Virally Induced Inflammation Triggers Fratricide of Fas-Ligand–Expressing β-Cells

Urs Christen,[1] Rima Darwiche,[2] Helen E. Thomas,[2] Tom Wolfe,[1] Evelyn Rodrigo,[1] Alexander Chervonsky,[3] Richard A. Flavell,[3] and Matthias G. von Herrath[1]

Tissue-specific expression of Fas-ligand (Fas-L) can provide immune privilege by inducing apoptosis of "invading" lymphocytes expressing Fas. However, accelerated diabetes has been reported in transgenic mice expressing Fas-L in islets (RIP-Fas-L) as a result of Fas-dependent fratricide of β-cells after transfer of diabetogenic clones. Here we studied whether Fas-L could protect islets from autoaggressive CD8 lymphocytes in a transgenic model of virally induced diabetes (RIP-LCMV-NP transgenic mice), in which the autoaggressive response is directed to a viral nucleoprotein (NP) expressed as a transgene in β-cells. Indeed, disease incidence after viral (lymphocytic choriomeningitis virus [LCMV]) infection was reduced by ~30%, which was associated with a decrease of autoaggressive CD8 NP-specific lymphocytes in islets and pancreatic draining lymph nodes. However, surprisingly, a high degree (50%) of diabetes was seen in mice that expressed only Fas-L but not the viral transgene (NP) in β-cells after infection with LCMV. This was due to induction of Fas on β-cells after LCMV infection of the pancreas, resulting in Fas/Fas-L–mediated fratricide. Thus, although Fas-L can lend some immune privilege to islet cells, local virus-induced inflammation will induce Fas on β-cells, leading to their mutual destruction if Fas-L is present. Expression of Fas-L therefore might not be protective in situations in which viral inflammation can be expected, resulting in Fas induction on the targeted cell itself. *Diabetes* 53:591–596, 2004

Expression of Fas-ligand (Fas-L) on the surface of cells has been shown to provide immune-privilege by inducing death of lymphocytes that express Fas (CD95). For example, expression of Fas-L is increased in the eye, and, consequently, local immunity is suppressed (1–3). It is not clear whether a similar protective mechanism is operative in other sites, for example, the brain. Therapeutically, this concept has elicited interest, because one could envision preventing immune-mediated destruction of transplanted cells or organs by expression of Fas-L (4–6). However, there has been only limited success using this strategy (4,5,7–9). Some insight into the potential problems with this approach was provided by a recent study in which Fas-L was expressed under the rat insulin promoter in β-cells (4,10). Unexpectedly, such mice were not protected from spontaneous diabetes and several NOD congenic lines even exhibited accelerated disease. In addition, diabetogenic T-cell clones were not eliminated by Fas-mediated killing but induced more rapid diabetes in RIP-Fas-L transgenics. The reason was that arrival of T-cells in the islets would induce upregulation of Fas on β-cells, which resulted in their ability to kill each other ("fratricide"). Thus, overexpression of Fas-L bears the inherent danger of locally increasing the risk of Fas-mediated death of any cell that expresses Fas. This can lead to undesirable demise of useful cells, among them possibly also regulatory lymphocytes.

For the pathogenesis of type 1 diabetes, the role of Fas/Fas-L interactions in β-cell destruction is controversial (11–15). It has been observed that Fas and Fas-L are upregulated on human islets (14), in particular after in vitro manipulation using cytokines (16) or nitric oxide (17). Furthermore, on the lymphocyte side, there is some evidence for altered apoptosis in patients with type 1 diabetes (18), which supports the therapeutic concept of inducing immune privilege by killing invading T-cells through apoptosis using Fas/Fas-L interactions (6).

We sought to evaluate two outstanding issues with this present investigation. First, we wanted to study the in vivo effect of RIP-expressed Fas-L on a defined population of autoaggressive "driver" CD8 lymphocytes. Second, we wished to test whether virally induced local inflammation of the pancreas would be capable of upregulating Fas on β-cells and in this way lead to their mutual demise in RIP-Fas-L transgenics. This allowed us to judge the effect of inflammation on cells or organs engineered to express Fas-L and capable of possibly expressing Fas as well, which is of clinical relevance. We chose to use the well-established RIP-LCMV model for virally triggered type 1 diabetes, because it was ideally suited to address these questions. These mice express the nucleoprotein (NP) of lymphocytic choriomeningitis virus (LCMV) in β-cells as an islet-specific autoantigen to which the immune response can be tracked easily (19–21). Diabetes usually develops only after infection with LCMV that activates NP-specific autoaggressive CD4 and CD8 lymphocytes,

From the [1]Division of Developmental Immunology, La Jolla Institute for Allergy and Immunology, San Diego, California; [2]St. Vincent's Institute, Fitzroy, Victoria, Australia; and the [3]Section of Immunobiology, Yale University School of Medicine, New Haven, Connecticut.

Address correspondence and reprint requests to Matthias G. von Herrath, MD, Division of Developmental Immunology, DI-3, La Jolla Institute for Allergy and Immunology, 10355 Science Center Dr., San Diego, CA 92121. E-mail: matthias@liai.org.

Received for publication 30 Jul 2003 and accepted in revised form 26 November 2003.

CTL, cytotoxic T-cell; Fas-L, Fas-ligand; IFN-γ, γ-interferon; LCMV, lymphocytic choriomeningitis virus; mAb, monoclonal antibody; NP, nucleoprotein; RPA, RNase protection assay.

© 2004 by the American Diabetes Association.

which both are required for disease (19). Indeed, cytotoxic killing of β-cells is a major requirement for diabetes development along with local inflammation of the pancreas and islets. As early as 2 days after LCMV infection, "preconditioning" of the pancreas is occurring, reflected by upregulation of major histocompatibility complex class II molecules on activated antigen-presenting cells (22). This LCMV-induced inflammation is transient (day 2 through 6 postinfection) and is alone insufficient to cause diabetes in nontransgenic mice (23,24). However, in RIP-LCMV mice this activation of APCs and secretion of interferon and other inflammatory molecules is important to "entice" autoaggressive (LCMV-NP specific) lymphocytes to the pancreas and islets, where they will continue to attack β-cells that express the NP antigen until all of them have been destroyed.

For the present study, RIP-LCMV mice ($L^dK^dIA^d$) were crossed to RIP-Fas-L transgenic mice ($D^bK^dIA^{g7}$). We present two interesting observations that clearly illustrate the dual and opposing mechanisms that local Fas-L expression can elicit. First, virally induced disease in RIP-NP mice is significantly delayed by Fas-L expression in β-cells mediated by reduction of autoaggressive CD8 cells locally in islets and pancreatic draining lymph nodes. However, LCMV infection can induce diabetes in Fas-L expressor mice in complete absence of the LCMV-NP transgene. This occurs in conjunction with Fas upregulation on β-cells that enables Fas-L transgenic β-cells to kill their neighbors/brothers (fratricide).

RESEARCH DESIGN AND METHODS

Mice breeding scheme and origin. Generation of H-2^b RIP-LCMV-NP transgenic mice used for this study has been described previously (19). RIP-Fas-L mice expressing Fas-L under the control of the rat insulin promoter had been generated previously (4). NOD ($D^bK^dIA^{g7}$) RIP-Fas-L mice were intercrossed with the H-2^d ($D^bK^dIA^d$) RIP-LCMV-NP line, resulting in double-transgenic mice. These mice were used for all studies here as littermates.

Mice genotyping. The presence of transgenic RIP-LCMV-NP or Fas-L sequences was determined by performing two independent standard PCR reactions with genomic DNA obtained from mouse tails as described (4,19).

Virus. Virus used was LCMV strain Armstrong (arm), clone 53b. LCMV was plaque purified three times on Vero cells, and stocks were prepared by a single passage on BHK-21 cells. Mice were infected with a single intraperitoneal dose of 10^5 pfu LCMV-arm unless indicated otherwise (20).

Viral titers. LCMV viral titers of organ homogenates were determined by infection of Vero cells as described elsewhere (18). Briefly, organ homogenates were diluted serially and cultured with Vero cells for 5 days. Viral titers were determined from the number of counted plaques (20).

Blood glucose measurements. Blood samples were obtained from the retro-orbital plexus, and plasma glucose concentration was determined using Accucheck III (Roche, Indianapolis, IN). Mice with blood glucose values >300 mg/dl were considered diabetic (24).

Islet cell enrichment. Islets were isolated as previously described (25). Briefly, the pancreas was removed, cut into little pieces, and digested with collagenase P (Roche). Islets were purified on histopaque-1077 density gradients (Sigma, St. Louis, MO). The obtained islet-enriched fraction and the remaining portion of the pancreas containing mainly acinar cells were either immediately homogenized in tri-reagent (Molecular Research Center) for subsequent isolation of total RNA or islets were further digested with trypsin (Sigma) to obtain single β-cell suspensions (26).

RNase protection assay. Total RNA was isolated either from whole pancreas homogenates or from islet or acinar cell–enriched fractions using tri-reagent (Molecular Research Center). RNA was extracted with chloroform followed by isopropanol precipitation and washing with ethanol. Twenty micrograms of total pancreatic RNA was used for hybridization with a ^{32}P-UTP–labeled multitemplate set containing specific probes for Fas, Fas-L, tumor necrosis factor-α, γ-interferon (IFN-γ), and lymphotoxin-β provided by a commercial kit (Riboquant, mCK-3b and mAPO-3; Pharmingen, La Jolla, CA). The RNase protection assay was carried out according to the manufacturer's guidelines. The resulting analytical acrylamide gel was scanned using a Storm-860 phospho-imaging system (Molecular Dynamics), and the intensity of bands corresponding to protected mRNAs was quantified using the Imagequant image analysis software (Molecular Dynamics) using L32 as a reference gene (23).

Immunohistochemistry. Organs were harvested at week 6 after LCMV infection unless indicated otherwise, immersed in Tissue-Tek OCT (Bayer, Elkhart, IN), and quick frozen on dry ice. Six- to 10-μm tissue sections were cut using a cryomicrotome and sialin-coated "superfrost plus" slides (Fisher Scientific, Pittsburgh, PA). Sections were fixed with 90% ethanol at −20°C, and after washing in PBS an avidin-biotin blocking step was included (Vector Laboratories, Burlingame, CA). Primary and biotinylated secondary antibodies (Vector Laboratories) were reacted with the sections for 30 min each, and color reaction was obtained by sequential incubation with avidin-peroxidase conjugate (Vector Laboratories) and diaminobenzidine-hydrogen peroxide. Primary antibodies were rat anti-mouse CD8a (ly2), rat anti-mouse CD8b (ly3), and anti–LCMV-NP Alexa 1.1.3 antibody (19,27).

Cytotoxicity assays. LCMV-specific cytotoxic T-cell (CTL) activity in spleen was analyzed in a 5-h in vitro ^{51}Cr-release assay (19). All samples were run in triplicate. Primary CTL activity was tested by harvesting spleens at day 7 after intraperitoneal infection with 10^5 pfu LCMV-arm. Splenocytes were co-incubated with major histocompatibility complex–matched (Balb/C17 H-2^d) and mismatched (MC57 H-2^b) target cells that had been loaded with ^{51}Cr. Target cells were either LCMV-infected, uninfected but coated with the immune dominant LCMV major histocompatibility complex class I peptide NP_{118} (RPQASGVYM), or uninfected and uncoated. For determination of secondary CTL activity, spleens were harvested 6 weeks after LCMV infection and splenocytes were cultured for 8 days on LCMV-infected, irradiated peritoneal exudate cells before testing CTL activity in a ^{51}Cr release assay. For precursor frequency analysis, spleen cells were serially diluted and cultured in 96-well flat bottom plates in the presence of T-cell growth factor (primarily containing interleukin-2), irradiated LCMV-infected peritoneal exudate cells and spleen feeder cells. After 8–10 days, cultures were assayed for CTL activity on LCMV-infected and uninfected target cells. Precursor frequencies were calculated as described (28).

Flow cytometry. Spleen and pancreatic draining lymph nodes were harvested at week 6 after LCMV infection. Single-cell suspensions were stimulated with 100 μ/ml interleukin-2 and 2 μg/ml Brefeldin A (Sigma) for 5–16 h at 37°C. Cells were stained for cell surface markers using monoclonal antibodies (mAbs) against CD8 and CD4, permeabilized and fixed with paraformaldehyde/saponin, and stained for intracellular cytokines using fluorescein isothiocyanate–conjugated anti-mouse tumor necrosis factor-α mAb and PE-conjugated anti-mouse IFN-γ mAb (Pharmingen). Cells were acquired and analyzed on a FACSort or FACS-Calibur flow cytometer (Becton Dickinson) using cell quest software (Becton Dickinson) (29).

RESULTS

Expression of Fas-L by β-cells leads to elimination of autoaggressive CD8 lymphocytes and lower diabetes incidence in RIP-LCMV mice. Littermates from F1 mating of RIP-LCMV-NP (H-2d) × RIP-Fas-L (NOD) transgenic were infected with LCMV. As shown in Fig. 1, LCMV infection resulted in 80% diabetes in RIP-NP^+ Fas-L^- controls within 2–8 weeks. In contrast, type 1 diabetes incidence was significantly reduced in littermates that expressed Fas-L in addition to NP antigen in β-cells. Thus, expression of Fas-L has a clear protective effect in the RIP-LCMV diabetes model. Importantly, systemic numbers of cytotoxic CD8 cells were equivalent when comparing Fas-L^+ versus Fas-L^- NP expressors (Table 1). Comparable numbers of CTL precursors and primary killing activity was detected in spleens. In contrast, locally in the pancreatic draining lymph node, we detected a significant reduction of NP pCTL (Table 1) and, accordingly, a lesser degree of CD8 infiltration in Fas-L^+NP^+ protected compared with Fas-L-NP^+ diabetic mice (Fig. 2*A* versus *B*). This reduction of NP CTLs was not only site specific but also antigen specific, because CD8 lymphocytes producing IFN-γ in response to LCMV NP but not glycoprotein peptides were reduced (Table 1). Thus, local expression of Fas-L by

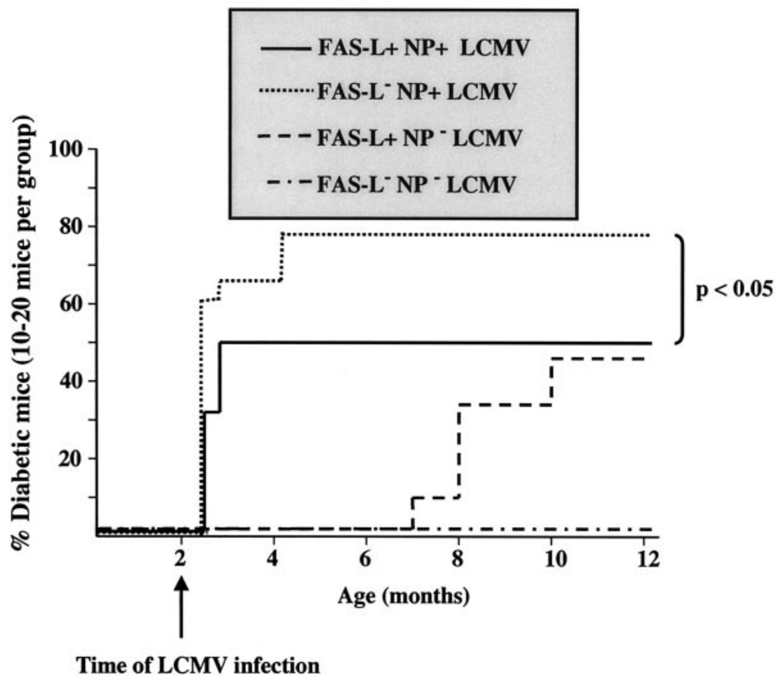

FIG. 1. Incidence of diabetes in RIP-Fas-L × RIP-LCMV-NP lines after LCMV infection. Ten to 20 mice per group were infected with 1 × 10^5 pfu LCMV intraperitoneally. All experimental mice are littermates from F_1 mating of RIP-LCMV-NP (H-2^d) × RIP-Fas-L (NOD) transgenics. Spontaneous diabetes (without LCMV infection) did consequently not occur in any of the offspring. Blood glucose was assessed once every 2 weeks, and mice with values >300 mg/dl were considered diabetic.

TABLE 1
Enumeration of autoaggressive (LCMV-NP–specific) CD8 lymphocytes in RIP-Fas-L × RIP-LCMV-NP transgenic mice after LCMV infection

	Fas-L$^-$/NP$^+$	Fas-L$^+$/NP$^+$
Spleen		
CTL, day 7		
LCMV	43 ± 7	39 ± 11
NP396	12 ± 2	11 ± 1
pCTL, day 60		
NP (%)	1/5,211 ± 18	1/4,850 ± 15
PDLN		
ICCS (IFN-γ), day 45		
GP33	1.7 ± 0.2	2.4 ± 0.4
NP396	1.12 ± 0.5*	0.19 ± 0.11*
pCTL, day 60		
NP	1/6,430*	1/15,000*

Data are means ± SE. Groups of three mice were infected with 1 × 10^5 pfu LCMV intraperitoneally, and CTL activities were determined at the times indicated. Lytic CD8 activities were determined from splenocytes on day 7 after LCMV infection by conventional Cr51 release assays using MC57 (H-2^b) target cells coated with the immune dominant LCMV NP peptide (NP396: FQPQNGQFI) or infected with LCMV 48 h before at a multiplicity of infection 1:1. Percentage of Cr51 release after a standard 5-h assay is shown ± SE. CTL precursors were assessed by limiting dilution as explained in detail in our previous publications (24). For detecting NP-specific precursors in limiting dilution assays (pCTL), target cells (MC57) were infected with vaccinia virus recombinants expressing LCMV-NP. On day 45 or 60 after LCMV infection pancreatic draining lymph nodes were harvested as described previously. Numbers of IFN-γ–producing CD8 lymphocytes specific for H-$2D^b$ restricted GP33 or NP396 peptides were assessed by intracellular cytokine staining (ICCS) as described by us previously (30). Lytic precursor CTLs were determined as described (29). *Significant differences are indicated ($P < 0.05$). Note that only in PDLN from Fas-L–expressing NP$^+$ mice CTLs directed to LCMV-NP are significantly reduced, indicating that the Fas-L–mediated effect on autoaggressive CD8 lymphocytes is antigen specific and locally restricted. PDLN, pancreatic draining lymph nodes.

β-cells can exert some protective capacity by eliminating autoaggressive CD8 lymphocytes in an islet antigen-specific manner. The antigen specificity of the process argues for the fact that interaction of Fas-expressing LCMV-NP–specific CD8 lymphocytes directly with Fas-L–expressing NP$^+$ β-cells leads to their demise.

Viral (LCMV) infection of the pancreas induces Fas on β-cells, leading to fratricide by Fas-L–expressing transgenic islet cells. To our surprise, we observed almost 50% diabetes development in Fas-L$^+$, NP-nonexpressing littermates 6–10 months after LCMV infection (Fig. 1). This was not a spontaneous disease comparable to that found in the original Fas-L$^+$ NOD lines, because the mice under observation were intercrosses between the original NOD and nondiabetes-prone Balb/c mice, and spontaneous disease without LCMV infection was never seen in these mice (data not shown). It is interesting that the diabetes in the Fas-L$^+$ NP$^-$ mice was not associated with marked CD8 (Fig. 2*C*) and other lymphocytic infiltration (not shown) as compared with more extensive CD8 infiltrates in NP$^+$ littermates (Fig. 2*A* and *B*) or in LCMV-infected RIP-NP Balb/c mice (Fig. 2*D*). This observation indicated that β-cell fratricide had occurred as a consequence of virally induced inflammation of the pancreas, because LCMV infects the pancreas and the islets directly as shown by immunohistochemical stains of pancreas sections with anti-NP antibody (clone 1.1.3.; Fig. 2*E*) (19,27). Because earlier studies had demonstrated expression of Fas by β-cells after transfer of diabetogenic clones, we hypothesized that LCMV-induced inflammation of the pancreas would lead to Fas induction by β-cells.

Indeed, we could detect Fas expression on both RNA and protein level by RNase protection assay (RPA) and flow cytometry of isolated islets, respectively. First, Fig. 3*A* shows that Fas gene expression, as detected by RPA, is seen on islet cells early after LCMV infection (day 4) immediately after viral antigen is first expressed (day 2 after infection) and interferons, chemokines, and other

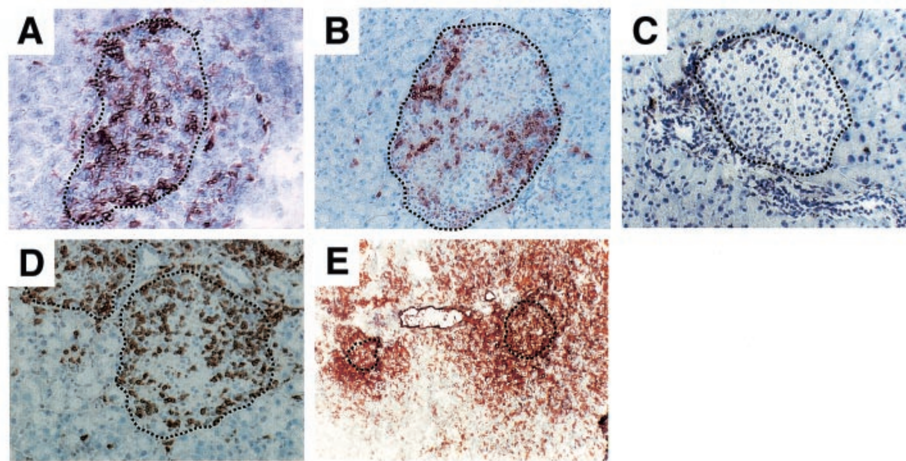

FIG. 2. Histologic findings in RIP-Fas-L × RIP-LCMV-NP transgenic mice after LCMV infection. Representative sections are shown in each panel; at least 10 islets and three mice for each group or time point were evaluated. Immunohistochemistry was performed as described by us previously. The positions of the individual pancreatic islets of Langerhans are indicated by dotted lines. *A*: CD8 lymphocytes in an islet of a RIP-NP$^+$ (H-2^d) × Fas-L$^-$ (NOD) F$_1$ mouse 2 weeks after LCMV infection, diabetic. *B*: CD8 lymphocytes in an islet of a RIP-NP$^+$ (H-2^d) × Fas-L$^+$ (NOD) F$_1$ mouse 2 weeks after LCMV infection, no diabetes. *C*: Lack of lymphocytic infiltration into islets of a RIP-NP$^-$ (H-2^d) × Fas-L$^+$ (NOD) F$_1$ littermate 5 days after LCMV infection, diabetic. *D*: Strong CD8 lymphocyte infiltration in an islet of a "regular" RIP-NP$^+$ (H-2^d) transgenic mouse at week 2 after infection with LCMV, diabetic. *E*: LCMV infection of pancreas and islets at day 2 after inoculation of 10^5 pfu LCMV intraperitoneally. LCMV-NP expression was detected by the anti-NP antibody (clone 1.1.3).

inflammatory mediators are induced. Because Fas upregulation is induced by IFN-γ and can be blocked by neutralizing antibodies (30), we next treated LCMV-infected mice with neutralizing antibodies against IFN-γ and analyzed Fas gene expression by RPA. As expected, Fas gene expression at day 4 after LCMV infection was significantly lower in the pancreatic draining lymph node of mice that received four doses of anti–IFN-γ mAb at days 0, 1, 2, and 3 after infection than in mice that received an isotype-matched control antibody (Fig. 3*B*). However, no change in Fas gene expression was observed in the pancreas (Fig. 3*B*). Second, for detection of Fas expression on the β-cell surface, whole islets obtained by collagenase P digestion of pancreata were further treated with trypsin to obtain a single-cell suspension. Islet cells were stained for Fas surface expression by flow cytometry, and viable β-cells were identified through their high autofluorescence detectable in the FL1 channel (Fig. 4*A*). Increased Fas expression was first noted at day 5 after LCMV infection, and it was >10-fold higher than on β-cells isolated from uninfected mice at day 7 after infection (Fig. 4*B*).

DISCUSSION

We demonstrate in this study a dual effect of Fas-L expression on β-cells. On one hand, Fas-L provides immune privilege by eliminating autoaggressive CD8 lymphocytes. This finding would encourage the use of Fas-L to protect β-cells and other cell types/organs from autoimmune attacks and fits well with the known role of Fas-mediated apoptosis in CD4 populations. On the other hand, viral infection of the pancreas but not the islets can

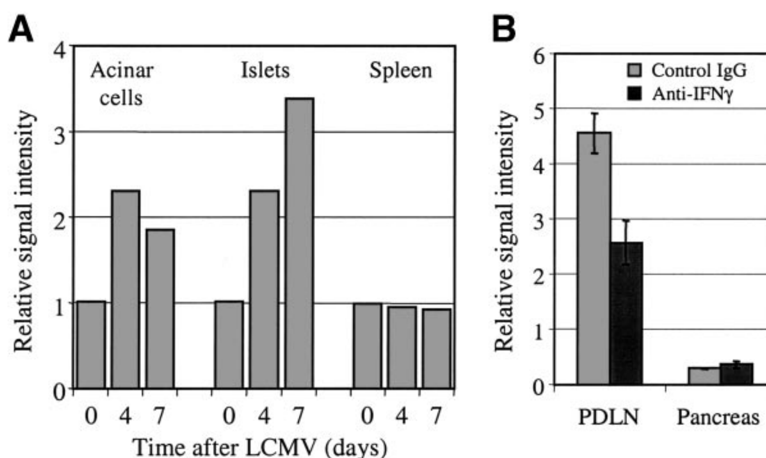

FIG. 3. Expression of Fas in pancreatic acinar and β-cells after systemic LCMV infection. *A*: Groups of three mice were infected with 10^5 pfu LCMV intraperitoneally, and RNA was collected from enriched fractions of isolated islets (>90% pure after handpicking of islets) and acinar cells and from whole spleen at the times indicated and was pooled for RPA as described in RESEARCH DESIGN AND METHODS. *B*: Groups of four mice were infected with 10^5 pfu LCMV intraperitoneally, and neutralizing anti–IFN-γ mAb (75 μg/dose intraperitoneally; Pharmingen #554409) was administered 6 h before and 1 day after LCMV infection. Groups of two LCMV-infected mice were administered with an isotype-matched control antibody at the same conditions. RPA was performed with total RNA isolated from pancreas and pancreatic draining lymph nodes (PDLN) at day 3 after infection. Elevation of Fas levels is statistically significant ($P < 0.05$).

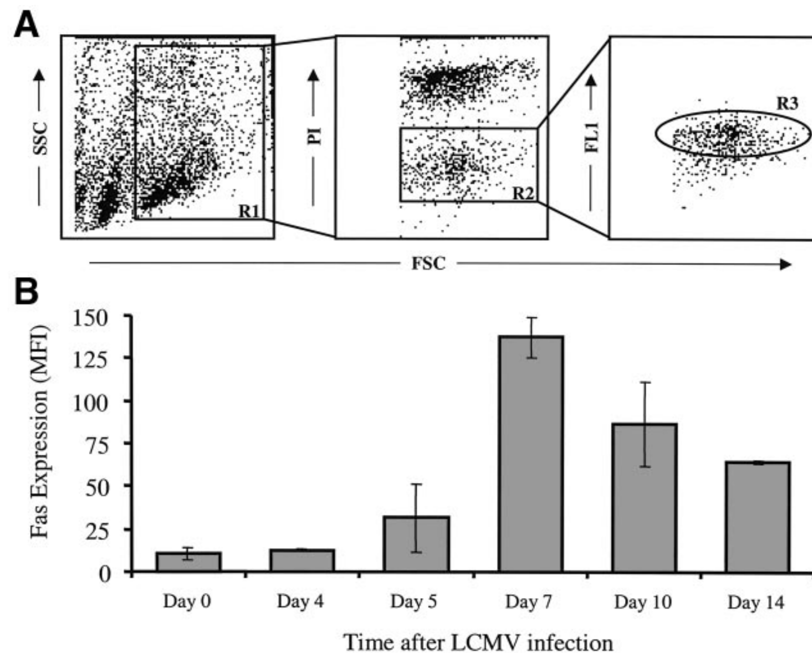

FIG. 4. Expression of Fas on the cell surface of isolated β-cells. *A*: Pancreatic islet cell preparations were stained with anti-Fas antibody and incubated with propidium iodine (PI). Viable β-cells were identified by flow cytometry on the basis of their side scatter (SSC) versus forward scatter (FSC) pattern (R1; *left*), low propidium iodine incorporation (R2; *middle*), and high autofluorescence in channel FL1 (R3; *right*). *B*: Fas expression was analyzed in β-cells (gated in R3) at several days after infection with 10^5 pfu LCMV. Data shown are average (±SE) of the mean fluorescence intensity (MFI) (n = 3–5 mice/group).

lead to induction of Fas on β-cells, which renders them susceptible to Fas/Fas-L–mediated apoptosis. This results in our system in a significant degree of clinically manifest diabetes after LCMV infection. The mechanism of virally mediated Fas upregulation in β-cells is not completely clear at this point but is likely not caused by direct viral infection of islets (Fig. 2*E*), because in vitro infected islets do not exhibit increased Fas levels (25). Rather, we propose an action of virally induced inflammatory mediators such as nitric oxide (NO) and/or cytokines or viral dsRNA (31). Experimentally, using neutralizing antibodies, we excluded a role for IFN-γ (Fig. 3*B*) or lymphotoxin-β (not shown), because no change in pancreatic/islet FAS levels was observed after their blockade. It is unlikely that virally induced NO plays a role in islet FAS upregulation as suggested by studies with islets from inducible NO synthase knockout mice (32). Our observation thus underlines an important additional caveat when using topical expression of Fas-L to provide immune protection. Viral infections of the target area might abolish any advantage by inducing Fas on selective cell types. This possibility should be taken into account before using Fas-L expression therapeutically.

ACKNOWLEDGMENTS

This is Publication No. 550 from the Division of Developmental Immunology, La Jolla Institute for Allergy and Immunology (San Diego, CA).

This work was supported by National Institutes of Health Grants U19 AI51973, 2 PO1 AG04342-19, and R29 DK51091 to M.G.V. and Juvenile Diabetes Research Foundation Grant 3-2000-510 to U.C.

We thank Diana Frye for excellent assistance with manuscript preparation.

REFERENCES

1. Kaplan HJ, Leibole MA, Tezel T, Ferguson TA: Fas ligand (CD95 ligand) controls angiogenesis beneath the retina. *Nat Med* 5:292–297, 1999
2. Ferguson TA, Green DR: Fas-ligand and immune privilege: the eyes have it. *Cell Death Differ* 8:771–772, 2001
3. Green DR, Ferguson TA: The role of Fas ligand in immune privilege. *Nat Rev Mol Cell Biol* 2:917–924, 2001
4. Chervonsky AV, Wang Y, Wong FS, Visintin I, Flavell RA, Janeway CA Jr, Matis LA: The role of Fas in autoimmune diabetes. *Cell* 89:17–24, 1997
5. Allison J, Georgiou HM, Strasser A, Vaux DL: Transgenic expression of CD95 ligand on islet beta cells induces a granulocytic infiltration but does not confer immune privilege upon islet allografts. *Proc Natl Acad Sci U S A* 94:3943–3947, 1997
6. Refaeli Y, Van Parijs L, London CA, Tschopp J, Abbas AK: Biochemical mechanisms of IL-2-regulated Fas-mediated T cell apoptosis. *Immunity* 8:615–623, 1998
7. Kang SM, Schneider DB, Lin Z, Hanahan D, Dichek DA, Stock PG, Baekkeskov S: Fas ligand expression in islets of Langerhans does not confer immune privilege and instead targets them for rapid destruction. *Nat Med* 3:738–743, 1997
8. Hsu PN, Lin HH, Tu CF, Chen NJ, Wu KM, Tsai HF, Hsieh SL: Expression of human Fas ligand on mouse beta islet cells does not induce insulitis but is insufficient to confer immune privilege for islet grafts. *J Biomed Sci* 8:262–269, 2001
9. Kang SM, Braat D, Schneider DB, O'Rourke RW, Lin Z, Ascher NL, Dichek DA, Baekkeskov S, Stock PG: A non-cleavable mutant of Fas ligand does not prevent neutrophilic destruction of islet transplants. *Transplantation* 69:1813–1817, 2000
10. Savinov AY, Tcherepanov A, Green EA, Flavell RA, Chervonsky AV: Contribution of Fas to diabetes development. *Proc Natl Acad Sci U S A* 100:628–632, 2003
11. Thomas HE, Darwiche R, Corbett JA, Kay TWH: Evidence that beta cell death in the nonobese diabetic mouse is Fas independent. *J Immunol* 163:1562–1569, 1999
12. Kim S, Kim KA, Hwang DY, Lee TH, Kayagaki N, Yagita H, Lee MS: Inhibition of autoimmune diabetes by Fas ligand: the paradox is solved. *J Immunol* 164:2931–2936, 2000
13. Itoh N, Imagawa A, Hanafusa T, Waguri M, Yamamoto K, Iwahashi H, Moriwaki M, Nakajima H, Miyagawa J, Namba M, Makino S, Nagata S, Kono N, Matsuzawa Y: Requirement of Fas for the development of

autoimmune diabetes in nonobese diabetic mice. *J Exp Med* 186:613–618, 1997
14. Moriwaki M, Itoh N, Miyagawa J, Yamamoto K, Imagawa A, Yamagata K, Iwahashi H, Nakajima H, Namba M, Nagata S, Hanafusa T, Matsuzawa Y: Fas and Fas ligand expression in inflamed islets in pancreas sections of patients with recent-onset type I diabetes mellitus. *Diabetologia* 42:1332–1340, 1999
15. Suarez-Pinzon WL, Power RF, Rabinovitch A: Fas ligand-mediated mechanisms are involved in autoimmune destruction of islet beta cells in non-obese diabetic mice. *Diabetologia* 43:1149–1156, 2000
16. Stassi G, Todaro M, Richiusa P, Giordano M, Mattina A, Sbriglia MS, Lo Monte A, Buscemi G, Galluzzo A, Giordano C: Expression of apoptosis-inducing CD95 (Fas/Apo-1) on human beta-cells sorted by flow-cytometry and cultured in vitro. *Transplant Proc* 27:3271–3275, 1995
17. Stassi G, De Maria R, Trucco G, Rudert W, Testi R, Galluzzo A, Giordano C, Trucco M: Nitric oxide primes pancreatic β cells for Fas-mediated destruction in insulin-dependent diabetes mellitus. *J Exp Med* 186:1193–1200, 1997
18. Hayashi T, Faustman DL: Implications of altered apoptosis in diabetes mellitus and autoimmune disease. *Apoptosis* 6:31–45, 2001
19. von Herrath MG, Dockter J, Oldstone MB: How virus induces a rapid or slow onset insulin-dependent diabetes mellitus in a transgenic model. *Immunity* 1:231–242, 1994
20. Oldstone MB, Nerenberg M, Southern P, Price J, Lewicki H: Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. *Cell* 65:319–331, 1991
21. Ohashi PS, Oehen S, Buerki K, Pircher H, Ohashi CT, Odermatt B, Malissen B, Zinkernagel RM, Hengartner H: Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. *Cell* 65:305–317, 1991
22. Holz A, Bot A, Coon B, Wolfe T, Grusby MJ, von Herrath MG: Disruption of the STAT4 signaling pathway protects from autoimmune diabetes while retaining anti-viral immune competence. *J Immunol* 163:5374–5382, 1999
23. Christen U, Wolfe T, Mohrle U, Hughes AC, Rodrigo E, Green EA, Flavell RA, von Herrath MG: A dual role for tnf-alpha in type 1 diabetes: islet-specific expression abrogates the ongoing autoimmune process when induced late but not early during pathogenesis. *J Immunol* 166:7023–7032, 2001
24. von Herrath MG, Guerder S, Lewicki H, Flavell RA, Oldstone MB: Coexpression of B7–1 and viral ("self") transgenes in pancreatic beta cells can break peripheral ignorance and lead to spontaneous autoimmune diabetes. *Immunity* 3:727–738, 1995
25. Seewaldt S, Thomas HE, Ejrnaes M, Christen U, Wolfe T, Rodrigo E, Coon B, Michelsen B, Kay TW, von Herrath MG: Virus-induced autoimmune diabetes: most β-cells die through inflammatory cytokines and not perforin from autoreactive (anti-viral) cytotoxic T-lymphocytes. *Diabetes* 49:1801–1809, 2000
26. Darwiche R, Chong MM, Santamaria P, Thomas HE, Kay TW: Fas is detectable on beta cells in accelerated, but not spontaneous, diabetes in nonobese diabetic mice. *J Immunol* 170:6292–6297, 2003
27. von Herrath MG, Efrat S, Oldstone MB, Horwitz MS: Expression of adenoviral E3 transgenes in beta cells prevents autoimmune diabetes. *Proc Natl Acad Sci U S A* 94:9808–9813, 1997
28. von Herrath MG, Yokoyama M, Dockter J, Oldstone MB, Whitton JL: CD4-deficient mice have reduced levels of memory cytotoxic T lymphocytes after immunization and show diminished resistance to subsequent virus challenge. *J Virol* 70:1072–1079, 1996
29. Homann D, Holz A, Bot A, Coon B, Wolfe T, Petersen J, Dyrberg TP, Grusby MJ, von Herrath MG: Autoreactive CD4+ T cells protect from autoimmune diabetes via bystander suppression using the IL-4/Stat6 pathway. *Immunity* 11:463–472, 1999
30. Mullbacher A, Lobigs M, Hla RT, Tran T, Stehle T, Simon MM: Antigen-dependent release of IFN-gamma by cytotoxic T cells up-regulates Fas on target cells and facilitates exocytosis-independent specific target cell lysis. *J Immunol* 169:145–150, 2002
31. Liu D, Cardozo AK, Darville MI, Eizirik DL: Double-stranded RNA cooperates with interferon-gamma and IL-1 beta to induce both chemokine expression and nuclear factor-kappa B-dependent apoptosis in pancreatic beta-cells: potential mechanisms for viral-induced insulitis and beta-cell death in type 1 diabetes mellitus. *Endocrinology* 143:1225–1234, 2002
32. Liu D, Pavlovic D, Chen MC, Flodstrom M, Sandler S, Eizirik DL: Cytokines induce apoptosis in β-cells isolated from mice lacking the inducible isoform of nitric oxide synthase (iNOS$^{-/-}$). *Diabetes* 49:1116–1122, 2000