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Mechanistic Assays (Part 7 of Safety and Efficacy of Imatinib for Preserving Beta-Cell Function in New-onset Type 1 Diabetes Mellitus)

In 1 collection

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ABSTRACT

This is Part 7 of "Safety and Efficacy of Imatinib for Preserving Beta-Cell Function in New-Onset Type 1 Diabetes Mellitus".

This clinical study is supported by JDRF. The aim of the collection is to determine whether imatinib will slow the progression of the autoimmune destruction of ß cells and lead to the preservation of C-peptide secretion in T1DM and to assess Diabetes-related objectives and safety of Imatinib in new-onset type 1 diabetes mellitus".

ATTACHMENTS

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COLLECTIONS

Collection of Protocols and Guidelines for Safety and Efficacy of Imatinib for Preserving Beta-cell Function in New-onset Type 1 Diabetes Mellitus

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Collection of Protocols and Guidelines for Safety and Efficacy of Imatinib for Preserving Beta-cell Function in New-onset Type 1 Diabetes Mellitus

GUIDELINES

The study team will partner with the ITN for mechanistic studies, taking full advantage of their systems for sample collection and archiving (see letter from Nepom). Following results of the study, various immunological assays will be considered through the available ITN cores, and beyond. We recognize that this field is rapidly evolving, and thus novel assays that are particularly relevant to this study population, and to this drug, will be considered. Our mechanistic assays plan is outlined below.

7.1 RATIONALE

The rationale for treating T1DM patients with imatinib stems from results in animal studies where imatinib therapy led not only to reversal of T1DM but also to reversal of the loss of tolerance, a process ultimately responsible for the pathology of the disease.

Treatment with imatinib potentially leads to a change from a pro-inflammatory to an anti-inflammatory state. In the NOD mouse model it was shown that both adaptive immune and inflammatory mechanisms directly trigger insulitis, insulin resistance, faulty insulin signaling and islet cell destruction, all leading to the loss of tolerance to islets.

It is anticipated that drug treatment is likely to affect T cells, B cells and mast cells directly, resulting in decreased numbers and function of islet antigen-specific T cells and perhaps changes in autoantibodies. In T1DM, there is a growing belief that generalized inflammation and adaptive immunity are major contributing factors to the induction and progression of this autoimmune process. Thus, a decrease in these cells or their function might help to eliminate activated autoreactive T cells resulting in tolerance to islet antigens in T1DM patients. In addition, we hypothesize that imatinib alters PDGF signaling that alters macrophage differentiation, affecting the balance of pathogenic versus alternatively activated macrophages and inflamed endothelial cells altering cell trafficking.

The goal of the mechanistic studies will be to determine if the biological effects of imatinib correlate with therapeutic efficacy in T1DM patients. The proposed immunologic mechanistic studies are designed to determine how imatinib therapy may arrest ongoing autoimmune pancreatic β-cell destruction and preserve insulin secretion in patients with T1DM. These will be conducted in the existing Immune Tolerance Network core laboratories. We recognize that this field is rapidly evolving, and thus novel assays that are particularly relevant to this population and this agent may be incorporated into the trial at a later date. Whenever possible, samples will be frozen and archived for batch analysis at a later date. Finally, since this trial has a safety component, emphasis will be placed on the study of immune responses to pathogens and overall immune competence.

7.2 RETENTION OF SAMPLES

Biological specimens collected in this trial may be used to re-evaluate biologic responses as new research tools become available. The specimens will be stored at the ITN sample repository until the end of the ITN contract. Residual specimens may be used by the investigators for development of new immunologic assays or for cross-

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trial comparisons. Specimens for mechanistic studies will be obtained throughout the study. While specimens are described in this protocol in the context of assays to be performed, it should be noted that not necessarily all assays will be performed for all participants at each time point. Decisions to perform assays will be made according to statistical and scientific planning, questions being asked, and current technologies to be utilized. Finally, clinical outcomes will be taken into account to determine the potential value of the assays. For example, if a clinical effect fails to occur, the assays performed by the ITN may be minimal.

7.3 FROZEN PBMCS

Peripheral blood will be collected at the sites, processed at an ITN core laboratory, and frozen cells stored in a central ITN repository.

7.3.1 Functional Cell-Based Assays

The functional status of lymphocyte subsets, specifically T/ B cells, mast cells and monocytes, can be assessed in various cell-based assays. Inflammatory and TCR-driven autoantigen-specific stimulations need to be examined to fully evaluate the effect of imatinib on immunological responses. To address the participants' overall inflammatory response potential during imatinib treatment, PBMCs or whole blood can be stimulated with anti-CD3 plus anti-CD28 and/or lipopolysaccharide (LPS) to determine the array of secreted cytokines and chemokines made by T and B cells, respectively. Since inflammatory cytokines direct the commitment of antigen-activated CD4 T cells to specific effector or FoxP3 regulatory cells, the overall pattern of secreted cytokines might indicate a preference towards specific CD4 T-cell commitment. T-cell assessments will include those measuring both the number and function of T cells. These measures may include the use of class I and class II tetramers to enumerate antigen-specific T cells. Additionally, in vitro cell culture techniques may be utilized to study T cell functionality. Readouts will include ELISPOT and intracellular staining technology for cytokine determination. Additional studies may be done to study regulatory T cells in these treated participants. Both carboxyfluorescein diacetate succinimidyl ester (CSFE) and ^{3}[H]-thymidine assays could be employed depending on cell numbers. Harvested blood cells will be separated by flow-based sorting using CD4, CD25 and CD127 antibodies and assayed for their ability to suppress polyclonal T cell responses. Follow-up experiments can include examination of Treg cell function as well as Foxp3 methylation. Several assays can address responses to autoantigens implicated in T1DM. Secretion of various cytokines can be determined, thus enabling characterization of overall phenotype (Th1/Th17/Th2/Treg) of T-cell responses, which might indicate skewing towards a more regulatory phenotype during imatinib treatment.

Imatinib can alter the differentiation of human and mouse DCs into mature APCs. The treated cells could not be induced to mature with IFNγ or CD40 agonists and were unable to stimulate T cells. In fact, the DCs look very much like the "tolerogenic" DCs defined in a number of settings. Other cell types may be analyzed that have been shown to play important roles in diabetes development and progression. These include B cells, NK cells, NKT cells and macrophages. We may perform functional analyses of these cells during and after therapy.

PBMCs will be isolated from blood, frozen by established ITN protocols, and studied in batches for quality control and standardization. These assays will be used in an attempt to test the hypothesis that imatinib treatment in humans, as in the NOD model, will tip the balance of inflammation toward an anti-inflammatory state, thus triggering beneficial changes in T cell-directed autoimmunity.

7.3.2 FLOW CYTOMETRY PANEL STAINING

It is essential that we examine both in vivo and in vitro the effects of imatinib on DC maturation and function. The ITN core will perform multi-parameter flow cytometry on frozen cells. Changes in the following sub-sets of cells during treatment may be analyzed using various cell surface markers: B-cells (IgM, CD21, CD23, MHC class II, B7, etc); NK and NKT cells; macrophages, and mast cells. Myeloid DC and lymphoid DC-specific markers may also be examined to determine if DC number and subsets are altered. In addition, expression of activation markers MHC class II, CD40 and CD80, and CD86 molecules may be assessed to determine the state of DC maturation. Of special interest would be examination of various T cell subsets based on phosphoprotein and transcription factor expression, after activation. Flow cytometric evaluations of T cell and antigen-presenting cell subsets might complement the functional studies, enabling a better understanding of adaptive and innate immune compartments. Flow cytometric evaluations may also include the use of class I tetramers for detection of CD8 T cells implicated in the pathogenesis of T1DM. Specifically, HLA-A2 tetramers that enable detection of CD8 cells specific for the following antigenic peptides could be used: GAD 65 114-123, preproinsulin 2-10, IGRP 228-236,

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insulin B chain 10-18, and insulin A chain 1-10. As shown in preliminary studies, these cells can be visualized directly ex vivo without a need for in vitro auto-antigen induced expansion. When possible other validated class I tetramers may be included as they become available that utilize other autoantigen specificities and HLA restriction elements. Alternatively, other tools for visualization of autoantigen specific T cells might be used, such as engineered antibodies containing dimeric MHC/peptide complexes or other MHC/peptide multimers. Finally, we will work with Drs. Eisenbarth and Kappler in Denver to use newly developed class II MHC tetramers that take advantage of mutations that fix autoantigenic peptides in specific registers to maximize TCR binding. Identification of autoantigen-specific CD4 or CD8 T cells directly ex vivo would provide a unique opportunity to reveal the function of these cells without manipulation. In combination with ex vivo polyclonal activation with agents like 4-phorbol 12-myristate 13-acetate (PMA) and ionomycin, it would be possible to also address the cytokine production profile of these cells along with expression of co-stimulatory and activation molecules.

7.3.3 GENOMICS AND PROTEOMICS

Imatinib has been shown to have several downstream targets. For instance, imatinib treatment of DCs resulted in decreased activation-induced upregulation of nuclearlocalized RelB, RelA, c-Rel, NFkB, p50 and reduced phosphorylation of AKT, suggesting that imatinib mediates its effects in part via the NFkB pathway (AKT is upstream of NFkB). A reduction of NFkB and phosphorylation of Lck and ERK1/2 were observed in PHA-stimulated T cells treated with imatinib. These biochemical results suggest a series of pathways to be investigated in cells isolated from treated individuals. We may analyze the biochemical effect of imatinib in T, B and dendritic cells, including the examination of the global effect of imatinib on tyrosine phosphorylation in T cells in vitro and in cells recovered from imatinib-treated patients. Studies will be designed to determine if imatinib affects phosphorylation of TCR, ZAP- 70 and LAT equally, differentially or not. More precise studies on important protein kinases in the TCR signaling pathway, notably ZAP-70, ERK1/2 and AKT, could be performed by immunoprecipitation followed by western blotting in order to track their phosphorylation state (intra-cellular phospho-flow cytometry may also be used). The active form of c-abl may also be assessed in these cells. Similar studies will be performed in B cells if data suggests that B cells are a major target for imatinib activity in T1DM. The ITN may also examine the effect of imatinib treatment on DCs. Purified splenic CD11c+ DCs could be cultured with LPS or CD40L and in the presence or absence of imatinib for 24 hours, then nuclear extracts prepared to assess translocation of NFkB

7.4 SERUM ASSAYS

7.4.1 SERUM-AUTOANTIBODY ANALYSES

Key markers for the presence of the autoimmune processes directed against pancreatic islets include assessing the presence and titers of anti-GAD65, anti-insulin, antiICA512/IA-2, and anti-ICA autoantibodies. Detection of these autoantibody combinations has proven to be an accurate predictor of T1DM in several natural history studies. In the DPT-1 prevention study, over half of the individuals who were positive for two of these antibodies progressed to full disease. Shifts in the titers of Ig isotypes may indicate a change in the type of T-helper cell responses to autoantigen. For example, increases in titers of anti-GAD IgE, IgG2, or IgG4 antibodies could indicate a shift to a more regulatory-type cytokine profile following drug administration. In summary, this study will test the hypothesis that successful treatment will be associated with a reduction in the titer or isotype of diabetes-related autoantibodies.

7.4.2 SERUM ARCHIVE

Patient serum will be archived for future studies. These studies might include measurements of cytokines and subsequent correlation with induction of clinical tolerance. The archived plasma samples could potentially be used for analysis of immune and inflammatory molecules at the proteosome and transcriptosome levels. In addition, expression of pro-inflammatory cytokines (PDGF, IL-1ß, IL-6, IL-12, TNFα), molecules that are induced by inflammation (SOCS1, 2, 3 and acute phase reactants, e.g., ceruloplasmin, SAA, CRP), and anti-inflammatory cytokines (TGF-ß, IL-10) could also be assessed to determine the effect of imatinib treatment. We anticipate that novel assays relating to beta cell death may also be run from archived samples using differentially methylated circulating DNA[180].

7.5 WHOLE BLOOD-GENE-EXPRESSION PROFILING

To further elucidate possible changes in cytokine and cellular profiles, gene-expression profiling analysis may be

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performed on RNA isolated from peripheral blood using microarray or high-throughput real-time polymerase chain reaction (RT-PCR). RTPCR can be used to compare the expression of several genes reported to play a role in T1DM, which might include IFNγ, TGFβ, IL-4, Tbet, RORγt, FOXP3, STAT molecules, IL-2, IL-5, IL-13, IL-15, IL-21, IL-23 and IL-25. The goal of these assays is to identify differences between a tolerant versus non-tolerant state and to find new genes that could serve as potential markers of disease. These types of analyses may also explain why some individuals respond better to this treatment or elucidate mechanisms resulting in adverse responses to treatment. This assay has proven informative in characterizing unique genes that determine the clinical course in systemic lupus erythematosus, and preliminary studies with this technology have enabled us to determine a distinct subset of genes that are either up- or down-regulated in those with new-onset T1DM as opposed to controls in other studies.

7.6 WHOLE BLOOD DNA-HLA GENOTYPES

DNA collected from participants will be used to perform sequence-based HLA typing. A complete class I and class II haplotype will be performed, including fine typing of the DQB and DRB regions. Genotyping for single nucleotide polymorphisms (SNPs) in selected immune-response genes may also be performed. The results of genotype analyses might be used to correlate with disease progression and therapeutic responses (tolerance induction). In addition, collected DNA may be used for epigenetic analysis that includes histone modification and DNA methylation studies.

7.7 CHANGE IN BETA CELL FUNCTION

Many of the measures that we will utilize in assessing changes in beta cell function are now standard assessments in new-onset T1DM trials (see Section 3.3). However, these studies have ignored changes in insulin sensitivity. Imatinib may have novel effects on beta cell function and insulin sensitivity, as noted by the pre-clinical studies and effects in subjects with type 2 diabetes. We recognize that euglycemic hyperinsulinemic clamps are the gold standard for assessment of insulin sensitivity, but pose significant additional subject and investigative team burden and expense. We will assess changes in adiponectin levels in study patients, as these have shown dramatic increases in patients with type 2 DM on imatinib[126]. We will also use data derived from MMTTs to model beta cell function and insulin sensitivity, as has been employed by others[181,182](see letter of collaboration from Ferrannini). In addition to glucose and cpeptide levels, plasma glucagon, and proinsulin levels will be measured to further explore the effects of Imatinib on insulin sensitivity.

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