Type C Retrovirus Production by Pancreatic Beta Cells

Association With Accelerated Pathogenesis in C3H-db/db ("Diabetes") Mice

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C3H.SW/SnJ females (haplotype *H-2*^{b}) were mated with C3HeB/FeJ males (haplotype *H-2*^{k}), which were heterozygous for the recessive mutation, diabetes (*db*). The object of the study was to analyze whether the *H-2*^{b} haplotype conferred diabetes resistance to *db/db* males in the F_2 generation. Severity of diabetes did not segregate with *H-2* haplotype in this mouse model of diabetes. Instead, all F_2 male mutants developed a much more severe diabetes syndrome than did "grandparental-type" C3HeB/FeJ-*db/db* males. Most surprisingly, unlike grandparental-type *db/db* females, which were uniformly resistant to the diabetogenic action of the *db* mutation, 75% of F_2 *db/db* females developed severe diabetes. Ultrastructural comparison of beta cells in islets of these diabetic females versus those in the diabetes-resistant F_2 mutants showed expression in the susceptible females of a type C retrovirus which budded extracellularly and intracellularly into vacuoles. No other islet endocrine cell type showed this expression, but intraislet exocrine cells as well as some ductal epithelial cells did produce type C particles. Macrophages were frequently observed in close association with the virus-expressing cell types. Failure of the diabetes acceleration factor(s) to segregate according to Mendelian expectations further suggested that type C retrovirus induction was linked to the accelerated pathogenesis of diabetes in the F_2 generation. (Am J Pathol 1985, 119:22–32)

THE MOUSE carries genomic information for producing at least three categories of endogenous retroviruses distinguishable on morphologic, serologic, and biochemical criteria. These are the type C particles associated with leukemogenesis and autoimmune diseases in mice, the type B particles (and their immature precursors, the intracytoplasmic type A particle) associated with mammary tumorigenesis, and the intracisternal type A particles (IAPs) which are normally expressed in early embryogenesis.[1,2] Both the type C and type B particles are shed from the cell surface, acquiring an outer unit (plasma) membrane envelope in the process. In contrast, IAPs remain sequestered intracellularly within the cisternae of the rough endoplasmic reticulum (RER) and do not bud from the plasma membrane. However, during a brief period of early embryogenesis, their 73,000-dalton group-specific antigen (p73) is expressed at the cell surface.[2]

Retroviral gene expression in pancreatic beta cells of adult mice may be of pathogenetic significance in the development of both chemically and genetically induced diabetes. Appel et al[3] described the induction of intracellular type C particles and IAP budding into the RER in prenecrotic beta cells of CD-1 male mice following diabetes induction by multiple low doses of streptozotocin. Intracellular type C particles have also been reported sequestered within the RER in prenecrotic beta cells of the nonobese diabetic (NOD) mouse.[4] When different inbred strains of mice were studied for their response to an autosomal recessive obesity mutation, diabetes (*db*), only those inbred strains constitutively able to express IAPs in the RER of their beta cells developed severe diabetes.[5] The appearance of increased numbers of morphologically identifiable IAPs in beta cells was accompanied by increased intracellular concentrations of p73; studies *in vitro* indicated that this induction was glucose-dependent.[5,6] The beta cell was the only islet cell type to exhibit these sequestered retroviruses in any of these models of diabetes, and in each model decrease in the size of the islets was pri-

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marily due to beta cell necrosis. Autoimmune mechanisms directed against beta cells have been implicated in the etiopathogenesis of diabetes in CD-1 males treated with multiple low doses of streptozotocin,[7] the NOD mouse,[8] and the C57BL/KsJ-*db/db* mouse.[9] Although type C particles have been observed to bud extracellularly from the pancreatic exocrine cells of inbred mice,[10,11] they have heretofore not been observed to bud from islet cells. Conceivably, retroviral proteins might pass into, or be secreted through, beta cell membranes in the absence of assembly of the entire particle at the cell surface, as occurs with p73 during early embryogenesis. If retroviral gene products at the surface of beta cells could indeed lead to immune sensitization of the host, then the presence of intact viral particles budding from beta cell surfaces might be expected to accelerate this process.

The unexpected discovery of accelerated diabetogenesis in a hybrid stock of C3H-*db/db* mice permitted an examination of this question in the present study. This stock was created for determination of whether the major histocompatibility complex (*H-2*) was an important modifier of inbred strain susceptibility to the diabetogenic action of the *db* mutation.[12] C3H.SW/SnJ females (haplotype *H-2*^{b}) were crossed with C3HeB/FeJ males (haplotype *H-2*^{k}) heterozygous for the "diabetes" mutation (+/*db*). The F_1 mice (all *H-2*^{b}/*H-2*^{k} heterozygotes) were intercrossed to produce an F_2 generation in which *H-2* and *db* would segregate independently. Since a sexual dimorphism in susceptibility was known to exist in "grandparental-type" *C3HeB/FeJ-db/db* mice (i.e., males susceptible, females resistant),[13] only F_2 *db/db* males were originally studied. All F_2 *db/db* males, regardless of *H-2* genotype (*H-2*^{b}/*H-2*^{b}, *H-2*^{b}/*H-2*^{k}, or *H-2*^{k}/*H-2*^{k}), showed an accelerated diabetogenesis, with 100% mortality before 6 months of age as compared with grandparental *db/db* males, which exhibited only 50% mortality by 8 months of age.[12] Failure to observe the expected segregation of these diabetogenic accelerating factors in the F_2 generation suggested that the C3H.SW/SnJ female grandparent had transmitted an extrachromosomal factor to all of her male progeny. Such transmission was very possible, because the C3HeB/FeJ subline carrying the *db* mutation was specifically created to eliminate certain C3H milk-borne retroviruses[14]; these would potentially have been reintroduced by mating with an unfostered C3H.SW/SnJ female. The accelerated diabetogenesis in F_2-*db/db* males led us to examine in the present study whether the diabetes resistance characterizing the grandparental-type C3HeB/FeJ-*db/db* females had been compromised in the F_2 generation and, if so, whether accelerated diabetogenesis was associated with shedding of retroviruses at the beta-cell surface.

Materials and Methods

Mice

The establishment and maintenance of a *db*-congenic stock of mice on the C3HeB/FeJ inbred background (*H-2*^{k} haplotype) has been described previously.[13] C3HeB/FeJ females are free of the Bittner agent (a milk-borne mouse mammary tumor virus) by virtue of an original transplantation of C3H/HeJ eggs into a C57BL/6J female.[14] Unfostered C3H.SW/SnJ females (*H-2*^{b} haplotype) were initially obtained from the research colony of Dr. Marianna Cherry, of the Jackson Laboratory. More recently, C3H.SW/SnJ$_f$ (caesarian-derived and fostered onto B6D2F$_1$ females) were obtained from the Animal Resources Department of the Jackson Laboratory. C3H.SW/SnJ-+/+ females were mated with C3HeB/FeJ-+/*db* males. The (C3H.SW/SnJ × C3HeB/FeJ-+/*db*)F$_1$ mice (all *H-2*^{b}/*H-2*^{k} heterozygotes) were progeny-tested for the presence of the *db* gene by mating to a known +/*db* heterozygote. The +/*db* *H-2*^{b}/*H-2*^{k} heterozygous F_1 mice thus identified were intercrossed to produce F_2 mice in which *db* and *H-2* haplotypes were segregating independently.

F_2 *db/db* mice were detected at weaning by their increased adiposity and were compared with a group of sex-matched lean littermate controls (designated as +/? because they include +/+ and +/*db* genotypes at a 1:2 ratio). Some of the +/*db* heterozygotes were identified by a test cross with a known +/*db* heterozygote followed by a progeny test for the appearance of *db/db* homozygotes. *H-2* typing to distinguish between parental (*H-2*^{b}/*H-2*^{b}, *H-2*^{k}/*H-2*^{k}) and recombinant (*H-2*^{b}/*H-2*^{k}) genotypes was performed by *in vivo* absorption of a monoclonal antibody against *H-2K*^{b} and a conventional anti-*H-2*^{k} alloantiserum as described in detail elsewhere.[12] Mating of F_2 +/*db* *H-2*^{b}/*H-2*^{b} males to C3H.SW/SnJ$_f$ females (Animal Resources Department, The Jackson Laboratory) and subsequent intercrossing of +/*db* heterozygotes (all *H-2*^{b}/*H-2*^{b}) followed by further backcrosses to the C3H.SW/SnJ$_f$ mice effected the "trapping" of the *db* mutation into the C3H/SW/SnJ subline.

Analytic Methods

Mice were allowed free access to water and food (diet 96W, Emory Morse Co., Guilford, Conn). Plasma glucose in nonfasted mice was determined monthly until death in males and for 12 months in females.[13] All females were sacrificed at 12 months of age. Pancreatic and plasma insulin were determined by radioimmunoassay.[13] An necropsy, pancreata were bisected (head to tail), one-half being extracted for insulin with the use of acidified ethanol and the other half being fixed in

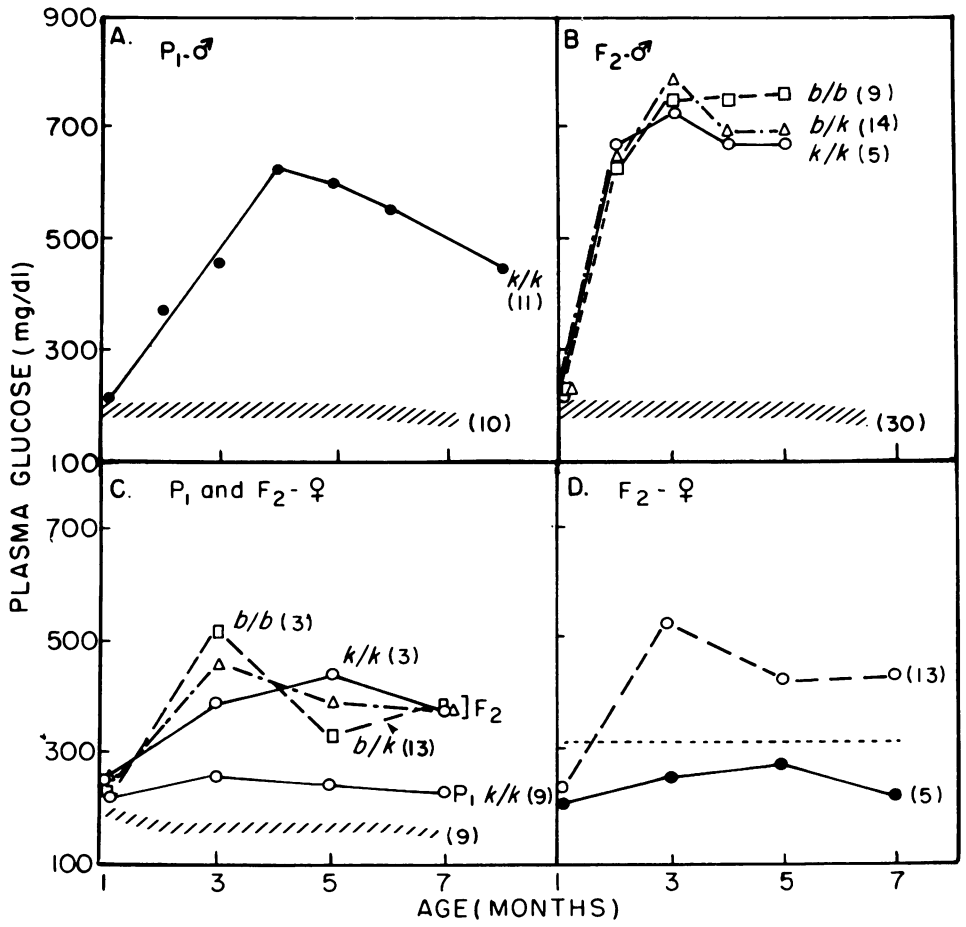

Figure 1—Changes in mean monthly plasma glucose in (**A**) P_1 (grandparental-type) C3HeB/FeJ-*db/db* males, (**B**) F_2 *db/db* males, and (**C**) P_1 and F_2 *db/db* females analyzed according to *H-2* genotypes ($H\text{-}2^b/H\text{-}2^b$, $H\text{-}2^b/H\text{-}2^k$, or $H\text{-}2^k/H\text{-}2^k$). In (**D**), the same F_2 *db/db* females as shown in (**C**) are analyzed not according to *H-2*, but according to whether mean monthly plasma glucose fell above or below 280 mg/dl. The *dotted line* drawn at 320 mg/dl represents this mean + 2 SD. Parentheses show the number of mice per group; *hatched lines* denote the mean plasma glucose levels of matched littermate controls.

Bouin's fluid for light-microscopic observation following aldehyde fuchsin staining of islets of 5-μ paraffin sections.[13] The protein content of the pancreatic extracts was also determined.[13]

Electron Microscopy

Pancreatic islets for ultrastructural observation were obtained from tribromoethanol-anesthetized +/? and *db/db* F_2 females between 5 and 8 months of age. Intraventricular perfusion of a rinse and fixative (2% glutaraldehyde, 1% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.2) was performed at a pressure of 70 mm of mercury as described previously.[15] Following perfusion, islets were hand-microdissected with the aid of a Wild dissecting microscope, were postfixed in 1% OsO_4, embedded, then sectioned, and examined with a Hitachi HU-11C transmission electron microscope operated at 75 kV.

Results

Diabetes Development in F_2 Mice

Age-related changes in plasma glucose in C3HeB/FeJ-*db/db* grandparental-type (P_1) and F_2 *db/db* mice of both sexes are shown in Figure 1. Input of diabetes-accelerating factor(s) from the C3H.SW/SnJ grandparental females to all F_2 *db/db* males, regardless of *H-2* genotype, has been described in an earlier report[12]; plasma glucose values and *H-2* genotypes for males are reproduced here to allow comparison to the pattern seen in F_2 females. Compared with C3HeB/FeJ-*db/db* grandparental-type (P_1) males (Figure 1A), F_2 *db/db* males developed a markedly accelerated hyperglycemia (Figure 1B). Grandparental-type (P_1) C3HeB/FeJ-*db/db* females were extremely resistant to the hyperglycemic action of the *db* genes (Figure 1C), whereas in F_2 *db/db* females a more severe hyperglycemia developed, again independent of *H-2* genotype (Figure 1C). In these females the obesity typically associated with the *db* mutation developed, and they weighed an average of 29 g more than +/? littermates at 12 months of age (69 ± 3 g for *db/db* versus 40 ± 2 g for +/? controls). The F_2 *db/db* females lived much longer than the F_2 *db/db* males. None in the group of F_2 *db/db* females described in Figure 1 had died of disease after 1 year of aging, whereas all F_2 *db/db* males were dead or moribund before the sixth month of age.[12] Although no segregation into diabetes resistant and diabetes susceptible classes were discerned when the hyperglycemia of

F_2 *db/db* females were grouped and analyzed according to *H-2* genotype (Figure 1C), a clear 3:1 segregation into susceptible versus resistant phenotypes could be demonstrated by computer sorting solely on the basis of whether an individual's mean monthly blood glucose fell above or below 280 mg/dl over an 8-month sampling period (Figure 1D). By the third month of age, in 13 of 18 females (roughly 75%) accelerated hyperglycemia developed, in comparison with C3HeB/FeJ-*db/db* grandparental-type females (P_1, Figure 1C), whereas 5 of 18 (roughly 25%) retained the grandparental resistance to induction of hyperglycemia in the presence of the *db* genes.

This 3:1 segregation pattern in F_2 *db/db* females clearly differentiated them from F_2 *db/db* males, all of which uniformly exhibited accelerated diabetogenesis. Such a 3:1 segregation suggested that the C3H.SW/SnJ female grandparent may have carried a dominant diabetes susceptibility gene, while the C3HeB/FeJ grandparental male carried the recessive allele. In males, this trait was presumably secondary to C3H.SW/SnJ-derived (extrachromosomal?) factors interactive with the male sex-associated traits (Y-chromosome genes?).[12] To determine whether a dominant gene for susceptibility (*S*) and its recessive allele for resistance (*s*) was segregating in the F_2 generation, we backcrossed two F_2-+ */db* $H\text{-}2^b/H\text{-}2^b$ males (potential genotypes at the *S* locus would be *SS*, *Ss*, or *ss* in 1/4:1/2:1/4 frequencies) to $C3H.SW_f$/SnJ females (putatively *SS* genotype). The probability that both F_2 males employed in this "trapping" cross was *ss* homozygotes would be 1/16; in this event the first backcross generation (BC_1) would all be *Ss*. Therefore, when BC_1 individuals were sib-mated for recovery of *db/db* mice (now all $H\text{-}2^b/H\text{-}2^b$ homozygotes and therefore called C3H.SW/SnJ-*db/db*), the maximum recovery of *ss* individuals in the *db/db* female group could be no greater than 1/64 (1/16 × 1/4, or 1.6%). The actual results obtained from these crosses are shown in Figure 2, with grandparental-type (P_1) mutant males (Figure 2A) and females (Figure 2C) compared with the C3H.SW/SnJ-*db/db* males (Figure 2B) and females (Figure 2D). The C3H.SW/SnJ-*db/db* males exhibited the same acceleration of diabetogenesis as had the F_2 mutant males; and again, no segregation equivalent to that observed for F_2 mutant females was observed. Instead, the C3H.SW/SnJ-*db/db* females continued to segregate into two phenotypes based on the extent of hyperglycemia and at the same 3:1 ratio previously observed in the F_2 generation (Figure 2D).

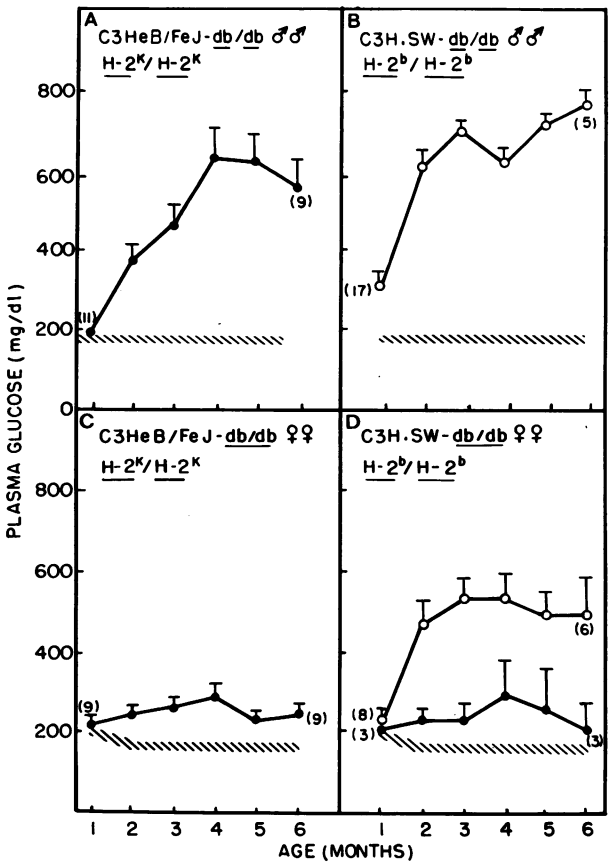

Figure 2—Effect of trapping the *db* mutation on the C3H.SW/SnJ inbred background (*H-2* haplotype). Mean monthly plasma glucose (+ SEM) for grandparental-type C3HeB/FeJ-*db/db* males (**A**) and females (**C**) are plotted for comparison to the C3H.SW/SnJ-*db/db* males (**B**) and females (**D**). In parentheses is the number of mice per group initially and surviving to 6 months; *hatched lines* denote the plasma glucose levels of matched littermate controls. The approximate 3:1 ratio of susceptible to resistant *db/db* females in **D** was considerably lower than expected (64/1) and was identical to that observed in the F_2 generation (see Figure 1D).

Light Microscopy of Islets and Islet Function

Figure 3 shows representative islet morphology in F_2 mice. Figure 3a illustrates normal, well-granulated beta cells in the islets of F_2 males determined by progeny testing to be +/+ homozygotes. Because F_2 *db/db* males died suddenly between 3 and 5 months of age, only five pancreases were obtained in adequate condition at necropsy for histologic analysis. The islets of Langerhans in these specimens were extremely degenerate in structure, containing fewer than 5% granulated beta cells (Figure 3b). In addition to aldehyde fuchsin-unstainable islet cells, the islet capsular space contained numerous enlarged intraislet ductular structures and exocrine cells. Mitotic figures were frequently observed in the duct cells; and, when an aldehyde fuchsin-positive beta cell was found, it was generally one cell removed from the ductal lumen. These lumens contained a mucuslike secretion that stained faintly with aldehyde fuchsin (Figure 3b); macrophages were often seen at the periphery of this secretion. Perivascular and ductular inflammatory cell infiltrates (small lymphocytes, macrophages, and a few polymorphonuclear neutrophils) were commonly observed; the inflammation in-

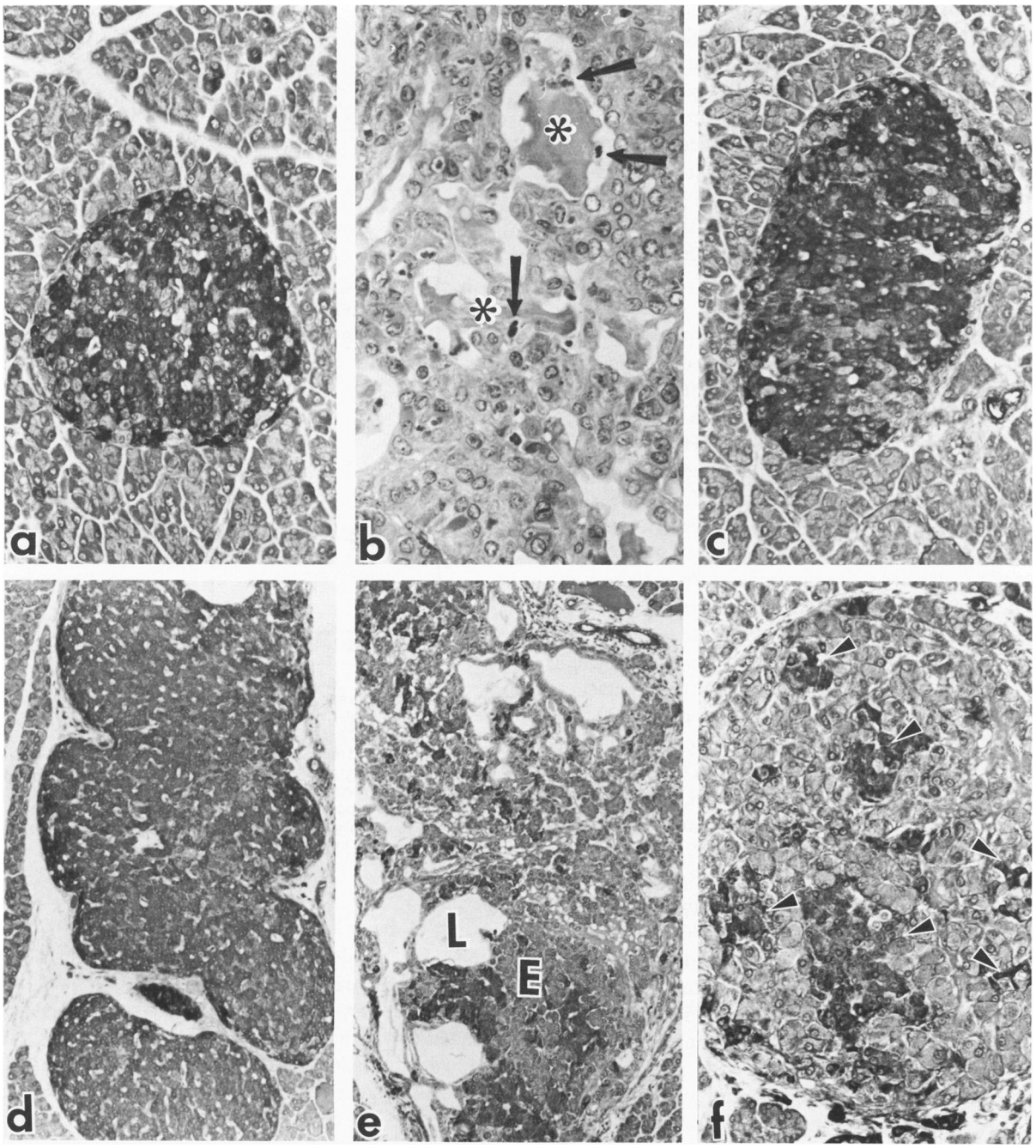

Figure 3—Light micrographs of aldehyde-fuchsin stained pancreatic islets in F_2 generation mice. **a**—Well-granulated islet in a +/+ male. (×400) **b**—Degenerate islet in pancreas of a 4-month-old *db/db* male. Signs of islet atrophy include absence of granulated beta cells and the proliferation of intraislet ducts. A weakly aldehyde fuchsin-positive stain is seen in a mucus secretion within the ductal lumen (*asterisk*); macrophages (*arrows*) are seen in association with this material. (×760) **c**—Well-granulated islet in a +/+ female. (×380) **d**—Hyperplastic, partially degranulated islets in pancreas of a hyperglycemia-resistant 12-month-old *db/db* female whose mean monthly plasma glucose over a 6-month period fell below 280 mg/dl (see Figure 1d). (×200) **e**—Hyperplastic islet undergoing necrosis in pancreas of a hyperglycemic 12-month-old *db/db* female whose mean monthly plasma glucose over a 6-month period fell above 280 mg/dl (see Figure 1D). Prominent lumens (*L*) created by intraislet duct proliferation, as well as infiltration of islet areas containing granulated beta cells by exocrine cells (*E*), and diffuse perivascular leukocytic infiltrates are seen. (×190) **f**—"Exocrinization" of an islet in the pancreas of a hyperglycemic 12-month old *db/db* female. The original islet zone is still demarcated from the exocrine parenchyma, but the islet itself is filled with exocrine cells, with only small rests of granulated beta cells (*arrows*) surviving. (×380)

volved the surrounding exocrine parenchyma as well as the damaged islets. Fibrotic changes were noted around the perimeter of some of these islets, but the most characteristic change was the complete replacement of islet tissue by exocrine cells which remained demarcated from the rest of the exocrine parenchyma by the islet capsular space (see, Figure 3f).

The histopathology of the pancreas of F_2 *db/db* females was consistent with plasma glucose concentration at necropsy (12 months). Islet structure and beta cell granulation in lean F_2 +/? females were normal (Figure 3c). Mutant females from the group whose mean monthly plasma glucose was below 280 mg/dl showed hypertrophy and hyperplasia of beta cells; aldehyde fuchsin staining of these beta cells was less intense, indicating partial degranulation. Radioimmunoassay of plasma insulin at sacrifice confirmed hypersecretion (mean ± SEM, 1009 ± 728 μU/ml, range, 118–3560, versus 49 μU/ml in +/? females). Similarly, total pancreatic insulin content was increased (21.7 ± 8 μg/mg pancreatic protein versus 6.4 ± 0.6 μg/mg in +/? controls), a reflection of the beta-cell hypertrophy and hyperplasia.

The histopathology observed in the hyperglycemic group of F_2 *db/db* females contrasted sharply with that observed in the hyperglycemia-resistant group. In the hyperglycemic group, the presence of greatly enlarged islets indicated that hypertrophy and hyperplasia of beta cells had occurred; however, most of these hyperplastic islets were at various stages of degeneration. One such islet mass illustrated in Figure 3e shows granulated beta cells scattered between intraislet ducts, exocrine cells, and leukocytes. An example of an "exocrinized" islet is shown in Figure 3f, wherein scattered groups of granulated beta cells are surrounded by exocrine cells within the islet capsule. Mean plasma insulin values obtained at sacrifice of these highly insulin-resistant mutants (mean ± SEM, 2080 ± 939 μU/ml; range, 246–5145) were twice as high as observed in the hyperglycemia-resistant *db/db* females, while pancreatic insulin content was only one-fifth as high (4.6 ± 0.8 μg/mg protein). This insulin content was 25% below the value for normal females (6.4 ± 0.6 μg/mg protein). Focal necrosis of the exocrine parenchyma, and fatty tissue replacement were frequently observed. In addition to these pancreatic lesions, hepatomas were found at a high incidence (65%) in these 12 month old females, and livers were fatty in all *db/db* mutants, regardless of sex or degree of hyperglycemia. The adrenal cortex and medulla were hypertrophied in females. Of the five *db/db* males studied, two had extensive nephritis in one kidney, consisting of multiple renal abscesses with surrounding granulomatous reaction.

Retroviral Activation in Beta Cells

Table 1 documents the types of retroviruses observed by electron microscopy in the pancreases of mice of the P_1 and F_2 generations. IAPs were constitutively expressed in small numbers in beta cells of C3HeB/FeJ-+/+ and C3H.SW/SnJ-+/+ parental generation mice. This expression was markedly enhanced by the hyperglycemia associated with diabetogenesis in the C3HeB/FeJ-*db/db* males. Expression was much less pronounced in the very mildly hyperglycemic C3HeB/FeJ-*db/db* females. No other islet endocrine cell type showed IAPs, and in none of these P_1 sublines was type C retrovirus ever observed in beta cells. However, immature type C particles were observed budding from exocrine cells, and mature type C were often seen in the intracellular spaces between exocrine cells. This type C expression was seen in exocrine cells of both normal and *db/db*

Table 1—Intracisternal Type A Particle (IAP) and Type C Retrovirus Expression in Pancreas of P_1 and F_2 Generation Mice

	Cell type and associated retrovirus					
	Beta		Exocrine		Ductal epithelial	
Genotype	IAP	Type C	IAP	Type C	IAP	Type C
P_1 Generation						
C3HeB/FeJ-+/+ males	+	–	+	+ +	–	–
C3H.SW/SnJ-+/+ males	+ +	–	+	+ +	–	–
C3HeB/FeJ-*db/db* males	+ + + +	–	+	+ + +	–	+ + +
C3HeB/FeJ-*db/db* females	+ +	–	+	+ +	–	–
F_2 generation						
+/? (both sexes)	+	–	+	+ +	–	–
db/db females, hyperglycemia-resistant	+ +	–	+	+ +	–	–
db/db females, hyperglycemia	+ + + +	+ + +	+ +	+ + + +	–	+ + +

Three mice of each category were sampled, with at least three islets and associated exocrine tissue examined from each individual. Relative abundance of retroviral particles for each cell type per grid was estimated on a scale in which – = absent, + = 1–5 particles, + + = 5–10 particles, + + + = 10–20 particles, and + + + + = more than 20 particles. F_2 *db/db* males were not analyzed because islet atrophy was too severe at the sampling points chosen (9 weeks and older).

P_1 generation mice. However, in C3HeB/FeJ-*db/db* males with severe hyperglycemia, type C viral expression was seen only in exocrine cells adjacent to or within the degenerate islets, and virus was also associated with the mucus secretion in the ductal lumens, apparently deriving from the ductal epithelial cells.

Neither normoglycemic F_2 +/? lean controls nor F_2 *db/db* females resistant to hyperglycemia differed from P_1 generation female mice with regard to retrovirus expression, with beta cells expressing IAPs, but not type C particles (Table 1). A striking difference, however, was noted in the F_2 *db/db* females which were hyperglycemic; the prenecrotic beta cells of these mice were expressing type C retrovirus that was being shed from the plasma membrane as well as budding intracellularly into vacuoles and lysosomelike structures (see Figure 5). Type C particles were also observed in the periinsular and intraislet exocrine cells, as well as the ductal lumens, apparently deriving from the ductal epithelial cells. IAP production was also greatly enhanced in these hyperglycemic mice.

Figure 4a contrasts the ultrastructural appearance of well-granulated beta cells in the enlarged islets of the diabetes-resistant F_2 *db/db* (type C-negative) with that of atrophic islets seen in hyperglycemic F_2 *db/db* females, in which both type C and IAP expression was high in beta cells (Figure 4b–d). This islet profile shown in Figure 4b is composed primarily of surviving alpha cells which are virus negative. An example of islet "exocrinization" (see Figure 3f) is shown in Figure 4c; a cluster composed of a well-granulated alpha cell and three beta cells at various stages of degranulation is completely enclosed by exocrine cells. A portion of a necrotic beta cell being engulfed by an intraislet macrophage is shown in Figure 4d.

The most noteworthy ultrastructural feature of the beta cells in these atrophic islets was the expression of type C particles typical of murine leukemia viruses at the beta cell surface (Figure 5a). The first distinct viral structures were electron-dense, crescent-shaped protrusions at the plasma membrane which developed short microvillous "stalks" as they budded (Figure 5a and inset). Mature virions, approximately 100 mμ in diameter, were observed in the intracellular spaces between beta cells. In addition to presentation at the cell surface, these particles budded intracellularly into cytoplasmic vacuoles (Figure 5b). Often these vacuoles were partially or completely filled with an amorphous, electron-dense substance such that they resembled lysosomes. Fusion of contiguous particles in areas of extensive virus budding from the vacuole membranes was sometimes observed (Figure 5c); presumably, such fused particles, in cross-section, produced the atypical tubular forms sometimes seen (Figure 5d). The above-described forms of type C retroviral expression were seen in approximately 10% of the beta cells surveyed in an islet. In contrast, the annular intracisternal type A particles (IAP) were present in nearly every beta cell examined (Figure 5e); the greatest concentrations were always observed in the most degranulated (hyperglycemia-stressed) beta cells.

The periinsular and intraislet exocrine cells in the hand-microdissected islet preparations expressed large numbers of type C particles along their cell surfaces (Figure 5f), without the intracytoplasmic budding forms of type C virus seen in beta cells. The intraislet ductal epithelial cells were filled with mucus droplets and appeared to be hypersecretory (Figure 5g): no intracytoplasmic type C or IAP retroviruses were noted. However, this cell type appeared to be shedding type C viruses, because the mucus secretion in the ductal lumens (see Figure 3b) sometimes contained considerable numbers of type C particles (Figure 5h).

Discussion

In a previous analysis of a sexually dimorphic expression of the *db* mutation in CBA/LtJ mice, we found that diabetes-resistant female mutants could not be converted to diabetes susceptibility by endocrinologic manipulation, even though the diabetes-susceptible males could be rendered diabetes-resistant by estrogen treatment.[15] In the present study, simply by mating C3HeB/FeJ-+/*db* males to C3H.SW/SnJ females and sib-mating the progeny, the female resistance was abrogated in 75% of the F_2 *db/db* females, and the male susceptibility was markedly exacerbated in 100% of the F_2 *db/db* males. The failure of the diabetogenic accelerator factor(s) to segregate in these males, as well as the failure to decrease the 25% frequency of *db/db* females retaining resistance following backcross to C3H.SW/SnJ females, strongly suggested congenital transmission of a retrovirus from C3H.SW/SnJ females into the C3HeB/FeJ subline derived originally to be free of such C3H maternally transmitted retroviruses. Congenital infection of offspring by the mother can occur via direct infection of ova, via intrauterine infection of the embryo, or via the milk at birth. Although the classic example of a milk-borne retrovirus is MMTV, ecotropic type C retrovirus may also be disseminated through milk to offspring. Melief et al[16] achieved transfer of high titers of MuLV by foster-nursing of neonates from a virus-negative stock (C57BL/10J) by females of a virus-positive congenic resistant stock (B10.A/SgSn). Interestingly, when the virus-positive B10.A females were mated with virus-negative B10 males, only 75% of the progeny were virus-positive, matching the figure of 75% of diabetes-accelerated female *db/db*

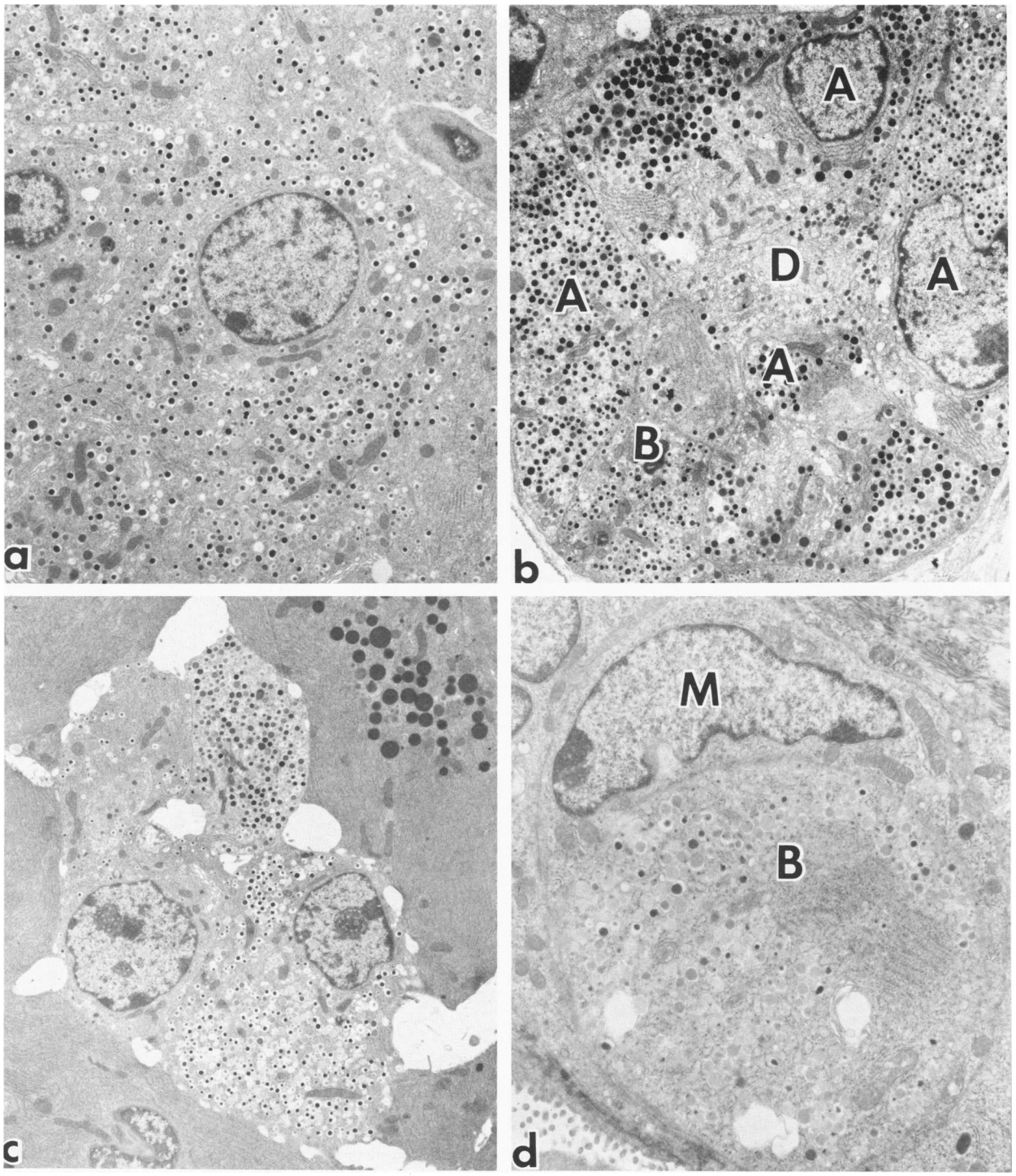

Figure 4—Electron micrographs of pancreatic islets in F_2 *db/db* female mice at 12 months of age. **a**—Well-granulated beta cells constitute the majority of cells in the hyperplastic islets of a diabetes-resistant female. (×2600) **b**—Atrophied islet in the pancreas of a hyperglycemic female. This field consists mainly of alpha (*A*) cells, a delta (*D*) cell, and a beta (*B*) cell. (×4200) **c**—A small rest of granulated islet cells remain within an islet capsule infiltrated by exocrine cells (see Figure 3) in the pancreas of a hyperglycemic female. (×8442) **d**—A portion of a beta cell (*B*) exhibiting degenerative cytoplasmic changes being enveloped by a macrophage (*M*) in a atrophic islet in the pancreas of a hyperglycemic female. (×2850)

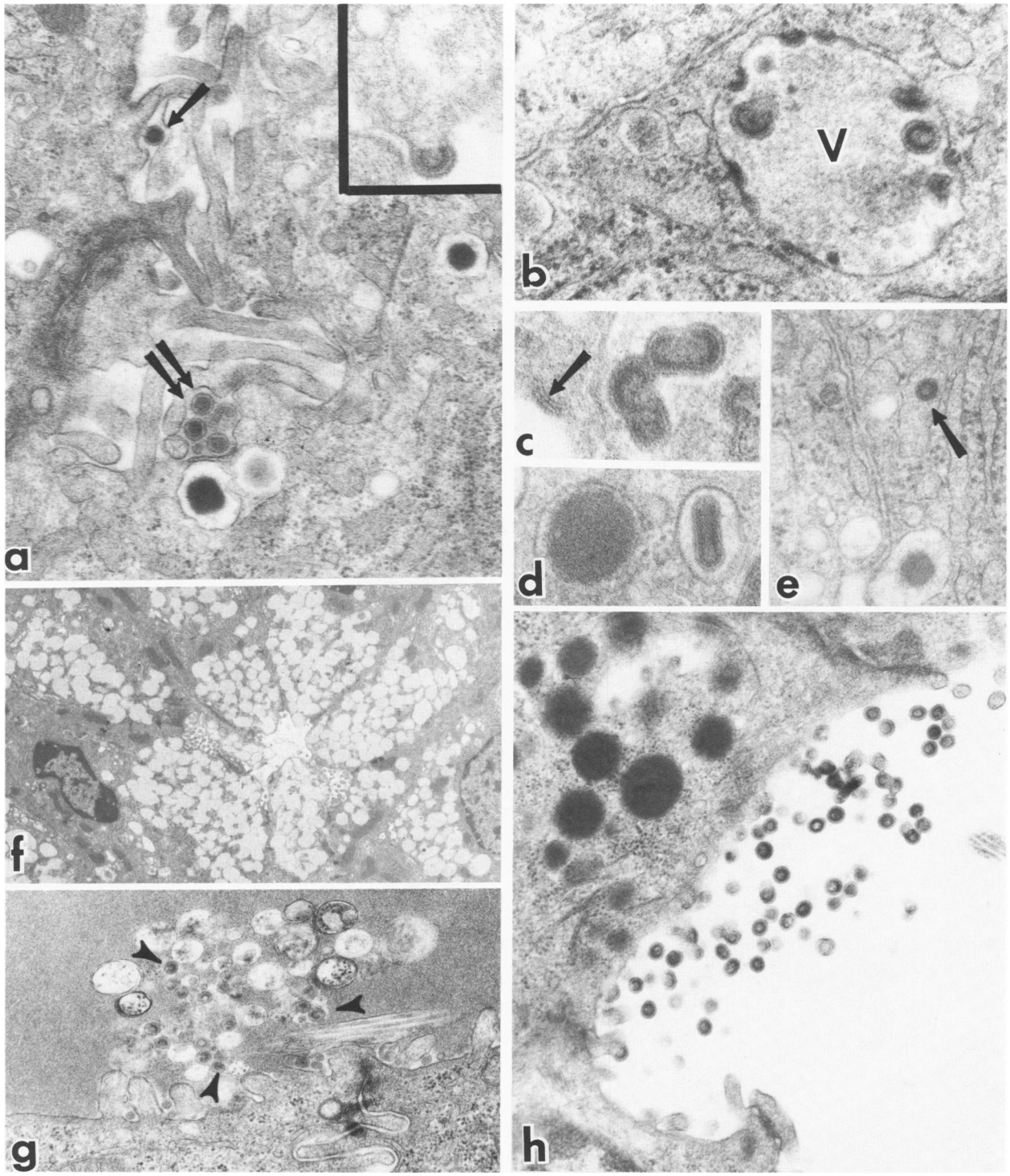

Figure 5—Retroviral expression in islets of hyperglycemic F_2 *db/db* females. **a**—Budding of immature type C particle (*single arrows*) from the plasma membrane of a beta cell. Mature virions (*double arrows*) are seen in the intracellular space. (×34,970) **Inset** shows detail of the semicircular viral nucleocapsid forming at the beta-cell surface (×72,792) **b**—Appearance of "aberrant" intracellular forms of the type C retrovirus budding into a vacuole (*V*) in the beta-cell cytoplasm. The vacuoles contain an amorphous, electron-dense substance and sometimes appear to be lysosomes. (×59,825) **c** and **d**—Intracellularly-budding type C particles apparently fusing by pairs to form elongated tubular elements. An early stage in the formation of a typical trilaminar viral nucleocapsid is seen in **c** (*arrow*). (**c**, ×91,680; **d**, ×64,640) **e**—An intracisternal type A particle (*arrow*) in the cytoplasm of the degranulated beta cells. This was the most commonly encountered retrovirus in beta cells of hyperglycemic mice and also was present at low density in beta cells of normoglycemic mice of both C3H sublines. (×45,450) **f** and **g**—Hypersecretory intraislet duct cells associated with an atrophic islet. Type C particles (*arrows*) are present in the ductal secretion. Macrophages were often seen in the ductal lumens apparently phagocytosing ductal secretions (see Figure 3b). (**f**, ×11,349; **g**, ×26,658) **h**—Extensive shedding of type C particles from the surface of an intraislet exocrine cell. Some of the budded virons are fusing. (×28,560)

progeny in the present cross. Thus, genomic integration of congenitally transmitted retrovirus appears not to occur in all individuals. The absence of mammary tumors in 12-month-old + /? and *db/db* females indicated that an exogenous MMTV had not been transmitted. Moreover, our ability to retain accelerated diabetogenesis on a line of C3H.SW$_f$/SnJ mice (fostered free of C3H milk factors) indicated that the milk was not the likely vector of transmission, and that the type C viral expression in beta cells probably reflected amplification of an endogenous C3H.SW retroviral genome. The high incidence of hepatomas found in the *db/db* females was probably a function of the hyperinsulinemic state produced by the obesity (*db*) mutation, because a similar high incidence was reported in obese C3H-A^{vy} male mice.[17] In comparing the severity of the diabetic syndrome in various inbred mouse strains, we had previously noted an association between the *H-2*^{b} haplotype and resistance to the diabetogenic action of the *db* mutation.[13] This association was apparently coincidental because the present study has clearly demonstrated lack of *H-2* influence on syndrome development on the C3H inbred strain background. In addition, we had previously noted a strong association between diabetes susceptibility in inbred mice and their ability to express IAP retroviral gene products in their beta cells.[5] Increased intracellular p73, demonstrable by immunocytochemical methods, as well increased numbers of IAPs, identified by electron microscopy, led us to propose that if exposed to cells of the immune surveillance system, p73 might be perceived as a neoantigen. If this hypothesis were correct, then a demonstration of increased retroviral activity at the beta cell surface should be reflected by an accelerated destruction of beta cells. Our finding of a type C particle being shed from mouse beta cells is novel; that this novel expression was observed only in beta cells of the *db/db* females with accelerated diabetogenesis and not in beta cells of the diabetes-resistant females suggests to us that elaboration of retroviral gene products at the surfaces of beta cells leads to immune sensitization of the host. Only the beta cells showed this activation of retroviral genomes, and the beta cells were the only endocrine islet cell type destroyed. It should be mentioned that certain retroviral proteins can be integrated into the plasma membrane at sites not associated with maturation of virus particles, eg, the type C *env* gene product, gp70, and the IAP gene product, p73, in early embryogenesis.[2,18] Elevated serum levels of gp70 have been associated with accelerated pathogenesis of lupus nephritis in autoimmune BXSB male mice.[19] A recent report has shown that a noncytopathic viral infection of mouse beta cells can lead to deterioration of beta cell function as a result of persistent infection.[20] Such a finding may explain the male glucose intolerance observed in the "Wellesley hybrid," a cross between C3H$_f$ females and I strain males[21]; ie, the I strain could have contributed a retroviral genome to the foster-derived C3H line. Further, the present study suggests a potential explanation for the observation that F$_1$ males of a cross between C3H/Tif females and DBA/2J males were sensitive to diabetes induction by multiple, low doses of streptozotocin, whereas the parental males were resistant.[22] The cytopathologic significance of the intracellular forms of type C virus in beta cells is unknown. The observation of aberrant type C particles budding into intracellular vacuoles is not unique to the present study; it has been described in the beta cells of outbred CD-1 male mice following administration of multiple low doses of streptozotocin.[3] Following infection of inbred mice with a type C retrovirus isolated from wild (Lake Cassitas) mice, lower limb paralysis develops which is characterized by intracellular budding of type C particles in the degenerating central nervous system myelinated neurons.[23] Perhaps noteworthy in this regard is the finding of accelerated thymus atrophy and T-lymphopenia in C57BL/KsJ-*db/db* mice.[9,24] Retroviruses have been reported to produce T-lymphocyte dysfunction in mice,[26] and an antiserum directed against a recombinant virus gp70 also recognizes a thymus differentiation antigen.[26] The recent demonstration of a human retrovirus associated with T-lymphopenia in humans with acquired immunodeficiency syndrome (AIDS) further emphasizes the cytopathic potential of certain retroviruses.[27]

The leukocyte most commonly observed by light and electron microscopy to have infiltrated into the islets in hyperglycemic *db/db* mice was the macrophage. Possibly these intraislet macrophages were processing antigens for presentation to lymphocytes. We have recently demonstrated that macrophages may spontaneously express cytotoxic activity against beta cells *in vitro*,[18] and so a destructive role for the macrophage itself must be considered. Screening sera from C3HeB/FeJ mice by indirect fluorescence microscopy, we have found autoantibodies reacting with cells in the islets of Langerhans in 40% of *db/db* males (all hyperglycemic) but in none of the nearly normoglycemic C3HeB/FeJ-*db/db* females (Ji-Won Yoon, E. H. Leiter, and D. L. Coleman, manuscript in preparation). Antibodies against insulin and p73 are being detected at serum dilutions of 1/100 and greater with the use of enzyme-linked immunosorbent assay (ELISA); studies to screen the sera collected from F$_2$ *db/db* mice for antibodies against type C antigens (p30, gp70) are now in progress, as are host range and tropism studies. Continued study of the kinds of endogenous virus gene expression that can be elicited in mammalian beta cells is clearly important.

References

1. Teich N: Taxonomy of Retroviruses. Molecular Biology of Tumor Viruses, RNA Tumor Viruses, 2nd edition. Edited by R Weiss, N Teich, H Varmus, J Coffin. Cold Spring Harbor, Cold Spring Harbor Laboratory Publications, 1982, pp. 25-207
2. Huang TTF, Calarco AG: Evidence for the cell surface expression of intracisternal A particle-associated antigens during early mouse development. Dev Biol 1981, 82:388-392
3. Appel MC, Rossini AA, Williams RM, Like AA: Viral studies in streptozotocin-induced pancreatic insulitis. Diabetologia 1978, 15:327-336
4. Fujita H, Fujino H, Nonaka K, Tarui S, Tochino Y: Retrovirus-like particles in pancreatic B-cells of NOD (non-obese diabetic) mice. Biomed Res 1984, 5:67-70
5. Leiter EH, Kuff EL: Intracisternal type A particles in murine pancreatic B cells: Immunocytochemical demonstration of increased antigen (p73) in genetically diabetic mice. Am J Pathol 1984, 114:46-55
6. Leiter EH, Bedigian HG: Intracisternal type A particles in genetically diabetic mice: Identification in pancreas and induction in cultured beta cells. Diabetologia 1979, 17:175-185
7. Rossini AA: Animal models of insulin dependent diabetes. Immunology in Diabetes. Edited by D Andreani, U DiMario, KF Federlin, LG Heding. London, Kimpton Medical Publications, 1984, pp 35-44
8. Kataoka S, Satoh S, Fujiya H, Toyota T, Suzuki R, Itoh K, Kumagai K: Immunologic aspects of the nonobese diabetic (NOD) mouse. Abnormalities of cellular immunity. Diabetes 1983, 32:247-253
9. Debray-Sachs M, Sai P, Boitard C, Assan R, Hamburger J: Antipancreatic immunity in genetically diabetic mice. Clin Exp Immunol 1983, 51:1-7
10. Boiocchi M, Della Torre G, Della Porta G: Genetic control of endogenous C-type virus production in pancreatic acinar cells of C57BL/He and C57BL/6J mice. Proc Natl Acad Sci USA 1975, 72:1892-1894
11. Harrison AK, Murphy FA: Murine oncornavirus activation in the pancreas during infection with Venezuelan equine encephalitis virus. J Natl Cancer Inst 1975, 55:917-923
12. Leiter EH: Genetic control of pathogenesis of diabetes in C3H mice: influence of the major histocompatibility complex. Diabetes 1984, 33:1068-1071
13. Leiter EH, Coleman DL, Hummel KP: The influence of genetic background on the expression of mutations at the diabetes locus in the mouse: III. Effect of H-2 haplotype and sex. Diabetes 1981, 30:1029-1034
14. Staats J: Standardized nomenclature for inbred strains of mice: seventh listing. Cancer Res 1980, 40:2083-2128
15. Leiter EH: The influence of genetic background on the expression of mutations at the diabetes locus in the mouse: IV. Male lethal syndrome in CBA/Lt mice. Diabetes 1981, 30:1035-1044
16. Melief CJM, Louie S, Schwartz RS: Ecotropic leukemia viruses in congenic C57BL mice: Natural dissemination by milk-borne infection. J Natl Cancer Inst 1975, 55: 691-698
17. Sass B, Vernon ML, Peters RL, Kelloff GJ: Mammary tumors, hepatocellular carcinomas, and pancreatic islet changes in C3H-A^{vy} mice. J Natl Cancer Inst 1978, 60:611-621
18. Dickson C, Eisenman R, Fan H, Hunter E, Teich N: Protein biosynthesis and assembly, Molecular Biology of Tumor Viruses, RNA Tumor Viruses. 2nd edition. Edited by R Weiss, N Teich, H Varmus, J Coffin. Cold Spring Harbor, Cold Spring Harbor Laboratory Publications, 1982, pp 513-648
19. Izui S, Hara I, Hang LM, Elder JH, McConahey RJ, Dixon FJ: Association of elevated serum glycoprotein gp70 with increased gp70 immune complex formation and accelerated lupus nephritis in autoimmune male BXSB mice. Clin Exp Immunol 1984, 56:272-280
20. Oldstone MBA, Southern P, Rodriguez M, Lambert P: Virus persists in B cells of islets of Langerhans and is associated with chemical manifestations of diabetes. Science 1984, 234:1440-1443
21. Like AA, Steinke J, Jones EE, Cahill GF: Pancreatic studies in mice with spontaneous diabetes mellitus. Am J Pathol 1965, 46:621-644
22. Kromann H, Christy M, Lernmark Å, Nedergaard M, Nerup J: The low dose streptozotocin murine model of type 1 (insulin dependent) diabetes mellitus: Studies in vivo and in vitro of the modulating effect of sex hormones. Diabetologia 1982, 22:194-198
23. Andrews JM, Gardner MR: Lower motor neuron degeneration associated with type C RNA virus infection in mice: Neuropathological features. J Neuropathol Exp Neurol 1974, 33:285-307
24. Dardenne M, Savino W, Gastinel L-N, Nabarra B, Bach J-F: Thymic dysfunction in the mutant diabetic (*db/db*) mouse. J Immunol 1983, 130:1195-1199
25. Copelan EA, Rinehart JJ, Lewis M, Mathes L, Olsen R, Sagone A: The mechanism of retrovirus suppression of human T cell proliferation in vitro. J Immunol 1983, 131:2017-2020
26. Johnson DA, Elder JH: Antibody directed to determinants of a Moloney virus derived MCF gp70 recognizes a thymic differentiation antigen. J Exp Med 1983, 159:1751-1756
27. Gallo RC, Salahuddin SZ, Popovic M, Shearer GM, Kaplan M, Haynes BF, Palker TJ, Redfield R, Oleske J, Safai B, White G, Foster P, Markham PD: Frequent detection and isolation of cytopathic retrovirus (HTLV-III) from patients with AIDS and at risk for AIDS. Science 1984, 224:500-503
28. Schwizer RW, Leiter EH, Evans R: Macrophage-mediated cytotoxicity against cultured pancreatic islet cells. Transplantation 1984, 37:539-544

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