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Ingested interferon α suppresses Type I diabetes in non-obese diabetic mice

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Summary Type I diabetes mellitus is a chronic disorder that results from autoimmune destruction of the insulin-producing pancreatic beta cell. The non-obese diabetic mouse is a model of the human autoimmune disease Type I diabetes [1–3]. We have previously shown that ingested type 1 interferon inhibits chronic relapsing experimental autoimmune encephalomyelitis and the adoptive transfer of experimental autoimmune encephalomyelites by T cells, and decreases both antigen-specific and mitogen-induced pro-inflammatory cytokine secretion in this disorder. We therefore tried to determine whether ingested murine interferon α inhibits insulinitis and suppresses Type I diabetes mellitus in non-obese diabetic mice. Murine interferon α, given daily, decreased islet inflammation and suppressed diabetes. It increased the concanavalin A and ionomycin plus myristic acid palmitic ester-induced production of interleukin 4 and 10 and interferon γ-secretion in spleen cells from treated mice. Adoptive transfer of unstimulated splenocytes secreting interleukin 4 and interleukin 10 from fed interferon α donors suppressed spontaneous diabetes mellitus in recipients. The protective effect of adoptively transferred unstimulated splenocytes shows the presence of ingested interferon α-activated regulatory splenic cell populations that may work via increased interleukin 4 or interleukin 10 production. Ingested interferon α administered during vulnerable periods in at-risk populations may potentially provide a continuous, convenient, non-toxic and effective treatment for Type I diabetes. [Diabetologia (1998) 41: 1227–1232]

Keywords Non-obese diabetic mouse, ingested interferon α, interleukin 4, interleukin 10, adoptive transfer

Many key features of human Type I diabetes are reflected in the non-obese diabetic (NOD) mouse model. These include the development of insulitis, with infiltration of lymphocytes that are selectively cytotoxic to the insulin producing beta cells into the pancreatic islets of Langerhans, the dependence of disease pathogenesis by T cells, and the transmission of Type I diabetes by haematopoietic cells in bone marrow [1–5].

Experimental autoimmune encephalomyelitis (EAE) is an animal model for the presumed autoimmune disease multiple sclerosis. We have previously shown that ingested type 1 interferon (IFN) inhibits chronic relapsing EAE, inhibits the adoptive transfer of EAE by T cells, decreases both antigen-specific and mitogen-induced pro-inflammatory cytokine secretion and decreases serum soluble intercellular adhesion molecule 1 (sICAM-1) levels, a marker for subclinical disease activity, in multiple sclerosis without the absorption of ingested IFN [6–8].

The NOD mouse [9–11] model is mechanistically analogous to the EAE animal model because both are presumed to be mediated by a T cell subset, and depend on restriction elements and inflammatory cy-

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Abbreviations: EAE, experimental autoimmune enecephalomyelities; NOD, non-obese diabetic; IL, interleukin; IFN, interferon; ConA, concavalan A; PMA, myristic acid palmitic ester; TNF, tumor necrosis factor

tokines for disease expression. Both Type I diabetes and EAE can be induced by T cells, primarily one of the two types of helper T cells – T helper cells type 1, which produce pro-inflammatory cytokines such as IFN-γ [12]. Administration or upregulation of the type 2 T helper-associated cytokines interleukin (IL-4) and IL-10 can ameliorate autoimmune disease [13]. Because multiple sclerosis and Type I diabetes share several features of immunopathogenesis, we decided to determine whether murine ingested IFN-α inhibits insulitis and suppress diabetes in NOD mice.

Material and methods

Animals and feeding regimen. Female NOD mice (NOD MrkTacfBR) were obtained from Taconic Farms at 8 weeks of age. Feeding with mock IFN ($n = 10$) or 10 U murine IFN-α ($n = 10$) every other day or daily was started at 9 weeks. The mice were maintained and fed under specific pathogen-free conditions in microisolators, and were handled under negative pressure sterile hoods. Surveillance mice were maintained with experimental mice and were examined regularly for routine murine pathogens.

Mice were followed up after initiation of feeding by weekly blood glucose determination (Life Scan One Touch II, Johnson & Johnson, USA) beginning at week 10. Animals were considered diabetic if two consecutive blood glucose determinations were > 11.1 mmol/l. Spontaneous diabetes (blood sugar > 11.1 mmol/l) occurs in 50% female NOD mice by age 19 weeks in our facility.

Ten units of mIFN-α (Cytimmune mouse IFN-α, 4.0×10^5 IRU/ml, Lee Biomolecular Research, Inc., San Diego, Calif., USA), or mock murine IFN-α (Cytimmune < 2 IRU/ml, Lee Biomolecular Research, Inc., San Diego, Calif., USA) was administered directly to the stomach and proximal small intestine using a 2.5 cm syringe fitted with a 22–24 gauge ball point needle (Thomas Scientific, Swedesboro, N.J., USA) every other day or daily to NOD mice beginning at age nine weeks. IFN-α was delivered directly to the distal oesophagus, stomach and proximal small intestine (as determined experimentally by injecting Evans' blue during routine feeding and subsequent sacrifice). An equal number of control animals were fed mock mIFN. We used mock murine IFN control. This preparation is identical to the IFN preparation, which is purified of virus, except that the mock control is not induced with Newcastle disease virus (according to Lee Biomolecular). Therefore the mock murine preparation has whatever normal cell components are contained in the Newcastle disease virus-induced preparation. Disease course was plotted as Kaplan-Meier curve with the y axis denoting the percentage of mice remaining non-diabetic.

Measurement of cytokine secretion (experiment 1). Splenocytes from individual non-diabetic untreated ($n = 4$) or non-diabetic treated mice ($n = 9$) were stimulated with concananalin A (Con A) or ionomycin plus myristic acid palmitic ester (PMA) mitogens. Because ingested type I IFNs are systemic immunomodulators, and mitogen stimulation reflects both the systemic and target-organ immune response in treated mice [6, 7, 14], Con A and ionomycin + PMA were used. Individual spleens were removed under aseptic conditions, single cell suspensions were prepared, and red cell lysis was performed by adding 2–3 ml sterile water to single cells for 5 s, and, once the solution had become transparent, adding serum-free media to a 50 ml tube. Splenocytes were stimulated with Con A at a final concentration of 2.5 μg/ml (Sigma Chemical Co., St Louis, Mo., USA) or ionomycin at 100 ng/ml (Calbiochem, La Jolla, Calif., USA) in combination with PMA at 1 ng/ml (Sigma Chemical Co., St. Louis, Mo., USA) for 48 h in serum free medium (AIM-V medium, Gibco BRL, Grand Island, NY) with 2×10^5 cells/200 μl in triplicate in 96 well U-bottomed plates in a humidified 5% CO_2/95% air incubator at 37 °C. Murine IL-1, IL-2, IL-4, IL-6, IL-10, IFN-γ kits (Biosource International, Camarillo, Calif., USA), TNF-α and transforming growth factor β (TGF-β) ELISA kits (Genzyme, Cambridge, Mass., USA) were used. Results were given as mean ± SEM of individual samples from mice fed mock IFN or mice fed with 10 mIFN-α and were expressed as pg/ml.

Islet histology (experiment 2). Mice were killed after reaching end point (week 24). Each pancreas from individual non-diabetic untreated ($n = 4$) and non-diabetic treated mice ($n = 9$) was harvested, fixed in 10% formalin, embedding in paraffin, sectioned at 10 μ, and stained with haematoxylin and eosin. A pathologist, blinded to the treatment groups, scored the pancreas. Coded slides were read by light microscopy. Islet inflammation (insulitis) was graded 0 to 4, according to the extent of peri-islet and intra-islet infiltration by mononuclear cells as follows: 0 = none; 1 = peri-islet leucocytes without islet infiltration; 2 = < 25% islet area infiltrated by leucocytes; 3 = 25–75% islet area infiltrated; and 4 = > 75% islet area infiltrated. A mean insulitis score was calculated for each pancreas by dividing the sum of the insulitis score for individual islets by the number of islets examined (> 40/pancreas) [15].

Adoptive transfer (experiment 3). After 15 weeks of ingested mock IFN or 10 U mIFN-α treatment (experiment number 2 above), spleens from non-diabetic donors were removed aseptically from donors, and single cell suspensions were prepared. Cells were collected, washed twice in phosphate buffered saline, and viability was determined by standard Trypan blue exclusion. Thirty million viable unstimulated lymphocytes in 1 ml Dulbecco's PBS were injected intraperitoneally into 8 week old NOD recipients from mock fed non-diabetic donors or IFN-α fed donors and were followed for 24 weeks.

Statistics. Statistical analysis was performed using one-tailed Student's t test or log rank (Cox method) (SyStat 7.0.1, SPSS Inc).

Results

In the first set of experiments, 20 female NOD mice were mock treated ($n = 10$) or treated with 10 U mIFN-α ($n = 10$) every other day starting at age 9 weeks. Mice treated with mIFN-α showed a delayed onset of Type I diabetes and a decreased frequency of diabetes compared with mock treated mice, but this did not reach statistical significance (Fig. 1).

Because daily administration of type I IFN protects against acute EAE in the Lewis rat model, we examined whether feeding every day would provide more protection against diabetes. In a second set of experiments, 20 female NOD mice were mock treated ($n = 10$) or treated with 10 U mIFN-α ($n = 10$) every day starting at age 9 weeks. Mice ingesting

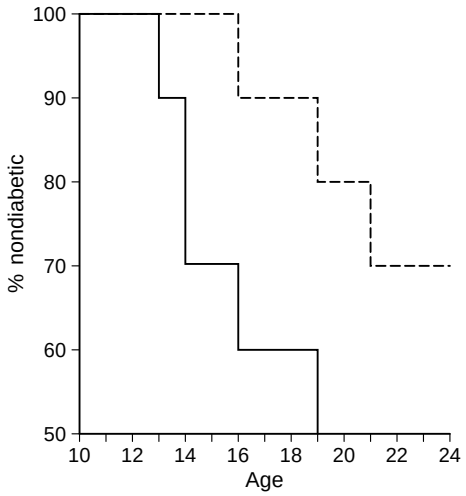

Fig. 1. Ingestion of mIFN-α on alternate days from age 9 weeks reduced the incidence of Type I diabetes in NOD mice. Twenty female NOD mice were fed mock IFN ($n = 10$) or 10 U mIFN-α ($n = 10$) three times per week starting at 9 weeks and followed with weekly blood glucose determination beginning at week 10. Data on time to overt diabetes in each group were analysed by Kaplan Meier survival curve showing the percentage remaining non-diabetic in relation to age. Mice fed IFN-α (dotted line) showed a delayed onset of diabetes and fewer of them became diabetic than the mock treated (solid line) animals

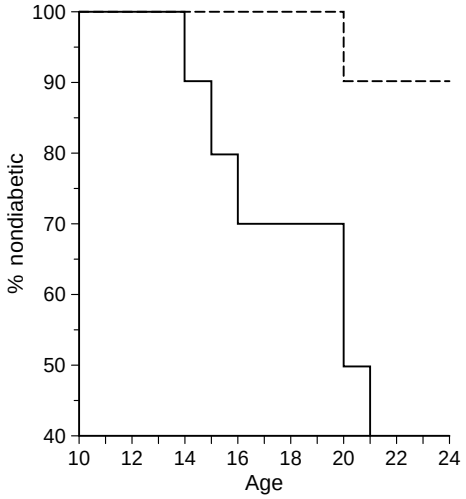

Fig. 2. Daily ingestion of mIFN-α from age 9 weeks enhanced the suppression of Type I diabetes in NOD mice. Twenty female NOD mice were fed mock IFN ($n = 10$) or 10 U mIFN-α ($n = 10$) daily from age 9 weeks. Mice were followed and data were analysed as described in Fig. 1. Mice fed IFN-α (dotted line) showed significantly greater delayed onset of Type I diabetes and fewer became diabetic than the mock treated (solid line) mice ($p < 0.014$ by log rank test). A representative experiment from two individual experiments is shown.

mIFN-α showed a more significant delay in the onset of Type I diabetes and a reduced frequency of diabetes ($p < 0.014$ by log rank test) (Fig. 2). There was also a significant decrease in islet inflammation in this experiment (3.0 ± 0.4 untreated mice vs 2.1 ± 0.3 treated, mice, $p < 0.05$).

Because mitogen stimulation reflects non-antigen and antigen-specific responses [6, 7, 14], we compared the cytokine profiles of Con A and ionomycin/PMA stimulated spleen cells in mock treated and oral mIFN-α treated mice. Whole splenocytes from non-diabetic mice from the second set of experiments were stimulated with Con A or ionomycin/PMA, and IL-1, IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α and TGF-β were measured. Splenocytes from IFN-α treated mice showed increased production of IL-2, IL-4, IL-6, IL-10 and IFN-γ after Con A stimulation, and increased production of IL-4, IL-10 and IFN-γ after ionomycin/PMA stimulation (Fig. 3). However, both Con A and ionomycin/PMA activation increased the production of proinflammatory IFN-γ and the counter-regulatory anti-inflammatory IL-4 and IL-10 cytokines. There were no significant changes in Con A or ionomycin/PMA stimulated TNF-α, IL-1 and TGF-β secretion (data not shown).

To determine whether the protective effect of the ingested IFN-α was transferred to spleen cell populations, we next examined whether adoptive transfer of splenocytes from mIFN-α fed donors could protect against diabetes. NOD mice from experiment 2 above, fed mock IFN or mIFN-α every day for 14 weeks, were used as donors. After 14 weeks of feeding, spleens were harvested from non-diabetic mice in both groups and single cell splenocytes were isolated. Unstimulated lymphocytes were injected intraperitoneally into 8 week old NOD recipients from mock fed non-diabetic donors ($n = 8$) or IFN-α fed donors ($n = 12$). Mice were followed up for 40 weeks thereafter. Development of Type I diabetes was delayed in 50 % of mice receiving cells from IFN-α fed donors (6/12) (Fig. 4) compared with mice receiving cells from mock fed donors (8/8), all of which became diabetic at 24 weeks ($p < 0.038$ by log rank test).

Discussion

Our data show that primary dysfunction of islets is prevented by treatment with ingested IFN-α, in spite of upregulation of IFN-γ. The ratio of counter-regulatory anti-inflammatory IL-4 or IL-10 to IFN-γ may be critically important in determining the balance between disease and protection in the NOD mouse [16]. Untreated non-diabetic mice produced no IL-4, but non-diabetic mice ingesting IFN-α produced 31 pg/ml of IL-4 after Con A stimulation.

Type 2 T helper-like cytokines can protect against the development of Type I diabetes in the NOD mouse. IL-4 is a potential counter-regulatory anti-inflammatory cytokine to type 1 T helper cells [13], and can inhibit the actions of IFN-γ [17–20]. The in vivo administration of recombinant IL-4 to predia-

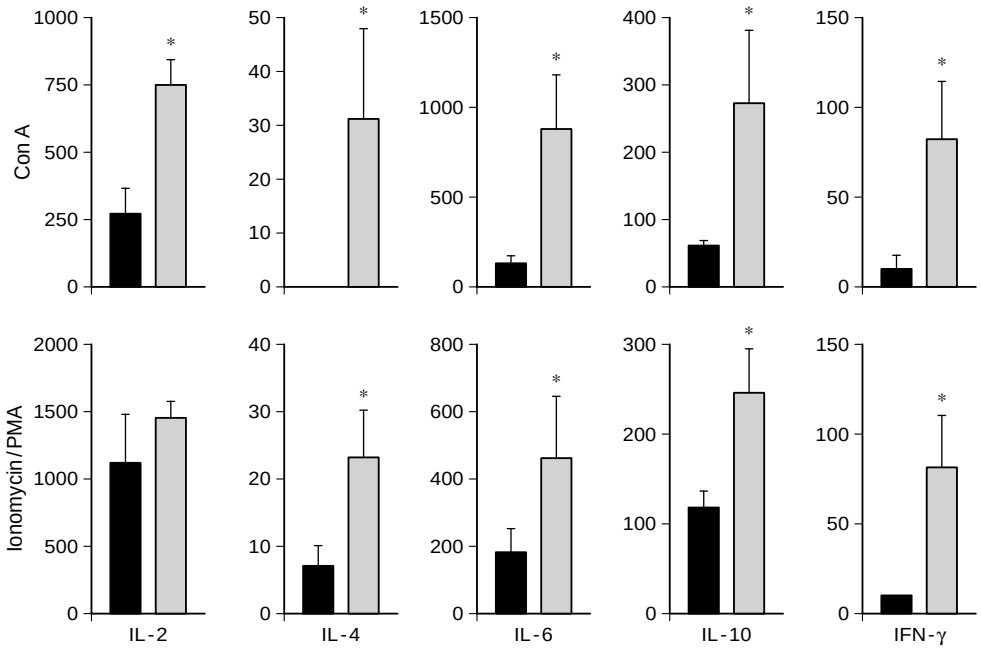

Fig. 3. Ingested mIFN-α increases the mitogen-induced production of IL-4, IL-10 and IFN-γ in spleen cells. Splenocytes from individual non-diabetic mock treated ($n = 4$) or non-diabetic treated mice ($n = 9$) were stimulated with Con A or ionomycin + PMA. Individual data correspond to mice ingesting 10 U mIFN-α (white bars) or animals ingesting mock IFN (black bars). Results are expressed as pg/ml, mean ± SEM. *Designates a significant difference between treated and mock treated animals. Con A: untreated vs treated mice: IL-2, $p < 0.025$; IL-4, $p < 0.05$; IL-6, $p < 0.02$; IL-10, $p < 0.04$; IFN-γ, $p < 0.05$. Ionomycin + PMA: untreated vs treated mice: IL-2, $p < 0.21$; IL-4, $p < 0.05$; IL-6, $p < 0.07$; IL-10, $p < 0.015$; IFN-γ, $p < 0.03$. Results are from the combined results of the two experiments outlined in Fig. 2 above

betic NOD mice protects them from diabetes [21]. Transgenic IL-4 NOD mice were protected from insulitis and diabetes, a significantly higher ratio of mitogen-induced IFN-γ/IL-4 was found in diabetic NOD mice but not in age-matched non-diabetic NOD mice and treatment of isologous islet transplants with the Type 2 T helper cell-associated cytokines IL-4 and IL-10 in spontaneously diabetic non-obese recipients restored immediate function of the grafts [16, 22, 23]. Induction of functional tolerance to islet antigens is indicated by the failure of diabetogenic spleen cells to induce diabetes in transgenic NOD-IL-4 recipients. IL-4 can prevent the development of autoimmunity and destructive autoreactivity in the NOD mouse [23].

Other type 2 T helper cell-like cytokines can modulate the onset of diabetes. Daily subcutaneous administration of IL-10 (a potent known inhibitor of IFN-γ production by type 1 T helper cells) to 9 and 10 week old NOD mice was shown to delay the onset of disease, significantly reduce the incidence of diabetes and reduce the severity of insulitis, to prevent cellular infiltration of islet cells and promote normal insulin production by beta cells [24]. Administration of non-cytolytic murine IL-10/Fc fusion protein from 5 to 25 weeks of age completely prevented diabetes in the NOD model. Immunohistochemistry studies showed that IL-10/Fc treatment promoted expression of IL-4 and IL-10, type 2 T helper cytokines, by islet-infiltrating leucocytes [25]. Primary non-function of islet grafts is prevented by treating the recipients with a combination of IL-4 and IL-10, via downregulation of type 1 T helper cytokines [22].

Our data also show that the adoptive transfer of splenocytes from donors who have ingested IFN-α and produced counter-regulatory anti-inflammatory IL-4 or IL-10 protects against diabetes in NOD recipients. Mice receiving unstimulated splenocytes from mock IFN ingesting donors had a faster onset of diabetes than the mock treated mice in experiments 1 and 2. The protective effect of adoptively transferred unstimulated splenocytes from donors that have taken IFN-α shows the presence of ingested IFN-α-activated regulatory splenic cell populations that may work via increased IL-4 or IL-10 production.

In contrast, intra-islet IFN-γ (type 1 T helper cell-like) has a diabetes-promoting role, while IL-4 and IL-10 (type 2 T helper cell-like) protect against diabetes [26, 27]. During a later phase of insulitis, there is a characteristic type 1 T helper cytokine profile, with production of IFN-γ occurring after stimulation [28]. IFN-γ has been detected in lymphocytes infiltrating the islets of humans with recent onset Type I diabetes. IFN-γ transgenic mice develop Type I diabetes, and antibodies against IFN-γ protect against diabetes development in NOD mice and BB rats [29–34]. However, IFN-γ may itself be immunosup-

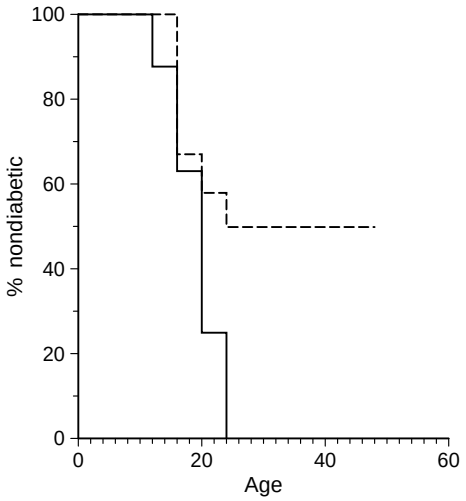

Fig. 4. Adoptive transfer of unstimulated splenocytes from mIFN-α fed donors suppressed spontaneous diabetes in recipients. Donor NOD mice were fed mock IFN or mIFN-α daily for 14 weeks. After 14 weeks, spleens were harvested from non-diabetic mice in both groups and single cell lymphocytes were isolated. Thirty million unstimulated lymphocytes were injected intraperitoneally into 8 week old NOD recipients from mock fed non-diabetic donors ($n = 8$) or IFN-α fed donors ($n = 12$). Mice were followed for 40 weeks thereafter. Mice given cells from IFN-α fed donors (dotted line) showed significantly greater delayed onset of Type I diabetes than mice given cells from mock fed donors (solid line) ($p < 0.038$ by log rank test)

pressive under certain conditions. Human CD8 + T cells activated via the autologous mixed lymphocyte reaction (AMLR) suppress CD3 mediated proliferation of autologous T cells through IFN-γ secretion [35]. IFN-γ can also mediate suppression by antigen-specific T suppressor clones [36].

IFN-α has been detected in beta cells of animals and people with recent onset Type I diabetes [37, 38]. Islet beta cell transgenic expression of IFN-α in mice elicits an immune mediated destruction of beta cells, and may recruit immune system cells to damage IFN-α-producing cells [39]. However, there is no evidence that the biologic effect of ingested IFN-α observed is dependent on its gastrointestinal absorption. Pharmacokinetics of IFNs delivered by various routes have shown that orally administered IFNs failed to appear in the bloodstream [40–46]. We have recently shown increased relative type I IFN-induced Mx mRNA levels, a type 1 IFN-specific induced message, using semi-quantitative reverse transcriptase polymer chain reaction on splenocytes after ingestion of mIFN-α (unpublished data). IFN-activated lymphocytes, by virtue of their ability to circulate through the body from the gut associated lymphoid system, have the potential to transfer their biological activities widely in the absence of circulating cytokines after contacting IFN in the gut associated lymphoid system [43, 47–49].

The natural history of the development of Type I diabetes includes a presymptomatic stage between the occurrence of insulitis and the onset of symptomatic hyperglycaemia, and provides a broad therapeutic window for intervention between T cell islet infiltration and beta cell numbers falling below that required to maintain normoglycaemia [50]. Ingested IFN-α may be an ideal treatment for Type I diabetes before or immediately after diagnosis. Ingested IFN-α may provide a continuous means of generating immunoregulation that is convenient, active at lower doses without toxicity, effective if given before the onset of clinical Type I diabetes or early in the disease course and provided enhanced efficacy via unique and potent immunoregulatory circuits originating in the gut associated lymphoid tissue [51].

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