Suppression of Hyperglycemia in NOD Mice After Inoculation With Recombinant Vaccinia Viruses

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Abstract

In autoimmune (type 1) diabetes, autoreactive lymphocytes destroy pancreatic β-cells responsible for insulin synthesis. To assess the feasibility of gene therapy for type 1 diabetes, recombinant vaccinia virus (rVV) vectors were constructed expressing pancreatic islet autoantigens proinsulin (INS) and a 55-kDa immunogenic peptide from glutamic acid decarboxylase (GAD), and the immunomodulatory cytokine interleukin (IL)-10. To augment the beneficial effects of recombinant virus therapy, the INS and GAD genes were fused to the C terminus of the cholera toxin B subunit (CTB). Five-week-old non-obese diabetic (NOD) mice were injected once with rVV. Humoral antibody immune responses and hyperglycemia in the infected mice were analyzed.

Only 20% of the mice inoculated with rVV expressing the CTB::INS fusion protein developed hyperglycemia, in comparison to 70% of the mice in the uninoculated animal group. Islets from pancreatic tissues isolated from euglycemic mice from this animal group showed no sign of inflammatory lymphocyte invasion. Inoculation with rVV producing CTB::GAD or IL-10 was somewhat less effective in reducing diabetes. Humoral antibody isotypes of hyperglycemic and euglycemic mice from all treated groups possessed similar IgG1/IgG2c antibody titer ratios from 19 to 32 wk after virus inoculation. In comparison with uninoculated mice, 11-wk-old NOD mice injected with virus expressing CTB::INS were delayed in diabetes onset by more than 4 wk. The experimental results demonstrate the feasibility of using rVV expressing CTB::INS fusion protein to generate significant protection and therapy against type 1 diabetes onset and progression.

Index Entries: Cholera toxin B subunit; autoimmunity; type 1 diabetes; IDDM; GAD; insulin; vaccinia virus.

1. Introduction

Type 1 diabetes is a Th1 lymphocyte-mediated autoimmune disease that destroys pancreatic islet insulin-secreting β-cells by inflammatory responses (insulitis), initiated by cytotoxic T lymphocytes and macrophages that infiltrate the pancreatic islets of Langerhans ***(1,2)***. Autoreactive T cells infiltrating the pancreas target multiple antigens, including glutamic acid decarboxylase (GAD) and insulin, which are known to be major β-cell autoantigens. Retardation or prevention of type 1 autoimmune diabetes onset by repeated oral inoculation with small amounts of pancreatic islet autoantigens has been demonstrated in animal models of autoimmune diabetes ***(3)***. Autoantigen conjugates with the cholera toxin B subunit (CTB) were shown to greatly enhance suppression of type

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1 diabetes insulitis and hyperglycemia symptoms in a variety of animal autoimmune disease models *(4–7)*.

Recent experiments have shown that adenovirus and adeno-associated viruses can deliver islet autoantigens *(8–10)* and the immunomodulatory cytokine interleukin (IL)-10 *(11–13)*, resulting in a reduction in the frequency of hyperglycemia in diabetic mice. More closely related to this work, in a transgenic, lymphocytic choriomeningitis virus (LCMV) associated mouse model of diabetes, vaccinia virus (VV) expressing the LCMV autoantigens were able to suppress diabetes onset *(14,15)*. Furthermore, vaccinia virus expressing GAD was shown to be effective in the prevention of diabetes in non-obese diabetic (NOD) mice *(16)*. Administration of helper cell cytokines such as IL-10 can induce Th2 lymphocyte mediated anti-inflammatory immune regulatory functions that prevent the onset of autoimmune diabetes in NOD mice. The NOD mouse is one of the best animal models for studying human type 1 diabetes as it spontaneously develops the disease with many features in common with human type 1 diabetes. Recently, we evaluated oral inoculation of juvenile NOD mice with recombinant VV (rVV) expressing fusion genes encoding CTB linked to the pancreatic islet autoantigens proinsulin (INS) and a 55 kDa C-terminal peptide from glutamate decarboxylase (GAD_{55}). Oral inoculation with rVV expressing the CTB fusion with islet autoantigens generated a significant reduction in insulitis *(17)*. These results demonstrate the feasibility of using vaccinia for delivery of immunogenic proteins while at the same time inducing tolerance. However, the virus uptake following oral administration is somewhat unpredictable and rather low. Therefore, in order to gain insight into mechanisms underlying immunotolerance, it would be important to deliver the therapeutic agent systemically with well controlled virus doses. In this study, we analyze the effect of rVV expressing two major pancreatic islet autoantigens, INS and GAD, fused with CTB and administered by intraperitoneal injection to NOD mice. Further, delivery of the immunomodulator cytokine IL-10 was also evaluated.

2. Methods

2.1. Viruses

African green monkey kidney fibroblast cells (CV-1 line) were obtained from the American Type Culture Collection. The CV-1 cells were maintained and grown as previously described *(17)*. The Lister vaccine strain of VV was used as the parental virus. Construction of rVV-CTB::INS, rVV-CTB::GAD was previously described *(17)*. The virus rVV-IL10 was constructed using the transfer plasmid pSIL10 and the conventional infection/contransfection of CV-1 cells as previously described *(18)*. The mouse cDNA of IL-10 (pCB-IL10) was kindly provided by Dr. Mark A. Atkinson (University of Florida, Gainesville, FL). To construct a transfer vector pSIL10, the BamHI fragment of pCB-IL10 containing IL-10 gene was inserted into the BglII site of pSC65 *(19)* obtained from Dr. B. Moss (NIH, Bethesda, MD). The pSIL10 contained the IL-10 gene driven by synthetic early/late promoter pE/L. Briefly, CV-1 cells were infected with VV and transfected with the transfer plasmid pSIL10 using the GenePORTER™ transfection reagent (Gene Therapy Systems Inc., San Diego, CA). Virus particles from individual recombinant plaques were amplified in CV-1 cells. Recombinant clones containing the *lacZ* reporter gene were selected by growth on CV-1 cells in the presence of 5-bromo-4-chloro-indolyl-β-D-galactosidase. The structure of the rVV was confirmed by PCR DNA amplification and sequencing methods. Recombinant viruses were propagated and purified as described earlier *(18)*. Virus titers were determined by plaque assay on CV-1 cells. The gene fusion constructs used in this study are presented in **Fig. 1**. A cDNA fragment encoding GAD55, a truncated form of human GAD65 minus the N-terminal membrane binding region (aa 89-585), and INS, a gene encoding human proinsulin, a second major diabetes autoantigen, were linked to the C-terminus of the CTB gene as previously described *(17)*.

2.2. Immunoblot Analysis of Recombinant CTB-Autoantigen Fusion and IL-10 Proteins

The synthesis of CTB::INS, CTB::GAD islet autoantigen and IL-10 fusion protein was ana-

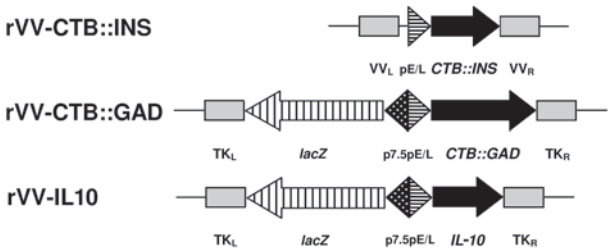


Fig. 1. Physical map of rVV strains used in the study. The TK_L and TK_R designate the flanking thymidine kinase sequences of the VV genome. The *lacZ* stands for the beta-galactosidase gene of *E. coli*; this reporter was used for selection of recombinant viruses. The autoantigen (CTB::INS, CTB::GAD) and IL-10 genes are driven by the synthetic early/late promoter pE/L, whereas the *lacZ* is driven by p7.5 promoter. For details of virus structure *see* **ref. *17***.

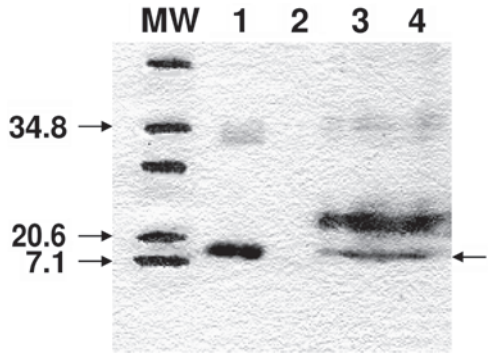


Fig. 2. Synthesis of IL-10 in CV-1 cells. MW = molecular weight standards, indicated values are in kDa; Lane 1, commercial IL-10 (Chemicon International); Lane 2, uninfected CV-1 cells; Lanes 3 and 4, two rVV infected CV-1 cell extracts; arrow indicates IL-10 (17 kDa).

lyzed in rVV infected CV-1 cells as described earlier ***(17)***. Autoantigen proteins were visualized by immunoblot analysis using anti-CTB, INS and GAD (Sigma-Aldrich Co, St. Louis, MO) as the primary antibodies. The cytokine IL-10 was identified on immunoblots by comparison with commercial IL-10 protein (Chemicon International, Temecula, CA) and rabbit anti-mouse IL-10 antibodies (Chemicon International) using goat anti-rabbit IgG conjugated to alkaline phosphatase as the secondary antibody (Sigma-Aldrich Co.), and incubation in a chemiluminescent substrate ***(20)***.

2.3. Detection of Hyperglycemia in NOD Mice Immunized With Recombinant Vaccinia Viruses

Four-week-old female NOD mice were purchased from Taconic Farms (Germantown, NY) and maintained in the Loma Linda University animal care facility. For measurement of hyperglycemia, the mice were subjected to intraperitoneal (i.p.) injection with viruses in 0.3 mL of phosphate-buffered saline (PBS). The control animals were mock-infected with PBS. To evaluate the efficacy of rVV treatment for arresting the progression of diabetes in NOD mice following insulitis onset, one group of 11-wk-old mice (n = 10) were subjected to a single i.p. injection of 5×10^7 plaque forming units (PFU) per mouse of rVV containing the CTB::INS fusion gene, in 0.3 mL of PBS. The mice were fed chow and water *ad libitum*. Starting at 15 wk of age for a total period of 31 wk, the mice in each experimental group (**Table 1**) were monitored bi-weekly with urinary glucose test strips (Clinistix® and Bayer Keto Diastix®, Bayer, Elkhart, IN) for development of diabetes related hyperglycemia. Glycosuric mice were bled from the tail vein every 2 wk to detect hyperglycemia onset using a glucose analyzer (Boehringer Mannheim, Mannheim, Germany). The onset of diabetes was confirmed by measurement of greater than 250 mg glucose/dL blood, for two consecutive weeks ***(21)***. We used the "Test for the Equality of Two Proportions," to assess the statistical significance of hyperglycemia onset incidence among experimental groups. The incidence of hyperglycemia was not considered to be significantly different between groups when the Z value calculated was between –1.96 and +1.96.

2.4. Enzyme Immunoassay Detection of CTB-Autoantigen, and Vaccinia Specific Antibodies

Enzyme-linked immunosorbent assay (ELISA) methods were used to estimate T-helper cell activation in response to CTB-autoantigen inoculation by measurement of CTB and VV specific IgG1 and IgG2c antibody isotype levels. Microtiter plate

Table 1
NOD Mouse Treatment Groups for rVV Suppression of Hyperglycemia

Animal group (n =10)	Treatment	rVV (PFU)	Age at injection
1.	rVV-CTB::INS	5×10^7	5 wk
2.	rVV-CTB::GAD	5×10^7	5 wk
3.	naïve	0	5 wk
4.	rVV-IL10	1×10^7	5 wk
5.	rVV-CTB::INS	5×10^7	11 wk

wells were coated overnight at 4°C with substrate antigen, e.g., 100 μL of CTB (3.0 μg/mL) (Sigma-Aldrich Co.) in PBS ***(22)*** or VV antigen (1×10^6 PFU) in PBS. The wells were then incubated sequentially with blocking solution 1% bovine serum albumin (BSA) in PBS containing 20% Tween-20 (PBST) at 37°C for 2 h and then with NOD mouse sera (diluted 1:5 with PBS) under the same conditions. After washing the wells three times with PBST, the wells were incubated with a 1:1000 dilution of alkaline phosphatase conjugated rabbit anti-mouse IgG1 (ICN/Cappel, Aurora, OH). To measure specific IgG2c levels, the wells were incubated with 1:1000 diluted mouse anti-mouse IgG2a^b (*Igh1-b* allotype, cat. no. 05031D, PharMingen Inc, San Diego, CA) monoclonal antibody, then incubated with a 1:5000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (whole molecule) (Sigma-Aldrich Co.), at 37°C for 2 h. Between each step the wells were rinsed three times with PBST. The chemiluminescent reaction was developed by addition of 100 μL Lumi-Phos Plus (Lumigen Inc., Southfeld, MI) luminescent substrate per well and the relative light units (RLU) were measured at 411–477 nm in a ML 3000 Microtiter Plate Luminometer (Dynatech Laboratories Inc., Chantilly, VA). The animals were sacrificed in a CO_2 atmosphere and blood was collected from the heart.

2.5. Histopathological Analysis of Pancreatic Islets

Pancreatic tissues were isolated from uninoculated NOD mice or mice inoculated at 5 wk of age with VV carrying the CTB::INS fusion gene. The mice were sacrificed from 19 to 32 wk of age. The pancreatic tissues were fixed in Bouin's fluid, embedded in paraffin, sectioned, stained with hematoxylin, and counterstained with eosin prior to double blind microscopic analysis. The levels of pancreatic islet inflammation (insulitis) were measured based on the extent of lymphocyte infiltration into the islets of Langerhans. The degree of insulitis was scored based on a six-level semi-quantitative scale ranging from 0–5 where a score of 0 is considered normal islet morphology with no sign of cytotoxic lymphocyte infiltration and scores of 1–5 indicate increasing levels of peri- and intra-insulitis, respectively ***(17)***. At least 15 islets were scored for each animal.

3. Results

3.1. Expression of Recombinant Proteins in Cell Culture

The rVVs expressing INS, GAD, and IL-10 fused to the C-terminus of CTB were constructed as previously described ***(17)*** and in the section on materials and methods (**Table 1**). All recombinant viruses expressed the ligand-autoantigen fusion proteins or IL-10 in rVV infected CV-1 cells under control of the strong synthetic vaccinia E/L promoter (**Fig. 2**). The biosynthesis of recombinant ligand–autoantigen fusion protein was monitored following rVV infection of cultured epithelial cell lines as previously described ***(17)***. Infection of CV-1 cells with two separate clones of rVV expressing CTB::INS showed in each case that the fused proteins assembled into pentameric structures containing GM1 ganglioside membrane receptor binding activity ***(17)***.

3.2. Detection of Hyperglycemia in NOD Mice Inoculated With rVV Expressing Ligand-Adjuvant–Autoantigen Fusions and IL-10

The onset of diabetes (hyperglycemia), was compared among 5-wk-old NOD mice injected with a single dose of rVV carrying the CTB::GAD and CTB::INS (5×10^7 PFU) fusion genes, or the IL-10 gene (1×10^7 PFU) (**Table 1**). A total of 70% of untreated NOD mice developed hyperglycemia through 29 wk of age based on blood glucose measurements (**Fig. 3A**). In contrast, by 31 wk of age, only 20% of the mice inoculated with rVV-CTB::INS developed hyperglycemia, whereas rVV delivered CTB::GAD and IL-10 generated more modest diabetes protection, in which 30 and 40% of the mice developed hyperglycemia, respectively (**Fig. 3A**). However, further analysis did not confirm the statistical significance of the latter values.

The efficacy of rVV mediated adjuvant–autoantigen suppression of type 1 diabetes was also measured in 11-wk-old NOD mice that had developed significant levels of islet inflammation (insulitis) prior to rVV inoculation. The results of this experiment showed that in comparison with naïve uninoculated mice, a single dose of rVV-CTB::INS can delay hyperglycemia onset by more than 4 wk (**Fig. 3B**). By 31 wk of age, hyperglycemia had progressed to only 40% in the rVV-CTB::INS inoculated mouse group, indicating that vaccinia mediated adjuvant–autoantigen treatment in addition to preventing or delaying diabetes onset, can provide a therapeutic protective effect in NOD mice in which insulitis has already progressed.

3.3. Effects of rVV Inoculation on Humoral Antibody Isotype Immune Responses in NOD Mice

Prevention of diabetes by vaccinia delivered CTB::INS, CTB::GAD fusion genes and the IL-10 gene was assessed by ELISA in euglycemic and hyperglycemic NOD mice 19 through 32 wk following rVV inoculation. The ratio of humoral IgG1 and IgG2c isotype levels in blood isolated

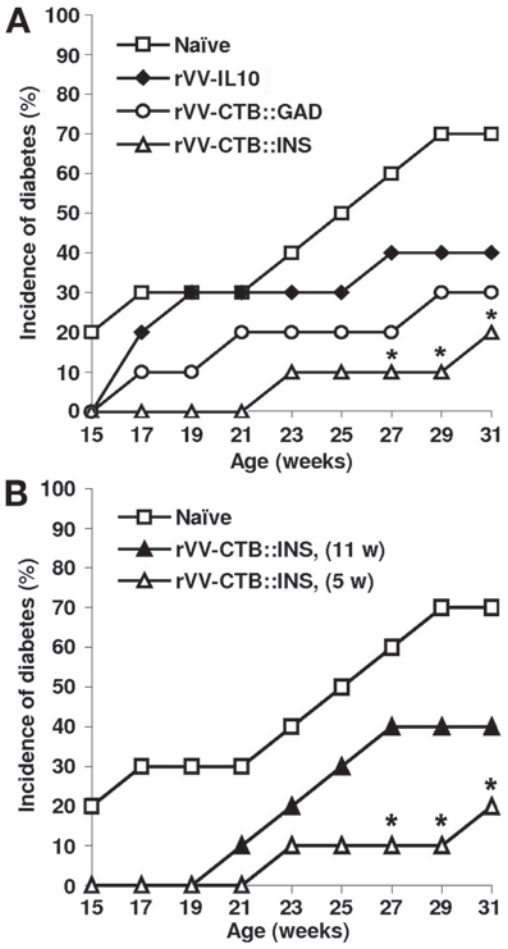


Fig. 3. Progression of hyperglycemia in NOD mice after rVV inoculation. Five wk-old female NOD mice (n = 10) were inoculated i.p. with a single injection of rVV (5×10^7 PFU mouse), expressing CTB::INS or CTB::GAD ligand-islet autoantigen fusion proteins, and with rVV (1×10^7 PFU mouse), expressing IL-10. Blood glucose levels were measured once per two wk, and the percentage of hyperglycemic mice (>250 mg glucose/dl blood), was monitored from 15 wk through 31 wk of age. **(A)** Uninoculated mice (□), mice inoculated with rVV-IL10 (◆), mice inoculated with rVV-CTB::GAD (○), and mice inoculated with rVV-CTB::INS (△). **(B)** Therapeutic protection against diabetes progression: uninoculated NOD mice (□), NOD mice inoculated once with 5×10^7 PFU/mouse of rVV-CTB::INS at 11 wk of age (▲), mice inoculated with 5×10^7 PFU/mouse of rVV-CTB::INS at 5 wk of age (△). * = statistically different from naïve animals, 27w, Z = 2.34; 29w, Z = 2.73; 31w, Z = 2.24.

from mice in the rVV-inoculated and uninoculated experimental animal groups was determined by ELISA using VV or CTB to capture anti-VV or anti-CTB antibodies present in immunized mouse sera (**Fig. 4A–C**). The experimental data showed that individual immunized mice that developed hyperglycemia had IgG1/IgG2c Ab isotype ratios in the range of 3.0 to 8.2 (**Fig. 4A**) or from 1.1 to 6.3 (**Fig. 4B**), depending on the capture molecule used in the assay. Three mice in panel A (CTB::INS,27; IL-10,19; and IL-10,21) had low levels of detectable IgG2c (>25 RLU), and their IgG1/IgG2c ratio values were not calculated. In panel B, only one mouse (CTB::INS,25) showed equally low antibody levels. Inoculated mice that did not develop hyperglycemia by the end of experiment (31 wk), generated about the same increase in IgG/IgG2c ratios (**Fig. 4C**) compared to the hyperglycemic animals (**Fig. 4A,B**). Antibody isotype ratios based on VV-specific IgG values from three treatment groups (CTB::GAD, CTB::INS, and IL-10) ranged from 3.8 to 4.9, whereas CTB-specific antibody values of CTB::GAD and CTB::INS groups were 4.3 and 1.5, respectively. Thus, VV- and CTB-specific IgG1/IgG2c ratios measured in hyperglycemic and healthy mice did not show statistically significant differences toward the end of the experiment and beyond (23–32 wk).

3.4. Development of Insulitis in NOD Mice

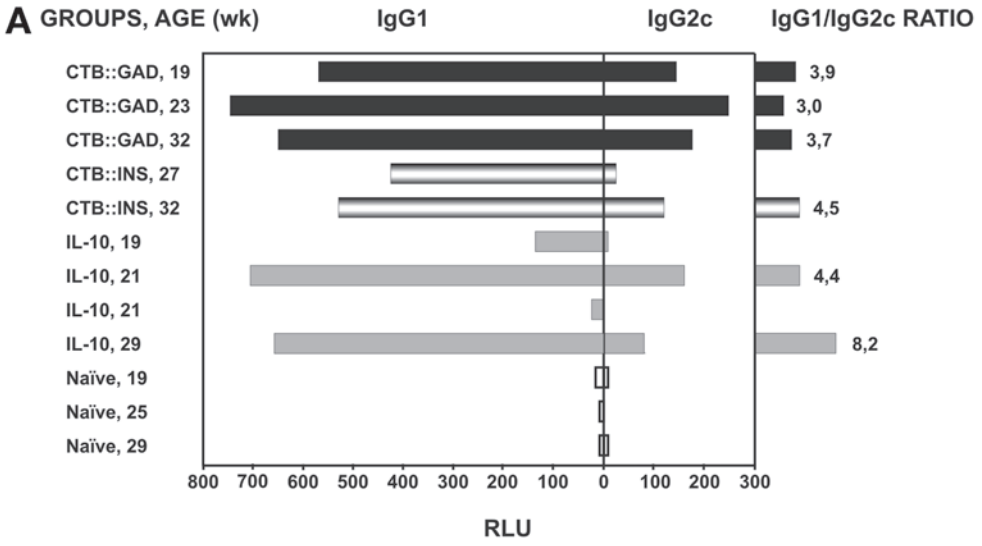
The effect of vaccinia delivered adjuvanted islet autoantigens on the progression of insulitis in immunized NOD mice was monitored following inoculation with rVV expressing the CTB::INS fusion protein (**Fig. 5**). In 27 and 32 wk old immunized hyperglycemic NOD mice different levels of cytotoxic lymphocyte invasion of the islets could be detected, e.g., islets surrounded by peri-islet lymphocytes (**Fig. 5B**), and occasional islets penetrated by numerous intra-islet cytotoxic lymphocytes (**Fig. 5C**). In contrast, the majority of immunized euglycemic mice showed no evident lymphocyte association with the islets (**Fig. 5A**). Extensive intra-islet lymphocyte invasion was observed in uninoculated naïve mice (**Fig. 5D**).

4. Discussion

Other than islet transplantation, which provides at present only a temporary cure for hyperglycemia, there is no treatment providing long-term relief from the progression of autoimmune diabetes. Given the extent of physical and psychological damage caused by type 1 diabetes, an effective form of prevention or therapy could be of special value for diabetic patients afflicted with this lifelong disease. To study alternative delivery and biosynthesis systems for adjuvanted-autoantigens, we have applied rVVs that upon systemic delivery synthesize pentameric cholera toxin B subunits linked to either INS or secreted GAD_{55} proteins in virus infected cells. Oral administration of rVV-CTB::INS to juvenile NOD mice was recently found to generate a marked reduction in pancreatic islet inflammation and hyperglycemia ***(17)***. In addition, in order to modulate the immune system toward an anti-inflammatory Th2 response, we analyzed the potential for reduction in inflammatory responses using rVV-mediated delivery of IL-10. The mechanism of IL-10-mediated protection is considered to be associated with inhibition of inflammatory cytokine production by cytotoxic macrophages and Th2 lymphocyte suppression of cytotoxic T cell activation ***(23)***.

We observed a significant reduction in hyperglycemia (50–60%) in NOD mice inoculated with rVV-CTB::INS compared to the naïve

Fig. 4. *(opposite page)* ELISA measurement of VV and CTB humoral antibody isotype ratios, in euglycemic and hyperglycemic NOD mice from 19 to 32 wk following inoculation with rVV expressing CTB::GAD, CTB::INS, and IL-10. **(A)** VV antibody isotype levels in individual hyperglycemic NOD mice. Ratios were not calculated for mice that had IgG2c level less than 25 RLU (10% of maximal value). **(B)** CTB antibody isotype levels measured in individual hyperglycemic NOD mice. Ratios were not calculated for mice that had the IgG2c level less than 0.7 RLU (10% of maximal value). **(C)** Average of VV antibody isotype ratios (left four groups) and CTB (right three groups) antibody isotype ratios in euglycemic NOD mice. n = 3 mice/group, except for naïve mice (n = 2). Bars represent standard deviation.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CTB::GAD, 19
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CTB::GAD, 32
CTB::INS, 27
CTB::INS, 32
IL-10, 19
IL-10, 21
IL-10, 21
IL-10, 29
Naïve, 19
Naïve, 25
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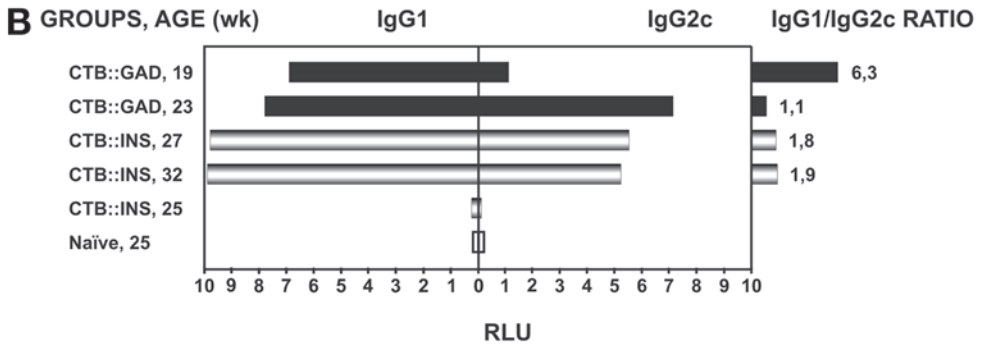
B
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CTB::GAD, 19
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CTB::INS, 32
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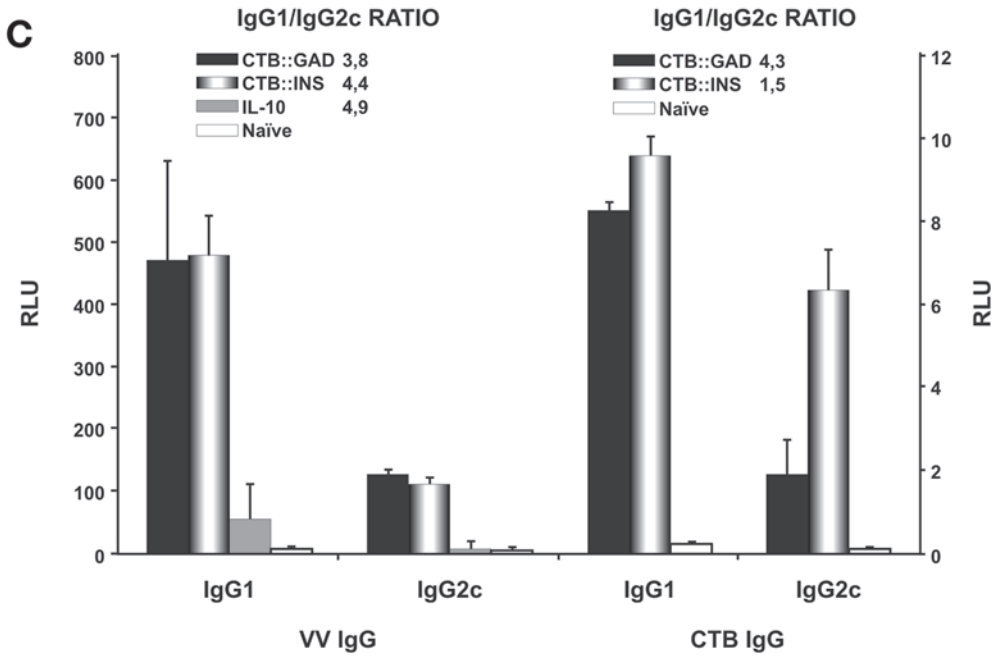
C
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IgG1/IgG2c RATIO
CTB::GAD 3,8
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IL-10 4,9
Naïve
CTB::GAD 4,3
CTB::INS 1,5
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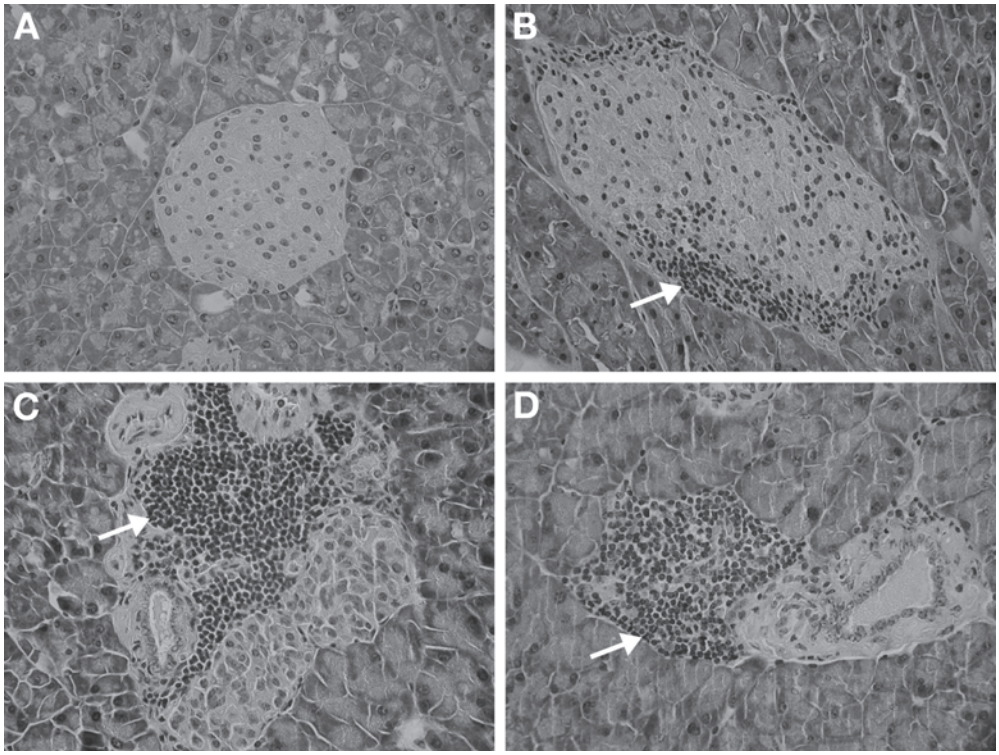

Fig. 5. Histology of NOD mouse pancreatic islets of Langerhans showing invasion with cytotoxic lymphocytes. **(A)** Cross section of a typical pancreatic islet from a 32-wk-old non-diabetic NOD mouse inoculated at 5 wk of age with rVV-CTB::INS, no islet inflammation, no autoreactive lymphocytes detected (insulitis score 0). **(B)** Islet from a 32-wk-old hyperglycemic NOD mouse inoculated with rVV-CTB::INS, autoreactive lymphocytes seen surrounding but remaining outside of the islet (peri-insulitis, insulitis score 1). **(C)** Islet from a 27-wk-old hyperglycemic NOD mouse inoculated with rVV-CTB::INS, autoreactive lymphocyte concentration increasing on the surface and entering the islet (partial intra-insulitis, insulitis score 3). **(D)** Typical islet from an uninoculated hyperglycemic NOD mouse (negative control) at 19 wk of age cytotoxic lymphocytes are invading islet tissues (intra-insulitis, insulitis score 4). Cytotoxic lymphocytes = small dark blue stained cells, white arrows indicate their location with respect to islet β-cells.

uninoculated animals, confirming a crucial role for the islet autoantigen insulin in diabetes development ***(24,25)***. Systemic inoculation with the rVV-CTB::GAD fusion caused a somewhat less pronounced decrease in hyperglycemia incidence than in mice inoculated with rVV-CTB::INS. Nevertheless, it is possible that a combination of both autoantigen-expressing viruses may increase immunological tolerance to autoantigens in inoculated mice. Similarly, combination of the adjuvanted autoantigens with the immunomodulatory cytokine IL-10 may also confer additional improvements in the efficacy of diabetes prevention, as a single administration of rVV-IL10 caused a detectable decrease in diabetes incidence at 15 and 27–31 wk of age. Previous experiments showed that CTB and VV alone do not significantly suppress hyperglycemia or insulitis in NOD mice ***(17,33)***. Presumably, protective efficacy may be further increased through selection of optimal rVV/cytokine dosages. Recently, it was confirmed that IL-10 can modulate the development of diabetes but this effect is dependent on applied IL-10 concentrations ***(13,26)***.

No additional increase or decrease in IgG1 to IgG2c ratios was detected in mice inoculated with

rVV containing CTB::INS, CTB::GAD, or the immunosuppressive cytokine IL-10 (**Fig. 4**) in later stages of the experiment suggesting that a Th2 helper cell driven anti-inflammatory response paradigm may also be the dominant immune response to rVV delivered CTB-islet autoantigen fusion proteins in NOD mice at early stages following virus inoculation. To confirm this hypothesis, repetition of the antibody isotype analysis should be performed at an earlier stage prior to insulitis development, e.g., in mice 5–10 wk of age, and with isotype antibodies adjusted for equal aviditiy for the autoantigens. Further confirmation of this hypothesis will be required based on splenocyte or pancreatic cell secreted cytokine assays of immunized mice.

Our experiments suggest that vaccinia delivery of autoantigen genes at later stages of insulitis onset (11 wk) can provide a therapeutic effect by suppressing the development of hyperglycemia. In comparison with unimmunized mice, NOD mice inoculated *orally* with rVV-CTB::INS after insulitis onset at 10–11 wk of age, showed a small reduction in development of hyperglycemia (10–20%), between 15 and 19 wk of age and also between 25 and 29 wk of age ***(17)***. Similarly, in comparison with naïve mice, systemic inoculation of 11-wk-old mice with rVV-CTB::INS partially protected immunized mice against hyperglycemia onset from 15 to 19, and from 27 to 31 wk of age. Interestingly, the reduction in diabetes incidence was higher after systemic ligand–autoantigen delivery (30%) than after oral delivery (20%). Further, inoculation of mice in which insulitis had already progressed provided significantly less protection from diabetes than mice inoculated prior to insulitis onset (5 wk old). This result was amplified at later stages of life (23–27 wk) where diabetes progressed to 40% of mice treated after 11 wk in contrast to only 10% diabetes progression in NOD mice inoculated at 5 wk of age. Thus, systemic inoculation of prediabetic mice with rVV-CTB::INS appears to reduce diabetes onset, especially at early stages of disease progression.

In comparison with unimmunized NOD mice in which most of the pancreatic islets develop peri- and especially intra-insulitis, rVV mediated immunization with adjuvanted autoantigens completely eliminated islet inflammation in most of the mice. However, occasionally mice (2 of 10) of the same group demonstrated the presence of a number of islets with varying degrees of inflammation suggesting that rVV mediated adjuvanted–autoantigen immunization does not completely eliminate islet inflammation in all mice. Further experiments in which mice are inoculated with vaccinia containing two or more adjuvant–autoantigen fusion genes may provide increased levels of immune suppression leading to complete inhibition of diabetes insulitis and hyperglycemia symptoms.

The application of VV as an autoantigen delivery vehicle and CTB as an adjuvant for enhanced suppression of autoimmune diabetes can provide several important advantages over existing methods of immunotolerization. Based on their wide host range, rapid infection, efficient expression of passenger transgenes, and production of foreign proteins that undergo mammalian post-translational modification, rVV is an attractive vehicle for transgene delivery into a variety of eukaryotic cells ***(27,28)***. Multiple antigen or autoantigen genes can be inserted into dispensable regions of the vaccinia genome without adverse effects on virus growth. The relative safety of live VV vaccines to humans has been clearly demonstrated in a major smallpox eradication program ***(27)***. To reduce possible side effects of live attenuated VV vaccines in immunocompromised individuals, the virus can be further attenuated by genetic manipulation ***(29,30)***. Several reports indicate that oral immunization with rVV can deliver antigens for protection against infectious diseases ***(31)*** and that the immune response can be intensified by co-immunization with cholera toxin as an adjuvant ***(32)***. These rVVs were successfully used for vaccination against a number of animal and human infectious diseases ***(28)***.

In summary, the results of preliminary experiments carried out in this study indicate that systemic delivery of rVV expressing the adjuvanted autoantigen CTB::INS can at a relatively low dose provide significant partial protection against type

1 autoimmune diabetes development in NOD mice. Further, a single recombinant virus inoculation late in the process of cytotoxic lymphocyte destruction of islet β cells may still confer a therapeutic effect in terms of reduction in hyperglycemia onset. Optimization of the virus dose and combination of autoantigens, along with IL-10 mediated anti-inflammatory responses, may lead to enhanced protection of prediabetic mice against the development of hyperglycemia. The design of future experiments will also include repeated inoculation dose responses to examine the possibility of additive or synergistic effects on enhanced protection against diabetes development.

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