# Virus-Induced Diabetes Mellitus
## Glucose Abnormalities Produced in Mice by the Six Members of the Coxsackie B Virus Group

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**SUMMARY**

**The capacity of Coxsackie B viruses (CBVs) to produce diabetes in mice was studied before and after passage in various cell types. CBVs that had been passaged in monkey kidney cells or in mouse embryo fibroblasts failed to produce abnormal glucose tolerance tests, whereas virus passaged five or more times in the pancreata of mice or in beta-cell cultures produced transient abnormal glucose tolerance tests. Immunofluorescence and histologic studies revealed that passage of CBVs in cultured beta-cells changed the tropism of these viruses from the acinar pancreas to the islets of Langerhans. Although all six CBV serotypes that had been passaged in beta-cell cultures behaved very similarly, substantial variation was observed with the different virus passages and in some experiments, beta-cell damage and the glucose abnormalities were minimal. From these and other experiments, we conclude that the six members of the CBV group have the potential for infecting and damaging pancreatic beta-cells in mice. DIABETES 31: 496–499, June 1982.**

In humans, members of the Coxsackie B virus group (CBV) produce a variety of clinical diseases, including epidemic pleurodynia, meningitis, encephalitis, pericarditis, myocarditis, orchitis, and pancreatitis.[1] Recently, evidence from several sources suggests that at least occasional cases of insulin-dependent diabetes mellitus (IDDM) may be caused by CBVs.[2] In newly diagnosed IDDM patients, an increase in antibody titer to CBVs has been reported by some, but not by other investigators.[3,4] Histologic examination of the pancreas from children dying of overwhelming CBV infections revealed evidence of insulitis and beta-cell damage.[5,6] CBVs also have been isolated or identified in the pancreas and stools of several children with acute-onset diabetes.[7–9] In two of these cases, the virus produced glucose abnormalities when injected into mice. Recently, in experimental animals, we showed that a standard laboratory strain of CBV-4, which initially failed to produce diabetes in mice, could be made to infect beta-cells and produce diabetes by first passaging the virus in cultures enriched in mouse beta-cells.[10] We now report that all six members of the CBV group will produce a mild and transient form of diabetes in mice, if first passaged in mouse beta-cell cultures.

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### METHODS

**Animals.** Male SJL mice (The Jackson Laboratory, Bar Harbor, Maine), 4–6 wk old, were infected intraperitoneally (i.p.) with $2 \times 10^5$ tissue culture infectious $doses_{50}$ ($TCID_{50}$) of virus.

**Virus passage and cell cultures.** CBV-1 (Conn-5), CBV-2 (Ohio-1), CBV-3 (Nancy), CBV-4 (JVB, Benschoten), CBV-5 (Faulkner), and CBV-6 (hemagglutinating strain 201869) were obtained from the American Type Culture Collection, Rockville, Maryland. The D variant of encephalomyocarditis virus (EMC-D) was prepared as previously described.[11] CBVs were assayed on monkey kidney cells (LLC-$MK_2$) by a microculture technique and the titer expressed as $TCID_{50}$. Cultures enriched for pancreatic beta-cells (i.e., between 15 and 30% beta-cells) were prepared from 3-wk-old SJL or NIH Swiss male mice as described elsewhere.[10] Viruses were serially passaged in monkey (LLC-$MK_2$) cells, secondary mouse embryo (SME) cells, mouse beta-cell cultures, or in vivo in the pancreata of weanling mice.

**Antisera.** For neutralization, reference horse antisera to CBVs 1–6 were obtained from the National Institute of Allergy and Infectious Diseases, and rabbit anti-EMC serum was produced in our laboratory. The antibodies used to detect viral antigens on frozen sections of pancreas[10] were prepared in SJL mice by repeated injections with prototype viruses and conjugated with fluorescein-isothiocyanate. Guinea pig anti-insulin serum was conjugated with tetramethyl-rhodamine-isothiocyanate.

**Histopathology.** Pancreata were fixed in Bouin's solution

5–20 days after infection, and sections stained with hematoxylin and eosin. Approximately 10 pancreata from mice infected with each serotype were studied and multiple sections from each pancreas were examined.

**Blood glucose assays.** Different passage levels of each CBV serotype were tested for their ability to induce diabetes. Mice were infected with passage 5, 10, 15, and 20 of beta-cell culture-grown viruses and with passage 3, 5, 10, and 12 of virus harvested from mouse pancreas. Each animal was bled on days 7 and 14 for nonfasting glucose (NFG), and on days 10 and 17 for glucose tolerance tests (GTT). GTTs were performed by i.p. injection of 2 mg glucose per gram body weight, and glucose levels were determined 60 min later.[10] The mean GTT level of 194 uninfected mice was 168 ± 26 mg/dl (mean ± SD). The mean NFG of 183 uninfected mice was 143 ± 24 mg/dl. Mice with glucose levels 3 SD above the mean (i.e., GTT > 246, NFG > 215) were considered diabetic. For brevity, only selected passages and the results of GTTs on day 10 are presented.

## RESULTS

The effect of virus passage on GTTs is illustrated in Figure 1. Except for an occasional animal, mice inoculated with CBVs that had been passaged in LLC-$MK_2$ or SME cells showed normal GTTs. In contrast, mice inoculated with CBVs that had been passaged in vivo in mouse pancreas or in cultured beta-cells showed abnormal GTTs, beginning at about the fifth passage. The most pronounced abnormalities occurred with viruses that had been passaged in beta-cell cultures 10 and 20 times (Figure 1D). Increased passage did not necessarily increase the severity of the hyperglycemia, and considerable variation was observed with the different viral passages, with some producing only minimal abnormalities. In general, the glucose abnormalities were transient, being detected at 10 and 17 days but returning to normal within 30 days. Elevated NFG levels were only occasionally seen in infected mice.

Evidence that at least some of the glucose abnormalities were secondary to beta-cell damage came from histologic studies. In agreement with already published reports,[12] prototype strains produced focal or diffuse pancreatic necrosis but spared the islets. Passage of prototype CBVs in vivo in mouse pancreas enhanced the tendency of these viruses to infect acinar cells, but also led to a low level of islet involvement. In contrast, the capacity of these viruses to infect acinar tissue was greatly reduced by passage in cultured SME cells. Passage of CBVs in beta-cell cultures also reduced their tropism for acinar tissue, but enhanced their capacity to infect islet cells. The beta-cell-passaged viruses produced varying degrees of islet damage, ranging from a few pycnotic nuclei in the majority of islets to focal necrosis and mononuclear cell infiltration in approximately 10–15% of the islets. Immunocytochemical staining with anti-insulin antibody 5 days after infection showed that the insulin content of some islets was grossly reduced, even in the absence of major pathologic changes. Islet alterations induced by the different serotypes (i.e., beta-cell-passaged) were indistinguishable, and the acinar tissue was, in general, remarkably preserved.

Immunofluorescence also showed that protype CBVs were acinar-tropic; however, after serial passage in beta-cell cultures, CBV-1 and -5 preferentially replicated in the

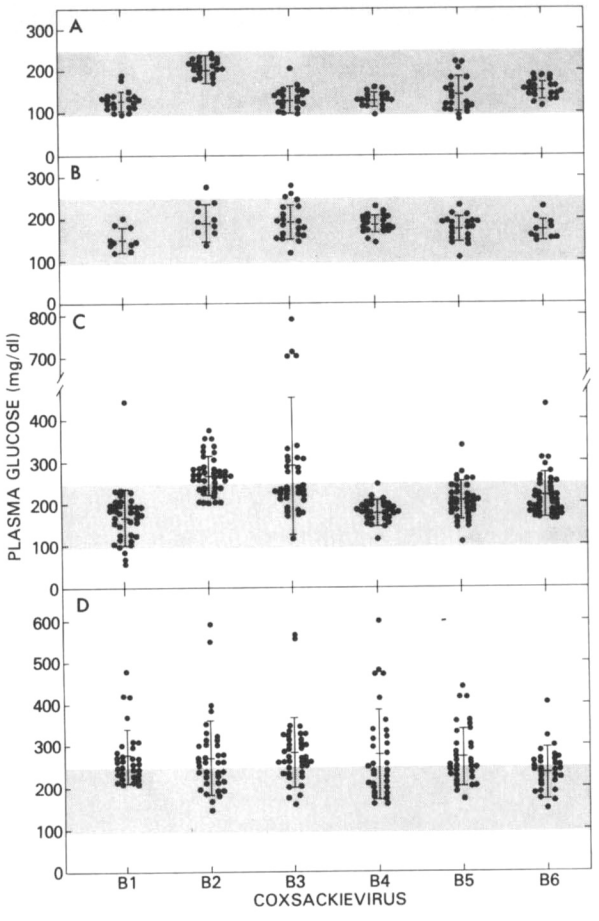


**FIGURE 1. Effect of virus passage on GTTs. Single-point GTTs in mice 10 days after infection with members of the CBV group. Each point represents an individual animal. The mean ± SD is shown by vertical lines and bars. Shaded areas represent the mean of 194 uninfected mice ± SD. Prototype CBVs: (A) passaged in LLC-$MK_2$ cells (two times); (B) passaged in SME cells (seven times); (C) passaged in vivo in mouse pancreas (combined results of 5th and 10th passage); (D) passaged in beta-cell cultures (combined results of 10th and 20th passage).**

islets of Langerhans with the exocrine tissue being relatively free of viral antigens (Figure 2). Similar observations, to a greater or lesser degree, were made with the other CBVs. Proof that beta-cells were actually infected, came from double staining with fluorescein-labeled antivirus antibody and rhodamine-labeled anti-insulin antibody (Figures 2D and E). The number of infected cells varied considerably among islets, but in general only 5–15% of the islet cells contained viral antigens.

To see if the degree of hyperglycemia produced by CBVs could be amplified, the beta-cell reserve was reduced by giving mice a subdiabetogenic dose of streptozotocin (SZ).[13] Figure 3 shows that GTTs of mice given SZ and subsequently infected with CBVs were considerably more elevated than those of infected mice not pretreated with SZ. Moreover, between 21% and 56% of SZ-treated and infected mice showed elevated NFG, and these elevated glucose levels persisted in some of the animals until the termination of the experiment on day 30 (data not shown).

To ensure that the elevated glucose levels were specifically induced by each of the six CBVs and not by inadvertent cross-contamination, a high passage level of each CBV

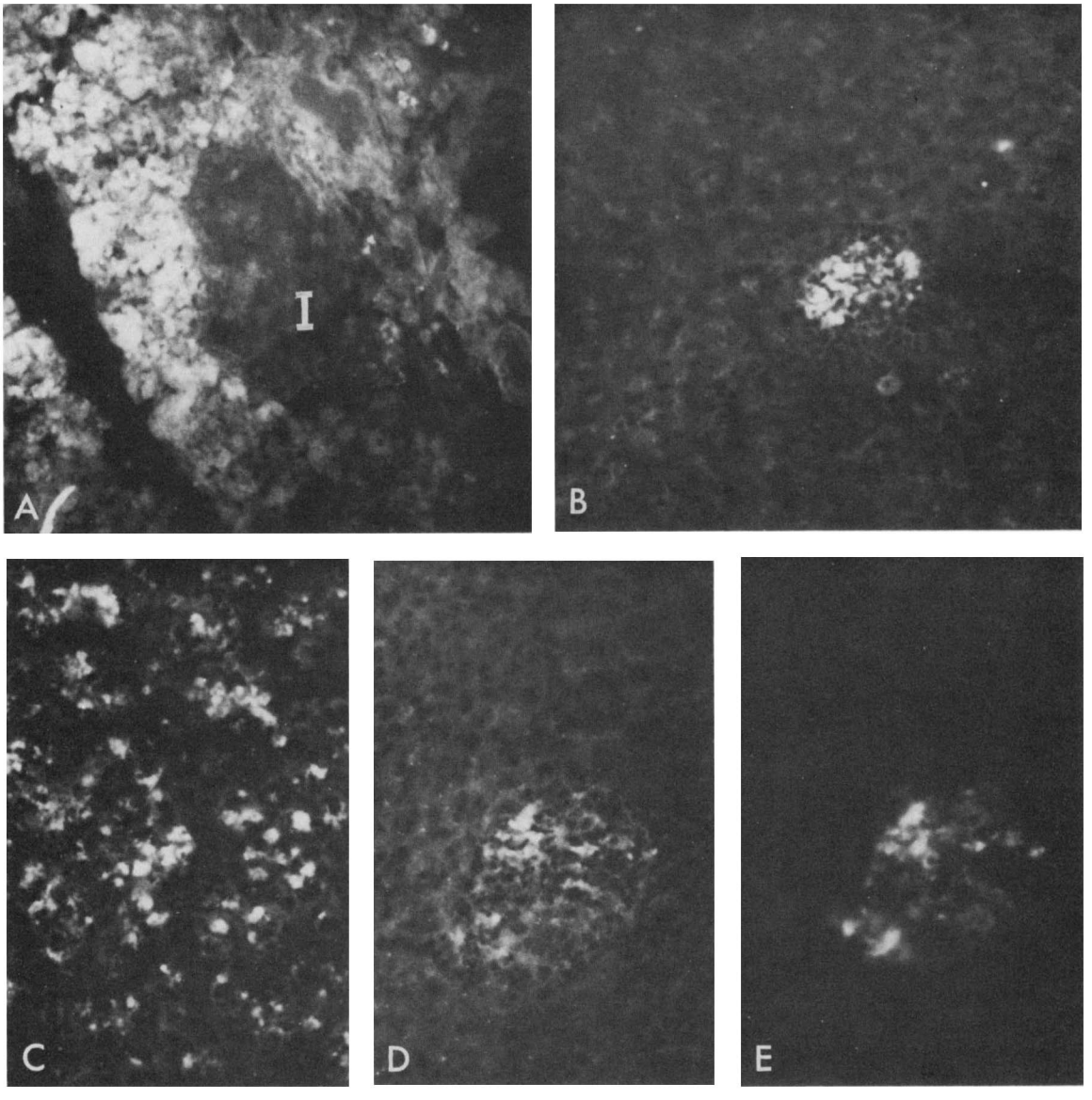


**FIGURE 2. Change in the tropism of CBVs after passage in beta-cell cultures. CBV-1 and CBV-5 were passaged 15 times in beta-cells. Pancreata were removed 4 days after infection and frozen sections were stained with the homologous fluorescein-labeled anti-virus antibody (×340). Panels A–D were viewed with fluorescein filters. (A) Prototype CBV-1: high concentration of viral antigens in acinar tissue, with little or no involvement of the islet (I); (B) beta-cell-passaged CBV-1: viral antigens in islet, but not acinar tissue; (C) prototype CBV-5: viral antigens scattered throughout acinar tissue; (D) beta-cell passaged CBV-5: viral antigens in islet, but not acinar tissue; (E) same islet as in panel D showing insulin when stained with rhodamine-labeled anti-insulin antibody (viewed with rhodamine filters).**

serotype (i.e., the 20th beta-cell culture passage and the 10th in vivo pancreatic passage) was tested in a checkerboard experiment with type-specific reference antisera. Each of the six CBVs was neutralized by homologous antiserum (titers > 1024), but not by heterologous antisera (titers < 16) or by anti-EMC serum (titers < 8). Similar specificity was observed by indirect immunofluorescence using type-specific reference antisera on SME and LLC-$MK_2$ cells infected with the different beta-cell-passaged CBVs. In other experiments, sera collected from mice that had been infected with beta-cell-passaged CBVs were tested in neutralization assays with prototype CBV strains; only type-specific antibody developed in the infected mice and none of the sera reacted with EMC virus (titers < 8). Further evidence against contamination came from experiments in which the induction of hyperglycemia in mice by beta-cell-passaged CBV-3 or -5 was specifically prevented by incubating these viruses with homologous type-specific reference antisera.

## DISCUSSION

Our experiments showed that serial passage in beta-cell cultures, but not in monkey kidney cells or secondary mouse embryo cells, increased the tropism of CBVs for pancreatic islet cells. Precisely how passage of CBVs in the pancreas of mice or in cultures enriched in beta-cells increases the diabetogenic capacity of these viruses, is not clear. A possible explanation is that serial passage in the presence of beta-cells selects for variants that bind to and replicate in beta-cells. This implies that CBV preparations

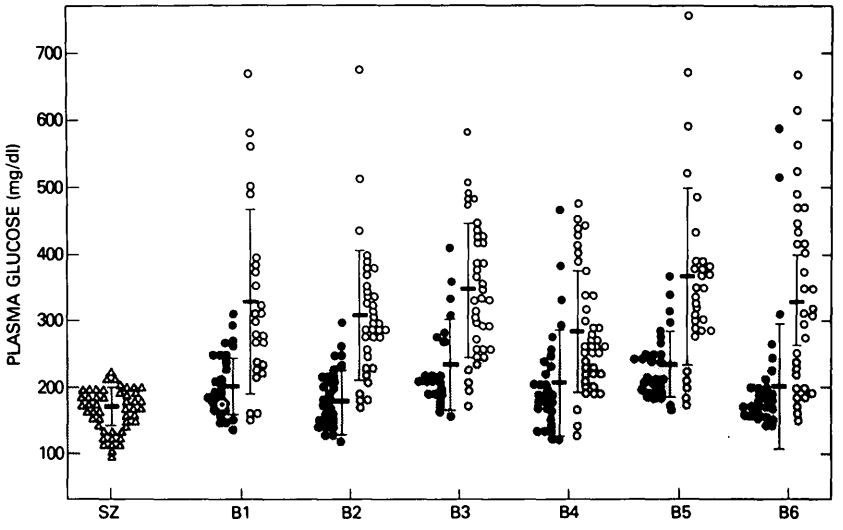


**FIGURE 3. Virus-induced glucose abnormalities enhanced by depletion of beta-cell reserve with SZ. Mice were injected with SZ (40 mg/kg) and 12 days later infected with CBVs that had been passaged in beta-cell cultures 13 or 17 times. GTTs were performed 10 days later. Mice given only SZ (△); mice given only virus (●); mice given both SZ and virus (○). Each point represents an individual animal. The mean ± SD is shown by vertical lines and bars. Shaded area represents the mean of untreated and uninfected mice ± 3 SD. Data pooled from two experiments.**

actually consist of a mixture of particles with different tropisms, or that during serial passage, mutants with different tropisms spontaneously develop. Support for this idea comes from studies with EMC virus which showed that EMC consisted of a mixture of diabetogenic and nondiabetogenic variants.[11]

Previous studies with EMC virus showed that the severity of the diabetes was directly related to the degree of beta-cell damage,[14] the size of the beta-cell reserve,[13] and the presence of interfering nondiabetogenic variants.[11] The present histologic and immunofluorescence studies, together with the enhancement of glucose levels produced by subdiabetogenic doses of SZ, suggest that the mild and transient nature of CBV-induced diabetes is due to the relatively small amount of beta-cell damage produced by these viruses. As in the case of EMC virus, it is possible that the wide variation in GTTs produced by the different preparations of passaged CBVs may also be due to the presence of interfering nondiabetogenic variants.[11]

The isolation of a CBV-4 and -5 from two patients with acute-onset diabetes and the demonstration that these isolates could produce abnormal glucose levels in mice suggest that diabetogenic variants of CBVs may exist in nature.[7,9] Moreover, recent experiments in our laboratory (B. Prabhakar and M. Haspel, personal communication) using monoclonal antibodies prepared against CBV-4 indicate that this serotype does not represent a single virus, but many variants which differ antigenically. Thus, the different clinical diseases produced by CBVs may be due to variants with different tropisms and biologic properties. Although CBVs do not appear to be a major cause of diabetes,[2] the demonstration here that the six members of the CBV group can produce transient GTT abnormalities in mice, if first passaged in beta-cell cultures, adds support to the idea that CBVs may be an occasional precipitating factor of IDDM.

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