● 学会発表

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Ⅳ. 研究成果の刊行物・別刷

3. 劇症1型糖尿病

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はじめに

劇症1型糖尿病は2000年に著者らが報告し，その後日本糖尿病学会による全国調査により161名の患者が報告され，臨床病型として確立された糖尿病のサブタイプである．その特徴として，①日本人急性発症1型糖尿病の約20%をしめる，②糖尿病関連自己抗体が陰性である，③ケトアシドーシスを伴って非常に急激に発症する，④発症時に著明な高血糖を認めるにも関わらず，HbA1cは正常または軽度上昇にとどまる，⑤発症時に既にインスリン分泌はほぼ完全に枯渇している，⑥発症時に血中膵外分泌酵素の上昇を認める，などがあげられる．2004年には同学会劇症型糖尿病調査研究委員会(当時)から，スクリーニング基準と診断基準が提示されている[1,2]．

本稿では，最近の報告に重点をおいて，reviewする．

疫　学

前述の全国調査では，日本における劇症1型糖尿病の発症率は急性発症1型糖尿病の19.4%であった．海外では，韓国において新規発症1型糖尿病患者99名中7名(7.1%)が劇症1型糖尿病であったことが報告されている．この7名はすべて18歳以上の成人であり，診断には日本と同じ基準が用いられている．また，中国からの症例も相次いで報告されている．最近Caucasianにおいても症例が報告されており[3]，劇症1型糖尿病が人種を越えた臨床病型であることが明らかにされつつある．

劇症1型糖尿病は，妊娠と関連して発症することも明らかにされており，その場合，発症時の代謝異常が高度であり，児の予後が非常に悪いことが報告されているが，妊娠関連発症症例以外では，発症年齢や性別に関わらず，臨床的特徴が比較的均質であることが最近報告された[4]．

遺伝因子

劇症1型糖尿病発症に関与する遺伝因子として，HLA(human leukocyte antigen)が報告されている．すなわち，クラスII HLAであるDR-DQのハプロタイプの中で，DR4-DQ4は劇症1型糖尿病患者の41.8%と高率に認められる．

このHLAに加え，最近2番目の遺伝因子としてCTLA-4(cytotoxic T lymphocyte antigen-4)が報告された．CTLA-4は抗原提示細胞(マクロファージや樹状細胞)上に発現しており，T細胞のB7-1(CD80)およびB7-2(CD86)と結合し，免疫機構を不活化させる刺激を伝達すると考えられている．

Kawasakiらは劇症1型糖尿病55名，自己免疫性1型糖尿病91名，健常者369名において，第1エクソンの+49 G>A多型(rs231775)と，3'非翻訳領域のCT60多型(rs3087243)について検討した．その結果，劇症1型糖尿病はCT60AA多型との関連を認めたが($p<0.05$，オッズ比2.68)，+49 G>A多型との関連を認めなかった．これに対し，自己免疫性1型糖尿病では+49GGおよびCT60GG多型との関連を認めた($p<0.001$)．今回の検討では，劇症1型糖尿病患者において血中可溶性CTLA-4濃度が低値であることも明らかになった．CT60遺伝子型と血中可溶性CTLA-4濃度には直接の相関は認められなかったものの，CTLA-4遺伝子多型が免疫応答の調節に関与し，劇症1型糖尿病発症に関与している可能性がある[5]．

ウイルス感染

劇症1型糖尿病発症にウイルス感染が関与することを示唆する事実として，次のようなことがあげられる．すなわち，①全国調査において劇症1型糖尿病の72%の症例に認められる先行感染症状，②広範囲のエンテロウイルスに反応する抗体を検出し得る測定系

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によるIg-A抗体価の上昇，ヒトヘルペスウイルス(HHV)6型，コクサッキーB3ウイルス，同B4ウイルス，Epstein-Barr(EB)ウイルス，インフルエンザBウイルス，ムンプスウイルスなどのウイルス抗体価上昇，あるいはウイルスDNAの増加に伴って劇症1型糖尿病を発症したという症例報告の存在，などである．

最近，劇症1型糖尿病調査委員会で行われた発症時の患者血清におけるウイルス抗体価の研究結果が報告された[6]．劇症1型糖尿病診断基準をみたす患者を対象に，合計23種類のウイルス抗体を測定したこの研究では，55名中9名において，合計13項目，すなわちコクサッキーウイルス(A4，A5，A6，B1)，ロタウイルス，サイトメガロウイルス，EBウイルス，HHV6型，HHV7型といったさまざまな既知のウイルス抗体価の有意な上昇を認めた．しかし，特定のウイルス抗体価の上昇は認められなかった．

この結果から，劇症1型糖尿病では，特定のウイルスにより直接的な膵β細胞傷害が生じるのではなく，ウイルス感染に伴って抗ウイルス免疫反応が惹起され，その反応にβ細胞が巻き込まれて傷害されるという仮説が考えられる．もちろん，現在までに抗体価を測定されていないウイルス，あるいは未知のウイルスによって直接β細胞傷害が生じる可能性もある．

膵組織所見

発症1～5カ月後に劇症1型糖尿病患者3名の生検膵組織を検討した著者らの最初の報告では，膵島への炎症細胞浸潤は認めなかった．しかし，最近，発症直後に死亡した劇症1型糖尿病患者3名の剖検膵組織を検討したところ，すべての症例において膵島(および膵外分泌領域)へのマクロファージとT細胞の浸潤を認めた．特徴的であったのは，自己免疫性1型糖尿病の場合，膵島浸潤細胞の主体はT細胞であるが，劇症1型糖尿病ではマクロファージが主体であることで，β細胞傷害の主役はマクロファージであるという仮説が想定される．膵島への炎症細胞浸潤については，Tanakaらも発症直後に死亡した劇症1型糖尿病患者1名の剖検膵組織においてその存在を報告している．これらの結果を総合すると，劇症1型糖尿病における炎症細胞浸潤(膵島炎)は時間経過とともに急速に消失することが推測される．ちなみに，自己免疫性1型糖尿病では，発症1～5カ月後の膵組織でも炎症細胞浸潤(膵島炎)を認める．また，自己免疫性1型糖尿病では，CD8＋T細胞が特異的に膵β細胞を認識し，最終的にFas-Fas ligandのinteractionにより膵β細胞が特異的に傷害されると考えられるが，劇症1型糖尿病患者膵ではマクロファージが浸潤細胞の主体であることやβ細胞のみならずα細胞も傷害されていることから，α細胞も含めた比較的非特異的な細胞傷害機構が存在するのではないかと考えられる．

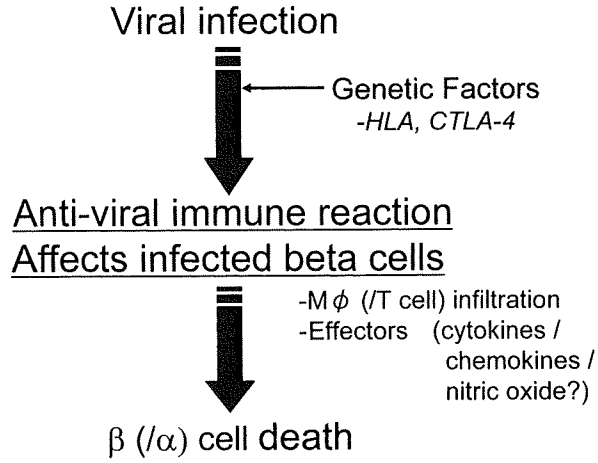

Fig. 劇症1型糖尿病における膵β細胞傷害(仮説)

その他

以前より，劇症1型糖尿病の患者血清中には膵外分泌組織に対する抗体の存在が報告されていたが，最近Endoらは新しい抗体としてamylase α-2Aに対する抗体が存在することを報告した[7]．その意義などについて，今後検討が進むことが期待される．

また，劇症1型糖尿病発症時に高率にtransaminaseの上昇と脂肪肝を合併すること[8]，劇症1型糖尿病では発症5年後に細小血管合併症が比較的高率に観察されること[9]，などが最近報告されている．

おわりに

現在までの研究結果から，劇症1型糖尿病では，HLAやCTLA-4などの遺伝因子を背景に，ウイルス感染が引き金となり，続いて生じる免疫担当細胞の活性化により，膵島へのマクロファージを主体とした細胞浸潤が生じ，(自己免疫性1型糖尿病と比べ)非特異的な機構を介して膵β細胞傷害が生じていると推測されている(Fig.)．

以上，最近明らかになった劇症1型糖尿病の知見について概説した．今後，β細胞傷害に関連する分子とその相互関係が一つ一つ明らかになることが期待される．

謝　辞

劇症1型糖尿病の確立とその後の研究において，日本糖尿病学会ならびに同学会会員の先生から多大なる御協力，御支援を頂きました．ここに深謝いたします．

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ORIGINAL

Expression of Toll-like Receptors in the Pancreas of Recent-onset Fulminant Type 1 Diabetes

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Abstract. Fulminant type 1 diabetes, established in 2000, is defined as a novel subtype of diabetes mellitus that results from remarkably acute and almost complete destruction of pancreatic beta cells at the disease onset. In this study, we aimed to clarify the pathogenesis of fulminant type 1 diabetes with special reference to insulitis and viral infection. We examined pancreatic autopsy samples from three patients who had died soon after the onset of disease and analyzed these by immunohistochemistry and *in situ* hybridization. The results were that both beta and alpha cell areas were significantly decreased in comparison with those of normal controls. Mean beta cell area of the patients just after the onset was only 0.00256 % while that of normal control was 1.745 %. Macrophages and T cells—but no natural killer cells—had infiltrated the islets and the exocrine pancreas. Although both of them had massively infiltrated, macrophages dominated islet infiltration and were detected in 92.6 % of the patients' islets. Toll-like receptor (TLR) 3, a sensor of viral components, was detected in 84.7±7.0 % of macrophages and 62.7±32.3 % of T cells (mean±SD) in all three patients. TLR7 and TLR9 were also detected in the pancreas of all three patients. Enterovirus RNA was detected in beta-cell positive islets in one of the three patients by *in situ* hybridization. In conclusion, our results suggest that macrophage-dominated insulitis rather than T cell autoimmunity contributes to beta cell destruction in fulminant type 1 diabetes.

Key words: Insulitis, Toll-like receptor, Enterovirus

TYPE 1 DIABETES, one of the two major forms of diabetes, results from nearly complete destruction of pancreatic beta cells [1]. We previously reported a novel subtype of type 1 diabetes that we called fulminant type 1 diabetes [2]. This subtype of diabetes is characterized by its clinical features, namely remarkably acute onset and absence of islet-related autoantibodies [2-4]. Fulminant type 1 diabetes has also shown high plasma glucose levels accompanied by ketosis or ketoacidosis. However, it also exhibits nearly normal glycosylated hemoglobin levels, a high serum pancreatic enzyme concentration and virtually no C-peptide secretion at the onset of disease.

A nationwide survey identified that this variant accounts for approximately 20 % of acute-onset type 1 diabetes cases in Japan [3]. Recently, 30.4 % of adult-onset type 1 diabetes was classified as fulminant type 1 diabetes in Korea according to the Japanese crite-

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Table 1. Patients' characteristics

	Age(yr)/Sex	Disease duration (days)*	Blood glucose (mg/dL)	HbA_{1c} (%)	Serum C-peptide (ng/mL)	GADAb/ IA-2Ab/ ICA	*HLA DRB1- DQB1*
1	29/Male	3+3	1585	5.8	undetectable	(-)/(-)/(-)	*0502***/-*
2	35/Male	3+0	1300	7.5	0.08	(-)/(-)/(-)	*0405-0401/1405-0503*
3	47/Male	5+0	660	6.2	0.39	(-)/(-)/(-)	*0404-0402/0802-0302*

*Disease duration refers to the period with hyperglycemic symptoms before the diagnosis of diabetes plus that with the treatment for diabetes. Patient 2 and 3 were diagnosed as having diabetes after death. ***DRB1* not available.

ria [5]. Several cases have been reported, not only in other Asian populations, but also in Caucasian populations [6, 7].

The destruction of beta cells was confirmed by virtually no C-peptide secretion in fulminant type 1 diabetes; however, the mechanism of beta cell death is largely unknown. For example, it remains controversial whether insulitis, mononuclear cell infiltration to the pancreatic islets, is characteristics of fulminant type 1 diabetes [3, 9, 10]. In addition, fulminant type 1 diabetes is different from classical type 1A diabetes with respects to the human leukocyte antigen (HLA)-DR/DQ and cytotoxic T lymphocyte antigen (CTLA)-4 gene polymorphisms [11, 12].

The possible involvement of viral infections has been suggested in the pathogenesis of fulminant type 1 diabetes. Flu-like symptoms are common and were exhibited just before the onset of overt diabetes in 71.2 % of patients diagnosed with fulminant type 1 diabetes [4]. IgA antibody titres to enterovirus common antigen were significantly higher in patients with recent-onset fulminant type 1 diabetes than in those with type 1A diabetes and in controls [13]. Finally, several cases have been reported in which the onset of diabetes was accompanied by a reactivation or an infection of human herpesvirus-6 and -7, herpes simplex virus, coxsackie A4, A5, A6 and B3 viruses, influenza B virus, mumps virus, rotavirus, Epstein-Barr virus and cytomegalovirus in fulminant type 1 diabetes [4, 6, 14-16]. However, there has been no direct evidence so far, for the pathogenic role of viruses in beta cell destruction.

To clarify the pathogenesis of fulminant type 1 diabetes with special reference to insulitis and viral infection, we investigated pancreatic autopsy specimens obtained from the patients who died soon after the disease onset.

Materials and Methods

Patients and samples

We examined three patients with fulminant type 1 diabetes who had died just after the onset of overt diabetes. Our inclusion criteria for fulminant type 1 diabetes matched those previously published by our group [4]. Namely, 1) presence of ketosis or ketoacidosis within a week after the onset of hyperglycemic symptoms, 2) urinary C-peptide excretion <10 μg/day or fasting serum C-peptide level < 0.3 ng/mL (0.10 nmol/L) and peak serum C-peptide level < 0.5 ng/mL (0.17 nmol/L) after glucagon (1 mg) or a meal load soon after disease onset; and 3) plasma glucose level ≥ 288 mg/dL (16.0 mmol/L) and HbA_{1c} level < 8.5 % at first visit.

In all three patients, postmortem dissections were performed, and we examined the pancreatic tissues. Patient 1 was diagnosed with diabetic ketoacidosis and died after 3 days of treatment. Patients 2 and 3 were diagnosed with diabetes after death. The direct causes of death of patient 2 and 3 were unknown at the time of autopsies. They ranged in age from 29 to 47 years, and the duration of disease was 3 to 6 days. Their blood glucose, HbA_{1c} and serum C-peptide levels were 660 to 1585 mg/dL, 5.8 to 7.5 % and less than 0.39 ng/mL, respectively. GAD_{65} antibody, IA-2 antibody and islet cell antibodies (ICA) were negative in all three patients. Patient 2 and 3 possessed HLADR4-DQ4 haplotype that is susceptible to fulminant type 1 diabetes (Table 1) [4]. The pancreatic tissues were fixed in 10 % formalin and embedded in paraffin. The normal pancreatic tissues of six individuals were examined as non-diabetic control samples. These individuals were free from pancreatic diseases.

Immunohistochemistry and morphometry

Formalin-fixed paraffin embedded pancreatic sections cut at a thickness of 4 μm were deparaffinized and rehydrated using xylene and graded descending series of alcohol. After washing in distilled water once for 5 min, the slides only for natural killer (NK) cells were exposed to microwave pretreatment in a target retrieval solution (Dako Japan, Kyoto, Japan) at 100 °C for 15 min to enhance antigenisity. Endogenous

peroxidase activity was blocked for all sections using ice-cold 3 % H_2O_2/methanol for 30 min. All slides were incubated for 30 min in 10 % normal serum. The slides were then incubated at room temperature for 1 hour with guinea pig anti-insulin antibody (1:1000; Dako Japan), rabbit anti-glucagon antibody (1:1000; Linco Research, Ellisville, MO, USA), rabbit anti-human CD3 antibody (1:100, Dako Japan), monoclonal mouse anti-human CD68 antibody (1:100, KP1, Dako Japan), monoclonal mouse anti-human CD56 antibody (1:100, SNCL-CD56-1B6, Novocastra, Newcastle, UK), goat anti-human Toll-like receptor (TLR) 3 antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-human TLR7 antibody (1:100, Santa Cruz) and monoclonal mouse anti-human TLR9 antibody (1:200, 26C593, Imgenex, San Diego, CA, USA). As a negative control for TLR9 antibody, the same concentration of mouse IgG1 (Dako, Japan) was applied as a primary antibody. As negative controls for TLR3 and TLR7, those antibodies incubated with 10-fold higher concentration of TLR3 and TLR7 blocking peptides (Santa Cruz) were applied. Then slides were incubated with secondary antibodies; mouse or rabbit Envision kit/HRP (Dako Japan), Elite ABC mouse or goat IgG kit (Vector Laboratories, Burlingame, CA, USA), following the manufacturers' instructions. Finally, antibody binding was detected using 3', 5'-diaminobenzidine (DAB) (Dako Japan). All the washes were performed in PBS (pH 7.4). Sections were counterstained with methyl green or hematoxylin and were mounted in oil mounting medium (Mount-quick, Tokyo, Japan) before microscopy (BH-2, Olympus, Tokyo, Japan).

The total areas of all sections, for both insulin- and glucagon-positive cells, were measured using Image-J and a digital light microscope (BZ-8000, Keyence, Osaka, Japan). For each subject, three sections separated by more than 250 μm were assessed to eliminate the risk of measurement dispersion. Insulin- and glucagon-positive cell areas were measured by two different observers and expressed as a percentage of the total area of each section.

We used a double-immunofluorescence method to detect insulitis. The sections were incubated at room temperature for 1 hour with rabbit anti-human CD3 antibody (1:25, Dako Japan) or monoclonal mouse anti-human CD68 antibody (1:25, KP1, Dako Japan). The sections were then incubated at room temperature for 30 min with biotinylated anti-rabbit or anti-mouse immunoglobulins (Vector Laboratories), and then for an additional 15 min with fluorescein avidin D (Vector Laboratories). These procedures were followed by incubation with guinea-pig anti-glucagon antibody (1:200) or guinea-pig anti-insulin antibody (1:200), and incubated with the secondary antibody, namely, Alexa Fluor goat anti-guinea-pig immunoglobulins (Molecular Probes, Carlsbad, CA, USA). Each section was washed in PBS and mounted in aqueous mounting medium (Perma Fluor, Immunon, Pittsburgh, PA, USA) prior to fluorescence microscopy (BX 50, Olympus). We examined more than 125 islets for each subject to detect insulitis. When we observed two or more mononuclear cells infiltrating an islet, we determined that the subject was insulitis-positive, as we had previously shown [17]. This criterion guarded against false negatives in evaluating human insulitis because massive infiltration of mononuclear cells is rare in comparison with non-obese diabetic (NOD) mice. The criterion was also robust to false positives because single mononuclear cells sometimes become lodged in an islet even in subjects who do not have diabetes. Infiltration rates are defined as the percentage of insulitis-positive islets out of total islets examined. To clarify the TLR3 expression on mononuclear cells, the staining by goat anti-human TLR3 antibody (1:200) was followed by the second staining by rabbit anti-human CD3 antibody (1:25) or monoclonal mouse anti-human CD68 antibody (1:25).

In situ hybridization

The used method was modified from a previously published method [18, 19] and has been described in detail by Oikarinen *et al.* [20]. An enterovirus-specific oligonucleotide probe designed to hybridize with the conserved 5' non-coding sequence was used (sequence from 5' to 3'GAA ACA CGG ACA CCC AAA GTA GTC GGT TCC GCT GCR GAG TTR CCC RTT ACG ACA) to detect all known enterovirus types. The probe was 3'end –labelled with digoxigenin using a kit (DIG oligonucleotide tailing kit; Roche Diagnostics, Welwyn Garden City, UK). A 10 pmol sample of the probe was used for one labelling reaction. Hybridization was performed using earlier published conditions [20]. The amount of probe in the hybridization cocktail was 250 ng, the hybridization time was 3 hour. Binding of the probes was revealed by anti-digoxigenin antibody, which was conjugated with alkaline phosphatase. This enzyme together with

Table 2. Results of the histological analysis

	Beta cell area	Alpha cell area	CD3+ insulitis	CD68+ insulitis	TLR3+/ CD3+ cells	TLR3+/ CD68+ cells
Fulminant						
No. 1	0.00259	0.0153	79.4	93.5	90.0	78.0
No. 2	0.00412	0.0430	78.9	94.7	27.0	92.0
No. 3	0.000970	0.0252	48.0	89.6	71.0	84.0
Mean±SD	0.00256±0.00158*	0.0278±0.0140*	68.8±18.0*	92.6±2.7*	62.7±32.3	84.7±7.0
Control (n=6)						
Mean±SD	1.745±0.336	0.266±0.049	2.8±1.9	1.3±1.2		

Islet cell areas are shown in percentage of insulin- and glucagon-positive cell area in total observed area. The results of insulitis and TLR3+ cells are shown in percentage. *$P<0.001$ *vs.* controls

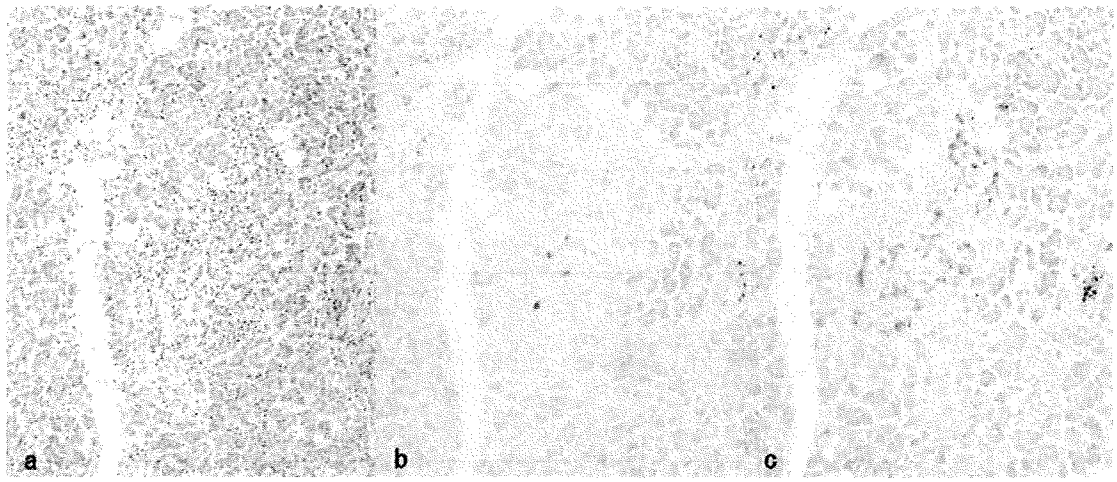

Fig. 1. Microphotographs of consecutive sections stained with H&E (a) and antibodies to insulin (b) and glucagon (c) in case 1 as a representative. Massive mononuclear cell infiltration was detected in and around the islet as well as in exocrine tissue by H&E stain. Decreased numbers of insulin+ cells and glucagon+ cells were seen in the islet. Original magnification: x300.

its substrate, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate yields an insoluble purple precipitate, which can be detected using a light microscope. Enterovirus-infected and mock-infected green monkey kidney cells were used as controls.

Statistical analysis

The significance of differences between the two groups was evaluated using Mann-Whitney's U-test. $P<0.05$ was considered statistically significant.

Results

Insulin- and glucagon-positive cell regions

Insulin- and glucagon-positive cells were markedly decreased in all patients with fulminant type 1 diabetes. The mean (±SD) insulin- and glucagon-positive cell areas were 0.00256±0.00158 % and 0.0278±0.0140 % in fulminant type 1 diabetes and 1.745±0.336 % and 0.266±0.049 % in normal control subjects, respectively (Table 2). In fulminant type 1 diabetes, both the beta and alpha cell regions were decreased significantly in comparison with those in normal control subjects ($P<0.001$).

Cellular infiltration of CD3+, CD68+ and CD56+ cells

We detected the infiltration of CD3+ cells and CD68+ cells in and around the islets as well as in the exocrine pancreas in all patients with fulminant type 1 diabetes. Insulitis were seen both in insulin-positive islets and insulin-negative islets (Figure 1, 2). CD56+ cells were not detected in mononuclear cells at all. Insulitis infiltrated by CD3+ cells was observed in 68.8±18.0 % (mean±SD) of islets examined in fulminant type 1 diabetes but only in 2.8±1.9 % in control subjects ($P<0.001$). Insulitis infiltrated by CD68+

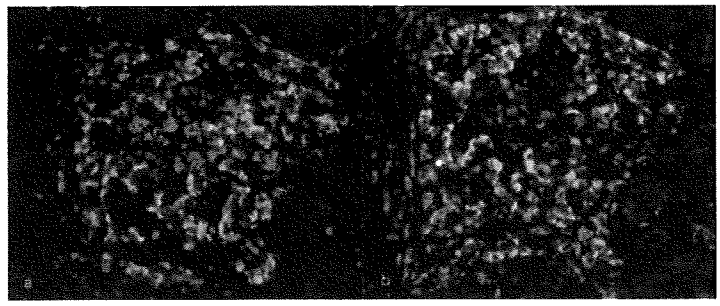

Fig. 2. Microphotographs of double staining sections for CD3/CD68 and glucagon in case 1 as a representative. CD3+ cells (red; a) and CD68+ cells (red; b) are infiltrating in and around the islet (green represents glucagon). Original magnification: x500.

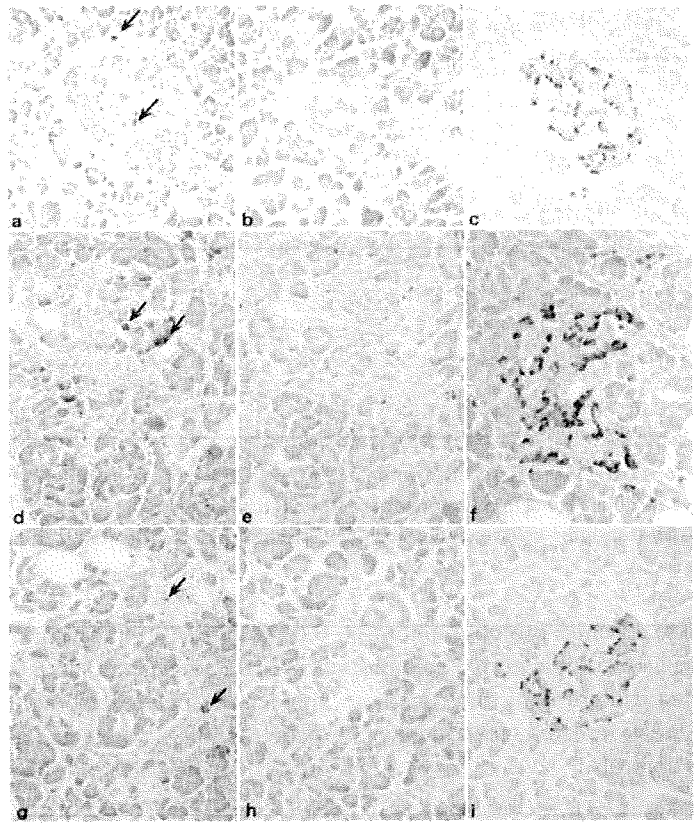

Fig. 3. Microphotographs of consecutive sections stained with antibodies to TLRs and controls for each other. The expressions of TLR3, its negative control and glucagon for patient 2 (a-c), TLR7, its negative control and glucagon for patient 3 (d-f), TLR9, its negative control and glucagon for patient 3 (g-i) in the consecutive sections are shown. Glucagon staining indicates the islet area. TLR3, TLR7 and TLR9 were expressed in monomuclear cells located both inside and outside an islet. Arrows (a, d, g) represent positive cells. Original magnification: x 200.

cells was observed in 92.6±2.7 % in fulminant type 1 diabetes but only in 1.3±1.2 % in control subjects ($P<0.001$) (Table 2).

Expression of TLRs

We detected the expression of TLR3, TLR7 and TLR9 in all patients with fulminant type 1 diabetes (Figure 3). Double staining method revealed that TLR3 was positive in 84.7±7.0 % of macrophages and 62.7±32.3 % of T cells (Table 2). TLR3-positive macrophages and T cells were detected both in the islets and exocrine areas (Figure 4). No positive cells were

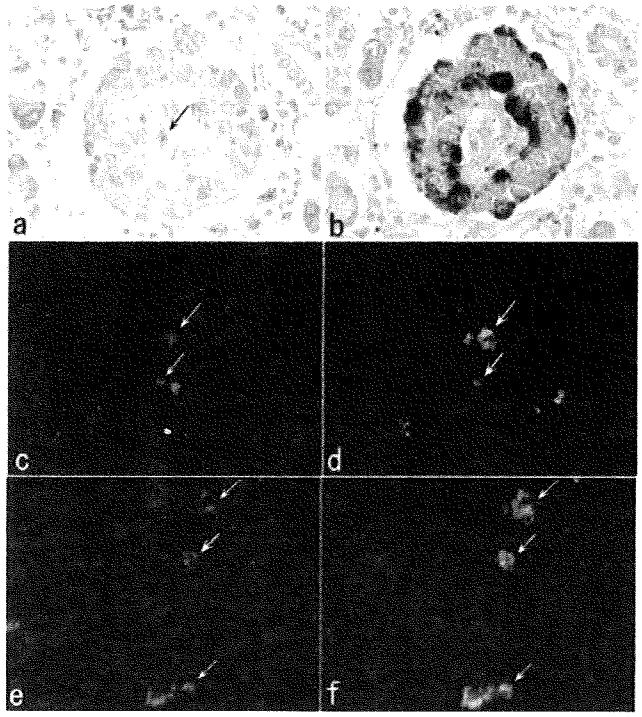

Fig. 4. Microphotographs of consecutive sections stained with antibodies to insulin (a), glucagon (b), CD3 (red; c), TLR3 (green, d), CD68 (red; e) and TLR3 (green; f) in case 2. Microphotographs of (c)(d) and (e)(f) are the same sections and visualized by a double staining method. Insulin+ and glucagon+ cells are detected respectively (a, b). The expression of TLR3 was detected in islet-infiltrating T cells and macrophages. Arrows (a), (c, d) and (e, f) represent insulin+ cell, TLR3+/CD3+ cells and TLR3+/CD68+ cells respectively. Original magnification: x 600.

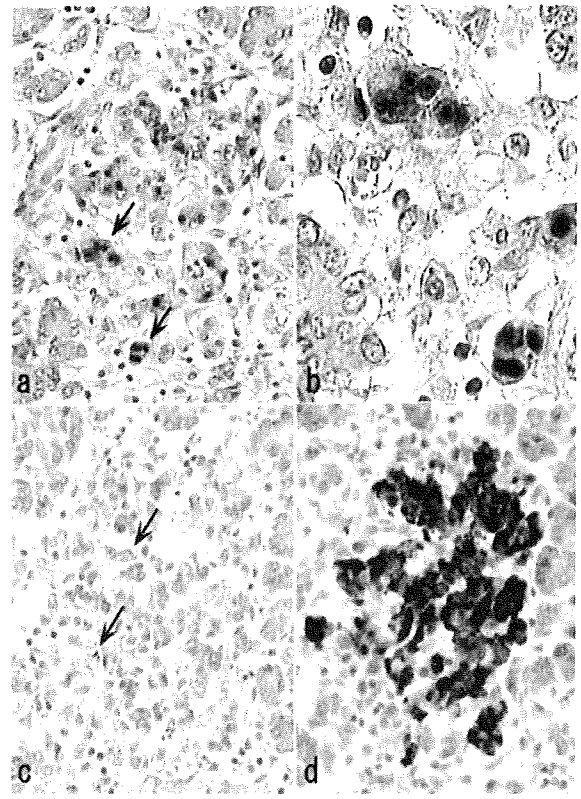

Fig. 5. Microphotographs of consecutive sections of enterovirus RNA (a, b), insulin (c) and glucagon (d) in Case 1. Enterovirus RNA was detected in the islets with insulin of case 1 but not in cases 2 and 3. No enterovirus RNA was detected in the exocrine pancreas. Arrows (a) (c) represent positive cells. Original magnification: x 500 (a, c, d), x 1500 (b).

detected in the control slides for any of the proteins TLR3, TLR7 or TLR9.

Expression of enterovirus RNA

We detected the expression of enterovirus RNA in 11 islets at case 1 by *in situ* hybridization. One islet contained insulin-positive cells (Figure 5). No enterovirus RNA was detected in the exocrine pancreas. We could not detect enterovirus RNA in case 2, case 3 and normal controls.

Discussion

In the present study, we have revealed the expression of multiple TLRs, especially TLR3, and enterovirus RNA in the pancreases of fulminant type 1 diabetes soon after the disease onset. Destruction of beta and alpha cells as well as macrophage predominant insulitis was also observed.

The expression of TLRs is an important finding from the view of viral infection. TLRs are pattern recognition receptors (PRRs) that detect conserved structures found across a broad range of pathogens and protect the gateways to innate immune systems. Of these, TLR3, TLR7/8 and TLR9 are known to recognize viral components and induce type I interferon for anti-viral defense. Notably, TLR 3 is upregulated when coxsackie B5 virus itself or interferon alpha, a cytokine induced by viral infection, is incubated with the isolated human islets [21, 22]. These findings suggest that the expression of TLRs, especially TLR3, indicates a signature of viral infection. The expressions of those TLRs detected in islet area in the patients with fulminant type 1 diabetes in this study may be related to activation of

the innate immune system by the virus. Moreover, the expression of TLR3 in T cells and macrophages infiltrating to the pancreas in those patients, is an important evidence of viral infection in fulminant type 1 diabetes. In addition, several TLRs and type I interferon are reported to express simultaneously in immune cells [23], suggesting different types of TLRs might be detectd in a same immunocyte in our specimens. The finding that enterovirus RNA was detected in a beta-cell-positive islet in one of the three patients by *in situ* hybridization is a direct evidence of enterovirus infection in this patient, while Ylipaasto *et al* studied autopsy pancreases from 65 type 1 diabetic patients (not subclassified into type 1A or type 1B) for presence enterovirus RNA by *in situ* hybridization and they found positive results in just 4 out of 65 patients. [24].

Second, both beta and alpha cell regions were decreased significantly in fulminant type 1 diabetes, even very soon after the onset of overt diabetes. The beta cell region in fulminant type 1 diabetes patients was 0.1 % of that in normal controls in our study. We previously reported that the beta cell area was decreased to 0.4 % of that of normal controls in pancreatic biopsy specimens with fulminant type 1 diabetes obtained 1 to 5 months after the onset of overt diabetes. We also reported that the beta cell area in autoimmune (type 1A) diabetes was decreased to only 14.5 % of that in normal controls [8]. These data indicated that almost all the beta cells were destroyed within a short period in fulminant type 1 diabetes. The result contrasts strikingly with type 1A diabetes where the process of beta cell destruction usually progresses gradually [25]. In addition, the alpha cell area in fulminant diabetes was also markedly decreased to 9.6 % of that in normal controls, indicating that both beta and alpha cells are damaged at the onset of fulminant type 1 diabetes. This finding also contrasts to the mild decrease of alpha cells in type 1A diabetes.

Third, we detected the infiltration of CD3+ cells and CD68+ cells in and around the islets, as well as in the exocrine pancreas, in all patients with fulminant type 1 diabetes just after the onset of clinical diabetes. CD68+ macrophages infiltrates predominantly and are observed in 92.6 % of islets examined in this study. In our previous report, no insulitis was observed in the biopsy specimens of three patients obtained 1 to 5 months after onset [2]. Overall, it is reasonable to believe that mononuclear cell infiltration into the islets exist at the time of disease onset but disappear soon after both beta cell destruction and elimination of possible viral antigens in fulminant type 1 diabetes.

From these results, we suggest that not autoimmunity but antiviral inflammation plays an etiopathological role in fulminant type 1 diabetes. In the classical type 1A diabetes, autoimmunity is believed to be an etiology and insulin is the most likely candidate as a primary antigen [25]. It is well known that T cells are dominated in insulitis lesion, alpha cells are not affected, and beta cells are specifically damaged because of selective recognition of beta cell autoantigens by T cells [26]. However, in fulminant type 1 diabetes, the infiltration of macrophages (but not T cells) is dominant. Macrophages are initially activated in viral infected lesion and generate inflammatory cytokines, and chemokines to kill the target cells [27], though it also observed in low-dose Streptozotocin-induced diabetes model mice [28]. They are less selective than T cell-oriented target cell death. This hypothesis of a less selective mechanism is in accord with the fact that both beta and alpha cell regions are decreased significantly in fulminant type 1 diabetes as shown in this study.

In conclusion, our study showed remarkably decreased numbers of pancreatic beta and alpha cells, macrophage-dominated insulitis and the expression of TLRs, a signature of viral infection, in fulminant type 1 diabetes soon after the disease onset. These results suggest a new mechanism of virus-induced macrophage-dominated inflammatory process, rather than autoimmune T cell response, plays a major role in beta cell destruction in this novel subtype of diabetes.

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Enterovirus Infection, CXC Chemokine Ligand 10 (CXCL10), and CXCR3 Circuit

A Mechanism of Accelerated β-Cell Failure in Fulminant Type 1 Diabetes

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OBJECTIVE—Fulminant type 1 diabetes is characterized by the rapid onset of severe hyperglycemia and ketoacidosis, with subsequent poor prognosis of diabetes complications. Causative mechanisms for accelerated β-cell failure are unclear.

RESEARCH DESIGN AND METHODS—Subjects comprised three autopsied patients who died from diabetic ketoacidosis within 2–5 days after onset of fulminant type 1 diabetes. We examined islet cell status, including the presence of enterovirus and chemokine/cytokine/major histocompatibility complex (MHC) expressions in the pancreata using immunohistochemical analyses and RT-PCR.

RESULTS—Immunohistochemical analysis revealed the presence of enterovirus-capsid protein in all three affected pancreata. Extensive infiltration of CXCR3 receptor–bearing T-cells and macrophages into islets was observed. Dendritic cells were stained in and around the islets. Specifically, interferon-γ and CXC chemokine ligand 10 (CXCL10) were strongly coexpressed in all subtypes of islet cells, including β-cells and α-cells. No CXCL10 was expressed in exocrine pancreas. Serum levels of CXCL10 were increased. Expression of MHC class II and hyperexpression of MHC class I was observed in some islet cells.

CONCLUSIONS—These results strongly suggest the presence of a circuit for the destruction of β-cells in fulminant type 1 diabetes. Enterovirus infection of the pancreas initiates coexpression of interferon-γ and CXCL10 in β-cells. CXCL10 secreted from β-cells activates and attracts autoreactive T-cells and macrophages to the islets via CXCR3. These infiltrating autoreactive T-cells and macrophages release inflammatory cytokines including interferon-γ in the islets, not only damaging β-cells but also accelerating CXCL10 generation in residual β-cells and thus further activating cell-mediated autoimmunity until all β-cells have been destroyed. ***Diabetes* 58:2285–2291, 2009**

Fulminant type 1 diabetes is characterized by abrupt onset of severe hyperglycemia and ketoacidosis preceded by flu-like symptoms including fever, abdominal pain, and headache (1–3). Due to the rushed clinical course in most cases, patients with fulminant type 1 diabetes are sometimes untreated until becoming comatose and/or entering a critical, life-threatening state (4). Endogenous insulin secretion is completely abolished over time and diabetic microangiopathies develop over a short duration (5,6). The mechanisms underlying the aggressive and rapid destruction of β-cells have remained one of the major questions regarding this subtype of type 1 diabetes. However, in situ human data on affected islets and pancreas and possible mechanisms have been completely lacking for fulminant type 1 diabetes.

Viral infection with subsequent immunological mechanisms represents one of the leading candidates for destruction of β-cells in fulminant type 1 diabetes (3,7). Some studies on the mouse model of lymphocytic choriomeningitis virus–induced type 1 diabetes have demonstrated that islet β-cells can be destroyed as follows: within 1 day after virus infection, CXC chemokine ligand 10 (CXCL10) (8), a key chemoattractant for activated T-cells and macrophages, is produced in β-cells and secreted from islets (9). Activated T-cells bearing the receptor for CXCL10, named CXCR3 (8), infiltrate and accumulate in islets secreting CXCL10 (10). Accumulated T-cells at the islets then destroy β-cells through cell-mediated mechanisms (11). With this mechanism, CXCL10 is necessary and sufficient for accelerated T-cell response with complete β-cell destruction and resulting type 1 diabetes (10,12,13). We have recently found that serum CXCL10 levels are increased at the onset of fulminant type 1 diabetes, suggesting a crucial role of the CXCL10-CXCR3 axis in the aggressive β-cell destruction in this syndrome (14). We therefore examined in situ status with regard to enterovirus infection, CXCL10-CXCR3 axis, major histocompatibility complex (MHC) molecule expression, and islet dysfunction in pancreata from patients with fulminant type 1 diabetes who died due to diabetic ketoacidosis within 2–5 days after outset of flu-like symptoms. Our in situ findings for affected pancreata provide new insights

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into understanding the pathogenesis of and developing interventional strategies against human type 1 diabetes.

RESEARCH DESIGN AND METHODS

Patients

Case 1. A 14-year-old boy with type 1 diabetes in a ketoacidotic coma was brought to our hospital and died 20 min later. He had developed headache and high fever (~38°C) 5 days earlier, with sudden onset of polyuria and polydipsia 1 day before arrival. Blood glucose and hemoglobin A1C levels were 70.3 mmol/l and 7.9%, respectively. Blood pH was 6.98 and plasma level of 3-hydroxybutyrate was 64,000 μmol/L. Serum C-peptide levels were <0.017 nmol/l. Negative results were obtained for autoantibody against GAD (GADAb) and IA-2Ab. Serum elastase-1 and amylase levels were 4.4 and 8.9 times above the upper limit of normal, respectively. HLA-DRB1 and DQB1 genotypes in this patient were *0405/*0803 and *0401/*0601, respectively.

Case 2. A 25-year-old man with diabetic ketoacidosis arrived at the hospital and died 40 min later. He had experienced symptoms of nausea and epigastralgia for 2 days before becoming comatose. Blood glucose concentration was 85.5 mmol/l, and A1C level was 5.1%. Blood gas analysis revealed acidosis (pH 6.91). Serum elastase-1 concentration was 3.1 times the upper limit of normal. Negative results were obtained for GADAb, IA-2Ab, and autoantibodies against insulin. The patient's HLA-DRB1 and DQB1 genotypes were *0101/*0405 and *0501/*0401, respectively. This case was partly reported previously (3).

Case 3. A 29-year-old man who collapsed with diabetic ketoacidosis was admitted to our hospital and died 1 h after arrival. Two days earlier he had experienced slight fever, nausea, and vomiting, followed the next day by severe thirst and polyuria. On the day of admission, his family had found him in a comatose state. Blood glucose level was 44.1 mmol/l, A1C level was 5.9%, blood pH was 6.99, and pancreatic-isoamylase level was 40 times the upper limit of normal. HLA-DRB1 and DQB1 genotypes in this patient were *0405/*0901 and *0401/*0303, respectively.

Pancreatic tissues from 7 male patients with pancreatitis ([means ± SD] aged 61 ± 20 years) and 10 nondiabetic male patients (aged 62 ± 10 years) with gastric carcinoma and had undergone partial pancreatectomy were used as inflammation control subjects and nondiabetic control subjects, respectively. In addition, pancreatic tissue from an autopsied patient (a 56-year-old woman who died due to cerebral infarction) with slowly progressive insulin-dependent type 1 diabetes (15) was also examined for presence of enterovirus and CXCL10 expression in the pancreas. She had been treated with insulin and had shown diminished urinary C-peptide secretion (1.1 nmol/day) and high serum GADAb titer (12.5 units/ml [221.4 WHO units/ml]).

Detection of viral RNA in pancreatic tissues. RNAs were extracted from two 5-μm paraffin sections using a RecoverAll total nucleic acid isolation kit (Ambion, Austin, TX) according to the protocol defined by the manufacturer. Nested RT-PCR targeting the 5′ nontranslated region and VP1 region was performed using the primers described previously (16–19). RT-PCR for CXCL10 and interferon-γ was performed using the primer described previously (20,21).

Immunostaining, immunofluorescent staining and morphometric analyses. Methods for immunohistochemical and morphometric analyses have been reported previously (22). In brief, serial sections (5 μm) were cut from 5% formaldehyde-fixed paraffin-embedded specimens, stained with hematoxylin and eosin, and then stained using indirect immunoperoxidase techniques and double- or triple-immunofluorescence techniques. Serial sections (5 μm) were deparaffinized, rehydrated, and subjected to antigen unmasking with citrate buffer (pH 6.0). Sections were processed using an Envision+ kit (Dako, Carpinteria, CA) or ABC Staining System (Santa Cruz Biotechnology, Santa Cruz, CA), then visualized with diaminobenzidine tetrahydrochloride or 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride according to the instructions from the manufacturer. Primary antibodies used in this study were guinea pig anti-swine insulin (Dako), rabbit anti-human glucagon (Dako), mouse monoclonal anti-enterovirus VP1 peptide (clone 5-D8/1; Novocastra, Newcastle Upon Tyne, U.K.; this antibody recognizes an epitope mapped to residue 40–48 at the NH_2-terminus of VP1 of enterovirus protein [23] and reacts with 36 enteroviral serotypes [24]), mouse monoclonal anti-CD8 (clone 144B; Dako), mouse monoclonal anti-CD4 (IF4; Novocastra), mouse monoclonal anti-CD56 (clone CD564; Novocastra), rabbit monoclonal anti-CD11c (EP1347Y; Abcam, Cambridge, U.K.), goat polyclonal anti-CXCL10 (R&D Systems, Minneapolis, MN), rabbit polyclonal anti-interferon-γ (Santa Cruz Biotechnology), mouse monoclonal anti-interferon-α (NYRhIFN-a; Abcam), rabbit anti-2′,5′-oligoadenylate synthetase-like protein (HPA001474; Sigma), mouse monoclonal anti-interferon-γ (clone 25718; R&D Systems), mouse monoclonal anti-CXCR3/CD183 (clone 1C6; BD Bioscience, San Jose, CA), mouse monoclonal anti-CD68 (clone PG-M1; Dako), mouse monoclonal anti–HLA class-I (clone EMR8-5; Hokudo, Sapporo, Japan), and mouse monoclonal anti–HLA-DR (clone TAL.1B5; Dako).

For immunofluorescent staining, sections were processed as described above then incubated with 7-amino-4-methylcoumarin-3-acetic acid-, Texas Red-, fluorescein isothiocyanate– or Rhodamin Red–conjugated secondary antibodies (all from Jackson ImmunoResearch Laboratories, West Grove, PA). Stained sections were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and analyzed on an IX71 microscope (Olympus, Tokyo, Japan). Phenotyping of mononuclear cells that had infiltrated islets was performed using serial pancreas sections, and more than 23 islets from each patient and control were examined. Immunostainings were carried out at least three times for each section of the pancreas. Some pancreatic sections were processed with isotype-matched control immunoglobulins (mouse IgG1, κ [DAK-GO1; Dako]; mouse IgG2a, κ [DAK-GO5; Dako]; and rabbit IgG [X0903; Dako]) or in the absence of primary antibody to confirm the specificity of immunostaining. We confirmed that each primary antibody was specific for each antigen (supplementary Fig. S1 [available at http://diabetes.diabetesjournals.org/cgi/content/full/db09-0091/DC1). Sections were processed for immunostaining in the same run.

Morphometric analyses were performed using NIH Image software (http://rsb.info.nih.gov/nih-image/). Photographs of histological specimens for each case were taken at magnifications of ×200 and ×400 for analysis. Twenty-two images were examined for each patient compared with 34 for each control subject. Percentage islet area was obtained by dividing the islet area by the area of the entire section examined.

The percentage β-cell area and percentage α-cell area were calculated by dividing each cell area by the area of the corresponding islet. β-Cell volume and α-cell volume were calculated using percentage islet area multiplied by percentage β-cell area or percentage α-cell area, respectively. According to previous criteria (25), insulitis was defined as infiltration of two or more mononuclear cells into the islet.

Serum CXCL10 assay. We examined serum CXCL10 levels in two fulminant type 1 diabetic patients (cases 1 and 2) using enzyme-linked immunosorbent assay, as previously described (26). Serum samples were obtained on arrival and stored at −80°C until assay.

Ethics. All procedures used in this study were approved by the ethical committee of the University of Yamanashi.

Statistical analysis. Differences in variables between groups were compared using the Student's *t* test. Fisher's exact test was used to compare the frequencies of positive immunostainings. Values are expressed as means ± SD, unless otherwise mentioned.

RESULTS

Enterovirus in the pancreas. Immunohistochemical staining showed the presence of enterovirus capsid protein (VP1) in the pancreas from all three patients with fulminant type 1 diabetes (Fig. 1*A–C*). Some proportion of islet cells was positive for VP1 (Fig. 1*A* and *B*). Some VP1-positive acinar cells showed degenerating pathological features (Fig. 1*C*). The number of VP1-positive cell clusters on examined sections was 892/cm^2, 470/cm^2, and 752/cm^2 in cases 1, 2, and 3, respectively. No VP1-positive cell clusters were found in the 10 nondiabetic control subjects (P = 0.004), 7 patients with pancreatitis (P = 0.008) (supplementary Fig. S2), or in the patient with slowly progressive type 1 diabetes.

We were unable to detect the enterovirus sequence, and we could not amplify 18S rRNA and/or glyceraldehyde phosphate dehydrogenase (GAPDH) cDNAs from the pancreatic sections of diabetic patients, although we could detect 18S rRNA and/or GAPDH sequences from control pancreata. We therefore assumed that enterovirus RNAs had already degraded.

CD8+ T-cells, macrophages, and CD11c+ dendritic cells in the pancreas. Marked mononuclear cell (MNC) infiltration into islets (insulitis) and around islets (periinsulitis) was observed in all three cases with fulminant type 1 diabetes (Fig. 2*A–C*). Frequency of insulitis per examined islet was almost 100% in all three cases (Table 1). Islet volume and β-cell volume were markedly decreased (Table 1). α-Cell volume was decreased in case 1.

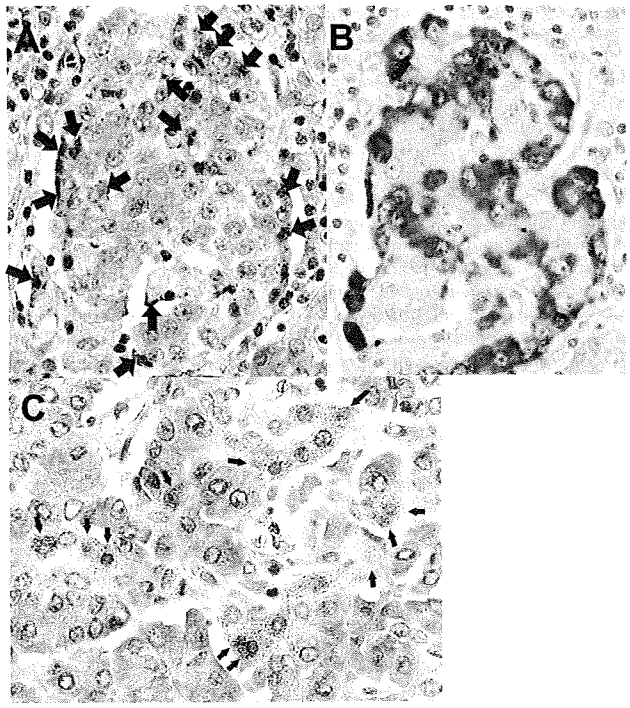

FIG. 1. *A*: Immunohistochemical demonstration of enterovirus-associated VP1 antigen in pancreatic islets (brown, arrows). Cells with shrunken and dark nuclei (arrows) suggestive of pyknosis, a sign of cell death, were observed (×400, case 1). *B*: Immunohistochemical staining for glucagon in serial sections of (*A*) (×400). Comparing (*A*) and (*B*) indicates enterovirus VP1 antigen residing on islet cells. *C*: Homogeneous staining for VP1 was observed in pancreatic acinar cell clusters (brown) with shrunken and darkly staining nuclei suggestive of pyknosis (arrows) (×400). (A high-quality digital representation of this figure is available in the online issue.)

Some exocrine pancreatic tissues, including acinar and ductal cells, were also surrounded by MNCs.

Predominant phenotypes of MNCs in islets with insulitis were macrophages and CD8+ T-cells (Fig. 2*B* and *C*) (Table 1). CD11c+ dendritic cells were detected in and around the islet with or without β-cells in cases 1 and 2 (Fig. 2*D*), while dendritic cell staining was less prominent in case 3. CD11c+ dendritic cell infiltration into islets was not observed in the 7 pancreatitis patients and 10 nondiabetic control subjects. B-lymphocytes, CD4+ T-cells, and NK cells were rare. VP1-positive pancreatic acinar cells were surrounded predominantly by macrophages (supplementary Fig. S3). In all three cases, MHC class II molecules were expressed on some residual β-cells (Fig. 2*E*). Macrophages did not show positive immunostaining for insulin (Fig. 2*F*), removing the possibility that macrophages with phagocytosed insulin vesicles from damaged β-cells represent MHC class II–expressing β-cells (27). Some vascular endothelium surrounding or inside the islets showed dilatation and enhanced expression of MHC class II molecules (Fig. 2*G*). MHC class I molecules were hyperexpressed on the pancreatic islet cells in three cases, while the islet cells of nondiabetic control pancreas showed only faint expression of MHC class I molecules in some islet cells (Fig. 2*H* and *I*). We could not detect interferon-α or 2′,5′-oligoadenylate synthetase–like protein on affected pancreata from patients with fulminant type 1 diabetes and control subjects, although these proteins represented markers of recent virus infection in pancreata affected by type 1 diabetes (28).

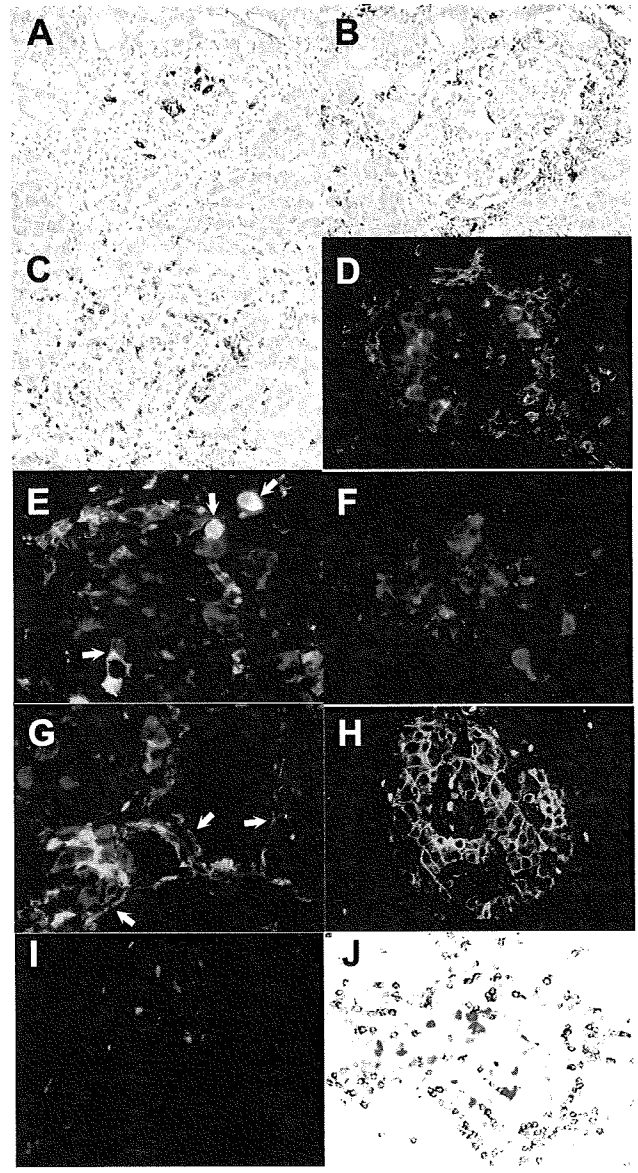

FIG. 2. Mononuclear cell infiltration into islets with residual β-cells (*A*) (brown), macrophages (*B*) (brown), and CD8+ T-cells (*C*) (brown) (×200, serial sections of case 1). *D*: Double immunofluorescent staining for CD11c+ dendritic cells (red) and insulin (blue) demonstrates that some dendritic cells surrounded and infiltrated into islets (×400, case 1). *E*: Double immunofluorescent staining for insulin (blue) and MHC class II antigen (green) demonstrates that some residual β-cells aberrantly express MHC class II molecules (light blue, arrows) (×400, case 1). *F*: Double immunostaining for CD68+ macrophages (red) and insulin (blue). Insulin was not stained in macrophages (×400, case 1). *G*: Double immunofluorescent staining for MHC class II molecules (green) and α-cells (blue) demonstrates aberrant expression of MHC class II molecules on vascular endothelium around and within the islets (arrows) (×400, case 1). *H*: Immunofluorescent staining demonstrates hyperexpression of MHC class I molecules (green) on islet cells (×200, case 1). *I*: Faint staining of MHC class I molecules (green) were observed on some nondiabetic control islet-cells (×200). *J*: Double immunostaining of the pancreatic section stained for CXCL10 (purple) and CXCR3 (brown). CXCR3-positive cells have infiltrated islet cells expressing CXCL10 (×200, case 1). (A high-quality digital representation of this figure is available in the online issue.)

CXCL10 expressed in all islet cell subsets, which were infiltrated by CXCR3+ T-cells. Double immunostaining demonstrated CXCL10 expression in pancreatic islets, while CXCR3-bearing MNCs that had infiltrated the

TABLE 1
Results on morohometric analysis, frequency of insulitis, and phenotypic analysis on three autopsied pancreata from patients with fulminant type 1 diabetes

	Morphometric analysis			Frequency of insulitis	Phenotype of MNCs infiltrating islets	
	Islet volume (%)	β-Cell volume (%)	α-Cell volume (%)	[% (*n*1/*n*2)]	Macrophage (%)	CD8+ T-cell (%)
Patient						
Case 1	0.46	0.006	0.073	100 (34/34)	42.3	38.4
Case 2	0.94	0.129	0.350	100 (39/39)	38.5	34.2
Case 3	0.39	0.001	0.174	95 (21/22)	70.2	24.6
Mean value in patients	0.60 ± 0.30*	0.045 ± 0.073†	0.199 ± 0.140	99 (94/95)	50.3 ± 17.3	32.4 ± 7.1
Mean value in nondiabetic control subjects (n = 10)	3.14 ± 1.85	2.233 ± 1.431	0.300 ± 0.079	0 (0/747)	—	—

Data are means ± SD, unless otherwise indicated. *n*1, number of the islets with insulitis; *n*2, number of the evaluated islets. *$P < 0.002$; †$P < 0.001$.

islets expressed CXCL10 in all three cases (Fig. 2*J*). β-Cells, α-cells, and other subsets of islet cells expressed CXCL10 in all three cases (Fig. 3*A–D*). The positive cells for CXCL10 were observed in 96% (44 of 46), 100% (34 of 34), and 83% (31 of 38) of islets in case 1, case 2, and case 3, respectively. No CXCL10 expression was found in pancreatic acinar or ductal cells, which were surrounded by CD8+ T-cells and macrophages. Neither control pancreata nor that from the patient with slowly progressive type 1 diabetes expressed CXCL10 in the islets or exocrine pancreas (supplementary Figs. S4 and S5).

Coexpression of CXCL10 and interferon-γ in islet cells. Interferon-γ was expressed in most β-cells, α-cells, and other types of islet cells from the cases with fulminant type 1 diabetes. Surprisingly, interferon-γ was coexpressed in CXCL10-positive islet cells (Fig. 4*A–D*). No CXCL10 or interferon-γ was expressed on affected exocrine pancreas or nondiabetic pancreas. RT-PCR could not

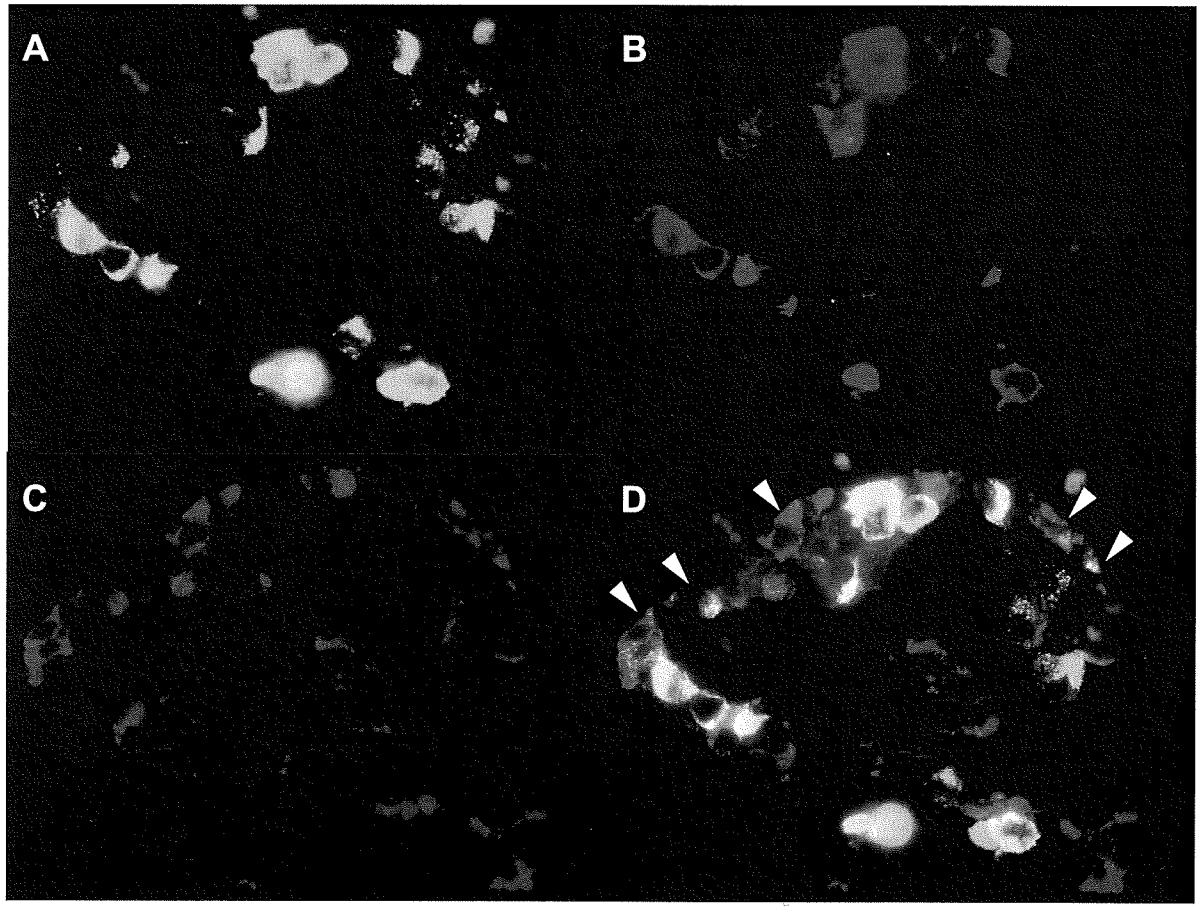

FIG. 3. Triple-immunofluorescent staining for CXCL10 (*A*), insulin (*B*), and glucagon (*C*). A merged image (*D*) demonstrates expression of CXCL10 on β-cells (light blue) (case 2). A proportion of α-cells (orange, arrowheads) and other types of islet cells (green) also express CXCL10 (×400, case 2). (A high-quality digital representation of this figure is available in the online issue.)

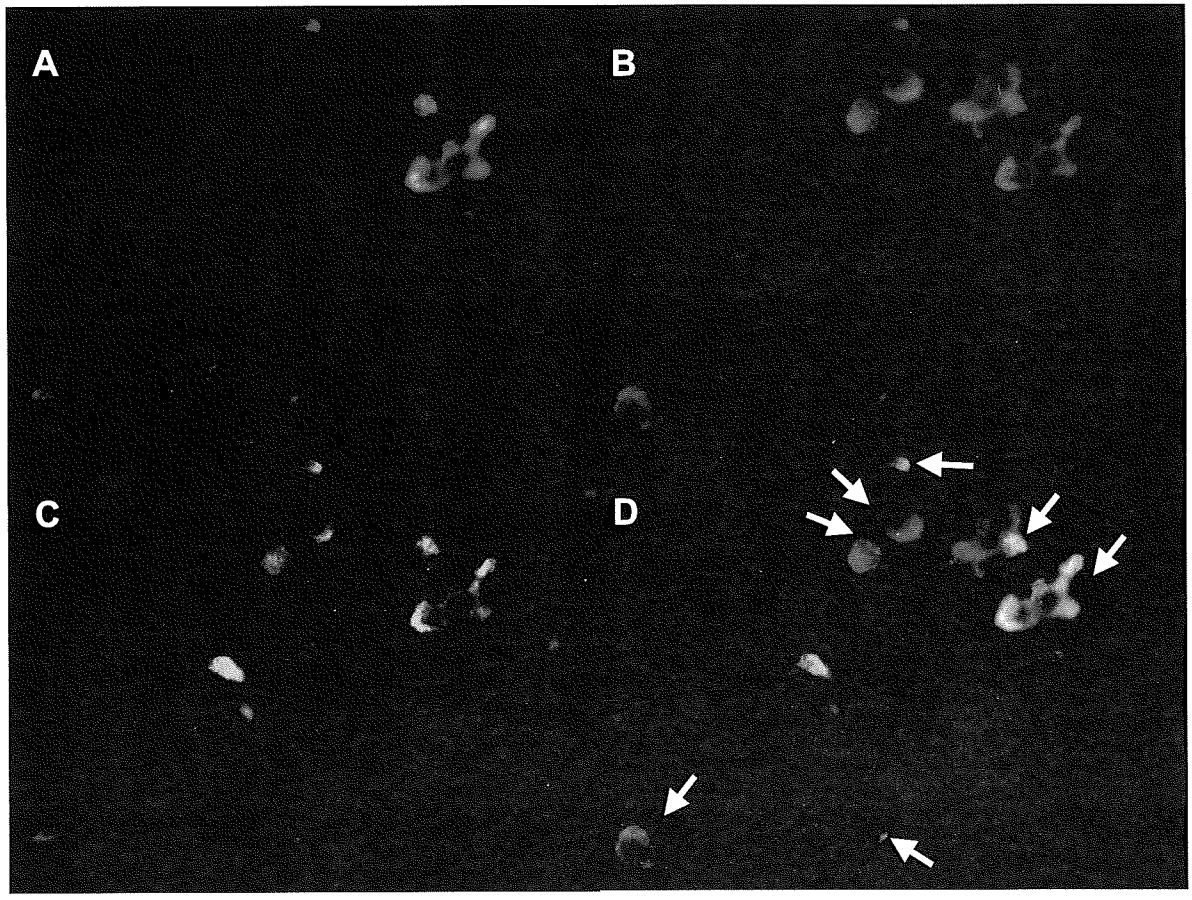

FIG. 4. Triple-immunofluorescent staining for CXCL10 (*A*), insulin (*B*), and interferon-γ (*C*) in case 3. *D*: Merged image shows that residual β-cells express both CXCL10 and interferon-γ (arrows) (×400, case 3). (A high-quality digital representation of this figure is available in the online issue.)

show CXCL10 or interferon-γ sequences in the affected pancreas by fulminant type 1 diabetes. As we were unable to amplify 18S rRNA or GAPDH cDNAs from pancreatic sections of diabetic patients as mentioned above, we assumed that CXCL10 and interferon-γ RNAs had already degraded.

Serum CXCL10 levels. Serum CXCL10 levels in cases 1 and 2 were 563 pg/ml and 622 pg/ml, respectively. Serum CXCL10 levels were 13.5 times (case 1) and 15.0 times (case 2) higher than the mean value for healthy subjects (26).

DISCUSSION

We demonstrated various novel findings in fulminant type 1 diabetes that have not previously been reported for typical human type 1 diabetes (29–31). First, extensive enterovirus infection with severe infiltration of MNCs into both islets (insulitis) and the exocrine pancreas was observed around VP1-positive cells (Fig. 1*A–C*) (supplementary Fig. S3). Typical type 1 diabetic pancreas showed mild to moderate insulitis, distributed in a patchy manner throughout the pancreas (29,30), and VP1-positive cells could not be found in the exocrine pancreas (31). However, Richardson et al. (32) recently reported a high prevalence of VP1 in the islets of young patients with recent-onset type 1 diabetes using the same monoclonal antibody applied in our study. The VP1-positive pancreatic endocrine and exocrine cells showed characteristic features of cell damage, including shrunken and darkly stained nuclei suggestive of pyknosis, which was reported in Coxsackie virus–infected islets (31). Elevated serum pancreatic enzyme levels and pathological changes observed in virus-infected cells (Fig. 1*C*) (supplementary Fig. S3) showed enterovirus-associated involvement of the exocrine pancreas in this syndrome. Second, CXCL10 and interferon-γ were extensively coexpressed in islet cells (Fig. 4*A–D*). Most MNCs infiltrating into islets were either CD8+ T-cells bearing the CXCL10 receptor, CXCR3, or macrophages.

CXCL10 is a chemokine that is inducible by interferon-γ and exerts key roles in the expansion and attraction of autoreactive and antigen-specific T-cells (10,12). This finding of the coexpression of CXCL10 and interferon-γ in β-cells suggests the presence of a unique immunological circuit for accelerating β-cell destruction. The initial event that trigged CXCL10 expression on islet cells may be enteroviral infection of β-cells and the exocrine pancreas surrounding the islets (Fig. 1*A–C*). In vitro studies showed that enterovirus infection of islet cells induced CXCL10 production within 1–2 days after infection (33,34). In our patients, serum CXCL10 levels were elevated to >10 times higher than levels in control subjects. CXCL10 from islet cells will preferentially activate autoreactive T-cells via CXCR3 and thus attract cells to the islets releasing islet-specific antigen (10,12,13). The presence of activated autoreactive T-cells reacting with insulin B9-23 peptide,

and GAD65 peptides has been reported in fulminant type 1 diabetes (35). Dendritic cells in the pancreas amplify immune responses to tissue antigens along with T-cells (36), thus contributing to rapid progression of β-cell failure. MHC class II molecules expressed on β-cells and dilated capillary endothelium around the islets (Fig. 2*G*) and aberrantly expressed MHC class II molecules on the islet cells will facilitate the "homing" process of activated T-cells and macrophages to islets (37–39). Our preliminary study examined the expression of another chemotactic protein besides CXCL10, namely monocyte chemotactic protein (MCP)-1. However, we failed to identify positive staining for MCP-1 on the affected pancreas, so CXCL10 and associated immunological cascades were studied. Chemoattracted autoreactive T-cells and macrophages brought to the islets will secrete interferon-γ and other inflammatory cytokines upregulating MHC class I molecules (Fig. 2*H*) and further destroy islet cells expressing CXCL10 (13,40). In such an extensively inflamed milieu in the islets, β-cells produce both CXCL10 and interferon-γ in the same cell (Fig. 4*A–D*). Interferon-γ in β-cells disturbs the function and viability of those cells and further accelerates CXCL10 generation and activation of autoreactive CXCR3-bearing T-cells and macrophages. These additional activated and accumulated T-cells and macrophages in the islets again secrete inflammatory cytokines including interferon-γ, inducing further CXCL10 generation in β-cells and CXCR3-mediated T-cell activation. This vicious cycle will continue until complete destruction of all β-cells has been achieved. The absence of expression of CXCL10 or interferon-γ in islets of the patient with slowly progressive type 1 diabetes (supplementary Fig. S5) supports the concept that CXCL10 and CXCR3 activation circuit represents a unique mechanism of rapid β-cell destruction in fulminant typel diabetes.

Another unique finding in patients with fulminant type 1 diabetes was that both α- and β-cells in islets were infected by enterovirus and expressed CXCL10 and interferon-γ. In Coxsackie B4 enterovirus–induced type 1 diabetes, β-cells are specifically involved (41). Impaired α-cell volume was observed in case 1 and has been reported in long-standing patients with fulminant type 1 diabetes (42). Inflammatory processes were observed in pancreatic exocrine tissues in our study, and pancreatic enzyme is specifically increased in fulminant type 1 diabetes (3,4). These findings suggest that enterovirus-causing diabetes will display a wide diversity of tropism from β-cell-specific, as in cases of typical type 1 diabetes (41), to other subsets of pancreatic endocrine and exocrine cells, as in cases of fulminant type 1 diabetes. The genetic bondage of the host may have influence on virus potency or tropism. We have already reported specific genetic backgrounds (i.e., HLA-DRB1*0405 and HLA-DQB1*0401, which was possessed in our cases) for this syndrome (43).

The present findings regarding the destruction of islet endocrine cells provide new insights into strategies for the treatment of fulminant type 1 diabetes. Development of antagonists and neutralizing agents for interferon-γ and the CXCL10/CXCR3 axis may represent one therapeutic option. In an experimental animal model of type 1 diabetes, neutralization of CXCL10 can cure virus-induced type 1 diabetes (44).

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No potential conflicts of interest relevant to this article were reported.

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