Innate Immune Responses to Adenoviral Vector-Mediated Acute Pancreatitis

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Objectives: The role of innate immunity in the development of acute viral pancreatitis is not well understood. The aim of the study was to characterize the role of the innate immune system, especially macrophages, natural killer (NK), and NK T (NKT) cells, in the generation of immune responses to intrapancreatic delivery of recombinant adenoviral vector.

Methods: Adenoviral vectors expressing β-galactosidase or green fluorescent protein genes with viral capsid conjugated covalently with carbocyanine dye were directly injected into the pancreas of C57Bl/6 mice.

Results: Fluorescent microscopy of the pancreas showed that 30 minutes after vector administration, adenoviral particles localized to cell membranes, internalized, and localized to the nucleus by 4 hours, and transgene expression began at 24 hours. Immunohistochemical staining showed macrophages entering the pancreas shortly after vector administration, with maximal infiltration at day 4, and then disappearing as antigen-expressing cells were eliminated. Intrapancreatic macrophages appeared to deliver viral capsid proteins to the spleen. Flow cytometry showed that NK and NKT cells migrate to the pancreas and persist. Serum cytokines IL-6, IL-10, and IL-12 were all elevated.

Conclusion: Macrophages and NK and NKT cells play a major role in the development of acute adenovirus-mediated pancreatitis.

Key Words: rodent, adenoviral vector, macrophages, natural killer cells, natural killer T lymphocytes, interleukin-6, interleukin-10, interleukin-12

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Viral infection of the pancreas is an uncommon but important cause of acute pancreatitis. Various viruses affect the pancreas, including adenovirus, Coxsackievirus, cytomegalovirus, and varicella.[1–3] In children, viral pancreatitis is proportionally more common than in adults and is often seen in severe combined immunodeficiency disease or hepatic transplantation.[4–6] In adult HIV patients, viral pancreatitis is also a concern.[7] In addition to spontaneous viral infection, it is important to understand mechanisms of viral infection as a consequence of the development of novel vector-based therapy for metabolic and neoplastic pancreatic disease.[8,9]

Intrapancreatic gene delivery using recombinant adenoviral vectors is a well-characterized method for gene expression in rodent pancreas. Previous work has shown that intrapancreatic injection of adenoviral vector containing a marker gene, β-galactosidase, efficiently enters pancreatic acinar, ductal, and endothelial cells.[10] Transgene expression begins about 24 hours after vector administration. Peak transgene expression occurs at about 4 days and then slowly regresses to complete elimination by 4 weeks.[11] Multiple studies show that the acquired immune system is involved in adenoviral clearance.[12] After the local delivery of adenoviral vector to the pancreas, significant lymphocytic infiltration is seen, which correlates with a decrease in transgene expression. Splenocytes are sensitized to the adenovirus and engage in increased cytotoxic and lymphoproliferative activity upon in vitro repeat exposure to the adenovirus. T_H1 and T_H2 subset cytokines are increased. Neutralizing IgG and IgM antibodies are formed that are predominantly systemic in nature. The systemic immune response limits the duration of expression and does not allow vector to be readministered.[13]

Although acquired immune responses to recombinant adenoviral vectors have been extensively characterized,[14–17] less is known about the role of the innate immune system in initiating responses to adenoviral vectors. Macrophages and NK and NKT cells are among the major cell types that are activated early in other types of antiviral immune response.[18] NK and NKT cells lyse the virus-infected cells and are a major source of potent antiviral cytokines such as IL-6 and IL-12, which can also regulate other components of the acquired immune system.[19,20] Macrophages take up viral particles, by receptor-mediated endocytosis, process them, and then act as antigen-presenting cells (APCs) to present antigen in the context of MHC class II to helper T cells.[21] At the same time, by utilizing MHC class I, macrophages activate cytotoxic T lymphocytes (CTL), which eliminate the virus-containing cells.[22–24]

The aim of these experiments was to more fully characterize the role of the innate immune system in the development of an antiadenoviral vector immune response. Using immunohistochemistry, Cy3 fluorescent labeled adenoviral

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particles could be observed in relation to transgene expression in the initial stages of pancreatic adenoviral vector transduction, including localization, internalization, and intracellular trafficking. Also, the cytokine profile after vector administration was studied. The experiments described here are designed to provide further insight into the possible mechanisms of virus-induced pancreatitis.

MATERIALS AND METHODS

Experimental Design

Four- to 8-week-old female, C57/Bl6 mice were purchased from Taconic or Charles River Laboratories. Mice were randomized into 1 of 3 groups: 2 experimental (intrapancreatic injection of rAdCMVlacZ and intrapancreatic injection of Cy3-labeled rAdCMVGFP) and a control group (intrapancreatic injection of normal saline solution). The Institutional Animal Care and Use Committee and Institutional Biosafety Committee of the University of Pennsylvania approved animal procedures and use of recombinant DNA.

On day 0, mice were anesthetized by intraperitoneal injection of ketamine (70 mg/kg) and xylazine (10 mg/kg). Through a midline laparotomy, the pancreas was identified and directly injected with 100 μL recombinant adenoviral suspension using a 27-gauge needle. For both vectors (AdCMVlacZ and AdCMVGFP) 1×10^{11} particles were used, based on our previously published work.[9–13] The fascia and skin were closed in separate layers with running 4-0 Vicryl suture (Ethicon, Inc., Somerville, NJ). Mice (N = 4) were killed at 30 minutes, 4 and 24 hours, and 4, 10, 21, or 28 days after vector injection. Serum (50 μL per cytokine) was collected for cytokine analysis, and the pancreas was divided longitudinally for immunohistochemistry and X-gal staining (for mice that received AdCMVlacZ or saline) and for paraffin sectioning (for mice injected with Cy3-labeled rAdCMVGFP).

Vector Construction, Dose, and Administration

H5.010CMVlacZ deleted in E1a and E1b was produced by homologous recombination in the human embryonic kidney cell line 293. This construct has a cytomegalovirus (CMV) promoter driving the *Escherichia coli* β-galactosidase gene (*lacZ*), as reported previously.[25] Expression of the *lacZ* gene makes it suitable for transgene product detection (β-galactosidase) with X-gal staining. H5.010CMVGFP deleted in E1a and E1b was produced by homologous recombination in the human embryonic kidney cell line 293. This construct has a CMV promoter driving the *GFP* gene. The adenoviral capsid was labeled with Cy3 conjugated covalently to the adenoviral capsid protein, as previously described.[26]

Microscopy, Histochemistry, and Immunohistochemistry

Detection of β-Galactosidase

Tissue was embedded in OCT Compound (Sakura Finetek USA, Inc., Torrance, CA) and frozen over dry ice and 2-methylbutane. Sections of 6 μm were cut on a cryostat, placed on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA), and fixed in 0.5% glutaraldehyde in PBS for 10 minutes. Then slides were rinsed with 1 mmol/L $MgCl_2$ in PBS for 10 minutes twice and incubated in X-gal solution at 37°C for 3–4 hours. After that, slides were rinsed with PBS, counterstained for 3 minutes in Nuclear Fast Red stain (ENG Scientific, Inc., Clifton, NJ), mounted with a media Permount (Fisher Scientific, Pittsburgh, PA), and covered with a glass coverslip.

Immunohistochemical Staining for Macrophages

Tissue was embedded in OCT Compound (Sakura Finetek USA, Inc.) frozen over dry ice and 2-methylbutane. Cryosections 5 μm thick were placed on Superfrost/Plus microscope slides (Fisher Scientific). Staining was performed with an automated immunohistochemistry machine NEXES IHC staining system (Ventana Medical Systems, Inc., Tucson, AZ). Slides were fixed in Morpho-Save (Ventana Medical Systems, Inc.) for 15 minutes and washed in APK wash buffer (Ventana Medical Systems, Inc.) for 5 minutes. After fixation and wash, tissue was incubated in 0.3% H_2O_2 for 3 minutes to rid the section of endogenous peroxidase. Sections were then blocked in Background Buster (Accurate Chemical and Scientific Corp., Westbury, NY) for 20 minutes, avidin block, and biotin block (Vector Laboratories, Inc.) for 15 minutes each. Sections were then incubated with primary antibody, F4/80 antimouse monoclonal antibodies (Serotec Inc., Raleigh, NC) for 32 minutes and diluted 1:50, followed by secondary antibody: a biotinylated anti-rat IgG (Vector Laboratories, Inc.) diluted 1:500. Using a Ventana DAB detection kit (Ventana Medical Systems, Inc.), slides were incubated for 8 minutes in avidin conjugated to horseradish peroxidase, and peroxidase was detected with 3,3-diaminobenzidine and hydrogen peroxide with a light hematoxylin and bluing reagent counterstain. For light and fluorescent microscopy for GFP expression and Cy3 fluorescence, 5-μm sections of tissue embedded in paraffin were deparaffinized and evaluated under a fluorescent microscope. Cy3 and GFP fluorescence was viewed under rhodamine/fluorescein filters. Quantitative morphometry was done by counting the total number of positively stained cells using Image Pro computer software under magnification of ×100. Five areas per slide, a minimum of 3 slides (1 slide per animal) per time point were analyzed.

TCR-β/CD3 (NK 1.1/NKT Cell) Assay

Lymphocytes were separated by straining through nylon mesh. Fifty microliters of PBS-FCS-NaN3 containing appropriately diluted NK 1.1-PE antibody for NK cells and CD3-PE or TCR-β-FITC antibody for T-cell detection was added to 0.25 μL of cells. Cells were incubated on ice for 30 minutes, washed twice with PBS-FBS-NaN_3 solution, and fixed in 2% paraformaldehyde in phosphate-buffered saline before FACS analysis.

Interleukin Assay by Enzyme-Linked Immunosorbent Assay

Cytokines (50 μL of serum per each cytokine) were assessed using commercially available kits for mouse cytokines (Genzyme, Cambridge, MA): IL-6, IL-10, and IL-12 assays were performed as described.[13]

RESULTS

Timing of Vector Binding, Internalization, and Expression After Intrapancreatic Injection of Cy3-Labeled AdCMVGFP

Thirty minutes after vector injection, Cy3-labeled viral particles (red) localize to the acinar cell membrane (Fig. 1A). Four hours after adenoviral injection, red fluorescence, which represents viral capsid, has internalized inside the acinar cells and localizes in a predominantly perinuclear position in a number of cells (Fig. 1B). At 24 hours after injection of AdCMVGFP-Cy3, transgene expression is seen (Fig. 1C). At this time, viral capsid proteins appear to have been exported outside the cell. Transgene expression was initially more intense in the nuclei with subsequent spreading to the acinar cell cytoplasm. At day 4 after intrapancreatic injection of AdCMVGFP-Cy3, there is good transgene expression with almost no viral particles or viral capsid proteins present (Fig. 1D). By 12 days, transgene expression is gone, and there are no fluorescent viral particles left in the pancreas (not shown).

Transport of Viral Capsid Protein Into the Spleen

The question of the fate of fluorescent Cy3-labeled capsid proteins led to an analysis of the spleen. Given the proximity of the spleen to the pancreas, it was of interest to determine whether capsid proteins might be transported to the splenic parenchyma. Fluorescent microscopy of the spleen after intrapancreatic injection of AdCMVGFP-Cy3 was compared with intravenous (tail vein) injection. At 30 minutes, minimal Cy3 fluorescence is seen in the spleen (Fig. 2A). At 24 hours after intrapancreatic vector injection, viral capsid proteins appear in the spleen with predominant localization at the marginal zone (Fig. 2B). No transgene expression was noted at any time point up to day 28 (data from later time points not shown). In contrast, intravenous injection of AdCMVGFP-Cy3 was associated with transgene expression (green fluorescence) at the marginal zone of the spleen as early as 24 hours after vector injection (Fig. 2C, D). Fluorescence colocalized at the same areas where we had seen red fluorescence for Cy3. These areas correspond to the normal localization of macrophages in the spleen in the marginal zone

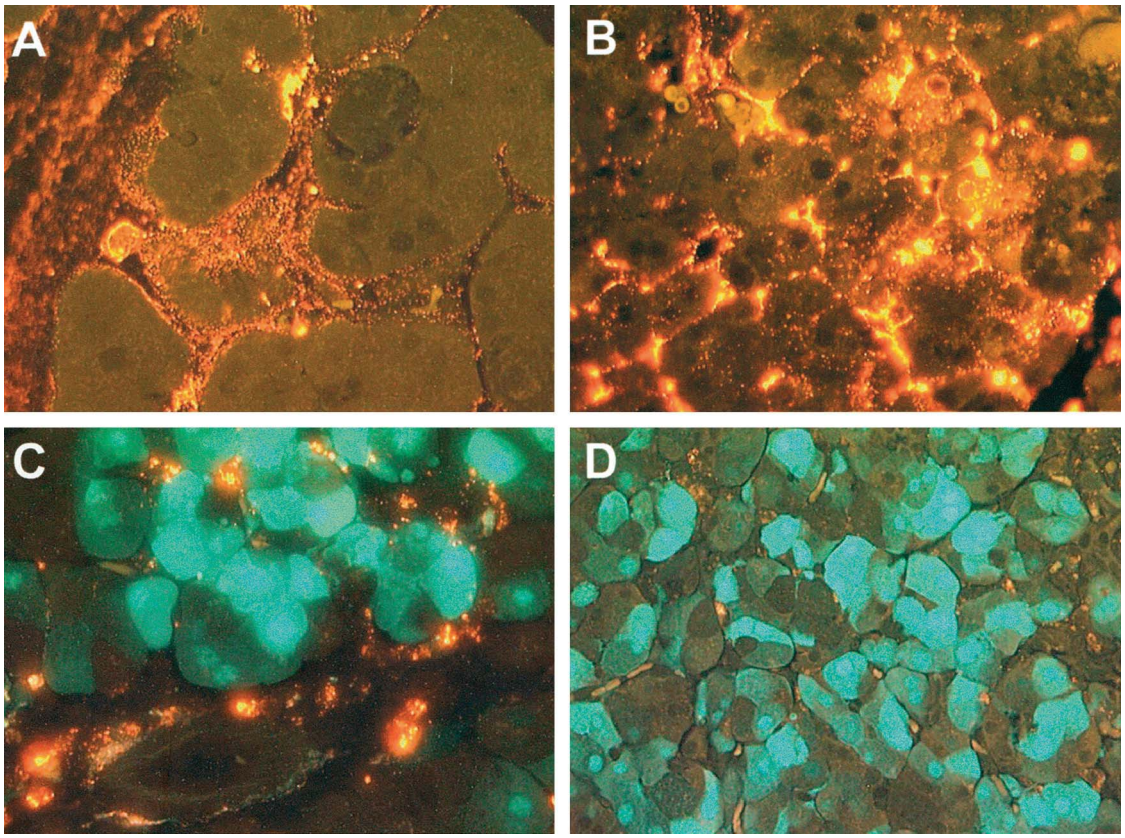

FIGURE 1. Fluorescence microscopy after intrapancreatic injection of AdCMVGFP-Cy3. Fluorescent filters: DAPI (blue: nuclei); rhodamine (red: Cy3-labeled viral capsid proteins); fluorescein (green: EGFP transgene product). A, Thirty minutes post-injection. Note how viral particles localize to the cell membrane and interstices of the acini. B, Four hours after injection. Note how viral capsid proteins have internalized to surround the nuclei. C, Twenty-four hours after injection. Note that viral capsid proteins have been exported outside the cell cytoplasm at a time when transgene expression begins. D, Four days after injection. Note transgene (green) expression with almost no viral particles or viral capsid proteins (red) outside the cells (objective ×20).

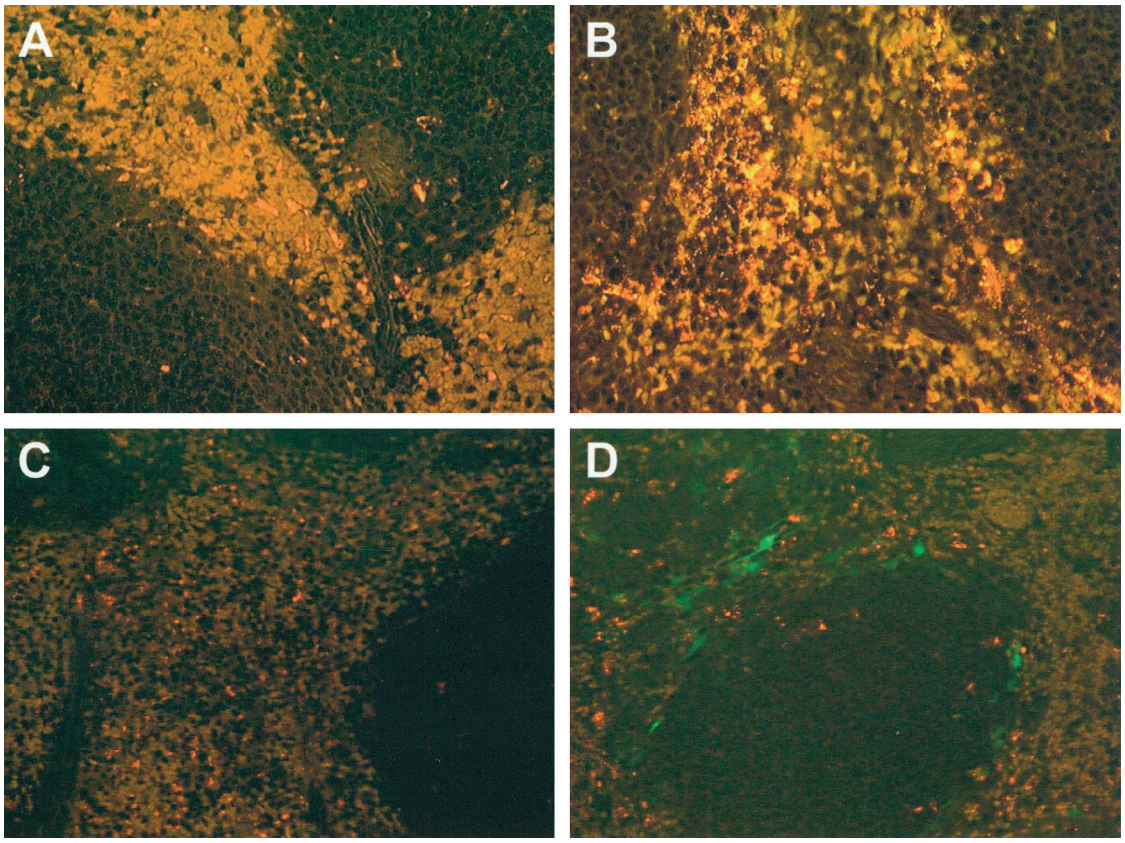

FIGURE 2. Fluorescent microscopy of the spleen after intrapancreatic and intravenous injection of AdCMVGFP-Cy3 vector. A, Thirty minutes after intrapancreatic injection. B, Twenty-four hours after intrapancreatic injection. C, Thirty minutes after intravenous injection. D, Twenty-four hours after intravenous injection. Red fluorescence represents viral capsid proteins labeled with Cy3 dye, with predominant localization at the marginal zone of the spleen. Note that after intrapancreatic injection, there is no transgene expression (green fluorescence) at either time point, suggesting the absence of vector DNA for transcription. By contrast, detectable splenic transgene expression fluorescence is seen after intravenous injection (objective ×10).

macrophages, marginal metallophilic macrophages, and red pulp macrophages.

Role of Macrophages in the Early Antivector Immune Responses

Using F4/80 anti-mouse macrophage antibody, macrophage accumulation in pancreatic parenchyma was qualitatively evaluated after adenoviral vector administration. Normal pancreatic parenchyma had almost no macrophages. The F4/80 anti-mouse macrophage antibody is very specific for mature, activated macrophages that express surface F4/80 antigen.[27–30] Macrophages are seen as large, elongated cells with abundant cytoplasm (light brown staining) and round or oval dark-stained nuclei. After intrapancreatic injection of vector, macrophages appear rapidly within the first 24 hours (Fig. 3A), and macrophage numbers reach a peak at day 4 (Fig. 3B). Macrophage infiltration of the pancreas persists up to day 10 (Fig. 3C) and then steadily decreases over the next 10–14 days. By day 28, macrophages are essentially gone (Fig. 3D). F4/80 anti-mouse macrophage antibody staining and X-gal staining for β-galactosidase expression colocalized at the same areas of the pancreas (not shown). Quantitative morphometry of macrophages in the pancreas was also performed in support of the qualitative data. In controls, pancreatic parenchyma has essentially no macrophages. Macrophage numbers increase after intrapancreatic injection of adenoviral vector, peaking at days 4–10 and declining by day 28 (Fig. 4).

NK/NKT Cells Infiltrate the Pancreas and Spleen After Intrapancreatic Injection of Recombinant Adenoviral Vector

Flow cytometric analysis of NK, NKT, and T cells from the pancreas and spleen after direct intrapancreatic injection of adenoviral vector in C57Bl/6 mice showed that in normal (PBS-injected) animals, there were no detectable NK 1.1 or NKT cells in the pancreas or spleen. Trafficking of NK 1.1 cells into the pancreas and the spleen begins by 2 days after intrapancreatic vector injection, is maximal at day 4, and remains to day 28, with splenic cells being greater in number. NKT cells follow the same pattern, but the cell number at day 4 in the pancreas is twofold higher than NK 1.1. The pattern in similar for both the pancreas and spleen (Fig. 5A, B). The number of T cells markedly increased after direct intrapancreatic injection of adenoviral vector in both the pancreas and spleen.

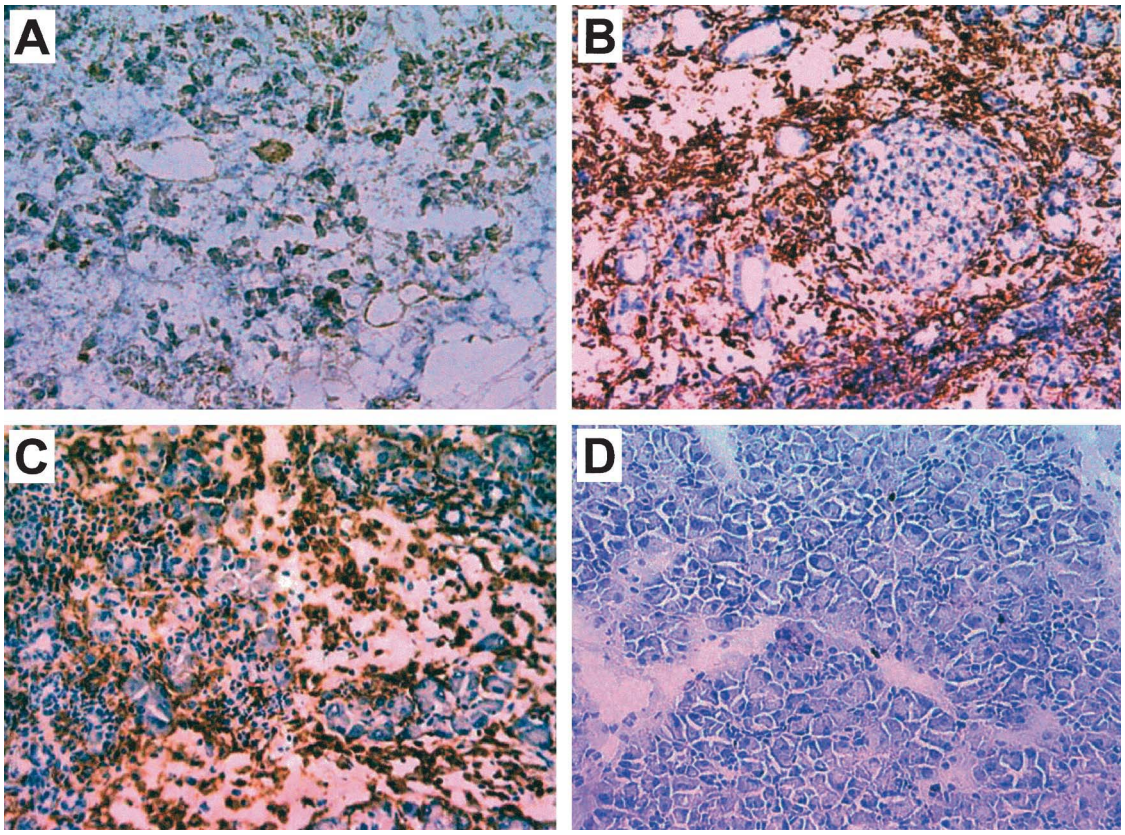

FIGURE 3. Qualitative immunohistochemistry of macrophage accumulation in pancreatic parenchyma after adenoviral vector administration. Using F4/80 anti-mouse macrophage antibody, brown staining (DAB) represents macrophages. A, Twenty-four hours after injection; B, 4 days after injection; C, 10 days after injection; D, 28 days after injection. Note that macrophage numbers increase from days 4–10 and that the islets of Langerhans are spared. By day 28, macrophages are essentially gone (objective ×10).

Quantity of TCRβ-Positive Cells Increased in the Pancreas After Intrapancreatic Injection of Recombinant Adenoviral Vector

Quantitative analysis of TCRβ/CD3 receptor–positive cells in the pancreas was evaluated at the several time points after intrapancreatic injection of adenoviral vector. The number of TCRβ-positive cells was undetectable in the pancreas of normal animals. At 2 days after intrapancreatic injection of adenoviral vector, they composed 9.6% (average per group) of the cells detected by the flow cytometry technique. It increased to 13.4% at day 4 and to 20.3% at day 10. The peak levels (23.6%) of TCRβ-positive cells in the pancreas were seen at day 28 after intrapancreatic adenoviral vector administration. In the spleen, the number of TCRβ-positive cells was undetectable in PBS-injected mice. Two days after intrapancreatic injection of adenoviral vector, TCRβ-positive cells composed 0.4% (average per group), increased to 45% at day 4, and peaked at 55.12% at day 10.

Serum Levels of IL-6, IL-10, and IL-12 After Intrapancreatic Administration of Recombinant Adenoviral Vector

Serum cytokine assays, by ELISA, after direct intrapancreatic injection of adenoviral vector was also performed. IL-6, IL-10, and IL-12 levels were undetectable in the serum of control animals or before intrapancreatic injection of adenoviral vector. Systemic secretion of the proinflammatory cytokine IL-12 begins within 30 minutes after intrapancreatic vector administration and remained elevated for 10 days (Fig. 6). The proinflammatory cytokine IL-6 peaked at 4 hours after intrapancreatic adenoviral vector injection. Levels sharply declined at 24 hours to undetectable by day 4. Peak secretion of the antiinflammatory cytokine IL-10 was delayed compared with elevation of serum IL-6 and IL-12 levels. The IL-10 level increased within 4 hours after vector injection and remain elevated for 4 days, returning to undetectable at 10 days.

DISCUSSION

The experiments described here detail the effects of direct pancreatic adenoviral transduction on cells of the innate immune system (ie, macrophages and APCs). Recent evidence suggests that the host innate immune system may moderate the severity of local acute pancreatitis, progression to multiorgan system failure, and death. For example, secretion of macrophage-derived IL-1 and TNF-α has been clearly correlated with damage of the acinar cells in the pancreas.[31–34] The present work complements our previously published observations regarding the acquired immune system ($CD4^+$, $CD8^+$ T cells). We have documented high level but transient

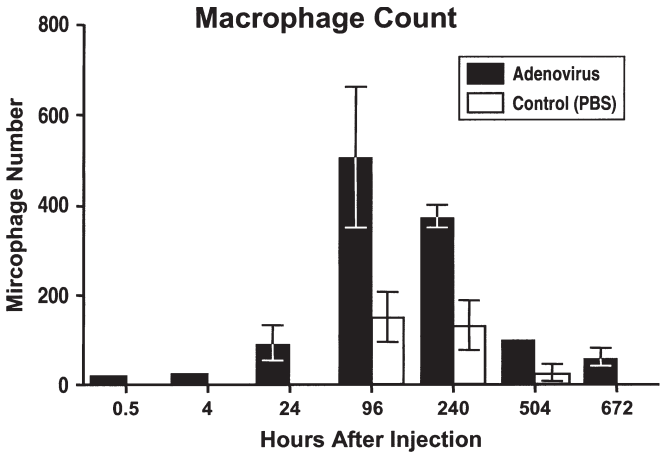

FIGURE 4. Quantitative morphometric analysis of intrapancreatic macrophages after adenoviral vector administration. In an unmanipulated state (0 hours), pancreatic parenchyma has essentially no macrophages. The number of macrophages increases after intrapancreatic injection of adenoviral vector at 4–10 days, and there is a marked decline by day 28.

expression of adenovirally transduced transgene product in rodent pancreas.[10] Levels of transgene expression correlate with pancreatic injury as measured by serum markers such as amylase and histologic parameters such as edema and necrosis.[12] As previously demonstrated in a variety of other tissues, pancreatic adenoviral gene transfer is associated with a brisk acquired immune response associated with a T-cell infiltrate.[1,13]

Very little is known about the pancreatic immune response to viral or other microbial infections. Intrapancreatic virus injection has been shown to cause a histopathologic picture similar to what we have seen with typical acute viral pancreatitis. Viruses known to be linked with the development of acute pancreatitis include cytomegalovirus, Epstein-Barr, Coxsackie, enterovirus, mumps, hepatitis B, herpes simplex,[35] and adenovirus.[36] Functional consequences of intrapancreatic injection of adenoviral vector consisted in elevation of serum and pancreatic tissue amylase levels, edema, cell destruction, inflammation, and vacuolization of the acinar cells.[12]

Despite the accumulating body of literature implicating host immune responses in the pathogenesis of acute pancreatitis, the role of innate immunity has not been systematically studied. Macrophages and NK and NKT cells are among the major cell types that respond early to a viral challenge. The innate immune system mediates protection against viral infection by inhibiting viral replication within host cells or eliminating virus-infected cells. Adaptive immune responses use the components of innate immunity to generate an antiviral effect.[37–39] Macrophages internalize viral particles by receptor-mediated endocytosis, a process that involves clathrin.[21] One of the components of viral protein–processing is MHC I presentation on APCs and activation of a CTL response during the viral infection. APCs serve as initial sites for viral entry. Viral particles then traffic to a variety of cell types to facilitate viral spread throughout the host.[40,41] Recent work suggests that activated macrophages play a more important role in antiviral immunity than in generation of a bacterial immune response.

The technique of labeling adenoviral capsid with covalently conjugated carbocyanine dye (Cy3) to adenoviral capsid protein is useful for studying early events in vector dissemination. As discussed in the Results section, the data suggest that macrophages present only viral capsid protein– or virus-inactivated in the process of viral presentation. Turnover of macrophages from the pancreas into the spleen is a fast process so that the macrophages would be able to constantly present the viral particles or viral capsid (which still has Cy3 fluorescence) to the spleen. This explained the fact that after injection of Cy3-labeled AdCMVGFP, we were able to detect viral particles in the interacinar space up to day 4, which were undetectable by day 12.

Macrophages were detected by using a monospecific polyclonal antibody directed against the macrophage-specific plasma membrane differentiation antigen F4/80. As shown above, at 96 hours, macrophage infiltration into pancreas is maximal (Fig. 4). Macrophage infiltration into the pancreas is diffuse; however, they do not appear to enter the islets of Langerhans. Macrophages that uptake the adenoviral particle

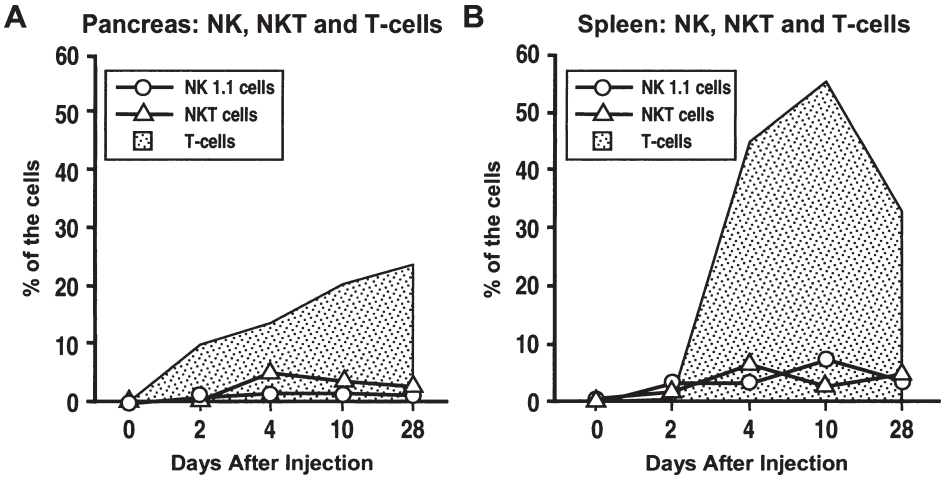

FIGURE 5. Flow cytometric analysis of NK, NKT, and T cells in the pancreas (A) and spleen (B) after direct intrapancreatic injection of adenoviral vector in C57Bl/6 mice. Trafficking of NK cells into the pancreas and spleen begins within 4 hours after intrapancreatic vector injection, is maximal at day 4, and remains until day 28. NKT cells follow the same pattern, but the cell number at day 4 in the pancreas is twofold higher than in the spleen. The number of T cells markedly increased after direct intrapancreatic injection of adenoviral vector in both pancreas and spleen.

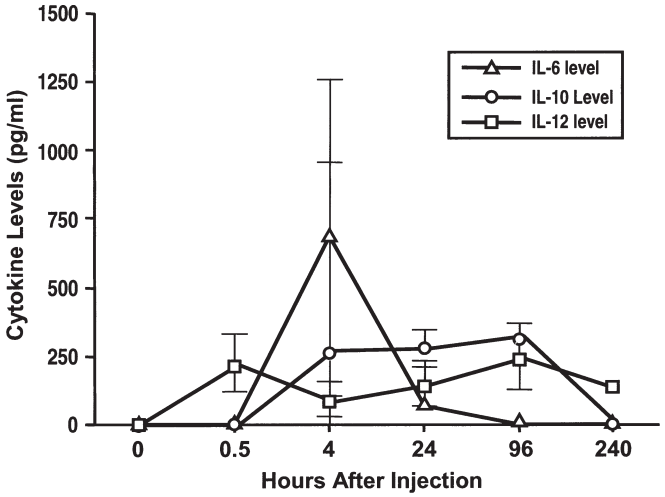

FIGURE 6. Serum cytokine assay (ELISA) after direct intrapancreatic injection of adenoviral vector. Systemic secretion of the proinflammatory cytokine IL-12 begins within 30 minutes. IL-6 peaks at 4 hours. Peak secretion of the antiinflammatory cytokine IL-10 is delayed compared with elevation of serum IL-6 and IL-12 levels.

capsid proteins appear to traffic to the spleen rapidly after injection. Despite trafficking of Cy3-labeled particles into the spleen after intrapancreatic injection, the transgene was not expressed. In contrast, direct intravenous injection of the vector was associated with transgene expression in the spleen. The co-localization of transgene expression in the spleen and staining of macrophages showed a similar pattern of distribution. NK and NKT cells are a second population of lymphocytes that can be activated in response to viral infection. With activation they produce certain cytokines including IL-6 and chemokines.[42] We have shown that trafficking of NK and NKT cells into the pancreas and spleen begins within 4 hours after intrapancreatic vector injection, peaks at days 4–10, and remains until day 28.

To determine whether the influx of macrophages had a functional correlation, levels of different cytokines were evaluated after vector administration. Detectable levels of IL-12 were noted within 30 minutes, and IL-6 was elevated beginning at 4 hours post-injection. The data are consistent with the fact that macrophages are the major source of IL-6 and IL-12. As a counterregulatory cytokine, IL-10 had a delayed onset and prolonged serum levels. It is secreted in response to elevated levels of IL-12, and its level remained elevated while IL-12 was present in the serum. The identification, in the transduced pancreas, of elevated levels of IL-10 suggests an antiinflammatory, pancreas-protective response of Th_2 cells.

In summary, Cy3-labeled recombinant adenoviral vector is a good model to study early events of viral vector uptake. In the first 30 minutes, virus binds to the cell membrane. By 4 hours, vector internalizes and localizes around the nucleus. Transgene expression begins in 24 hours and continues for 2 weeks. Transgene expression occurs both in the nucleus and cytoplasm. Viral capsid proteins, but not transgene-containing DNA, traffic to the spleen, presumably in macrophages. After transgene expression begins, viral capsid proteins are exported outside the cell and cleared from the tissue by the macrophages into the marginal zone of the spleen. Normal pancreas has almost no macrophages or NK or NKT cells. Shortly after vector injection, macrophages infiltrate pancreatic parenchyma. Trafficking of macrophages and NK/NKT cells into the pancreas begins as early as 30 minutes after intrapancreatic vector injection and is maximal at day 4. By day 28, macrophages in the spleen increase, and there are almost no macrophages in the pancreas. Early systemic secretion of the proinflammatory cytokines IL-6 and IL-12 correlates with APC appearance in the pancreas and is followed by elevated levels of the antiinflammatory cytokine IL-10. Future work will be directed toward elimination or suppression of macrophages and other APCs before stimulation with intrapancreatic injection of recombinant adenoviral vector. The goal will be to prolong transgene expression and diminish the severity of virus-mediated—and potentially other causes of—acute pancreatitis.

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