A Histological Study of Fulminant Type 1 Diabetes Mellitus Related to Human Cytomegalovirus Reactivation

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Context: Fulminant type 1 diabetes mellitus (T1DM) is thought to be partly caused by virus infection.

Objective: This study investigated the mechanism of β cell destruction in fulminant T1DM after drug-induced hypersensitivity syndrome (DIHS).

Methods: We determined the localization of human cytomegalovirus (HCMV), human herpesvirus 6 (HHV-6), and Epstein-Barr virus (EBV) and the expression of interferon regulatory factor 3 (IRF3) and viral receptors of Z-DNA binding protein 1 (ZBP1) and retinoic acid-inducible gene I (RIG-I), together with inflammatory cells, by immunohistochemistry of the autopsy pancreas of a patient with fulminant T1DM with DIHS and in seven subjects with normal glucose tolerance who underwent pancreatectomy.

Results: HCMV-positive cells were detected in islets and exocrine areas in the patient with fulminant T1DM. Greater numbers of macrophages and $CD4^+$ and $CD8^+$ T lymphocytes had infiltrated into HCMV-positive islets than into HCMV-negative islets, and 52.6% of HCMV-positive cells were also positive for IRF3. α Cells expressed IRF3, ZBP1, or RIG-I. No HCMV-positive cells were detected in the control subjects. HHV-6−positive, but not EBV-positive, cells were present in the patient and the control subjects.

Conclusions: These findings indicate that the immunoresponse caused by HCMV infection was associated with β cell injury. (***J Clin Endocrinol Metab*** **102: 2394–2400, 2017)**

Fulminant type 1 diabetes mellitus (T1DM), characterized by the extremely rapid progression of hyperglycemia and ketosis/ketoacidosis caused by the destruction of almost all pancreatic β cells, has been established as a subtype of idiopathic T1DM (1, 2). The etiology is thought to be viral infection because preceding flulike symptoms are frequently observed (2). In addition, there have been several reports of elevated broadly reactive anti-enterovirus immunoglobulin A (3) and antivirus antibody to human herpesvirus 6 (HHV-6), mumps virus, Coxsackie virus B4, and Epstein-Barr virus (EBV) in fulminant T1DM (4–7). However, only enterovirus has been detected in the autopsy pancreas of a patient with fulminant T1DM (8–10).

Drug-induced hypersensitivity syndrome (DIHS) is a severe adverse drug reaction caused by carbamazepine, allopurinol, or mexiletine; it is characterized by visceral organ involvement and the reactivation of various human herpesviruses, such as HHV-6, human cytomegalovirus (HCMV), and EBV (11). Although the frequency of fulminant T1DM in DIHS is only 0.54%, this is much higher than the frequency in the general Japanese population

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA

Received 26 December 2016. Accepted 5 April 2017.
First Published Online 10 April 2017

Abbreviations: ABC, avidin-biotin complex; DAB, 3,3-diaminobenzidine; DIHS, drug-induced hypersensitivity syndrome; EBV, Epstein-Barr virus; HCMV, human cytomegalovirus; HHV-6, human herpesvirus 6; IFN, interferon; IRF3, interferon regulatory factor 3; RIG-I, retinoic acid-inducible gene I; T1DM, type 1 diabetes mellitus; ZBP1, Z-DNA binding protein 1.

(0.010%) (12), indicating that virus reactivation might be associated with the onset of fulminant T1DM.

In this study, we performed immunohistochemical examination of an autopsy pancreas from a patient who developed fulminant T1DM after DIHS.

Patient and Methods

Case presentation

A 69-year-old male was prescribed allopurinol for hyperuricemia. After 2 weeks, he had exanthema, fever, and cervical lymphadenopathy, and elevated hepatic enzyme and C-reactive protein levels were observed in his blood test results. He was then admitted to a hospital. Although he was treated with prednisolone, the exanthematous eruption persisted. At 3 weeks after admission, his levels of plasma glucose, glycoalbumin, and serum C-peptide were 836 mg/dL, 15.7% (normal range, 12.4–% to 16.3%), and 0.15 ng/mL (normal range, 0.61 to 2.09 ng/mL), respectively. A diagnosis of diabetic ketoacidosis was made on the basis of an arterial blood pH of 6.998, bicarbonate level of 2.1 mmol/L, anion gap of 34.9 mEq/L, 3-hydroxybutyrate level of 8.1 mmol/L (normal range, 0 to 0.074 mmol/L), and lactic acid level of 5.5 mmol/L (normal range, 0.44 to 1.78 mmol/L). After 1 week, his hemoglobin A1c level (NGSP) was 7.7%, and HCMV pp65 antigen was detected in his blood. He was diagnosed with fulminant T1DM after DIHS. After 3 weeks, he died of septicemia and heart failure.

Control subjects

Seven patients with normal glucose tolerance who had undergone pancreatic resection between 2009 and 2013 in the Department of Gastroenterological Surgery, Osaka University Hospital, were evaluated as control subjects. The tissues examined in this study were identified as noncancerous lesions by hematoxylin and eosin staining.

Immunohistochemistry

The primary and secondary antibodies and chromogenic substrates used in this study are listed in Supplemental Tables 1–3. To measure relative β or α cell areas, pancreatic sections were stained for insulin or glucagon using an avidin-biotin complex (ABC) and 3,3-diaminobenzidine (DAB) tetrahydrochloride substrate (13). Next, we used double immunofluorescence staining (14) for HCMV and insulin, glucagon, or amylase.

To evaluate a relationship between the infiltration of inflammatory cells into islets and HCMV infection, pancreatic sections were stained for glucagon and HCMV by immunofluorescence staining and for CD68 by the ABC-DAB method (triple staining). In addition, we performed double immunofluorescence staining for glucagon and HCMV or glucagon and CD4 or CD8 on serial sections. For control tissues, we performed double immunofluorescence staining for insulin and CD68 or CD3.

To evaluate antivirus immune responses to the virus infection, we also determined the expression of interferon regulatory factor 3 (IRF3), a transcription factor essential for type I interferon (IFN) production, using ABC-DAB staining and double immunofluorescence staining with HCMV or glucagon. The same method was used to examine the expression of Z-DNA binding protein 1 (ZBP1) and retinoic acid-inducible gene I (RIG-I), which are representative DNA and RNA sensors, respectively.

To screen for multiple infections, we stained for HHV-6, EBV, and enterovirus.

Morphometric analysis

Relative β or α cell area, the area of tissue containing insulin- or glucagon-positive cells in the entire pancreatic section, was quantified digitally using the WinROOF software program (Mitani Corporation, Japan) as previously reported (15). We counted all HCMV-, insulin-, or glucagon-positive cells within one section for analysis of the double immunofluorescence staining.

To evaluate the infiltration of inflammatory cells into islets, islets >150 μm in diameter were examined as analysis objects, and we regarded inflammatory cells found in the islet periphery and throughout the islet parenchyma as "infiltrating" (16). To evaluate the localization of HHV-6 and enterovirus, we examined islets consisting of four or more endocrine cells.

Statistical analysis

Data that were normally distributed are presented as the mean ± standard deviation unless otherwise noted. Data for continuous variables with a skewed distribution are presented as the median (minimum, maximum). The number of inflammatory cells infiltrating islets with or without HCMV infection was analyzed by the Student t test if continuous and parametric and by the Mann-Whitney U test if continuous and nonparametric. Differences were considered significant for values of $P < 0.05$. All statistical analyses were performed with the StatView software program (Statistical Analysis System Inc., Cary, NC).

Study approval

The study protocol was approved by the Ethics Committee of Osaka University and was conducted according to the principles of the Declaration of Helsinki. All patients provided informed consent before their participation.

Results

Relative β and α cell areas

In this case, the total pancreas area was 263 mm^2. Relative β and α cell areas were 0.017% and 0.084%, respectively. These areas were significantly reduced compared with those of the control subjects (total pancreas area, 8.4 ± 3.3 mm^2; relative β cell area, 1.56% ± 0.50%; relative α cell area, 0.17% ± 0.09%).

Localization and prevalence of HCMV in β and α cells and acinar cells

A representative image of HCMV infection by ABC-DAB staining is shown [Fig. 1(A)]. Overall, 907 ± 22 HCMV-positive cells were detected per section in β cells, α cells, and acinar cells: 0%, 0.8%, and 91.6%, respectively [Fig. 1(B–D)]. The prevalence of HCMV in β cells, α cells, and acinar cells was 0% (n = 54), 0.2%

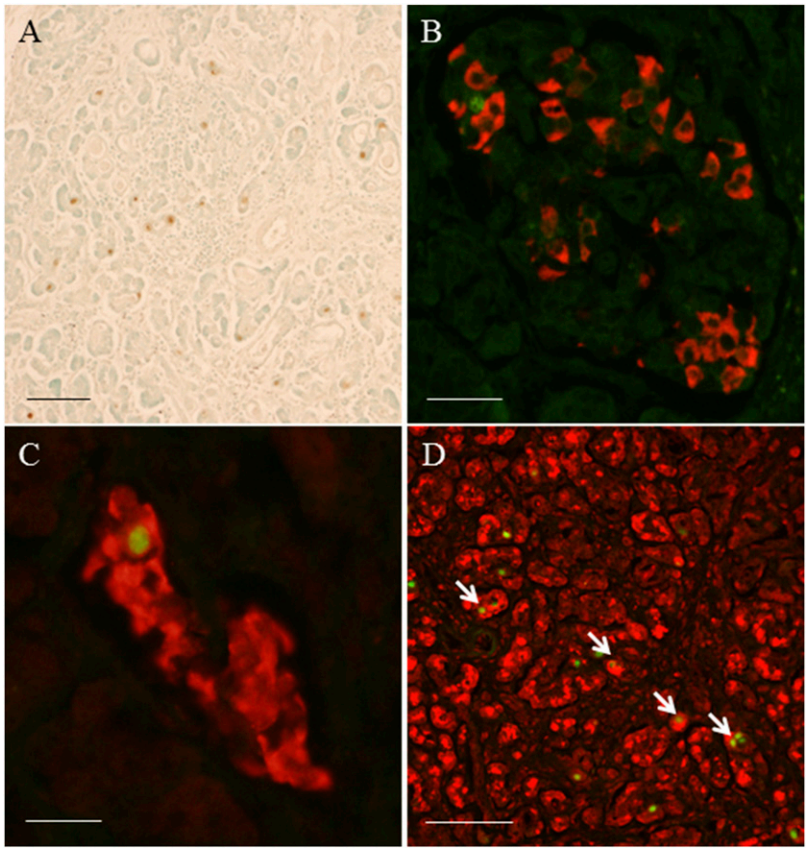

Figure 1. HCMV infection and localization in the pancreas. (A) Representative image of HCMV infection in the pancreas of this case. Double immunofluorescence staining for (B) HCMV and insulin, (C) HCMV and glucagon, and (D) HCMV and amylase. (B) HCMV (green) was not detected in residual β cells (red); (C) however, it was detected in α cells (red). (D) High levels of HCMV (arrows) were detected in acinar cells (red). Bars = (A, D) 200 μm, (B) 100 μm, and (C) 50 μm.

(n = 3078), and 1.6% (n = 1000), respectively. Notably, no HCMV-positive cells were detected in the pancreases of the control subjects.

Infiltration of inflammatory cells

We separately evaluated the infiltration of inflammatory cells into HCMV-negative and HCMV-positive islets. The numbers of $CD68^+$ cells infiltrating HCMV-negative and HCMV-positive islets with residual α cells were 4.2 ± 5.1 (n = 239) and 6.5 ± 6.5 (n = 11) (P = 0.154), respectively [Fig. 2(A–C)]. The numbers of infiltrating $CD4^+$ cells were 0 (0, 5) (n = 271) and 1 (0, 6) (n = 17) (P = 0.0023), respectively [Fig. 2(D–F)], and the numbers of infiltrating $CD8^+$ cells were 0 (0, 10) (n = 267) and 3 (0, 20) (n = 16) (P = 0.0012), respectively [Fig. 2(G–I)]. In the control subjects, the numbers of $CD68^+$ or $CD3^+$ cells in and around the islets (n = 16 ± 12) were 0 ± 0 and 0.07 ± 0.10, respectively.

IRF3, ZBP1, and RIG-I expression

In this case, double staining for IRF3 and HCMV revealed that 52.6% of the HCMV-positive cells (n = 1559) were also positive for IRF3 [Fig. 3(A–C)]. The rate of IRF3 positivity in α cells was 0.3% (n = 2872) (Fig. 3(D–F)]. In the control subjects, the number of IRF3-positive cells was 2.4 ± 2.8 (per section per subject) detected in the nonislet and nonexocrine regions. The rates of ZBP1 and RIG-I positivity in α cells were 0.4% (n = 2904) and 1.1% (n = 2987), respectively [Fig. 3(G–L)]. In the control subjects, there was no expression of ZBP1 or RIG-I.

Presence of HHV-6, EBV, and enterovirus in the pancreas

The numbers of HHV-6−positive cells in one pancreatic section of this case and in the control subjects were 422 [Fig. 4(A)] and 87 ± 87 [Fig. 4(B)], respectively. Double immunofluorescence staining for HHV-6 and glucagon or insulin revealed that all of the HHV-6 infected cells were present in nonislet areas [Fig. 4(C)] (numbers of islets in the case and in the control subjects were 181 and 31 ± 14, respectively). No EBV-positive cells were detected in the case or in any control subjects. For enterovirus infection, only one VP1–positive cell was detected in one islet of two sections widely separated [Fig. 4(D) and 4(E)], into which no $CD8^+$ cells had infiltrated [Fig. 4(F)]. VP1-–positive cells were not detected in any control subjects.

Discussion

In the patient who developed fulminant T1DM after DIHS, many HCMV-positive cells were detected in islet cells and exocrine lesions of the autopsy pancreas. Although HCMV-positive β cells were not detected because of their almost complete destruction, there were significantly increased numbers of α cells expressing IRF3, ZBP1, or RIG-I; there were also increased numbers of infiltrating macrophages and $CD4^+$ and $CD8^+$ T lymphocytes in HCMV-positive islets compared with negative islets, indicating that β cells were injured by HCMV-specific immunoresponses.

We detected HCMV infection in the islet cells of an autopsy pancreas with fulminant T1DM. To date, only enterovirus has been detected in the fulminant T1DM pancreas (8–10). In the current case, HCMV infection

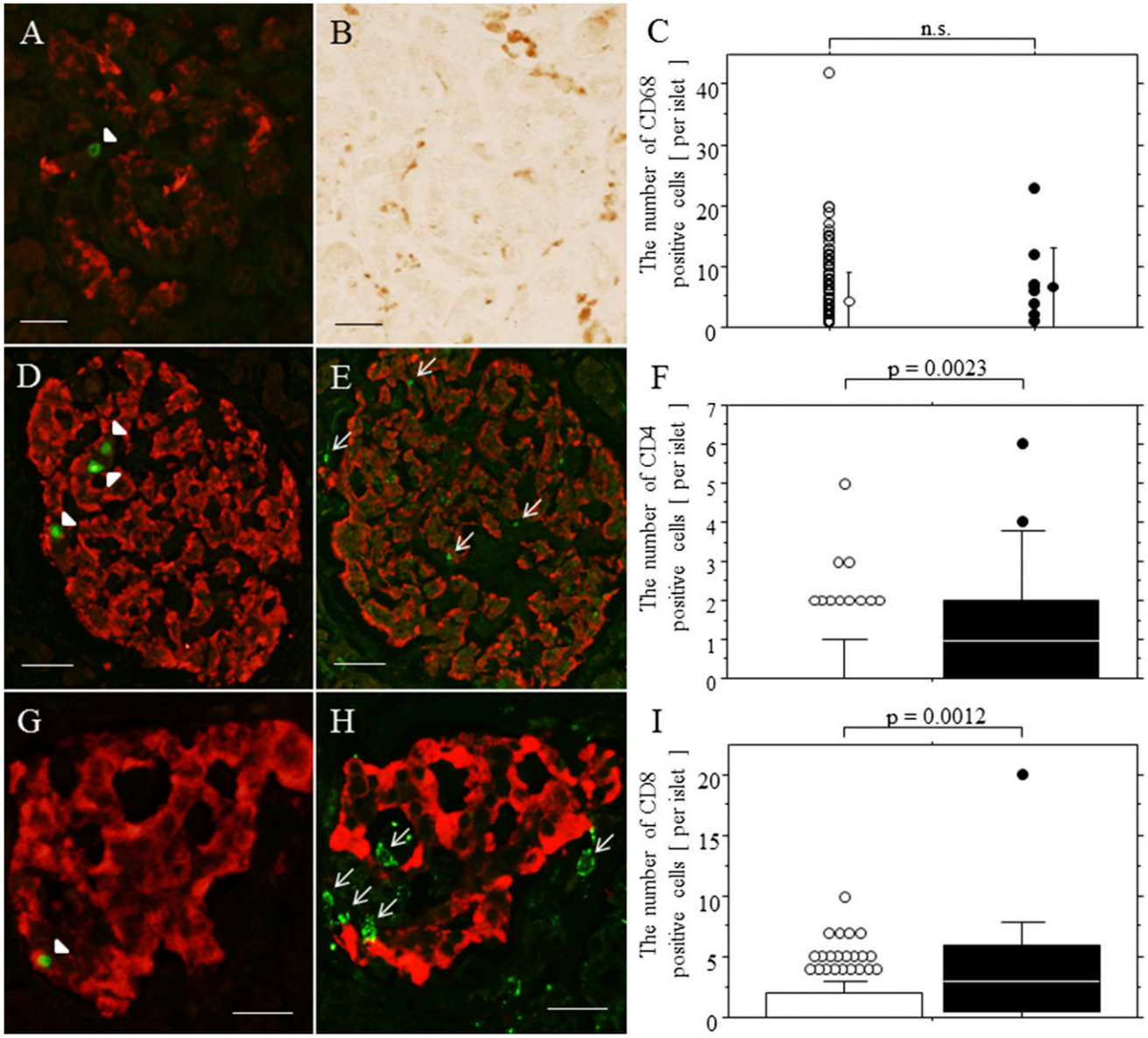

Figure 2. Infiltration of CD68$^+$, CD4$^+$, and CD8$^+$ cells. (A, B) Triple staining of the same section for HCMV (green, arrowhead), glucagon (red), and CD68 (DAB). Double immunofluorescence staining for (D, G) HCMV (green; arrowheads) and glucagon (red); (E) CD4 (green; arrows) and glucagon (red); and (H) CD8 (green; arrows) and glucagon (red) in serial sections (D, E and G, H). (C, F, I) Infiltration of macrophages and CD4$^+$ and CD8$^+$ T lymphocytes was increased in HCMV-positive islets (black circle and black bar) compared with HCMV-negative islets (white circle and white bar). Bars = (A, B, G, H) 50 μm and (D, E) 100 μm. Error bars indicate standard deviation (C) and 95% confidence interval (F, I). n.s., not significant.

was detected in 0.2% of α cells, and the infiltration of inflammatory cells was increased in HCMV-positive islets compared with HCMV-negative islets, suggesting that the specific immunoresponse for HCMV was generated in HCMV-infected α cells and then spread to nearby β cells or possibly directly in HCMV-infected β cells.

Because the relative β cell area was decreased by 97% compared with that of control subjects and few residual β cells remained, we could not detect HCMV-positive β cells in this case. It was previously reported that a human β cell could be infected with HCMV (17, 18). HCMV-infected CM insulinoma cells showed enhanced expression of major histocompatibility complex class I and intercellular adhesion molecule-1, the production of inflammatory cytokines such as interleukin-6 and interleukin-8, and the activation of the peripheral blood mononuclear leukocytes (18). Although CM insulinoma cells are different than native β cells regarding the function of insulin secretion, they have a high homology with β cells concerning their basic cell structure, including surface markers that have a role in virus infection. Thus, it is possible that in this case β cells might have been infected with HCMV

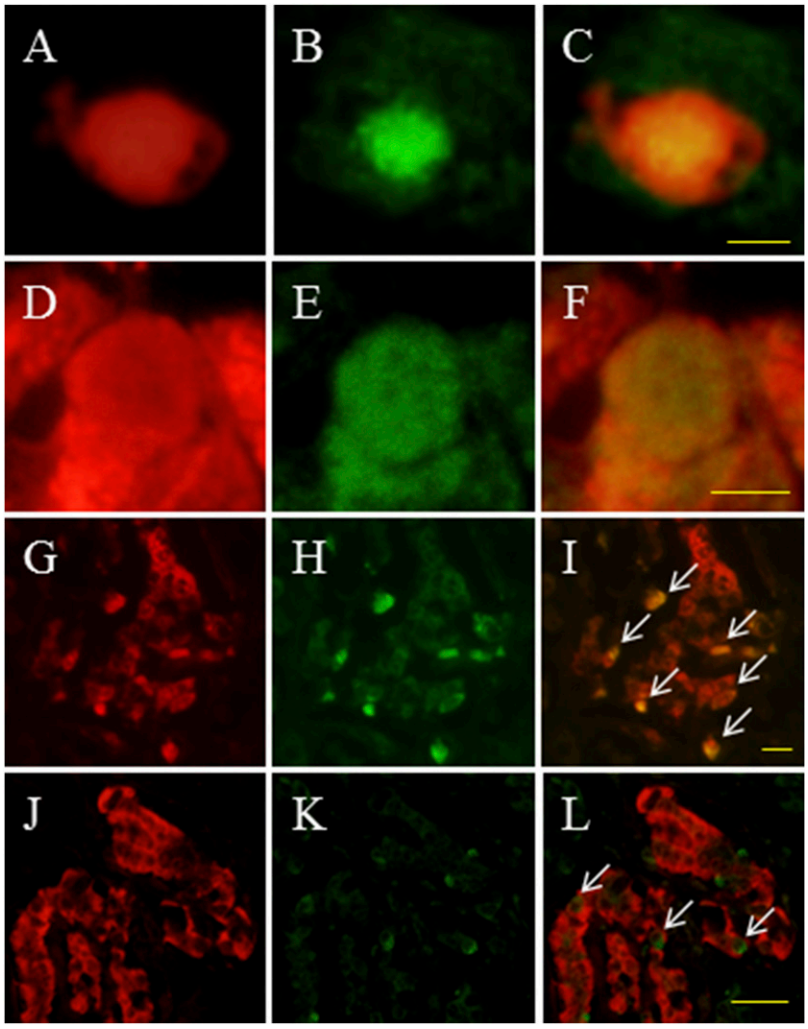

Figure 3. Expression of IRF3, ZBP1, and RIG-I. Double immunofluorescence staining for (A) HCMV (red) and (B) IRF3 (green). (C) HCMV-infected cells expressed IRF3 as shown in the merged image. Double immunofluorescence staining for (D) glucagon (red) and (E) IRF3 (green). (F) Some α cells expressed IRF3 as shown in the merged image. Double immunofluorescence for (G, J) glucagon (red) and (H) ZBP1 (green) or (K) RIG-I. (I, L) α cells expressing ZBP1 or RIG-I are shown in the merged images (arrows). Bars = (C, F, I) 10 μm and (L) 50 μm.

and injured by HCMV-specific immunoresponses, which is distinct from autoimmune T1DM characterized by β cell−specific immunoresponse. The other possible mechanism in fulminant T1DM in this case could be a bystander effect. Coxsackie B4 virus infection in nonobese diabetic mice, which carry the nonobese diabetic major histocompatibility complex allele to which presentation of the cross-reactive epitope is restricted, did not change the course of diabetes, but Coxsackie B4 infection of transgenic mice, which harbor a transgene encoding a diabetogenic T cell receptor specific to an islet antigen and not cross-reactive with Coxsackie B4, resulted in the rapid onset of diabetes (19). This indicates that Coxsackie B4 virus might induce tissue damage through bystander T cell activation. Similarly, HCMV infection in islet cells might induce the activation of bystander lymphocytes, including autoreactive T cells, which might contribute to β cell death in this case.

It was suggested that antivirus immunoresponses mediated by IRF3 expression had a function in the HCMV infection of pancreatic islets. Type I IFN, a representative factor of the antivirus immunoresponse, is important for the infiltration and activation of macrophages and $CD4^+$/$CD8^+$ T cells. In this case, the expression of IRF3 was detected in 52.6% of HCMV-infected cells and some α cells, suggesting that type I IFN anti-virus immunoresponses occurred in HCMV-infected islet cells. Generally, following the infection of a DNA virus such as HCMV, the expression of IRF3 occurs via ZBP1, which is a DNA sensor (20); in this case, however, the expression of RIG-I, an RNA sensor, was detected in α cells in addition to ZBP1. Similar to a study that reported that HCMV-infected fibroblasts expressed RIG-I (21), double immunofluorescence staining for HCMV and RIG-I in the adrenal gland of this case revealed that 13.7% of HCMV-positive cells expressed RIG-I (data not shown). In a previous study of a fulminant T1DM patient, RIG-I was expressed in the islet cells of the enterovirus-infected pancreas; however, activation of the DNA sensor pathway was not observed (10). By contrast, because both ZBP1 and RIG-I were detected in the islet cells in this case, HCMV infection might be involved in IRF3 expression via both DNA and RNA sensors, partly sharing a common pathway with the enterovirus-infected subject who developed fulminant T1DM.

We evaluated infiltrating $CD68^+$, $CD4^+$, and $CD8^+$ cells as markers for macrophages, helper T cells, and cytotoxic T cells, respectively. Insulitis is occasionally diagnosed with infiltrating $CD45^+$ cells as a marker for immune cells (16). CD45 is a leukocyte common antigen, and inflammation mediated by $CD45^+$ cells, mainly $CD68^+$ macrophages, was found in type 2 diabetic pancreases (22). Thus, this definition might not distinguish pancreases retrieved from individuals with T1DM from those with type 2 diabetes.

Although HHV-6 and EBV are reactivated in sequence in DIHS and enterovirus may be related to the onset of

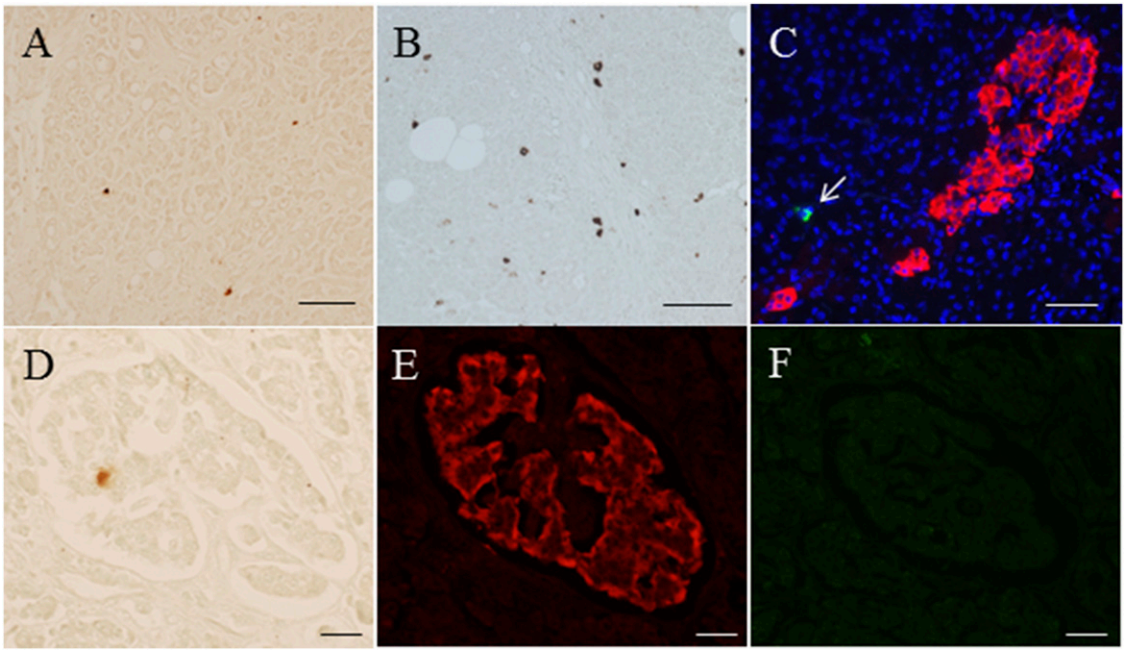

Figure 4. HHV-6 and enterovirus infection in the pancreas. HHV-6 infection was detected in the pancreas of (A) the case and (B) control subjects. (C) HHV-6 (green; arrow) was detected in nonislet lesions (insulin, red). Staining for (D) VP1 and (E) glucagon in serial sections of this case. (F) Enterovirus infection was detected in islets with no infiltration of $CD8^+$ cells. Bars = (A) 200 μm, (B, C) 100 μm, and (D–F) 50 μm.

fulminant T1DM, none of these viruses is likely to be related to the onset of fulminant T1DM, at least in this case. IFN (23) and checkpoint inhibitors (24) have been reported to be related to the development of T1DM, but these drugs were not used in this patient.

In conclusion, more HCMV-positive cells were detected in the autopsy pancreas of a patient who developed fulminant T1DM after DIHS. Furthermore, α cells expressing IRF3, ZBP1, or RIG-I and significantly increased numbers of inflammatory cells were observed in HCMV-positive islets compared with HCMV-negative islets. These findings suggest that the immunoresponse caused by HCMV infection was associated with β cell injury.

Acknowledgments

A.I. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. The author thanks Dr. Hidetoshi Eguchi, Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, for the resected pancreatic tissues of the control subjects; Dr. Yasuko Mori, Division of Clinical Virology, Graduate School of Medicine, Kobe University, for the HHV-6B antigen; and Misako Kobayashi, Department of Metabolic Medicine, Graduate School of Medicine, Osaka University, for her excellent technical assistance.

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This work was supported by KAKENHI Grant Number JP15K09429.

Author Contributions: S.Y. researched data and wrote the manuscript. A.I. and K.F. contributed to the discussion and reviewed/edited the manuscript. S.U., J.K., and H.I. contributed to the discussion. I.S. contributed to the discussion and reviewed the manuscript. M.S. and T.Y. were engaged in the treatment of this case.

Disclosure Summary: The authors have nothing to disclose.

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