JOP. J. Pancreas (Online) 2002; 3(3):76-82.

A Single Treatment with IL-4 via Retrovirally Transduced Lymphocytes Partially Protects Against Diabetes in BioBreeding (BB) Rats

Danny Zipris, Eddy Karnieli

The Institute of Endocrinology, Diabetes, and Metabolism, Rambam Medical Center. B. Rappaport Faculty of Medicine, Technion-Israel Institute of Technology. Haifa, Israel

ABSTRACT

Context Type 1 diabetes mellitus is a T cell mediated autoimmune disease with no known methods of prevention. The BioBreeding rat is used as an animal model for the study of human Type 1 diabetes. In spite of a severe lymphopenia, these animals develop spontaneous diabetes at the age of 10-12 weeks.

Objective To examine whether anti-inflammatory gene therapy could be used to prevent autoimmune diabetes in the BioBreeding rat.

Design A retroviral DNA vector, MSCVneo.IL-4, carrying the DNA sequence encoding the rat interleukin-4, was designed to transfer interleukin-4 to BioBreeding rats. Spleen cells of prediabetic animals were activated and transduced *in vitro* with replication-defective retroviruses expressing the MSCVneo.IL-4 vector. These lymphocytes were subsequently administered intraperitoneally to 3-4 week old prediabetic BioBreeding rats. Control animals were reconstituted with spleen cells transduced with MSCVneo vector.

Results The *neo* gene marker was detectable by RT-PCR in rat spleen cells of up to 6 to 12 months after treatment. Fifty percent (6 out of 12) of the animals treated were protected from autoimmune disease development.

Conclusion Our results suggest that the BioBreeding rat can be used as a useful model to develop gene therapy regimens for diabetes. These studies provide further support for the hypothesis that interleukin-4 based gene therapy may have potential clinical value for preventing autoimmune diabetes in humans.

INTRODUCTION

Type 1 diabetes mellitus is a T cell-mediated autoimmune disease, leading to the specific destruction of insulin producing beta cells [1]. The BioBreeding (BB) rat develops spontaneous autoimmunity against insulin-producing beta cells at the age of 12-14 weeks, and is therefore used as an animal model for diabetes [2]. Infiltration of macrophages into the pancreas occurs early in the development of diabetes in the BB rat. Subsequently, $CD4^+$ and $CD8^+$ lymphocytes, NK cells and B cells are recruited to the autoimmune lesion [1, 2, 3, 4, 5]. The BB rat is severely lymphopenic, and a single transfusion of lymphocytes from a normal histocompatible donor rat can protect against development of autoimmune disease [6].

It is thought that autoimmune diabetes in the BB rat and the non-obese diabetic (NOD) mouse model result from an immunological imbalance between Th1 and Th2 lymphocytes in pancreatic islets [7, 8, 9]. Th1 cells produce interferon gamma (IFNγ), interleukin-2 (IL-2), and tumor necrosis factor beta (TNFβ), which regulate cell-mediated immunity. Th2 cells produce IL-4, IL-5, and IL-10, which mediate humoral immunity (for review, see [10]). Studies of the BB rat and the NOD mouse models of autoimmune diabetes have shown that destruction of islet cells is associated with the presence of Type 1 cytokines in pancreatic beta cells. In contrast, an increase in the expression of Type 2 cytokines in islets protects against development of autoimmune diabetes [8, 11, 12]. Based on these and other findings, it has recently been suggested that cytokine-based gene therapy can be used as a preventive approach against autoimmune diabetes [13]. In this study, we tested the hypothesis whether anti-inflammatory IL-4 based gene therapy could prevent diabetes in the BB rat.

METHODS

Animals and Diagnosis of Diabetes

BB breeding pairs were obtained from the University of Massachusetts Medical Center, and were bred under specific pathogen-free conditions at the Technion, B. Rappaport Faculty of Medicine Animal Facilities, Haifa, Israel. Spleen cells from major histocompatibility complex (MHC)-compatible Wistar Furth (WF) normal rats were transferred to BB breeding pairs, to protect them against diabetes. Rats were checked for glycosuria twice weekly beginning at about 60 days of age. A glucose analyzer was used to determine plasma glucose levels. Glycosuric rats were designated as diabetic if two successive daily plasma glucose measurements were greater than 11.1 mmol/L.

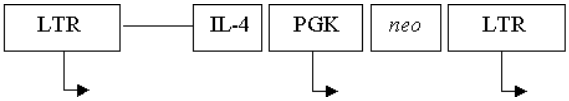

Figure 1. Schematic diagram of the MSCVneo vector containing the rat IL-4 coding sequence. Arrows indicate long terminal repeats and phosphoglycerate kinase (PGK) promoters utilized for the expression of the IL-4 and *neo* genes, respectively.

Experimental Design

We examined whether IL-4 based retroviral-mediated gene therapy is feasible and could interfere with the process of autoimmunity in the BB rat model. We aimed to develop a relatively simple gene therapy protocol, using whole spleen cells as carriers for the transfer of IL-4 to BB rats. Spleen cells from prediabetic rats were transduced with retroviruses expressing IL-4 and then injected into BB rats.

Construction of Retroviral Vector MSCVneo.IL-4 and Production of High Titers of Retroviruses

Total RNA was isolated from Con A activated spleen cells from control WF rats. The RNA was subjected to reverse transcription and amplification with specific oligonucleotides derived from the rat IL-4 gene [14]. The PCR fragment (442 nucleotides) was then cloned into the PCRII cloning vector (Invitrogen), and subjected to DNA sequencing, to confirm the identity of IL-4. The resulting PCRII construct was subjected to enzymatic digestion with EcoRI and ligated into the EcoRI site of MSCVneoEB (kindly provided by Dr. Robert Hawley, Division of Cancer Research, University of Toronto, Canada; see Figure 1 and reference [15]). The MSCVneo.IL-4 retroviral vector was transfected into the GP+E-86 packaging cell line, which provides the necessary proteins required for the generation of replication-defective viruses expressing the IL-4 gene [15]. Using G418, we selected GP+E-86 cells that produced high titers of retroviruses (10^7 CFU/mL).

Supernatants derived from these packaging cells were subsequently used to infect the amphotropic PA317 cell packaging cell line, which also generated high titers of retroviruses (10^7 CFU/mL).

Transduction of Cells with Retroviral Vectors

The transduction of peripheral lymphocytes with retroviruses has previously been described [16]. Briefly, spleen cells were activated with Con A in the presence of 20 U/mL of human recombinant IL-2 for 48 h. Cells were then harvested, washed, and resuspended in retrovirus-containing supernatants at a density of $2x10^6$ cells/mL. Cells were incubated in the presence of rIL-2 and 8 mg/mL polybrene, to facilitate viral infection. Cells were then placed in 6-well tissue culture plates and centrifuged at 2,500 rpm for 90 min at 32 ºC. After overnight incubation at 37º C, the cells were washed and then replated in fresh media containing rIL-2. Five days later, $5\text{-}10x10^6$ lymphocytes were harvested and administered intraperitoneally into 3-4 week old animals.

Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted by the guanidine thiocyanate phenol-chloroform method and subjected to reverse transcription to generate cDNA [8]. cDNA was incubated with sense and anti-sense primers in a thermocycler, using one cycle at 94º C for 5 min, 35 cycles at 94º C for 30 sec, 60º C for 60 sec, and 72º C for 60 sec. Agarose gel electrophoresis and ethidium bromide staining were used to visualize the PCR fragments. Primer sequences were derived from online searches of the GenBank and were checked for potential areas of dimerization. To exclude the possibility that amplification resulted from a contamination of the RNA preparations with genomic DNA, control experiments were performed in which RNA alone was amplified. RNA from Con A-activated spleen cells was used as a positive control. The sequences of oligonucleotides used to detect β-actin and IL-4 have previously been published [8].
The sequences of primers used for the detection of *neo* mRNA are as follows: Forward 5'-CCG GTG CCC TGA ATG AAC TGC-3'; Reverse 5'-CAA TAT CAC GGG TAG CCA ACG-3'.

Sandwich ELISA

IL-4 levels were determined using an ELISA designed with matched Ab pairs (PharMingen, San Diego, CA, USA). In short, 96-well plates were coated with mouse anti-rat IL-4 (2 μg/mL) overnight at 4 °C in coat buffer (50 mM H_3BO_3 and 120 mM NaCl, pH 8.6), and washed with wash buffer (PBS and 0.05% Tween-20). Wells were blocked with block buffer (PBS and 2% BSA) for 1 h at 37 °C and washed. Recombinant rat IL-4 (R&D Systems, Minneapolis, MN, USA) were added to the wells in duplicate for 3 h at 37 °C, followed by washing. Biotinylated rabbit anti-rat IL-4 (1 μg/mL) in block buffer was incubated for 1 h at 37 °C and washed, followed by incubation with streptavidin-HRP (PharMingen, San Diego, CA, USA; 1/10,000) in block buffer. After 30 min, the wells were washed, and HRP was detected with 3,3',5,5'-tetramethyl-benzidine liquid substrate system (Sigma, St. Louis, MO, USA). Color development was terminated with 0.5 M H_2SO_4, and the plates were read at 450 nm.

ETHICS

The experiments described in this report were approved by our institutional animal research committee. The animals used were cared for in accordance with the principles of the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences (NIH publication 86-23 revised 1985).

STATISTICS

Differences in diabetes development were analyzed using Kaplan Meier survival analysis with log rank test with the level of significance set as P less than 0.05.

RESULTS

Expression of IL-4 Gene and Protein

We initially tested whether MSCVneo.IL-4 (shown in Figure 1) can drive the expression of IL-4 in NIH 3T3 cells. Target cells were infected with retroviruses expressing the MSCVneo.IL-4 vector and the infected cells were then selected for neomycin-resistance. We found that IL-4 is expressed in IL-4 transduced NIH 3T3 cells, as determined by RT-PCR (Figure 2). Similarly, we found IL-4 mRNA in PA317 packaging cells that were transduced with MSCVneo.IL-4 expressing

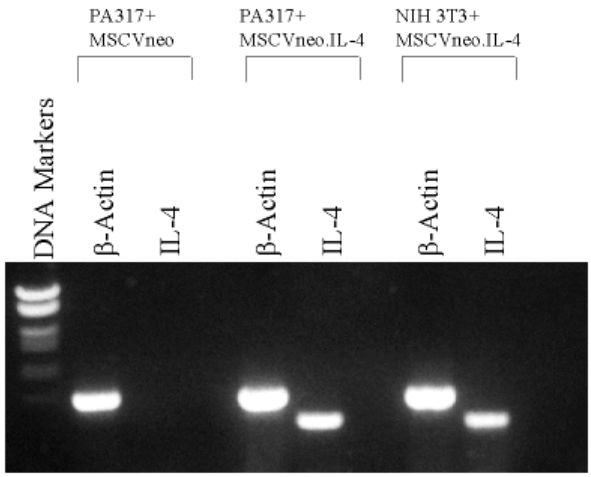

Figure 2. Expression of IL-4 mRNA in PA317 packaging and NIH 3T3 cells transduced with MSCVneo or MSCVneo.IL-4 retroviral vectors, as outlined. MSCVneo.IL-4 or control MSCVneo retroviral vectors were transfected into the GP+E-86 packaging cell line, as described in Materials and Methods. Using G418, high titers of retrovirus producing GP+E-86 cells were selected and supernatants derived from these cells were subsequently used to infect the amphotropic PA317 packaging cell line and NIH 3T3 target cells. Total RNA was extracted from transduced cells and RT-PCR was used to detect IL-4 mRNA. β-Actin was used as a positive control in all experiments. PCR products were visualized on 2% agarose gels with ethidium bromide.

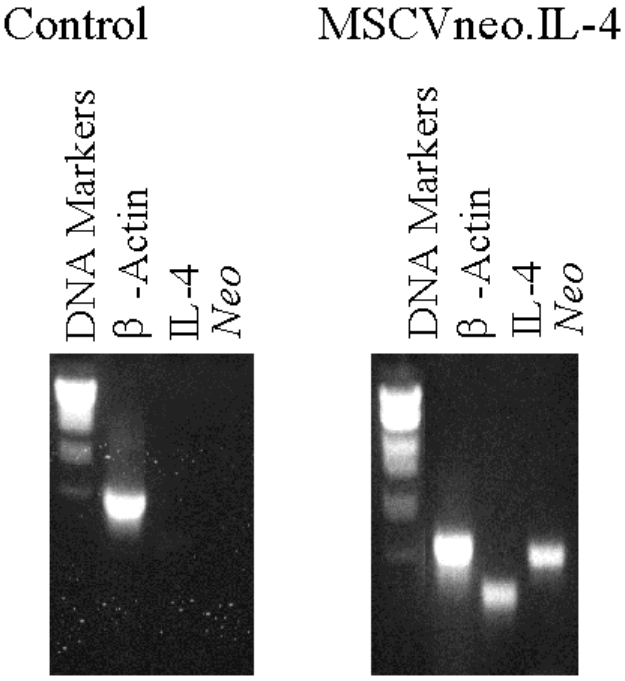

Figure 3. Expression of IL-4 mRNA in BB spleen cells transduced with MSCVneo or MSCVneo.IL-4 retroviral vectors, as outlined. BB spleen cells were activated for 48 h with Con A in the presence of rIL-2, harvested and subsequently incubated in the presence of MSCVneo.IL-4-containing supernatants, as described in Materials and Methods. Total RNA was extracted from transduced cells and the presence IL-4 mRNA was examined by RT-PCR. β-Actin was used as a positive control. PCR products were visualized on 2% agarose gels with ethidium bromide.

retroviruses. We next determined the level of IL-4 expression in IL-4 transduced NIH 3T3 cells and found that IL-4 protein was present in supernatants derived from these cells at levels of about 2 μg/mL, as determined by an ELISA (data not shown).

Transduction of BB Spleen Cells with MSCVneo.IL-4

We further examined whether whole BB spleen cells can be transduced with the IL-4 gene. Forty-eight hours after activation in the presence of IL-2, whole spleen cells were infected with retroviruses expressing MSCVneo.IL-4. Five days later, we examined the expression of IL-4 and *neo* mRNA using RT-PCR. Spleen cells transduced with MSCVneo.IL-4 expressed both IL-4 and *neo*

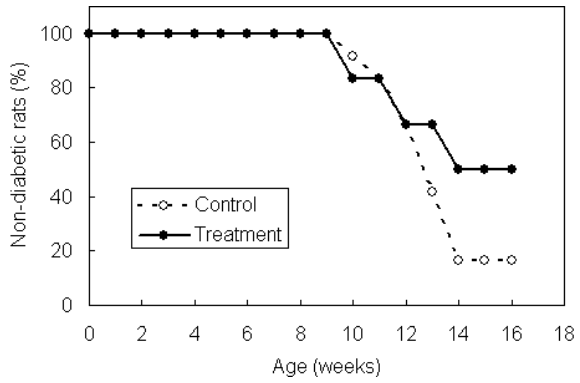

Figure 4. The incidence of diabetes in BB rats treated with whole spleen cells transduced with MSCVneo.IL-4 retroviral vector. Twelve prediabetic 3-4 wk old BB rats were injected with 5-10x10^6 MSCVneo.IL-4 transduced cells (Treatment) and 12 rats were treated with control cells containing MSCVneo control vector (Control). Blood glucose measurements (two successive daily plasma glucose measurements greater than 11.1 mmol/L) were used to assess diabetes. Using Kaplan Meier survival analysis with log rank test no significant difference (P=0.150) was found between rats treated with transduced cells and the control group.

(Figure 3), suggesting that the IL-4 gene was transcribed in these cells.

Treatment of BB Rats with Transduced Spleen Cells

Five to ten million transduced spleen cells were administered to 3-4 week old prediabetic BB rats. We observed that 50% (6/12) of the rats treated with cells that had been infected with the MSCVneo.IL-4 vector developed hyperglycemia at the age of 10-14 weeks. In contrast, 83.3% (10/12) of rats treated with the control vector developed hyperglycemia at the same age (Figure 4). Using log rank analysis, this difference failed to reach statistical significance, probably because of the small number of rats used in each group (P=0.150).

Expression of *neo* in Treated BB Rats

However, in order to support our conclusion, we then tested whether the *neo* gene is expressed in BB rats treated with MSCVneo.IL-4 transduced spleen cells. Total RNA was extracted from the spleens of BB rats 6 and 12 months following treatment. Using RT-PCR analysis, we readily detected *neo* mRNA in the spleens of animals that had received MSCVneo.IL-4. Message encoding *neo* was not detectable in the spleen cells of the control naïve animals (Figure 5).

DISCUSSION

In this study, we developed a relatively simple gene therapy protocol, using whole spleen cells as carriers for the transfer of IL-4 to BB rats which are a model of Type 1 diabetes mellitus. Our findings demonstrate that BB rat spleen cells can be transduced with the rat IL-4 gene and express IL-4 mRNA and that a single administration of IL-4 transduced cells to BB rats can lead to long-term expression of the transgene and a partial protection against the disease. The data are not conclusive because of the small number of animals used.

The mechanism by which IL-4 transduced cells protect from the onset of the disease is still unclear. However, our data suggest that

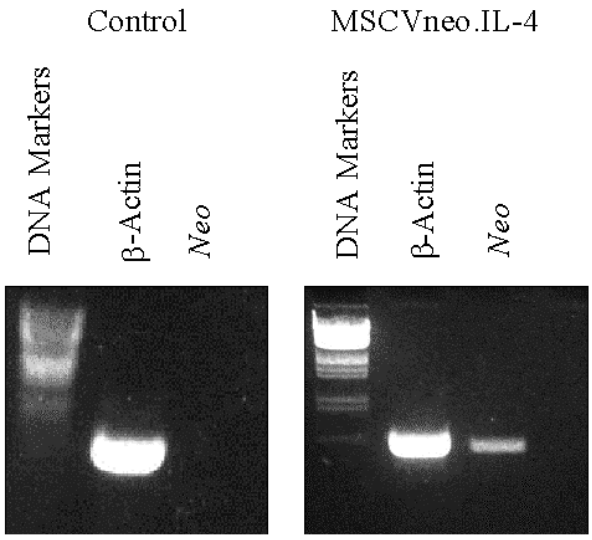

Figure 5. Expression of *neo* mRNA in spleen cells of BB rats treated 6 months previously with either untransduced cells or with MSCVneo.IL-4 transduced spleen cells. Total RNA was extracted from the spleens of treated rats and expression of the *neo* marker gene was determined by RT-PCR. β-Actin was used as a positive control. PCR products were visualized on 2% agarose gels with ethidium bromide.

the level of IL-4 expressed by the transferred cells is sufficient to modulate the immune response of BB rats. Our findings are consistent with previous reports that the transfer of spleen cells transduced with retroviruses expressing IL-4 are protected from diabetes [17, 18, 19]. It has been proposed that these cells protect from this disease by homing to pancreatic islets and downregulating autoreactive T cells [17, 18, 19].

In conclusion, our findings suggest that the BB rat can be used as a useful model to study gene therapy for diabetes. In addition, our findings demonstrate that IL-4 based gene therapy has potential therapeutic value for preventing Type 1 autoimmune diabetes in humans.

Received January 8th, 2002 - Accepted April 23rd, 2002

Key words Diabetes Mellitus, Insulin-Dependent; Gene Therapy; Interleukin-4; Primary Prevention; Rats, Inbred BB

Abbreviations BB: BioBreeding; IFN: interferon; IL: interleukin; MHC: major histocompatibility complex; NOD: non-obese diabetic; PGK: phosphoglycerate kinase; WF: Wistar Furth

Acknowledgments This study was supported by a grant from the Chief Scientist of the Ministry of Health of Israel, and supported in part by grants (to EK) from the L.R. Diamond Fund.

Correspondence
Eddy Karnieli
Institute of Endocrinology, Diabetes, and Metabolism
Rambam Medical Center
Haifa 31096
Israel
Phone: +972-4- 854.2706
Fax: +972-4-854.2746
E-mail address: eddy@tx.technion.ac.il

References

1. Bach JF. Insulin-dependent diabetes mellitus as an autoimmune disease. Endocrinol 1994; 15:516-42. [AN 95080168]

2. Rossini AA, Mordes JP, Like AA. Immunology of insulin-dependent diabetes mellitus. Annu Rev Immunol 1985; 3:289-320. [AN 86050993]

3. Crisa L, Mordes JP, Rossini AA. Autoimmune diabetes mellitus in the BB rat. Diabetes Metab Rev 1992; 8:4-37. [AN 92339302]

4. Hanenberg H, Kolb-Bachofen V, Kantwerk-Funke G, Kolb H. Macrophage infiltration precedes and is a prerequisite for lymphocytic insulitis in pancreatic islets of pre-diabetic BB rats. Diabetologia 1989; 32:126-34. [AN 89252531]

5. Weringer EJ, Like AA. Identification of T cell subsets and class I and class II antigen expression in islet grafts and pancreatic islets of diabetic BioBreeding/Worcester rats. Am J Pathol 1988; 132:292-303. [AN 88292511]

6. Burstein D, Mordes JP, Greiner DL, Stein D, Nakamura N, Handler ES, Rossini AA. Prevention of diabetes in BB/Wor rat by single transfusion of spleen cells. Parameters that affect degree of protection. Diabetes 1989; 38:24-30. [AN 89078800]

7. Mordes JP, Bortell R, Doukas J, Rigby M, Whalen B, Zipris D, et al. The BB/Wor rat and the balance hypothesis of autoimmunity. Diabetes Metab Rev 1996; 12:103-9. [AN 97031354]

8. Zipris D, Greiner DL, Malkani S, Whalen B, Mordes JP, Rossini AA. Cytokine gene expression in islets and thyroids of BB rats: IFN-gamma and IL-12p40 mRNA increase with age in both diabetic and insulin-treated BB rats. J Immunol 1996; 156:1315-21. [AN 96144359]

9. Zipris D. Evidence that Th1 lymphocytes predominate in islet inflammation. J Autoimmun 1996; 9:315-9. [AN 96413831]

10. Paul WE, Seder RA. Lymphocyte responses and cytokines. Cell 1994; 76:241-51. [AN 94123332]

11. Rabinovitch A, Suarez-Pinzon WL, El-Sheikh A, Sorensen O, Power RF. Cytokine gene expression in pancreatic islet-infiltrating leukocytes of BB rats: Expression of Th1 cytokines correlates with beta-cell destructive insulitis and IDDM. Diabetes 1996; 45:749-54. [AN 96220075]

12. Rabinovitch A, Suarez-Pinzon WL, Sorensen O, Bleackley RC, Power RF. IFN-gamma gene expression in pancreatic islet-infiltrating mononuclear cells correlates with autoimmune diabetes in nonobese diabetic mice. J Immunol 1995; 154:4874-82. [AN 95238990]

13. Efrat S. Prospects for gene therapy of insulin-dependent diabetes mellitus. Diabetologia 1998; 41:1401-9. [AN 99083095]

14. McKnight AJ, Barclay AN, Mason DW. Molecular cloning of rat interleukin 4 cDNA and analysis of the cytokine repertoire of subsets of CD4+ T cells. Eur J Immunol 1991; 21:1187-94. [AN 91243732]

15. Hawley RG, Lieu FH, Fong AZ, Hawley TS. Versatile retroviral vectors for potential use in gene therapy. Gene Ther 1994; 1:136-8. [AN 96050932]

16. Rudoll T, Phillips K, Lee SW, Hull S, Gaspar O, Sucgang N, et al. High efficiency retroviral vector mediated gene transfer into human peripheral blood CD4+ T lymphocytes. Gene Ther 1996; 3:695-705. [AN 97006819]

17. Cameron MJ, Arreaza GA, Waldhauser L, Gauldie J, Delovitch TL. Immunotherapy of spontaneous type 1 diabetic mice by systemic interleukin-4 treatment employing adenovirus vector-mediated gene transfer. Gene Ther 2000; 7:1840-6. [AN 20560366]

18. Chang Y, Prud'homme GJ. Intramuscular administration of expression plasmids encoding interferon-gamma receptor/IgG1 or IL-4/IgG1 chimeric proteins protects from autoimmunity. J Gene Med 1999; 1:415-23. [AN 20214741]

19. Yamamoto AM, Chernajovsky Y, Lepault F, Podhajcer O, Feldmann M, Bach JF, Chatenoud L. The activity of immunoregulatory T cells mediating active tolerance is potentiated in nonobese diabetic mice by an IL-4-based retroviral gene therapy. J Immunol 2001; 166:4973-80. [AN 21186220]