JOURNAL OF VIROLOGY, Feb. 2001, p. 1949–1957 Vol. 75, No. 4
0022-538X/01/$04.00+0 DOI: 10.1128/JVI.75.4.1949–1957.2001


# Role of Hck in the Pathogenesis of Encephalomyocarditis Virus-Induced Diabetes in Mice

K. S. CHOI,[1] H. S. JUN,[2] H. N. KIM,[1] H. J. PARK,[1] Y. W. EOM,[1] H. L. NOH,[3] H. KWON,[2] H. M. KIM,[3] AND J. W. YOON[1,2]*

*Laboratory of Endocrinology, Institute for Medical Sciences,[1] and Department of Endocrinology and Metabolism,[3] Ajou University School of Medicine, Suwon, Korea, and Laboratory of Viral and Immunopathogenesis of Diabetes, Julia McFarlane Diabetes Research Centre, Department of Microbiology and Infectious Diseases, Faculty of Medicine, The University of Calgary, Calgary, Alberta, Canada[2]*

Received 10 August 2000/Accepted 27 November 2000

**Soluble mediators such as interleukin-1β, tumor necrosis factor alpha (TNF-α), and inducible nitric oxide synthase (iNOS) produced from activated macrophages play an important role in the destruction of pancreatic β cells in mice infected with a low dose of the D variant of encephalomyocarditis (EMC-D) virus. The tyrosine kinase signaling pathway was shown to be involved in EMC-D virus-induced activation of macrophages. This investigation was initiated to determine whether the Src family of kinases plays a role in the activation of macrophages, subsequently resulting in the destruction of β cells, in mice infected with a low dose of EMC-D virus. We examined the activation of p59/p56$^{Hck}$, p55$^{Fgr}$, and p56/p53$^{Lyn}$ in macrophages from DBA/2 mice infected with the virus. We found that p59/p56$^{Hck}$ showed a marked increase in both autophosphorylation and kinase activity at 48 h after infection, whereas p55$^{Fgr}$ and p56/p53$^{Lyn}$ did not. The p59/p56$^{Hck}$ activity was closely correlated with the tyrosine phosphorylation level of Vav. Treatment of EMC-D virus-infected mice with the Src kinase inhibitor, PP2, resulted in the inhibition of p59/p56$^{Hck}$ activity and almost complete inhibition of the production of TNF-α and iNOS in macrophages and the subsequent prevention of diabetes in mice. On the basis of these observations, we conclude that the Src kinase, p59/p56$^{Hck}$, plays an important role in the activation of macrophages and the subsequent production of TNF-α and nitric oxide, leading to the destruction of pancreatic β cells, which results in the development of diabetes in mice infected with a low dose of EMC-D virus.**

Insulin-dependent diabetes mellitus results from the destruction of insulin-producing pancreatic β cells. Encephalomyocarditis (EMC) virus induces diabetes in genetically susceptible strains of mice by infecting and destroying pancreatic β cells (6, 24, 26). We have established two distinct animal models for EMC virus-induced diabetes. One model consists of mice infected with a high titer of the D variant of EMC (EMC-D) virus ($5 \times 10^5$ PFU/mouse), in which diabetes develops by the destruction of β cells through the replication of the virus in the β cells (25–27). The other animal model consists of mice infected with a low titer of EMC-D virus ($5 \times 10^1$ to $1 \times 10^2$ PFU/mouse), in which diabetes develops by the destruction of β cells primarily through the action of soluble mediators released from macrophages that are infected and activated by the EMC-D virus (1, 2, 12–14). Naturally occurring viral infections in animals and humans are more likely to involve exposure to relatively low numbers of viruses than to the high viral titers used in experimental studies. Thus, the latter model is likely to be more appropriate for the study of virus-induced diabetes in animals and for possible application to humans.

EMC-D virus has been proven to be β-cell trophic in the pancreatic islets. This virus infects β cells but does not infect alpha cells, delta cells, pancreatic polypeptide-producing cells, or exocrine acinar cells. However, EMC-D virus infects and activates macrophages but does not replicate in the macrophages. The infection of mice (DBA/2) with a very low titer of EMC-D virus does not result in sufficient β-cell destruction to cause the development of diabetes prior to the induction of anti-EMC-D viral neutralizing antibodies. However, diabetes does develop later as a result of the recruitment of activated macrophages to the pancreatic islets as scavengers as a consequence of some β-cell damage resulting from the limited replication of the virus in the β cells. The inactivation of macrophages prior to infection with a low dose of EMC-D virus results in the prevention of diabetes, while the activation of macrophages prior to viral infection results in the enhancement of β-cell destruction (1, 2). Soluble mediators, including nitric oxide (NO), interleukin-1 (IL-1), and tumor necrosis factor alpha (TNF-α), secreted from the EMC-D virus-activated macrophages destroy β cells in the islets (12). Thus, in this animal model, macrophages play a major role in the destruction of β cells through their soluble mediators, leading to the development of diabetes.

Recent studies suggest that the tyrosine kinase signaling pathway is involved in macrophage activation and the production of soluble mediators (13). It is known that Src-related tyrosine kinases are involved in signaling pathways in the hematopoietic lineage (23) and lipopolysaccharide (LPS)-induced activation of macrophages (3). This investigation was initiated to determine whether a Src family protein kinase might be involved in EMC-D virus-induced activation of mac-

* Corresponding author. Mailing address: Laboratory of Viral Immunopathogenesis of Diabetes, Julia McFarlane Diabetes Research Centre, Faculty of Medicine, The University of Calgary, 3330 Hospital Dr. NW, Calgary, Alberta, Canada T2N 4N1. Phone: (403) 220-4569. Fax: (403) 270-7526. E-mail: yoon@ucalgary.ca.

rophages, and if so, whether blocking the Src kinase might prevent diabetes induced by a low dose of EMC-D virus. We now report that only hematopoietic cell kinase (p59/p56$^{Hck}$), among the Src family of tyrosine kinases, showed a significant increase in both autophosphorylation and kinase activity in macrophages infected with EMC-D virus. In addition, we found that the administration of PP2, a Src kinase inhibitor, prior to the infection of DBA/2 mice with EMC-D virus decreased the incidence of diabetes by blocking the activation of p59/p56$^{Hck}$ and the subsequent production of inducible nitric oxide synthase (iNOS) and TNF-α by the macrophages. These results suggest that the p59/p56$^{Hck}$ signaling pathway plays a critical role in the activation of macrophages by EMC-D virus infection, leading to the destruction of pancreatic β cells and subsequent development of diabetes in mice.

## MATERIALS AND METHODS

**Virus.** The source and preparation of EMC virus have been described elsewhere (25). The viral titer was determined by plaque assay on L929 cells (25).

**Mice.** DBA/2 mice were obtained from Jackson Laboratory (Bar Harbor, Maine). The animals were housed in an animal facility at the Health Science Centre, University of Calgary, Calgary, Alberta, Canada, and at the Institute for Medical Sciences, Ajou School of Medicine, Suwon, Korea. Male mice were used at 6 to 8 weeks of age.

**Reagents.** Antibodies against Lyn, c-Fgr, Hck, Blk, Yes, and Lck were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). Substrates for Src family kinases, Sam68 and enolase, were purchased from Santa Cruz Biotechnology and Calbiochem, Inc. (La Jolla, Calif.), respectively. Phosphotyrosine-specific antibody and anti-phosphotyrosine agarose conjugate were purchased from Upstate Biochemical Institute (Lake Placid, N.Y.). An inhibitor of the Src family of protein tyrosine kinases, PP2, was purchased from Calbiochem, Inc.

**Measurement of blood glucose.** Blood glucose levels from nonfasting mice were measured with a one-touch Basic glucometer (Lifescan, Burnaby, British Columbia, Canada). The mean blood glucose level of 30 uninfected DBA/2 male mice was 139 ± 29 mg/dl (mean ± standard deviation [SD]). In these experiments, nonfasting animals with blood glucose levels greater than 226 mg/dl (3 SD above the mean) were scored as diabetic.

**Infection of DBA/2 mice with EMC-D virus and macrophage preparation.** DBA/2 mice were injected intraperitoneally (i.p.) with 2 ml of 3% thioglycolate (Difco Laboratories, Detroit, Mich.). After 3 days, the mice were infected with EMC-D virus (100 PFU/mouse, i.p.) and peritoneal macrophages were harvested at 0, 24, 48, and 72 h postinfection. The cells were washed and resuspended in RPMI 1640 medium supplemented with 5% fetal calf serum, 2 mM L-glutamine, 50 U of penicillin per ml, and 50 μM streptomycin per ml for 2 h at 37°C in an incubator with 5% $CO_2$. Nonadherent cells were removed by suction and adherent cells were washed with phosphate-buffered saline (PBS) three times to remove residual nonadherent cells. More than 95% of the adherent cells were macrophages on the basis of morphologic criteria and immunocytochemical staining with anti-Mac-1 antibody.

**Reverse transcription (RT)-PCR.** To minimize the variation among animals and to increase the confidence of the data, 20 mice/time point were sacrificed. Total RNA was extracted from peritoneal macrophages and prepared as described above with RNAzolB (Tel-test, Inc., Friendwood, Tex.). The cDNA was synthesized with 2 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) of deoxynucleotide triphosphate, and 20 U of Moloney murine leukemia virus reverse transcriptase (TaKaRa-Korea Biomedical Inc., Seoul, Korea). PCR was performed with 5 μl of cDNA with pairs of oligonucleotide primers corresponding to the cDNA sequences. The following oligonucleotide sequences were derived from the sequences at GenBank: for β-actin, CATGTTTGAGACCTTCAACACCCC and GCCATCTCCTGCTCGAAGTCTAG; for iNOS, CCCTTCGAAGTTTCTGG CAGCAGC and GGCTGTCAGAGCCTCGTGGCTTTGG; for TNF-α, CTTA GACTTTGCGGACCAGTATAAGGCAAGCA and GGGACAGTGACCTG GACTGT; for IL-1β, GGAATGACCTGTTCTTTGAAGTT and GGCTCCGA GATGAACAACAAAA; for gamma interferon (IFN-γ), AGCTCTGAGACAA TGAACGC and GGACAATCTCTTCCCCACCC; for transforming growth factor β (TGF-β), CCCACTCCCGTGGCTTCTAGTGC and GATGGCGTTG TTGCGGTCCACC; and for IL-10, TGCCTTCAGTCAAGTGAAGAC and TTTCAGTGTTGTGAGCGTGGA. PCR amplification was carried out in 50 μl of the reaction mixture containing 50 pmol of sense and antisense primer, 0.2 mM (each) deoxynucleoside triphosphate, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 0.1% Triton X-100 with denaturation at 94°C for 1 min, annealing at 60°C, and extension at 72°C with a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). The product was run on a 1.8% agarose gel and detected by ethidium bromide staining.

**Immune complex kinase assays of the Src family kinases.** Peritoneal macrophages from at least 40 mice were pooled and cells ($10^7$) were lysed in 1 ml of lysis buffer (10 mM Tris-HCL [pH 7.5], 1% Triton X-100, 10 mM $MgCl_2$, 1 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, 2 μM pepstatin, 0.1% aprotinin) for 20 min on ice. The lysate was clarified by centrifugation (Microfuge 18; Beckman, Fullerton, Calif.) at 13,000 rpm for 15 min and the protein concentration was determined using a Bio-Rad protein assay (Hercules, Calif.). Aliquots of protein (200 μg) were precleared with 20 μl of 10% pansorbin (Calbiochem) for 30 min at 4°C and immunoprecipitated with 1 μg of Lyn-, c-Fgr-, Hck-, c-Src-, or Blk-specific antibody (Santa Cruz Biotechnology, Inc.) for 3 h and incubated with 30 μl of 10% pansorbin (Calbiochem) as a carrier for 30 min on ice. The immunoprecipitated pellets were washed four times in lysis buffer and once with phosphorylation buffer (20 mM HEPES [pH 7.4], 10 mM $MgCl_2$, 10 mM $MnCl_2$, 200 μM sodium vanadate, 0.1% aprotinin). The kinase reaction was performed by the addition of a solution containing 10 μCi of [γ-$^{32}$P]ATP and Sam68 or enolase as a substrate in 0.5× phosphorylation buffer for 10 min at 30°C. Following separation of the denatured samples on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, the phosphorylated proteins were detected by autoradiography. In addition, the extent of phosphorylation was measured by liquid scintillation counting of gel slices of the autophosphorylated proteins and the substrate, Sam68.

**Western blot analysis.** Peritoneal macrophages from at least 10 mice were pooled and the cells ($10^6$) were lysed by adding 100 μl of 2× SDS sample buffer (100 mM Tris-HCl [pH 6.8], 4% SDS, 4% β-mercaptoethanol, 20% glycerol, 0.1% bromophenol blue) and sonicated for 2 s. Samples were boiled for 5 min and centrifuged for 5 min. Proteins were electrophoresed on SDS–12% PAGE and transferred to Immobilon membranes (Millipore Corp., Bedford, Mass.). Immunoblotting was carried out using specific antibodies and detection was performed according to the manufacturer's instructions with an enhanced chemiluminescence detection system (Amersham Life Science Inc., Arlington Heights, Calif.). Some blots were deprobed with 0.2 N NaOH for 10 min and reprobed using other specific antibodies.

**Measurement of virus replication.** The virus concentrations of the pancreatic tissues from EMC-D virus-infected mice were determined by plaque assay using L929 cells, as described previously (25).

**Treatment of EMC virus-infected DBA/2 mice with a Src kinase inhibitor, PP2.** Male DBA/2 mice infected with 100 PFU of EMC-D virus per mouse were injected i.p. with 20 μg of the Src family protein kinase inhibitor PP2 in 100 μl of 10% dimethyl sulfoxide (DMSO)–PBS. Daily administration of PP2 was initiated on the same day as EMC-D viral infection and continued for 9 days. Blood glucose was measured at 2, 4, 6, and 8 days postinfection. RT-PCR analyses of cytokines and iNOS in macrophages were performed at 0, 1, 2, and 3 days postinfection. Histological examination of the pancreata was performed at 8 days postinfection. As a control, vehicle (100 μl of 10% DMSO–PBS) was injected instead of PP2.

**Passive transfer of macrophages.** Peritoneal macrophages were isolated from PP2-treated (20 μg/mouse), EMC-D virus-infected (100 PFU/mouse) mice or 10% DMSO–PBS-treated, EMC-D virus-infected (100 PFU/mouse) mice and treated with a high titer of anti-EMC-D virus antibody (>2,560 neuralizing antibody [NA] titer) to neutralize any residual infectious virus. The macrophages were injected intravenously ($10^7$ cells in PBS/mouse) into recipient mice (6-week-old male DBA/2 mice), which were injected with a subdiabetogenic dose of streptozotocin (50 mg/kg of body weight) on two consecutive days beginning 1 day prior to the passive transfer of macrophages. Blood glucose levels were measured every other day until 12 days after the transfer of macrophages. Uninfected DBA/2 mice and DBA/2 mice injected with streptozotocin (50 mg/kg) on two consecutive days were used as controls.

**Histological examination.** At least eight mice/group were sacrificed at 8 days postinfection and the pancreata were fixed in 10% buffered neutral formalin. Paraffin-embedded sections were stained with hematoxylin and eosin (H and E) and examined. The classifications of "peri-islet infiltration," "mild to moderate insulitis," "severe insulitis," and "atrophied morphology" were used to describe the histological changes of the pancreatic islets. Islets with peri-islet infiltration had infiltrating mononuclear cells around them. The architecture of islets having mild to moderate insulitis was preserved, but 1 to 49% of these islets exhibited

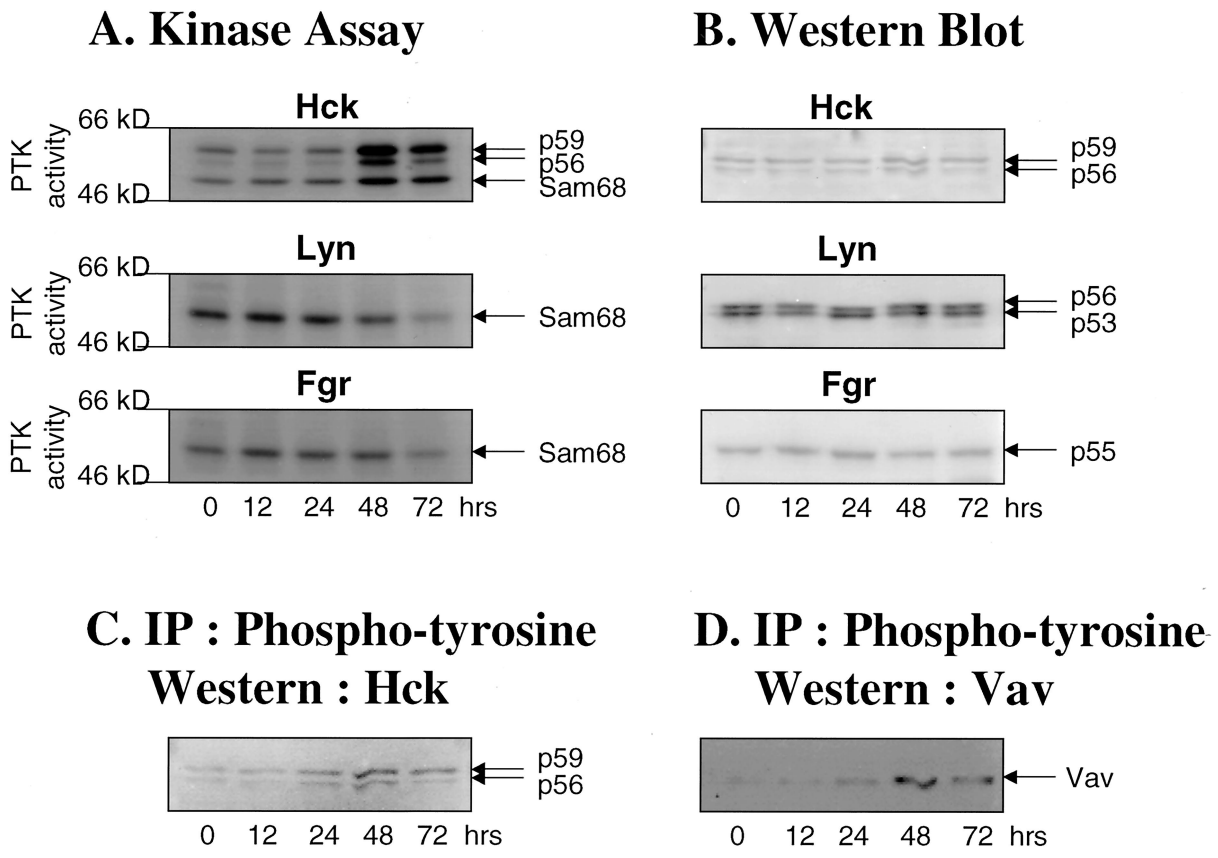


FIG. 1. Kinase activities of immunoprecipitated p59/p56$^{Hck}$, p56/p53$^{Lyn}$, and p55$^{Fgr}$ and their expression in macrophages from mice infected with EMC-D virus. (A) Immune complex kinase assay. Cell extracts were prepared from isolated macrophages after virus infection at the indicated time points. The activated p59/p56$^{Hck}$, p56/p53$^{Lyn}$, or p55$^{Fgr}$ was immunoprecipitated with anti-p59/p56$^{Hck}$, anti-p56/p53$^{Lyn}$, or anti-p55$^{Fgr}$ antibodies, respectively, and an in vitro kinase assay was performed in the presence of recombinant Sam68 as substrate. PTK, protein tyrosine kinase. (B) Immunoblot of whole-cell lysates of macrophages from mice infected with EMC-D virus. Cell extracts were prepared from the isolated macrophages at the indicated times after virus infection, and the expression level of Src family kinases was determined by immunoblotting with specific antibodies. (C) Changes in the tyrosine phosphorylation level of p59/p56$^{Hck}$. Cell extracts of macrophages from mice infected with EMC-D virus at the indicated times after viral infection were immunoprecipitated with agarose-conjugated phosphotyrosine antibody. Immune complexes were separated on SDS–10% PAGE and transferred to nitrocellulose. Western blot analysis was performed using anti-p59/p56$^{Hck}$ antibody. (D) Changes in the tyrosine phosphorylation level of Vav. Western blotting of Vav was performed using the same blot as that prepared for panel C after deprobing. Similar results were obtained from a total of three independent experiments. IP, immunoprecipitation.

lymphocytic infiltration within the islets. Severe insulitis was characterized by morphological damage to the pancreatic β cells, with 50 to 100% of these islets exhibiting lymphocytic infiltration. Islets with atrophied morphology were small and retracted, exhibiting severe β-cell necrosis with or without residual lymphocytic infiltration.

**Immunohistochemical staining.** The pancreata were rapidly removed from the mice and fixed overnight in a solution of 4% paraformaldehyde. Fixed tissues were processed for paraffin embedding. Paraffin sections (4 μm) were deparaffinized and rehydrated. These sections were immunohistochemically stained with polyconal antibody against insulin (DAKO, Carpinteria, Calif.) and detected by the avidin-biotin peroxidase complex method with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, Calif.).

**Statistical analysis.** Statistical analyses were conducted using the Student's *t* test. For statistical analysis of the incidence of diabetes, the Kruskall-Wallis one-way analysis of variance on ranks was used.

## RESULTS

**The selective activation of p59/p56$^{Hck}$, among the Src family of protein tyrosine kinases in macrophages, by infection with EMC-D virus.** Three members of the Src family of protein tyrosine kinases, p59/p56$^{Hck}$, p55$^{Fgr}$, and p56/p53$^{Lyn}$, are strong candidates for being primary signal transducers of LPS responses (3, 4, 10, 11, 21, 22), and their increased kinase activities have been correlated with upregulation of inflammatory cytokines and iNOS (3, 8, 15, 19). To investigate whether these kinases are activated in macrophages infected with EMC-D virus, we examined the kinase activity of p59/p56$^{Hck}$, p55$^{Fgr}$, and p56/p53$^{Lyn}$ in macrophages from DBA/2 mice infected with EMC-D virus at 24, 48, and 72 h after viral infection. To amplify the macrophages for the signaling studies, we injected thioglycolate at 3 days before EMC-D viral infection. We isolated macrophages from EMC-D virus-infected, thioglycolate-treated DBA/2 mice and performed an immune complex kinase assay using Sam68 as a substrate (21). We found that p59/p56$^{Hck}$, among the Src family kinases tested, showed a 7.5-fold increase in autophosphorylation and a 3.1-fold increase in kinase activity at 48 h postinfection (Fig. 1A and B). In contrast, autophosphorylating activity in p56/p53$^{Lyn}$ and p55$^{Fgr}$ was barely detected and significant activation of these kinases was not observed (Fig. 1A and B). The same result was obtained from the immune complex kinase assay using denatured enolase as a substrate (data not shown). When we per-

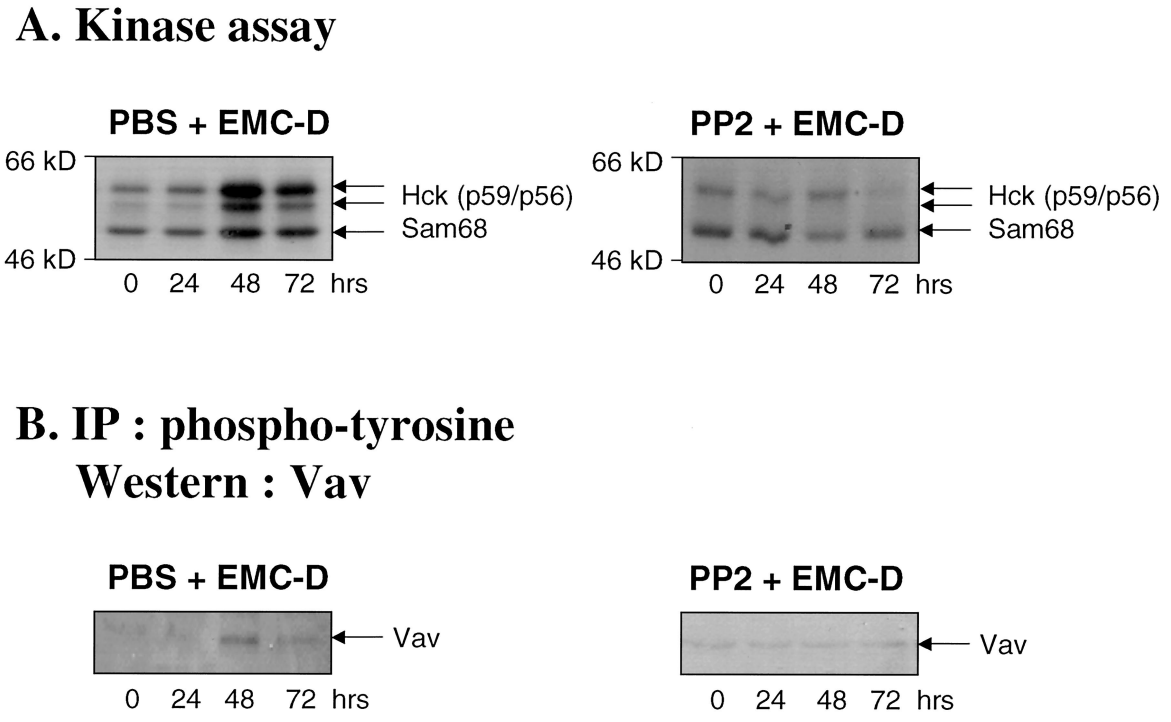


FIG. 2. Blocking of p59/p56$^{Hck}$ and Vav activation in macrophages by treatment with PP2. DBA/2 mice were treated with PP2 (20 μg in 10% DMSO–PBS/mouse) or 10% DMSO–PBS at 1 day prior to EMC-D virus infection and daily thereafter. Cell extracts were prepared from isolated macrophages after infection at the indicated time points. (A) In vitro kinase assays were performed in the presence of recombinant Sam68 as a substrate. (B) The tyrosine phosphorylation level of Vav was determined. Representative data from three independent experiments are shown. IP, immunoprecipitation.

formed Hck immune complex kinase assays using cell extracts from macrophages treated only with thioglycolate (without viral infection) for 0, 1, 2, or 3 days, we found that there was no increase in either Hck autophosphorylation or kinase activity from 0 to 3 days in uninfected, thioglycolate-treated macrophages (data not shown). These results suggest that EMC-D virus selectively activates p59/p56$^{Hck}$, among the Src family of protein tyrosine kinases, in murine macrophages.

To determine whether the activation of p59/p56$^{Hck}$ in macrophages from EMC-D virus-infected mice is correlated with tyrosine phosphorylation, we isolated macrophages from EMC-D virus-infected mice at different times after infection and immunoprecipitated macrophage extracts with antibody against phosphotyrosine. We then performed Western blotting of the precipitated immune complex with anti-Hck antibody. We found that the tyrosine phosphorylation level of p59/p56$^{Hck}$ increased significantly, peaking at 48 h after EMC-D viral infection (about a fourfold increase compared to 0 h postinfection) with the same kinetics as shown in the Hck immune complex kinase assay (Fig. 1C). These results indicate that increased tyrosine phosphorylation of p59/p56$^{Hck}$ correlates with p59/p56$^{Hck}$ activation. It has been suggested that p59/p56$^{Hck}$ may mediate tyrosine phosphorylation of an adaptor protein, Vav, during macrophage activation induced by LPS and IFN-γ (9). To determine whether Vav is activated in macrophages infected by EMC-D virus, we isolated macrophages from EMC-D virus-infected mice, immunoprecipitated the macrophage extracts with anti-phosphotyrosine antibody, and performed Western blotting of the precipitated immune complex with anti-Vav antibody. We found that tyrosine phosphorylation levels of Vav increased significantly (about a sixfold increase compared to 0 h postinfection) at the time of highest p59/p56$^{Hck}$ activity at 48 h postinfection (Fig. 1D), suggesting that p59/p56$^{Hck}$ signaling may be mediated by Vav during EMC-D virus-induced activation of macrophages.

**Inhibition of EMC-D virus-induced p59/p56$^{Hck}$ activation in macrophages by a Src family kinase inhibitor, PP2.** To determine whether p59/p56$^{Hck}$ activity is inhibited by a Src family kinase inhibitor, PP2, we administered PP2 into DBA/2 mice prior to viral infection, isolated peritoneal macrophages at 24, 48, and 72 h after viral infection, and examined p59/p56$^{Hck}$ activity in macrophage extracts by the p59/p56$^{Hck}$ immune complex kinase assay. We found that p59/p56$^{Hck}$ activity significantly decreased at 72 h after PP2 treatment (Fig. 2A). In addition, there was no increase in tyrosine phosphorylation levels of Vav in response to EMC-D virus infection in PP2-treated mice (Fig. 2B).

**Decrease in the expression of inflammatory cytokines and iNOS in macrophages in EMC-D virus-infected mice by treatment with a Src family kinase inhibitor, PP2.** It was previously found that IL-1β, TNF-α, and iNOS produced by activated macrophages made a significant contribution to the destruction of β cells, leading to the development of diabetes in mice infected with a low dose of EMC-D virus (12). To determine whether the treatment of EMC-D virus-infected DBA/2 mice with PP2 affects the induction of inflammatory cytokines and iNOS, we isolated macrophages from PP2-treated, EMC-D virus-infected DBA/2 mice or 10% DMSO–PBS-treated, EMC-D virus-infected DBA/2 mice (control) at various times after virus infection and analyzed the expression of cytokines

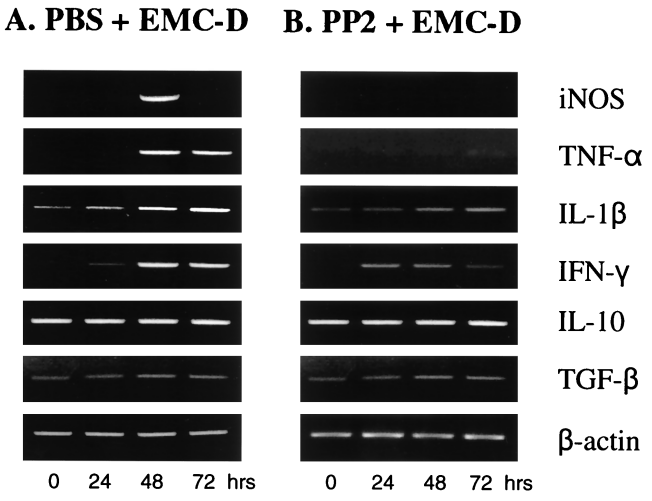


FIG. 3. The effect of PP2 treatment on the expression of macrophage-derived cytokines and iNOS in macrophages of EMC-D virus-infected DBA/2 mice. Macrophages were isolated from 10% DMSO–PBS-treated, EMC-D virus-infected (A) or PP2-treated EMC-D virus-infected (B) DBA/2 mice at 0, 24, 48, and 72 h after viral infection. Total RNA was extracted from the isolated macrophages and the expression of the mRNA of iNOS and cytokines was analyzed by RT-PCR. Similar results were obtained in three separate experiments.

and iNOS by RT-PCR. We found that the expression of IL-1β and IFN-γ continuously increased up to 48 h postinfection in the EMC-D virus-infected control mice. The expression of TNF-α also significantly increased from 48 h postinfection and then was maintained up to 72 h postinfection. The expression of iNOS peaked at 48 h postinfection. However, the expression of IL-10 and TGF-β was not altered (Fig. 3A). When we analyzed the expression of these cytokines and iNOS in the macrophages of PP2-treated, EMC-D virus-infected mice, we found that the induction of iNOS and TNF-α expression was almost completely inhibited. The expression profiles of IL-1β and IFN-γ were decreased, but TGF-β and IL-10 were not significantly altered compared with the vehicle-treated, EMC-D virus-infected control mice (Fig. 3B).

**Prevention of EMC-D virus-induced diabetes in mice by treatment with a Src family kinase inhibitor, PP2.** The Src kinase inhibitor, PP2, was shown to almost completely inhibit the induction of iNOS and TNF-α, which are involved in the destruction of pancreatic β cells. To determine whether PP2 can prevent the development of diabetes in mice infected with a low dose of EMC-D virus, we administered PP2 to DBA/2 mice infected with 100 PFU of EMC-D virus per mouse and examined the incidence of diabetes. We found that treatment of EMC-D virus-infected DBA/2 mice with PP2 significantly decreased the incidence of diabetes. Twenty-eight percent (5 of 18) of the mice treated with PP2 became diabetic by 8 days postinfection, while 74% (14 of 19) of 10% DMSO–PBS-treated mice became diabetic (Fig. 4). Histological examination of the pancreatic islets revealed a significant decrease in mononuclear cell infiltration in PP2-treated mice compared with vehicle-treated control mice. The majority of pancreatic islets (83%) from the EMC-D infected, PP2-treated mice showed peri-insulitis (24%) or mild to moderate insulitis (59%). Only 17% of the islets from the PP2-treated mice showed severe insulitis (12%) or islet atrophy (5%). In contrast, the majority of the islets (84%) from EMC-D virus-infected control mice showed atrophy (55%) or severe insulitis (29%). Only 16% of the examined islets showed mild to moderate insulitis (15%) or peri-insulitis (1%) (Fig. 5A, C, and E and Table 1). When we examined the insulin-containing cells in the islets from PP2-treated or control mice by immunohistochemical staining of pancreatic sections with anti-insulin antibodies, we found that the majority of islet cells in the PP2-treated, EMC-D virus-infected mice were insulin positive (67%), which was less than for the uninfected normal control mice (84%). In contrast, only 20% of the examined islet cells in DMSO-PBS-treated, EMC-D virus-infected mice were insulin positive (Fig. 5B, D and F and Table 2). To determine whether there was any difference in viral replication in the pancreatic islets between PP2-treated, EMC-D virus-infected mice and control 10% DMSO–PBS-treated, EMC-D virus-infected mice, we measured viral titers in the pancreatic tissues at 4 days after infection. We found that there was no significant difference in the viral titer between these two groups ($4.95 \pm 0.16$ $\log_{10}$ PFU/g of tissue in PP2-treated mice; $5.28 \pm 0.21$ $\log_{10}$ PFU/g of tissue in 10% DMSO–PBS-treated mice). This result suggests that the prevention of diabetes by PP2 does not result from the inhibition of EMC-D virus replication in the β cells but from the inhibition of the production of soluble mediators such as iNOS and TNF-α.

**Loss of ability of macrophages from PP2-treated, EMC-D virus-infected mice to transfer diabetes to recipient mice.** To determine whether the EMC-D virus-activated macrophages play a role in the destruction of β cells through the p59/p56$^{Hck}$ signaling pathway, we infected DBA/2 mice with 100 PFU of EMC-D virus and isolated the peritoneal macrophages. We then transferred the macrophages into DBA/2 mice treated with a subdiabetogenic dose (50 mg/kg) of streptozotocin twice

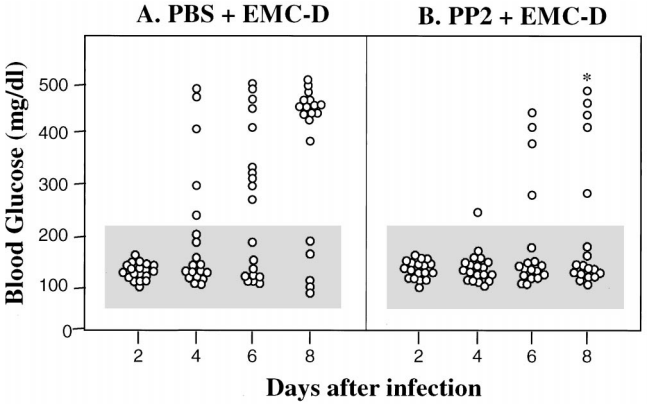


FIG. 4. Effect of PP2 treatment on the development of EMC-D virus-induced diabetes in DBA/2 mice. EMC-D virus-infected mice (100 PFU/mouse) were injected with (A) 10% DMSO–PBS ($n = 19$) or (B) 20 μg of PP2 in 10% DMSO–PBS ($n = 18$) and the development of diabetes was monitored by checking blood glucose levels. Shaded areas represent the mean ± 3 SD from 30 uninfected DBA/2 mice. Any mouse with a nonfasting blood glucose level greater than 226 mg/dl (3 SD greater than the mean of the uninfected controls) was scored as diabetic. Each circle represents an individual animal. *, $P < 0.05$ compared with the incidence of diabetes in 10% DMSO–PBS-treated control mice at 8 days postinfection.

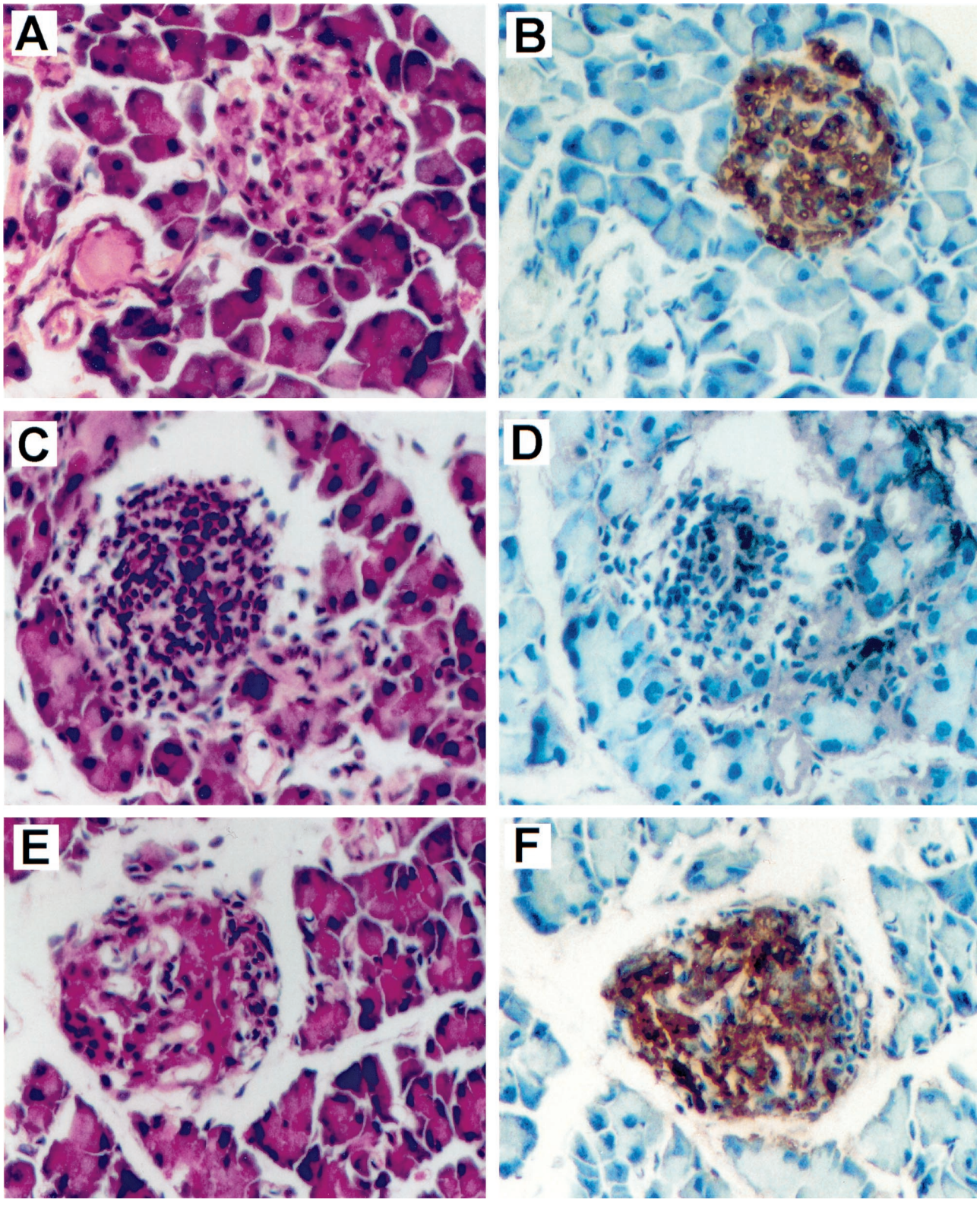


FIG. 5. Histological and immunohistochemical examination of pancreatic islets. H and E staining of pancreatic islets from uninfected mice showing an intact islet (A), and anti-insulin antibody staining of the islet showing insulin-producing β cells throughout the islet (B); H and E staining of an islet from 10% DMSO–PBS-treated, EMC-D virus-infected mice showing severe lymphocytic infiltration and necrosis (C), and anti-insulin antibody staining of the islet showing only a few insulin-producing β cells (D); H and E staining of an islet from PP2-treated, EMC-D virus-infected mice showing mild insulitis, particularly in the periphery (E), and anti-insulin antibody staining of the islet showing insulin-producing β cells in the major portion of the islet, particularly the center (F). Representative pictures from each group are shown (magnification, ×400).

TABLE 1. Histological analysis of the pancreatic islets from PP2-treated, EMC-D virus-infected mice[a]

| Treatment | No. of islets examined[b] | Islet histology (%)[c] | | | |
|---|---|---|---|---|---|
| | | P | M | S | A |
| 10% DMSO–PBS | 220 | 1 | 15 | 29 | 55 |
| PP2 | 185 | 24 | 59 | 12 | 5 |

[a] Mice were infected with 100 PFU of EMC-D virus per mouse and treated daily with 10% DMSO–PBS ($n = 8$) or PP2 ($n = 8$) for 8 days. The mice were sacrificed and histological changes in islets were examined at 8 days postinfection.
[b] Combined data from diabetic and nondiabetic animals (20 to 30 islets/mouse).
[c] P, peri-islet infiltration; M, mild to moderate insulitis; S, severe insulitis; A, atrophied morphology.

on consecutive days in order to induce minor damage sufficient for the recruitment of macrophages and examined the development of diabetes in the recipients. Treatment of mice with a subdiabetogenic dose of streptozotocin did not result in the development of diabetes but did result in the recruitment of macrophages into the pancreatic islets. We found that only 11% (1 of 9) of the recipients of macrophages from PP2-treated mice developed diabetes, whereas 56% (5 of 9) of the recipients of macrophages from 10% DMSO–PBS-treated mice became diabetic (Table 3). These results suggest that the p59/p56$^{Hck}$ signaling pathway plays an important role in the activation of macrophages in mice infected with a low dose of EMC-D virus and the activated macrophages contribute to the destruction of β cells, resulting in the development of diabetes.

## DISCUSSION

The selective infection of pancreatic β cells with EMC-D virus results in an initial recruitment of macrophages into the islets followed by infiltration of other immunocytes, including T cells, natural killer cells, and B cells (1). The activation of macrophages prior to EMC-D viral infection results in a significant increase in the incidence of diabetes, whereas inactivation of macrophages prior to viral infection almost completely prevents the development of diabetes in mice infected with a low dose of EMC-D virus (2). The inhibition of macrophage-produced soluble mediators such as IL-1β, TNF-α, and iNOS results in the prevention of EMC-D virus-induced diabetes, indicating that the destruction of β cells in mice infected with a low dose of EMC-D virus is due to macrophage-derived soluble mediators (12). However, the mechanism by which macrophages are activated and produce the soluble mediators as a result of EMC-D viral infection was unknown.

TABLE 2. Immunohistochemical analysis of the pancreatic islets from PP2-treated, EMC-D virus-infected mice

| Treatment | Total no. of islets examined[a] | Avg. no. of cells per islet[b] | Insulin-positive cells per islet (%) |
|---|---|---|---|
| 10% DMSO–PBS | 80 | 214 ± 37 | 84 |
| 10% DMSO–PBS plus EMC-D | 101 | 78 ± 26 | 20 |
| PP2 plus EMC-D | 75 | 219 ± 40 | 67 |

[a] Pancreatic sections from at least eight mice were analyzed for immunoreactive insulin.
[b] Values represent the means ± SD.

TABLE 3. Loss of ability of macrophages from PP2-treated, EMC-D virus-infected mice to transfer diabetes to recipient mice

| Treatment of macrophage donors[a] | Cumulative incidence of diabetes (%) ($n = 9$) on the following days:[b] | | | | | |
|---|---|---|---|---|---|---|
| | 2 | 4 | 6 | 8 | 10 | 12 |
| 10% DMSO–PBS plus EMC-D virus | 0 | 0 | 1 (11) | 4 (45) | 5 (56) | 5 (56) |
| PP2 plus EMC-D virus | 0 | 0 | 1 (11) | 1 (11) | 1 (11) | 1 (11) |

[a] DBA/2 mice were infected with 100 PFU of EMC-D virus and treated with PP2 (20 μg/mouse in 10% DMSO–PBS) or 10% DMSO–PBS. The peritoneal macrophages ($10^7$ cells/mouse) were isolated and injected intravenously into DBA/2 mice treated with a subdiabetogenic dose of streptozotocin (50 mg/kg on two consecutive days beginning 1 day prior to the transfer of macrophages). None of the DBA/2 mice injected with the subdiabetogenic dose of streptozotocin became diabetic.
[b] The development of diabetes was monitored by checking blood glucose after transfer of macrophages on the indicated day. Any mouse with a nonfasting blood glucose level greater than 226 mg/dl (3 SD of the mean blood glucose level of the untreated control mice) was scored as diabetic.

It was recently found that the tyrosine kinase signaling pathway is involved in the activation of macrophages by EMC-D virus, specifically activation of the mitogen-activated protein kinases, such as extracellular regulated kinase 1/2, p38 mitogen-activated protein kinase, and c-Jun N-terminal kinase. Furthermore, treatment of EMC-D virus-infected mice with a tyrosine kinase inhibitor, AG126, significantly reduced the incidence of diabetes (13). Recent studies have implicated the Src-related tyrosine kinases as critical signaling pathways in the hematopoietic lineages. Src family members mediate relevant functions, such as the induction of NO production by bacterial LPS (15), phagocytosis, cell spreading (5, 17, 19), and FcγI receptor signaling (7). Recently, it was reported that p56$^{Lck}$ is implicated in Coxsackievirus B3-mediated heart disease, demonstrating a novel function of a Src family kinase as an essential host factor involved in viral pathogenicity (16). The hematopoietic cell kinase (p59/p56$^{Hck}$) is a member of the Src family of tyrosine kinases, and p59/p56$^{Hck}$ expression has been reported to correlate with terminal differentiation in both monocytes/macrophages and granulocytes (23). Functional activation of human cultured macrophages with LPS augmented the expression of p59/p56$^{Hck}$ transcripts and p59/p56$^{Hck}$ protein (8, 28). Expression of a constitutively active mutant of p59/p56$^{Hck}$ in macrophages augments TNF-α production, whereas inhibition of endogenous p59/p56$^{Hck}$ expression interferes with LPS-mediated TNF synthesis. Chronic exposure of macrophages to LPS and IFN-γ induces increased synthesis of p59/p56$^{Hck}$ and p59/p56$^{Lyn}$, which correlates with the ability of LPS and IFN-γ to prime macrophages for a respiratory burst (4). Lowell et al. (18) demonstrated that phagocytosis is impaired in p59/p56Hck$^{-/-}$ mutant mice. Moreover, Hck$^{-/-}$ Lyn$^{-/-}$ double-mutant animals have a novel immunodeficiency characterized by an increased susceptibility to infection with *Listeria monocytogenes*, indicating that either Hck or Fgr is required to maintain a normal natural immune response. Taken together, these observations suggest that Hck is an important component of the signal transduction pathways in activated macrophages (8). Thus, we examined whether Src kinases are

involved in the activation of macrophages by EMC-D viral infection. We isolated peritoneal macrophages from mice infected with a low dose of EMC-D virus and analyzed the Src kinase activity. We found that only p59/p56$^{Hck}$ of the Src kinase family members examined demonstrated a clear increase in both kinase and autophosphorylating activity after EMC-D viral infection. In addition, tyrosine phosphorylation levels of Vav, which has been postulated as a mediator of p59/p56$^{Hck}$ signaling (18), significantly increased at the time of the highest p59/p56$^{Hck}$ activity.

Next, we examined whether p59/p56$^{Hck}$ is involved in the production of toxic soluble mediators by macrophages from EMC-D virus-infected mice. We treated EMC-D virus-infected mice with a Src kinase inhibitor, PP2, and isolated macrophages. We first examined whether PP2 could inhibit p59/p56$^{Hck}$ activation in the macrophages and found that p59/p56$^{Hck}$ activity was abrogated by PP2 treatment. In addition, there was no increase in tyrosine phosphorylation levels of Vav in response to EMC-D virus infection in PP2-treated mice. We then examined the expression of inflammatory cytokines and iNOS and found that the induction of TNF-α and iNOS expression was almost completely suppressed in PP2-treated mice compared with vehicle-treated control mice. However, the induction of IL-1β mRNA was not completely suppressed in PP2-treated mice. These results suggest that p59/56$^{Hck}$ may be involved in the EMC-D virus-induced production of TNF-α and iNOS in the macrophages, and that the signaling pathway to produce IL-1β might be different from those of TNF-α and iNOS. Consistent with these results, a recent report demonstrated that the Src family tyrosine kinase inhibitor, PP1, blocks LPS- and IFN-γ-mediated phosphorylation of Hck, resulting in the inhibition of the production of TNF and iNOS in RAW 264.7 murine macrophages (20). However, we cannot exclude the possibility that PP2 may also inhibit the production of some as-yet-unknown protein induced by EMC-D viral infection.

Since PP2 suppresses the EMC-D virus-induced production of TNF-α and iNOS in activated macrophages, which are known to be toxic to β cells, the next question was whether the inhibition of these macrophage-derived soluble mediators by PP2 might contribute to the prevention of the development of diabetes in EMC-D virus-infected mice. When we examined the effect of PP2 on the development of diabetes in DBA/2 mice infected with a low dose of EMC-D virus, we found that the mean blood glucose level and the incidence of diabetes were significantly decreased in PP2-treated mice in a dose-dependent manner compared with control mice. In addition, histological examination revealed that the insulin-producing pancreatic β cells were well preserved in PP2-treated mice compared with untreated controls when they were infected with a low dose of EMC-D virus.

Finally, we determined whether macrophages activated through p59/p56$^{Hck}$ signaling play a critical role in the destruction of pancreatic β cells in mice infected with a low dose of EMC-D virus. We found that macrophages from mice treated with the Src family kinase inhibitor PP2 lose the ability to transfer diabetes to recipient mice, whereas macrophages from 10% DMSO–PBS-treated control mice are able to transfer diabetes. These results indicate that macrophages activated by EMC-D virus infection through the p59/p56$^{Hck}$ signaling pathway contribute to the destruction of pancreatic β cells, resulting in the development of diabetes, although this may not be the sole contributing factor for the destruction of pancreatic β cells by EMC-D viral infection.

On the basis of these observations, we conclude that EMC-D viral infection of macrophages activates a Src tyrosine kinase, p59/p56$^{Hck}$, and induces the production of β-cell-toxic soluble mediators such as TNF-α and iNOS, resulting in the destruction of pancreatic β cells. Blocking this pathway, by treatment with the Src kinase inhibitor PP2, results in the suppression of the production of TNF-α and iNOS and the subsequent prevention of EMC-D virus-induced diabetes in mice.

## ACKNOWLEDGMENTS

This work was supported by grants from the Korea Science and Engineering Foundation (97-0403-0101-3) to K.S.C. and J.W.Y. and from the Canadian Institutes of Health Research (MOP-13224) and the Canadian Diabetes Association to J.W.Y. J.W.Y. is a Heritage Medical Scientist awardee of the Alberta Heritage Foundation for Medical Research.

We are grateful to Yup Kang for technical assistance.

## REFERENCES

1. **Baek, H. S., and J. W. Yoon.** 1991. Direct involvement of macrophages in destruction of β-cells leading to development of diabetes in virus-infected mice. Diabetes **40:**1586–1597.
2. **Baek, H. S., and J. W. Yoon.** 1990. Role of macrophages in the pathogenesis of encephalomyocarditis virus-induced diabetes in mice. J. Virol. **64:**5708–5715.
3. **Beaty, C. D., T. L. Franklin, Y. Uehara, and C. B. Wilson.** 1994. Lipopolysaccharide-induced cytokine production in human monocytes: role of tyrosine phosphorylation in transmembrane signal transduction. Eur. J. Immunol. **24:**1278–1284.
4. **Boulet, I., S. Ralph, E. Stanley, P. Lock, A. R. Dunn, S. P. Green, and W. A. Phillips.** 1992. Lipopolysaccharide- and interferon-gamma-induced expression of hck and lyn tyrosine kinases in murine bone marrow-derived macrophages. Oncogene **7:**703–710.
5. **Chiaradonna, F., L. Fontana, C. Iavarone, M. V. Carriero, G. Scholz, M. V. Barone, and M. P. Stoppelli.** 1999. Urokinase receptor-dependent and -independent p56/59(hck) activation state is a molecular switch between myelomonocytic cell motility and adherence. EMBO J. **18:**3013–3023.
6. **Craighead, J. E., and M. F. McLane.** 1968. Diabetes mellitus: induction in mice by encephalomyocarditis virus. Science **162:**913–914.
7. **Durden, D. L., H. M. Kim, B. Calore, and Y. Liu.** 1995. The Fc gamma RI receptor signals through the activation of hck and MAP kinase. J. Immunol. **154:**4039–4047.
8. **English, B. K., J. N. Ihle, A. Myracle, and T. Yi.** 1993. Hck tyrosine kinase activity modulates tumor necrosis factor production by murine macrophages. J. Exp. Med. **178:**1017–1022.
9. **English, B. K., S. L. Orlicek, Z. Mei, and E. A. Meals.** 1997. Bacterial LPS and IFN-gamma trigger the tyrosine phosphorylation of vav in macrophages: evidence for involvement of the hck tyrosine kinase. J. Leukoc. Biol. **62:**859–864.
10. **Henricson, B. E., J. M. Carboni, A. L. Burkhardt, and S. N. Vogel.** 1995. LPS and Taxol activate Lyn kinase autophosphorylation in Lps(n), but not in Lps(d), macrophages. Mol. Med. **1:**428–435.
11. **Herrera-Velit, P., and N. E. Reiner.** 1996. Bacterial lipopolysaccharide induces the association and coordinate activation of p53/56lyn and phosphatidylinositol 3-kinase in human monocytes. J. Immunol. **156:**1157–1165.
12. **Hirasawa, K., H. S. Jun, K. Maeda, Y. Kawaguchi, S. Itagaki, T. Mikami, H. S. Baek, K. Doi, and J. W. Yoon.** 1997. Possible role of macrophage-derived soluble mediators in the pathogenesis of encephalomyocarditis virus-induced diabetes in mice. J. Virol. **71:**4024–4031.
13. **Hirasawa, K., H. S. Jun, H. S. Han, M. L. Zhang, M. D. Hollenberg, and J. W. Yoon.** 1999. Prevention of encephalomyocarditis virus-induced diabetes in mice by inhibition of the tyrosine kinase signaling pathway and subsequent suppression of nitric oxide production in macrophages. J. Virol. **73:**8541–8548.
14. **Hirasawa, K., S. Tsutsui, M. Takeda, M. Mizutani, S. Itagaki, and K. Doi.** 1996. Depletion of Macl-positive macrophages protects DBA/2 mice from encephalomyocarditis virus-induced myocarditis and diabetes. J. Gen. Virol. **77:**737–741.
15. **Kuo, M. L., Y. P. Chau, J. H. Wang, and P. J. Lin.** 1997. The role of Src kinase in the potentiation by ethanol of cytokine- and endotoxin-mediated nitric oxide synthase expression in rat hepatocytes. Mol. Pharmacol. **52:**535–541.

16. **Liu, P., K. Aitken, Y. Y. Kong, M. A. Opavsky, T. Martino, F. Dawood, W. H. Wen, I. Kozieradzki, K. Bachmaier, D. Straus, T. W. Mak, and J. M. Penninger.** 2000. The tyrosine kinase p56lck is essential in coxsackievirus B3-mediated heart disease. Nat. Med. **6:**429–434.
17. **Lowell, C. A., L. Fumagalli, and G. Berton.** 1996. Deficiency of Src family kinases p59/61hck and p58c-fgr results in defective adhesion-dependent neutrophil functions. J. Cell Biol. **133:**895–910.
18. **Lowell, C. A., P. Soriano, and H. E. Varmus.** 1994. Functional overlap in the src gene family: inactivation of hck and fgr impairs natural immunity. Genes Dev. **8:**387–398.
19. **Meng, F., and C. A. Lowell.** 1998. A β1 integrin signaling pathway involving Src-family kinases, Cb1 and PI-3 kinase is required for macrophage spreading and migration. EMBO J. **17:**4391–4403.
20. **Orlicek, S. L., J. H. Hanke, and B. K. English.** 1999. The src family-selective tyrosine kinase inhibitor PP1 blocks LPS and IFN-gamma-mediated TNF and iNOS production in murine macrophages. Shock **12:**350–354.
21. **Pillay, I., H. Nakano, and S. V. Sharma.** 1996. Radicicol inhibits tyrosine phosphorylation of the mitotic Src substrate Sam68 and subsequent exit from mitosis of Src-transformed cells. Cell Growth Differ. **7:**1487–1499.
22. **Stefanova, I., M. L. Corcoran, E. M. Horak, L. M. Wahl, J. B. Bolen, and I. D. Horak.** 1993. Lipopolysaccharide induces activation of CD14-associated protein tyrosine kinase p53/56lyn. J. Biol. Chem. **268:**20725–20728.
23. **Willman, C. L., C. C. Stewart, T. L. Longacre, D. R. Head, R. Habbersett, S. F. Ziegler, and R. M. Perlmutter.** 1991. Expression of the c-fgr and hck protein-tyrosine kinases in acute myeloid leukemic blasts is associated with early commitment and differentiation events in the monocytic and granulocytic lineages. Blood **77:**726–734.
24. **Yoon, J. W., and H. S. Jun.** 2000. Role of viruses in the pathogenesis of type I diabetes mellitus, p. 419–430. *In* D. LeRoith and S. I. Taylor (ed.), Diabetes mellitus: a fundamental and clinical text. Lippincott, Williams and Wilkins, Philadelphia, Pa.
25. **Yoon, J. W., M. A. Lesniak, R. Fussganger, and A. L. Notkins.** 1976. Genetic differences in susceptibility of pancreatic β-cells to virus-induced diabetes mellitus. Nature **264:**178–180.
26. **Yoon, J. W., P. R. McClintock, T. Onodera, and A. L. Notkins.** 1980. Virus-induced diabetes mellitus. XVIII. Inhibition by a nondiabetogenic variant of encephalomyocarditis virus. J. Exp. Med. **152:**878–892.
27. **Yoon, J. W., M. M. Rodrigues, C. Currier, and A. L. Notkins.** 1982. Long-term complications of virus-induced diabetes in mice. Nature **296:**566–569.
28. **Ziegler, S. F., C. B. Wilson, and R. M. Perlmutter.** 1988. Augmented expression of a myeloid-specific protein tyrosine kinase gene (hck) after macrophage activation. J. Exp. Med. **168:**1801–1810.