Prevention of autoimmune diabetes by immunogene therapy using recombinant vaccinia virus expressing glutamic acid decarboxylase

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Abstract

Aims/hypotheses. Type I (insulin-dependent) diabetes mellitus results from T-cell-mediated autoimmune destruction of pancreatic beta cells. Among the beta-cell autoantigens that have been implicated in triggering of beta-cell-specific autoimmunity, glutamic acid decarboxylase (GAD) is a strong candidate in both humans and the NOD mouse. We aimed to determine whether treatment with a recombinant vaccinia virus expressing GAD (rVV-GAD65) could prevent the development of diabetes in NOD mice.

Methods. Three-eight-to-nine-week-old female NOD mice were injected with various doses of rVV-GAD65 or rVV-MJ601 as a control. We then examined the incidence of diabetes, T-cell proliferative response to GAD, amounts of anti-GAD IgGs, cytokine production and generation of regulatory cell populations.

Results. Administration of rVV-GAD65 to NOD mice prevented diabetes in an age-dependent and dose-dependent manner. Splenic T cells from rVV-GAD65-treated mice did not proliferate in response to GAD65.

The amount of IgG1 was increased, whereas IgG2a amounts did not change in rVV-GAD65-treated NOD mice. The production of interleukin-4 increased, whereas the production of interferon- γ decreased in rVV-GAD65-treated mice after stimulation with GAD. Furthermore, splenocytes from rVV-GAD65-treated NOD mice prevented the transfer of diabetes by splenocytes from acutely diabetic NOD mice in NOD.*scid* recipients.

Conclusion/interpretation. Immunogene therapy using a recombinant vaccinia virus expressing GAD results in the prevention of autoimmune diabetes in NOD mice by the induction of immunological tolerance through active suppression of effector T cells, and this treatment might have therapeutic value for the prevention of Type I diabetes. [Diabetologia (2002) 45:668–676]

Keywords Type I diabetes, NOD mice, recombinant vaccinia virus, glutamic acid, decarboxylase immunogene therapy, immunological tolerance, regulatory cells, effector T cells, Th2 immune response cytokine.

Received: 5 November 2001 and in revised form: 16 January 2002

Published online: 4 April 2002

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Abbreviations: GAD, Glutamic acid decarboxylase; IDDM, insulin dependent diabetes mellitus; NOD, non-obese diabetic; NS1, non-structural protein 1; PFU, plaque-forming units; rVV, recombinant vaccinia virus; scid, severe combined immunodeficiency

Type I (insulin-dependent) diabetes mellitus, results from the destruction of pancreatic beta cells by T cell-mediated, beta-cell-specific autoimmune responses [1, 2, 3, 4, 5, 6]. The non-obese diabetic (NOD) mouse is one of the best animal models for human Type I diabetes; it spontaneously develops the disease with many features in common with human Type I diabetes [7, 8, 9]. It has been shown that the destruction of pancreatic beta cells is mediated by both CD4⁺ and CD8⁺ T cells [10, 11, 12]. Therefore, various strategies, particularly autoantigen-based strategies, have been used to tolerise these autoreactive T cells and prevent autoimmune Type I diabetes in NOD mice. In NOD mice

and human Type I diabetes, glutamic acid decarboxylase (GAD) and insulin are known to be major beta-cell autoantigens that play an important role in the development of diabetes [13, 14].

To explore the potential of immunogene therapy based on GAD for the prevention of autoimmune diabetes, we developed a recombinant vaccinia virus (rVV) expressing GAD. Vaccinia virus has been used as a live vaccine against smallpox [15, 16]. Of particular importance are the findings that rVV can induce humoral and cell-mediated immune responses to a target protein and that the induced immune responses are long lived [17, 18, 19, 20]. For example, animals immunised with rVV expressing a protein from a pathogenic virus are protected from the disease caused by challenge with that virus [21, 22]. Of note too, in Phase I clinical trials with vaccinia virus expressing the human immunodeficiency virus 1 envelope gene, no serious side effects were found in healthy subjects that received percutaneous administration [23, 24]. These advantages of rVV would make it valuable for clinical use in Type I diabetes, if the administration of rVV expressing beta-cell autoantigen proteins such as GAD can tolerate the autoreactive T cells and prevent the development of diabetes. In this study, we attempted to prevent autoimmune Type I diabetes in NOD mice using rVV expressing GAD65 protein (rVV-GAD65).

Materials and methods

Animals. NOD mice were obtained from Taconic (Germantown, N.Y., USA) and NOD.severe combined deficiency (*scid*) mice were obtained from Jackson Laboratory (Bar Harbor, Me., USA). Mice were bred and housed under specific pathogen-free conditions and provided with sterile food and water at the Animal Resources Centre of the University of Calgary. Female NOD mice were used throughout the experiments. The use and care of the animals in this study were approved by the Animal Care Committee of the University of Calgary.

Construction of recombinant vaccinia virus expressing mouse GAD65 protein. The mouse cDNA coding for GAD65 [25] was cloned into the vaccinia virus vector pMJ601 [26] under the strong synthetic late promoter using the *NheI* and *BamHI* sites (pMJ-GAD65). The rVV was produced by homologous recombination, as described elsewhere [17, 18]. The recombinant plasmid (10 µg) was transfected into CV-1 cells (ATCC, Manassas, Va., USA), which had previously been infected with wild type vaccinia virus (0.05 plaque forming units [PFU]/cell), using the calcium phosphate method. As a control, the plasmid pMJ601 was transfected into CV-1 cells, instead of pMJ-GAD65, to produce rVV-MJ601. The rVV was plaque purified on HuTK-143B cells (ATCC) in the presence of 5-bromodeoxyuridine in agar overlay three times and amplified.

Immunisation with recombinant vaccinia viruses. Three-week-old female NOD mice were injected with 1×10^7 , 2.5×10^7 or 5×10^7 PFU i.p. of rVV-GAD65. As controls, age-matched and sex-matched NOD mice were injected with 1×10^7 , 2.5×10^7 or 5×10^7 PFU of rVV-MJ601 or rVV expressing an unrelated

protein, the non-structural protein (NS)-1 of the Kilham rat virus (rVV-NS1) [27]. To determine whether the effect of rVV-GAD65 is age-dependent, 8–9 week-old female NOD mice also were injected with 5×10^7 PFU i.p. of rVV-GAD65 or rVV-MJ601. The development of diabetes was monitored by the measurement of urine glucose using Diastix (Miles, Ont., Canada) and confirmed by measuring blood glucose using a one-touch Basic glucometer (Lifescan, Milpitas, Calif., USA). Any mouse with a blood glucose concentration of more than 16.7 mmol/l on three consecutive days was scored as diabetic.

In vitro proliferation assay. Single cell splenocytes were prepared from NOD mice 8 weeks after injection of 5×10^7 rVV-GAD65 or 5×10^7 rVV-MJ601 and from age-matched and sex-matched uninjected NOD mice. The cells (5×10^5) were cultured in 200 µl of complete RPMI-1640 medium supplemented with 10% fetal bovine serum, 1 mmol/l L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µmol/l beta-mercaptoethanol in a 96-well microplate in the presence of recombinant human GAD65 protein (produced in baculovirus) or ovalbumin for 72 h. The cells were then pulsed with ^3H -thymidine (1 µCi/well) for 16–18 h. The proliferation was quantified by determining the ^3H -thymidine incorporation using a liquid scintillation counter.

Assay of antibodies against GAD. Sera from NOD mice were collected 8 weeks after injection with 5×10^7 rVV-GAD65 or 5×10^7 rVV-MJ601 and reacted with recombinant human GAD65 protein (produced in baculovirus) as described elsewhere [28, 29]. GAD65 protein was coated onto a 96-well microplate (10 µg/ml in 0.1 mol/l NaHCO_3 , pH 8.5) at 4°C overnight. Sera from age-matched and sex-matched NOD mice were used as a control. After washing, the bound antibody was reacted with alkaline phosphatase-conjugated goat anti-mouse IgG, anti-mouse IgG1 or anti-mouse IgG2a antibodies. The colour was developed by incubation with 4-nitrophenyl phosphate. The optical density was read at 405 nm with an ELISA reader.

RT-PCR analysis. Total RNA was isolated from splenocytes of 5×10^7 rVV-GAD65- and 5×10^7 rVV-MJ601-immunized mice at 1, 2, 3 and 4 weeks after immunisation using a RNA extraction kit (Qiagen, Mississauga, Ont., Canada) according to the manufacturer's protocol. RNA from the splenocytes of age-matched and sex-matched NOD mice was used as a control. Two µg of total RNA was converted to cDNA using Superscript II reverse transcriptase (Gibco BRL, Gaithersburg, Md., USA) and oligo (dT)_{12–18}. PCR was carried out using specific primers for various cytokine genes, as described previously [30]. The primers used were as follows: *IL-2*: sense – CTTGCCCAAGCAGGCCACAG, antisense – GAGCCTTATGTGTTGTAAGC; *IFN-γ*: sense – AGCTCTGAGACAATGACGC, antisense – GGACAATCTCTTCCCCACCC; *IL-4*: sense – TCTTTCTCGAATCTACCAGG, antisense – CATGGTGGCTCAGTACTACG; and *IL-10*: sense – CAAACAAAGGACCAGCTGGAC, antisense – TTGACCTCAGCGCTGAGTTG. Hypoxanthine phosphoribosyl transferase (HPRT) mRNA was amplified as an internal standard; the primers used for HPRT were: sense – GTAATGATCAGTCAACGGGGGAC, antisense – CAAGCAAGCTTGCAACCTTAACCA. The PCR conditions were optimised for each set of primers. PCR was done using different numbers of cycles to ensure that amplification occurred in a linear range. The PCR mixture (50 µl) contained 0.2 mmol/l of deoxynucleotide triphosphate, 1 µmol/l of each specific primer, 1.5 mmol/l or 2 mmol/l MgCl_2 , 50 mmol/l KCl, 10 mmol/l Tris-Cl (pH 9.0) and 2.5 units of *Taq* polymerase (Pharmacia Biotech, Uppsala, Swe-

Fig. 1A, B. **A** Schematic structure of GAD-expressing vaccinia virus vector. Mouse GAD65 cDNA was inserted into the multiple cloning site of the vector, pMJ601, using *Bam*HI and *Nhe*I restriction enzyme sites under the late synthetic promoter of vaccinia virus. *TK_L*, thymidine kinase left region; *TK_R*, thymidine kinase right region; *LacZ*, beta-galactosidase; *GAD65*, mouse GAD65 cDNA. **B** Immunohistochemical staining of GAD65 expression in recombinant vaccinia virus. HeLa cells were infected with rVV-GAD65 or rVV-MJ601; the GAD65 protein was stained using an antibody to GAD, GAD1, and counterstained with haematoxylin and eosin. Left panel: HeLa cells infected with rVV-MJ601; Right panel: HeLa cells infected with rVV-GAD65. Brown precipitate indicates the presence of immunoreactive GAD

den). After amplification, the products were subjected to electrophoresis on a 1.5% agarose gel and detected by ethidium bromide staining.

ELISA of cytokine production. The production of cytokines in the splenic T cells of rVV-injected NOD mice was measured as described previously [30]. Splenocytes were isolated 8 weeks after injection of 5×10^7 rVV-GAD65 or 5×10^7 rVV-MJ601. The cells were cultured in 24-well plates (2×10^6 cells/well) in complete RPMI-1640 medium in the presence or absence of GAD protein for 72 h. The supernatant was collected and IFN- γ and IL-4 production was measured by sandwich ELISA using a Quantikine kit (R & D Systems, Minneapolis, Minn., USA).

Adoptive transfer of diabetes. Three week-old female NOD mice were injected with rVV-GAD65 or rVV-MJ601 (5×10^7 PFU/mouse). At 20 weeks of age, splenocytes (1×10^7 cells) were isolated from each donor and injected i.p. with or without splenocytes (1×10^7 cells) from uninjected, acutely diabetic NOD mice into 6 to 8 week-old NOD.*scid* mice. The development of diabetes was determined by the measurement of urine and blood glucose every other day. As controls, age-matched NOD.*scid* mice received splenocytes (1×10^7 cells) from uninjected, acutely diabetic NOD mice.

Immunohistochemistry. The expression of GAD65 protein was examined in rVV-GAD65-infected cells by immunohistochemistry using GAD1 monoclonal antibody (ATCC). Briefly, HeLa cells were cultured in Lab-Tek slide flasks (Nunc, Naperville,

Ill., USA) and infected with rVV-GAD65 or rVV-MJ601. At 3 days after infection, the cells were washed and fixed with acetone, incubated with GAD1 monoclonal antibody followed by horseradish peroxidase-conjugated secondary antibody, visualized using 3-3'-diaminobenzidine as a substrate (Vector Laboratories, Burlingame, Calif., USA) and counterstained with haematoxylin and eosin.

Histology. Mice that had been immunised with 5×10^7 rVV-GAD65 or 5×10^7 rVV-MJ601 at 3 weeks of age were killed at 25 and 40 weeks of age (5 mice/group) and the pancreata and salivary glands were removed. Each pancreas was fixed with 10% buffered formalin, embedded in paraffin, sectioned at 4.5 μ m and stained with haematoxylin and eosin [31]. Insulitis grade was determined as follows: 0, normal islet; 1, mononuclear infiltration, largely in the periphery, in less than 25% of the islet; 2, 25%–50% of the islet showing mononuclear infiltration; 3, over 50% of the islet showing mononuclear infiltration; 4, small, retracted islet with few mononuclear cells.

Statistical analyses. Statistical analyses were performed using Student's *t* test. A *p* value of 0.05 or less was considered to be statistically significant.

Results

Prevention of autoimmune diabetes and insulitis, but not sialitis, in NOD mice by immunisation with recombinant vaccinia virus expressing GAD. We cloned mouse GAD65 cDNA [25] into the vaccinia virus vector pMJ601 under the late synthetic promoter [26] (Fig. 1A) and produced rVV-GAD65. The expression of GAD protein was confirmed by immunohistochemistry; HeLa cells that had been infected with rVV-GAD65 expressed GAD65 protein (Fig. 1B, right panel), while those infected with rVV-MJ601 (control rVV without GAD65 cDNA) did not (Fig. 1B, left panel). To determine whether rVV-GAD65 prevented the development of autoimmune diabetes in NOD mice, we injected rVV-GAD65 (5×10^7 PFU) i.p. into 3 week-old female NOD mice and examined the development of diabetes. Only 7% (2/29) of the NOD

Fig. 2. Treatment of NOD mice with rVV-GAD65 at a young age prevented the development of diabetes. Three week-old female NOD mice were injected with rVV-GAD65 (—●—, $n = 29$), rVV-MJ601 (—○—, $n = 13$) or rVV-NS1 (—▲—, $n = 10$) (5×10^7 PFU) and the development of diabetes was monitored until 40 weeks of age by observing the onset of hyperglycaemia. Uninjected mice (—△—, $n = 9$) were used as a control

mice that received rVV-GAD65 developed diabetes, whereas 85% (11/13) and 80% (8/10) of the NOD mice that received rVV-MJ601 or rVV-NS1, respectively, developed diabetes by 40 weeks of age. Similarly, 89% (8/9) of the uninjected control NOD female mice also developed diabetes by the same age (Fig. 2). These data suggest that the prevention of diabetes by rVV-GAD65 is GAD antigen-specific, and not simply a non-specific effect of the vaccinia virus infection itself. However, the onset of diabetes in the rVV-MJ601- or rVV-NS1-infected mice was somewhat delayed compared with that in uninjected control NOD mice. The mean age of diabetes onset was 26 weeks in both the rVV-MJ601- and rVV-NS1-infected mice as compared to 21 weeks in the uninjected animals.

To determine whether the slight delay in the onset of diabetes in rVV-MJ601-injected or rVV-NS1-injected NOD mice could be attributed to a non-specific immune disturbance caused by rVV infection, we examined the cytokine gene expression of splenocytes at different times (1–4 weeks) after infection with the control virus. The expression of interleukin (*IL*)-2, interferon (*IFN*)- γ , *IL*-4 and *IL*-10 increased substantially 1 week after rVV-MJ601 or rVV-NS1 infection compared to uninfected control mice, thereafter cytokine expression gradually declined, reaching normal amounts 3 weeks after infection, similar to the amounts seen in uninfected mice (data not shown). This result suggests that the modest delay in disease onset in rVV-MJ601- or rVV-NS1-injected NOD mice could be due to a non-specific, transient immune disturbance.

The development of insulinitis in NOD mice injected with rVV-GAD65 was reduced compared to those in-

Fig. 3. Treatment of NOD mice with rVV-GAD65 prevented the development of insulinitis. Three-week-old female NOD mice were injected with rVV-GAD65 or rVV-MJ601 (5×10^7 PFU). Non-diabetic animals ($n=5$) were killed at 25 weeks of age and histological examination of pancreatic islets (at least 20 islets for each mouse) was performed. The insulinitis score was determined as described in Methods

jected with rVV-MJ601. A total of 87% of the examined islets from rVV-GAD65-injected NOD mice were intact (Figs. 3 and 4A), whereas 83% of the islets from rVV-MJ601-injected NOD mice showed moderate to severe insulinitis, similar to that found in uninjected control NOD mice, at 25 weeks of age (Figs. 3 and 4B, 4C). At 40 weeks of age, when the experiment was terminated, 42% of the examined islets from rVV-GAD65-injected mice remained intact, 50% of the islets showed mild peri-insulinitis or moderate insulinitis, and only about 8% of the islets showed severe insulinitis.

To determine whether the immunisation of NOD mice with rVV-GAD65 specifically affects beta-cell-specific autoimmunity, we examined the salivary glands, which show lymphocytic infiltration in NOD mice. There was equally severe infiltration of lymphocytes in the salivary glands of both rVV-GAD65- and rVV-MJ601-infected NOD mice at 25 weeks of age (Fig. 4D, E), similar to that of uninjected NOD mice (Fig. 4F). These results indicate that the suppression of autoimmunity induced by immunisation with rVV-GAD65 encompasses only the pancreatic islets, and not other target tissues that do not express GAD protein.

Preventive effect of rVV-GAD65 is dependent upon both dosage and age of immunised NOD mice. The influence of rVV-GAD65 dosage on disease expression was evaluated by immunising three-week-old NOD female mice with three different dosages of rVV-GAD65 (1×10^7 , 2.5×10^7 and 5×10^7 PFU/mouse, i.p.). Diabetes occurred in 67% (10/15) of the low dose (1×10^7 PFU) and 53% (8/15) of the intermediate dose (2.5×10^7 PFU) groups, as compared to 7% (2/29) in the high dose

Fig. 4A–F. Photomicrographs of representative islets from: **A** rVV-GAD65-injected mice, **B** rVV-MJ601-injected mice and **C** uninjected mice, and salivary glands from: **D** rVV-GAD65-injected mice, **E** rVV-MJ601-injected mice and **F** uninjected mice at 25 weeks of age. Sections were stained with haematoxylin and eosin

(5×10^7 PFU) rVV-GAD65-immunised NOD mice. In addition, the time of rVV-GAD65 immunisation altered its effectiveness. When older NOD mice (8 to 9 weeks of age) were immunised with rVV-GAD65 (5×10^7 PFU/mouse), 73% (8/11) became diabetic, values comparable to those of the unimmunised control NOD mice (75%, 9/12). Thus, the dose of GAD-expressing recombinant vaccinia virus and the age of the NOD mice at the time of immunisation determine the effect of the vaccine on the prevention of diabetes.

rVV-GAD65 immunisation of NOD mice tolerises T cell immune response to GAD and induces Th2 immune response. To determine the mechanism by which rVV-GAD65 prevented the development of diabetes, we first examined the proliferative response to GAD protein of splenocytes collected 8 weeks after injection with rVV-GAD65. These splenocytes did not respond to GAD (Fig. 5). In contrast, GAD stimulated the proliferation of splenocytes collected from rVV-MJ601-injected mice and uninjected control NOD mice (Fig. 5). Splenocytes from all three groups showed no proliferative response against ovalbumin, an antigen unrelated to beta-cell proteins. These results imply that GAD-reactive T cells are tolerated in rVV-GAD65-injected animals. We also examined the humoral immune response against GAD in the sera of NOD mice 8 weeks after injection of rVV-GAD65 or rVV-MJ601. There was an in-

Fig. 5. The T-cell immune response to GAD was diminished in rVV-GAD65-treated NOD mice. Splenocytes isolated from NOD mice 8 weeks after injection of rVV-GAD65 or rVV-MJ601 were reacted with GAD protein (GAD, cross-hatched columns), ovalbumin (OVA □) or control media (media ►). The cells were pulsed with 1 μ Ci of [3 H]-thymidine. Proliferation was determined by [3 H]-thymidine uptake. Results are shown as cpm $\pm$ SD of three experiments using two mice for each group. The proliferative response of splenocytes from uninjected mice were examined as a control. * p < 0.01 as compared to rVV-MJ601-injected mice

Fig. 6. Treatment of NOD mice with rVV-GAD65 increased IgG1 anti-GAD antibodies. Sera from rVV-GAD65-(►) and rVV-MJ601-vaccinated (cross-hatched columns) NOD mice and uninjected (□) NOD mice were analysed for anti-GAD IgG, IgG1 and IgG2a antibodies by ELISA as described in the Methods. Values are shown as means $\pm$ SD of 7 mice. * p < 0.001 as compared to rVV-MJ601-injected mice

crease in the amount of IgG antibodies to GAD in the serum of rVV-GAD65-injected mice as compared to rVV-MJ601-injected and uninjected mice. When the isotypes of the IgG antibodies to GAD were examined, the amount of the IgG1 subtype was specifically increased in rVV-GAD65-immunized NOD mice, whereas the IgG2a subtype did not change compared to rVV-MJ601-injected NOD mice

Fig. 7. Treatment of NOD mice with rVV-GAD65 increased IL-4 production and decreased IFN- γ production. Splenocytes were isolated from five mice from each group 8 weeks after vaccination and stimulated with (+GAD ►) or without (-GAD □) recombinant GAD65 protein for 3 days. The production of IFN- γ and IL-4 was determined in the culture supernatant by ELISA. Values are shown as mean $\pm$ SD of 5 mice. * p < 0.05, ** p < 0.001 as compared to rVV-MJ601-injected mice

(Fig. 6). These observations suggest that the rVV-GAD65 immunisation enhanced the Th2 immune response. This possibility was supported when we analysed IFN- γ and IL-4 release in vitro following GAD stimulation of splenocytes collected from NOD mice 8 weeks after rVV-GAD65- or rVV-MJ601 injection. The production of IL-4 increased, whereas the production of IFN- γ decreased in the splenocytes of rVV-GAD65-injected mice compared to splenocytes from rVV-MJ601-injected NOD mice (Fig. 7). These results suggest that immunisation of NOD mice with rVV-GAD65 induces a Th2 immune response in an antigen-specific manner.

rVV-GAD65 immunisation of NOD mice induces regulatory cells. Finally, we examined whether immunisation of NOD mice with rVV-GAD65 might promote the generation of regulatory cells, resulting in the protection of beta cells from destruction by effector T cells. We isolated splenocytes from rVV-GAD65- or rVV-MJ601-injected mice at 20 weeks of age and transfused these splenic lymphocytes alone, or together with an equal number of splenocytes from acutely diabetic NOD mice, into 6- to 8-week-old NOD.scid mice. We found that 13% (1/8) of the NOD.scid recipients of splenic lymphocytes from rVV-GAD65-treated NOD mice developed diabetes, whereas 86% (6/7) of NOD.scid recipients of splenic lymphocytes from

Table 1. Splenocytes from rVV-GAD65-treated NOD mice prevented the adoptive transfer of diabetes

Donor splenocytes	Incidence of diabetes – weeks after transfer							
	1	2	3	4	5	6	7	8 (%)
rVV-MJ601	0/7	0/7	1/7	3/7	5/7	6/7	6/7	6/7 (86)
rVV-GAD65	0/8	0/8	0/8	1/8	1/8	1/8	1/8	1/8 (13)
Diabetic	0/7	0/7	1/7	1/7	3/7	6/7	7/7	7/7 (100)
rVV-MJ601 + Diabetic	0/11	0/11	1/11	3/11	6/11	10/11	10/11	10/11 (91)
rVV-GAD65 + Diabetic	0/9	0/9	0/9	1/9	2/9	2/9	3/9	3/9 (33)

Splenocytes (1×10^7 cells) isolated from rVV-GAD65-treated or rVV-MJ601-treated NOD mice at 20 weeks of age were transferred alone or together with splenocytes (1×10^7 cells)

from acutely diabetic NOD mice into NOD.*scid* mice. The development of diabetes in the recipients was monitored

rVV-MJ601-treated NOD mice developed diabetes (Table 1). In addition, 33% (3/9) of the NOD.*scid* recipients of splenic lymphocytes that included cells from rVV-GAD65-injected NOD mice developed diabetes, whereas 91% (10/11) of the NOD.*scid* recipients of splenic lymphocytes that included cells from rVV-MJ601-injected mice developed diabetes (Table 1). These results imply that the vaccine might alter disease expression in NOD mice by altering cellular immune function, such as by inducing a regulatory T cell population.

Discussion

Tolerance induction of autoreactive T cells against specific target autoantigens has been attempted as a method to prevent autoimmune disease. In Type I diabetes, GAD and insulin have been identified as major autoantigens and tolerisation against these autoantigens has been attempted as a method for the prevention of the disease [14, 32]. It has been reported that administration of purified GAD protein or peptide or insulin protein or peptide to NOD mice by a variety of routes can tolerate the T cell-mediated immune response against pancreatic beta cells, resulting in the prevention or delay of the development of insulinitis and diabetes [28, 29, 33, 34, 35, 36, 37, 38, 39]. In many cases, the preventive effect was found to be associated with a Th2 shift [14, 32].

An extension of this idea is the vaccination of NOD mice with plasmid DNA encoding GAD or insulin although such studies have inconsistent results. Some showed that intramuscular injection of a plasmid encoding GAD65 resulted in the prevention of diabetes [40, 41]; others showed that injection of a plasmid encoding GAD65 was not effective [42, 43, 44]. However, a plasmid encoding GAD65 was effective only if the plasmid also contained DNA encoding IL-4 [42, 44]. Some studies showed that an insulin gene vaccine had a preventive effect [43, 45]; another showed that an insulin gene vaccine accelerated the onset of diabetes [42]. These different results might be due to different

amounts of autoantigen gene expression in the vaccinated mice, as the transfection efficiency by intramuscular injection is quite low. Therefore, in our study, we used vaccinia virus as a carrier for the GAD65 autoantigen gene for the vaccination of NOD mice because the vaccinia virus vector has a high transduction efficiency and induces a strong immune response. We found that a single immunisation of three-week-old NOD mice with rVV-GAD65 resulted in the almost complete prevention of autoimmune diabetes.

Our first question was whether vaccinia virus itself might cause a non-specific immune disturbance that might result in the prevention of autoimmune diabetes in NOD mice immunised with rVV-GAD65. To exclude this possibility, we immunised NOD mice with recombinant vaccinia virus containing the vector pMJ601 without the GAD65 cDNA insert. A total of 85% of these mice became diabetic, an incidence similar to that in untreated NOD mice, although the onset of disease was delayed. Similarly, 80% of mice that received another control treatment, rVV expressing the unrelated protein NS1 of the Kilham rat virus, developed diabetes. This result indicates that the prevention of autoimmune diabetes by rVV-GAD65 immunisation is not due to a non-specific immune disturbance but an antigen-specific effect.

Our second question was whether the prevention of autoimmune diabetes by immunisation with GAD-expressing vaccinia virus is dose-dependent or age-dependent. To answer the question of age, we immunised female NOD mice with rVV-GAD65 before (3 weeks of age) or after (8–9 weeks of age) the development of insulinitis. We found that immunisation with rVV-GAD65 before the development of insulinitis almost completely prevented diabetes, whereas immunisation after the development of insulinitis did not prevent diabetes indicating that immunisation with this vaccine at an early age, before insulinitis, is required for the preventive effect to occur. The lack of preventive effect by rVV-GAD65 in 8–9 week-old NOD mice might be due to the pre-existing increase in the Th1 immune response at this age and the failure to sufficiently induce a Th2 immune response in these

animals. To determine whether the dose of rVV-GAD65 is critical for the prevention of diabetes in NOD mice, we immunised three-week-old NOD female mice with 3 different doses of rVV-GAD65. We found that only 7% of mice immunised with a dose of 5×10^7 PFU of rVV-GAD65 developed diabetes, while lower dosages (2.5×10^7 PFU and 1×10^7 PFU) resulted in a reduced effect (53% and 67% diabetic, respectively). This result indicates that the dose of GAD-expressing vaccinia virus is critical for the prevention of autoimmune diabetes.

Our third question was what mechanisms are involved in the prevention of autoimmune diabetes in NOD mice by rVV-GAD65 treatment. We examined the proliferative response of splenocytes from NOD mice infected with rVV-GAD65 to GAD protein. We found that these splenocytes did not respond to GAD, implying that GAD-reactive T cells were tolerised in the rVV-GAD65-treated mice. We then examined the humoral immune response against GAD in the sera of NOD mice infected with rVV-GAD65. We found that the amount of the IgG1 subtype was increased in the rVV-GAD65-infected mice ($p < 0.001$), whereas the IgG2a subtype was not changed, suggesting that immunisation of NOD mice with rVV-GAD65 results in the enhancement of the Th2 immune response. To determine whether the Th2 immune response is truly involved in the disease process, we examined IFN- γ and IL-4 release in vitro following GAD stimulation of splenocytes obtained from NOD mice infected with rVV-GAD65. We found that the production of IL-4 increased, whereas the production of IFN- γ decreased, indicating that the immunisation of NOD mice with rVV-GAD65 induced a Th2 immune response in an antigen-specific manner. Our finding is consistent with the results of previous reports on NOD mice treated with GAD protein systemically, nasally or orally [28, 29, 33, 34, 35] or vaccinated with GAD encoding plasmid DNA [41]. Th2 immune responses induced by rVV-GAD immunisation could play an important role in the prevention of autoimmune diabetes in this animal model. In addition, the increase in anti-GAD antibodies could synergise the preventive effect, as the administration of anti-GAD antibody has been shown to prevent the development of diabetes in NOD mice [46].

We then examined whether rVV-GAD65 treatment induced the generation of regulatory cells that could suppress diabetogenic effector T cells. We adoptively co-transferred splenocytes from rVV-GAD65 immunised NOD mice with splenocytes from acutely diabetic NOD mice into NOD.scid mice. The splenocytes from the rVV-GAD65 immunised mice were found to inhibit the transfer of diabetes by splenocytes from acutely diabetic NOD mice. This result suggests that immunisation with rVV-GAD65 could generate a regulatory population. GAD expressed in rVV-GAD65-infected cells could present GAD peptide in conjunction with MHC antigens and stimulate the regulatory

cells. However, the precise mechanism involved in the generation of regulatory T cells in rVV-GAD65-treated mice remains to be determined.

In conclusion, we have developed a recombinant vaccinia virus expressing GAD65. Immunogene therapy using this rVV-GAD65 resulted in the almost complete prevention of autoimmune diabetes in NOD mice by a single injection. Further studies on the mechanisms for this preventive effect suggest that the therapeutic effect of rVV-GAD65 was due to the induction of immunological tolerance through the active suppression of effector T cells by the induction of Th2 immune responses, rather than clonal deletion or clonal anergy. As no serious side-effects were found in healthy human subjects that received percutaneous administration of vaccinia virus [23, 24], a recombinant vaccinia virus expressing GAD might have therapeutic value for the prevention of Type I diabetes.

Acknowledgements. We gratefully acknowledge B. Moss for providing the pMJ601 vector, D. Kaufman for providing the mouse GAD65 cDNA, K. Hirasawa for performing the immunocytochemistry, and A. L. Kyle for editorial assistance. This work was supported by grants from the Canadian Institutes of Health Research (MA 9584), the Juvenile Diabetes Foundation International, the Korean Green Cross Corporation, the National Institutes of Health (DK 45735 and DK 53015-01) and the Alberta Heritage Foundation for Medical Research. J. W. Yoon holds a Canada Research Chair in Diabetes.

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