Tyrosine kinase inhibitors reverse type 1 diabetes in nonobese diabetic mice

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Contributed by Arthur Weiss, October 14, 2008 (sent for review July 19, 2008)

The recent development of small-molecule tyrosine kinase (TK) inhibitors offers increasing opportunities for the treatment of autoimmune diseases. In this study, we investigated the potential of this new class of drugs to treat and cure type 1 diabetes (T1D) in the NOD mouse. Treatment of prediabetic and new onset diabetic mice with imatinib (Gleevec) prevented and reversed T1D. Similar results were observed with sunitinib (Sutent), an additional approved multikinase inhibitor, suggesting that the primary target of imatinib, c-Abl, was not essential in blocking disease in this model. Additional studies with another TK inhibitor, PLX647 (targeting c-Kit and c-Fms) or an anti-c-Kit mAb showed only marginal efficacy whereas a soluble form of platelet-derived growth factor receptor (PDGFR), PDGFR β lg, rapidly reversed diabetes. These findings strongly suggest that inhibition of PDGFR is critical to reverse diabetes and highlight a crucial role of inflammation in the development of T1D. These conclusions were supported by the finding that the adaptive immune system was not significantly affected by imatinib treatment. Finally, and most significantly, imatinib treatment led to durable remission after discontinuation of therapy at 10 weeks in a majority of mice. Thus, long-term efficacy and tolerance is likely to depend on inhibiting a combination of tyrosine kinases supporting the use of selective kinase inhibitors as a new, potentially very attractive approach for the treatment of T1D.

autoimmunity | diabetes | tyrosine kinase inhibitor | imatinib | PDGFR

Imatinib (Gleevec) is a 2-phenylaminopyrimidine derivative designed as a specific inhibitor of the inactive conformation of Abl protein tyrosine kinases (1). Imatinib's activity against cells bearing the Bcr-Abl translocation has yielded remarkable results in the treatments of chronic myeloid leukemia (CML) with minimal side effects (2). In addition, imatinib has been found to be active against other tyrosine kinases such as platelet-derived growth factor receptor (PDGFR), c-Kit (CD117), macrophage colony stimulating factor receptor (c-Fms) and, perhaps, Lck (3–6). The inhibitory activity against c-Kit and PDGFR has enabled the development of effective treatments for gastrointestinal stromal tumors (7), eosinophilic disorders, and systemic mast cell disease (8, 9).

In addition to its direct effect on malignant cells, it has been suggested that imatinib treatment can have immunomodulatory activity. Studies have shown that imatinib treatment can result in impaired adaptive and innate immunity including T cell, dendritic cell (DC), and monocytes/macrophages function (10). Notably, several investigators have demonstrated antiinflammatory effects in various mouse models. Wolf *et al.* showed in a mouse model of acute hepatic inflammation that imatinib exhibited a strong antiinflammatory role by inhibiting TNF- α production in macrophages (11). Dietz *et al.* demonstrated that delayed-type hypersensitivity was reduced in mice treated with imatinib (12). Finally, imatinib has been shown to be effective in a number of rodent studies of autoimmunity. Imatinib efficiently prevented disease and induced remission in an autoimmune arthritis model (13, 14) and ameliorated autoimmune nephritis

in a mouse model of lupus (15). These data are consistent with case reports and phase I studies in humans demonstrating a positive effect of imatinib on rheumatoid arthritis (16, 17), psoriasis (18), spondyloarthritis (19), and Crohn's disease (20).

Type 1 diabetes (T1D) is an autoimmune disease dependent on T cell-mediated destruction of insulin-producing β cells. Disease progression is strongly dependent on T cells, B cells, macrophages, and DCs. Importantly, recent studies have emphasized a role of inflammatory processes in β cell destruction and insulin resistance. To date there is no good immunotherapy to treat or prevent the development of this disease. T1D is characterized by the development of autoreactive antibodies and destructive T cell infiltration of insulin-producing islet β cells. The NOD (nonobese diabetes) mouse is an important model of autoimmune diabetes. Disease occurs spontaneously and shares many phenotypic and genetic similarities with T1D in human subjects (21). Lymphocyte infiltration of the islets of Langerhans begins at 2–4 weeks of age, progressing from periinsulitis to severe insulitis by 10 weeks of age. Diabetes onset typically occurs at 12–14 weeks in most female NOD mice. Given the overlap between the multiple targets of imatinib, previous results in other models of autoimmunity and the pathogenesis of autoimmune diabetes, we set out to test the hypothesis that this drug might be effective in preventing or treating this autoimmune disease. We show here that imatinib treatment can prevent and even reverse diabetes when administered to NOD mice. Furthermore, imatinib can be administered for as short as 10 weeks with long-lasting effects working through the inhibition of PDGFR. These results, coupled with recent studies demonstrating a direct protective effect of imatinib on type 2 diabetes in rodents (22) suggests that this molecule and other kinase inhibitors such as sunitinib have potential as a therapeutic to treat patients with this disease.

Results

Imatinib Prevents Development of Autoimmune Diabetes. To test whether imatinib could alter diabetes development, we treated prediabetic NOD mice and followed incidence of diabetes. Commercially available Gleevec tablets were ground and suspended in peanut oil and given orally once a day at a dose of 1.5 mg/mouse. Treatment was initiated at 12 weeks, a prediabetic stage when a high degree of insulitis is already evident. During the 7 weeks of treatment, none of the imatinib-treated mice developed diabetes. By comparison, by 19 weeks, $\approx 40\%$ of the oil-treated mice had developed disease (Fig. 1A). After cessation

Author contributions: A.W. and J.B. designed research; C.L., G.L.S., J.L., M.R.L., N.M., G.B., and S.Z. performed research; G.B. contributed new reagents/analytic tools; C.L., G.L.S., S.Z., and J.B. analyzed data; and C.L. and J.B. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0810246105/DCSupplemental.

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Fig. 1. Imatinib prevents and reverses development of autoimmune diabetes. (A) Prediabetic NOD mice (12 weeks of age) were treated (gavage) daily with imatinib ($n = 20$) or oil ($n = 14$) for 7 weeks (gray shaded area). Diabetes incidence is shown. Mice were determined diabetic with two consecutive readings of blood glucose >250 mg/dL. (B) Prediabetic female NOD mice were treated daily with imatinib ($n = 10$) or oil ($n = 8$). Cyclophosphamide (Cy, 300 mg/kg) was injected i.p., 2.5 weeks after beginning of treatment. Diabetes incidence is shown. (C) Imatinib or oil treatment of NOD mice was initiated at the time of disease onset (blood glucose >250 mg/dL) and continued for 3 weeks. Individual glucose readings and percentages of diabetic mice for imatinib-treated ($n = 28$) and oil-treated ($n = 15$) mice are shown. (D and E) Imatinib treatment (gray shaded area) was initiated at the time of disease onset and discontinued after 3 weeks ($n = 8$) or 10 weeks ($n = 17$). Percentages of diabetic mice are shown.

of treatment, 20% of the animals treated with imatinib became progressively diabetic by 30 weeks of age, as opposed to 71% of the oil-treated mice. The majority of imatinib-treated mice remained nondiabetic at >50 weeks of age, suggesting that the short-term therapy had long-term effects on the development of diabetes in this setting. We then assessed the effect of imatinib on cyclophosphamide (Cy)-induced diabetes. Cy-induced diabetes is a rapid onset robust model of disease, which has been linked to increased islet cell apoptosis and perhaps altered immune regulation (21). In this setting, only 30% of the 15-week-old animals treated with imatinib developed diabetes within 3 weeks after Cy injection (Fig. 1B). This result contrasted with the control oil-treated group of mice where fully 85% of the animals became diabetic during the same period. These results show that imatinib can prevent T1D in NOD mice.

Imatinib Induces Remission of Established Diabetes. Multiple approaches have been used to prevent spontaneous diabetes in the NOD mouse. However, there has been substantially less success in curing overtly diabetic NOD mice (23). Therefore, we tested whether imatinib could reverse diabetes when given at the time of new onset. Treatment with imatinib led to remission in 80% of new-onset diabetic mice. One week after initiation of treatment, imatinib reversed diabetes in $>40\%$ of the mice (Fig. 1C) but by 2 weeks, remission was observed in $\approx 80\%$ of the mice ($n = 28$). Oil treatment did not reverse diabetes as all of the mice were diabetic ($n = 15$) 2 weeks after the beginning of the treatment (Fig. 1C). The imatinib effect was most notable in mice with blood glucose levels between 250 and 350 mg/dL at diagnosis but also in mice exhibiting higher glycemia (>300 – 450 mg/dL) although somewhat less effective. This difference most likely reflects the fact that efficacy of the imatinib may require several days of treatment, thus favoring remission in mice with substantial islet reserve at the time of the initiation of therapy.

Next, we examined whether imatinib treatment could be discontinued and maintain its efficacy. After cessation of therapy at 3 weeks, all mice became hyperglycemic by 10 weeks postdiagnosis (Fig. 1D). However, increasing the duration of treatment from 3 weeks to 10 weeks led to long-lasting diabetes remission. In the experiment depicted in Fig. 1E, a majority of the mice remained normal glycemic after cessation of therapy 10 weeks postdisease onset without the need of additional treatment.

Imatinib Does Not Significantly Diminish Leukocyte Accumulation Within the Pancreas. Prediabetic NOD mice were treated with imatinib or oil. After 2 weeks of treatment, mice were examined for the presence of mononuclear cell infiltrates. Although severe and mild insulitis were generally diminished in imatinib-treated mice compared with oil-treated mice (Fig. 2A), these differences were not statistically significant. Furthermore, longer treatment of 5 weeks did not reveal significant differences (data not shown). Finally, pancreatic islets from long-term normoglycemic mice (10-week treatment, Fig. 1E) were also examined. Although these mice were not diabetic, significant insulitis was still observed (data not shown). Thus, while preventing clinical disease, imatinib diminishes to some extent, but does not eliminate leukocyte infiltration of the pancreas.

Effect of Imatinib on T Cells *in Vivo*. Both $CD4^+$ and $CD8^+$ T cells play an important role in the development of diabetes (21). Although several studies reported that imatinib could alter T cell function (12, 24), most of these effects were described when imatinib was added *in vitro* and using a relatively high concentration (13). Treatment of prediabetic NOD mice with the therapeutic dose of imatinib for 5 weeks did not change the $CD4^+/CD8^+$ T cell ratio in the spleen or pancreatic lymph nodes, nor did the therapy alter the absolute cell numbers of each subset when compared with oil-treated mice (data not shown). Simi-

Fig. 2. Effect of imatinib on insulinitis and T cells *in vivo*. (A) Prediabetic NOD mice (10 weeks of age) were treated daily with imatinib or oil for 2 weeks. Quantification of >280 pancreatic islets as a percentage for each histological score is shown ($n = 8$ mice/group). (B) Prediabetic NOD mice were treated with imatinib or oil for 5 weeks and spleen cell proliferation to p31 peptide (1–1000 μ M) or anti-CD3 (0.1 μ g/ μ L) was examined *in vitro*. Representative response from one mouse of each group is shown. (C) Prediabetic NOD mice were pretreated daily with imatinib or oil for 1 week before adoptive transfer of CD25-depleted CFSE-labeled BDC2.5-Thy1.1 congenic spleen and lymph node cells. Treatment was continued for 4 days and pancreatic (PancLN) and inguinal (IngLN) lymph nodes were subsequently collected to measure CFSE dilution ($n = 9$ –19 mice/group, from five independent experiments). * $P < 0.05$. (D) Prediabetic NOD mice were pretreated with imatinib or oil for 1 week before adoptive transfer of CD4⁺CD25⁺ YFP⁺ T cells from BDC2.5.Thy1.1.Yeti mice. Treatment was continued for 1 week. Single-cell suspensions from isolated pancreatic islets were subsequently prepared to measure induction of YFP expression (reporter for IFN- γ expression). Gated on CD4⁺ cells.

larly, the percentage of Foxp3⁺ among CD4⁺ T cells was not modified (data not shown). CD8⁺ T cells specific for islet-specific glucose-6-phosphatase catalytic subunit-related protein (25) were detectable in the islets of both oil- and imatinib-treated mice ([supporting information \(SI\) Fig. S1](#)). Antigen-specific CD4⁺ T cell proliferation was examined using the p31 peptide (1040-p31), a mimotope for an unknown islet antigen recognized by the diabetogenic BDC2.5 T cell (26). Spleen cells from imatinib- and oil-treated mice proliferated similarly when stimulated *in vitro* with p31 peptide (Fig. 2*B*). Furthermore, *in vivo* proliferation of the islet antigen-specific TCR transgenic mouse BDC2.5 was not affected in imatinib-treated mice compared with oil-treated mice (Fig. 2*C*). Finally, to determine whether imatinib could affect IFN- γ expression in T cells, we took advantage of the IFN- γ reporter mice known as “Yeti mice” (27). As shown in Fig. 2*D*, YFP expression was induced in $\approx 45\%$ of the BDC2.5 Thy1.1⁺CD4⁺ T cells that infiltrated the islets in oil-treated mice. When recipient mice were treated with imatinib, the percentage of YFP⁺ cells decreased to $\approx 35\%$, although this difference did not reach significance ($P = 0.059$) when compared with oil-treated mice. Thus, at the dose that enables remission in new onset diabetic mice, imatinib marginally affects pathogenic T cell function, suggesting that imatinib does not

Fig. 3. Effect of PLX647 and anti-c-Kit treatments on diabetic mice. (A) New onset diabetic NOD mice were fed with mouse chow containing PLX647 ($n = 9$, 600 mg/kg of chow) or control chow ($n = 6$). Percentages of diabetic mice are shown. (B) New onset diabetic NOD mice received anti-c-Kit mAb (ACK2, 1 mg/mouse/day, i.v.) and Benadryl injection (30 mg/kg, i.p.) for 10 days (gray shaded area) ($n = 11$). Control mice received either rat IgG2b isotype plus Benadryl ($n = 8$) or Benadryl alone ($n = 8$).

achieve its effect on T1D through potent inhibition of Abl or Lck expressed in T cells.

Discrimination of Key Tyrosine Kinase Targets in the Diabetes Remission Induced by Imatinib. To identify which tyrosine kinase pathway(s), inhibited by imatinib, are key in the reversal of diabetes, we tested different TK blocking agents that share targets with imatinib (Table S1). First, we tested a small molecule, PLX647, which has been developed to inhibit c-Fms and c-Kit, specifically. As seen in Table S2, the IC50 for PLX647 suggests strong activity against KIT and CSF1R but not PDGFR- β when compared to imatinib and sunitinib allowing for separation of these kinase activities. Although there was a small effect of the ability of this drug to reverse diabetes after onset (Fig. 3A), the therapy was not nearly as efficacious as imatinib and not nearly as durable with the majority of mice relapsing while undergoing treatment. Next, we tested a mAb directed at the c-Kit cell surface molecule, ACK2. This mAb has previously been shown to block c-Kit and eliminate c-Kit⁺ cells *in vivo* (28). Unfortunately, treatment of NOD mice with ACK2 led to immediate hypersensitivity responses that required the coadministration of Benadryl to prevent anaphylaxis. Under these conditions, there was transient reversal of hyperglycemia in ~60% of mice, however, a similar transient reduction in hyperglycemias was observed in control Ig plus Benadryl or Benadryl only-treated mice suggesting a transient effect on blood glucose because of the antihistamine (Fig. 3B). Thus, targeting c-Kit, either by PLX647 or anti-c-Kit mAb, was not sufficient to block disease progression.

To test the effect of PDGF blockade, we generated a PDGFR β Ig fusion protein. Specific inhibition of PDGFR signaling through PDGF neutralization was verified *in vitro* using NIH 3T3 cells (data not shown). As shown in Fig. 4A, diabetes remission was noted in five of six mice treated with PDGFR β Ig for 10 days, whereas only one of six mice treated with control Ig transiently returned to normal glycemia. After cessation of therapy, all of the PDGFR β Ig-treated mice relapsed to diabetes within 2 weeks. Unfortunately, continued treatment with the PDGFR β Ig was not feasible as the human protein was quite immunogenic and the mice all developed a potent antibody response against the foreign protein.

Finally, we tested sunitinib, a small molecule potentially inhibiting c-Kit, PDGFR and c-Fms (29) in an attempt to look directly at c-Kit and PDGFR kinases, which had shown some effect individually. As seen in [Table S2](#), unlike PLX647, sunitinib strongly inhibited PDGFR- β . In this regard, treatment of diabetic mice with sunitinib resulted in remission of disease in 100%

Fig. 4. Effect of PDGF neutralization and sunitinib treatment on diabetic mice. (A) New onset diabetic NOD mice received either PDGFR β Ig fusion molecule ($n = 6$) or control human Ig ($n = 6$) at the dose of 200 μ g/mouse/day, i.p., for 10 days (gray shaded area). Individual glucose readings and percentages of diabetic mice are shown. (B) Sunitinib treatment (gavage, 2 mg/mouse) of NOD mice ($n = 6$) was initiated at the time of disease onset and continued for 5 weeks.

of treated mice within 1 week after initiation of therapy (Fig. 4B). Only one of six treated mice relapsed to diabetes within the continuous 9-week treatment. The suppressive effects continued for the duration of therapy, suggesting that efficacy did not depend on blocking Abl (or Lck) but is most likely targeting PDGFR, and to some extent c-Kit. Taken together, these data strongly suggest that short-term imatinib or sunitinib treatments affect the inflammatory response involved in the development and progression of diabetes in NOD mice.

Discussion

Type 1 diabetes remains an intractable disease with a limited number of treatment options that can reverse disease once diagnosed. In this study, we demonstrate that treatment with imatinib, an anticancer drug used to treat patients with CML and other tumors, prevented and reversed the development of autoimmune diabetes in the NOD mouse. This rapid and robust effect is a significant result given the paucity of existing treatments to induce remission in overtly diabetic mice (23). The immunosuppressive activity is reminiscent of recent case reports in patients with rheumatologic and other autoimmune diseases and a collagen-induced mouse model of rheumatoid arthritis. However, one unexpected observation was that limiting treatment to 8–10 weeks after disease onset was sufficient to reverse diabetes and induce long-term remission consistent with reestablishment of tolerance in these NOD mice. This observation provides a new perspective for the use of this kinase inhibitor or other similar drugs for the treatment of type 1 diabetes or other autoimmune diseases.

From a mechanistic point of view, the pleiotropic effect of the drug on multiple kinases had made it difficult to determine the biochemical basis of imatinib's efficacy. In fact, in the study by Paniagua *et al.*, the investigators showed the marked effects of imatinib on several kinases (13); however, the precise mechanism of action of the drug in this and other models remained

elusive. Because imatinib inhibits both c-Abl and Lck kinases, which are expressed in both B and T cells of the adaptive immune system, it seemed possible that these kinases might be involved as primary imatinib targets. Abl kinase is functional in both B and T cells, which have been shown to be critically involved in NOD diabetes (30). Moreover, c-Abl has been implicated in T cell activation and thymocyte development (31). Furthermore, imatinib has been shown to inhibit Lck (6), although to a lower extent than Abl. *In vitro*, imatinib inhibits T cell proliferation likely because of reduced phosphorylation of ZAP-70, LAT (24), Lck, and ERK1/2, and reduced levels of activated NF κ B (12). However, in the current study, the imatinib effect did not appear to express through direct function on B or T cells. Imatinib treatment did not significantly alter insulinitis, T cell number, or antigen-specific T cell proliferation. In addition, adoptive transfer of spleen cells from imatinib-treated mice into (untreated) severe combined immunodeficiency (SCID) mice resulted in a similar or only slightly delayed development of diabetes compared with spleen cells from oil-treated mice (data not shown). Finally, imatinib did not alter CD25 or Foxp3 expression in CD4⁺ Tregs in treated mice. The significant effects of imatinib on T cells *in vitro* likely reflect the relatively high concentrations of imatinib used (13). In fact, *in vivo* studies showed that imatinib treatment did not affect primary T cell responses (32, 33) or T cell function in patients treated with imatinib (34, 35). Thus, it would appear that T cells are not the primary target of imatinib activity.

The observation that imatinib weakly modulated IFN- γ expression in T cells (Fig. 2D) is consistent with a recent study (33) suggesting that drug treatment might decrease effector function through indirect effects on inflammation or non-T cells such as APCs. In this regard, some studies had suggested that imatinib negatively affected DC numbers and function (10, 36). However, 2 weeks of imatinib did not alter DC numbers or expression of MHC class II, B7-2 and IL-12p40 (37) (Fig. S2) or alter islet antigen processing and presentation by DCs (Fig. 2C).

was performed as previously described (30). Insulinitis index (I) was calculated as previously described (49).

Adoptive Transfer. For *in vivo* proliferation assays, BDC2.5 spleen and lymph node cells were depleted of CD25⁺ cells, labeled with 2.5 μ M CFSE, and one million cells were transferred into NOD mice by retroorbital injection. For *in vivo* assessment of IFN- γ production, CD4⁺CD25⁻YFP⁺ T cells from NOD.BDC2.5.Th1.1.Yeti mice were sorted on a MoFlo cytometer high speed cell sorter (DakoCytomation, Beckman Coulter) to $\geq 99\%$ purity. One million cells were subsequently transferred into NOD mice by retroorbital injection.

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Statistical Analysis. All statistical analyses were performed using GraphPad Prism version 5.00. Statistical comparison of insulinitis index, thymidine incorporation, CFSE dilution, and cell marker expression were performed using an unpaired two-tailed Mann-Whitney *U* test. Values of $P \leq 0.05$ were considered significant.

ACKNOWLEDGMENTS. The authors thank P. Koudria for technical assistance. We thank H. Bour-Jordan and members of the Bluestone laboratory for helpful discussions. We thank Dr. Richard Locksley for generously providing the NOD.Yet40 mice. This work was supported by National Institutes of Health Grants R01 AI50834, R37 AI46643, and N01 AI15416 (to J.B.), P30 DK63720 (to J.B.), and the Juvenile Diabetes Research Foundation (JDRF) (3-2006-710 to C.L.).