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Possible Role of Macrophage-Derived Soluble Mediators in the Pathogenesis of Encephalomyocarditis Virus-Induced Diabetes in Mice

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Pancreatic islets from DBA/2 mice infected with the D variant of encephalomyocarditis (EMC-D) virus revealed lymphocytic infiltration with moderate to severe destruction of pancreatic β cells. Our previous studies showed that the major population of infiltrating cells at the early stages of infection is macrophages. The inactivation of macrophages prior to viral infection resulted in the prevention of diabetes, whereas activation of macrophages prior to viral infection resulted in the enhancement of β-cell destruction. This investigation was initiated to determine whether macrophage-produced soluble mediators play a role in the destruction of pancreatic β cells in mice infected with a low dose of EMC-D virus. When we examined the expression of the soluble mediators interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), and inducible nitric oxide synthase (iNOS) in the pancreatic islets, we found that these mediators were clearly expressed at an early stage of insulitis and that this expression was evident until the development of diabetes. We confirmed the expression of these mediators by in situ hybridization with digoxigenin-labelled RNA probes or immunohistochemistry in the pancreatic islets. Mice treated with antibody against IL-1β or TNF-α or with the iNOS inhibitor aminoguanidine exhibited a significant decrease in the incidence of diabetes. Mice treated with a combination of anti-IL-1β antibody, anti-TNF-α antibody, and aminoguanidine exhibited a greater decrease in the incidence of disease than did mice treated with one of the antibodies or aminoguanidine. On the basis of these observations, we conclude that macrophage-produced soluble mediators play an important role in the destruction of pancreatic β cells, resulting in the development of diabetes in mice infected with a low dose of EMC-D virus.

Insulin-dependent diabetes mellitus (IDDM) results from the destruction of insulin-producing pancreatic β cells. Genetic factors, autoimmunity, and viral infection have been extensively studied as possible causes of IDDM (26, 31, 33, 38, 45–47). The best experimental evidence that viruses play an etiological role in the pathogenesis of IDDM appears to be found in mice infected with encephalomyocarditis (EMC) virus (17, 49). EMC virus induces a diabetes-like syndrome characterized by hypoinsulinemia, hyperglycemia, glycosuria, polydipsia, and polyphagia (9, 44, 49). Whereas molecular identification of EMC virus genes responsible for the induction of diabetes (1–3, 18, 50) and genetic factors of the host have been extensively studied (30, 48, 51, 52), immune mechanisms involved in the pathogenesis of diabetes in EMC virus-infected animals have not been clearly elucidated.

Early studies suggested that T lymphocytes may be involved in the destruction of β cells in diabetic mice infected with the M variant of EMC (EMC-M) virus (11, 12). However, later studies revealed that a depletion of T lymphocytes failed to alter the incidence of diabetes (53). In addition, treatment of EMC virus-infected mice with cyclosporine increased the incidence and severity of diabetes rather than preventing the disease (41). Thus, the emphasis of the search for immune mechanisms involved in the pathogenesis of diabetes in EMC virus-infected animals has shifted from T lymphocytes to macrophages as more evidence supporting the devastating role that macrophages play in the destruction of pancreatic β cells has accumulated (4, 5, 22, 23).

Several studies have indicated that macrophages are involved in the destruction of pancreatic β cells in EMC-D virus-infected mice (5, 23). Macrophages are predominant at an early stage after infection with EMC-D virus, whereas mixed immunocytes such as macrophages, $CD4^+$ T lymphocytes, and $CD8^+$ T lymphocytes are present at intermediate and late stages after virus infection (4). The inactivation of macrophages with antibody against macrophages, or long-term treatment with silica prior to the infection of mice with a low dose of EMC-D virus, resulted in the prevention of diabetes (5). In contrast, the incidence of diabetes increased when macrophages were activated with a single administration of silica followed by a low dose of EMC-D virus. Macrophage depletion with antimacrophage monoclonal antibodies resulted in the near complete prevention of β-cell destruction in silica-treated (single administration) EMC-D-infected mice (5).

Although previous studies have demonstrated that macrophages are involved in the destruction of pancreatic β cells, the role macrophages play in the destruction of these cells is not fully understood. Since macrophages are known to produce

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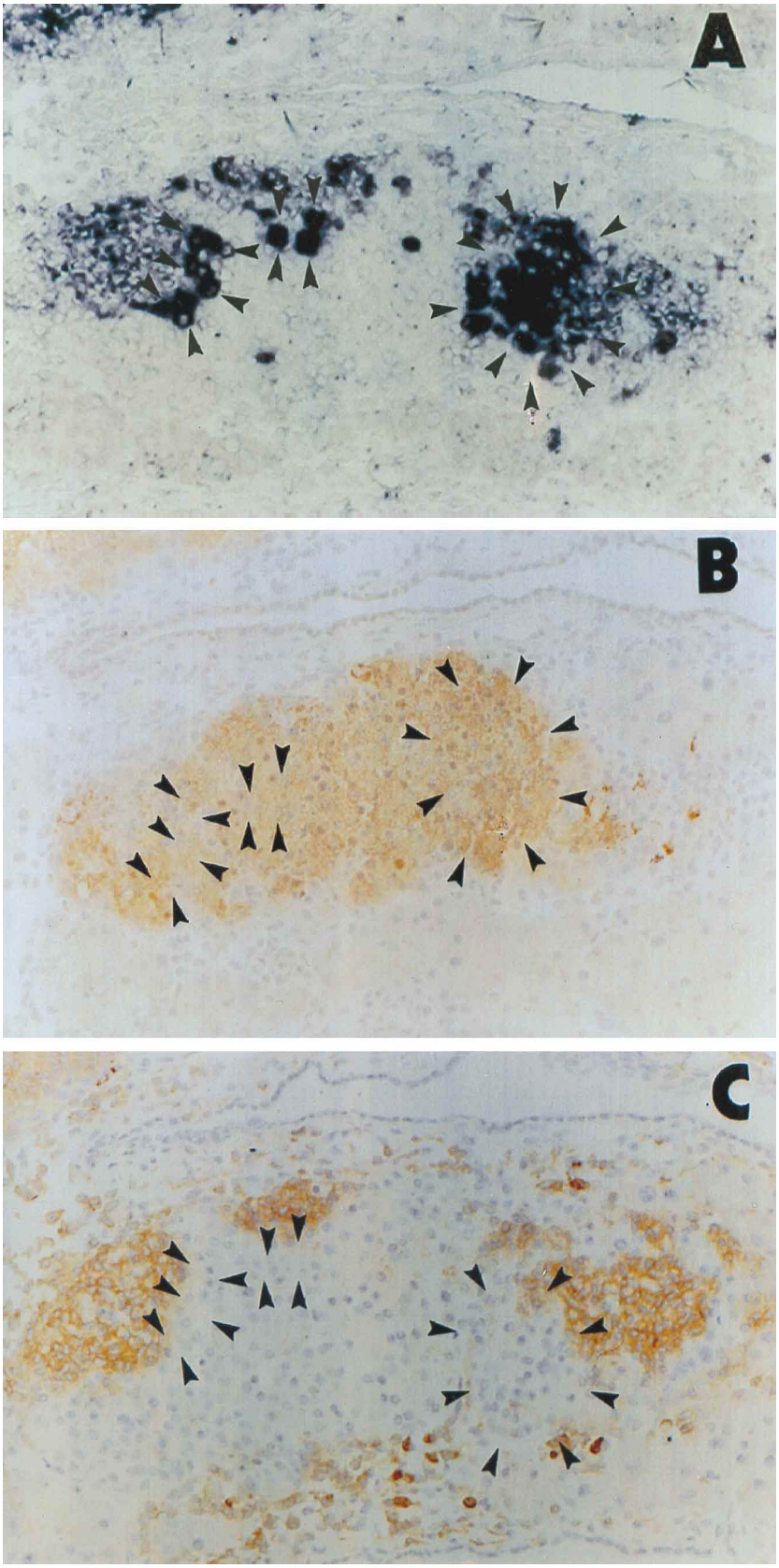

FIG. 1. Serial sections of pancreatic islets prepared from DBA/2 mice at 3 days after infection with EMC-D virus. Sections of pancreas hybridized with the antisense RNA probe of EMC-D virus (A) and sections of pancreas stained with anti-insulin antibody (B) and antimacrophage antibody (C) are shown. Arrowheads indicate corresponding locations between sections.

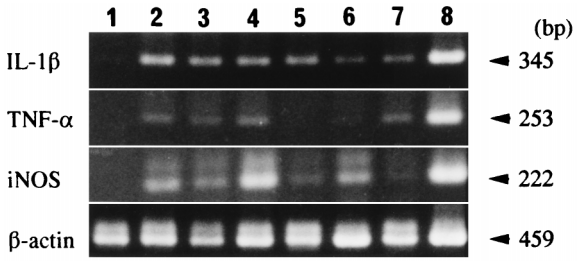

FIG. 2. RT-PCR analysis of IL-1β, TNF-α, iNOS, and β-actin mRNA expression in the pancreatic islets and spleen cells (positive control) of DBA/2 mice infected with EMC-D virus at 2, 3, and 4 days postinfection. Lane 1, pancreas of uninfected mouse (negative control); lanes 2 and 3, pancreas at 2 days postinfection; lanes 4 and 5: pancreas at 3 days postinfection; lanes 6 and 7, pancreas at 4 days postinfection; lane 8, spleen at 4 days postinfection.

soluble mediators, such as interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), and inducible nitric oxide synthase (iNOS), we investigated whether these mediators are involved in the destruction of β cells in EMC-D virus-infected mice. We now report that IL-1β, TNF-α, and iNOS, produced by activated macrophages, play a critical role in the destruction of pancreatic β cells, leading to the development of diabetes in EMC-D-infected DBA/2 mice.

MATERIALS AND METHODS

Virus. The source and preparation of EMC virus have been described elsewhere (49). Virus pools were prepared from L929 cells, and the virus titer was determined with a plaque assay on L929 cells (50).

Mice. DBA/2 mice were obtained from Charles River Japan Inc. (Kanagawa, Japan) and Jackson Laboratory (Bar Harbor, Maine). The animals were housed in an animal facility at the Health Sciences Centre, University of Calgary, Calgary, Alberta, Canada, and the University of Tokyo, Tokyo, Japan. Eight-week-old male mice were used. EMC-D virus was injected intraperitoneally (100 PFU/mouse).

Measurement of virus replication in the pancreas. The pancreas was removed from each mouse at 4 days after virus infection, and virus concentration in the pancreatic tissue was determined by plaque assay on L929 cells as described previously (22, 50).

Measurement of blood glucose. Blood glucose levels of nonfasting mice were measured every other day after infection for up to 14 days. The mean blood glucose level of 38 nonfasting unaffected DBA/2 male mice was 167 ± 16 mg/dl (mean ± standard deviation [SD]). In this experiment, nonfasting animals with blood glucose levels greater than 215 mg/dl (3 SD above the mean) were scored as diabetic.

Immunohistochemical staining of pancreatic sections. For immunohistochemical examination of pancreatic sections, mice were sacrificed at 3 days after infection and the pancreas was removed from each animal. Small pieces of the pancreas from each mouse were immediately frozen with dry ice and ethanol. Pancreatic sections (thickness, 4.5 μm) were fixed with acetone or 4% paraformaldehyde in phosphate-buffered saline (PBS). These sections were immunohistochemically stained with monoclonal antibody against macrophages (Anti-Mac-1; Boehringer Mannheim, Yamanouchi, Japan), polyclonal antibody against iNOS (Santa Cruz Biochemistry Inc., Santa Cruz, Calif.), and polyclonal antibody against insulin (Funakoshi Co., Tokyo, Japan) by the avidin-biotin-peroxidase complex method with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, Calif.).

Histological examination. Several mice per group were sacrificed at 14 days after infection, and 50% of each pancreas was fixed in 6% formalin. Paraffin-embedded sections were stained with hematoxylin and eosin and examined. The classifications "normal morphology," "mild to moderate insulitis," "severe insulitis," and "atrophied morphology" were used to describe histological changes of the pancreatic islets. Normal islets exhibited normal morphology. The architecture of islets having mild to moderate insulitis was well preserved, but 1 to 49% of the islets exhibited lymphocytic infiltration. Severe insulitis was characterized by morphological damage to pancreatic β cells, and 50 to 100% of these islets exhibited lymphocytic infiltration. Atrophied islets were small and retracted, exhibiting severe β-cell necrosis with or without residual lymphocytic infiltrate.

Preparation of digoxigenin-labelled RNA probes. A 1,000-bp cDNA fragment of a specific region of the EMC-D virus (VP1 to VP3) was subcloned into a pBluescript SK(−) vector (Stratagene, La Jolla, Calif.) by standard techniques (37). A 675-bp cDNA fragment of IL-1β (nucleotides 683 to 1358) and a 1,180-bp cDNA fragment of TNF-α (nucleotides 1 to 1180) (Genentech Inc., San Francisco, Calif.) were subcloned into pGEM3Z (Promega, Madison, Wis.) in the same way. After the plasmids were linearized with the appropriate restriction endonuclease, digoxigenin-labelled antisense and sense RNA probes were prepared with T7, T3, and SP6 RNA polymerase by using a DIG RNA labelling kit (Boehringer Mannheim).

In situ hybridization. In situ hybridization was performed as described elsewhere with some modifications for cryosections (23). Briefly, cryosections were fixed with 3% paraformaldehyde for 60 min, 0.2 N HCl for 8 min, 0.25% acetic anhydride–0.1 M triethanolamine-HCl (pH 8.0) for 15 min, and 2× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) for 10 min. These sections were then prehybridized with 50% deionized formamide–2× SSC for 60 min. After pretreatment, the sections were hybridized with the EMC-D virus RNA probe, an IL-1β RNA probe, or a TNF-α RNA probe for 18 h at 50°C in the following solution: 50% deionized formamide, 100 μg of yeast tRNA/ml, 10% dextran sulfate, 1× Denhardt's solution, 0.05 M Tris-HCl (pH 7.5), 5 mM EDTA, and 0.6 M NaCl. After hybridization, the sections were rinsed with 5× SSC, washed with 50% formamide–2× SSC for 30 min at 50°C, and incubated with a buffer (2 μg of RNase A/ml, 10 mM Tris-HCl [pH 7.5], 0.5 M NaCl) for 30 min at 37°C. The sections were then washed twice with 2× SSC and 0.2× SSC for 30 min each at 48°C.

For colorimetric detection after hybridization, the sections were treated with 1% blocking reagent (Boehringer Mannheim) in DIG buffer 1 (0.1 M Tris-HCl [pH 7.5], 0.15 M NaCl) for 30 min and then incubated with alkaline phosphatase-conjugated antidigoxigenin antibody (1:500) (Boehringer Mannheim) overnight. Sections were colored with nitroblue tetrazolium chloride–5-bromo-4-chloro-3-indolylphosphate toluidinium and counterstained with hematoxylin.

Reverse transcription (RT)-PCR analysis. Total RNA was extracted from the pancreata of DBA/2 mice infected with EMC-D virus 2 to 4 days after infection as described previously (20). The cDNA was synthesized with 4 μg of RNA in 20 μl of reaction mixture containing 50 pmol of oligo$(dT)_{12–18}$ primer, 10 mM dithiothreitol, 75 mM KCl, 50 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 15 U of RNase inhibitor, 0.2 mM (each) deoxynucleoside triphosphate, and 20 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL Life Technologies Inc., Gaithersburg, Md.). PCR was performed with 4 μl of cDNA with pairs of oligonucleotide primers corresponding to the cDNA sequences. The following oligonucleotide sequences were derived from a sequence at GenBank: for β-actin, GTTACCAACTGGGACGACA and TTCGAGCAGGAGATGGC CA; for IL-1β, GGAATGACCTGTTCTTTGAAGTT and GGCTCCGAGAT GAACAACAAAA; for TNF-α, CTTAGACTTTGCGGAGTCCG and GGGA CAGTGACCTGGACTGT; and for iNOS, GCATGGACCAGTATAAGGCA AGCA and TTGCTCATGACATCGACCAGAAGC. PCR amplification was carried out in 50 μl of the reaction mixture containing 50 pmol of sense and antisense primer, 0.2 mM deoxynucleoside triphosphate, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 0.1% Triton X-100 (Perkin-Elmer Cetus, Norwalk, Conn.) for 45 cycles with denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min with a DNA thermal cycler (Perkin-Elmer Cetus). The product was run on a 2% agarose gel and detected by ethidium bromide staining.

Treatment of DBA/2 mice with an iNOS inhibitor and antibody against IL-1β and TNF-α. Male DBA/2 mice were injected intraperitoneally with the iNOS inhibitor aminoguanidine (AG) (Sigma, St. Louis, Mo.), anti-IL-1β antibody, anti-TNF-α antibody (R&D Systems, Minneapolis, Minn.), or a combination of the three. Concentrations of 0.5 mg of antibody or 5 mg of AG in 200 μl of PBS were used for treatment with a single substance, and the same concentrations were used in combination. As a control, 0.5 mg of normal goat immunoglobulin G (IgG) in 200 μl of PBS and 200 μl of PBS alone were injected into male DBA/2 mice. Daily administration of antibody or AG was initiated on the same day as EMC-D virus infection and continued for 10 days. Blood glucose was measured every other day for 2 weeks after antibody and virus infection. Virus titer was determined at 4 days after infection. Histological examination was performed 2 weeks after infection.

Statistical analysis. Statistical analysis was conducted by Fisher's exact test.

RESULTS

Correlation of the infiltration of macrophages into the pancreatic islets with the infection of pancreatic β cells by using EMC-D virus. To determine whether EMC-D virus infects pancreatic β cells, subsequently resulting in the recruitment of macrophages, sections of serial mounts of the pancreata from EMC-D virus-infected mice were hybridized with the antisense RNA probe of the EMC-D virus. Consecutive sections of pancreata were immunohistochemically stained with anti-insulin antibody and antimacrophage antibody. We found that the major portion of EMC-D virus RNA-positive cells were also stained by anti-insulin antibody (Fig. 1A and B), indicating that β cells were infected by the EMC-D virus. In addition, we found that the infiltration of macrophages was localized adja-

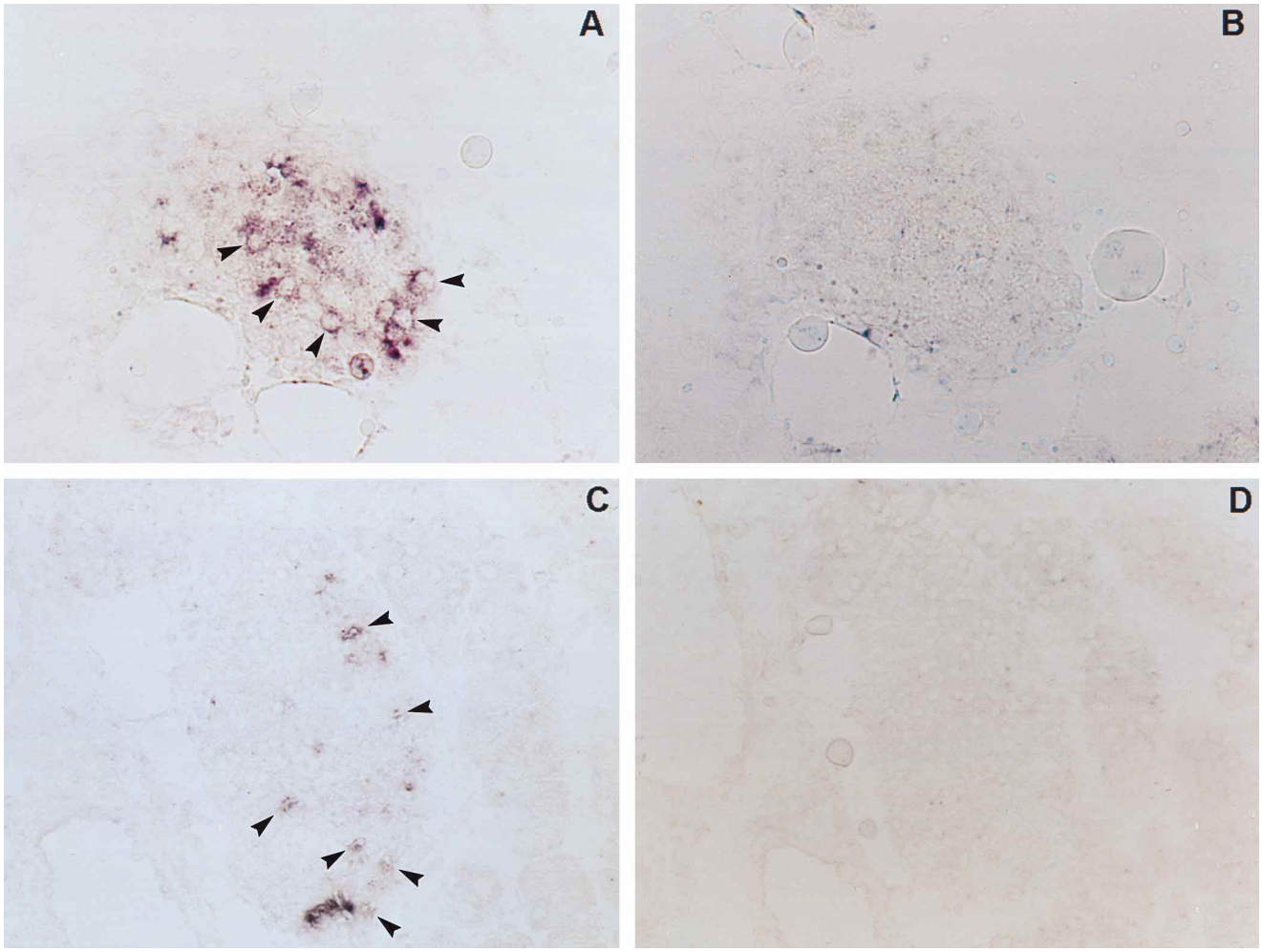

FIG. 3. Localization of cytokine gene expression in cryosections of pancreata from mice at 3 days after infection with EMC-D virus. Pancreatic sections hybridized with the digoxigenin-labelled IL-1β antisense probe (A), IL-1β sense probe (B), TNF-α antisense probe (C), and TNF-α sense probe (D) are shown. Arrowheads indicate IL-1β and TNF-α mRNA-positive cells.

cent to the pancreatic β cells in which EMC-D virus RNA was detected (Fig. 1A and C), indicating that EMC-D virus infection recruits macrophages to the pancreatic islets.

Macrophage-derived cytokine and iNOS gene expression in the pancreata of EMC-D virus-infected mice. To determine whether macrophages in the pancreatic islets express cytokines and iNOS, we examined the expression of IL-1β, TNF-α, iNOS, and β-actin (as a control) with RT-PCR. We found that there was constitutive expression of IL-1β, TNF-α, and iNOS mRNA in the pancreatic islets of EMC-D virus-infected mice (Fig. 2, lanes 2 to 7). Most of the animals tested expressed IL-1β, TNF-α, and iNOS in the pancreatic islets, which contained infiltrated immunocytes at 2, 3, and 4 days postinfection, although some variation among animals was evident. For example, cytokine gene expression varied from weak to strong with the exception of the expression of TNF-α at 3 days postinfection, when no expression was evident for one of the mice. These variations may be a result of different numbers of infiltrated macrophages in the pancreatic islets when samples were prepared for RT-PCR analysis.

Islet cell-specific expression of cytokine genes and iNOS in EMC-D virus-infected mice. To determine the location of cytokine gene expression and iNOS expression, cryosections of the pancreata from mice infected with EMC-D virus were hybridized in situ with digoxigenin-labelled anti-RNA probes for IL-1β and TNF-α mRNA or stained with anti-murine-iNOS polyclonal antibody. We found IL-1β mRNA-positive cells (Fig. 3A) and TNF-α mRNA-positive cells (Fig. 3C) in the intraislet infiltrates but not in exocrine acinar cells, indicating that IL-1β and TNF-α are specifically expressed in the intraislet infiltrates. We did not find any cytokine-positive cells in the pancreatic sections hybridized with the sense probes of IL-1β (Fig. 3B) or TNF-α (Fig. 3D). Furthermore, we found that iNOS-positive cells were localized to the pancreatic islets upon examination of the same animals (Fig. 4).

Effect of anticytokine antibodies and the iNOS inhibitor AG on the prevention of EMC-D virus-induced diabetes in mice. To determine whether IL-1β, TNF-α, or iNOS plays any role in the destruction of pancreatic β cells of mice infected with a low dose of EMC-D virus, we treated EMC-D virus-infected mice with anti-IL-1β antibody, anti-TNF-α antibody, the iNOS inhibitor AG, or a combination of the three for up to 10 days. We found that the incidence of diabetes decreased significantly in the animals treated with antibody or AG (Fig. 5 and Table 1).

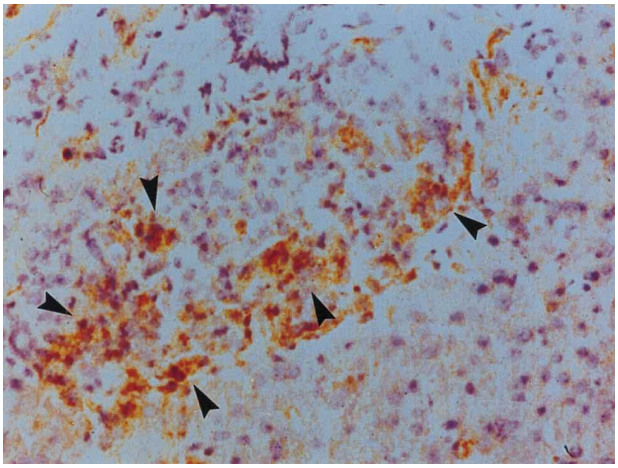

FIG. 4. Localization of iNOS expression in cryosections of pancreata from mice at 3 days after infection with EMC-D virus. Pancreatic sections were stained with anti-iNOS antibody. Arrowheads indicate iNOS-positive cells.

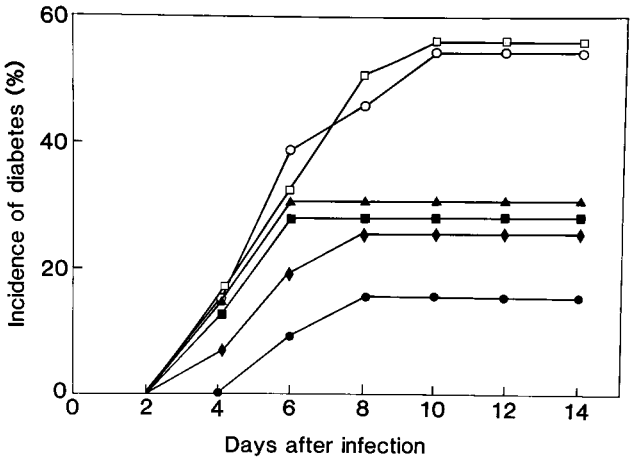

FIG. 5. Cumulative incidence of diabetes in EMC-D virus-infected mice (100 PFU/mouse) treated with PBS (○), normal goat IgG (□), anti-IL-1β antibody (■), anti-TNF-α antibody (▲), the iNOS inhibitor AG (◆), or anti-IL-1β antibody plus anti-TNF-α antibody plus AG (●). On the days indicated, the mice were bled and the blood glucose levels of nonfasting mice were determined. Each group contained 12 to 15 mice. The mean blood glucose level of 38 nonfasting unaffected DBA/2 male mice was 167 ± 16 mg/dl (mean ± SD). Nonfasting animals with a blood glucose level greater than 215 mg/dl (3 SD above the mean) were scored as diabetic. Each point represents the percentage of diabetic animals in that group.

We observed that 29% of the mice treated with anti-IL-1β antibody, 31% of the mice treated with anti-TNF-α antibody, and 27% of the mice treated with AG developed diabetes compared with 54% of the PBS-treated animals and 58% of the goat IgG-treated animals. Furthermore, only 17% of the mice treated with all three substances developed diabetes.

To determine whether the treatment of animals with anti-IL-1β and anti-TNF-α antibodies or an iNOS inhibitor influences replication of the virus in the pancreatic islets, we measured the virus titers in the pancreata of mice from groups treated with anti-IL-1β antibody, anti-TNF-α antibody, the iNOS inhibitor, and all three substances. We found that the virus titer decreased slightly in the anti-IL-1β antibody-treated group, the anti-TNF-α antibody-treated group, and the iNOS inhibitor-treated group compared with the PBS- or goat IgG-treated control groups. The greatest decrease of virus titer was observed in mice treated with all three substances (Table 1).

Examination of β-cell architecture revealed a significant reduction in β-cell destruction when mice were treated with antibodies against IL-1β or TNF-α or with the iNOS inhibitor (Table 2). A combination of the three substances resulted in minimal destruction to the β cells compared to results in mice treated with a single antibody or AG and in untreated animals (Table 2).

DISCUSSION

Infection of mice with diabetogenic EMC virus results in selective destruction of pancreatic β cells and a diabetes-like syndrome characterized by hyperglycemia, glycosuria, polydipsia, polyphagia, and hypoinsulinemia (9, 44, 49). The severity of the diabetes-like syndrome correlates with the degree of virus-induced β-cell destruction (21). Our earlier studies showed that over 90% of mice infected with a high titer of EMC-D virus (5×10^5 PFU/mouse) developed diabetes within 4 days after infection (49). However, infection of mice with a low dose of EMC-D virus (10^2 PFU/mouse) resulted in a significant decrease in the incidence of diabetes (approximately 50% became diabetic) (4, 5). When the macrophages of mice were activated with a single injection of silica, complete Freund's adjuvant, or type 1 carrageenan prior to a low dose of EMC-D virus, the incidence of diabetes increased significantly (100% became diabetic) (5). When peritoneal macrophages were isolated from uninfected SJL/J mice (mice had been treated once with silica for the activation of macrophages) and transferred into SJL/J mice 2 days before infection of the mice with a low dose of EMC-D virus, approximately 90% of the mice developed diabetes (5). In contrast, depletion of macrophages with a treatment of antimacrophage monoclonal antibody following a single administration of complete Freund's adjuvant, type 1 carrageenan, or silica resulted in the near complete prevention of EMC-D virus-induced diabetes (5). These results indicate that the activated macrophages of mice infected with a low dose of EMC-D virus are directly involved in the destruction of pancreatic β cells. However, the precise mechanisms involved in the destruction of β cells in EMC-D virus-infected mice have not been elucidated. We have previously hypothesized that macrophages may contribute to β-cell destruction as a result of a release of cytokines and free radicals (4, 5). In this study, we present for the first time clear evidence that the soluble mediators IL-1β, TNF-α, and iNOS, known as major factors produced from activated macrophages, contribute to the destruction of pancreatic β cells in mice infected with a low dose of EMC-D virus, leading to the development of IDDM.

First, we examined the expression of macrophage-derived soluble mediators in the pancreatic islets of mice infected with a low dose of EMC-D virus, since macrophages are the primary infiltrates into the islets (4). We found that IL-1β, TNF-α, and iNOS are selectively expressed in the pancreatic islets of mice infected with EMC-D virus. We then inactivated these soluble mediators with monoclonal antibodies against IL-1β and TNF-α and with an iNOS inhibitor in order to examine their role in the development of diabetes. We found that the incidence of diabetes was significantly reduced, perhaps due to the protection of β cells from the toxic effects of IL-1β, TNF-α, and NO, indicating that these soluble mediators, derived from activated macrophages, are involved in the destruction of pancreatic β cells in EMC-D virus-infected mice.

Studies utilizing spontaneously diabetic animal models, such

TABLE 1. Effects of anti-IL-1β antibody, anti-TNF-α antibody, and the iNOS inhibitor AG on the development of EMC-D virus-induced diabetes in DBA/2 mice[a]

Treatment[b]	Mean virus titer ± SD (log_{10} PFU/g of pancreas)	Mean blood glucose level ± SD (mg/dl)[c]	Incidence of diabetes (%)[d]
None (uninfected controls)		167 ± 16	0
PBS	5.7 ± 0.80	256 ± 70	54
Normal goat IgG	5.8 ± 0.97	263 ± 69	58
Anti-IL-1β antibody	5.2 ± 0.87	200 ± 63[e]	29
Anti-TNF-α antibody	5.0 ± 0.95	203 ± 61[e]	31
AG	5.1 ± 0.83	202 ± 62[e]	27
Anti-IL-1β antibody + anti-TNF-α antibody + AG	4.7 ± 0.94[e]	189 ± 55[f]	17

[a] Mice were in groups of 12 to 15 (except the uninfected control group, which contained 38 mice).
[b] All mice except uninfected controls were infected with EMC-D virus (100 PFU/mouse) and treated daily with the indicated substance(s) for 10 days.
[c] All mice were nonfasting.
[d] Any mouse with a glucose level greater than 215 mg/dl (3 SD above the mean of uninfected control mice) was scored as diabetic.
[e] $P < 0.05$ compared with controls (mice injected with EMC-D virus and PBS or goat IgG).
[f] $P < 0.01$ compared with controls (mice injected with EMC-D virus and PBS or goat IgG).

TABLE 2. Histological change in the pancreatic islets of mice treated with anti-IL-1β antibody, anti-TNF-α antibody, and the iNOS inhibitor AG after infection with EMC-D virus

Treatment[a]	No. of islets examined	% of islets with histology[b]			
		N	M	S	A
None (uninfected controls)	94	100	0	0	0
PBS	89	6.7	24.7	52.8	15.8
Normal goat IgG	91	6.6	25.3	51.6	16.5
Anti-IL-1β antibody	102	12.7	48.0	31.4	7.8
Anti-TNF-α antibody	78	11.5	44.9	34.6	9.0
AG	98	14.3	50.0	28.6	7.1
Anti-IL-1β antibody + anti-TNF-α antibody + AG	113	30.9	54.9	12.4	1.8

[a] All mice except uninfected controls were infected with EMC-D virus (100 PFU/mouse) and treated daily with the indicated substance(s) for 10 days. At 14 days after infection the mice were sacrificed and histological changes in the islets of Langerhans were examined.
[b] Combined data from diabetic and nondiabetic animals (20 to 30 islets per mouse and two diabetic and two nondiabetic mice per group). Category abbreviations are as follows (see the text for details): N, normal morphology; M, mild to moderate insulitis; S, severe insulitis; A, atrophied morphology.

as the NOD mouse and the BB rat, have suggested that macrophage-produced IL-1β, TNF-α, and NO play a role in the destruction of pancreatic β cells (19, 40, 43), even though conflicting observations exist (14, 24). In vitro studies have shown that IL-1 is selectively cytotoxic to isolated human and rat pancreatic β cells (7, 8, 15, 28, 34, 35). TNF-α inhibits insulin release and decreases the amount of insulin in isolated mouse islets (13). In addition, high levels of TNF-α were detected in the sera of prediabetic NOD mice and BB rats (6, 32, 39). On the basis of these studies, it was suggested that IL-1β and TNF-α, produced during the early stages of insulitis, may be effector molecules which mediate the disruption of β cells in these animals (19). The cytokines IL-1β, TNF-α, and gamma interferon are known to induce iNOS, which in turn generates the free radical nitric oxide (15, 16, 19, 29). The expression of iNOS (42), a macrophage activation marker, was not detected in pancreatic islets without lymphocytic infiltration (25). However, islets with advanced infiltration highly expressed iNOS, suggesting that expression of iNOS may be associated with the development of diabetes in the BB rat (25). Furthermore, treatment with N^G-nitro-L-arginine-methylester significantly reduced the incidence of diabetes in DP-BB rats (27), and treatment of NOD mice with AG, an iNOS inhibitor, caused a delay in the onset of diabetes in adoptive-transfer models (10). Thus it appears that NO (36), generated by iNOS, may contribute to β-cell destruction and that macrophage-derived soluble factors may be involved in the destruction of β cells in the spontaneously diabetic animal model. However, systemic administration of IL-1β or TNF-α results in the prevention rather than the induction of diabetes in NOD mice (14, 24). Therefore, it may be difficult to substantiate the precise role of these macrophage-derived proinflammatory cytokines in the development of IDDM in spontaneously diabetic animal models because cytokines, secreted from both T cells and macrophages, appear to regulate the immune system in a complex manner.

Differences between the mechanisms involved in the destruction of β cells in spontaneously diabetic animal models and in the EMC-D virus-induced-diabetes animal model exist. It is well known that the development of diabetes in NOD mice and BB rats is T-cell mediated, since depletion of T cells results in the prevention of diabetes in these models (31, 45). Development of autoimmune IDDM in these animals may depend upon the delicate balance of cytokines produced by macrophages and T cells (20, 45). If the balance is tipped in favor of destructive cytokines (IL-1, TNF-α, gamma interferon, and IL-2, etc.), the autoimmune processes leading to β-cell destruction may be enhanced. In contrast, protective cytokines (e.g., transforming growth factor β) may inhibit the destructive process when the balance is tipped in their favor (20). The interaction between cytokines determines the direction the delicate balance may swing. Thus, it may not be easy to accurately evaluate the role of macrophage-derived cytokines and iNOS in spontaneously diabetic animal models. In contrast, T cells are not involved in the EMC-D virus-induced-diabetes animal model in the same manner because a depletion of T cells does not cause an alteration in the incidence of diabetes (53). Therefore, the EMC-D virus-induced-diabetes animal model is less complex than the spontaneously diabetic animal model, which is an advantage when the roles of macrophage-derived soluble mediators are studied.

Although β-cell protection was greatly enhanced after mice were treated with anti-TNF-α antibody, anti-IL-1β antibody, or AG, these substances did not completely protect β cells or entirely arrest the incidence of diabetes. In a parallel experiment, treatment of mice with anticytokine antibodies and/or an iNOS inhibitor resulted in a decrease of virus titer compared to that of mice treated with PBS or goat IgG. Therefore, the destruction of β cells in EMC-D virus-induced diabetes in mice is not solely a result of cytotoxic soluble mediators produced from macrophages; the replication of EMC-D virus in β cells could be another contributing element (48). On the basis of these observations, we conclude that the synergistic effect of macrophage-derived soluble mediators and the replication of EMC-D virus results in the destruction of pancreatic β cells, leading to IDDM in mice.

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