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Short-Term High Expression of Interferon-Alpha Modulates Progression of Type 1 Diabetes in NOD Mice

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Type I diabetes (T1D) is an organ-specific autoimmune disease caused by the T cell-mediated destruction of the insulin-producing β cells in the pancreatic islets. The onset of T1D is the consequence of a progressive destruction of islet β cells mediated by an imbalance between effector $CD4^+$ T helper (Th)1 and regulatory $CD4^+$ Th2 cell function. Since interferon-alpha (IFN-α) has been known to modulate immune function and autoimmunity, we investigated whether administration of adenoviral-mediated IFN-α gene would inhibit the diabetic process in NOD mice. The development of diabetes was significantly inhibited by a single injection of adenoviral-mediated IFN-α gene before 8 weeks of age. Next, we examined the hypothesis that Th2-type cytokines are associated with host protection against autoimmune diabetes, whereas Th1-type cytokines are associated with pathogenesis of T1D. The expression of IFN-α induced increase of serum IL-4 and IL-6 (Th2 cytokines) levels and decrease of serum IL-12 and IFN-γ (Th1 cytokines) levels. Therefore, overexpression of IFN-α by adenoviral-mediated delivery provides modulation of pathogenic progression and protection of NOD mice from T1D.

Key Words: IFN-α, T1D, Adenoviral-mediated delivery, Immunomodulation

INTRODUCTION

Type 1 diabetes (T1D) is an organ-specific autoimmune disease caused by the T cell-mediated destruction of the insulin-producing β cells in the pancreatic islets of Langerhans (Atkinson and MacLaren, 1994; Bach, 1994). T1D patients can depend on insulin treatment for their survival, however, this treatment does not prevent eventual complications such as blindness, nephropathy, atherosclerosis, and microvascular disease. An important and well-characterized experimental animal model of T1D is the nonobese diabetic (NOD) mouse. NOD mice spontaneously develop T1D remarkably similar to that seen in humans (Delovitch and Singh, 1997). The onset of T1D is the consequence of a progressive destruction of islet β cells mediated by an imbalance between effector $CD4^+$ T helper (Th)1 and regulatory $CD4^+$ Th2 cell function (Daniel et al, 1995; Pilstrom et al, 1995; Rabinovitch et al, 1996; Shimada et al, 1996; Heurtier and Boitard, 1997). Th1 cells produce IFN-γ and Th2 cells secrete IL-4, and these cytokines can counterregulate development of the opposing cellular subset. This process in NOD mice is manifested by the fact that a period of local cytokine imbalance appears to cause polarization and emergence of either Th1 or Th2 response (Pilstrom et al, 1995; Rabinovitch et al, 1996; Shimada et al, 1996). Polarization to Th1 response induces rapid progression to T1D (Pilstrom et al, 1995), and the destructive insulitis of diabetes-prone NOD mice is associated with a relatively higher frequency of IFN-γ producing cells and lower frequency of IL-4-producing cells than found in mice protected from this disease (Rabinovitch et al, 1995). Whereas Th1 responses seem to produce disease, Th2-like responses have been associated with protection (Cameron et al, 1997; Gallichan et al, 1999), although homogeneous Th2 populations are unable to mediate protection from diabetogenic lymphocytes and, in fact, can cause disease under certain conditions (Pakala et al, 1997). The prospect of counterregulating pathological autoimmune Th1 cells in diabetes by promoting a protective (Th2) phenotype has generated considerable interest (Mueller et al, 1996; Cameron et al, 1997; Mueller et al, 1997).

Therefore, we sought to find a counterregulator of pathological autoimmune Th1 cells in diabetes by promoting a protective (Th2) phenotype. It has been reported that systemic injection of female NOD mice with recombinant IFN-α limits damage to islet β cells and prevents Th1-mediated destructive insulitis and T1D. We developed a gene therapy strategy using a replication-deficient adenovirus (Ad) vector expressing IFN-α as a more efficient and optimal means of sustaining cytokine expression and reducing pancreas inflammation. Ad vectors are well suited to

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ABBREVIATIONS: T1D, type 1 diabetes; NOD, non-obese diabetic; Th1, T helper cell type 1; Th2, T helper cell type 2; Ad, adenovirus; IFN, interferon.

efficiently deliver transgenes as therapeutic agents for T1D (Giannoukakis et al, 1999). Replication-deficient Ad vectors can be grown at very high density and maintain their genomes as episomal DNA in the nucleus of both proliferating and non-proliferating cells (Graham and Prevec, 1991). Systemic transient Ad-based cytokine overexpression has been successful in the treatment of several inflammatory disease models, including collagen-induced arthritis (Parks et al, 1998) and experimental inflammatory bowel disease (Hogaboam et al, 1997). In this study, we demonstrate that systemic IFN-α gene transfer achieved by injection of a recombinant replication-deficient human adenoviral-mediated IFN-α-expressing vector (Ad.hIFN-α) can protect NOD mice from the spontaneous development of T1D and thus establish a principle for the future development of adenoviral-based immunotherapy.

METHODS

Ad vectors

A E1-deleted recombinant serotype 5 adenovirus (Ad), containing a reporter beta-galactosidase (beta-Gal) gene under CMV promoter control (Ad.lacZ) was used for concurrent control. To generate our E1-deleted recombinant adenoviral vector encoding hIFN-α (Ad.hIFN-α), hIFN-α cDNA was introduced into the shuttle plasmid, pAvCvSv, under the transcriptional control of the cytomegalovirus (CMV) immediate early enhancer/promoter. The recombinant shuttle plasmid was co-transfected with the E1-deleted adenovirus serotype 5 genome, pJM17, into 293 cells (McGrory et al, 1988; Teng et al, 1994). Viruses were stored at $-80^{\circ}C$ until use. The number of viral particles was assessed by measurement of the optical density at 260 nm.

Mice

NOD mice were bred in a specific pathogen-free barrier facility at KRIBB (Daejeon, South Korea). Islet infiltration begins at 4~6 weeks of age in our colony of female NOD mice, and progression to destructive insulitis and overt diabetes occurs by 3~6 months of age. The incidence of diabetes in female NOD mice in our colony is 40~50% at 17 weeks of age and 80~90% by 30 weeks.

Treatment of NOD mice with Ad.hIFN-α

Seven weeks old NOD mice were intravenously injected via the tail vein with either 1×10^{11} particles of Ad.hIFN-α or Ad.lacZ. Blood glucose levels were weekly monitored with a Accutrend Sensor (Roche Diagnostics, Indianapolis, USA). Mice with a blood glucose levels >11.1 mmol/l (200 mg/dl) for 2 consecutive weeks were considered diabetic.

Western immunoblotting for the expression of IFN-α

Sera from NOD mice of each groups were mixed with Laemmli buffer. After SDS-polyacrylamide gel electrophoresis, the resolved proteins were transferred onto nitrocellulose membrane. Transferred membrane was incubated with polyclonal antibodies of hIFN-α and then incubated with HRP-conjugated IgG. After wash, Immunostaining with antibodies was performed using Chemiluminescent Substrate (Amersham Biosciences, Pittsburgh, PA, USA) and detected by LAS-1000PLUS (Fujifilm, Japan).

Histopathological analysis

Pancreatic tissue was removed, fixed with 10% buffered formalin, embedded in paraffin and sectioned at 5 um intervals. The incidence and severity of insulitis were examined by hematoxylin and eosin. The immunohistochemical identification of IFN-γ was performed using an anti-IFN-γ antibody and avidin-biotin peroxidase detection system (Dako, Carpinteria, CA, USA). Tissue sections with 5μm thickness were deparaffinized and rehydrated through a series of graded alcohols. The sections were processed in 0.05 M sodium citrate buffer (pH 6.0) and heated in a microwave for 10 min for antigene retrieval. The peroxidase was visualized with DAB (3,3'-diaminobenzidine tetrahydrochloride). Sections were counterstained with Mayer's hematoxylin and then coverslipped.

Measurement of serum IL-4, IL-10, IL-12 and IFN-γ

Serum pooled from three female NOD mice was collected after each inoculation *in vivo* with either Ad.hIFN-α or the Ad.lacZ control vector. It was necessary to pool the sera to meet the volume requirement of multiple assays described below. Sera were appropriately diluted and assayed for IL-4, IL-10, IL-12 and IFN-g content by ELISA (Pharmingen, San Diago, CA, USA).

Statistical analysis

Data were presented as means$\pm$SEM. Statistical comparisons between groups were performed with two-tailed Students's *t*-test. A value of $p<0.05$ was considered statistically significant.

RESULTS

Ad.hIFN- treatment elicits detectable levels of IFN-α in the sera of treated NOD mice

We tested the ability of intravenous injection of Ad.hIFN-α to yield high levels of serum IFN-α production in female NOD mice. Peak level of IFN-α (125 ± 48 ng/ml) was transiently expressed in the serum of NOD mice on the 3rd day and detected up to 6 days following Ad.hIFN-α injection. IFN-α was undetectable in sera from Ad.lacZ control vector-treated NOD mice (Fig. 1A). We also detected serum IFN-α in time series by Western blotting (Fig. 1B).

Gene transfer of IFN-α reduces destructive insulitis and protects against T1D in female NOD mice

The ability of Ad.hIFN-α to yield high levels of serum IFN-α production in female NOD mice prompted us to determine whether inoculation of this vector protects the mice from insulitis and/or T1D. A comparison of histological sections of pancreas from 30-week-old non-diabetic NOD female mice treated with Ad.hIFN-α or Ad.lacZ control revealed significant difference in the severity of insulitis of pancreatic islets (Fig. 2). In the pancreas of 30-week-old NOD mice treated with Ad.hIFN-α, almost all of the islets were either normal or showed only peri-insulitis whereas

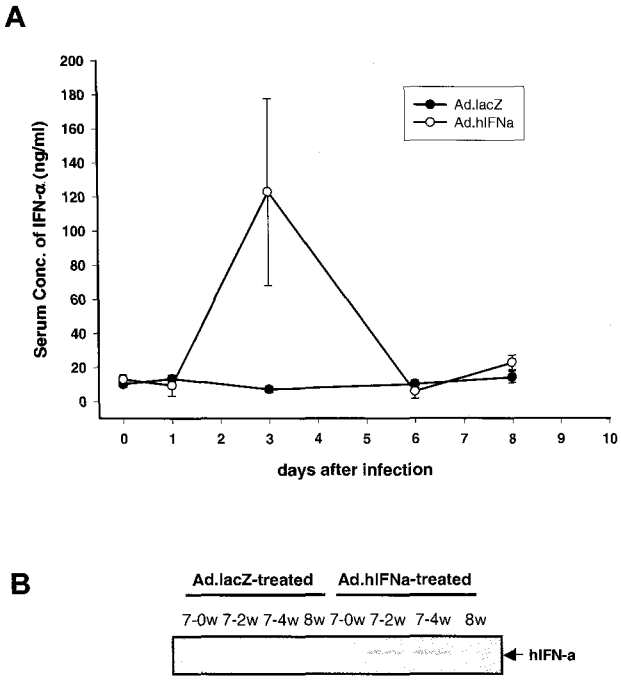

Fig. 1. Expression of IFN-α by injection of Ad.IFN-α. (A) Concentration of IFN-α measured by ELISA. The peak serum level of IFN-α was on the 3rd day after infection of Ad.hIFN-α. The expression of IFN-α was maintained for only one week. However, the concentration of IFN-α was much higher than control mice. (B) Western blotting by anti-IFN-α antibody. The expression of IFN-α was detected on the 2nd (7-2 w) and 4th (7-4 w) days after treatment with Ad.hIFN-α.

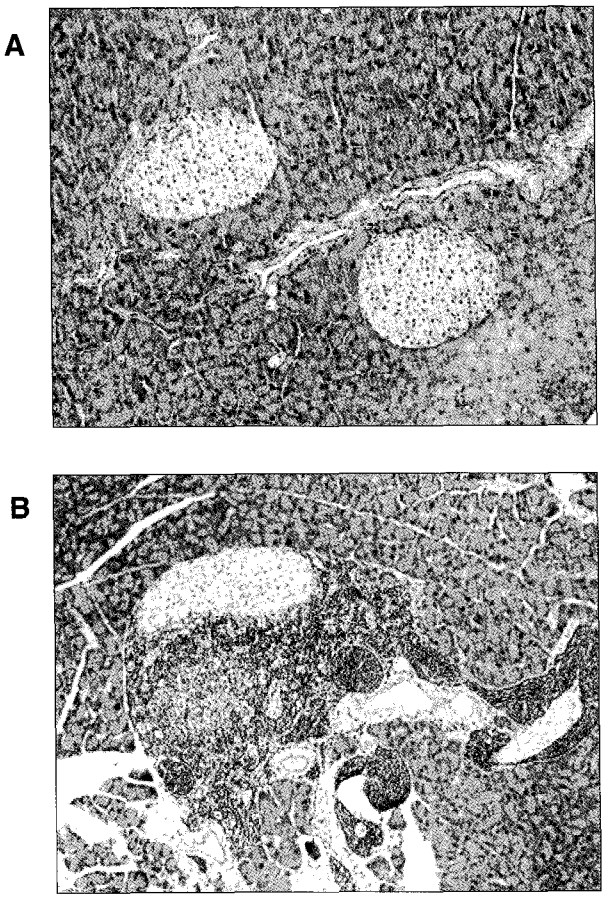

Fig. 2. H&E Staining of pancreatic islets of NOD mice aged 30 weeks ($\times$100). Ad.hIFN-α-infected mice shows intact pancreatic islets, while Ad.lacZ-infected mice show severe infiltration of lymphocytes. Infiltrating lymphocytes induce cell death in pancreatic islets and make progress to overt diabetes.

severe insulitis was observed in 85~90% of islets from Ad.lacZ control NOD mice. Thus, delivery of IFN-α using a replication-deficient Ad vector reduced destructive insulitis in NOD mice. The cumulative incidence of diabetes was lower in Ad.hIFN-α administered mice (20% vs 80% in control) (Fig. 3).

Ad.hIFN-α treatment modulates the production of Th1/Th2 cytokines

The onset of T1D is the consequence of a progressive destruction of islet β cells mediated by an imbalance between effector $CD4^+$ Th1 and regulatory $CD4^+$ Th2 cell function. We examined the levels of IL-12 and IFN-γ produced by Th1 cells, and those of IL-4 and IL-6 secreted by Th2 cells. The manifestation of local cytokine imbalance appears to cause polarization and emergence of either a Th1 or a Th2 response. Th1 cytokines, IL-12 and IFN-γ, gradually increased in Ad.lacZ-infected control mice around 9~13 weeks old and 9~15 weeks old, respectively, while Ad.hIFN-α infected mice showed the inhibition of secretion of Th1 cytokines (Fig. 4A, B). On the other hand, Th2 cytokines, IL-4 and IL-6, were increased in the serum after Ad.hIFN-α administration on 7 weeks of NOD mice, and the Ad.lacZ-infected control mice showed no modulation of Th2 cytokines (Fig. 4C, D).

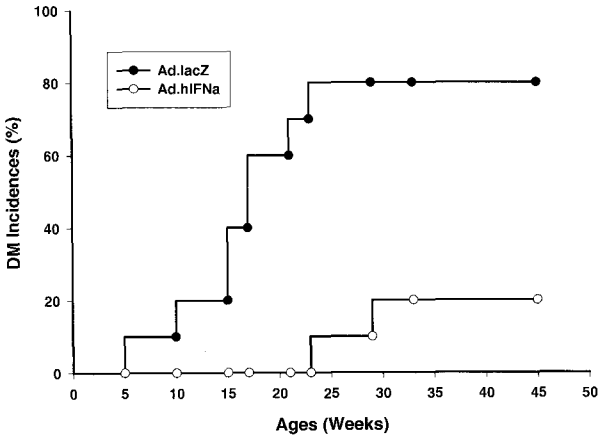

Fig. 3. A single administration of Ad.hIFN-α reduced the incidence of Type I diabetes in NOD mice. Twenty female NOD mice were injected with Ad.hIFN-α (n=10) or Ad.lacZ (n=10) at 7 week old and followed with weekly blood glucose determination. Data on time to overt diabetes in each group were analyzed by Kaplan-Meier survival curve which shows the percentage of diabetes occurring in relation to age.

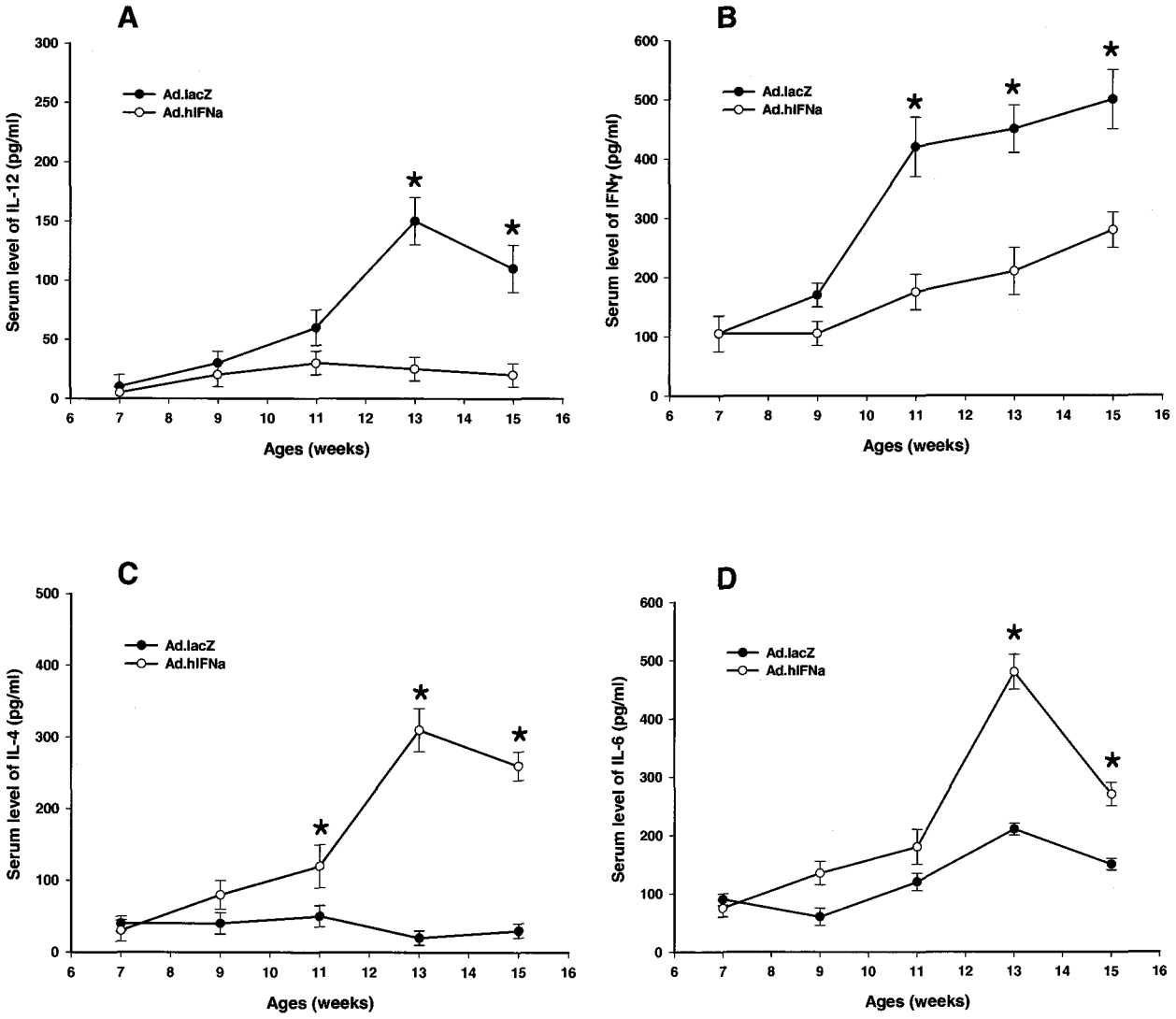

Fig. 4. Cytokine profiles in serum from Ad.lacZ-injected (closed circles) or Ad.hIFN-α-injected (open circles) NOD mice. (A,B) IL-12 and IFN-γ, gradually increased in Ad.lacZ-infected control mice around 9~13 weeks old and 9~15 weeks old, respectively, while Ad.hIFN-α infected mice showed the inhibition of secretion of Th1 cytokines. (C,D) Th2 cytokines, IL-4 and IL-6, were increased in the serum after Ad.hIFN-α administration on 7 weeks of NOD mice, and the Ad.lacZ-infected control mice showed no modulation of Th2 cytokines. *$p<0.05$.

Detection of IFN-γ in destructive islets of T1D

The progressive destruction of pancreatic islets was caused by infiltration of T lymphocytes and secretion of Th1 cytokines. Immunohistochemical analysis revealed that IFN-γ was detectable in infiltrating area of T lymphocytes and remnant β-cells of destructive islets (Fig. 5). However, IFN-γ was not detected in pancreatic islets of IFN-α infected mice.

DISCUSSION

Multiple low doses of recombinant IFN-α administered intraperitoneally or ingestion of IFN-α protect NOD mice from T1D (Brod et al, 1998; Sobel et al, 1998). The short *in vivo* half-life of IFN-α necessitates that it should be injected frequently, ie three times a week for 8~10 weeks. To enhance the potential efficacy of systemic IFN-α therapy of T1D, we took an approach to reduce pancreas inflammation and onset of overt diabetes by replication-deficient adenoviral-based hIFN-α (Ad.hIFN-α) gene delivery. Here, we demonstrated that only a prophylactic injection of Ad.hIFN-α could transiently increase *in vivo* serum IFN-α to high levels and profoundly reduced the incidence of T1D in female NOD mice. The transient high level and one week presence of IFN-α provided a notable improvement over the short serum half-life of an injected recombinant IFN-α. Several properties make the replication-deficient Ad vectors good candidates for the expression of transgenes. However, the main problem associated with the therapeutic use of Ad vectors is their immunogenicity (Giannoukakis et al, 1999). Anti-viral cellular and humoral immune responses may preclude the stable gene expression

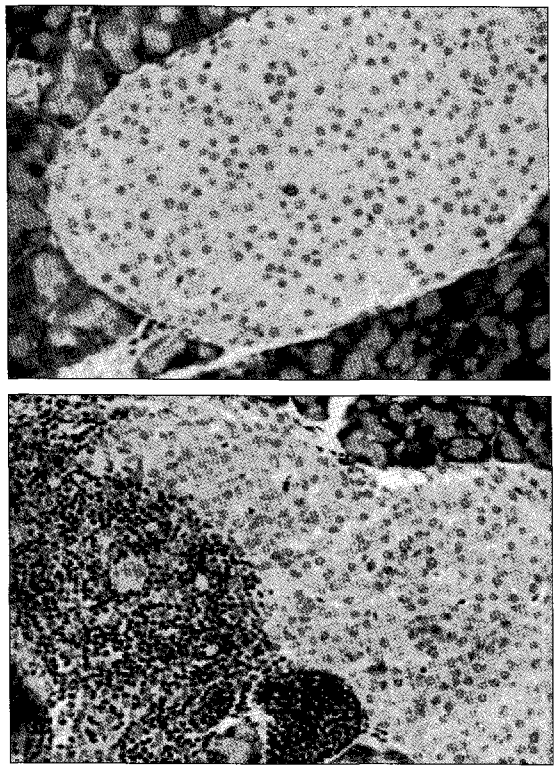

Fig. 5. Immunohistochemical analysis for secretion of IFN-γ in pancreatic islets of 15 week old NOD mice ($\times$400). IFN-γ could not be detected in pancreatic islets of Ad.hIFN-α-infected mice, while Ad.lacZ-infected mice showed production of IFN-γ in pancreatic islet cells.

and repeated dosing that treatment of chronic diseases may require. However, in the present study, a single injection of Ad.hIFN-α elicited high level of serum IFN-α and transient expression reduced diabetes incidence. Therefore, we suggest that immunological modulation can be induced by a potent stimulation for a relatively short period.

Ad.hIFN-α treatment appears to regulate autoreactive T cells in the pancreas to suppress islet β cell destruction and progression to overt T1D. Evidence in support of this notion is derived from analyses of the levels of expression of Th1/Th2 cytokines in the serum of Ad.hIFN-α treated NOD mice at 7~15 weeks of age. The increase of IL-4 and IL-6 and significant down-regulation of IL-12 and IFN-γ expression elicited by Ad.hIFN-α treatment may explain its ability to protect NOD mice from diabetes. The persistent effect of an apparent Th2 class shift noted here is consistent with reports demonstrating that the presence of specific cytokines at the initiation of an immune response can lead to the generation of both effector and long-lived memory T cell populations (Swain, 1994).

Future experiments may benefit from the use of less immunogenic Ad-based gene transfer vehicles (Giannoukakis et al, 1999). Helper-dependent Ad vector systems that have reduced immunogenicity and rely on a complementing virus to provide the necessary proteins in *trans* for packaging are also available. This system utilizes a helper virus that has packaging sequences flanked by *lox*P sites, therefore, in transduced cells that stably express the Cre recombinase, the packaging signal is efficiently excised, thus rendering the helper virus unpackagable (Parks et al, 1999). In addition, adeno-associated virus (AAV) vectors possess low immunogenicity, but generally afford low-level gene transfer. Transduction by AAV vectors can be enhanced in the presence of Ad gene products through the formation of double-stranded, non-integrated AAV genomes, which elicit high-level and stable transgene expression in mice after intramuscular injection of recombinant AAV (Fisher et al, 1997).

The objective of this study was to induce immune deviation and modify the pathological mechanisms occurring in the development of T1D by gene transfer of IFN-α. To our best knowledge, this is the first report to demonstrate that IFN-α can protect NOD mice from spontaneous diabetes by an adenovirus-based systemic gene delivery approach. Our experimental approach, which makes use of transient IFN-α gene transfer by means of a replication-deficient Ad vector, offers the prospect of studies on the effects of cytokines as well as approaches that may favorably modify autoimmune responses with minimal intervention.

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