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Diabetes Mellitus

ANTONIO TONIOLO, GIOVANNI FEDERICO, FULVIO BASOLO, and TAKASHI ONODERA

1. INTRODUCTION

The term diabetes refers to a group of disorders having in common hyperglycemia and an elevated prevalence of serious complications affecting many organ systems. Studies of the natural history and pathogenesis of hyperglycemia have led to a widely accepted classification developed by the National Diabetes Data Group. Among the various diabetic syndromes that are now recognized, insulin-dependent diabetes mellitus (IDDM) and noninsulin-dependent diabetes mellitus (NIDDM) account for the great majority of cases, the former being less prevalent. It is now clear that IDDM derives from a deficiency of insulin production due to the loss of pancreatic β-cells, while NIDDM is mostly due to a reduced effect of this hormone on peripheral tissues. Because coxsackieviruses (CV), and in particular the members of group B (CVB), have been implicated only in the etiopathogenesis of IDDM, this discussion refers exclusively to this form of diabetes.

All patients with IDDM share a common clinical finding: dependence on exogenous insulin for survival. This results from the near-total disappearance of the insulin-producing β-cells of the islets of Langerhans. It is generally believed that genetic, immunologic, and environmental factors all play a role in the irreversible loss of β-cells. There is,

ANTONIO TONIOLO • Institute of Microbiology and Virology, University of Sassari Medical School, 07100 Sassari, Italy. GIOVANNI FEDERICO • Department of Pediatrics, University of Pisa, I-56100 Pisa, Italy. FULVIO BASOLO • Department of Pathology, University of Pisa, I-56100 Pisa, Italy. TAKASHI ONODERA • Laboratory of Viral Immunology, National Institute of Animal Health, Tsukuba, Ibaraki 305, Japan.

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however, considerable uncertainty regarding the relative importance of these factors and how they interact to result in what is suspected to be an etiologically heterogeneous disease.[1] Until a few years ago, IDDM was believed to be an acute-onset disease, but recent prospective studies of individuals at high risk of IDDM have indicated that a long euglycemic period, characterized by immune and metabolic abnormalities, precedes the appearance of hyperglycemia. Notably, islet cell antibodies (ICA) and T-lymphocyte abnormalities are present in most patients for months or years before the onset. This, together with the finding of a strong genetic association with the histocompatibility markers HLA-DR 3/4 and with other autoimmune diseases, led to the hypothesis that IDDM is an organ-specific autoimmune disorder[2] and that an autoimmune process is responsible for the chronic and irreversible loss of β-cells. Because genetic factors alone are not sufficient to explain the production of this disease, environmental factors must be implicated.[3] What triggers pancreatic autoimmunity in genetically predisposed individuals is unknown, but many lines of evidence point to the critical role of infectious agents and environmental toxins. More than two decades of study into the mechanisms of virus-induced pancreatic damage have failed to clarify the role of biologic agents in the etiopathogenesis of diabetes.[4] However, many impressive observations indicate that, even if acute virus-induced diabetes is rare, IDDM as other autoimmune diseases may be due to a chronic inapparent viral infection or may represent the consequence of a more classic infectious event. It is the purpose of this chapter to review the large body of evidence linking CVB infections to IDDM.

2. EPIDEMIOLOGY OF INSULIN-DEPENDENT DIABETES MELLITUS

Most studies of the prevalence or incidence of IDDM in young people (i.e., approximately younger than 18) suggest that the prevalence of the disease varies between 5 and 30 in 10,000 and that in Western countries the annual incidence can be estimated at 7–12 in 100,000 in the age group 0–16 years.[5] The highest incidence has been reported from Finland, while Japanese, Indians, Chinese, Eskimos, South African blacks, and Polynesians have a relatively low incidence. Both sexes are approximately equally affected, although some studies indicate a slight preponderance of males in the first 4 years of life. IDDM is extremely rare until 9 months of age, after which its frequency increases abruptly. The peak age of onset is 10–14 years of age, and a secondary peak occurs at 5–8 years. These observations are consistent with the epidemiologic pattern of many infections, which are prevented by maternal

immunity and are more frequent at the time of entry into school. The peak frequency at the age of about 12 years (earlier in females) suggests that susceptibility increases at puberty. The higher incidence after puberty of orchitis and paralytic disease due to mumps and polioviruses, respectively, is a well-known example of age-modulated susceptibility to the viral invasion of particular tissues.

Seasonal variation in the onset of IDDM has been reported from several countries,[5–7] with the greatest number of new cases seen in autumn and winter. Seasonality is not very pronounced and apparently does not occur in tropical countries.[8] As before, these observations are consistent with the epidemiologic pattern of several infections; it is unclear, however, as to how seasonality can be maintained supposing that the infectious event precedes diabetes by several months or years. An alternative possibility is that common viruses having a seasonal distribution may merely act as precipitating factors of an ongoing diabetogenic process.

The relative importance of socioeconomic and climatic factors in the incidence of IDDM is unclear, as it is why fluctuations of the incidence rate seem to occur with cycles of several years. All these aspects would merit further investigations in the light of an infectious etiology of IDDM.

3. SEROEPIDEMIOLOGIC STUDIES

Since 1969, when Gamble suggested for the first time the possibility of a link between CVB infections and human diabetes,[9] a large number of serologic investigations were initiated to confirm his findings.[10–54] Two excellent reviews by Gamble *et al.*[5] and Barrett-Connor[11] have attempted a comprehensive interpretation of the available data. We review these findings in an effort to compare those studies that have been done with similar virologic methods and with appropriately selected groups of patients and controls. In addition, some recent data on the immunoglobulin class of antibodies against CVB is reviewed, together with the results of a study we have conducted on our diabetic patients.

3.1. Neutralizing Antibodies

Based on the notion that a growing number of viruses can infect the pancreas of both animals and humans[10] and on sporadic reports attributing juvenile diabetes to mumps or to other infections,[4] Gamble and colleagues[9] initiated a systematic search for antiviral antibodies in the sera of diabetic patients using complement fixation (CF) and comparing

the prevalence of these antibodies to that observed in healthy controls. Although the spectrum of infectious agents used for this survey was large (including influenza, parainfluenza, RSV, HSV, mumps, measles, adeno, and CV), significant differences in the prevalence of viral antibodies were found only with regard to influenza C and parainfluenza (reduction), and to CVB (increase). No difference between diabetics and controls was detected in the proportion of sera positive to group A CV (types 2, 5, 10, and 16). Since the CF antibody response to CV infections is often nonspecific, the same sera were examined for neutralizing titers to CVB1–5. Insulin-dependent diabetics within 3 months of onset were found to have higher antibody titers to CVB4 than did either normal subjects or patients with diabetes of longer duration. This finding thus suggested that in the small group of diabetics studied there was in some cases a temporal association of CVB infection (probably type 4) with the onset of diabetes. In this study, the control and patient groups were not appropriately matched and most of the patients were over 15 years of age (i.e., they were not typical IDDM patients).

A second study of 162 patients (with controls matched for age, sex, geographic area, and time of bleeding) was published by Gamble *et al.*[12] in 1973. Again, antibody to CVB4 was found more often in diabetics than in controls, particularly in the age group 10–19 and >20 years. By contrast, children aged 0–9 years had a reduced frequency of antibodies as compared to controls. A reduction of the antibody response to CVB in young diabetic children was later confirmed by two reports from Copenhagen and Seattle[20,21] and is also suggested by a recent study from Pittsburg.[27] This may mean either than individuals with a genetic predisposition to IDDM have a reduced immunologic competence against CVB or that young diabetic children have had less previous experience with CVB infections as compared with nondiabetics and consequently have a more severe disease when they contract CVB infections at later ages (akin to the polio model).

Investigations of this type have been reported from several countries with controversial results.[12,17–23,25,27,40–42,45,47–49,53] It is not surprising, however, that different conclusions have been reached, since it is obviously difficult to establish, by serology alone, cause-and-effect relationships between widespread infectious agents as CVB and a disease with a prevalence of about 1 in 1000 population. In addition, because of the cost and length of these studies, many of the published reports have been rather limited in scope. Table I presents the averaged results obtained by various investigative groups on the prevalence of CVB-neutralizing antibodies in more than 400 newly diagnosed diabetics. Within 4 months of onset, the proportion of patients positive for the CVB4 antibodies is significantly increased as compared with normal

TABLE I
Prevalence of Neutralizing Antibodies to CVB in Insulin-Dependent Diabetics under 20 Years of Age and Matched Controls[a]

Group	Time from onset (months)	% positive and number tested[d]					
		B1	B2	B3	B4	B5	B6
Controls[b]	—	19.9 (347)	39.1 (327)	40.3 (417)	45.1 (483)	34.6 (347)	10.0 (80)
IDDM[b]	<4 (1–4)	23.1 (428)	38.2 (408)	40.8 (441)	59.5 (501)[f]	30.1 (428)	9.4 (203)
IDDM[c]	>6 (6–24)	29.0 (186)[e]	54.8 (186)[f]	46.8 (186)	64.0 (186)[f]	41.9 (186)	3.7 (107)

[a]Cumulated data from the literature.
[b]From refs. 12, 18–20, 25, 39–41, and 52–54.
[c]From refs. 12, 14, and 54.
[d]Percentage of sera with NA titers of ≥1:8 and, in parentheses, the number of individuals tested.
[e]Significantly more prevalent in diabetics than in controls at $p < 0.025$.
[f]$p < 0.001$.

controls, confirming the early data of Gamble's group. When antibodies are measured 6–24 months from onset, the high prevalence of CVB4 infections is still observed, but evidence is also obtained for an elevated incidence of CVB2 and CVB1 infections.

We have carried out a study of this kind on our own patients aged 1–14 years and in a comparable group of healthy children.[54] Because only one method was used for measuring antibody titers and its sensitivity was accurately monitored, we were able to compare not only the proportions of positive individuals in the two groups, but also the mean titers of positives (Table II). Again, within 1 month of onset, evidence for a somewhat increased prevalence of CVB4-neutralizing antibodies was obtained and, more important, significantly altered neutralizing antibody titers were found only to this agent. The finding of a reduced antibody responsiveness to CVB4 was confirmed with sera obtained 6–20 months after diagnosis. Thus, our data suggest that early-onset diabetes is associated with frequent CVB4 infections but that young diabetics may have a reduced ability to produce neutralizing antibodies to this virus. This has been already proposed by others.[12,20,21,27] Interestingly, Tables I and II show that, at onset, the immune response of diabetic children to CVB types other than CVB4 is essentially normal.

The recurring observations of an altered antibody response to CVB4 in these patients has prompted some authors to postulate that antigenic similarities exist between this virus and pancreatic β-cells and that ICA would simulate CVB4 antibodies. This point has been recently addressed by Schernthaner *et al.*,[35] who measured anti-CVB antibodies and ICA in newly diagnosed diabetics. It was concluded that it is unlikely that CVB-specific IgM and CF-ICA antibodies are crossreactive, because by no means every patient who was CVB-IgM positive had CF-ICA and vice versa. It should also be considered that more than 40% of normal children have CVB4 antibodies, whereas less than 1% are ICA positive and that sera of normal individuals with high titers of CVB antibodies do not react with islet cells.[31] These observations, however, do not exclude that some individuals may recognize common epitopes on these antigens.

3.2. Immunoglobulin Class Specificity of CVB Antibodies

Additional serological evidence for a role of CVB in diabetogenesis is provided by recent investigations of the immunoglobulin class of CVB antibodies in young diabetics. Following a report by the group of Banatvala,[29] several investigators studied this point using solid-phase immunoglobulin-capture methods.[27,30–35,37–39,43] These studies provided

TABLE II
Prevalence and Titer of CVB Neutralizing Antibodies by Duration of IDDM in Children under 14 years of Age and Matched Controls[a]

Group	Time from onset (months)	No. tested	% positive and mean titer of positives[b]					
			B1	B2	B3	B4	B5	B6
Controls	—	48	16.7 (43.2)	60.4 (156.3)	35.4 (42.8)	54.2 (328.5)	58.3 (53.1)	4.2 (8.0)
IDDM	0–1	81	25.9 (78.5)	48.1 (134.8)	46.9 (67.0)	65.4 (239.9)[c]	43.2 (72.8)	6.2 (16.7)
IDDM	6–20	107	25.2 (61.4)	45.8 (139.2)	47.7 (44.9)	66.4 (219.1)[c]	46.7 (79.3)	3.7 (19.2)

[a]Patients at onset: mean age 10.2 years (range 1–14); patients several months after onset: mean age 11.3 years (range 2–15).
[b]Percentage of sera with NA titers ≥1:8 and, in parentheses, geometric mean titer of positive sera. Adapted from Toniolo *et al.*[54]
[c]Mean titer of diabetic patients significantly reduced as compared with controls ($p < 0.025$).

TABLE III
CVB Antibodies of Different Immunoglobulin Classes in Children with Newly Diagnosed IDDM[a]

		IDDM	Controls
Virus	Ig class	Positive/no. tested	Positive/no. tested
CVB1–5	IgM[b]	87/247[e]	34/550
CVB4	IgG[c]	97/210	107/210
CVB4	IgM[c]	29/210	26/210
CVB4	IgA[c]	79/210[d]	56/210

[a]Children aged <15 years old; time from diagnosis of less than 3 months. Control subjects matched by age, time, and geographic area. Antibody determinations by Ig-capture ELISA or RIA.
[b]Cumulated data from refs. 29, 30, 32–34, and 39.
[c]Data from Hyoty *et al.*[37]
[d]Significantly more prevalent in diabetics than in controls at $p < 0.025$.
[e]$p < 0.001$.

results that are less controversial than those obtained with tests for neutralizing antibodies.

Table III presents the averaged results obtained by various investigators within a short period of diagnosis in children younger than 15 years. First, it is apparent that most studies have documented a high frequency of CVB-specific IgM antibodies in these patients.[29,30,32–34,39] Second, these antibodies (when homotypic) were shown to react mostly with CVB4, but also with CVB5, CVB2, and rarely CVB3. Third, limited studies suggest that the CVB-specific IgM response of diabetic patients is not unduly prolonged (this tends to exclude the possibility of a persistent infection). Fourth, not all studies support the idea of a central etiologic role of CVB4 in IDDM.[33,37] In fact, a recent study from Finland[37] failed to show an increased frequency of IgM responses to this virus but indicated that diabetic children may have a higher prevalence and mean levels of IgA antibodies to CVB4 as compared with controls. The same has already been observed in young diabetics with regard to mumps virus[55,56] and might reflect the establishment of persistent infections in these patients or, alternatively, the presence of immunoregulatory abnormalities associated with the predisposing genotype.

Thus, collectively, these studies suggest once again that up to one third of new cases of diabetes in children are preceded by an acute CVB infection.

3.3. Genetic Control of the Immune Response to CVB

In 1977, Cudworth *et al.*[16] noted for the first time that diabetic patients who were positive for the HLA antigen B15, and in particular

those who were both B8 and B15 positive, had higher neutralizing antibody titers to CVB1–4 than did patients negative for these alleles. This observation stimulated investigations on the influence of HLA loci on the immune response to CVB. It is now clear that the association of IDDM with the HLA class I alleles B8 and B15 is mostly due to a linkage disequilibrium occurring between these markers and the class II alleles DR3 and DR4, which are believed to represent markers of closely linked, but as yet untypable, diabetogenic genes. Thus, it was found that male patients who are negative for DR4 (most of whom are DR3 positive) show a lower frequency of recent CVB infection at diagnosis.[27] This finding may reflect a failure of serologic response to these viruses and seems to confirm indirectly, the results of Cudworth's group. Collectively, these data indicate that the markers B8 and/or DR3 are associated with a reduced antibody response to CVB, whereas patients positive for B15 and DR4 produce increased titers of antibodies to these viruses. Patients with the combined genetic predisposition to IDDM (i.e., DR3 and DR4 positive) seem to have the highest antibody titers. Positivity for either DR3 or DR4, or both, not only appears to influence the levels of neutralizing antibodies to CVB but is also associated with an increased frequency of CVB-specific IgM at onset.[35]

These and other observations stimulated *in vitro* studies of the proliferative response of T lymphocytes to CVB and other diabetogenic viruses. In agreement with what was observed *in vivo,* Bruserud *et al.*[57] found, by limiting-dilution analysis, that DR3-positive individuals had a lower frequency of T lymphocytes proliferating in response to CVB4 and mumps virus as compared with subjects with DR determinants other than DR3. By contrast, DR4-positive individuals had an increased frequency of these lymphocytes. These results were similar in diabetics and healthy individuals, and no differences were found in the frequency of T lymphocytes responding to varicella–zoster virus or tuberculin. Supposing that these differences were mediated by antigen-specific T-suppressor (Ts) cells, peripheral T lymphocytes were deprived of Ts cells and cultured in the presence of antigen-presenting cells. Analysis of the proliferative response to mumps and CVB4 antigens showed that the DR3-mediated hyporesponsiveness is not due to Ts cells.[58] Because the suppression exerted by antigen-presenting cells is believed not to be antigen specific, the reduced proliferative ability of lymphocytes from DR3-positive individuals appears to be most probably caused by an intrinsically low frequency of antigen-reactive helper/inducer T lymphocytes or by a regulatory influence of a subset of these cells.

The significance of this hyporesponsiveness in diabetogenesis, if any, remains unclear, but a recent study from Gorsuch *et al.*[59] tends to exclude the possibility that diabetics are hyporesponsive to picor-

naviruses in general. Antibody titers to poliovirus types 1–3 were measured in HLA-typed diabetics and their discordant siblings; there was no convincing association of titers with HLA phenotypes. However, statistical analysis suggested that immune-response genes may play some role in viral diabetogenesis.

4. FOLLOW-UP STUDIES

Evidence to associate CVB infections and the clinical onset of IDDM has been also searched for by studying naturally infected populations. Thus, 24 British patients clinically infected with CVB2 or 4 were followed for 3 years, but no one developed diabetes.[48] More interestingly, a negative report came from the Pribilof islands within 5 years of a major CVB4 epidemic.[49] Neither diabetes nor glucose intolerance developed in infected persons aged 5–20 years at the time of the epidemic, nor among any of the approximately 70 children under 5 years at that time. It was therefore concluded that the risk of developing diabetes 5 years after CVB4 infection is less than 3% in Aleuts under 20 years of age. This study also pointed out the extreme difficulty of preventive measures, unless markers are found to identify persons susceptible to diabetes. Four years after an outbreak of CVB3 and CVB4 infection in a children's home, Hierholzer and Farris[51] measured the fasting plasma

TABLE IV
Incidence of Diabetes in Individuals with Recognized CVB Infections: Follow-up Studies

Infection	IDDM cases/ no. infected	Incubation time	Notes	Ref.
CVB2 and CVB4	0/24	—	3-year observation	48
CVB4	0/166	—	5-year observation (OGTT in population)[a]	49
CVB3	0/17	—	4-year observation	51
CVB4	0/32	—	4-year observation	51
CVB3 and CVB4	0/39	—	4-year observation (1 case altered OGTT)	51
CVB4	1/4	>3 year	Family members; patient positive for ICA and CF-ICA	36
CVB4	1/22	10 weeks	U.N. soldiers; patient negative for ICA and CF-ICA	46

[a]OGTT, oral glucose-tolerance test.

glucose and insulin levels of 88 previously infected children. Only one case of suspected chemical diabetes was reported in a girl who had been infected with both CVB3 and CVB4. These data are summarized in Table IV. After clinical infection with CVB4, however, one case of overt diabetes has been described in a family[36] and another in a group of 22 U.N. soldiers serving in Egypt.[46] In the first case, the time between infection and clinical onset was more than 3 years; the child had ICA 3 years before the onset. By contrast, in the second case, the time between infection and hyperglycemia was 10 weeks, and this patient was ICA negative, suggesting an unusual form of diabetes. In none of these cases was it possible to demonstrate that hyperglycemia was indeed related to the infectious event. Investigations of this kind are ill suited to the study of rare diseases such as IDDM and would require the observation of large numbers of infected individuals (possibly more than 1000).

5. CLINICAL OBSERVATIONS

The best way to establish a direct link between viral infections and human diabetes is to isolate viruses from diabetic patients and to show that the recovered agents can cause diabetes in experimental hosts. So far, this goal has been achieved only in two cases. The first is that of a 10-year-old boy who developed diabetic ketoacidosis 3 days after the onset of a flulike illness. Despite intensive therapy, his condition deteriorated, and he died 7 days later. At autopsy, insulitis and necrosis of β-cells were observed. Yoon *et al.*[60] were able to isolate a variant of CVB4 from the pancreas. Studies of the patient's serum showed a rise of neutralizing antibodies to the isolate from a titer of less than 1 : 4 on the second hospital day, to 1 : 32 on the day of death. Viral antigens were detected in various organs, including the child's brain stem, demonstrating a severe CVB infection. Sections of the pancreas were characterized by lymphocytic infiltrates immediately surrounding the islets of Langerhans, often in a perivascular distribution. Complete loss of islet architecture and severe β-cell degeneration were present in scattered islets.

To prove that this agent was truly related to the child's diabetes, several inbred strains of mice were inoculated with the isolate. Mice of strains susceptible to encephalomyocarditis virus-induced diabetes (SJL) developed hyperglycemia. Viral antigens were found by immunofluorescence in pancreatic islets, and insulitis with β-cell necrosis was detected in infected mice. All these data indicated that the patient's diabetes was virus induced. However, subsequent histopathologic studies showed that some pseudoatrophic islets, composed mainly of α- and Δ-cells but lacking β-cells, were present in the child's pancreas. This finding is typical of

a long diabetogenic process and might suggest that the CVB4 infection did not cause, but only precipitated, diabetes in this patient.

The second case is that of a 16-month-old French girl who was hospitalized for thrombocytopenic purpura that appeared after 1 week of fever. Laboratory evidence of diabetes was present from day 13 to 25; then a remission ensued, but 2 months later, the child developed overt diabetes. On the eighth day after the onset of fever, CVB5 was isolated from stools.[61,62] Antibody titers to this virus rose from less than 1 : 10 to 1 : 640, showing that in the period preceding the onset of diabetes the baby had been infected with CVB5. When this isolate was inoculated into various strains of mice, glucose intolerance was produced in susceptible strains (DBA and SJL). Since the common strains of CVB5 do not produce diabetes in mice, it was concluded that this child had been infected with a diabetogenic variant of this virus. The girl had the high-risk genetic markers DR3 and BfF1, and at the time of hospitalization high levels of ICA were already present in her serum. Thus, it appears that CVB infection collaborated with genetic and immunologic factors in producing diabetes in this child.

In other cases, proof that the isolated virus could induce diabetes in experimental animals was not obtained. Nevertheless, various CBV have been isolated and their relationship to the disease has been inferred by histopathology. A 5-year-old girl developed myocarditis and acute diabetes 7 days after open-heart surgery; she died of diabetic ketoacidosis a few days later. At necropsy, degeneration and necrosis of islet cells and an associated insulitis were found; more importantly, CVB4 antigens were detected by immunofluorescence in pancreatic islets.[63] As in the previous cases, high titers of neutralizing antibodies were detected, confirming that the girl had had a recent CVB4 infection.

One case of diabetes attributed to CVB1 occurred in a newborn twin who acquired the virus from the mother a few days before birth (transplacental infection has been documented in a few cases). Immediately after birth, the infant's condition deteriorated, and he developed thrombocytopenia and myocardiopathy. On day 12 blood glucose rose to over 500 mg/dl, and he died 4 days later.[64] Pancreatic lesions consisted of insulitis with islet cell degeneration, and CVB1 was recovered from the pancreas. It is of interest that his twin brother, although developing a severe disease due to CVB1, did not become diabetic and could be discharged from the hospital 25 days after birth. This case strongly suggests that CVB1 can indeed produce diabetes in humans, as it is hardly conceivable that the diabetes of this baby could recognize a nonviral etiology. This case also illustrates that clinical infection with a diabetogenic virus does not necessarily produce diabetes; it is therefore unfortunate that these twins and their mother were not HLA typed.

Other cases of IDDM have been associated with CVB infections on less solid grounds. A 4-year-old boy was hospitalized for hyperpyrexia, diarrhea, lymphocytosis, and meningeal involvement. A few days later, he developed mild hyperglycemia and died 20 days after hospitalization.[65] CVB6 was isolated from the spinal fluid and stools. Several serum samples were analyzed for IGM against CVB and other viruses, but only CVB6-specific IgM were found. More interestingly, on the first hospital day the boy was negative for ICA and CF-ICA, but ICA appeared on day 7 and CF-ICA on day 15. In contrast with the French case reported by Champsaur *et al.*,[62] in which ICA were found on the first hospital day (leaving aside the possibility that pancreatic autoantibodies were already present before CVB5 infection), this case strongly suggests that ICA can indeed be induced as a consequence of CVB infection.

An 18-month-old boy developed acute diabetes after 1 week of fever, diarrhea, and polyuria without cardiac involvement.[66] He had lymphocytosis and high titers of neutralizing antibodies to CVB2, but not to other CVB; in addition, low titers of CVB-specific IgM were detected by immunofluorescence in the acute phase of the disease. Both parents and two siblings were also found to have high titers of antibodies to CVB2, confirming a recent familial infection. A similar case was described by Gamble's group[67] in a 10-year-old boy from a diabetic family who developed clinical diabetes 1 month after acute Bornholm disease. Because of the presence of high antibody titers to CVB1 and CVB5, this case was tentatively attributed to these viruses. Interestingly, ICA were not detected in this patient or in family members.

Two additional cases attributed to CVB4 on serologic basis have been reported from Japan in adult patients.[68] One patient underwent a complete remission, while the other developed a mild diabetes, clinically similar to the noninsulin-dependent form.

Although it is impossible to establish whether CVB infections played a direct etiologic role in these patients or acted merely as triggering factors of diabetes in individuals who had an already reduced insulin reserve, these observations are consistent with the view that CVB may damage, at least occasionally, insulin-producing pancreatic β-cells in humans.

6. POSTMORTEM STUDIES

This view is reinforced by numerous histopathologic reports on the effects of CVB on the islets of Langerhans in patients dead of severe infections with or without clinical diabetes. It has long been recognized that CVB can infect and damage the exocrine pancreas;[69,70] more re-

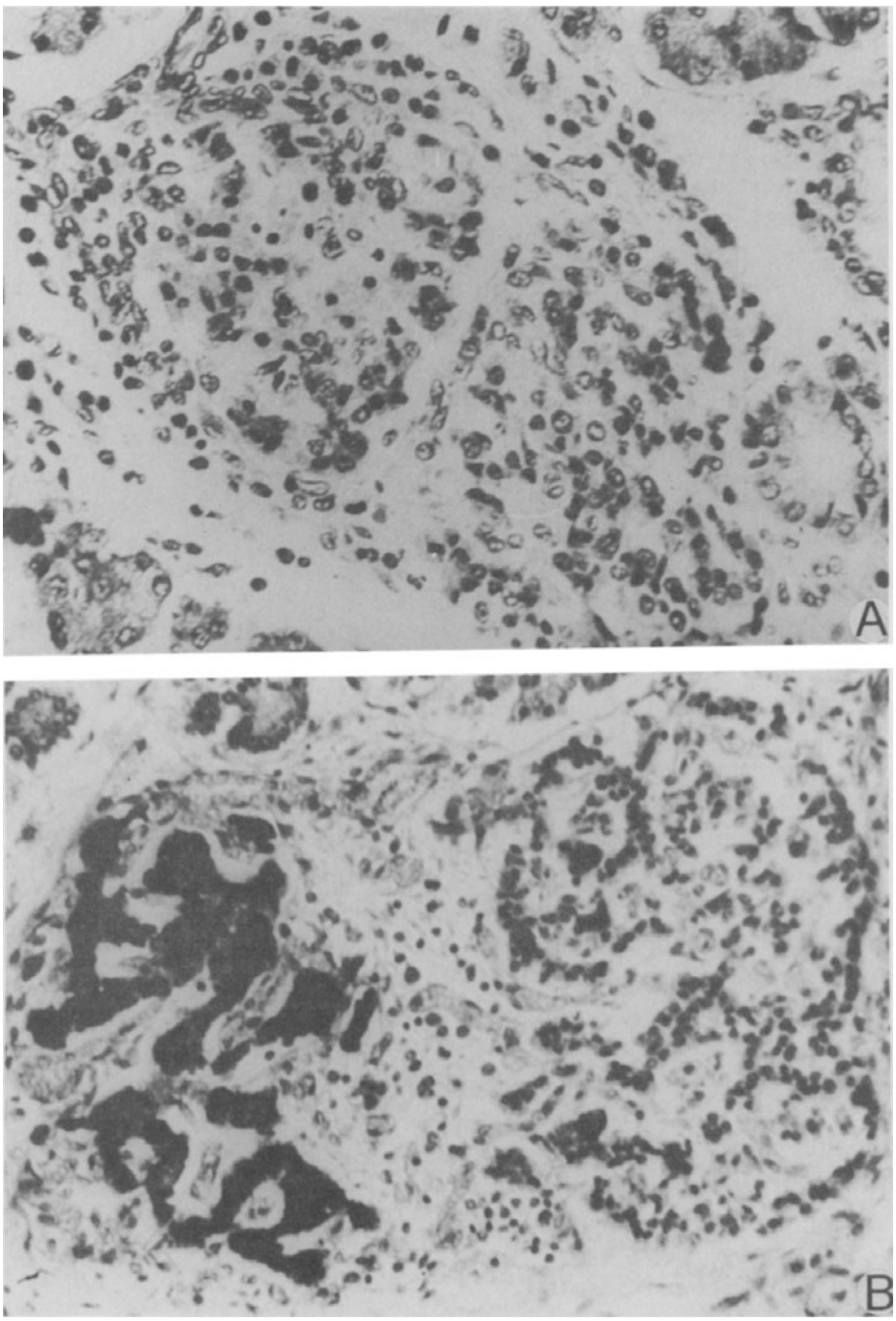

FIGURE 1. Histopathology of pancreatic islets of a neonate who died of disseminated CVB4 infection without evidence of diabetes. (Courtesy of Dr. A. B. Jenson.) (**A**) Degeneration and necrosis without lysis of islet cells is characterized by pyknotic nuclei and condensed eosinophilic cytoplasm. (**B**) Immunologic staining for insulin from the same case (immunoperoxidase method); insulin-containing cells are marked by the dark staining. Note that more than one half the islet is completely degranulated of insulin.

cently it has become apparent that these agents can also damage pancreatic endocrine cells, especially during the neonatal period. Thus Jenson *et al.*,[71] Kaplan *et al.*,[64] and Ujevich and Jaffe[72] examined the pancreas of neonates dying of overwhelming CVB infections and found that 17 of 53 cases had islet cell lesions of probable viral origin. Only one of these babies had hyperglycemia; actually, most of them were hypoglycemic before death. It is known, however, that it may take several days or months for clinical diabetes to develop after CVB infection, while all these babies died within a few days of infection. Another three cases with histopathologic reports were contributed by Yoon *et al.*,[60] Gladisch *et al.*,[63] and Ahmad and Abraham.[73] All these cases were examined during the period of acute disease and were characterized by degeneration and necrosis of islet cells accompanied by the infiltration of lymphocytes and macrophages. Necrotic islet cells were most prominent in islets containing large numbers of neutrophils. In most cases, no more than one third of islets were involved, and the large islets were frequently bisected into segments by a mixed inflammatory cell infiltrate; thus, one part of the islet appeared normal, whereas islet cells of the other segment were degenerated or had condensed eosinophilic cytoplasm and small dark nuclei. This lobular appearance of the damage appears to be characteristic of CVB infections (Fig. 1). When pancreas sections were examined by immunohistochemistry with antibody to insulin, mild to severe loss of insulin (degranulation) was apparent in those cells with eosinophilic cytoplasm and in frankly necrotic cells. Staining for other pancreatic hormones showed, however, that virus-induced damage was not confined to β-cells alone and also that α- and Δ-cells were sometimes lost or degranulated.[72] It is noteworthy that particularly in premature babies, islet cell necrosis was not accompanied by any cellular response. The extent of the exocrine damage was somewhat variable, and in many cases the endocrine damage predominated. Altogether, these cases have been attributed to CVB1 (three cases), CVB2 (two), CVB4 (four), CVB5 (one), and to untyped CVB (seven). Thus, these data suggest that all the CVB types can infect and damage pancreatic islets in newborn children and that, presumably, islet-cell involvement during CVB infection is more widespread than was commonly suspected. However, because only extremely severe infections have been studied, the possible relevance of neonatal CVB infection to the eventual development of diabetes in adulthood is not clear.

7. EXPERIMENTAL MODELS

The diabetogenic properties of CVB have been closely examined in mice because (1) these viruses may be implicated in human diabetes; (2)

unlike mumps and rubella, the host range include both humans and mice; (3) like the D and M variants of encephalomyocarditis virus, they produce a systemic infection in the animal model; and (4) it has long been known that they can infect the pancreas of mice.[74] All these reasons prompted numerous investigators to search for pancreatic endocrine damage in infected animals using histologic or metabolic criteria. Early studies, however, produced conflicting reports, probably because of the pronounced diversity of CVB isolates and of the genetic differences between various strains of test mice.

7.1. CVB-Induced Pancreatitis and Insulitis

In 1951, Pappenheimer and colleagues[74] showed that the Connecticut-5 strain of CVB1 induced pancreatitis in adult mice. This finding could not be confirmed by Gifford and Dalldorf[75] in newborn mice of a different strain, but 1 year later the same workers were able to adapt this virus to the pancreatic tissue of adult mice by repeated passage *in vivo*.[76] Infection with *in vivo* or *in vitro* grown CVB1 caused extensive destruction of pancreatic acinar tissue, but the islets of Langerhans were spared. While the hepatic damage was more pronounced in male than in female CD-1 mice, the degree of pancreatic involvement was independent of sex.[77]

Early electron microscopic studies suggested that CVB could occasionally produce alterations of pancreatic endocrine cells.[78] This finding was later confirmed by various investigators who studied the pancreatic pathology produced by CVB4, CVB1, and CVB3 in neonate and young adult outbred HaM/ICR or CD-1 mice.[79–81,83,84] Collectively, these studies indicated that after infection some islets of Langerhans underwent not only scattered degeneration and necrosis, but subtle atrophic changes as well. Light microscopic examination showed that, especially in areas of extensive exocrine damage, islets were reduced in size, and some islet cells had more eosinophilic cytoplasm with smaller and darker nuclei. Mononuclear cells were seen in the islets, but in no case was this condition pronounced. Electron microscopic changes were noted as early as 1 day postinfection. Minor damage consisted of vacuolation of cytoplasm with formation of membranous vesicles, with the rough endoplasmic reticulum still intact. Severe damage consisted of breakdown of cells, so that their type was difficult to ascertain: the cristae of mitochondria were swollen, the nuclear chromatin was marginated, and a marked loss of electron density was apparent. Loss of specific hormone granules was occasionally seen in β- and α-cells; Δ-cells appeared intact. Viral crystals of CVB1 were seen in a few mice both in acinar cells and in β-cells.[80] The individual particles measured approximately 210 Å in diameter and were separated by a distance of 60 Å. This observation is of

great significance in establishing a direct relationship between CVB infection and endocrine damage, as many of the subtle electron microscopic changes are compatible with the local effects of toxins or enzymes released during the acute destruction of acinar tissue. For unknown reasons, crystals of CVB other than type 1 were not detected during the course of these studies.

Not all studies, however, confirmed these findings. Lansdown[82] as well as the Notkins's group[85,86] could not find histopathologic changes in the endocrine pancreas of mice infected with laboratory strains or fresh isolates of the six CVB. Outbred (Swiss and CD-1) and inbred (DBA/2, BALB/c, A, CBA, and C57BL/6) mice were used. Although mild to severe pancreatitis was consistently produced by most virus–host combinations, the islets of Langerhans escaped severe damage. Acutely, capillary dilation and a slight disarray of islet cell architecture were observed. Rarely, β-cells had vacuolated cytoplasms and small pyknotic nuclei. Several months postinfection, the islets remained intact, although the acinar tissue had often been replaced by adipose tissue. Comparable results were obtained in pregnant mice and in mice given cortisone, silica, or other immunosuppressive treatments[82,86] (A. Toniolo, unpublished data). It is unfortunate that these mice were not studied by electron microscopy, as even these negative results suggest that CVB frequently produce minimal pathologic changes in the islets of Langerhans. Fluorescein-labeled polyclonal antibodies to CVB were used to ascertain whether these viruses were replicating in islet cells, but negative results were obtained.[85]

By contrast, islet cell lesions were easily observed when male mice of strains susceptible to the diabetogenic effects of encephalomyocarditis virus (SJL, SWR) were infected with preparations of CVB4 that had repeatedly been passaged in cultures enriched for pancreatic β-cells.[87] Mild inflammatory infiltrates were seen in pancreatic islets within 3–5 days of infection, and the severity of pathologic changes varied considerably among animals and within a single pancreas. Normal-appearing islets were often seen adjacent to islets showing extensive infiltrates. In some cases, only a portion of the islet was involved. Rarely, coagulation necrosis was observed. These findings are reminiscent of those described in the pancreas of neonates dying of severe CVB infections. Within 3 days postinfection, viral antigens were seen in the cytoplasm of islet cells and maximal involvement occurred at 4 days, with some islets containing only a few positive cells; others had viral antigens in most cells. Interestingly, acinar and ductal cells were almost free of infection. Comparable results were obtained in mice inoculated with the CVB4 strain that had been isolated from the acute case of diabetes reported by Yoon *et al.*[60]

That all CVB types can indeed infect and damage the endocrine

pancreas of mice was shown by Notkins's group in a subsequent study.[88] Prototype strains of CVB were serially passaged *in vitro* in monkey kidney cells, mouse embryo cells, or mouse β-cells and *in vivo* in the mouse pancreas. When susceptible SJL mice were inoculated with these virus stocks and their pancreata examined by immunofluorescence with type-specific sera, it was apparent that all six types had the potential of infecting some islet cells, had they been passaged in β-cell cultures (Fig. 2). Although the number of infected cells varied considerably among islets, in general 5–15% of islet cells contained viral antigens 4 days postinfection. Double-label technique with antiviral and anti-insulin antibodies showed that most infected cells were indeed insulin-containing β-cells. It is remarkable that only virus stocks grown in β-cell cultures or in the pancreas *in vivo* had the capacity of infecting appreciable numbers of islet cells. Virus preparations grown in monkey cells, in fact, had a remarkable tropism for the acinar tissue, whereas stocks obtained from mouse embryo cultures were somewhat attenuated and failed to induce viral antigens in exocrine and endocrine cells. β cell-passaged viruses produced mild islet damage, ranging from capillary dilation and few pyknotic nuclei to focal necrosis with mononuclear cell infiltration in approximately 10% of islets. Staining with anti-insulin antibody 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 types were indistinguishable.

Recent results obtained with fluorescein-labeled monoclonal antibodies indicate that various laboratory strains of CVB3 have the capacity of infecting minimal numbers of islet cells in mice without causing appreciable histopathologic changes (A. Toniolo and C. Garzelli, unpublished data). Thus, it appears that all CVB may have some tropism for islet cells but that significant endocrine damage is produced only by strongly β-tropic variants.

7.2. Metabolic Studies

In agreement with these pathologic findings, Ross *et al.*[86] reported that infection of different mouse strains with CVB1–6 caused elevations of serum amylase of as much as 10-fold and a reduction of the amylase content of the pancreas by more than 95%. In their studies, however, blood glucose levels were significantly reduced during the acute infection, and at no time was evidence of hyperglycemia found. These findings were in contrast to those of Coleman *et al.*,[83,84] who found CVB4 infection of CD-1 mice to result in a transient elevation of glucose (i.e., 15–20 days postinfection) in the absence of an absolute decrease in the concentration of insulin in blood. For this reason, both Notkins's group

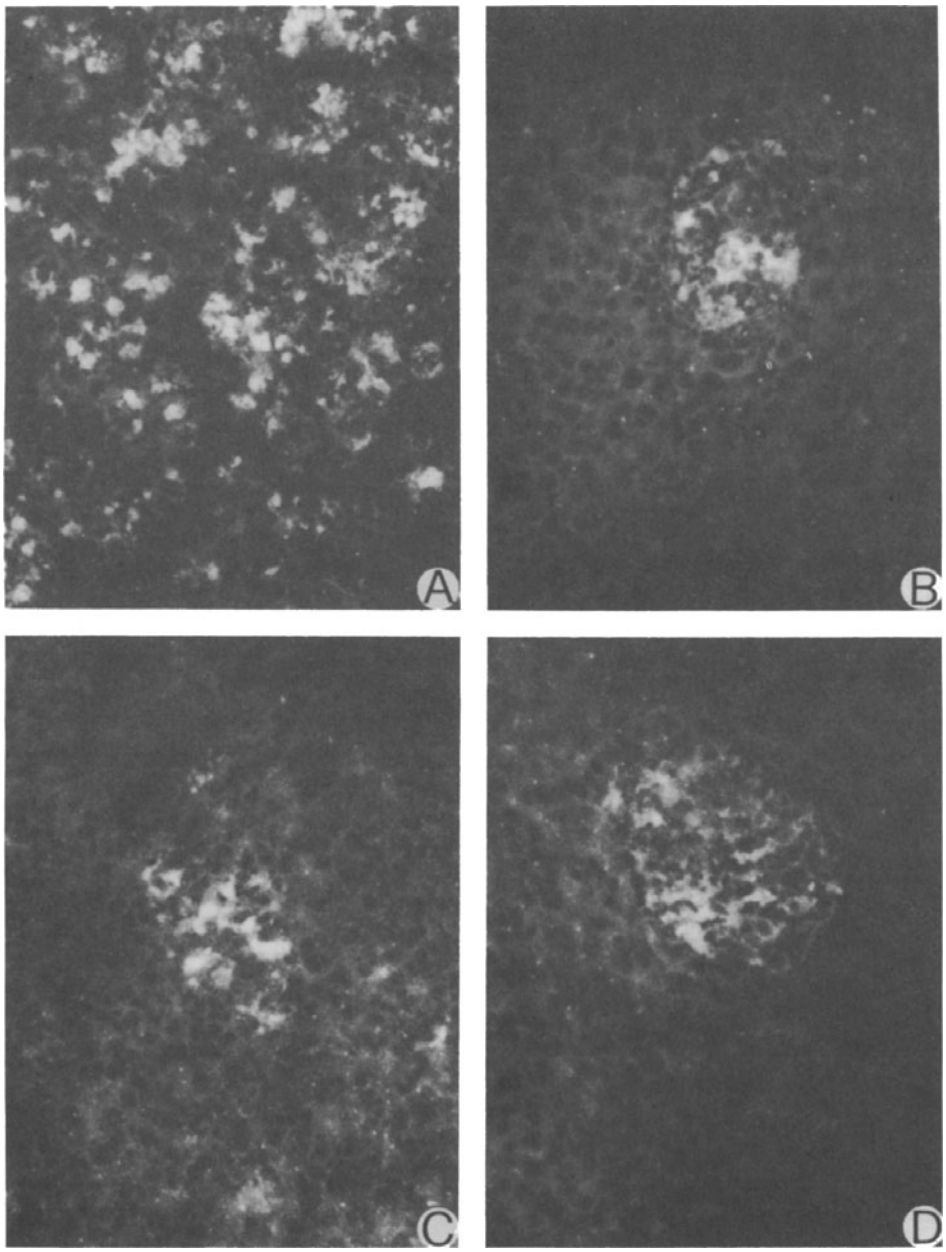

FIGURE 2. Change in the tropism of CVB after passage in mouse β-cell cultures. Four days postinfection, the pancreas was removed from infected mice, and frozen sections were stained with fluorescein-labeled antivirus antibody (×340). Infection with a prototype strain of CVB5 grown in monkey kidney cells. High concentrations of viral antigens in acinar cells (**A**). Antigens of CVB1 (**B**), CVB2 (**C**), and CVB5 (**D**) in the islets of Langerhans. These strains were passaged 15 times in β-cell cultures. Note that the original tropism for acinar cells is virtually lost.

and Craighead and co-workers examined fresh isolates and prototype strains of CVB4 (including the strain employed by Coleman in his studies) for their ability to produce hyperglycemia in mice.[85] Again, nonfasting glucose levels and glucose tolerance tests remained within the normal range. These contradictory results were tentatively attributed to environmental or dietary differences between test mice.

Profiting from the large experience gained in the study of diabetes induced by the encephalomyocarditis virus, Yoon *et al.*[87] used pancreatic β-cell cultures to increase the diabetogenic potential of CVB4 and susceptible male SJL mice as test animals. Four days after infection, the concentration of immunoreactive insulin (IRI) started to decrease both in the pancreas and in blood. Glucose levels correlated inversely with the amount of IRI, and more than 80% of animals were found to be hyperglycemic within 14 days of infection. The degree of hyperglycemia was highly variable, and the percentage of animals with elevated glucose concentrations decreased with time; thus, after 60 days less than 5% of animals were still hyperglycemic. However, many of the euglycemic mice were metabolically abnormal when evaluated by glucose-tolerance tests. It was not investigated whether, in addition to β-cells, CVB4 damaged glucagon- or somatostatin-secreting cells as well. Alterations of the functional capability of these cells, in fact, might also contribute to the production of hyperglycemia.

Precisely how passage in β-cell cultures can increase the diabetogenic capacity of CVB is not known, but alterations in the tropism of viruses after serial passage in animals or tissue culture is a widely recognized phenomenon. Recent studies[89,90] on CVB4 have demonstrated that antigenic changes at the epitope level occur at a frequency of $>10^{-2}$. This points to the possibility that even within the same virus pool there are many genetic variants and that variants with the appropriate tropism for β-cells are selected for during replication in β-cell-enriched cultures. Thus, the great difficulty in obtaining relatively pure β-cell cultures might contribute to explain why experiments with truly diabetogenic strains of CVB were carried out so rarely. It should be borne in mind, however, that about one third of field strains may induce mild glucose abnormalities in susceptible mice.[91]

Only mild alterations of glucose tolerance were produced in mice even by prototype strains of the six CVB that had been serially passaged in pancreatic β-cell cultures.[88] By contrast, remarkable elevations of glucose levels resulted when these strains were injected into mice whose β-cell reserve had been reduced with small doses of streptozotocin, a potent β-cell toxin.[88,92] This observation, together with direct counts of the islet cells expressing viral antigens, indicates that CVB usually do not damage enough β-cells to produce overt diabetes. Hyperglycemia is in-

stead produced in mice with reduced numbers of functional β-cells. This principle was recently confirmed by Yoon *et al.*[93] in patas monkeys. Infection of these animals with CVB4 resulted in transient elevations of glucose-tolerance tests and depressed insulin secretion and glucose in the urine. All these effects were markedly enhanced by pretreatment of monkeys with a subdiabetogenic dose of streptozotocin, but no ICA were detected in these animals. Comparable cumulative effects have been obtained when BALB/c mice, a strain resistant to virus-induced diabetes, were infected with CVB4.[94] Our own experience and that of Jordan *et al.*[91] with mice, together with a few data obtained with monkeys,[93] support the possibility that the diabetogenic potential of CVB not only can be increased by pretreatment with β-cell toxins, but also by sequential infections of the same host with different CVB.

7.3. Genetic Aspects

Numerous studies indicate that the capacity of CVB to induce diabetes is influenced by the genetic background of the host. Yoon *et al.*[87] found that mouse strains susceptible to diabetes caused by encephalomyocarditis virus were also susceptible to CVB4-induced hyperglycemia and that male mice developed more severe diabetes than did females. This finding has been confirmed by others,[88,91,92,95] and it is now believed that strains such as SJL, SWR, and NFS are susceptible and that DBA/2, Swiss, and CD-1 intermediate, C57BL/6, CBA, AKR, BALB/c, and C3H are resistant. Susceptibility does not seem to correlate with MHC type. Recent experiments[93] suggest that even the susceptibility of monkeys to CVB4 is under genetic control and that so far, pancreatic endocrine damage has been induced only in patas monkeys.

Considerable efforts were also spent to assess the role of certain loci conferring predisposition to diabetes in the susceptibility of mice to CVB. Most of these studies were carried out in C57BL mice homozygous or heterozygous for the diabetes-associated genes *db* or *ob*.[95–98] Susceptibility to CVB4-induced pancreatic disease and mortality rate were compared with those of the parental background strains. It was generally seen that genetic traits predisposing to diabetes were associated with an increased mortality after infection and with the development of more severe pancreatic disease. However, because of the low diabetogenic potential of the infecting virus, clear-cut data on the precise relationships between genetic traits and the metabolic response to CVB4 infection were not obtained. No genotypic differences were seen in the virus levels attained in the pancreas, but β-cells of mice carrying the *db* gene underwent severe degranulation during acute infection. This alteration, however, was not followed by overt hyperglycemia, and the

most reproducible finding was an acute release of insulin accompanied by hypoglycemia during the early phase of infection.[95,96] Studies of these and other strains of mice demonstrated an inverse relationship between the effects of CVB4 on the exocrine and the endocrine pancreas. In other words, mouse strains susceptible to viral diabetes had less severe acinar pancreatitis than did mice resistant to viral diabetes.[95] The phenotypic basis of this phenomenon is unknown. More recently, it was shown that C57BL/Ks mice homozygous for the *db* gene fail to produce neutralizing antibodies to CVB4 and have other immune abnormalities reminiscent of those described in the prediabetic period in humans.[99]

Progress in these and other genetic aspects of CVB-induced hyperglycemia will depend, however, on the development of reliable methods for obtaining truly diabetogenic variants of CVB. No useful markers for such variants have yet been detected,[100] and empirical methods for the plaque purification of diabetogenic variants based only on tests for the induction of diabetes *in vivo* have been unsuccessful so far (A. Toniolo, unpublished data).

7.4. CVB Infection of Cultured Pancreatic Endocrine Cells

According to one study,[101] mouse pancreatic organ cultures can support CVB replication only if obtained from mice younger than 3 weeks. After that age, no production of infectious virus occurred. Thus, it appears that multiplication in isolated mouse organs ceases at a much earlier age than in the intact animal. In our experience, *in vitro* cultured pancreatic β-cells can support the replication of all CVB, even if taken from mice older than 2 months of age. Within 2–6 days postinfection, CVB produce a rapid lytic effect in these cultures.[87,88] To date, no functional evaluations of CVB-infected mouse β-cell cultures have been reported. By contrast, insulin release has been measured in a continuous line of rat insulinoma cells (RIN cells) persistently infected with CVB4.[101] High titers of infectious virus were continuously released from infected cultures without cytopathology, and the total release of insulin in response to both low and high concentrations of glucose was not reduced by CVB4. These investigators proposed that CVB-induced diabetes might be initiated by a chronic infection, possibly leading to immunopathologic damage of β-cells. However, caution should be exercised in interpreting these data, since persistent CVB infections of human β-cells have not been documented, and it is known that rats are highly resistant to CVB. Thus, this condition seems totally different from that of mice and human subjects, where β-cell permissiveness to CVB is the rule.

An *in vitro* model has been developed to determine whether viruses are capable of infecting human β-cells. By use of double-label immu-

nofluorescence with antibodies to insulin and CVB, it was shown that CVB3 and CVB4 do replicate in insulin-containing β-cells and that these cells are killed by the virus.[60,103] Radioimmunoassay (RIA) showed that intracellular IRI decreased rapidly, beginning at 24 hr after CVB3 infection, and that the decrease in insulin roughly paralleled the increase in viral titer. Only a few human pancreas were used for these studies, precluding any conclusions as to the influence of the HLA phenotype on β-cell susceptibility.

7.5. Mechanisms of Endocrine Damage

What is the pathogenesis of CVB-induced hyperglycemia? Two major pathways may lead from islet cell infection to diabetes: (1) direct lysis of target cells, and (2) virus-induced immunopathology.[104]

The first possibility is suggested by the type of histopathologic damage seen in the pancreas of neonates dying of overwhelming CVB infections and of rare cases of acute diabetes induced by CVB.[60,71] Experimental studies suggest that β-cell lysis is most probably involved as well in the histologic lesions observed in mice and in the acute release of insulin occurring in the early phase of infection.[87,95] That CVB cause only mild metabolic effects is probably due to the fact that these agents usually infect and damage only small numbers of β-cells. It is more difficult to interpret the long-term defects of insulin production seen not only in mice,[87,106] but also *in vitro* in isolated islets of Langerhans[105] and in cultured β-cells[106] obtained from CVB4-infected mice. The studies mentioned above suggest that, after infection, subtle long-term alterations of β-cell functions do occur in the pancreas of mice. Although releasing unchanged or greater than normal amounts of insulin in response to glucose *in vitro,* β-cells isolated from infected mice had a reduced synthesis of total proteins and, in particular, of pro-insulin and insulin. The biochemical basis of these defects is unknown, but it is likely that they contribute to an altered control of glucose homeostasis.

The possibility of an immune-mediated pathogenesis of CVB-induced β-cell damage would strengthen the association of these agents with human diabetes. It is known that most cases of IDDM are preceded by a long prediabetic period characterized by metabolic and immunologic abnormalities and, in particular, by autoimmune reactions to the endocrine pancreas. What triggers pancreatic autoimmunity is, however, unknown.

An hypothesis connecting viruses with the origin of endocrine autoimmunity has been recently proposed.[3] Briefly, it has been postulated that, as a consequence of local damage, an inflammatory response may be induced in endocrine tissues. Interferon or other mediators released

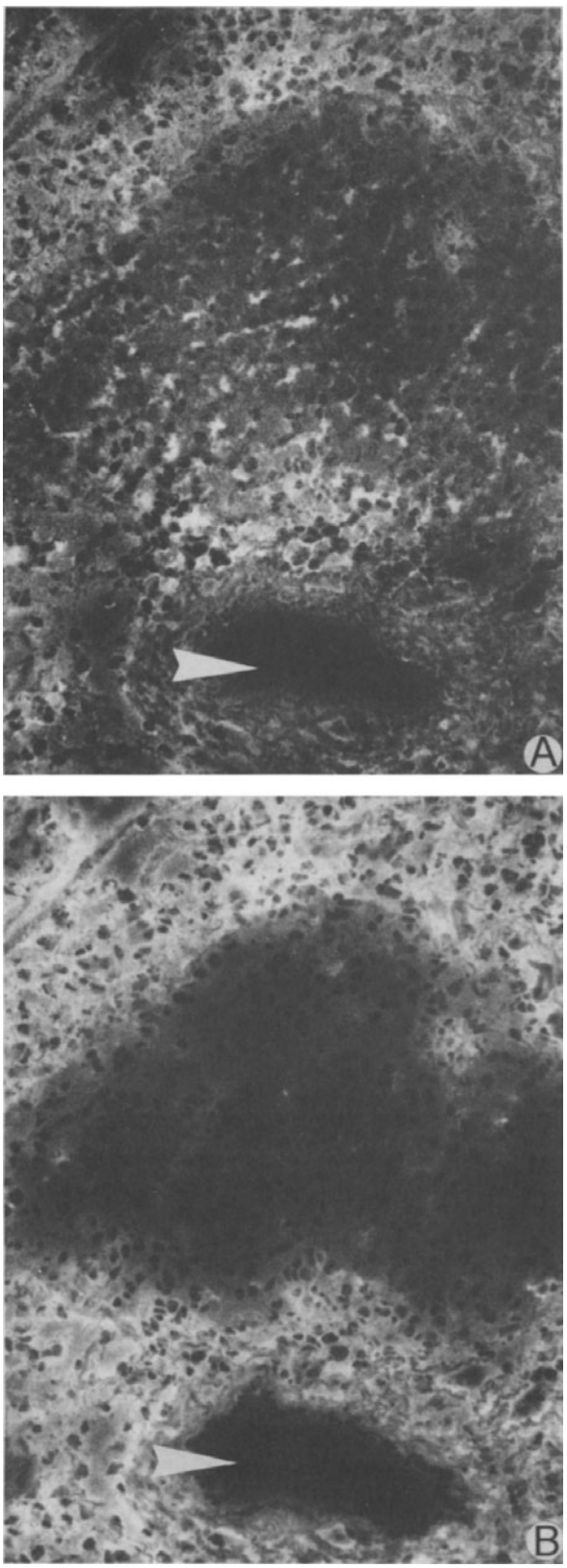

FIGURE 3. Lack of expression of Ia antigens in endocrine pancreatic cells of DBA/2 mice infected with CVB3. Four days postinfection with prototype CVB3, frozen sections of pancreas were stained with fluorescein-labeled monoclonal antibody to CVB3 and rhodamine-labeled monoclonal antibody to murine Ia d (×200). With fluorescein filters, small amounts of viral antigens were seen in islet cells and substantial amounts in the surrounding acinar tissue (**A**). When the same section is seen with rhodamine filters it appears that high concentrations of Ia molecules are expressed in exocrine cells, but not in islet cells (**B**). The arrows point to the lumen of a pancreatic duct.

in situ would induce the aberrant expression of the major histocompatibility complex class II molecules (Ia antigens) on endocrine epithelial cells. Ia-positive epithelial cells, in turn, would become capable of presenting autoantigens to infiltrating lymphocytes, triggering an organ-specific autoimmune response.

It has been shown *in vitro* that this sequence of events may be initiated by coronavirus infection of rat astrocytes[107] and, more importantly, high levels of Ia antigens have been demonstrated *in vivo* in the islets of Langerhans of individuals dying in the early phase of acute diabetes.[108]

To see whether CVB infection of mice is followed by the induction of Ia antigens on islet cells, we infected mice with CVB3 and searched for the expression of viral and murine Ia antigens on frozen sections of pancreas. Direct staining with fluorescein-labeled monoclonal antibody to CVB3 and rhodamine-labeled monoclonals to the Ia antigen of BALB/c and DBA/2 mice showed that moderate CVB3 infection of islet cells does not induce the *de novo* expression of Ia antigens on endocrine cells. By contrast, virus-infected exocrine epithelial cells were strongly Ia positive (Fig. 3). Control studies showed that normal pancreatic acinar cells do not express Ia antigens and that these molecules appear on acinar cells approximately 2 days postinfection. Our observations, however, were limited to the early phase of infection, characterized by the expression of viral antigens in islet cells (2–6 days postinfection). Further studies are in progress. These results are in agreement with those of Bottazzo and colleagues,[109] showing that human endocrine pancreatic cells fail to express Ia molecules under a variety of physiologic stimuli *in vitro*. Under the same conditions, pancreatic exocrine cells were strongly Ia positive.

As it has been reported that ICA may be induced by CVB infections in humans,[65] and it is known that ICA may appear in virus-infected mice,[110] we also searched for ICA in the serum of CVB-infected mice. Pancreatic autoantibodies were not detected. Thus, experimental evidence linking CVB infections to the production of autoimmune diabetes is lacking. However, as already observed in CVB-induced cardiac autoimmunity,[111] it is likely that only a few strains of mice are capable of mounting an autoimmune response against islet cells as a consequence of CVB infection. Newer techniques, and in particular hybridoma technology, will permit assessment of the possible role of molecular mimicry and of anti-idiotypic antibodies in the pathogenesis of CVB-induced endocrine disease.[112]

8. SUMMARY AND CONCLUSIONS

What can we learn from these studies on viral diabetes? First, it has been shown that common human viruses, such as the six CVB, have the

potential of infecting and damaging pancreatic endocrine cells causing hyperglycemia. Second, serologic studies demonstrated that up to one third of cases of IDDM in the young are probably preceded by CVB infection. Third, experimental studies have shown that the genetic makeup of the host, as well as phenotypic factors, can modulate the endocrine effects of CVB infections. Fourth, the tropism of CVB to pancreatic islet cells may be increased by *in vitro* passage in β-cell cultures. Fifth, the roles of persistent infections and autoimmunity in CVB-induced endocrine damage have just begun to be investigated. Progress on this point would help explain the reported high frequency of CVB infections in the prediabetic period.

We are confident that newer methods to detect viral antibodies, as well as the use of immunologic and genetic viral probes for the histopathologic analysis of the pancreas from cases of recent-onset diabetes, will permit more precise evaluation of the role of CVB in human diabetes. This knowledge will be of utmost importance should preventive measures for IDDM be worked out. At the very least, these studies on the viral etiology of diabetes should serve as a guide for future investigations on idiopathic diseases of suspect viral origin.

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