Intracisternal Type A Particles in Murine Pancreatic B Cells

Immunocytochemical Demonstration of Increased Antigen (p73) in Genetically Diabetic Mice

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Intracisternal Type A particles (IAPs) are retroviruslike structures identified by a core protein antigen (p73) and found in mouse embryos, in many mouse tumor cells, and in pancreatic B cells of some strains of genetically diabetic mice. Using both peroxidase–antiperoxidase and protein A–gold immunocytochemical techniques to localize p73, the authors have observed differences in intracellular antigen distribution between MOPC-104E, a mouse tumor cell line rich in IAP, and B cells from genetically diabetic (*db/db*) mice of the CBA/LtJ and C57BL/KsJ strain. In MOPC-104E cells studied by electron microscopy, localization of protein A–gold complex label was almost exclusively limited to IAP and their sites of assembly on the rough endoplasmic reticulum. In contrast, p73 appeared widely distributed throughout the cytoplasm of B cells from hyperglycemic *db/db* mice but not normal littermate controls. In addition to distribution over budding IAP, label was also found dispersed through other cytoplasmic organelles involved in secretion, including Golgi complexes and secretory granules. Patch labeling of B cell surfaces was sometimes observed. An ultrastructural survey of islets isolated from normal mice of 7 inbred genetic backgrounds on which the "diabetes" (*db*) gene has been studied showed that constitutive ability to produce IAP was associated with strain susceptibility to severe diabetes (eg, C57BL/KsJ, DBA/2J, CBA/LtJ, and C3HeB/FeJ). Strains whose B cells failed to show constitutive expression *in situ* or glucose-inducible expression in cell culture were resistant to the diabetogenic action of *db* genes. The possibility is discussed that p73 may represent a "neoantigen" which sensitizes the diabetic mouse to reject, by autoimmune mechanisms, the B cells expressing it. (Am J Pathol 1984, 114:46–55)

THE AUTOSOMAL recessive gene "diabetes" (*db*) is an obesity-causing mutation in mice.[1] In certain inbred strains of mice (C57BL/KsJ, DBA/2J, C3HeB/FeJ, and CBA/LtJ), the *db* gene can interact with genetic background modifiers to effect a severe, insulin-resistant diabetes syndrome characterized by B cell necrosis and pancreatic islet atrophy.[2] In other inbred strains (C57BL/6J, 129/J, MA/J), B cells are not destroyed, but are able to compensate for the diabetogenic stress by sustaining insulin secretion, active growth, and cell division.[2]

The expression of retroviral genes has been an enigma in the pathogenesis of genetic diabetes in the mouse. The appearance of retrovirus in prenecrotic B cells has been pathognomonic of genetically diabetic (*db/db*) mice with established hyperglycemia. Although originally described as type C retrovirus,[3] subsequent morphologic and biochemical characterization has shown it to be intracisternal type A particle (IAP).[4] The biologic significance of these vertically transmitted viruslike particles is unknown. The 73,000-dalton core protein (p73) of IAPs may be a differentiation antigen, because IAPs normally show a stage-specific appearance in 2- to 8-cell stage preimplantation embryos, and during this period, the cell surface appearance of the p73-related antigen can

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be demonstrated.[5] IAPs are only occasionally expressed in undiseased tissues from adult mice. If p73 indeed is a differentiation protein normally expressed prior to the constitution of an immune surveillance system, then its expression by B cells in adult hyperglycemic mice might lead to its perception as a "neoantigen." This, in turn, could lead to an autoimmune reaction against the B cells. Altered immunologic responsiveness has been found in *db/db* mice.[6] Immune complex deposition in kidneys of hyperglycemic *db/db* mice has been reported,[7] as has evidence for both cell-mediated and humoral autoimmunity against B cells.[8]

The purpose of the present study was to employ immunocytochemical staining techniques at the light- and electron-microscopic levels to analyze p73 distribution in B cells of severely diabetic *db/db* mice. Additionally, inbred strains were surveyed for constitutive ability to show IAP expression of B cells for determination of whether this expression was associated with susceptibility to genetic diabetes.

Materials and Methods

Mice

Genetically diabetic (*db/db*) male mice with established hyperglycemia were obtained from C57BL/KsJ-+/*db* and CBA/LtJ-+/*db* breeding colonies at the Jackson Laboratory.[2] In this study, pancreases from 8 C57BL/KsJ-*db/db* males between the ages of 12 and 20 weeks and 11 CBA/LtJ-*db/db* males between the ages of 8 and 12 weeks were studied. Equal numbers of age- and sex-matched normal littermate controls (either +/*db* or +/?) were also studied. For a survey of constitutive expression of IAPs in B cells in normoglycemic (+/+) mice, pancreases were sampled from 8-week-old males from the C57BL/KsJ, DBA/2J, C57BL/6J, 129/J, CBA/J, C3HeB/FeJ, and MA/J colonies maintained by the Animal Resources Department of the Jackson Laboratory.

Rabbit Antiserums Against p73

IAPs were isolated from the MOPC-104E plasmacytoma of BALB/c mice and purified by isopyknic banding as previously described.[9] The IAPs were washed with 1% SDS in 0.02 M sodium phosphate, pH 7.2, to yield a particulate preparation of disulfide-linked internal structural protein (SDS-cores) consisting of p73 and lesser amounts of several other structurally and antigenically related polypeptides.[10] The SDS-cores were dissociated by heating in 0.1 M β-mercaptoethanol and fractionated by electrophoresis in SDS-polyacrylamide gels. Gel regions containing p73 were located by ultraviolet-scanning and excised. Rabbit antiserums were prepared by repeated injections of the gel slices emulsified with complete (primary immunization) or incomplete (boosts) Freund's adjuvant; each rabbit received a total of about 200 μg of p73. These antiserums were active in a dilution of 1:10,000 in radioimmunoassays of p73.[11] When tested against extracts from IAP-rich myeloma or neuroblastoma cells that had been labeled in culture with ^{35}S-methionine, the antiserums precipitated the major 73,000-dalton component plus an additional 120,000-dalton polypeptide that shared tryptic peptides with p73 (J. Fewell and E. Kuff, unpublished observations). These components were not precipitated by preimmunization serums or by antiserums previously absorbed with purified p73.

Immunocytochemical Staining for Light Microscopy

Expression of p73 antigen in 5-μ paraffin sections of Bouin's-fixed pancreases was detected by the indirect peroxidase–antiperoxidase technique of Sternberger.[12] Three serial sections cut from each pancreas were placed on a slide. Endogenous peroxidase activity was blocked by methanol and hydrogen peroxide–sodium borohydride treatment.[13] One section per slide was incubated in normal rabbit serum diluted 1:50 in phosphate-buffered saline (PBS), pH 7.4, and containing 1% normal goat serum as a negative control. Another section was incubated in a 1:50 dilution of rabbit anti-p73 serum. As a control for specificity, the third section was incubated in a 1:50 dilution of rabbit anti-p73 serum that had been preabsorbed in a 500-μl volume with 60 μg of p73 immunogen purified from MOPC-104E myeloma cells.[14] Incubation was for 48 hours at 4 C. This incubation was followed by sequential 30-minute incubations in goat anti-rabbit gamma globulin serum and rabbit peroxidase–antiperoxidase complex (both diluted 1:50 and obtained from Polysciences, Inc., Warrington, Pa). Following three rinses, slides were developed for 3 minutes in a large volume of 0.05% diaminobenzidine·HCl/0.01% H_2O_2 in 0.1 M sodium phosphate buffer, pH 6.0. The slides were then washed, stained in hematoxylin and eosin (H&E), dehydrated, and mounted.

Immunocytochemical Staining for Electron Microscopy

Pancreatic islets for ultrastructural observation were obtained from tribromoethanol-anesthetized mice by intraventricular perfusion of fixative (2% glu-

taraldehyde, 1% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) exactly as described previously.[15] Ultrastructural localization of the p73 antigen was performed with the protein A–gold technique of Roth et al.[16] Thin sections were mounted on 200-mesh, Formvar-coated nickel grids; and because the tissues had been postfixed in osmium, the sections were either etched in 10% hydrogen peroxide as described by Roth et al[16] or treated with sodium metaperiodate for 60 minutes as described by Bendayan.[17] Following washes in PBS containing 1.6% bovine serum albumin (BSA), sections were floated in drops of primary antiserum or control serums for 24–48 hours in humidified chambers at 4 C. Normal rabbit serum, anti-p73 immune serum, and immune serum preabsorbed with p73 as described above were employed at a 1:250 dilution. After 3 rinses in distilled water and a 30-minute incubation in PBS containing 1.6% BSA, the grids were floated for 1 hour at room temperature in protein A–gold complex (used at a 1:4 dilution). Following further rinsing and poststaining in uranyl acetate and lead citrate, the grids were examined in a Hitachi HU-IIC microscope. Specificity controls included the omission of primary antiserum (labeling with protein A–gold only). Antiserum titers and specificity to localize to IAPs at the ultrastructural level were tested by staining thin sections of cultured MOPC-104E myeloma cells (kindly donated by Dr. H. J. Heiniger of The Jackson Laboratory). Cells of this tumor line contained numerous IAPs and, growing as subcutaneous transplants, were the source of the p73 immunogen.

To compare the distribution of p73 in B cells with that of insulin, thin sections semi-adjacent to those incubated in anti-p73 serum were incubated in guinea pig anti-insulin serum (diluted 1:500, gift of Dr. P. H. Wright, Indiana University, Indianapolis, Ind) and then processed with protein A–gold complex as described above.

Strain Survey of Constitutive or Inducible IAP in B Cells

Islets from normoglycemic (+/+) male C57BL/KsJ, C57BL/6J, DBA/2J, 129/J, C3HeB/FeJ, CBA/LtJ, and MA/J mice, fixed by perfusion as de-

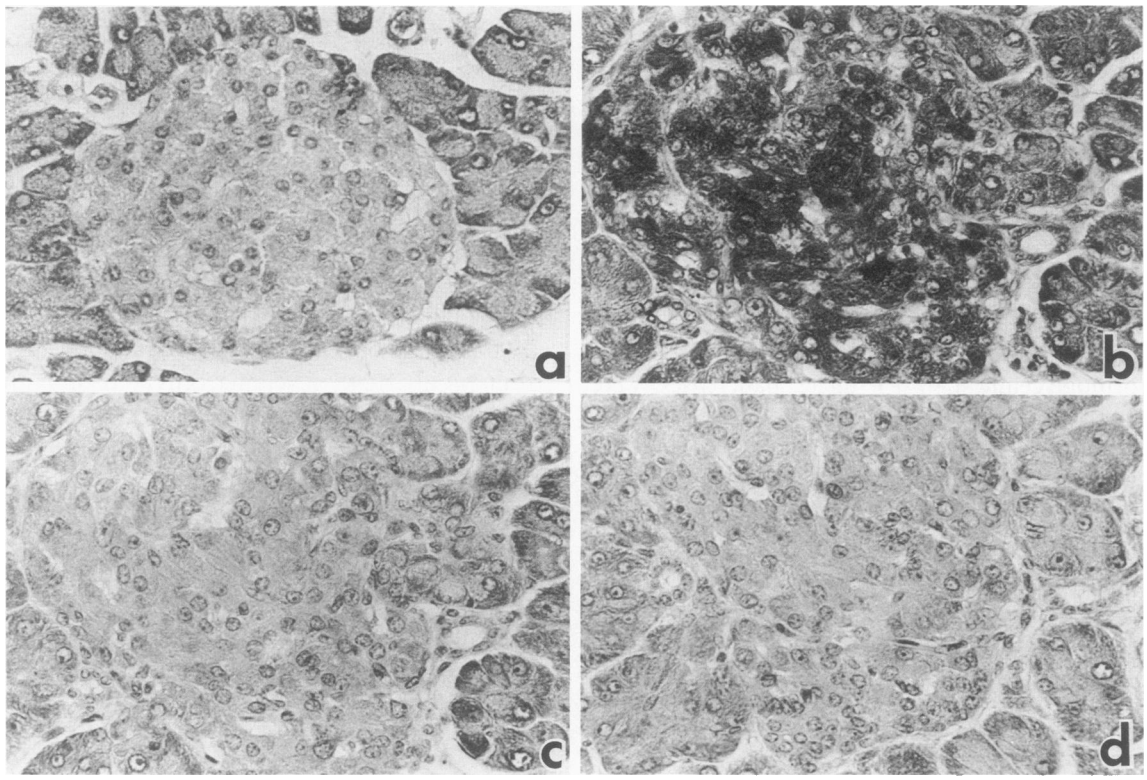


Figure 1 — Immunoperoxidase staining of p73 in islet cells. (×900) **a** — Pancreas of a 9-week-old CBA/LtJ – +/? normoglycemic male stained with rabbit anti-p73. A single islet cell shows a positive reaction. **b** — Anti-p73 stained pancreas of the *db/db* male littermate of the above mouse. The mutant had a ninth week blood glucose of 445 mg/dl. Most of the islet cells contain a positive peroxidase reaction product diffusely distributed throughout the cytoplasm. **c** — Adjacent section of same islet shown in **b** but stained with p73-preabsorbed immune serum. The peroxidase-positive staining has been abolished. **d** — Adjacent section of same islet shown in **c**, but stained with nonimmune rabbit serum. Only slight staining above background is seen.

scribed above, were examined by electron microscopy for constitutive presence of IAPs. At least three islets from each genotype were sampled, with all B cells in the thin sections studied. Since we had previously shown that cultured B cells from C57BL/KsJ normoglycemic mice showed enhanced expression in response to elevated medium glucose concentration,[4] we also screened (by electron microscopy) monolayers of islet cells established from collagenase-isolated islets and incubated at either low (5.5 mM) or high (16.5 mM) glucose collagenase-isolated islets containing Dulbecco's modified Eagle's medium (DMEM medium) as described previously.[18] Approximately 50–100 B cell profiles were scanned in thin sections of cultured islets from each group.

Results

Light-Microscopic Localization of p73

Immunohistochemical staining for p73 in pancreases from normoglycemic CBA/LtJ and C57BL/KsJ male mice showed only a weakly positive staining reaction within most of the islet cells; an occasional islet cell showed strong staining (Figure 1a). Pancreases of hyperglycemic *db/db* mice of both inbred strains incubated with anti-p73 gave a strongly positive cytoplasmic staining reaction in up to 80% of the cells within the pancreatic islets (Figure 1b). This staining was specific for p73, as evidenced by its reduction to near-background level when p73-preabsorbed immune serum was used (Figure 1c). Normal rabbit serum exhibited essentially background staining in sections of either control or *db/db* pancreas (Figure 1d). These observations were based upon staining of four matched pairs (genotype normal and mutant) of pancreases from strain CBA/LtJ and three from strain C57BL/KsJ.

Ultrastructural Localization of p73

The anti-p73 antiserum was highly specific when used to stain IAP-rich MOPC-104E myeloma cells (Figure 2). Almost 70% of all gold particles were localized over nascent IAPs budding from the inner surface of the membranes of the rough endoplasmic reticulum (RER). Background levels of gold particles were very low, and essentially "background" levels of labeling were observed over mitochondria, Golgi elements, and plasma membrane. As shown in Figure 2, the heaviest labeling was observed in the nascent (budding) IAP. The fully mature (budded) forms within the cisternae of the RER contained only 0–2 gold particles per particle, indicating that the p73

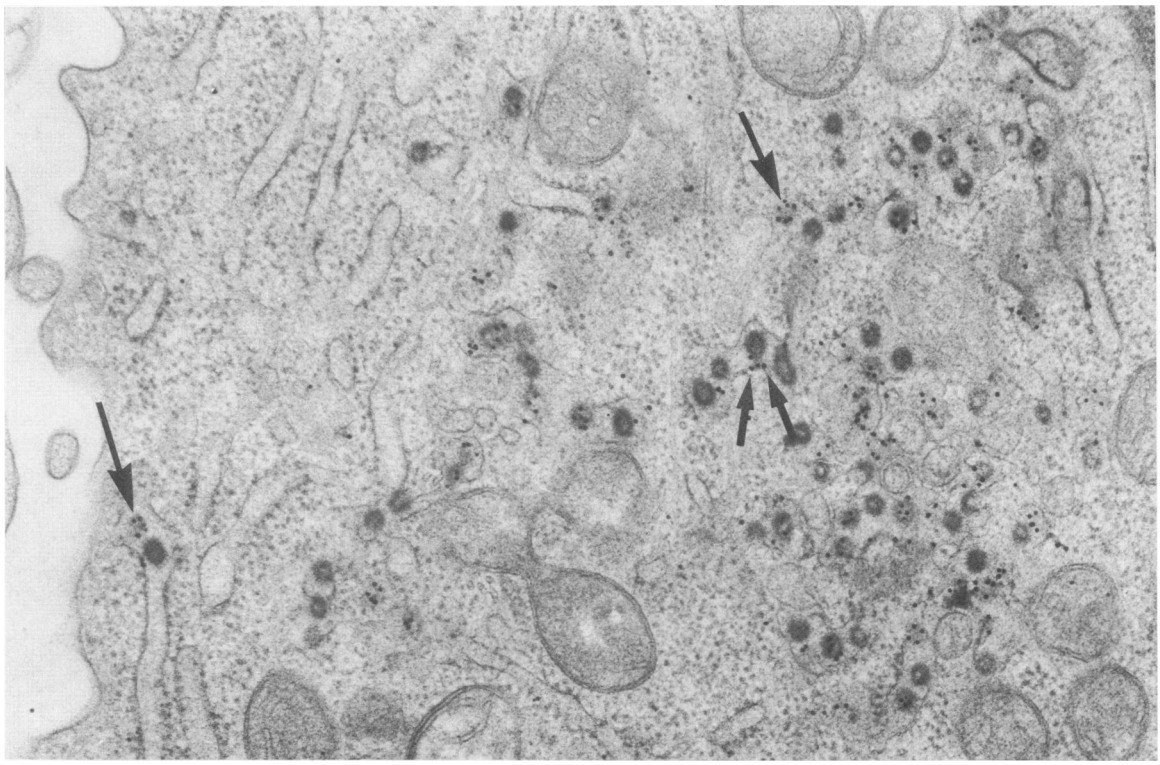


Figure 2 — Ultrastructural localization of p73 antigen in a cultured MOPC-104E myeloma cell by the protein A-gold technique. The gold particles (small black grains) are almost exclusively localized to nascent (budding) IAPs (*single arrow*) or to the surface of RER (*double arrows*). (×41,328).

antigen was most accessible to antibody during early assembly stages and became less accessible as the particle formed. This ultrastructural observation confirmed earlier biochemical studies showing p73 latency in the core of fully assembled IAPs.[11]

B cells of normoglycemic littermate control CBA/LtJ mice were unexceptional in appearance; IAPs were constitutively expressed, but at a low frequency (approximately 1 IAP per 8 B cell profiles screened). Immunocytochemical staining of these sections with the anti-p73 serum produced only a very light labeling above background of the RER, Golgi, and secretion granules (Figure 3a). The alterations in the ultrastructure of B cells in hyperglycemic CBA/LtJ-*db/db* mice has been described in detail elsewhere.[15] The increased concentration of IAP in a hyperglycemia-stressed (eg, degranulated) B cell in an 8-week-old *db/db* male is illustrated in Figure 3b. Anti-p73 serum stained nascent IAPs in B cells (Figures 3b and 4a). However, unlike the MOPC-104E myeloma cells, in which label was primarily concentrated over IAP in the RER, label over beta cells from older *db/db* mice with established hyperglycemia indicated a much wider distribution of p73 that was independent of the presence of morphologically distinct IAPs. The heaviest distribution of colloidal gold in beta cells of *db/db* mice older than 8 weeks was always observed on the membranes or within the cisternal matrix of RER and its transitional elements. Weaker labeling over Golgi elements, mitochondria, and secretory granules were detected (Figure 5a). Labeling of B cell plasma membranes was only slightly above background but was differentiated from nonspecific background labeling (in which individual gold particles were uniformly distributed) by its occurrence in small patches (inset, Figure 5a). B cells stained with nonimmune or preabsorbed immune serum did not show this labeling. Further, the B cell was the only islet cell type to show specific labeling (Figure 5b). The only other cells observed with higher than background gold deposition were macrophages that had infiltrated into the interior of *db/db* islets in which B cells appeared to be undergoing degenerative changes. These observations were made from an ultrastructural survey of five CBA/LtJ-*db/db* and 3 C57BL/KsJ-*db/db* pancreases.

When B cells from normoglycemic mice stained with guinea pig anti-insulin serum were examined, the

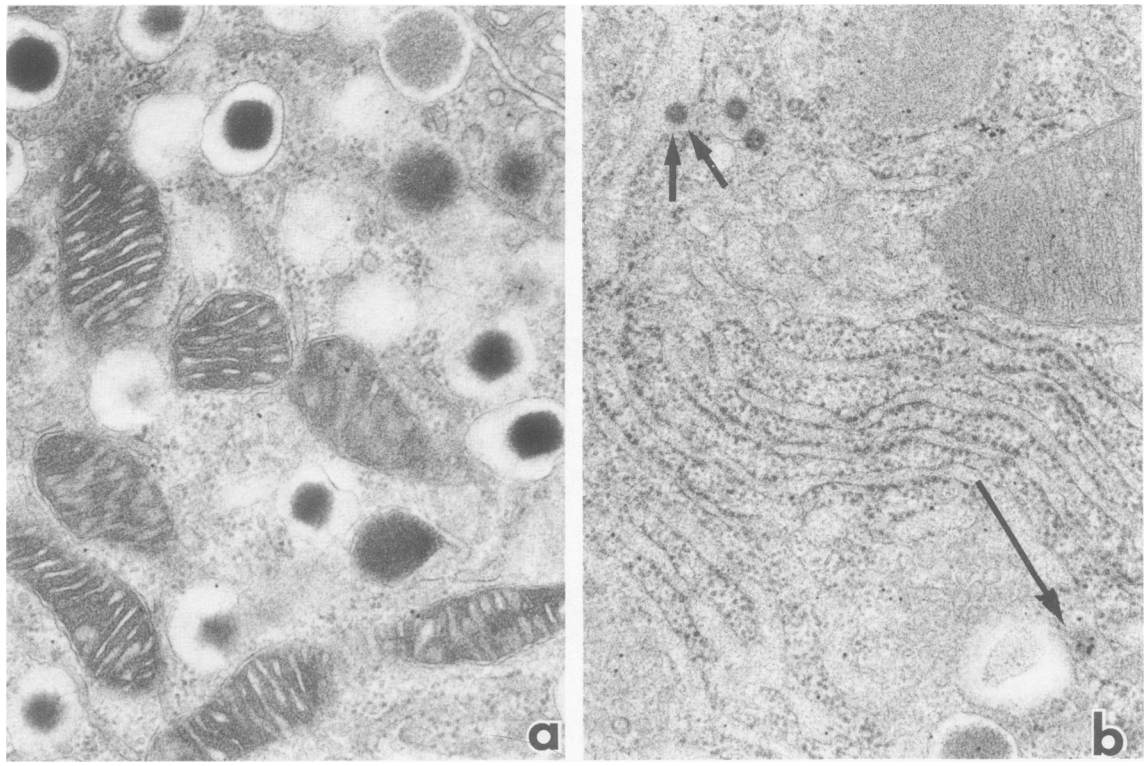


Figure 3a — Appearance of a well granulated B cell from a 12-week-old normoglycemic C57BL/KsJ male mouse. This section, although stained with p73 antiserum, shows only low (background) distribution of gold particles. (×35,310) **b** — Anti-p73 stained B cell in an islet from *db/db* male littermate of the control depicted in **3a**. Typical ultrastructural changes associated with the establishment of chronic hyperglycemia are beta-degranulation, mitochondrial enlargement, and dilatation of RER cisternae, which often contain small numbers of IAPs. The nascent particle at the lower right (*single arrow*) is showing gold labeling of its core. Mature particles are less heavily labeled (*double arrow*). (×36,330)

insulin-containing cores of the beta-granules were heavily stained by the protein A–gold complexes, and very few gold particles were observed elsewhere over the cytoplasmic matrix (data are not shown); this has been previously reported by others.[16] However, in heavily degranulated B cells from *db/db* mice with established hyperglycemia, a much more widespread distribution of (pro)insulin-antibody complexes was observed, similar to that observed when one is staining with anti-p73 serum. In addition to localization to beta granules, heavy (pro)insulin labeling was observed within the dilated cisternae of the RER (Figure 6).

Strain Survey of IAP in B Cells

Table 1 shows the results of an electron-microscopic survey of B cells observed *in situ* and in cell cultures. The seven inbred strains examined have been grouped in the table according to their previously reported susceptibility or resistance to the induction of genetically induced diabetes.[2] Although the results in Table 1 are limited to a survey of normal +/+ (and not *db/db*) mice, it is clear that both constitutive expression of IAPs as well as glucose induction of enhanced expression of B cells correlate with diabetes susceptibility, but not with a single H-2 haplotype.

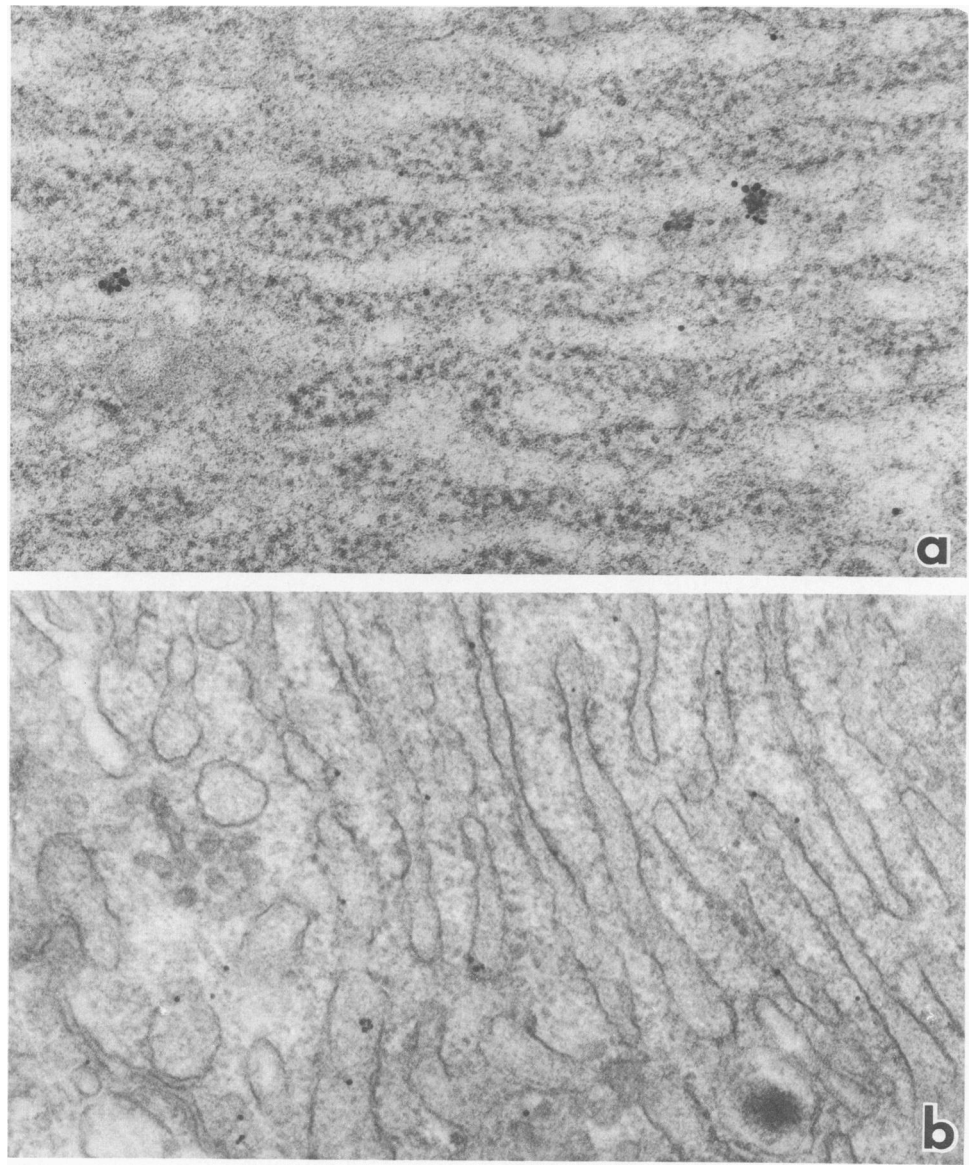
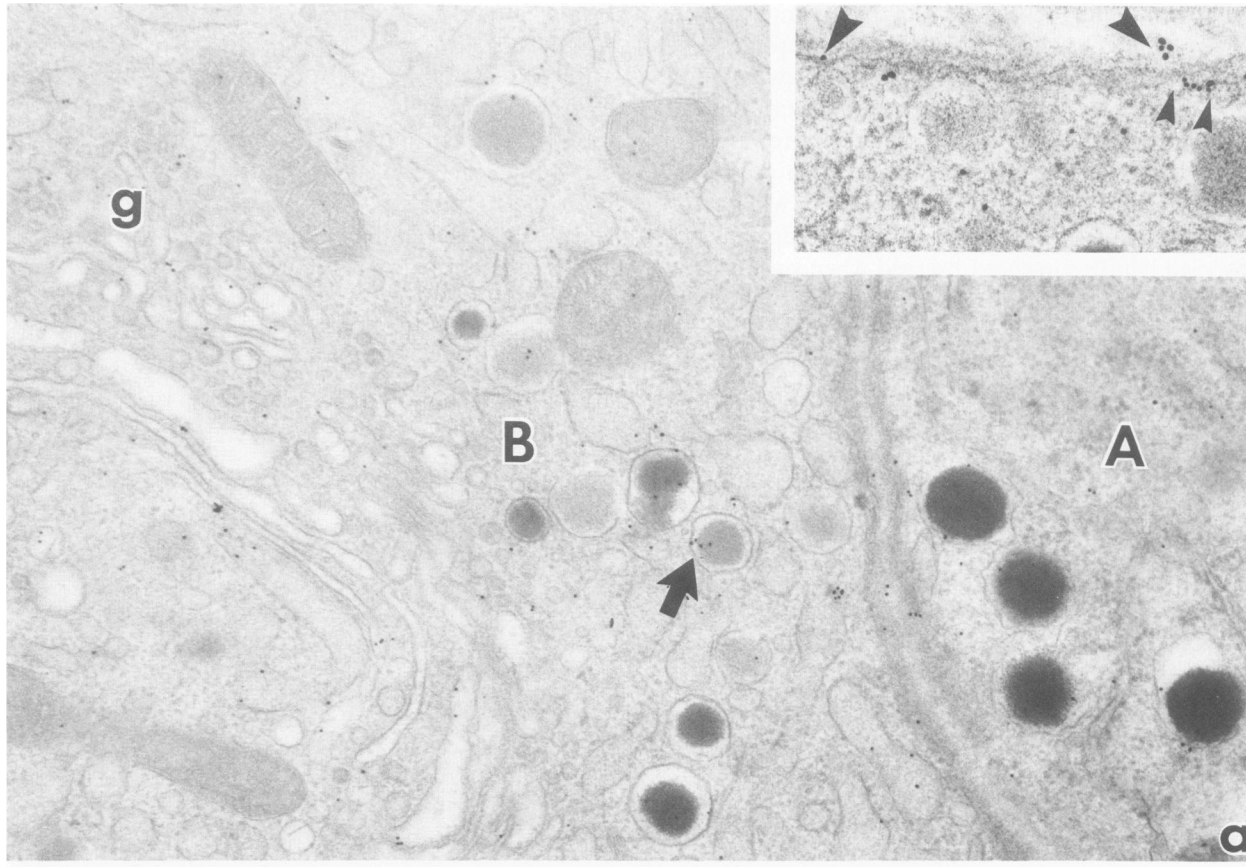


Figure 4 — Non-IAP localization of p73 antigen in anti-p73 stained B cells from CBA/LtJ-*db/db* mice. **a** — Heavy labeling by protein A–gold complex of p73 at the sites of IAP formation in the RER. (×58,620) **b** — In contrast to the IAP-associated label distribution in **a**, this micrograph shows considerable p73 distribution in RER cisternae and membrane without obvious association with IAPs. (×61,650)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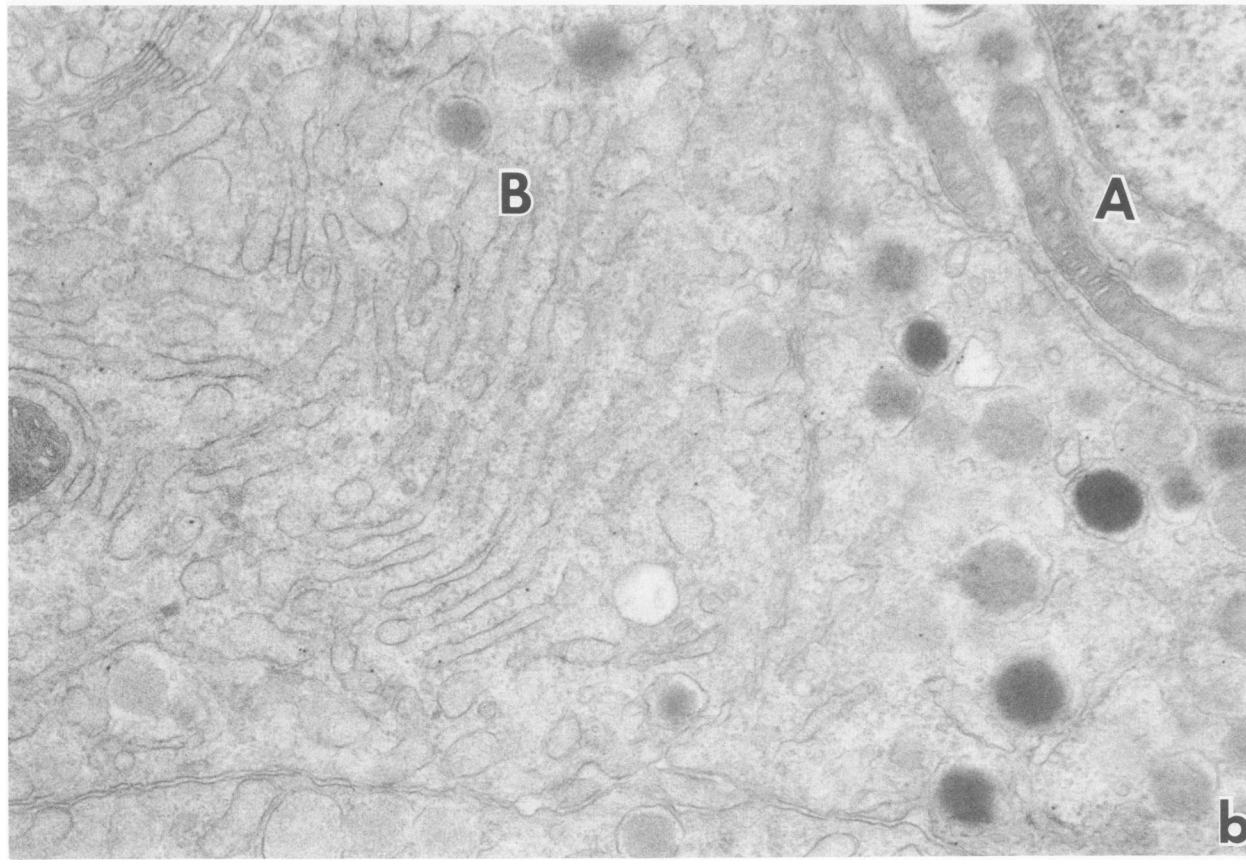
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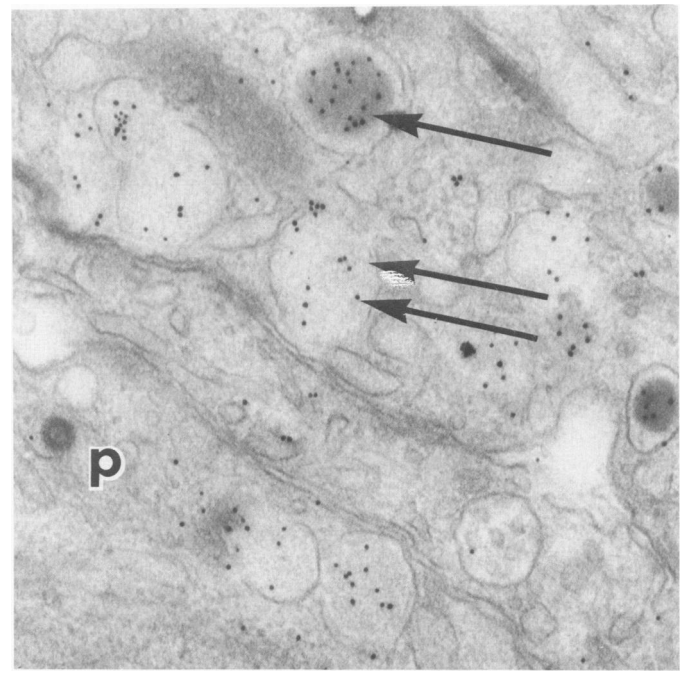

Figure 6—Ultrastructural localization of insulin by protein A-gold in beta cells from a 20 week-old C57BL/KsJ-*db/db* mouse. Note that following staining with anti-insulin serum, gold particles are heavily distributed not only over the cores of secretion granules, when present (*single arrow*), but also over the electron-dense cisternal matrix of dilated RER (*double arrows*). An IAP (*p*) seen at the left-hand margin is not labeled by the guinea pig anti-insulin serum. (×55,590)

Thus, B cells from diabetes-susceptible C57BL/KsJ, DBA/2J, CBA/LtJ, and C3HeB/FeJ male mice expressed IAPs *in vivo* and *in vitro*, whereas those from diabetes-resistant C57BL/6J, 129/J, and MA/J males did not. Glucose-enhanced production of IAP in cultured C57BL/KsJ, CBA/Lt, and C3HeB/FeJ B cells was not beta-cytotoxic. Although ultrastructural examination showed the glucose-stressed B cells to be degranulated, no cytopathic changes were evident.

Discussion

Immunocytochemical staining at both light- and electron-microscopic levels showed that pancreatic B cells in chronically hyperglycemic *db/db* mice contained considerable levels of p73 antigen in excess of that localized within the inner particle shells of mature IAPs. The strong anti-p73 antibody staining of islets as detected by the immunoperoxidase method indicated that p73, or a precursor, or a fragment sharing the same antigenic determinant, may be a major biosynthetic product of glucose-stressed *db/db* B cells. The high levels of non-particle-associated p73 in glucose-stressed B cells contrasted with IAP-expressing tumor cells, in which this pool is very small.[19] The distribution of p73 in these B cells was remarkably similar to that observed for (pro)insulin. B cells from hyperglycemic C57BL/KsJ-*db/db* mice, although degranulated, retain considerable potential for proinsulin/insulin biosynthesis.[20] The model proposed by Lacy[21] for translocation of newly synthesized (pro)insulin to the cell surface via transit through transitional elements of the RER to Golgi and hence into secretory granules has been largely substantiated.[22] If p73 molecules (in excess of those assembled into the retroviruslike particles) were accumulating within the RER cisternae, as suggested by the immunocytochemical staining, then it is certainly conceivable that translocation of p73 through the cytoplasm might be effected by the same glucose-responsive mechanisms that lead to insulin secretion. If some p73 were packaged into secretory vesicles, it could either be shed by emiocytosis into the extracellular space or intercalated into the plasma membrane. Although small clusters of gold particles sometimes indicated localization of p73 very near to or at the B cell surface, the morphologic approach employed does not allow the conclusion that IAP-expressing B cells have a changed membrane structure due to insertion of microsomal antigens. Indeed, increased IAP and intracellular p73 in B cells of severely diabetic mice might only reflect changes in genetic expression

Figure 5—Alpha (**a**) and degranulated beta (**b**) cell in an islet from a 4-month-old C57BL/KsJ-*db/db* male mouse. **a**—The section, stained with p73 antiserum, shows diffuse, non-IAP-associated distribution of gold over the cytoplasm, including Golgi (*g*) and secretory granule (*arrow*) compartments in the B cell. The A cell shows background labeling. (×42,385) The inset (*upper right*) shows patch-labeling by protein A-gold complex of p73 distributed just beneath (*double arrows*) and on (*single arrow*) the cell surface adjacent to a capillary space. (×31,260) **b**—Semiadjacent sections of the islet depicted in **a**, but stained with nonimmune rabbit serum as a control. (×42,385)

Table 1—Association between B Cell IAP Expression and Susceptibility to Diabetes*

Inbred strain	H-2 haplotype	Constitutive IAP expression in B cells of normal mice	Glucose-inducible IAP expression in B cell cultures
Diabetes-resistant			
C57BL/6J	*b*	–	–
129/J	*b*	–	–
Ma/MyJ	*k*	–	ND†
Diabetes-susceptible			
C57BL/KsJ	*d*	+	+
DBA/2J	*d*	+	ND
CBA/LtJ	*k*	+	+
C3HeB/FeJ	*k*	+	+

* Strain groupings pertain to inbred strain response to homozygous expression of mutations at the "diabetes" (*db*) locus (see Leiter et al[2]).
† Not done.

associated with impending cell death. In this regard, a significant increase in the number of IAPs was observed in CBA/J B cells undergoing complement-mediated immune lysis *in vitro* within a 4-hour period.[18] Even if immunogenic quantities of non-IAP-associated p73 were not actively shed from, or incorporated into, the cell surface, presentation of the antigen might nevertheless occur at the time of B cell destruction. For example, phagocytosis of these necrotic B cells by intraislet macrophages is a consistent finding[3,15]; this might serve as a means of "processing" the antigen for presentation to the immune system.

We have previously shown that diabetes-susceptible *db/db* mice are extremely sensitive to dietary carbohydrate, with B cell destruction being circumvented when carbohydrate-free diets are fed.[23,24] Thus, high-carbohydrate diets in some way interact with both the *db* gene and background genetic factors to elicit B cell necrosis. The appearance of increased numbers of IAPs in B cells appears to be glucose-dependent.[4] Glucose is clearly an important regulator of genetic expression in B cells, influencing the level of preproinsulin mRNA.[25,26] It is perhaps not surprising, then, that all four inbred strains with diabetes-susceptible backgrounds (ie, those that developed severe chronic hyperglycemia in response to the diabetogenic stress imposed by the *db* genes) were constitutive producers of IAPs in B cells (Table 1). At the present time, no causal relationship between IAP induction in glucose-stressed, prenecrotic B cells and their eventual necrosis can be established on the basis of these purely morphologic studies. Conceivably, IAPs could be involved in pathogenesis through eliciting systemic (autoimmune?) reactions such as reported in *db/db* mice by Debray-Sachs et al.[8] This prediction could be tested by making diabetes-prone neonates "tolerant" to p73 and then determining whether the course of diabetes would be altered from a severe to a mild syndrome. It should be noted that the presence of intracellularly budding retroviruslike particles has been associated with cell destruction in two other murine disease models. Expression of "aberrant" Type C retrovirus within lower motor neurons of mice has been associated with a "slow virus" type of neuropathy.[27] In CD-1 mice, treatment with multiple low doses of streptozotocin induced a similar "aberrant" Type C particle in B cells several days prior to the onset of B cell necrosis and insulitis; in inbred C57BL/KsJ mice, the same streptozotocin treatment induced IAPs.[28] IAP genes in *Mus musculus* constitute one of the most reiterated sets of structural genes yet observed.[29] The present study has demonstrated that the constitutive ability to express some of this genetic information in B cells characterizes those inbred mouse strains susceptible, but not those resistant, to the diabetogenic action of the "diabetes" gene.

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