SUSCEPTIBILITY OF MICE TO GROUP B COXSACKIE VIRUS IS INFLUENCED BY THE DIABETIC GENE*

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It is generally accepted that diabetes mellitus is a heritable disease, but the pattern of inheritance remains to be precisely defined. Recent epidemiological reports have indicated that infection with group B Coxsackie viruses may also play a role in some cases of this disease in man (1–3). These two observations suggest that a host carrying the gene(s) for diabetes may react differently to infection with these viruses than a similar host who is lacking this gene(s). The present report describes experiments to test this hypothesis using Coxsackie virus B4 (CB4),[1] C57BL/Ks mice, and the genetic variants derived from this murine strain which are homozygous and heterozygous for the diabetic gene, *db* (4).

Materials and Methods

Virus. The virus used in this study was isolated in rhesus renal cell cultures from myocardial tissue of an infant with encephalohepatomyocarditis and focal necrosis and inflammation of the pancreas (5). It was identified as Coxsackie virus group B, type 4 (CB4; Edwards strain) by neutralization tests with specific antiserum in monkey renal cell cultures. The isolate was passed once intraperitoneally in the suckling mouse, then inoculated into monkey renal cell cultures. This culture fluid was stored in 1958 in sealed ampuls at −55°C until recently, when it was passed once in HeLa cells to check its infectivity. Virus from the HeLa cell cultures was then passed three times sequentially by intraperitoneal inoculation into outbred adult male and female CD-1 mice (Charles River Breeding Laboratories, Wilmington, Mass.). Each successive inoculum consisted of a cell-free suspension of homogenized pancreatic tissue taken 3 days after the previous inoculation. A virus pool was then prepared in HeLa cells and stored at −70°C as a cell-free suspension. This constituted the virus inoculum for the present study. The titer of this working pool was 2×10^8 plaque-forming units (PFU)/ml for HeLa cells. Preparation of tissues for viral assay and techniques for viral quantitation have previously been described (6).

Animals. Three genetic variants of the inbred murine strain C57BL/Ks (The Jackson Laboratory, Bar Harbor, Maine) were used. These differed only in the presence of the unit autosomal recessive gene for diabetes, *db*, located on chromosome 4 (linkage group VIII). They consisted of (*a*) the parental background strain, C57BL/Ks +/+, which has no reported predisposition to diabetes; (*b*) mice heterozygous for the diabetic gene, C57BL/Ks *db*/+, which are phenotypically and physiologically similar to the +/+ variant; and (*c*) mice homozygous for the diabetic gene,

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[1] *Abbreviations used in this paper:* CB4, Coxsackie virus, group B, type 4; EMC virus, encephalomyocarditis virus; PFU, plaque-forming units.

C57BL/Ks *db/db*. The mode of inheritance and phenotypic expression of the *db* gene in this host have already been described (7–11).

Mice were received at 3½–5 wk of age and treated as follows. The +/+ and *db*/+ mice were housed four per cage and allowed free access to water and Purina Lab Chow® (Ralston Purina Co., St. Louis, Mo.). The *db/db* mice, which are hyperphagic, were kept in individual cages and allowed water *ad lib*, but their food consumption was restricted. The daily food allotment for the *db/db* mice was determined by measuring the amount of food consumed each day by age- and sex-matched +/+ mice housed in individual cages. The average food allotment for the hyperphagic mice was as follows: 10–22 g mice received 4 g/day; 22–27 g mice received 3 g/day; mice larger than 27 g received 2 g/day. This dietary regime maintained noninfected *db/db* mice at near normal weight without hyperglycemia throughout the experimental period.

Experimental Procedure. Male mice, 6-wk old (±3 days), from each of the three genotypes were given single intraperitoneal inoculations with 0.5 ml of a suspension containing either 10^4, 10^6, or 10^8 PFU of CB4. All animals were weight matched in addition to being age and sex matched. Controls consisted of age-, sex-, and weight-matched uninfected mice of each respective genotype.

The mice were observed for up to 17 wk with mortality rate recorded daily. Representative mice from groups which had been inoculated with 10^8 PFU of CB4 were selected for histopathological examination during the acute stage of infection (3 days postinoculation) and also during the convalescent stage at 6 and 12 wk postinoculation. Animals from the corresponding uninfected control groups were also sacrificed at these times. Mice were killed by etherization and the dissected tissues fixed in buffered formalin. Paraffin-sectioned tissues were mounted and stained with hematoxylin and eosin.

Results

Mortality. The mortality response elicited by infection was different for the three variants (Fig. 1). All *db/db* variants died within 13 days after inoculation with each dose of virus administered, but the mean survival times were dependent upon the magnitude of the dose. The mean survival time of *db/db* mice inoculated with 10^8 PFU virus was only 3.3 days, while it was 5.6 and 7.4 days, respectively, for *db/db* mice inoculated with 10^6 and 10^4 PFU virus. Almost 90% (23/26) of the *db*/+ mice died after inoculation with 10^8 PFU virus. 21 of these died within the first 11 days. Administration of 10^6 PFU resulted in mortality of 50% of the *db*/+ mice, whereas only 10% of the +/+ mice died when given the equivalent dose. The 10^4 PFU dose killed no *db*/+ mice and only one from the +/+ group. Of the 124 mice inoculated in all three groups only 3 of the 79 cumulative deaths occurred after the 13th day. These occurred on days 20 and 120 in the *db*/+ group and on day 17 in the +/+ group; all of these were inoculated with 10^8 PFU CB4.

A more revealing comparison of the total mortality of the variants at each virus dose is presented in Fig. 2. With 10^4 PFU CB4 the total mortality of the *db*/+ variant is similar to that of the +/+ variant. Conversely, after inoculation with 10^8 PFU the response of the *db*/+ variant more closely resembles the *db/db* variant than the +/+ variant. This genotypic dependent mortality is best seen at the 10^6 PFU dose. Here the total mortality of the *db*/+ variant is almost equidistant between the *db/db* and +/+ variants.

Histopathology

ACUTE STAGE. Histopathological examinations revealed no significant findings in the tissues examined, including the pancreas, from noninfected +/+, *db*/+, and *db/db* mice. During the acute stage of infection histological examination

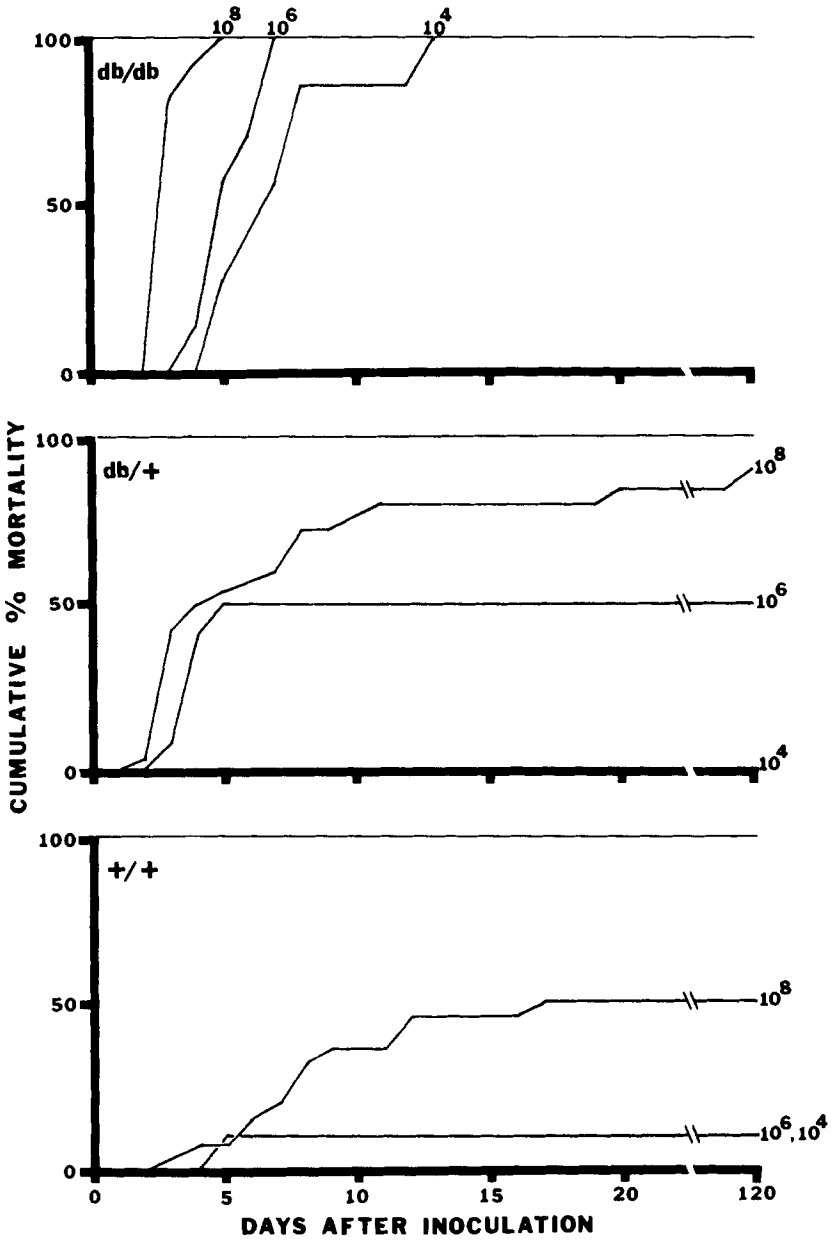

FIG. 1. Cumulative percent mortality of the three genetic variants of the murine strain, C57BL/Ks, after intraperitoneal inoculation with either 10^4, 10^6, or 10^8 PFU CB4 per mouse. The mice were either homozygous for the diabetic gene (*db/db*), heterozygous (*db/+*), or lacked the gene (+/+).

revealed no substantial differences in pathological findings in the heart, brain, or adipose tissue of the three genetic variants. The myocardia contained small necrotic lesions, while there were none observed in the endocardia or pericardia. These lesions were accompanied by a cell infiltrate which consisted primarily of

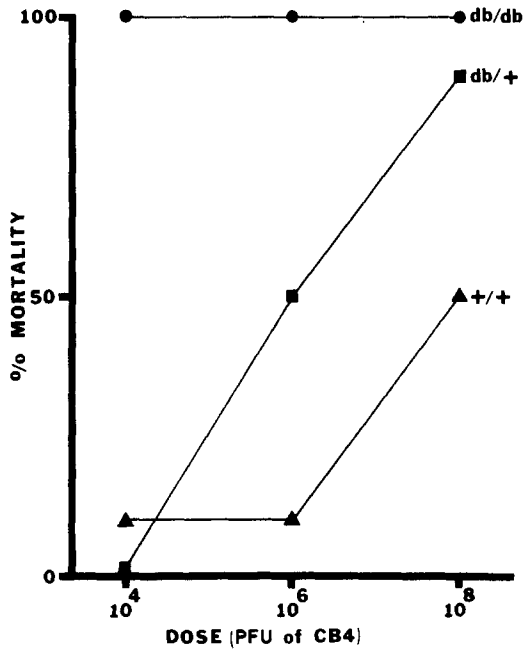

FIG. 2. Total mortality of the three genetic variants of the murine strain, C57BL/Ks, 120 days after intraperitoneal inoculation with either 10^4, 10^6, or 10^8 PFU CB4 per mouse. The mice were either homozygous for the diabetic gene (*db/db*), heterozygous (*db/+*), or lacked the gene (+/+).

lymphocytes and plasma cells. No lesions were observed in the brains, but a small infiltrate of chronic inflammatory cells was commonly found beneath the leptomeninges. Necrosis of perirenal and inguinal fat was a consistent observation by both gross examination and microscopically; the necrosis was accompanied by inflammation.

Livers of all three infected variants also showed necrosis. This was more central than periportal and the bile ducts were preserved. This process was most severe in the *db/db* mice and most of the hepatocytes were destroyed. It was less severe in the livers of *db/+* and +/+ mice. No cellular infiltrate was noted in this organ. Inclusion-like bodies were seen in the hepatocytes of one infected +/+ animal. Their staining properties and appearance were consistent with the coalesced fuchsinophile ("F") granules described by Pappenheimer (12).

Significant pathological differences were observed in the pancreases of the infected mice. In contrast to the pancreas of an uninfected +/+ variant (Fig. 3), the pancreas of the infected +/+ variant consistently exhibited acute and chronic pancreatitis (Fig. 4). Much of the exocrine tissue had been affected, but many islands of acinar cells filled with zymogen granules remained. Many acinar cells were degranulated and others had been destroyed and replaced with an infiltrate made up primarily of lymphocytes, plasma cells, and a few polymorphonuclear leukocytes and macrophages. The pancreatic ducts and islets of Langerhans appeared normal in the infected +/+ animals.

The exocrine pancreas of the *db/+* variant showed all degrees of degeneration

from complete degranulation to necrosis (Fig. 5). The ducts appeared normal and only subtle changes were apparent in some islets of Langerhans. Islet cell nuclei stained more darkly than those of the infected +/+ variant. This slight difference was most probably due to release of digestive enzymes from necrosis of the acinar portion.

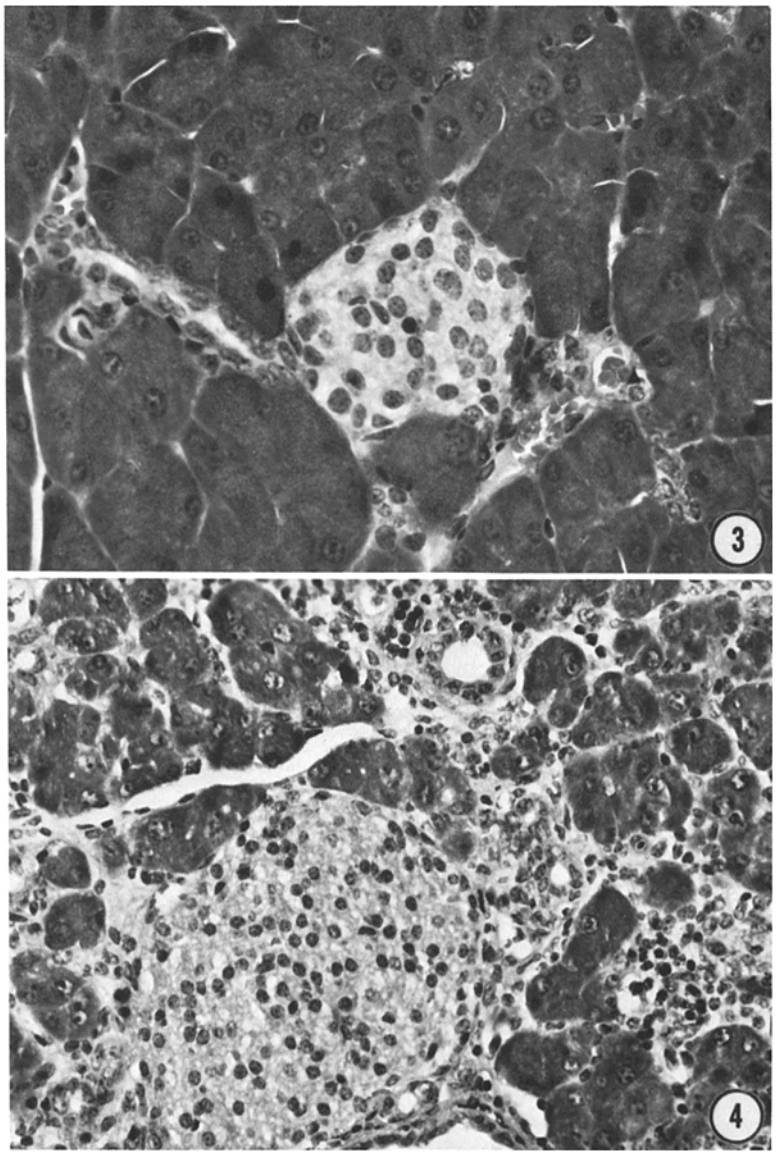

FIG. 3. Normal islet of Langerhans and surrounding exocrine tissue in the pancreas of a 6-wk-old male, C57BL/Ks mouse. Hematoxylin and eosin stain. × 375.

FIG. 4. Islet of Langerhans in pancreas of a 6-wk-old male, C57BL/Ks mouse, 3 days after CB4 inoculation. Note the loss of acinar tissue due to necrosis and the cellular infiltrate in the surrounding exocrine tissue. Hematoxylin and eosin stain. × 375.

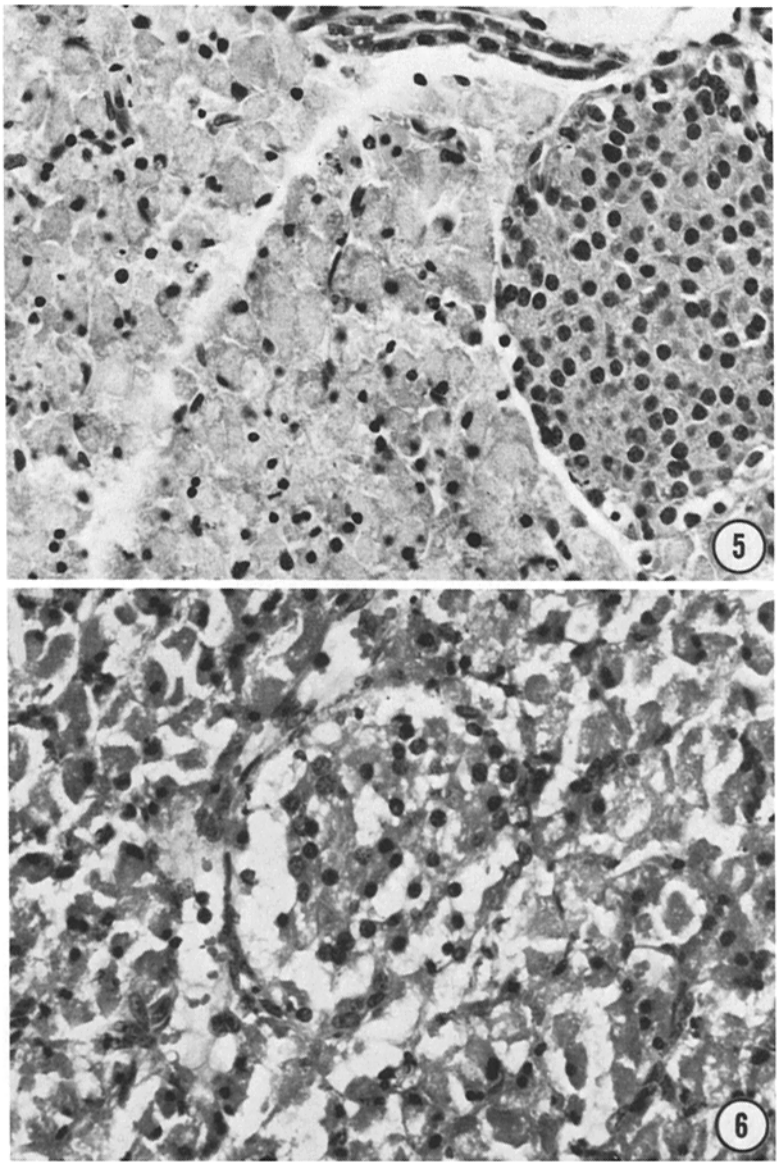

FIG. 5. Islet of Langerhans in pancreas of a 6-wk-old male, C57BL/Ks mouse, which is heterozygous for the diabetic gene, 3 days after CB4 inoculation. Note complete degranulation and extensive focal necrosis of the surrounding exocrine tissue. Hematoxylin and eosin stain. × 250.

FIG. 6. Islet of Langerhans in pancreas of a 6-wk-old male, C57BL/Ks mouse, which is homozygous for the diabetic gene, 3 days after CB4 inoculation. Note loss of morphological integrity of islet in the center of the field, with focal necrosis of individual islet cells. The exocrine tissue shows degranulation and focal cellular necrosis. Hematoxylin and eosin stain. × 375.

In contrast to findings in the +/+ and *db*/+ variants, the pancreas of the infected *db*/*db* variant exhibited both exocrine and endocrine involvement (Fig. 6). The exocrine portion showed massive necrosis with collapse of the cell outline and loss of lobular pattern. Most acinar cells were destroyed and the remaining cells were in various stages of degeneration. The ducts, again, appeared unaffected, but some of the islets of Langerhans had distinctive changes. Many islets were morphologically normal, but others exhibited indistinct cytoplasmic borders and necrosis of individual islet cells. Unlike the infected +/+ variants, there was no inflammatory cell infiltrate in the pancreases of any of the infected *db*/*db* or *db*/+ mice examined. No lesions were noticed during this acute stage in the spleen, bowel, lungs, skeletal muscle, kidney, adrenal gland, or testes, and spermatogenesis appeared active.

The cause of death in CB4-infected mice was not conclusively determined. Massive liver necrosis and fat necrosis were probably of sufficient magnitude to be primary contributing factors. Urinalysis revealed no glucose or ketones in moribund animals and their blood glucose levels were either normal or low at this time.

CONVALESCENT STAGE. There were no survivors remaining in the *db*/*db* group for histopathological examination at 6 and 12 wk after inoculation. No differences were observed between the *db*/+ or +/+ mice that survived the infection. All tissues examined in these mice appeared normal except for the heart and pancreas. The myocardia of infected *db*/+ and +/+ mice exhibited sparse areas of cytolysis with lymphocyte infiltration at 6 and 12 wk postinoculation, similar to that seen during the acute stage. Although the livers of both groups exhibited substantial necrosis during the acute stage, they appeared normal at this time. The exocrine pancreas, however, had not regenerated or recovered and was almost completely replaced with fatty tissue. Small areas of granulated acinar cells remained, but these comprised only a small proportion of the original volume of the pancreas. Ducts and islets of Langerhans were present in the fatty infiltrate; other than being surrounded by fat instead of acinar cells, the cells of the islets of Langerhans appeared normal (Fig. 7). A slight inflammatory cell infiltrate was present in the exocrine pancreases of both *db*/+ and +/+ variants during the convalescent stage. Its significance was not readily apparent.

Discussion

This host-virus system provides a useful model for examining the relative roles of genetic and nongenetic determinants in a viral disease. It differs from most others used for such studies in that the genetic factor (the *db* gene), its general mechanism of action, and its mode of inheritance are known (4, 7–11). We found that susceptibility of the murine strain C57BL/Ks to infection with group B Coxsackie virus was influenced by the autosomal recessive gene for diabetes. In addition, this genetically determined effect was expressed even though overt phenotypic expression of the gene was lacking in the heterozygous variant. Both mortality data and pathological findings were consistent with such an effect. The response of each genotype to the virus was dose dependent.

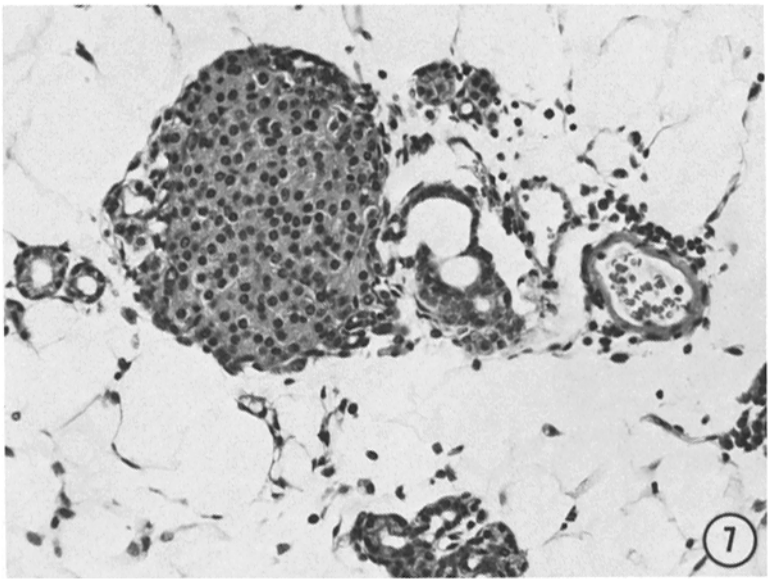

FIG. 7. Islet of Langerhans in pancreas of a male, C57BL/Ks mouse which is heterozygous for the diabetic gene, 12 wk after CB4 infection. Note normal appearance of islet and ducts surrounded by fat cells which have replaced the acinar cells. Hematoxylin and eosin stain. × 375.

Another picornavirus, encephalomyocarditis (EMC) virus, has been employed extensively in recent years to help define genetic and nongenetic factors which influence hosts' susceptibility to picornaviruses. In a recent review, Craighead compiled an impressive compendium of data on possible diabetogenic viruses (13). The "M" variant of EMC unquestionably produces a diabetes mellitus-like syndrome in mice which is influenced by nonspecified genetic factors as well as known genetic (sex) and nongenetic factors. Admittedly, however, the basis for the differences in response between mouse strains to EMC virus infection are not well defined and the pattern of inheritance of the predisposition to diabetes is not clear. Recent evidence, however, suggests that it is transmitted as an autosomal recessive trait (14).

EMC virus is not a common human pathogen and the M variant now being used in several laboratories has undergone considerable selection through consecutive passage in mice. The M variant is not known to be pathogenic for man, but it exhibits a specific tropism for cells of the islets of Langerhans in mice (15). The Coxsackie virus used in this study has less specific tissue tropism than EMC virus, but is a common human pathogen and has undergone little selection in the laboratory. The entire history was presented above (see Materials and Methods) since virus passage has been shown to influence the tissue tropism of Coxsackie viruses (16). Coxsackie virus has also been suggested as a diabetogenic agent. The possible etiological role of CB4 in diabetes mellitus has previously been reported in both man (1–3) and animals (17). Its etiological role in both, however, has not been confirmed.

The diabetic mutant mouse, C57BL/Ks *db/db*, has been one of several animal

models presented that might suffice as an experimental model for the study of diabetes mellitus in man. Mice homozygous for the gene are hyperglycemic and glucose intolerant and exhibit insulin insensitivity. They also exhibit glycosuria, polydipsia, polyuria, polyphagia, and a shortened life span. The heterozygous animals were originally thought to be normal, but older males (3- to 16-mo old) do exhibit slightly higher blood glucose and serum immunoreactive insulin levels than the normal C57BL/Ks mice and tend to attain greater body weights (11).

In as much as this animal represents a plausible experimental model for diabetes mellitus, these data support the contention that genetic predisposition to diabetes also conveys a greater susceptibility to Coxsackie virus infection. However, this is the first report of infection of the homozygous or heterozygous variant with a pathogen. The mortality response of these mice to other pathogens is unknown and deserves further study. Nevertheless, three facts suggest that the results reported here are probably specific to this system and not a generalized response of the genetic mutants to infection with a pathogen. They are (*a*) the primary difference in the pathological findings here was in the pancreas, (*b*) one of the primary abnormalities of this mutant involves glucose metabolism, and (*c*) Coxsackie virus B4 is pancreatropic in man and laboratory animals.

Despite the obvious shortcomings of animal models to describe disease states in man, the data support previous conclusions that environmental (viral) stress on a host is influenced by its genetic background. The host-virus system presented here should serve as an additional tool with which to describe and delineate genetic determinants for host susceptibility. Preliminary studies in our laboratories have shown that this system provides a model for viral-induced diabetes.

Summary

A positive correlation was found between genetic predisposition to diabetes in the mouse and susceptibility to group B Coxsackie virus in this host. Male mice of the inbred strain C57BL/Ks and the following genetic variants were used; mice homozygous for the autosomal recessive gene for diabetes (*db*/*db*), the phenotypically normal heterozygous (*db*/+), and the normal mice which lacked the diabetic gene (+/+). The mortality response of the +/+ mice to intraperitoneal inoculation with Coxsackie virus B4 differed from the response of the two genetic variants (*db*/*db* and *db*/+) derived from this strain. The *db*/+ variant was more susceptible to Coxsackie virus B4 than the parental background strain (+/+). The *db*/*db* variant was more susceptible than either of the other genotypes. Pathological findings of the pancreas of the three genotypes during the acute stage of infection closely paralleled the genotypically dependent susceptibility of the host.

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