Adenovirus Early Region 3 Antiapoptotic 10.4K, 14.5K, and 14.7K Genes Decrease the Incidence of Autoimmune Diabetes in NOD Mice

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Genes in the early region 3 (E3) of the adenovirus genome allow the virus to evade host immune responses by interfering with major histocompatibility (MHC) class I–mediated antigen presentation and tumor necrosis factor-α (TNF-α)– or Fas-induced apoptosis of infected cells. Autoimmune type 1 diabetes (T1D) is inhibited in NOD mice transgenically expressing all E3 genes under control of a rat insulin promoter (RIPE3/NOD). For dissecting the protective mechanisms afforded by various E3 genes, they were subdivided into RIP-driven transgene constructs. Strong T1D protection mediated at the β-cell level characterized DL704/NOD mice lacking the E3 gp19K gene suppressing MHC class I expression but retaining the 10.4K, 14.5K, and 14.7K genes inhibiting Fas- or TNF-α–induced apoptosis and TNF-α–induced NF-κB activation. Much weaker protection characterized DL309/NOD mice expressing the gp19K but not the 10.4K, 14.5K, and 14.7K genes. While RIPE3/NOD splenocytes had an unexpected decrease in ability to adoptively transfer T1D, splenocytes from both the DL704 and DL309 stocks efficiently did so. These findings indicate that all E3 genes must be expressed to inhibit the diabetogenic potential of NOD immune cells. They also demonstrate that the antiapoptotic E3 genes most effectively protect pancreatic β-cells from diabetogenic immune responses. Diabetes 52:1119–1127, 2003

Type 1 diabetes (T1D) is caused by T-cell–mediated autoimmune destruction of insulin-producing pancreatic β-cells within the islets of Langerhans (1). Currently, treatment of patients with T1D involves administration of exogenous insulin to control hyperglycemia. However, this therapy cannot accurately control glycemia; hence, complications associated with such abnormal regulation are difficult to avoid. The NOD mouse spontaneously develops T-cell–mediated autoimmune T1D similar to the human disease (rev. in 2).

The destruction of pancreatic β-cells in NOD mice requires contributions from both major histocompatibility (MHC) class I– and class II–restricted autoreactive T-cells. Pancreatic β-cells express MHC class I but not class II molecules (3). Hence, β-cells can directly present internally derived MHC class I–bound antigenic peptides to autoreactive CD8 T-cells that then exert cytotoxic activity. In contrast, the MHC class II–bound antigenic peptides recognized by diabetogenic CD4 T-cells are displayed on the surface of hematopoietically derived antigen-presenting cells (APCs), which generate such structures through an ability to internalize and process externally derived pancreatic β-cell proteins. Once stimulated, such CD4 T-cells produce cytokines that provide a source of help for the activation of the cytotoxic CD8 T-cells. There are also additional mechanisms by which the cytokines produced by CD4 T-cells, as well as macrophages, contribute to pancreatic β-cell destruction in NOD mice. Locally released cytokines in the insulitic lesions include IL-1β, tumor necrosis factor-α (TNF-α), and γ-interferon as well as the free radical nitric oxide, all of which have been implicated as contributors to β-cell destruction (4–6). IL-1β, TNF-α, and γ-interferon have been shown to upregulate Fas levels on the surface of β-cells and to increase β-cell sensitivity to apoptotic death induced by FasL molecules expressed by both autoreactive CD4 and CD8 T-cells (7–9). Exacerbating this issue is the fact that pancreatic β-cells are particularly sensitive to cytokine-induced oxidative stress because they normally express very low levels of antioxidant enzymes (10). Hence, if a genetic engineering approach that renders pancreatic β-cells resistant to this collection of autoimmune processes could be developed, then it could provide a means of preventing T1D or reversing it through improved islet transplantation protocols.

The adenovirus genome contains at least five genes within the early region 3 (E3) that encode proteins used by the virus to evade the host immune system (rev. in 11). Within the E3 is a gene encoding a 19-kDa glycoprotein (gp19K) that downregulates the MHC class I presentation of antigenic peptides by 1) binding to the heavy chain of the class I MHC retaining it in the endoplasmic reticulum and 2) preventing tapasin processing of peptides that bind to MHC class I molecules (11). The combined functions of the gp19K glycoprotein impairs the transport of MHC class I–bound antigenic peptides to the cell surface, thus limiting cytotoxic T-cell responses. Also encoded within the E3 are the 14.7K, 10.4K, and 14.5K proteins, which serve to
The 14.7K protein has been shown to decrease TNF-α–induced apoptosis (12), although its exact mechanism of action has not been fully elucidated. Inhibition of apoptosis by the 14.7K protein does not involve alterations in the expression pattern of TNF-α receptors (13). However, it does seem to inhibit the synthesis of arachidonic acid induced by TNF-α, which could affect the apoptotic pathway as well as the inflammatory response (11,12). The AdE3 10.4K and 14.5K proteins form a heterotrimer (two molecules of 10.4K and one molecule of 14.5K) that inhibits FasL-mediated apoptosis by internalizing Fas receptors on the surface of target cells and subsequently causing their degradation in lysosomes (11,14). As a result of this mechanism, the 10.4K and 14.5K proteins have been renamed RIDo and RID6 to better describe their function of receptor internalizing and degradation, which extends to the TRAIL but not to the TNF receptor (15). Recently, the Ad E3 10.4K/14.5K complex was shown to inhibit the activation of NF-κB, and there is an additional antipapoptotic E3 function that prevents the induction of chemokines by TNF-α (16,17). In contrast to the other E3 encoded proteins, the 11.6K protein, also known as adenovirus death protein, induces cell death. The regulation of 11.6K expression is different from that of the antipapoptotic proteins in the E3 because it is turned on by a strong late promoter identical to what upregulates the synthesis of structural proteins necessary to make progeny virions (11). Among its functions, adenovirus death protein has been shown to facilitate viral exit from the nucleus of the infected cell.

Given the importance of both MHC class I–restricted T-cells and apoptosis inducing cytokines in the development of T1D in NOD mice, we previously tested whether disease protection could be achieved by inducing adenovirus E3 gene expression in pancreatic β-cells. This was indeed the case, because insulitis and T1D development are decreased in NOD mice transgenically expressing all E3 genes under control of a rat insulin promoter (designated RIPE3/NOD mice) (18). As hoped for, disease protection was associated with E3 gene expression rendering β-cells resistant to the autoreactive effector mechanisms used by NOD mice. Unexpectedly, transgene expression also directly or indirectly impaired the development or functional capacity of β-cell autoreactive effectors, because splenocytes from RIPE3/NOD mice were less efficient than those from standard NOD donors in adoptively transferring T1D to lymphocyte-deficient NOD-scid recipients (18). Hence, we wished to assess the relative contributions of E3 genes suppressing MHC class I antigen presentation (gp19K) versus those inhibiting cytokine-induced apoptosis (10.4K, 14.5K, and 14.7K) in inducing T1D resistance at either the β-cell target or immune effector cell level. This was done through analyses of NOD mice expressing different subsets of RIP-regulated E3 transgenes.

**RESEARCH DESIGN AND METHODS**

**Mice.** NOD/Lt mice are maintained at The Jackson Laboratory by brother-sister mating. Currently, T1D develops in ~90% of female and ~70% of male NOD/Lt mice by 30 weeks of age. The previously described stock of T- and B-cell–deficient NOD-scid mice (official designation NOD.CB17-Prkdcscid) are maintained at the N11 backcross generation (19). Generation of an N12 backcross stock of NOD mice congenic for a RIP-driven E3 transgene cassette has also been previously described (18). At the eighth backcross generation (N9), these mice were typed by PCR analyses for a previously described set of polymorphic microsatellites (20) and confirmed for E3 loci contributing to diabetes susceptibility or resistance (Idd loci). All RIPE3/NOD mice used in this study originated from N9 females found to be homozygous for markers delineating all known Idd loci of NOD origin.

Two other RIPE3 deletion constructs were transgenically introduced directly into fertilized NOD oocytes (Cell Biology and Microinjection Service, The Jackson Laboratory, Bar Harbor, ME). The RIPE3DL704 construct was derived from an Ad 2/5 recombinant virus that has a deletion of 292 bp in the Ad gp19K gene (21). The Ad DL704 genomic DNA was digested with KpnI/NheI, which cuts well upstream of the first open reading frame of Ad E3 and downstream of the poly A site, respectively. This Ad DNA fragment was subcloned into pUC18 at the SalI site and then removed by Bsp120I/SalI digestion. The resulting Ad fragment was inserted behind the RIP promoter by cutting the RIP-I/PA construct with XbaI/SalI, and subsequent ligation of the Ad fragment into the 3′ SalI site and blunt ended ligation into the 5′ site. The construct was linearized through an upstream BamHI site before yzotic microinjection. Litters were screened for transgenic founders by the same PCR protocol used to detect the original RIPE3 construct (22).

The RIPE3DL299 construct is derived from an Ad5 virus that has a 750-bp deletion disrupting the 10.4K, 14.5K, and 14.7K genes. The deletion removes base pairs 2674–5242 in the E3, with an insertion of 642 bp of salmon sperm DNA sequences. The AdDL309 construct also has a 642-bp deletion in the Ad 6.7K gene. This construct was digested with SalI, and the resulting fragment was cloned into the pUC18 SalI site. The Ad DNA was removed by Smal/SalI digestion and inserted behind the RIP promoter as described for the DL704 construct and linearized with BamHI before yzotic microinjection. Litters were screened by PCR for founders using the following primer set: Int309s 5′-CTCCTGCCCAGTGTTGCTACCAT-3′ and Int309as 5′-GACCTACGGGAGAATGCAT-3′.

**Analysis of E3 gene expression.** Islets were isolated from pancreata of RIPE3/NOD, DL309/NOD, or DL704/NOD mice as previously described (24). Islets from four mice of each strain were pooled, and RNA was extracted using TRI REAGENT (Sigma, St. Louis, MO) according to the manufacturer’s protocol. Total RNA was treated with DNAase 1 (Invitrogen, Carlsbad, CA) to remove any contaminating genomic DNA and reverse-transcribed using an Advantage RT-For PCR Kit (Clontech, Palo Alto, CA). The previously described Ad2–53 and Ad2–88 primers (25) were used to detect gp19K (1,390 bp) and 11.6K (601 bp) mRNA transcripts in RIPE3/NOD and DL704/NOD mice. Because of the presence of an Ad5 rather than an Ad2 backbone, gp19K (1,390 bp) and 11.6K (601 bp) mRNA transcripts in DL309/NOD mice were detected with the previously described primer pair Ad 5–53 5′-CTACGTGAAATTTAAAGGAGGAG-3′ and Ad5–53R 5′-AACCTGCGTTGAGCTTGATGAG-3′. The previously described Ad2–53 and Ad2–99 primers (25) were used to detect 10.4K (436 bp) mRNA transcripts in RIPE3/NOD and DL704/NOD mice. The presence of 14.5K (844 bp) mRNA transcripts in RIPE3/NOD mice were detected with the previously described Ad2–53 primer (25) and primer Ad2–91 5′-ACCTGTGGCATGATCATGAGGAG-3′. Because of its recombinant Ad5/25 backbone, 14.5K (844 bp) mRNA transcripts were not detected in DL704/NOD mice. The presence of 14.7K (286 bp) mRNA transcript in RIPE3/NOD was detected with the primer pair Ad2–98F 5′-AAAAAGGCGAAAGAGGGCGGGGCT-3′ and Ad2–98R 5′-GGTCTCTGTGATATTGAGG-3′. Because of its Ad5 backbone, the presence of 14.7K (292 bp) mRNA transcripts in the DL704/NOD mice was detected with the primer pair Ad5–95F 5′-GAGAACAGAGCAGGCGGGGCT-3′ and Ad5–95R 5′-GGTCTCTGTGATATTGAGG-3′. As noted above, a deletion mutation has disrupted the 10.4K, 14.5K, and 14.7K genes in DL309/NOD mice. The analyses were controlled by confirming the presence of β-actin (348 bp) mRNA transcripts in all samples using the primer pair ActinF 5′-GCTGCTGACGATCTTATAACTCAGG-3′ and ActinR 5′-TTAAAACGGAGCTGACTAAGACTCCG-3′. Transcripts were resolved in 1.5% agarose gels and visualized by ethidium bromide staining.

**Analysis of MHC class I expression on β-cells.** For determining MHC class I expression levels on pancreatic β-cells from RIPE3/NOD and DL309/NOD mice, isolated islets were dispersed using Cell Dissociation Buffer (Invitrogen). The single-cell suspensions were then stained with the I-25C6 MHC class I specific monoclonal antibody SF1-1:1 conjugated to a green fluorescent FITC tag. Stained cells were analyzed on a FACScan flow cytometer using the Cell Quest data reduction software (BD Biosciences, San Jose, CA).

**Analysis of diabetes development.** The indicated mice were monitored for development of diabetes by weekly for glycosuria with Ames Diastix (supplied by Miles Diagnostics, Elkhart, IN). Values ≥3 were considered diagnostic for the onset of T1D. Rates of T1D development in the indicated experimental groups were assessed by competitors' study design.
Adoptive transfer of splenocytes. Splenic leukocytes were isolated as previously described (26). In one set of experiments, $1 \times 10^7$ splenocytes from prediabetic NOD female donors (6–8 weeks of age) were injected intravenously into sublethally irradiated (750 rads) 4- to 6-week-old NOD, RIPE3/NOD, DL704/NOD, or DL309/NOD female mice. In other experiments, $1 \times 10^7$ splenocytes from 6- to 8-week-old female NOD, RIPE3/NOD, DL704/NOD, or DL309/NOD mice were injected intravenously into 4- to 6-week-old NOD-scid mice. Splenocyte recipients were monitored for T1D through 17 weeks after transfer.

Bone marrow reconstitution studies. Bone marrow reconstitution was performed as previously described (27). Briefly, 4- to 6-week-old NOD or RIPE3/NOD female recipients were lethally irradiated (1,200 rads) and then reconstituted with $5 \times 10^6$ bone marrow cells from either NOD or RIPE3/NOD female donors. Bone marrow recipients were monitored for T1D through 22 weeks after reconstitution.

Adoptive transfer of purified T-cells. CD4 and CD8 T-cells from female NOD or RIPE3/NOD mice were purified from splenic leukocyte preparations using the previously described (28) streptavidin-conjugated magnetic bead system (Miltenyi Biotec, Auburn, CA). Subsequent flow cytometric analyses indicated that the purity of the resulting purified CD4 or CD8 T-cells was >90%. Admixtures of $2.5 \times 10^6$ purified CD4 and CD8 T-cells from NOD or RIPE3/NOD donors were then injected intravenously into female NOD-scid recipients.

RESULTS

E3 gene expression in RIPE3/NOD, DL309/NOD, and DL704/NOD mice. DL309/NOD mice carrying the gp19K and 11.6K genes and DL704/NOD mice carrying the 10.4K, 14.5K, 14.7K, and 11.6K genes were characterized by RT-PCR for intra-islet expression of these genes. Standard and RIPE3/NOD mice, respectively, served as negative and positive controls. The samples analyzed by the E3 gene-specific primers consisted of cDNA reverse-transcribed from total RNA obtained from pooled islets of four mice per strain. As expected, mRNA transcripts for all E3 genes (gp19K, 11.6K, 10.4K, 14.5K, and 14.7K) were detected in islets from the RIPE3/NOD-positive control strain (Fig. 1A). The antiapoptotic 10.4K, 14.5K, and 14.7K genes, as well as the 11.6K gene, were expressed in the islets of DL704/NOD mice. This stock did not express the gp19K gene with a putative ability to inhibit MHC class I–mediated antigen presentation. In contrast, the DL309/NOD stock expressed mRNA transcripts for the gp19K and 11.6K genes but not the 10.4K, 14.5K, and 14.7K genes. Thus, the pancreatic islets of DL309/NOD and DL704/NOD mice demonstrated the patterns of E3 transgene expression anticipated from the known deletions in sequence of each of these E3 mutants.

Greater protection from T1D in DL704/NOD than DL309/NOD mice. For examining the individual effects of genes in the RIPE3DL309 (gp19K and 11.6K) and RIPE3DL704 (10.4K, 14.5K, and 14.7K) constructs on T1D development in NOD mice, female transgene-positive segregants were monitored for disease onset from 6 through 30 weeks of age. Controls consisted of pooled nontransgenic segregants from both lines (standard NOD females). As a further control, we monitored a cohort of female RIPE3/NOD mice expressing all E3 genes, which we knew from previous work to suppress strongly their rate of T1D development (18). By 30 weeks of age, T1D had developed in most (95%) of the standard nontransgenic NOD female controls (Fig. 1B). As expected, RIPE3/NOD female mice were strongly protected from T1D, with 64% of the mice remaining disease-free over the same period of time ($P \leq 0.0001$ vs. NOD controls). Female DL704/NOD mice were also strongly protected from T1D, with 63% of mice remaining disease-free at 30 weeks of age ($P \leq 0.0001$ vs. NOD controls). In contrast, while still developing at a significantly lower rate than in the standard NOD controls ($P \leq 0.01$), DL309/NOD female mice were more weakly protected from T1D (33% disease-free at 30 weeks of age) than either the RIPE3/NOD or the DL704/NOD stocks. Furthermore, it should be noted that with the exception of one mouse, the minority subset of RIPE3/NOD female mice developing TID did so at later ages than either disease-affected DL309/NOD or DL704/NOD female mice. Hence, it seems that genes in both the RIPE3DL309 and

FIG. 1. Correlation of intra-islet E3 transgene expression patterns with alterations in T1D and insulitis development in NOD mice. A: Intra-islet expression of various RPIII-driven E3 transgenes in NOD mice. Total RNA was extracted from the pooled islets of four mice per strain. RT-PCR using the primers described in RESEARCH DESIGN AND METHODS was then used to detect transcripts from E3 genes contributing to the inhibition of apoptosis (10.4K, 14.5K, and 14.7K) or MHC class I expression (gp19K). The primers used to detect transcripts from the MHC class I inhibitory gp19K gene also generate an amplicon from an alternative splice variant encoding the E3 11.6K gene product. As a positive control, β-actin mRNA transcripts were identified in all samples. B: Effects of expressing different sets of RPIII-driven E3 genes on T1D development in NOD mice. Female RIPE3/NOD (n = 14, expressing all E3 genes), DL704/NOD (n = 16, expressing E3 apoptosis-inhibitory genes), DL309/NOD (n = 18, expressing E3 MHC class I suppressive gene), and nontransgenic controls (n = 41) were monitored for T1D development through 30 weeks of age. Compared with the standard NOD controls, TID development was strongly inhibited in both the RIPE3/NOD and the DL704/NOD stocks ($P \leq 0.0001$, Life Table Analysis), whereas the DL309/NOD stock was characterized by a weaker degree of protection ($P \leq 0.01$, Life Table Analysis).
the RIPE3DL704 constructs additively but unequally contribute to T1D resistance. Our collective data indicate that the strongest of these additive protective effects is provided by some combination of the antiapoptotic 10.4K, 14.5K, and 14.7K genes present in the RIPE3DL704 construct.

The gp19K transgene equally suppresses β-cell MHC class I expression in RIPE3/NOD and DL309/NOD mice. The data described above indicated that stronger degrees of T1D protection were conferred to NOD mice expressing RIP-driven E3 genes suppressing apoptosis rather than MHC class I expression. However, we recognized that one alternative explanation for the weaker degree of protection from T1D in DL309/NOD mice was that MHC class I expression on β-cells was more strongly inhibited by the gp19K transgene present in the latter strain. Hence, we used flow cytometry to assess the levels at which Kd MHC class I molecules are expressed on the surface of pancreatic islet cells in these two stocks (>90% β-cells). To date, all autoreactive diabeticogenic CD8 T-cells cloned from NOD mice have been restricted to Kd MHC class I molecules (29–31). As illustrated in Fig. 2, compared with standard NOD mice, there were respective 32.4 and 34.6% decreases in the mean fluorescence intensity (MFI) of Kd staining of islet cells from RIPE3/NOD (A) (dashed lines) and DL309/NOD (B) mice (dashed lines) were decreased by 32.4 and 34.6%, respectively.

**FIG. 2.** Expression of Kd MHC class I molecules is equally reduced on the islet cells of RIPE3/NOD and DL309/NOD transgenic mice. Dispersed pancreatic islet cells separately pooled from two NOD, RIPE3/NOD, and DL309/NOD mice were stained with a FITC-conjugated monoclonal antibody specific for Kd MHC class I molecules (clone SF1–1.1) and then analyzed by flow cytometry. Compared with islet cells from NOD controls (solid lines in both A and B), the mean fluorescence intensity (MFI) of Kd staining of islet cells from RIPE3/NOD (A) (dashed lines) and DL309/NOD (B) mice (dashed lines) were decreased by 32.4 and 34.6%, respectively.
gene inhibits MHC class I expression on β-cells (~30%) does afford weak but significant protection from T1D. **T1D protection by E3DL704 genes is partially manifest at the level of the pancreatic β-cell target.** We previously found that the diminished rate of T1D development in RIPE3/NOD mice is associated with an increased resistance of their pancreatic β-cells to destruction by autoimmune effector mechanisms (18). However, in this earlier study, we also unexpectedly found that splenic leukocytes from RIPE3/NOD mice were less efficient than those from standard NOD donors in adoptively transferring T1D to NOD-scid recipients. Hence, it seemed that in addition to rendering β-cells partially resistant to destruction by diabetogenic immune cells, expressing all E3 genes under control of the RIPII promoter also inhibits the development or function of such effectors. For distinguishing between the E3 gene products conferring T1D resistance at the level of the β-cell target from those involved in altering immune effector functions, a series of adoptive transfer experiments were performed. For examining the effects of various E3 genes at the β-cell level, 1 × 10^7 splenic leukocytes from 6- to 8-week-old prediabetic NOD female donors were transplanted into sublethally irradiated NOD, RIPE3/NOD, DL309/NOD, and DL704/NOD female recipients. As previously observed (18), T1D was transferred less efficiently (P ≤ 0.0001) to RIPE3/NOD than standard NOD recipients (Fig. 3). There was also a significant delay of T1D onset in the DL704/NOD versus standard NOD recipients (P ≤ 0.01). Although the onset of adoptively transferred T1D was significantly delayed in RIPE3/NOD and DL704/NOD recipients, they ultimately developed disease at a final frequency not statistically different (χ² analysis) from standard NOD recipients. These results indicated that at least some component of T1D resistance mediated by E3 antiapoptotic genes (10.4K, 14.5K, and 14.7K) in NOD mice is manifest at the level of the pancreatic β-cell. In contrast, the rate of adoptively transferred T1D did not statistically differ in standard NOD and DL309/NOD recipients (Fig. 3). Although this indicated that the E3 gp19K and 11.6K genes do not confer strong T1D resistance at the β-cell level, it cannot be completely ruled out that these genes exert some weakly protective target cell effects. This possibility is suggested by the observation that the rate of adoptively transferred T1D in the DL309/NOD recipients was intermediate between that of standard NOD and DL704/NOD recipients, albeit at levels not statistically differing from either. These collective data raised the question of why unmanipulated RIPE3/NOD and DL704/NOD mice were more resistant to T1D than those that received adoptively transferred spleen cells from standard NOD donors. We reasoned that this could result from the previously observed ability of E3 genes to impair the development or function of diabetogenic immune cells in NOD mice, and this effect is overridden by the introduction of effectors that had matured in an E3-negative environment. **RIPE3DL704 and RIPE3DL309 region genes must act in concert to suppress diabetogenic immune effectors in NOD mice.** As an initial way of deciphering how RIP-driven E3 genes may inhibit the development or function of diabetogenic immune cells, we determined which ones contribute to this process. To do this, 1 × 10^5 splenocytes from NOD, RIPE3/NOD, DL309/NOD, or DL704/NOD female donors were transferred into NOD-scid recipients. As shown in Fig. 4, during a 17-week period, T1D developed in significantly fewer NOD-scid recipients of splenocytes from RIPE3/NOD (10%) than NOD donors (90%; P ≤ 0.001). However, the proportion of NOD-scid mice that developed T1D after repopulation with splenocytes from DL309/NOD (89%) or DL704/NOD mice (67%) did not differ from those that received NOD splenocytes. These findings suggest that all E3 genes need to be present and expressed in a RIP-driven manner to suppress diabetogenic immune functions in NOD mice. **RIPE3 genes directly exert T1D protective effects in hematopoietically derived cell types.** We reasoned that RIP-driven E3 genes could dampen the diabetogenic capacity of NOD immune cells in several ways. One possibility was that this occurs in an indirect manner whereby the functional capacity of NOD immune cells is suppressed through unknown mechanisms after encountering E3-expressing β-cells. However, insulin gene expression has also been detected in the thymus (32,33). Hence, RIP-driven E3 transgenes might also be expressed in...
non-β-cells, including those of hematopoietic origin, and this somehow inhibits the development or function of diabetogenic effectors. To test these possibilities, we generated reciprocal bone marrow chimeras between RIPE3/NOD and NOD mice. Controls consisted of irradiated NOD mice reconstituted with syngeneic marrow. As shown in Fig. 5, during a 22-week period after reconstitution with NOD bone marrow, T1D developed in a significantly higher portion of syngeneic (100%) than RIPE3/NOD recipients (54%; \( P < 0.005 \)). This provided further indication that some portion of E3-induced T1D resistance in NOD mice is mediated at the level of the β-cell target, which, based on experiments described earlier, is likely a function of the subset of genes exerting antiapoptotic activity. However, T1D also developed at a significantly slower rate \( (P < 0.005, \text{Life Table Analysis}) \) and final frequency \( (P < 0.05, \chi^2 \text{analysis}) \) in NOD mice reconstituted with RIPE3/NOD rather than syngeneic marrow.

Although undetectable by RT-PCR analyses, it is possible that RIP-driven E3 genes are expressed in the thymus, and this inhibits the development of diabetogenic T-cells in NOD mice. To test this possibility, we compared the ability of purified CD4 and CD8 T-cells from standard NOD or RIPE3/NOD mice to co-transfer T1D to NOD-scid recipients. In contrast to what was observed with NOD and RIPE3 splenocytes, admixtures of purified CD4 and CD8 T-cells from these two strains transferred T1D at an equivalent rate and final frequency to NOD-scid recipients (Fig. 6). This indicated that the unexpected ability of RIP-driven E3 genes to suppress diabetogenic immune responses in NOD mice directly at the level of hematopoietically derived cell types does not block the development of pathogenic T-cells but instead limits their functional activation. One possibility is that diabetogenic T-cell function is suppressed through alterations induced in other hematopoietic cell types, such as APC, that express the greater degree of T1D resistance in unmanipulated RIPE3/NOD mice than in the reciprocal bone marrow chimeras.
RIP-driven E3 transgenes at low levels. This will be an area of future investigation.

DISCUSSION

These studies delineate the subset of adenovirus E3 genes that when expressed in pancreatic β-cells of NOD mice decreases the rate and final frequency of T1D development. Specifically, those E3 genes encoding proteins with antiapoptotic functions (10.4K, 14.5K, and 14.7K) and that inhibit the activation of NF-κB by TNF-α (10.4K/14.5K) contribute the greatest amount of disease protection. It should be noted that transgenic expression in β-cells of the CrmA protein, which inhibits apoptosis by blocking the activity of several caspases, did not decrease the rate or incidence of spontaneous T1D development in NOD mice (34). Hence, it seems to be important to inhibit β-cell apoptosis and/or NF-κB through the mechanisms induced by Ad E3 genes to most efficiently block diabetogenic immune responses.

Determining the exact mechanism by which antiapoptotic Ad E3 gene products protect β-cells from autoimmune destruction will require continued analyses. However, previous studies focused on the molecules that contribute to β-cell death in NOD mice may lend insight to how antiapoptotic E3 proteins exert their T1D protective effects. Many of these have examined the role that TNF-α and Fas-induced apoptotic pathways may play in the development of insulitis and T1D in NOD mice (7–9,35,36). One study demonstrated that expression of a RIP-regulated FasL transgene resulted in an accelerated rate of T1D development in NOD mice (7). Further illustrating the pathogenic importance of the Fas-FasL pathway is that Fas-deficient NOD mice are T1D-resistant (7,37). It has also been reported that the NOD-derived diabeticogenic CD4 T-cell clone NY4.1 could not exert pathogenic effects against β-cells that had not upregulated Fas expression (8). Studies focused on the TNF-α apoptotic pathway have also implicated this cytokine in β-cell death. TNF-α receptor deficiency has been found to render islets somewhat resistant to the β-cell autoreactive CD4 T-cell clone BDC 2.5 (35). Moreover, treatment of NOD mice with a neutralizing TNF-α antibody inhibited the development of insulitis and T1D (36). Consequently, the reduction of Fas receptors on the surface of β-cells as a result of the function of the E3 encoded 10.4K/14.5K complex, as well as the TNF-α inhibitory action of the 14.7K protein, could render β-cells resistant to apoptotic destruction mediated by these cytokines.

In addition to suppression of apoptotic pathways mediated by the other E3 transgenes, we reasoned that the gp19K protein expressed in RIPE3/NOD and DL309/NOD mice might also confer disease protection by downregulating MHC class I–mediated presentation of β-cell autoantigens. Previous studies have demonstrated that MHC class I–restricted CD8 T-cells are required for all but the end stages of T1D development (31). Hence, reducing the level of MHC class I–bound autoantigens on the surface of β-cells could potentially limit their ability to be targeted by autoreactive CD8 T-cells. However, the ~30% reduction in MHC class I expression on β-cells elicited by the gp19K transgene in DL309/NOD mice was sufficient to confer only a marginally significant delay in T1D onset. This provided further evidence that blocking apoptotic effector pathways, rather than the original presentation of MHC class I–restricted autoantigens, might ultimately provide the most effective way of protecting endogenous or engrafted β-cells from diabetogenic immune responses.

The current study also provided some further insight to our previously unexpected finding that in addition to effects mediated at the β-cell level, RIP-driven expression of all E3 genes limits the ability of NOD immunological effectors to induce T1D (18). Here, we found through the use of reciprocal bone marrow chimeras that this immunological effect was indeed directly mediated at the level of hematopoietically derived cell types. There is also an indication that all E3 genes must be expressed in a RIP-regulated manner to reduce the diabetogenic capacity of NOD immune cells because this effect was lost in stocks carrying the DL309 and DL704 E3 transgene deletion variants. Furthermore, RIP-driven expression of all E3 genes does not seem to impair directly the diabetogenic capacity of NOD T-cells. This was demonstrated by the fact that although unfractionated splenocytes from RIPE3/NOD mice had an impaired ability to transfer T1D to NOD-scid recipients, disease was efficiently induced by purified T-cells from the same donors. A possible explanation for this finding is that E3 gene expression alters some NOD APC population in a way that impairs their...
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ability to support the functional activation of diabetogenic T-cells but does not induce an increase in their capacity to delete or anergize such pathogenic effectors. However, this protective effect may be partially overridden when APC from nontransgenic mice are also present. This possibility is supported by several observations. First is that although still significantly protected compared with standard NOD recipients, subletally irradiated RIPE3/NOD mice infused with NOD spleen cells develop a higher rate of T1D than those that have remained unmanipulated (compare Figs. 3 and 1). Similarly, the presence of somewhat lesser residual levels of host-type APC could account for the higher levels of T1D in irradiated NOD recipients of RIPE3/NOD bone marrow than in NOD-scid mice repopulated with spleen cells of the same donor type (compare Figs. 5 and 4). Finally, it cannot be ruled out that APC function has been impaired by an insertional mutagenesis event mediated by the RIPE3 but not the DL309 or DL704 transgene constructs.

Additional ways to optimize the E3 system could include increasing the expression of the antiapoptotic 10.4K, 14.5K, and 14.7K genes as well as deleting the proapoptotic 11.6K gene that may have deleterious effects when expressed in β-cells. If this provides an additional palliative effect in the NOD mouse model, then it could increase the attractiveness of ultimately determining whether the introduction of antiapoptotic E3 genes enhances the ability of islet cells transplanted into T1D patients to reverse disease. In vitro gene therapy of β-cells before transplantation with replication-defective viral vectors could have several advantages. These include efficiently reaching the intended target with relatively low levels of virus and not systemically exposing the patient to gene therapy vectors that may exert undesirable effects, for example triggering innate immune response at unintended target sites. Furthermore, an in vitro–based gene therapy approach would allow for a careful examination of stability and function of the manipulated β-cells before their transplantation into the patient. Because of the immunoevasive nature of the E3 genes, they could also serve to facilitate β-cell transplantation without significant levels of immunosuppression.

REFERENCES


