VIRUS-INDUCED DIABETES MELLITUS

XV. Beta Cell Damage and Insulin-Dependent Hyperglycemia in Mice Infected with Coxsackie Virus B4

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Since the turn of the century, numerous case reports have appeared showing a temporal relationship between the onset of certain viral infections and the subsequent development of diabetes (1). In the late 1960s, Gamble and co-workers reported that newly diagnosed juvenile-onset diabetics had higher neutralizing antibody titers to Coxsackie virus B4 than a comparable group of nondiabetic patients (2–4). Reports from other laboratories, however, failed to confirm these observations and solid evidence that viruses produce diabetes in humans is still lacking (5–8).

In contrast to the data from humans, there is good evidence that at least one virus, the M-variant of encephalomyocarditis (EMC)[1] virus, can infect and destroy pancreatic beta cells in certain inbred strains of mice and produce an insulin-dependent hyperglycemia (1). Coxsackie viruses also have been implicated as a possible cause of diabetes in mice, but the data is more controversial (1). Since the early 1950s, it has been known that members of the Coxsackie virus B group can infect and destroy pancreatic acinar cells while leaving the adjacent islets of Langerhans intact (9–13). However, in 1971 Burch and associates (14) reported that infection of suckling mice with Coxsackie virus B4 resulted in degranulation of a variable number of beta cells and, by electron microscopy, they observed some alterations in the architecture of the islets. Similar types of observations were made with Coxsackie viruses B1 and B3 (15, 16), but in none of these cases was the degree of beta cell damage quantitated nor were blood glucose levels reported. Coleman and colleagues (17) then reported that infection of CD-1 mice with Coxsackie virus B4 resulted in a transient elevation of glucose, (i.e., 15–20 days after infection), in the absence of an absolute decrease in the concentration of insulin in the blood. Subsequently, Coleman et al., were unable to reproduce their initial observations (personal communication), and extensive studies by Ross et al. and Ross and Notkins also failed to demonstrate elevated blood glucose levels, abnormal glucose tolerance tests, beta cell damage, or viral antigens in islets of mice inoculated with various members of the Coxsackie virus B group (11, 12). The most plausible explanation for these conflicting reports would appear to be that, in mice, most strains of Coxsackie virus are minimally beta-tropic, and that if beta cells are damaged, the number is usually insufficient to produce substantial alterations in glucose metabolism.

Recently, it was shown that beta cells grown in culture would support the replication of certain viruses (18–21) and that the tropism of these viruses for beta cells could be increased by serial passage in beta cell cultures (21, 22). The present experiments were initiated to evaluate both the effect of passage in beta cell cultures and the influence of the host on the diabetogenic capacity of Coxsackie virus B4.

Materials and Methods

Pancreatic Beta Cell Cultures. Pancreas were obtained from suckling SJL/J mice, and beta cell monolayers were prepared essentially as described (18). Staining of the monolayer with

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[1] *Abbreviations used in this paper:* EMC, encephalomyocarditis; FITC, fluorescein isothiocyanate; IRI, immunoreactive insulin; pfu, plaque-forming units; SME, secondary mouse embryo.

fluorescein isothiocyanate-labeled antibody to insulin indicated that 40–70% of the cells were beta cells (18).

Monolayers of SJL/J secondary mouse embryo (SME) fibroblasts were prepared by standard methods.

Virus.   Coxsackie virus B4 (JVB) was originally obtained from the Research Resources Branch of the National Institute of Allergy and Infectious Diseases. Stock pools of Coxsackie virus B4 were prepared either in SME cell cultures (unpassaged virus) or by serial passage in beta cell cultures (passaged virus), using a multiplicity of infection of 10. Virus was harvested 48–72 h after inoculation. The source and preparation of the M-variant of EMC virus are described elsewhere (18, 23). The titer of these viruses, expressed as plaque-forming units (pfu/ml), was determined on SME monolayers.

Preparation of Antiserums.   Antiserums to Coxsackie virus B4 and EMC virus were prepared in rabbits and mice, respectively. Neutralization titers are expressed as the reciprocal of the highest dilution of serum that inhibited plaque formation by 50%. Antiserum prepared against unpassaged Coxsackie virus B4 gave the same 50% neutralization titer (i.e., 500) when tested against either unpassaged virus or virus that had been passaged 14 times in beta cell cultures.

Fluorescein isothiocyanate (FITC)-labeled antiserums to virus and insulin were obtained and prepared as described previously (18).

Mice.   Unless otherwise indicated, 5- to 6-wk old SJL/J male mice, obtained from The Jackson Laboratory, Bar Harbor, Maine, were used in all experiments and the animals were inoculated intraperitoneally with $10^{5.0}$ pfu of Coxsackie virus B4 which had been passaged 14 times in beta cell cultures.

Glucose and Insulin Assays.   Blood glucose levels and glucose tolerance tests were performed as described previously (24). Nonfasting glucose levels were measured 7 and 14 days after infection and the 60-min glucose tolerance tests were performed 10 and 17 days after infection. The data obtained on these 4 days were used to calculate the glucose index (24) for each mouse. The mean glucose index of 80 uninfected mice was 162 ± 16 mg/dl. Any mouse with a glucose index greater than 210 mg/dl, which was 3 SD above the mean, was scored as diabetic.

In some experiments, only nonfasting glucose levels were determined. The mean nonfasting glucose of 100 uninfected animals was 151 ± 22 mg/dl. In these experiments, any mouse with a nonfasting glucose greater than 217 mg/dl (3 SD above the mean) was scored as diabetic.

The concentration of insulin in the pancreas and plasma of infected and uninfected mice was measured by radioimmunoassay (25) using mouse insulin as the standard.

Results

Passage of Coxsackie Virus B4 in Pancreatic Beta Cell Cultures.   Coxsackie virus B4 that had been grown in SME cells was serially passaged in beta cell cultures. Virus from selected passages was titrated and $10^{5.0}$ pfu were inoculated into mice. The data in Fig. 1. show that unpassaged virus failed to raise the glucose index above control values. Beginning, however, with the third passage, a number of animals showed elevated glucose indexes. By the fifth passage, over 35% of the inoculated animals became diabetic and by the 14th passage, close to 80% of the inoculated animals became diabetic. In all subsequent experiments, virus from the 14th passage was used.

Virus-Induced Metabolic Alterations.   To see whether the severity of the diabetes was dependent upon the dose of the virus, animals were inoculated with 10^5–10^7 pfu of passaged or unpassaged virus. Approximately 40% of the mice inoculated with 10^4 pfu of passaged virus developed diabetes. This increased to 80% when mice were inoculated with 10^5 pfu of passaged virus. No further increase was observed when animals were inoculated with 10^6 or 10^7 pfu of passaged virus. In contrast, unpassaged virus did not produce diabetes even when mice were inoculated with 10^7 pfu of virus.

The duration of the hyperglycemia induced by Coxsackie virus B4 is illustrated in Fig. 2. At 13 days after inoculation, the mean nonfasting glucose was 292 mg/dl and 86% of the animals were hyperglycemic. The severity of the hyperglycemia and the
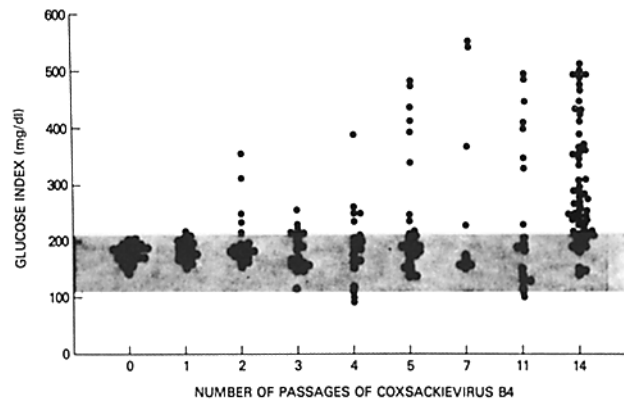
FIG. 1.   Effect of passage of Coxsackie virus B4 in beta cell cultures on the diabetogenic capacity of the virus. Virus harvested after each passage was titered in SME cells and $10^{5.0}$ pfu were inoculated intraperitoneally into mice. Shaded horizontal area represents the mean glucose index of 80 uninfected mice $\pm$ 3 SD. Each point represents the glucose index of an infected animal.

percentage of hyperglycemic animals, however, declined rapidly. At 30 days after inoculation, the mean nonfasting glucose was about 200 mg/dl and only 43% of the animals were hyperglycemic. At 80 days, only 2% of the animals still had elevated glucose levels. Mice inoculated with unpassaged virus had glucose levels similar to uninfected mice.

When the nonfasting glucose of individual animals was examined over a period of 80 days, three general patterns were observed (Fig. 3). The most common pattern, found in up to 80% of the animals, was a transient hyperglycemia lasting from 2- to 8-wk. The second pattern, occurring in less than 5% of the animals, was a severe and persistent hyperglycemia lasting for at least 12 wk. The third pattern, found in 15–30% of the animals, was characterized by a failure to develop hyperglycemia. Despite the failure of these latter animals to develop hyperglycemia, many had distinctly abnormal glucose tolerance tests. Close to 45% of the mice that had normal nonfasting glucose levels 7 days after infection showed an abnormal response when administered a glucose load (data not shown). In contrast, only 1% of uninfected mice responded abnormally. Abnormal glucose tolerance tests were also found at 3 wk after infection in approximately 20% of the animals in which the nonfasting glucose levels had returned to normal.

To study the relationship between virus-induced hyperglycemia and immunoreactive insulin (IRI), mice were infected with Coxsackie virus B4 and, at different times thereafter, the concentration of IRI in the pancreas was determined and plotted against the concentration of nonfasting glucose in the blood (Fig. 4A). The mean IRI in the pancreas of uninfected animals was 163 $\pm$ 15 μg/gm. Within 2 days after infection, the concentration of IRI began to decline and at 4 days, 50% of the animals had less than 50 μg IRI per g/pancreas. Depressed IRI was even more apparent on days 6, 7, and 14. A number of animals had less than 20 μg IRI per g/pancreas. When nonfasting glucose levels were plotted against insulin levels, some of the animals were found to be slightly hypoglycemic at 2 days after infection. Beginning at 4 days, however, a number of animals became hyperglycemic with glucose levels between 200 and 500 mg/dl. On 6, 7, and 14 days after infection, the animals could be segregated
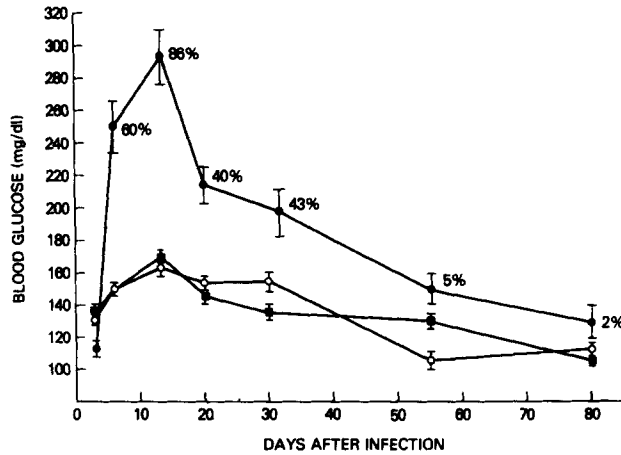
Fig. 2.   Duration of Coxsackie virus B4-induced hyperglycemia. Mice were inoculated with $10^{5.0}$ pfu of Coxsackie virus B4 and at different times thereafter, nonfasting blood glucose levels were determined and the percentage of animals with blood glucose levels 3 SD above the mean of uninfected animals was calculated. (-●-) passaged Coxsackie virus B4 (60 mice); (-O-) unpassaged Coxsackie virus B4 (20 mice); (-■-) uninfected (20 mice). Points represent the mean nonfasting glucose and vertical bars the standard error.
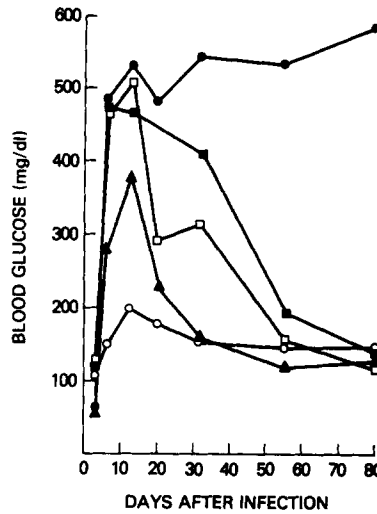

Fig. 3.   Representative glucose patterns of individual mice infected with Coxsackie virus B4. Mice were inoculated with $10^{5.0}$ pfu of Coxsackie virus B4 and at different times thereafter nonfasting glucose levels were determined. Each line represents a single mouse.

into two groups: one with normal pancreatic IRI and normal glucose levels; the other with depressed pancreatic IRI and elevated glucose levels.

The concentration of IRI in the plasma and its relationship to nonfasting glucose is plotted in Fig. 4 B. The mean concentration of IRI in the plasma of uninfected mice was 4.3 ± 0.23 ng/ml. Within 2 days after infection, a number of animals showed signs of hyperinsulinemia (IRI between 5 and 6 ng/ml), probably secondary to the release of insulin from damaged beta cells. This was followed, beginning on day 4, by hypoinsulinemia and hyperglycemia. As in Fig. 4 A, the animals could be segregated
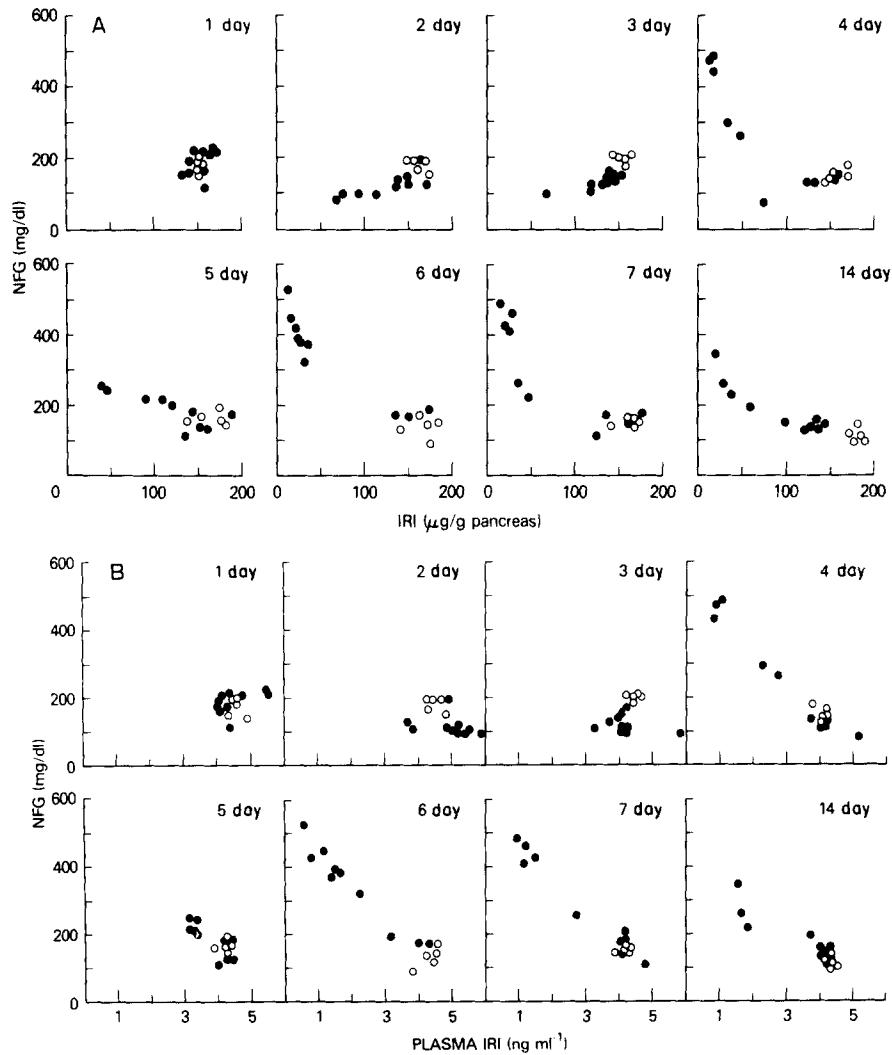
Fig. 4. Relationship of concentration of nonfasting glucose (NFG) in the plasma to the concentration of IRI in (A) pancreas and (B) plasma at different times after infection with 10^5 pfu of Coxsackie virus B4. Each point represents an individual mouse. (O) uninfected mice; (●) infected mice.

into two groups: one with normal plasma IRI and normal glucose levels and the other with depressed plasma IRI and elevated glucose levels.

Destruction of Beta Cells and Demonstration of Viral Antigens. To determine whether the alterations in insulin might be secondary to Coxsackie virus-induced beta cell damage, sections of pancreas from infected and uninfected mice were examined microscopically. Islets from animals infected with Coxsackie virus B4 revealed infiltration of mononuclear cells and disruption of the architecture of the islets of Langerhans (Figs. 5 A-C). Mild inflammatory changes were seen within 3-4 days after infection and the inflammation was maximal at about 5 days. The severity of the inflammatory changes varied considerably among animals and within a single pan-
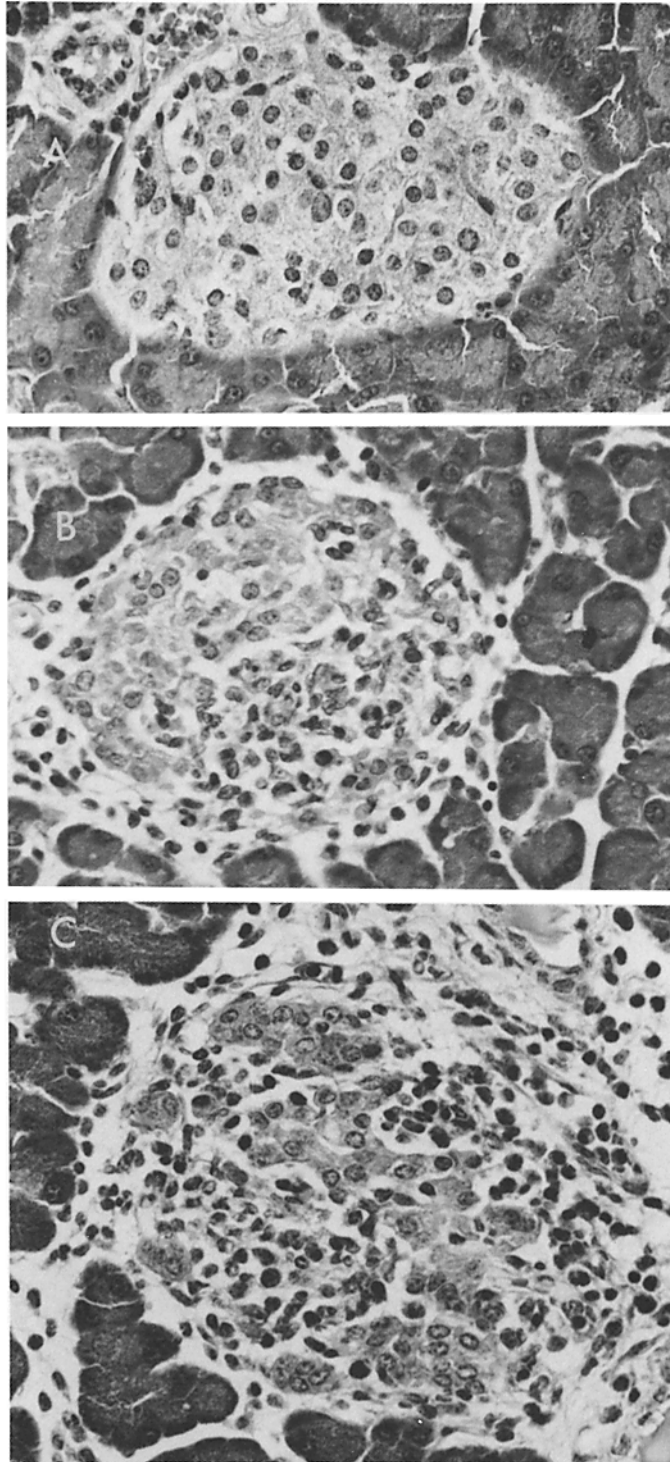
FIG. 5. Pathologic changes in the islets of Langerhans after infection with Coxsackie virus B4. (A) Section of pancreas from uninfected mouse showing a normal islet surrounded by acinar cells (H and E × 550). (B) Section of pancreas 5 days after infection showing approximately one-third of the islet infiltrated with mononuclear cells (H and E × 550). (C) Section of pancreas 5 days after infection showing extensive infiltration of entire islet with mononuclear and occasional polymorphonuclear leukocytes (H and E × 550).
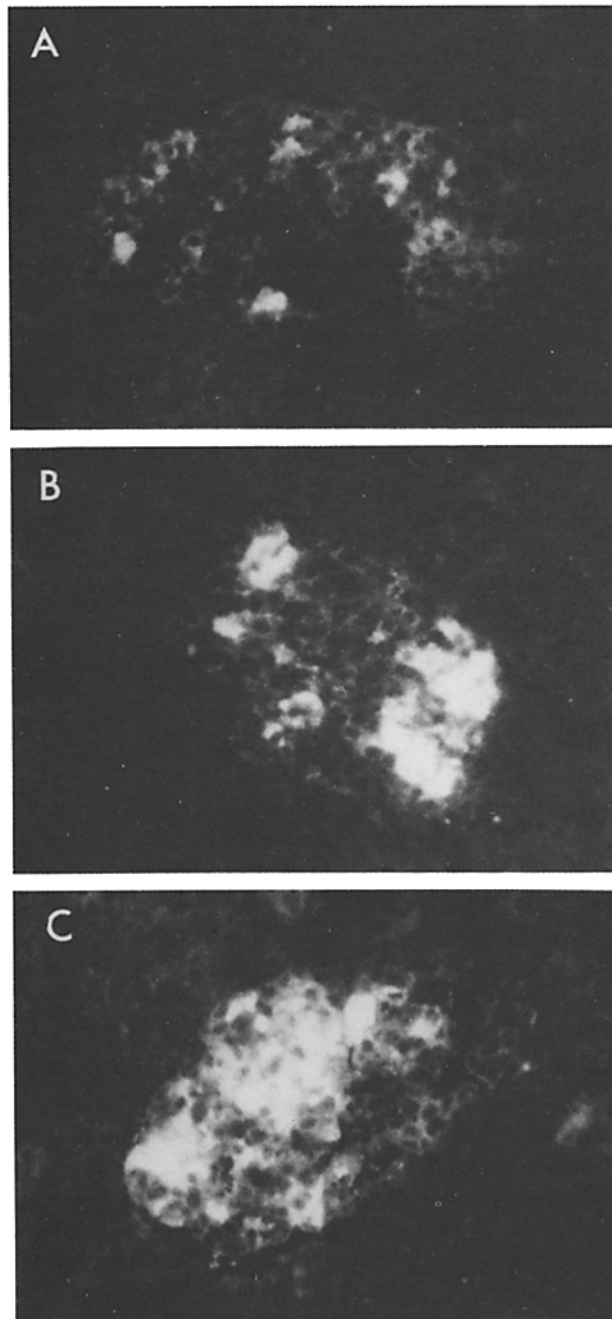
FIG. 6.   Coxsackie virus B4 antigens in islets of Langerhans. Frozen sections from pancreas taken 4 days after infection and stained with FITC-labeled antibody to Coxsackie virus B4 (× 340). (A) Section showing an islet with scattered cells containing viral antigens in the cytoplasma. (B) Section showing an islet with focal areas of infection. (C) Section of an islet with viral antigens in most of the cells. The surrounding acinar cells are relatively free of viral antigens in all three sections.

TABLE I

*Prevention of Virus-Induced Diabetes by Specific Anti-Viral Serum**

Antiserum	Coxsackie-virus B4		EMC Virus	
	Glucose‡ index	Diabetic	Glucose‡ index	Diabetic
	Mean ± SD	%	*Mean ± SD*	%
None	338 ± 116	88	264 ± 90	66
Anti-Coxsackie virus B4§	148 ± 12	0	242 ± 89	58
Anti-EMC Virus§	301 ± 116	60	168 ± 52	5

* Coxsackie-virus B4 (1.0 × 10$^{6.0}$ pfu/ml) or EMC virus (1.0 × 10$^{5.0}$ pfu/ml) was incubated with an equal volume of antiserum or control medium for 40 min at room temperature. Each mouse then received 0.3 ml of the appropriate reaction mixture intraperitoneally and glucose indexes were determined.

‡ Each group contained approximately 15 mice.

§ The 50% neutralization titer was adjusted to 512.

creas with some islets showing little if any change while others showed moderate to extensive infiltration of cells. Normal appearing islets often were seen adjacent to islets showing extensive infiltrates. In some cases, only a portion of the islet was involved. In the more severe cases, beta cell destruction, and coagulation necrosis were observed. At 2 wk after infection, atrophic islets were sometimes seen.

To show that the virus was actually replicating in beta cells, sections from the pancreas of infected mice were stained with FITC-labeled antibody to Coxsackie virus B4 (Figs. 6 A-C). Within 3 days after infection, viral antigens were seen in the cytoplasma of beta cells and maximal involvement occurred at about 4 days. After 7 days, the viral antigens were faint and difficult to detect. The degree of beta cell involvement varied considerably with some islets showing only a few cells and others showing almost all the cells containing viral antigens. Occasional acinar and ductal cells also contained viral antigens.

Evidence that the histologic and metabolic changes were not due to inadvertent contamination of beta cell-passaged Coxsackie virus with EMC virus comes from immunofluorescence and neutralization studies. Islets from animals infected with Coxsackie virus B4 stained strongly positive when incubated with FITC-labeled antibody to Coxsackie virus B4, but not when incubated with FITC-labeled antibody to EMC virus (data not shown). Conversely, islets from animals infected with EMC virus stained strongly positive when incubated with FITC-labeled antibody to EMC virus, but not when incubated with FITC-labeled antibody to Coxsackie virus B4. In addition, as seen in Table I, animals inoculated with Coxsackie virus B4 that had been incubated with antibody to Coxsackie virus B4 did not develop diabetes, whereas animals inoculated with Coxsackie virus B4 that had been incubated with antibody to EMC virus did develop diabetes. Conversely, incubation of EMC virus with the homologous, but not the heterologous, antibody prevented the development of diabetes.

Influence of the Host on the Development of Diabetes. To see whether the induction of diabetes by Coxsackie virus B4 was influenced by the host, several different inbred strains of mice were infected and glucose indexes determined. The data in Table II show that male SWR/J, SJL/J, and NIH Swiss mice readily developed diabetes, whereas C57BL/6J, CBA/J, AKR, BALB/c, C3H/J, DBA/1J, and DBA/2J mice

TABLE II

Induction of Diabetes by Coxsackie Virus B4 in Different Inbred Strains of Mice*

Strain of mouse	Sex	Glucose index	Diabetic
		Mean ± SD	%
SJL/J	M	253 ± 83	72
SWR/J	M	270 ± 91	75
NIH Swiss	M	325 ± 119	80
C57BL/6J	M	129 ± 27	0
CBA/J	M	157 ± 15	0
AKR	M	113 ± 32	0
BALB/C	M	92 ± 40	0
C3H/J	M	87 ± 33	0
DBA/1J	M	159 ± 25	0
DBA/2J	M	140 ± 25	0
SJL/J	F	216 ± 87	33
SWR/J	F	233 ± 25	50
NIH Swiss	F	174 ± 33	10
CBA/J	F	153 ± 17	0
AKR	F	125 ± 14	0
DBA/1J	F	100 ± 30	0
DBA/2J	F	158 ± 10	0

* Mice, 5- to 6-wk old, were infected intraperitoneally with $5 \times 10^{5.0}$ pfu of Coxsackie virus B4. Glucose indexes were determined and the percentage of diabetic animals calculated. Each group contained between 30 and 40 mice.

failed to develop diabetes. A similar pattern was observed in females of these strains, except the severity of the hyperglycemia and the percentage of diabetic animals were lower. This was especially the case with NIH Swiss females. These studies suggest that the genetic background of the host influences the development of diabetes.

Discussion

The present experiments show that Coxsackie virus B4 can infect and destroy pancreatic beta cells in certain inbred strains of mice. The destruction of beta cells results in a decrease in the insulin content of the pancreas. This in turn leads to hypoinsulinemia and the subsequent development of hyperglycemia. Thus, the destruction of beta cells by the virus appears to be responsible for the insulin-dependent hyperglycemia. Whether Coxsackie virus B4 also infects and damages the other hormone-producing cells in the islets of Langerhans, such as the glucagon-producing alpha cells or the somatostatin-producing delta cells has not yet been determined. Virus-induced alterations in the functional capacity of these cells might also contribute to the hyperglycemic state.

As in the case of EMC virus (1), the degree of beta cell damage produced by Coxsackie virus B4 varies considerably among animals, even though inbred strains of mice of the same age and sex are used. This variation in beta cell damage is, in all probability, responsible for the observed differences in the metabolic response of individual animals (Fig. 3). In the majority of the animals, the hyperglycemia is transient. This may very well be due to the fact that a sufficient number of beta cells are left intact after the infection so that proliferation and/or hypertrophy of these cells results in metabolic compensation.

The similarities of the diabetes-like syndrome produced by EMC virus and Coxsackie virus B4 were so striking that a number of steps were taken to rule out the possibility of inadvertent contamination of our beta cell-passaged Coxsackie virus B4 pool with EMC virus. First, sections of pancreas from animals infected with Coxsackie virus B4 or EMC virus were found to stain strongly positive with the homologous, but not heterologous, FITC-labeled antiserums. Second, incubation of Coxsackie virus B4 or EMC virus with the homologous, but not heterologous, antiserums before inoculation of these viruses into animals prevented the development of diabetes. Third, neutralization tests showed that antiserum prepared against Coxsackie virus B4 or EMC virus were highly specific for the homologous virus, although a low degree of cross-reactivity was observed (unpublished data). Fourth, a second isolate of Coxsackie virus B4, obtained from another laboratory, failed to produce diabetes when inoculated into SJL/J mice, but upon serial passage in beta cell cultures, also became diabetogenic (unpublished data). Taken together, these studies strongly argue against inadvertent contamination of the beta cell-passaged Coxsackie virus B4 pool with EMC virus.

Precisely how passage of Coxsackie virus B4 in cultures enriched for beta cells increases its diabetogenic capacity is not known, but alterations in the tropism of viruses after serial passage in animals or tissue culture is a widely recognized phenomenon. In the case of Coxsackie virus B4, there are at least several ways in which this could have occurred. First, the original stock virus pool may have contained two populations of virus; one tropic for beta cells and the other not tropic for beta cells. Growing the stock virus in cultures enriched for beta cells may have favored the replication of the beta-tropic virus. Second, mutation or recombination may have taken place during serial passage of the virus in culture and the presence of beta cells may have favored the selection of the beta-tropic virus. Third, nongenetic adaptation (e.g., host-controlled alterations in viral antigens or coat) may have occurred, thereby increasing the capacity of the virus to bind to beta cells. By plaque-purifying the virus and by passaging the virus in beta cell cultures from different inbred strains of mice, it might be possible to distinguish between a stable mutant and nongenetic adaptation. These experiments are now underway.

The present study also shows that the capacity of Coxsackie virus B4 to induce diabetes is influenced by the genetic background of the host. As in the case of EMC virus, only certain inbred strains of mice were found to develop diabetes when exposed to Coxsackie virus B4 and male mice developed more severe diabetes than female mice. Moreover, the strains of mice known to be susceptible to EMC-induced diabetes (18, 24, 26–28) were found to be the strains susceptible to Coxsackie virus B4-induced diabetes (Table II). Similarly, the strains of mice that did not develop diabetes when exposed to EMC virus, did not develop diabetes when exposed to Coxsackie virus B4. The only exception, thus far, appears to be DBA/1J and DBA/2J mice which developed diabetes when exposed to EMC virus (26), but did not develop diabetes when exposed to Coxsackie virus B4. In the case of EMC virus, it is known that susceptibility to diabetes is inherited as an autosomal recessive trait (24, 28), and that beta cells from strains of mice that develop diabetes are more susceptible to EMC infection than beta cells from strains of mice that do not develop diabetes (18, 27, 29). Moreover, recent data suggest that a single locus may control susceptibility (28). Based on these observations, it has been postulated (but not proven) that the gene product controlling susceptibility might be a receptor for the virus on the surface of

beta cells (29). If this turns out to be the case, then it would not be unreasonable to suspect that host-determined differences in the induction of diabetes by Coxsackie virus B4 also might be related to viral receptors on the surface of beta cells. In fact, one explanation for the similarity in the responses of inbred strains of mice to the diabetes-inducing capacity of Coxsackie virus B4 and EMC virus might be the existence of a common receptor on the surface of beta cells which is used by both viruses. The sharing of a common receptor by apparently unrelated viruses has recently been reported (30).

The experiments described here with Coxsackie virus B4, together with the recent demonstrations that reovirus type 3 also can infect mouse beta cells and cause metabolic alterations (22), show that two viruses which are widely disseminated in the human population are capable of producing a diabetes-like syndrome in mice. What role, if any, these viruses actually play in the etiology of juvenile-onset diabetes is still not known.

Summary

Coxsackie virus B4 that had been passaged in cultures enriched for pancreatic beta cells produced a diabetes-like syndrome when inoculated into SJL/J mice. The infection resulted in insulitis and destruction of beta cells. Viral antigens were found in beta cells by staining with fluorescein-labeled antibody to Coxsackie virus B4. The destruction of beta cells led to a decrease in the immunoreactive insulin content of the pancreas and hypoinsulinemia. The reduction in immunoreactive insulin correlated inversely with the elevation of glucose in the blood and over 80% of the animals were found to be hyperglycemic within 14 days after infection. The percentage of animals with hyperglycemia decreased with time and at the end of 60 days, less than 5% of the animals were still hyperglycemic. However, many of the normoglycemic mice were found to be metabolically abnormal when evaluated by glucose tolerance tests. Studies on the susceptibility of the host showed that only certain inbred strains of mice became diabetic when infected with Coxsackie virus B4. It is concluded that both the passage history of the virus and the strain of the host influence the development of diabetes.

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