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Temporal Changes in Pancreatic Islet Composition in C57BL/6J-*db/db* (Diabetes) Mice

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Summary. Temporal changes in non-B cell populations were determined during the period of B cell hyperplasia in diabetes-resistant C57BL/6J mice. Pancreases from normal and *db/db* mice between 3 and 20 weeks of age were stained immunocytochemically for glucagon, somatostatin and pancreatic polypeptide (PP), and changes in A, D and PP cell volume densities quantified by image analysis. Further, islet volumes, D cell volumes and actual D cell numbers per islet were determined by analysis of serial sections through entire islets. The volume of *db/db* islets was three- and ten-fold elevated above normal by 8 and 20 weeks, respectively, due mainly to B cell hyperplasia. D cell volume density exhibited a transient increase during the initial phase of B cell hyperplasia, but then showed a gradual reduction; the average number and absolute volume of D cells per islet was comparable in *db/db* and normal islets from older mice. In contrast, PP cell volume density remained stable throughout, suggesting that this cell type kept pace with B cell hyperplasia. A cells showed a reduced volume density throughout and were distinguished from other islet cells which all responded positively to a degree, albeit non-coordinately, to the mitogenic stimulus exerted by *db* gene expression. The finding that A cells shared with certain neuroectodermally-derived cell types a differentially high concentration of *sn*-glycerol-3-phosphate dehydrogenase further underscored the uniqueness of the A cell from other cell types.

Key words: Diabetes mice, *db* gene, pancreatic islets, hyperplasia, morphometric analysis, A, B, and D cells, pancreatic polypeptide cells, *sn*-glycerol-3-phosphate dehydrogenase.

The diabetes (*db*) gene in the mouse leads to obesity associated with hyperinsulinaemia and insulin resistance which may progress to severe diabetes characterized by B cell necrosis, islet atrophy and relative insulinopenia depending upon a complex interaction between the *db* gene, the genetic background and the diet [4, 9, 10]. In two closely related strains, C57BL/6J (BL/6) and C57BL/KsJ (BL/Ks), *db* gene expression produces two markedly different obesity syndromes with diabetes-resistant BL/6 mutants exhibiting only mild, transient hyperglycaemia as opposed to permanent hyperglycaemia and severe diabetes in BL/Ks mutants. The pronounced increase in islet size attributable to B cell hypertrophy and hyperplasia is sustained in BL/6-*db/db* mice but reversed in BL/Ks-*db/db* mice by B cell necrosis and atrophy occurring between 12 and 20 weeks of age [2, 4]. These changes produce marked alterations in the volume densities of constituent cell types which may perturb normal paracrine control of islet hormone secretion [14]. Comparison of B cell to non-B cell populations in 6–16 month mutants showed that non-B cell volume densities increased in BL/Ks-*db/db* mice where B cell mass was decreased, but in BL/6-*db/db* mice non-B cell volume densities were decreased proportionately where B cell hyperplasia is unrestricted by necrosis [2]. In a subsequent morphometric analysis we have confirmed not only the loss of normal topographic arrangements of the non-B cells in BL/Ks-*db/db* islets but also demonstrated an absolute increase in the number of D cells per islet [11]. The increased D cell volume density observed in B cell-depleted islets of older BL/Ks-*db/db* mice [2, 11] versus a decrease in the volume density of B cell-enriched older BL/6-*db/db* mice [2] suggests a reciprocal regulation of B cell versus non-B cell populations. However, since major changes in islet volume occur in the mouse prior to 20 weeks of age, the purpose of this study was to investigate whether non-B cells were regulated in a coordinate manner in BL/6-*db/db* islets during the period of pronounced B cell hyperplasia.

Materials and Methods

Animals

Diabetic BL/6 mice between 3 and 20 weeks of age and their normal littermate controls of both sexes were bred in our research colonies at The Jackson Laboratory and were fed ad libitum on diet 96W (Emory

Morse, Old Guilford, Connecticut, USA) until sacrifice. The littermate control mice represented a mixture of homozygous (+/+) and heterozygous (+/*db*), and thus were designated as +/?.

Procedures

The splenic and mid-body portions of the pancreas were removed from mice killed by cervical dislocation, fixed in Bouin's solution and embedded in paraffin. Serial sections (5 μm thick) were cut from each sample and stained immunocytochemically for somatostatin by the peroxidase-antiperoxidase method of Sternberger et al. [20] as described previously [11]. Briefly, hydrated sections were incubated for 30 min at room temperature with 3% normal goat serum, followed sequentially by incubation with rabbit anti-somatostatin serum (1:5000, lot no. 101, from Dr. A. Arimura, Tulane University Medical School, New Orleans, Louisiana, USA) for 36–48 h at 4 °C, goat anti-rabbit gamma globulin serum (1:100, Miles Biochemicals, Elkhart, Indiana, USA) and rabbit peroxidase-anti-peroxidase (1:50, Polysciences, Warrington, Pennsylvania, USA) for 30 min each at room temperature. All antisera were prepared in a 5 mmol/l Tris/0.15 mol/l NaCl (pH 7.6) containing 1 ml/100 ml normal goat serum. The resulting immune complex was detected with 50 mg/100 ml diaminobenzidine·4HCl and 0.01 ml/100 ml H_2O_2 dissolved in 100 mmol/l phosphate buffer, pH 6.0.

Islets for analysis were chosen randomly with the exception that those which were obviously a product of islet fusion were not analyzed. A total of three to four islets per animal were analyzed, and two to four animals per age and phenotype were used. Individual islets from 8, 10, 12 and 20 week age groups were analyzed in their entirety through the immunocytochemically stained sections. Islet area and D cell area were quantified as described previously [11] employing an Optomax II image analyzer (Optomax, Hollis, New Hampshire, USA). Nucleated D cells were counted in each section, totalled for the entire islet and divided by two to correct for the mean diameter (10 μm) of the D cell nucleus. For each islet analyzed, islet volume, D cell volume (total D cell mass), D cell volume density and the number of D cells per islet were determined to assess the question of D cell hypertrophic and/or hyperplastic changes during the development of the obesity syndrome.

In addition to morphometric analysis of entire islets for alterations in D cell populations, additional sections from each pancreas sampled between 8 and 20 weeks of age, as well as from 3–5 and 6–7 week age groups were chosen at random, and semi-adjacent sections were stained immunocytochemically for glucagon, somatostatin and pancreatic polypeptide employing specific antisera as described previously [11]. The volume density was determined for as many islet profiles as possible within a section stained for a given cell type. Adjacent and semi-adjacent sections were stained for the individual hormones so that the data represent volume density measurements for each hormone within the same islets. Since standard tinctorial or immunocytochemical procedures for staining insulin may lead to inaccuracies due to the partial or complete degranulation of B cells in young BL/6-*db/db* mice, the volume density of B cells (and capillary space) was derived by summing the volume density measurements for A, D and PP cells for each islet profile analyzed and subtracting from 1.

Cytoplasmic *sn*-glycerol-3-phosphate dehydrogenase (GPDH, EC 1.1.1.8) in islets was stained immunocytochemically as described by Fisher et al. [6] employing antiserum to GPDH at a 1:5000 dilution (kindly provided by Dr. L. P. Kozak of The Jackson Laboratory, Bar Harbor, Maine, USA). Controls for specificity of staining included adsorption of the antiserum with excess purified GPDH (also provided by Dr. L. P. Kozak) or with excess porcine glucagon (a gift from Dr. Mary Root, Eli Lilly, Indianapolis, Indiana, USA).

Results

Immunocytochemically stained D cells were localized to the islet periphery in islets of both normal (+/?) and *db/db* mice at all ages analyzed. However, D cell vol-

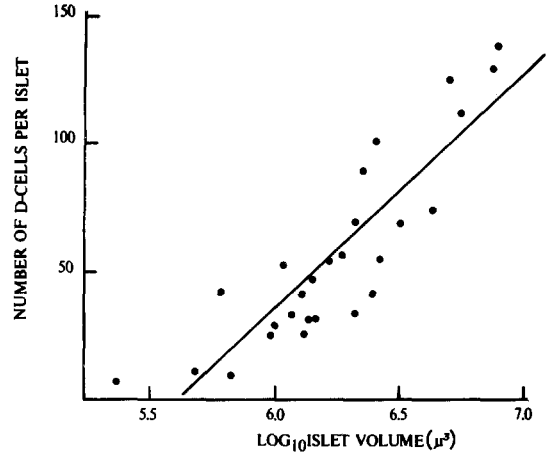

Fig. 1. Regression of D cells per islet against $\log_{10}$ islet volume for control islets pooled from all age groups. $Y = -524.7 \pm 93.2x$; $r = 0.865$. Analysis of variance for the regression line showed $F_{1,26} = 74.3$, $p < 0.001$

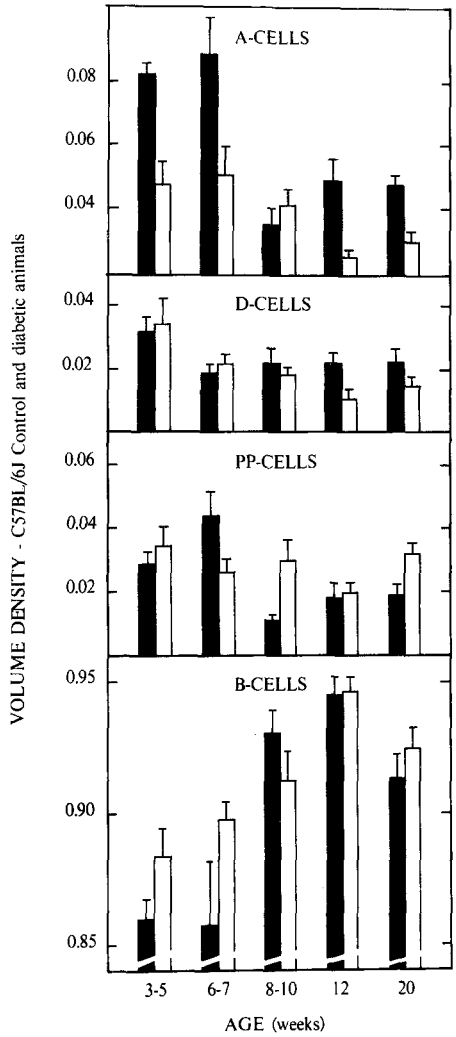

Fig. 2. Volume density (cell type area/islet area) of A, D, PP and B cells in control (■) and *db/db* (□) C57BL/6J mice of various ages. Values represent mean ± SEM of islet measurements in a sequence of random serial sections stained individually for each respective cell type as described in Materials and Methods

Table 1. Morphometric analysis of control and diabetic islets in C57BL/6J mice

Age (weeks)	Genotype	N[a]	Islet volume ($\mu^3 \times 10^6$)	D cell volume ($\mu^3 \times 10^5$)	D cell volume density	D cells per islet
8	+/?	3,9	1.94 ± 0.47[b]	0.52 ± 0.11	0.0288 ± 0.0037	54 ± 10
	db/db	3,9	7.18 ± 1.57	1.54 ± 0.29	0.0230 ± 0.0029	116 ± 20
	p[c]		0.006	0.004	0.232	0.014
10	+/?	2,6	3.49 ± 0.91	0.75 ± 0.19	0.0212 ± 0.0099	76 ± 18
	db/db	2,8	8.13 ± 2.02	0.81 ± 0.27	0.0095 ± 0.0011	102 ± 34
	p		0.087	0.865	0.0001	0.551
12	+/?	3,9	2.09 ± 0.64	0.41 ± 0.11	0.0204 ± 0.0036	54 ± 15
	db/db	3,9	6.33 ± 1.38	0.41 ± 0.10	0.0062 ± 0.0008	55 ± 13
	p		0.013	0.992	0.001	0.964
20	+/?	3,10	1.74 ± 0.43	0.67 ± 0.21	0.0523 ± 0.0110	121 ± 35
	db/db	3,12	15.96 ± 2.12	0.92 ± 0.18	0.0055 ± 0.0007	81 ± 11
	p		0.0001	0.371	0.0001	0.253

[a] N = number of animals followed by the total number of islets analyzed; [b] results expressed as mean ± SEM; [c] *p* values determined using non-paired t-test

umes did not change commensurately with age-dependent increases in total volumes of *db/db* islets. Islets from 8 week-old *db/db* mice exhibited an almost fourfold and threefold increase above +/? mice in total islet volume and D cell volume, respectively (Table 1). Further, Table 1 shows that an initial increase in D cell mass had occurred in *db/db* islets by 8 weeks as evidenced by an increase in D cell number per islet and by a D cell volume density not significantly different from that of +/? islets in spite of a threefold increase in total volume of *db/db* islets. As shown in Figure 1, a plot of D cell number versus the logarithm of islet volume for +/? islets (combined for all age groups) showed a significant linear correlation ($p < 0.01$). When the equation derived from the regression line was used to predict D cell number in a *db/db* islet of a given volume, the equation predicted accurately for younger *db/db* mice (with smaller islet volumes), but frequently over-estimated D cells per islet for older *db/db* mice (with larger islet volumes), Table 1 shows that as total *db/db* islet volume increased from approximately 7 to 16×10^6 μ^3 between 8 and 20 weeks of age, D cell volume density declined progressively, indicating under-replication of this cell type.

Volume density changes of A, D, and PP cells between 3 and 20 weeks of age are shown in Figure 2. A cells in *db/db* islets showed a reduced volume density compared to +/? islets, a decrease being apparent already in the 3–5 week age group. Confirming the data for the D cell volume density changes in Table 1 obtained by the more laborious section-by-section analysis, D cells in *db/db* animals maintained a normal volume density until the 8–10 week period, and then exhibited a declining volume density with increased age. In contrast, PP cells showed no pronounced changes in volume density (Fig. 2) which, given the great increase in islet size over the age period studied, suggests a marked hypertrophy and/or hyperplasia of PP cells in these islets. Analysis of B cell volume density (Fig. 2) showed that there was increased volume density in *db/db* islets until the 8–10 week period, but thereafter, B cell volume density was not markedly different between +/? and *db/db* islets.

Immunocytochemical staining for GPDH was localized to a peripheral population of cells that immunostained for glucagon in adjacent sections (Fig. 3). Staining for GPDH could be eliminated completely by preincubation of the antiserum with purified enzyme but not with glucagon.

Discussion

Baetens et al. [2] concluded that volume density changes in non-B cell populations were inversely related to changes in the B cell mass in islets of old BL/6 and BL/Ks *db/db* and *ob/ob* mice. The present study, based on a longitudinal survey of islet cell changes during early adulthood instead of a single sampling toward the end of mutant lifespan, showed that each non-B cell type has its own unique temporal response to the presence of the *db* gene and to the mitogenic stimulus that it exerts on the B cell mass. Thus, in 8 week-old BL/6-*db/db* mice in which B cell hyperactivity had already been initiated during the period immediately after weaning, the total number of D cells per islet, as well as the actual volume of somatostatin-containing cytoplasm per islet were increased above +/? values. PP cell volume density in *db/db* islets was also elevated significantly above control values at this time and would suggest that the initial mitogenic stimuli elicited by *db* gene expression and underlying B cell proliferation also stimulated D and PP cell hypertrophy and/or hyperplasia. Eventual under-replication of D cells vis a vis the continuously increasing B cell mass represented an inability of the D cell population to sustain replication matching that of the B cells rather than a decrease in ab-

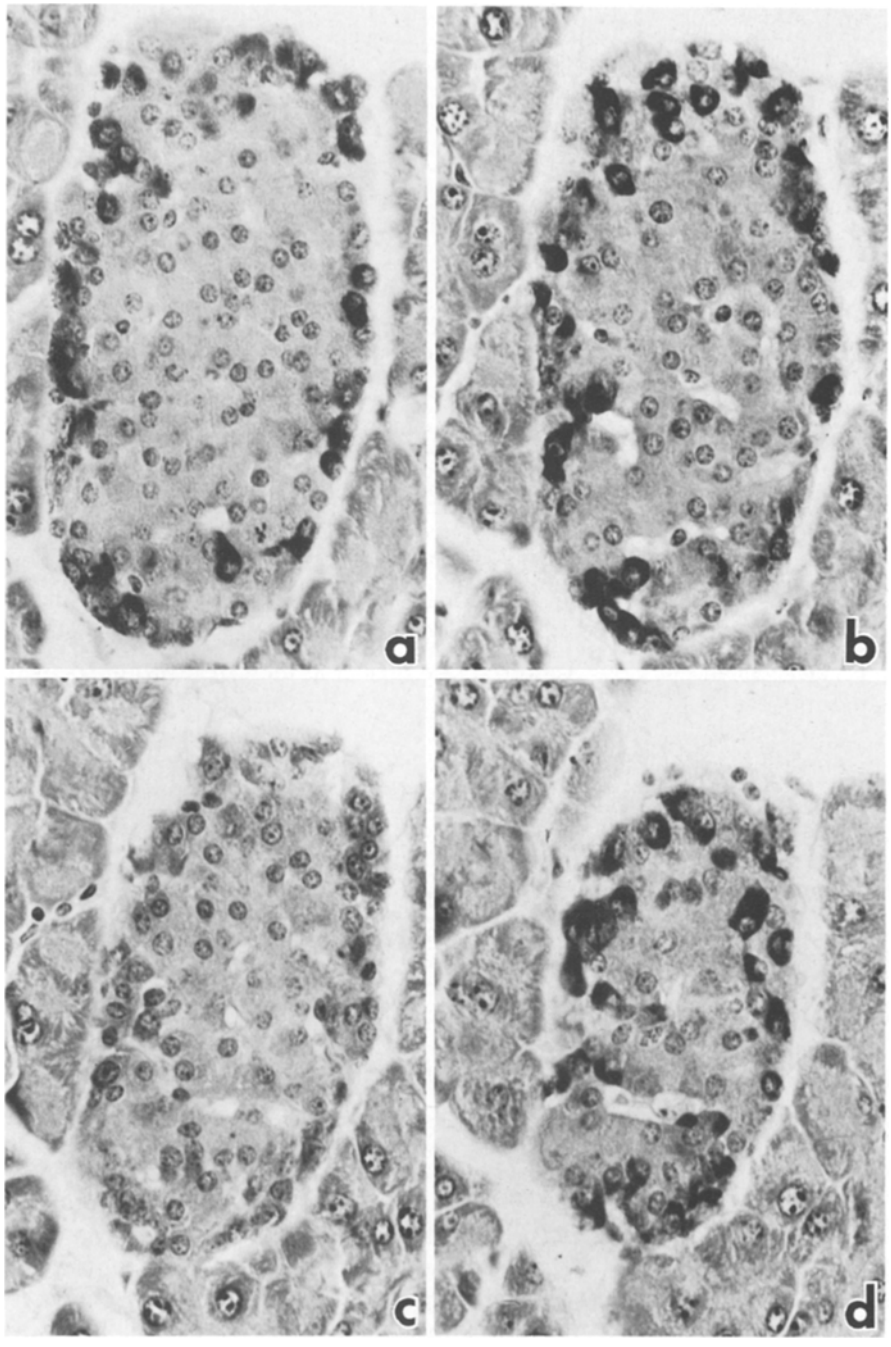

Fig. 3. Immunocytochemical staining for *sn*-glycerol-3-phosphate dehydrogenase in normal C57BL/6J mouse pancreas (all magnifications × 300). **a** Islet stained with anti-glucagon serum to show A cell distribution. **b** Adjacent section stained with antiserum to GPDH. Differentially high concentrations of GPDH are localized in the cytoplasm of A cells within the islet. **c** Section adjacent to **b** stained with anti-GPDH pre-absorbed with 50 Units of purified enzyme. Enzyme-specific staining is abolished. **d** Section adjacent to **c** stained with anti-GPDH pre-absorbed with 100 ng of porcine glucagon. Enzyme-specific staining is unaffected

solute D cell number or mass. This is supported by the longitudinal study of Berelowitz et al. [3] who showed that pancreatic somatostatin content became elevated by 10 weeks of age and continued to rise thereafter, being approximately 40% above control levels at 24 weeks. Thus, increases in pancreatic somatostatin content due to initial D cell hypertrophy and hyperplasia were not reversed at later stages when D cells appear to be under-replicated.

In contrast to the transiently hyperplastic D cells, the PP cell volume density in BL/6-*db/db* mutants did not decline with age [this study and 2]. Therefore, PP and D cell volume densities did not remain coordinately regulated over time, with PP cells in mutants not exhibiting the later under-replication (vis a vis B cells) that apparently typified D cells. Although age-related changes in pancreatic PP content have not yet been reported for either BL/6- or BL/Ks-*db/db* mice, Gingerich et al. [8] have shown an increase in pancreatic PP content, but a reduced volume density within the hyperplastic islets of 20 week BL/6-*ob/ob* mice. This report conflicts with the finding in the present study of an increased volume density in 20 week BL/6-*db/db* islets, but this is probably attributable to a difference in *db* versus *ob* gene effects on the pancreas as has been demonstrated by the striking differences in age-related changes in the total pancreatic somatostatin content of *ob/ob* and *db/db* mice [3]. This gene effect on islet hormone content may hold for pancreatic polypeptide as well.

Volume density changes in the A cell population separated this cell type clearly from other non-B cells in BL/6-*db/db* mice since it was the only one to vary as a simple reciprocal function of increased B cell mass. A reciprocal relationship also has been shown for the A cells in the B cell-depleted islets from BL/Ks-*db/db* mice [2, 12] and has been demonstrated experimentally in normal 129/J mice where a high protein diet favours a markedly reduced B cell/A cell ratio without affecting D or PP cell populations [13].

The uniqueness of A cell resistance to *db* gene-generated mitogenic stimuli is underscored further by the restricted localization of cytoplasmic GPDH to this cell type [7 and this study] and has implications for the embryonic origins of pancreatic endocrine cells. Islet cells are proposed to belong to the APUD series of cells derived from the neuroectoderm [17, 18], and the presence of neuron-specific enolase in all islet cells [16] as well as a transient catecholaminergic phenotype demonstrated for A cells [21] tend to support this hypothesis. Various studies employing neuroectoderm or neural tube ablation [19] or employing neural tube grafting [1] have not supported the APUD theory but rather support the concept of islet cell differentiation resulting from the interaction between endoderm and mesoderm [22]. In view of the controversy concerning the embryonic origin of islet cells, the presence of marked concentrations of GPDH restricted to A cells may suggest alternatives. Although the structural gene for GPDH is expressed in most tissues, the enzyme appears in higher concentrations in mesenchymally derived cells such as differentiating 3T3-L1 fibroblasts [15] and adipocytes [5] as well as certain cerebellar cell types [8] of presumed neuroectodermal origin. If a differentially high concentration of GPDH is indeed a valid marker for protodifferentiated or differentiated cells of mesodermal origin, this apparently high concentration of GPDH in the pancreatic A cell may suggest a unique (mesenchymal?) embryonic origin for this islet cell type.

In summary, it appears from the findings presented here that each islet cell type has its own characteristic temporal and absolute response to the mitogenic stimulus exerted by the homozygous expression of the *db* gene. Consequently, if volume density measurements are used to assess changes in islet cell constituents, it is essential that longitudinal studies be performed.

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