

Adenovirus Early Region 3 (E3) Immunomodulatory Genes Decrease the Incidence of Autoimmune Diabetes in NOD Mice

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The early three (E3) region of the adenovirus (Ad) encodes a number of immunomodulatory proteins that interfere with class I major histocompatibility-mediated antigen presentation and confer resistance to cytokine-induced apoptosis in cells infected by the virus. Transgenic expression of Ad E3 genes under the rat insulin II promoter (RIP-E3) in β -cells in nonobese diabetic (NOD) mice decreases the incidence and delays the onset of autoimmune diabetes. The immune effector cells of RIP-E3/NOD mice maintain the ability to infiltrate the islets and transfer diabetes into NOD-*scid* recipients, although at a significantly reduced rate compared with wild-type littermates. The islets of RIP-E3/NOD mice can be destroyed by adoptive transfer of splenocytes from wild-type NOD mice; however, the time to onset of hyperglycemia is delayed significantly, and 40% of these recipients were not diabetic at the end of the experiment. These findings suggest that expression of E3 genes in β -cells affects both the activation of immune effector cells and the intrinsic resistance of β -cells to autoimmune destruction. *Diabetes* 50:980–984, 2001

Type 1 diabetes is caused by a cell-mediated autoimmune destruction of the insulin-producing pancreatic islet β -cells (1). At present, there is no available treatment that can prevent this autoimmune process in humans. The current treatment of type 1 diabetes involves the administration of exogenous insulin, which does not accurately control glycemia and cannot avoid hypoglycemic episodes or complications caused by hyperglycemia. Genetic engineering of β -cells with immunomodulatory genes holds the promise of de-

velopment of novel approaches for prevention of type 1 diabetes as well as for β -cell replacement by transplantation. A number of genes have been shown to increase the resistance of β -cells to immune attacks or provide them with the ability to downregulate immune effector mechanisms (2). Genes evolved by viruses to evade immune responses against virus-infected cells represent an attractive source for such immunomodulatory elements (3).

The early three (E3) region of an adenovirus (Ad) includes a number of genes that encode proteins that prevent antigen presentation on infected cells and inhibit cytokine-induced apoptosis of such cells (4). A 19-kDa glycoprotein (gp19) downregulates class I major histocompatibility complex (MHC)-mediated antigen presentation to cytotoxic T-lymphocytes (CTL) by two mechanisms. The viral protein binds to the heavy chain of the class I MHC molecule and prevents its transport from the endoplasmic reticulum to the cell surface (4); in addition, gp19 inhibits the transporter associated with antigen presentation (TAP) and prevents loading of class I MHC molecules (5). Three other proteins encoded in the E3 region (10.4K, 14.5K, and 14.7K) inhibit tumor necrosis factor- α (TNF- α)-induced apoptosis (4,6,7). The 10.4K and 14.5K proteins function as a complex to inhibit both TNF- α - and Fas-induced apoptosis (8,9). We have demonstrated that expression of E3 genes in β -cells in transgenic mice under control of the rat insulin II promoter (RIP) prolongs the survival of islet allografts (10). In addition, expression of the E3 transgenes prevented autoimmune cytolysis of β -cells transgenically expressing individual antigens of the lymphocytic choriomeningitis virus (LCMV) when these mice were infected with LCMV (11). These studies suggest that expression of E3 genes in β -cells may prevent their destruction by class I MHC-, TNF- α - and Fas-dependent mechanisms and/or alter the ability of such islets to stimulate immune responses.

Here we describe the effect of E3 gene expression in pancreatic β -cells on a mouse model of spontaneous autoimmune diabetes, the nonobese diabetic (NOD) mouse. NOD female mice develop a disease very similar to human type 1 diabetes, initiated by infiltration of inflammatory cells into the islets (insulinitis) at 4–8 weeks of age (12). Recognition of both MHC class I- and class II-restricted antigens triggers the local release of cytokines (such as interleukin-1 β , TNF- α , and interferon- γ) and free radicals (such as nitric oxide) from the inflammatory cells, which are thought to induce β -cell apoptosis (13). β -Cells

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Ad, adenovirus; AECOM, Albert Einstein College of Medicine; CTL, cytotoxic T-lymphocytes; E3, early three; gp19, 19-kDa glycoprotein; TJL, The Jackson Laboratory; LCMV, lymphocytic choriomeningitis virus; MHC, major histocompatibility complex; PCR, polymerase chain reaction; RIP, rat insulin II promoter; TAP, transporter associated with antigen presentation; TNF- α , tumor necrosis factor- α .

are particularly sensitive to the effects of free radicals because of their low expression of antioxidant enzymes (14). In addition, the expression of TNF- α in islets has been shown to promote diabetes in neonatal NOD mice by enhancing islet antigen presentation (15,16). Our findings demonstrate that expression of the E3 genes in NOD mice decreases the destruction of β -cells and slows the development of diabetes. Although insulinitis occurs, it is significantly reduced, and destruction of the target cells by infiltrating inflammatory cells appears to be similarly decreased. However, the immune system in these mice maintains its capacity to destroy β -cells, as demonstrated by adoptive transfer experiments. The ability of E3 gene expression in β -cells to both prevent islet damage in NOD and LCMV murine models of type 1 diabetes and facilitate survival of allogeneic transplanted islets (10) makes it a potential candidate for gene therapy of type 1 diabetes.

RESEARCH DESIGN AND METHODS

Generation of RIP-E3/NOD mice. The original RIP-E3 transgenic line was generated in B6D2/F2 mice and maintained by back-crossing into C57BL/6 (B6) background (10). To generate RIP-E3/NOD mice, the RIP-E3 B6 mice were backcrossed with NOD mice for 11 generations. The presence of the transgene was determined by polymerase chain reaction (PCR) analysis of tail DNA, as previously described (10). At the eighth backcross generation, RIP-E3 transgenic mice were typed by PCR for the previously described set of microsatellite markers (17) delineating all known genetic loci of NOD origin that contribute to diabetes susceptibility. All mice used in this study (from the 9th to 11th backcross) were derived from two 8th backcross females found to be homozygous for NOD alleles of all of these markers. Animals were followed until 30 weeks of age. Rates of type 1 diabetes development in the indicated experimental groups were assessed for statistically significant differences by Kaplan Meier life table analysis using the Statview 4.5 computer software program (Abacus Concepts, Berkeley, CA).

Glucose measurements. Starting at 2 months of age at the Albert Einstein College of Medicine (AECOM), RIP-E3/NOD and nontransgenic littermate female mice were monitored weekly for plasma glucose levels using Glucometer II strips. Values >400 mg/dl on two consecutive weekly determinations were considered to be diabetic. At The Jackson Laboratory (TJL), glycosuria was monitored with the Ames Diastix (supplied by Miles Diagnostics, Elkhart, IN). Values ≥ 3 were considered diagnostic for the onset of diabetes.

Histological analyses. The pancreas was removed from killed animals, fixed in buffered formaldehyde, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin.

Adoptive transfer of splenocytes. Splenic leukocytes were isolated as previously described (18) from the indicated donors. In some experiments, 1×10^7 splenic leukocytes from prediabetic (6 weeks old) or overtly diabetic NOD female donors were injected intravenously into sublethally irradiated (700R) 6- to 8-week-old NOD or RIP-E3/NOD female recipients. In other experiments, 2×10^7 splenic leukocytes isolated from 6-week-old NOD or RIP-E3/NOD female donors were injected intravenously into 4- to 6-week-old T- and B-cell-deficient NOD/*scid* female recipients. These experiments were terminated at 21 weeks posttransplantation. All animal studies were approved by the animal institute committees of AECOM and TJL.

RESULTS

Incidence of diabetes in RIP-E3/NOD mice. To examine the effect of the E3 gene products on the development of diabetes in NOD mice, plasma glucose levels were monitored in 9th–11th backcross generation transgenic and nontransgenic littermate female mice. These animals were demonstrated to be homozygous for NOD alleles at markers delineating all known genetic loci that contribute to development of the disease (data not shown). The animals were evaluated for the protective effects of the Ad E3 genes in separate experiments either at AECOM or at TJL, where the incidence of diabetes in NOD controls was known to be higher. As seen in Fig. 1, by 21 weeks of age,

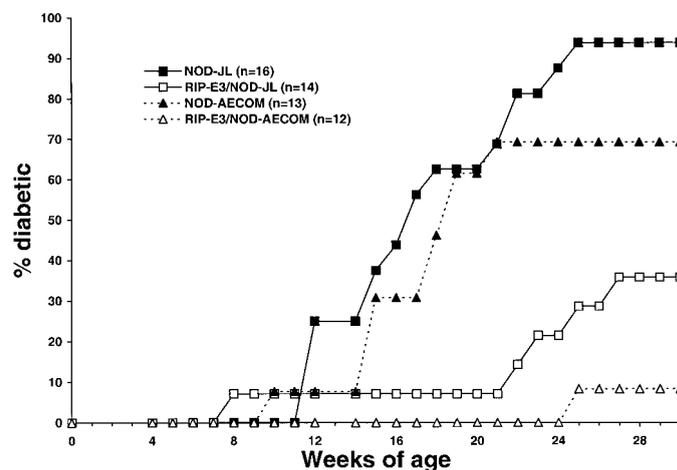


FIG. 1. Decreased incidence of diabetes in the RIP-E3/NOD transgenic mice. RIP-E3/NOD and nontransgenic NOD littermate female mice in the 11th backcross were monitored weekly for plasma glucose levels or glycosuria. Mice were considered diabetic according to criteria detailed in RESEARCH DESIGN AND METHODS. AECOM, AECOM colony; JL, TJL colony.

67% of the nontransgenic NOD females in the 11th backcross at AECOM developed hyperglycemia. In contrast, all of the RIP-E3/NOD females at AECOM remained euglycemic until 25 weeks of age, when 1 of 13 became diabetic. The incidence of diabetes at TJL was higher both in the NOD controls (93%) and in the RIP-E3/NOD animals (36%), but the delayed onset in the latter group was again noted. There was a statistically significant difference between RIP-E3/NOD and wild-type littermates at each institution ($P < 0.0001$ at TJL, $P = 0.0012$ at AECOM). Subsequent constructs of new transgenic NOD mice containing E3 genes have proven that the E3 effect on the incidence of diabetes is not attributable to a positional insertion effect of the transgene.

Histological analysis of RIP-E3/NOD mice. To determine whether the E3 gene products also retarded the development of insulinitis, the pancreases from 14-week-old prediabetic female mice in the 11th backcross at AECOM were subjected to histologic analysis. The pancreases of both transgenic (Fig. 2) and nontransgenic littermates (data not shown) contained islets with varying degrees of insulinitis as well as islets free of inflammatory cells. Quantitation of islets into four grades of insulinitis revealed a lower insulinitis score for the transgenic mice compared with the nontransgenic littermates (Table 1). Because these two groups developed insulinitis at quantitatively different rates, a qualitative comparison by histochemistry of cell types comprising the insulinitic lesions in age-matched standard and RIP-E3 transgenic NOD mice would not reveal mechanistic information about the basis of E3-induced type 1 diabetes resistance. Hence, alternative studies of mechanism were pursued.

Adoptive transfer experiments. Prevention of the development of diabetes in the RIP-E3/NOD mice might result from an aberrant stimulation of the immune system by the β -cells or from an increased resistance of β -cells to immune effector mechanisms. To evaluate both of these possibilities, several types of adoptive transfer experiments were performed. Splenocytes from 6-week-old RIP-E3/NOD or standard NOD female donors were transferred

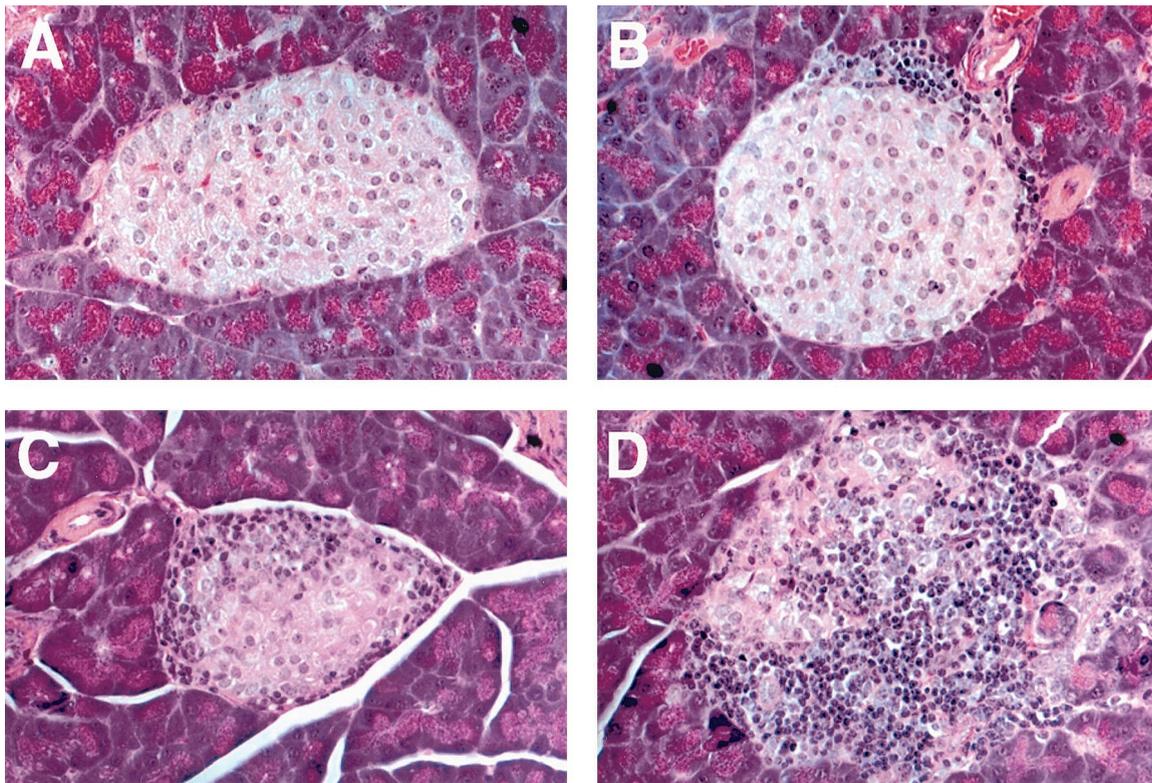


FIG. 2. Histological analysis of pancreatic islet sections from 14-week-old RIP-E3/NOD female mice. The pancreases were fixed in formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The islets shown represent four grades of insulinitis: *A*, grade 0, absence of insulinitis; *B*, grade 1, peri-insulinitis; *C*, grade 2, light infiltration involving <25% of the islet area; *D*, grade 3, heavy infiltration involving >25% of the islet area. Magnification is $\times 217$.

into NOD-severe combined immunodeficient (*scid*) female mice, which do not develop diabetes spontaneously due to their immunodeficiency (19). As seen in Fig. 3 data obtained in the TJL colony, ~80% of recipients transplanted with splenocytes from euglycemic nontransgenic NOD donors developed hyperglycemia within 15–16 weeks. Among recipients transplanted with splenocytes from RIP-E3/NOD donors, 40% of the mice developed hyperglycemia within the same period of time. These differences, which are statistically significant ($P = 0.042$), demonstrate that immune effector cells that are primed and mature in animals expressing the E3 genes can transfer diabetes into the NOD-*scid* recipients, but they do so at the reduced rate characteristic of their endogenous function. These results support the conclusion that there are changes in the donor

cell-mediated immune capacity as a result of expression of the RIP-E3 transgene. However, the following experiments suggest that there were also local effects of the RIP-E3 transgene in islet β -cells.

TABLE 1
Quantitation of insulinitis in RIP-E3/NOD mice

Genotype	Mice	Islets	Insulinitis grades (%)				Insulinitis score
			0	1	2	3	
Nontransgenic	3	45	46	9	7	38	1.32
RIP-E3	4	67	69	12	6	13	0.63

The pancreases from 14-week-old RIP-E3/NOD and nontransgenic littermate female mice from the 11th backcross were subjected to histological analysis. Insulinitis grades (shown in Fig. 2) were defined as follows: 0, no inflammatory cells visible inside the islet or in its proximity; 1, peri-insulinitis; 2, light infiltration involving <25% of the islet area; 3, heavy infiltration involving >25% of the islet area. A comparison of variance in the incidence of insulinitis grades between the two groups showed a significant difference ($P = 0.0006$).

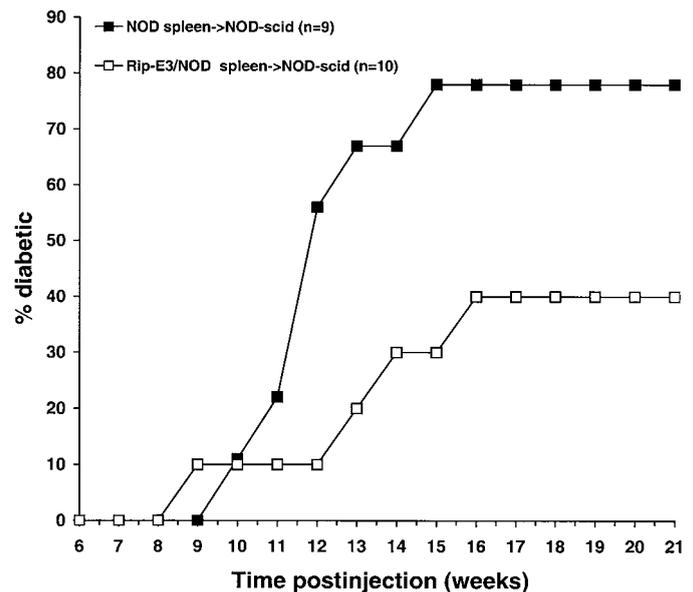


FIG. 3. Incidence of diabetes using splenocytes transferred from RIP-E3/NOD into NOD-*scid* mice. Splenocytes were harvested from 6-week-old NOD mice before the onset of hyperglycemia and from age-matched RIP-E3/NOD littermates as described in RESEARCH DESIGN AND METHODS. NOD splenocytes or RIP-E3/NOD splenocytes were injected into NOD-*scid* mice. Glycosuria was monitored weekly as described in RESEARCH DESIGN AND METHODS.

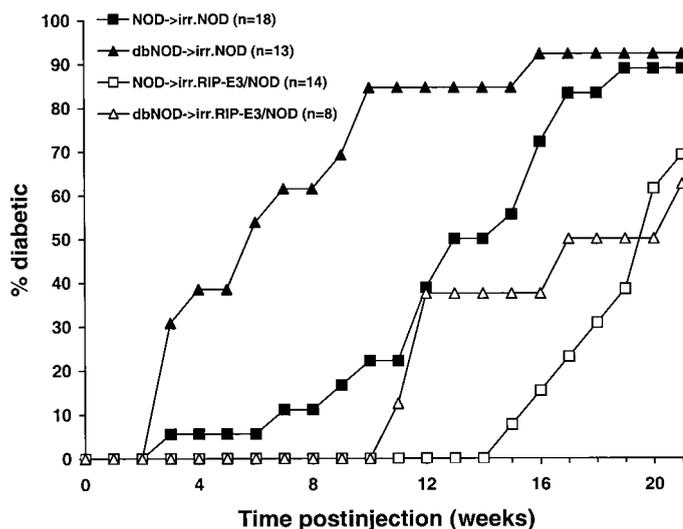


FIG. 4. Incidence of diabetes using splenocytes transferred from NOD mice into irradiated RIP-E3/NOD mice. RIP-E3/NOD animals and NOD littermate controls were irradiated (irr.) with 700R at 6 weeks of age and transplanted with splenocytes from either prediabetic or diabetic (db) NOD animals. Urinary glucose levels were measured at the indicated times after cell transfer.

To test whether the transgenes reduced the susceptibility of β -cells to immune destruction, the E3-expressing β -cells were exposed to immune effector cells that developed in the wild-type NOD environment. For this purpose, the RIP-E3/NOD transgenic mice and nontransgenic NOD controls were sublethally irradiated and transplanted with splenocytes harvested from either young prediabetic or overtly diabetic NOD female donors. As expected, splenocytes from overtly diabetic donors transferred disease more rapidly than those from prediabetic donors. However, in both cases, the onset of diabetes in RIP-E3 recipients was significantly delayed compared with standard NOD recipients ($P = 0.0049$ for prediabetic donor cells, and $P = 0.035$ for overtly diabetic donor cells). The results of these experiments, which were performed at TJL, are shown in Fig. 4.

DISCUSSION

These results demonstrate that expression of adenovirus E3 genes in β -cells in the NOD background decreases the incidence and delays the development of diabetes. These results were obtained with identical transgenic animals housed in two different institutions at which the rate of diabetes differed between 67 and 92% for the parental female NOD strain. The E3 gene products did not prevent the development of some insulinitis in the RIP-E3/NOD animals, even when the rate of diabetes was reduced to <10% in this population. However, the amount of insulinitis was significantly reduced in the presence of the E3 genes. Because it was unlikely that analysis of the quantitatively heterogeneous mononuclear infiltrates would yield an understanding of the Ad E3 regulatory process, we are performing other types of experiments to determine the nature of the effector cells that are altered in the presence of β -cell Ad E3 gene expression.

The mechanism of protection against diabetes by the Ad E3 gene products is not known, in part because of the multigenic nature and multiple pathways potentially al-

tered by the E3 genes. In addition to the inhibitory effects on cell death by inhibiting class I MHC-mediated antigen presentation and TNF- α - and Fas-initiated apoptosis by various E3 proteins, the E3-14.7K protein has been shown to interact with important regulatory molecules on the nuclear factor- κ B signal transduction pathway (20,21) and with a small guanosine 5'-triphosphatase that can complex to a component of dynein (22). Mapping studies are in progress to dissect the Ad E3 genes responsible for the inhibition of autoimmune diabetes in the LCMV-induced model as well as for the decrease of disease in the NOD mice. Animals that are transgenic for the Ad E3 cassette designed with various deletions have been constructed for these experiments, which are in progress. Although these experiments are not yet completed, it is clear that NOD transgenics expressing new constructs of E3 genes are also protected from diabetes. These recent results indicate that the effects of the E3 transgenes demonstrated in the current article are not attributable to insertional mutagenesis.

None of the Ad E3-coded proteins has been shown to be secreted. Thus, they should effect only cells in which they are synthesized or immunological reactions that depend on recognition of such cells. The insulin promoter is primarily active in pancreatic β -cells, but it has shown some activity in testis; the RIP II version used here has also been shown to be active at low levels in thymus (23,24). However, from adoptive transfer experiments, it appears that the RIP-driven E3 proteins affect both the activation and/or development of immune effector cells as well as the susceptibility of β -cell targets to destruction by fully activated immune effector cells. The two effects are likely to be interrelated, because the initial damage to β -cells may reinforce the immune response by providing further stimuli to immune effector cells. Adoptive transfer studies have been initiated to determine which immunologic cell types are altered during the reduction of the diabetogenic phenotype in NOD mice as a result of RIP-E3 transgene expression.

Whereas the reduction in diabetes by Ad E3 gene products is complete in the LCMV model and readily apparent in the NOD model, it is clear that the activity of the viral genes must be further enhanced before a useful prophylactic or therapeutic approach to diabetes in humans would be feasible using these principles. Although the system might be further optimized by increased expression of the E3 proteins, it also may be improved by deletion of E3 genes with potentially harmful effects. One example is an 11.6-kDa proapoptotic protein, also called the "adenovirus death protein," that is thought to function in viral release from the nucleus at the end of the infectious cycle (25). Thus, viral genes evolving with an infectious agent to modify the host immune and cytokine responses to favor survival of the pathogen might prove to be useful for controlling the host response to endogenous stimuli that promote autoimmune diseases. Unlike immunomodulation approaches based on gene targeting, such as the β_2 -microglobulin knockout animals (26,27), the E3 genes could be introduced into β -cells as dominant transgenes using viral vectors to modulate the immune response to human islets before transplantation.

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