Macrophage ablation attenuates adenoviral vector–induced pancreatitis

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Background. *The objective of these studies is to determine the effects of macrophage ablation on the course of acute viral pancreatitis. Macrophages secrete proinflammatory cytokines triggering local pancreatic and systemic inflammation in the acute phase of virus-induced pancreatitis. We hypothesized that ablation of macrophages should attenuate the host inflammatory response in a mouse model of adenovirus-induced pancreatitis.*
Methods. *Liposome-encapsulated dichloromethylene-diphosphonate, a macrophage-depleting agent, was used before direct pancreatic injection of a recombinant adenovirus expressing a marker gene in C57Bl/6 and IL-6 knockout (KO) mice.*
Results. *C57Bl/6 mice depleted of macrophages had diminished pancreatic inflammation in the first 24 hours after vector administration. IL-6 KO mice depleted of macrophages had more severe inflammation than similarly treated C57Bl/6 mice. C57Bl/6 mice depleted of macrophages, and IL-6 KO mice had prolonged transgene expression and diminished cytotoxic T lymphocyte responses to adenoviral vector. Mortality was highest in IL-6 KO mice depleted of macrophages. Depletion of macrophages also prevented detectable serum IL-6, IL-10, or IL-12 levels in C57Bl/6 mice.*
Conclusions. *The data suggest that macrophages play a role in the acute inflammatory response to viral vector–induced pancreatitis and that IL-6 may be protective. Understanding of the mechanisms that initiate the host immune cascade will allow more effective use of adenoviral vector–based pancreatic gene delivery.* (Surgery 2005;137:545-51.)

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THE PANCREATIC IMMUNE RESPONSE to viral or other microbial infectious diseases is not well characterized. Recent evidence suggests that host immunity may modulate the severity of acute pancreatitis and the progression to multiple organ system failure and death. Viruses known to cause the development of acute pancreatitis include cytomegalovirus, Epstein-Barr virus, the Coxsackie viruses, enterovirus, mumps, hepatitis B, herpes simplex, and adenovirus.[1,2] The host response to adenoviruses is of particular interest because recombinant adenoviral vectors are being used in the gene therapy of solid tumors, including pancreatic cancer.[3] Direct injection of vector into the

pancreas causes a vigorous immune response and inflammation with leukocyte infiltration when compared with saline-vehicle-injected controls.[4-6] One of the cytokines postulated to play an important role in the pathogenesis of acute pancreatitis is interleukin (IL)-6. IL-6 is a cytokine produced by a number of cell types, but an early and major source is activated macrophages.[7]

Taken together, available data suggest that transgene expression might be prolonged by the depletion of macrophages in C57Bl/6 and IL-6 knockout ([-/-]; KO) mice before adenoviral vector injection into the pancreas by intraperitoneal and intravenous injection of liposome-encapsulated dichloromethylene-diphosphonate (DMDP). We hypothesized that cytokines derived from activated macrophages in response to local tissue damage may play an integral role in the evolution of viral vector–induced acute pancreatitis. In experiments detailed here, we have shown that C57Bl/6 mice depleted of macrophages have no cytotoxic T lymphocyte (CTL) response to adenoviral vector, diminished pancreatic inflammation, and prolonged transgene expression. In contrast, IL-6 KO

mice depleted of macrophages have a higher mortality than vector-treated IL-6 KO mice or C57Bl/6 mice. These findings support the hypothesis that macrophage ablation in otherwise intact mice can attenuate the host inflammatory response and that IL-6 has an acinar protective effect early in the development of adenovirus-induced acute pancreatitis.

MATERIAL AND METHODS

Animals. Female 4- to 8-week-old C57Bl/6 (Taconic Laboratories, Albany, NY) or IL-6 KO mice bred on a C57Bl/6 background (Jackson Laboratory, Bar Harbor, Me) were randomized into 2 groups: control mice, which received intrapancreatic injection of a recombinant adenoviral vector expressing the marker gene β-galactosidase (AdCMVLacZ), and experimental mice, which received intraperitoneal and intravenous injections of liposome-encapsulated DMDP, followed by intrapancreatic injection of AdCMVLacZ. Animal procedures and use of recombinant DNA were approved by the Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee of the University of Pennsylvania.

Intrapancreatic injection of AdCMVLacZ. At day 0, mice were anesthetized by intraperitoneal injection of ketamine (70 mg/kg) and xylazine (10 mg/kg), which was followed by left side subcostal laparotomy. The pancreas was identified and injected directly with 100 μL of recombinant adenoviral suspension at a dose of 1×10^{11} particles per mouse. The fascia and skin were closed in separate layers with running 4-0 Vicryl suture (Ethicon, Somerville, NJ). Necropsy was performed at 30 minutes, 4 hours, 24 hours, and 4, 10, 21 or 28 days after vector injection; 10 to 15 animals per group per time point were killed humanely. Serum was collected for cytokine (50 μL per cytokine), amylase, and lipase assays, as well as immuno- and X-gal histochemical staining. Spleens from animals killed humanely at day 10 were processed for CTL assay by dispersion through nylon mesh.[8] The AdCMVLacZ adenoviral vector has been well characterized.[4-6,9] Prior publications from our laboratory (not repeated here) have documented the absence of inflammation with intrapancreatic saline injection.

Liposome-encapsulated DMDP preparation. DMDP encapsulation was performed as described.[10] Briefly, 75 mg of phosphatidylcholine (Sigma, St. Louis, Mo) and 19 mg of cholesterol (Sigma) were dissolved in 5 mL of chloroform/methanol (2:1). The chloroform phase was removed by low vacuum rotation/evaporation in a 500 mL bottle.

After the phospholipid layer formed, it was dispersed in 10 mL of phosphate-buffered saline (PBS) containing 1.89 g of DMDP (Sigma). The suspension was kept at room temperature under nitrogen gas for 2 hours, treated for 3 minutes in a water bath sonicator, and centrifuged $10,000g$ for 15 minutes. The liquid phase, containing excess DMDP, was removed with a Pasteur pipette. The remaining liposomes were resuspended and washed by centrifugation ($25,000g$) in PBS for 30 minutes. The pellet was suspended in a final volume of 4 mL of PBS.

Macrophage depletion in vivo and immunohistochemical staining for macrophages. To deplete macrophages, mice were injected intravenously on days −2 and −1 with 200 μL of liposome-encapsulated DMDP and 100 μL intraperitoneally. On day 0, the animals received an intrapancreatic injection of adenoviral vector. DMDP was reinjected every 4 to 5 days. Macrophage depletion in the liver, spleen, and pancreas was confirmed histologically by staining with an anti-mouse macrophage antibody. Tissue was embedded, frozen over dry ice and 2-methylbutane. Staining was performed on 6-μm cryosections with an automated immunohistochemistry machine NEXES IHC staining system (Ventana Medical Systems, Tucson, Ariz). After fixation and wash, tissue was incubated in 0.3% H_2O_2, then blocked in Background Buster (Accurate Chemical and Scientific, Westbury, NY), Avidin Block, and Biotin Block (Vector Laboratories, Burlingame, Calif). Sections were then incubated with a 1:50 dilution of F4/80 anti-mouse monoclonal antibody (Serotec, Raleigh, NC), followed by a biotinylated anti-rat IgG (Vector) diluted 1:500. Lastly, slides were incubated in horseradish peroxidase conjugated avidin. Peroxidase was detected with 3,3-diaminobenzidine and hydrogen peroxide. For X-gal staining, tissue was fixed in 0.5% glutaraldehyde in PBS for 10 minutes, rinsed twice with 1 mmol/L $MgCl_2$ in PBS, incubated in X-gal solution at 37°C for 3 to 4 hours, and then counterstained for 3 minutes in Nuclear Fast Red stain (ENG Scientific, Clifton, NJ). Quantitative analysis was done by counting the total number of positively stained cells using Image Pro computer software under magnification of ×100. Five areas per slide, with a minimum of 3 slides (1 slide per animal) per time point, were analyzed.

CTL and interleukin assays. CTL activity was measured by the ability of splenocytes to kill major histocompatibility complex (MHC)–matched adenovirus-infected target cells, as described previously.[4,5,9] Briefly, a single-cell suspension of spleen
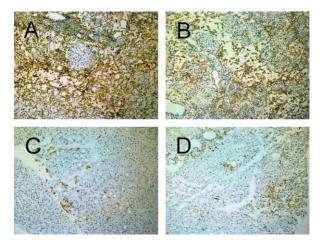
Fig 1. Immunohistochemical staining of the pancreas of C57Bl/6 mice with F4/80 anti-mouse macrophage antibody after intrapancreatic administration of AdCMV-LacZ. **A**, day 4, and, **B**, day 10, after vector administration. Note the intense macrophage infiltration (*brown staining*) and sparing of the islets of Langerhans. **C**, day 4, and, **D**, day 10, after macrophage depletion with DMDP before AdCMVLacZ vector administration. Note the almost complete absence of macrophages. Objective ×20.
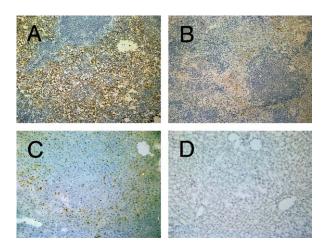

Fig 2. Immunohistochemical staining of the spleen and liver of C57Bl/6 mice with F4/80 anti-mouse macrophage antibody at day 4 after intrapancreatic administration of AdCMVLacZ. Splenic sections of C57Bl/6 mice with saline (**A**) or DMDP (**B**) administration before injection with AdCMVLacZ, then staining with F4/80 anti-mouse macrophage antibody (note abundant *brown staining* for macrophages at the red pulp and marginal zone of the spleen and Kupffer cells in the liver). Liver sections of C57Bl/6 mice with saline (**C**) or DMDP (**D**) administration before injection with AdCMVLacZ, then staining with F4/80 anti-mouse macrophage antibody (note abundant brown staining for macrophages in hepatic Kupffer cells). Objective ×20.

cells from pooled groups of 3 mice from day 10-11 was cultured for 5 days at a concentration of 5×10^6 cells per well in a 24-well plate. Purified AdCMV-LacZ was added at a multiplicity of infection (MOI) of 0.8. After secondary in vitro stimulation, non-adherent spleen cells were harvested and assayed on MHC-compatible target cells using serial effector-to-target-cell ratios. Target cells were infected with adenovirus at an MOI of 100 overnight; 1×10^6 cells were labeled with 100 mCi of $Na_2{}^{51}CrO_4$, (^{51}Cr; New England Nuclear, Boston, Mass) for 1 hour. The cells were washed 3 times with 10 mL Dulbecco modified Eagle medium and resuspended in assay medium at 5×10^4/mL. Aliquots of 100 μL of target cells were plated with 100 μL of serial spleen cell dilutions at various effector-to-target-cell ratios in V-bottom-shape microliter plates. The plates were spun down for 3 minutes at 1100 rpm and incubated for 6 hours at 37°C and 10% CO_2. A 100-μL sample of supernatant was removed from each well and counted in a gamma counter. The percentage of specific ^{51}Cr release was calculated. For the cytokine analysis, 50 μL of serum was assayed using commercially available kits for the mouse cytokines IL-6, IL-10, and IL-12 (Genzyme, Cambridge, Mass).

Serum amylase and lipase assay. Blood samples were obtained at 4 and 24 hours and 3, 4, 10, 14, and 21 days after vector administration. Twenty microliters of serum was used for each sample. Serum amylase activity was determined at 30°C spectrophotometrically by measuring the rate of change absorbance at 405 nm for 2 minutes with 4,6-ethylidene-G_7PNP as a substrate (Sigma Diagnostics Amylase Kit). Serum lipase activity was measured at 30°C by a titrimetric method that quantifies fatty acid formation with dilution of a standardized sodium hydroxide solution (Sigma Diagnostics Lipase Kit).[9]

Statistical analysis. Statistical analysis was performed with the Student unpaired *t* test.[11]

RESULTS

Macrophage depletion in vivo. Pancreas, spleen, and liver sections were evaluated for macrophages and Kupffer cells by staining with F4/80 anti-mouse macrophage monoclonal antibodies. Intrapancreatic injection of adenoviral vectors into the pancreas was associated with macrophage accumulation predominantly in the areas of injection with maximal presence at day 4. This infiltration persisted for about 10 days (Fig 1, *A* and *B*) and was gone by day 28. Adenoviral vector–associated macrophage accumulation in the pancreas was eliminated almost completely by administration of
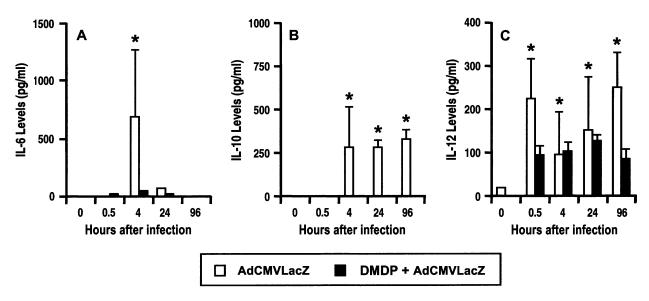
Fig 3. Serum cytokine levels (**A**, IL-6; **B**, IL-10; and **C**. IL-12) in C57Bl/6 mice after intrapancreatic admin-istration of AdCMVLacZ vector in C57Bl/6 mice with and without DMDP macrophage depletion ($N \geq 3$ per time point, *$P < .05$ vs time 0).

liposome-encapsulated DMDP before virus injec-tion into the pancreas (Fig 1, *C* and *D*).

When compared with a normal spleen, all 3 major subsets of macrophages—marginal zone, marginal metallophilic, and red pulp—were sub-stantially depleted at day 4 after liposome-DMDP injection (Fig 2, *A* and *B*). Liver was evaluated in a way similar to pancreas and spleen. DMDP depleted Kupffer cells in the liver effectively at day 10 after injection of adenoviral vector into the pancreas (Fig 2, *C* and *D*).

Macrophage depletion prevents elevation of serum levels of IL-6, IL-10, and IL-12 after intra-pancreatic adenoviral vector administration. In intact C57Bl/6 mice treated with intrapancreatic adenoviral vector, serum IL-6 levels peaked at 4 hours after vector injection. Depletion of macro-phages before vector administration eliminated the peak in serum IL-6 (Fig 3, *A*). Depletion of macrophages resulted in no detectable IL-10 up to day 4, although mice with intact macrophage populations had marked elevations in serum IL-10 beginning at 4 hours and persisting to 96 hours (Fig 3, *B*). Intrapancreatic vector injection resulted in increase of serum IL-12 levels. Depletion of macrophages when compared with controls con-cerning IL-12 levels did not reach statistical signif-icance (Fig 3, *C*).

Serum amylase and lipase activity and mortality after vector administration in C57Bl/6 and IL-6 KO mice with and without macrophage depletion. Administration of adenoviral vector into the pan-creas resulted in high amylase and lipase serum

levels (Fig 4, *A* and *B*). There was no difference in amylase or lipase activity in C57Bl/6 or IL-6 KO mice. Enzyme activity rose significantly in the first 4 hours after intrapancreatic injection of adeno-viral vector and returned to baseline by day 10. Interestingly, C57Bl/6 mice depleted of macro-phages had significantly decreased serum amylase and lipase activity when compared with IL-6 KO mice (Fig 5, *A* and *B*).

C57Bl/6 mice treated with vector alone had 100% survival to day 25 compared with mice depleted of macrophages, which had 70% survival at day 10 and 25% at day 25. IL-6 KO mice that received intrapancreatic injection of adenoviral vector had a 100% survival rate. With depletion of macrophages, IL-6 KO mice had a survival rate of 50% at day 4 and 17% at day 11. No IL-6 KO mice depleted of macrophages and injected with vector survived to day 25.

Absence of CTL response and enhanced transgene expression in DMDP-treated C57Bl/6 and IL-6 KO mice. CTL response was evaluated at day 11 after intrapancreatic injection of adenoviral vector. In the presence of intact macrophages, C57Bl/6 mice developed a strong CTL response to adenoviral vector at day 11 (Fig 6). With macro-phage depletion, no CTL response was seen. IL-6 KO mice had essentially no CTL response to the intrapancreatic injection of adenoviral vector, with or without depletion of macrophages (Fig 6).

β-Galactosidase expression was evaluated in pan-creatic frozen sections at day 11 and day 21 after intrapancreatic adenoviral vector administration.
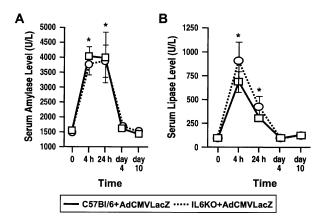
Fig 4. Serum amylase (**A**) and lipase (**B**) activity in C57Bl/6 mice (*solid line*) and IL-6 KO mice (*dotted line*) after intrapancreatic administration of AdCMVLacZ. Both mice strains demonstrate substantial pancreatic inflammation (N ≥ 3 per time point, *$P < .05$ vs time 0).
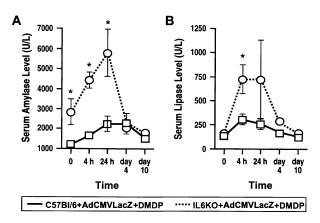

Fig 5. Serum amylase (**A**) and lipase (**B**) activity in C57Bl/6 mice (*solid line*) and IL-6 KO mice (*dotted line*) after depletion of macrophages with DMDP and intrapancreatic administration of AdCMVLacZ (N ≥ 3 per time point, *$P < .05$ vs time 0).

C57Bl/6 mice depleted of macrophages showed a 6-fold increase in transgene expression at day 11 (269 ± 38 cells per high power field vs 40 ± 4 cells per high power field, $P < .05$). There was no difference in transgene expression at day 21, when expression was essentially gone in normal and macrophage-depleted mice (2 ± 1 cells per high power field vs 5 ± 2 per high power field). The high mortality rate in IL-6 KO mice depleted of macrophages prevented the objective assessment of the transgene expression at the later time points.

DISCUSSION

These studies have identified a critical interaction between macrophages and IL-6 in adenoviral
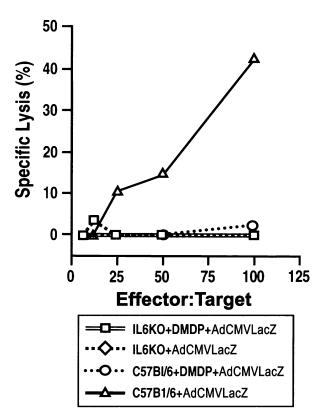

Fig 6. CTL response in C57Bl/6 mice and IL-6 KO mice with and without macrophage depletion by DMDP at day 11 after intrapancreatic injection of AdCMVLacZ. In the presence of macrophages, C57Bl/6 mice developed a strong CTL response to adenoviral vector at day 11. When macrophages were depleted, the CTL response was abolished. In contrast, IL-6 KO mice had no CTL response to the intrapancreatic injection of adenoviral vector with or without macrophages. The data are representative of 3 separate experiments.

vector–induced acute pancreatitis: (1) When macrophages are depleted, transgene expression is enhanced and pancreatic injury is lessened; (2) when IL-6 is absent, the mortality of vector administration in the absence of macrophages is exacerbated. Recent evidence suggests that cells of the innate immune system, such as macrophages, have a direct cytotoxic effect important in the development of local and systemic inflammation.[12] Previous work from our laboratory has shown that introduction of vector into the pancreas causes high-level gene transfer in the rat and mouse, but also a vigorous immune response with acute inflammation and local leukocyte infiltration.[4-6,9] Functional consequences of vector administration include increase in serum and pancreatic tissue amylase activity, edema, cell destruction, inflammation, and vacuolization of the acinar cells.[13]

Recently we have demonstrated that intrapancreatic injection of adenoviral vector is associated with macrophage infiltration, which is maximal at days 4 to 10. On accumulation in the pancreas, macrophages secrete IL-6, IL-10, and IL-12.[14] Here we describe the consequences of macrophage depletion. Several laboratories have shown that depletion of macrophages, such as splenic macrophages and Kupffer cells, prolongs transgene expression, prevents the release of macrophage-derived cytokines, and reduces mortality during acute hemorrhagic pancreatitis in mice.[15,16] Data also support macrophage blockade to decrease severity and improve survival of animals with chemically induced pancreatitis.[17] One agent that can block activity of the reticuloendothelial system by depleting macrophages selectively is liposome-encapsulated DMDP.[18]

Adoptive transfer experiments have shown that CTLs to viral antigens are sufficient to destroy virus-transduced cells, indicating that CTLs to transgene product, such as β-galactosidase, do not solely account for the observed cellular destruction that has characterized the use of adenoviral vectors. B cell–mediated events have also been shown not to participate in destruction of transduced cells in vivo, despite the production of virus- and transgene-specific antibodies.[19] Splenocytes are sensitized to the adenovirus and engage in increased cytotoxic and lymphoproliferative activity upon repeat in vitro exposure to the adenovirus. The data reported here are consistent with the postulated effects of innate immunity on intrapancreatic vector administration.

As noted above, intrapancreatic vector administration results in increased levels of serum IL-6 (Fig 3). The source of IL-6 documented in these studies is not identified definitively. The elimination of peak IL-6 levels by macrophage ablation suggests that macrophages and other dendritic cells are prime sources of this important cytokine; however, a contribution from acinar cells has not been excluded. Further, levels of pancreatic cytokines—including IL-6—increase early in the course of pancreatitis but are much lower than subsequent increases in other organs, such as liver, lung, and spleen.[20]

Levels of IL-6 correlate with the severity of acute pancreatitis; however, the exact role of IL-6 in acute pancreatitis has not been elucidated.[21-23] Recently, Dusetti et al[24] have shown that there are 2 IL-6 responsive elements in the pancreatitis-associated protein 1 gene. Thus, IL-6 may serve a cytoprotective role in the attempt to attenuate inflammation. The concept of IL-6 as a cytopro-

tective cytokine has been well worked out in liver.[25] As noted here, the absence of IL-6 in C57Bl/6 mice depleted of macrophages, as well as in IL-6 KO mice, is associated with increased mortality. Mortality in DMDP-treated C57Bl/6 mice was similar to that of IL-6 KO mice without DMDP, consistent with the hypothesis that IL-6 attenuates injury. The absence of both IL-6 and macrophages increased mortality profoundly. The mortality did not appear to be due to the pancreatitis but rather to a more diffuse peritonitis, suggesting an overall immunosuppressed state. We believe the eradication of fixed macrophages results in global immunosuppression state and inability to fight infection. Literally, the cure (immunocompromise caused by macrophage ablation and absence of IL-6) is worse than the disease (decreased vector-related inflammation).

In conclusion, we have shown that macrophages play an important role in the immune response to adenovirus-mediated pancreatitis. Strategies to eliminate or suppress mechanisms responsible for the initial activation of the immune system need to be developed to assist in attenuating both acute and chronic pancreatic inflammation. As viral vectors capable of efficient pancreatic transduction become available, control of pancreatic inflammation will become important. Although the global elimination of macrophages may not be a safe strategy for prolonged transgene expression, further refinement of current compounds, like DMDP, or development of agents targeted to only those elements responsible for virus-specific immunity may provide a more selective alternative. Further research on the intracellular mechanisms responsible for viral-mediated host inflammatory responses is necessary.

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