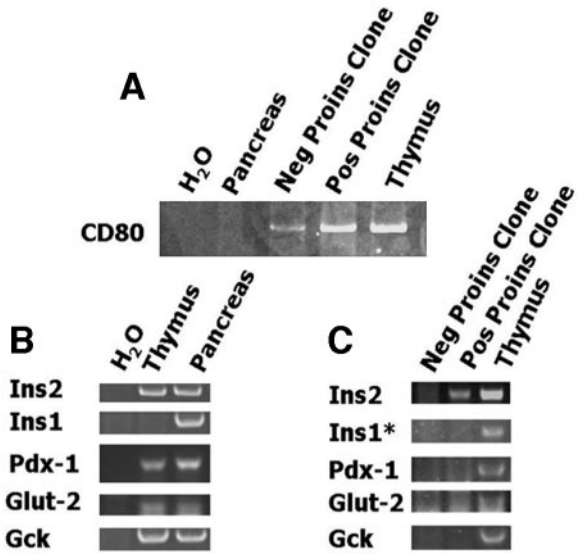

FIG. 5. *A*: RT-PCR for CD80 in INS2KO/Imm pancreas, proinsulin-negative mTEC colony, proinsulin-positive mTEC colony, and INS2KO/Imm thymus. *B*: RT-PCR for *Ins2*, *Ins1*, *Pdx-1*, *Glut-2*, and *Gck* in INS2KO/Imm pancreas and thymus. *C*: RT-PCR for *Ins2*, *Ins1*, *Pdx-1*, *Glut-2*, and *Gck* in the proinsulin-negative and -positive mTEC colonies. Pancreas and thymus RNA was obtained from a fourth 10-week-old INS2KO/Imm mouse. Cyclophilin was used to control for loading (data not shown).

nase), two proteins essential in the sensing of systemic glucose levels and the corresponding β-cell insulin response (36–38).

Interestingly, *Pdx-1*, *Glut-2*, and *Gck* transcripts were all detectable in whole thymus but were not detectable in either the proinsulin- or non–proinsulin-producing mTECs (Fig. 5*B* and *C*). This suggests that these molecules are expressed in the thymus as antigens rather than active insulin regulators.

DISCUSSION

To our knowledge, this is the first time proinsulin-producing mTEC cell lines have been established. Several important pieces of evidence suggest that our proinsulin-producing mTEC cells are representative of their in vivo counterparts in the murine thymus. In addition to having an appearance characteristic of epithelial cells, they also have a surface phenotype characteristic of mTECs with their UEA-1^+ phenotype, suggesting that they are mature mTECs. This mature mTEC phenotype is associated with the expression of Aire and CD80, two proteins that characterize cells that express a number of tissue-restricted antigens, including insulin (35). mTECs from *Aire*-deficient mice show a greatly reduced level of proinsulin expression, consistent with the lower *Ins2* expression in our mTECs compared with fresh whole thymus (which also explains the low β-gal expression we encountered during the cell cloning). As this reduction of *Ins2* expression was partly reversed by inducing Aire expression, we can conclude that loss of Aire in culture is, at least partly, to blame. The reason for such loss is not clear, but absence of stimulation by soluble factors and/or cell-to-cell interactions in the thymus environment is a very plausible explanation. Lymphotoxin, the endogenous LTBR ligand, is a prime candidate in this respect.

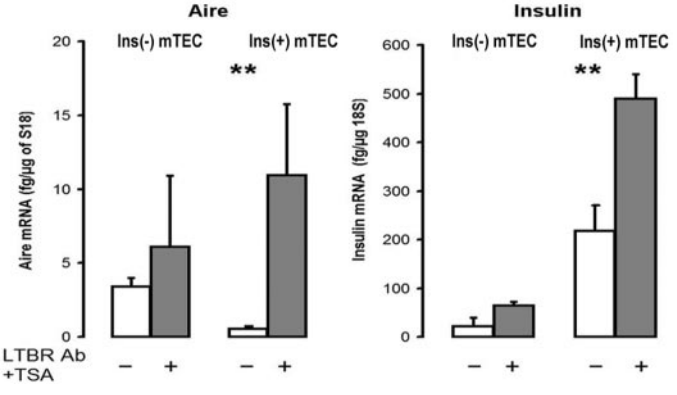

FIG. 6. The effect of preincubation with anti-lymphotoxin B antibody and trichostatin A (LTBR-Ab + TSA) on *Aire* and insulin by medullary thymic epithelial cell clones expressing [Ins(+) mTEC] or not expressing [Ins(-) mTEC] insulin. The means and SEs of seven (*Aire*) or four (insulin) separate preincubations are shown. □, untreated cells; ■, treated cells. **$P \leq 0.01$; otherwise NS.

As a histone deacetylase inhibitor, TSA might turn genes on in a nonspecific fashion. We do not believe this to be an explanation for the effect we saw for several reasons. First, in the absence of LTBR-Ab, no effect was seen. Second, the specificity of our *Ins2*-positive versus *Ins2*-negative mTECs was maintained after LTBR-Ab+TSA treatment, which caused a negligible (in absolute terms) increase in insulin mRNA in the negative clone (Fig. 6). Third, the values shown in Fig. 6 have been normalized for total RNA, using 18S RNA determined by real-time RT-PCR.

The fact that our proinsulin-positive cells express *Ins2* at a much higher level than *Ins1* is also very encouraging evidence that we indeed isolated the true proinsulin-positive mTECs. *Ins1* is expressed at very low levels; indeed, it is sometimes undetectable in thymus, whereas, in pancreas, its expression level is of the same order of magnitude as that of *Ins2*. These findings suggest that the mechanism of proinsulin expression in these cell lines has remained the same as what takes place in thymic mTECs.

Of much interest are the results with regards to *Pdx-1*, *Glut-2*, and *Gck* expression. The expression of these three proteins in the β-cell is crucial for β-cell formation and proinsulin production (*Pdx-1*) and for proinsulin level modulation with regards to peripheral glucose levels (*Glut-2* and *Gck*). Thus, we confirm a previously reported finding that thymic proinsulin expression is *Pdx-1* independent (32). However, our finding of *Pdx-1*, *Glut-2*, and *Gck* in whole thymus, but not in our proinsulin-producing mTECs, also suggests that proinsulin expression in the epithelial cultures and thymus is not *Pdx-1* dependent and that proinsulin expression is unresponsive to systemic glucose levels, consistent with our previous finding of compensatory *Ins1* and *Ins2* upregulation in the pancreas but not in the thymus in heterozygous knockout mice (8). These results also suggest that *Pdx-1*, *Glut-2*, and *Gck* may be expressed in thymus as self-antigens rather than functional regulatory molecules. A previous study (31) failed to detect *Pdx-1* in the thymus, a finding that is probably only a question of sensitivity, as that study used multiplex PCR for detection of several pancreatic transcription factors simultaneously and PCR was limited to 27 cycles.

A considerable amount of work remains to be done to truly characterize these proinsulin-producing mTEC cell lines. Microarray expression profiling will reveal other tissue-restricted antigens coexpressed with insulin along with, perhaps, important regulatory molecules

and transcription factors. Such profiling with or without prior induction of *Aire* will allow us to directly study the importance of *Aire* expression on tissue-restricted antigens and any corresponding transcriptions factors. It will be also interesting to see how our mTEC cultures react to a variety of environmental stimuli in order to further understand their regulation in the thymus. Does the expression profile of the cells change in the presence of cytokines and other regulatory molecules that are likely present in the thymic medulla? These cells will also be used to see whether proinsulin is secreted as intact peptide or directly processed into antigenic epitopes. Preliminary work (not shown) with enzyme-linked immunosorbent assay using monoclonal antibodies against two different regions of the molecule shows very low and inconsistent levels of immunoreactivity, suggesting that the cells do not store or secrete intact protein. We are in the process of developing a polyclonal competitive assay that can detect fragments, in conjuction with high-performance liquid chromatography analysis, to answer the question of whether insulin mRNA is indeed translated in thymus stroma, as is indirectly but strongly supported by recent evidence that the previously reported loss of tolerance to insulin in mice with thymus-specific insulin deficiency (8,9,11) is transferable by thymus transplants (10). Thus, the cell lines we generated can be used to address a number of questions that are very important in understanding the role of the thymus in self-tolerance to tissue-specific antigens, in general, and to diabetes pathogenesis in relation to insulin autoreactivity, in particular.

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