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Evaluating the Immunopathogenesis of Diabetes Following Acute Pancreatitis in the *D*iabetes *RE*lated to *A*cute Pancreatitis and Its *M*echanisms (DREAM) Study: From the Type 1 Diabetes in Acute Pancreatitis Consortium (T1DAPC)

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On behalf of the Type 1 Diabetes in Acute Pancreatitis Consortium (T1DAPC)

Abstract

The association between acute pancreatitis (AP) and diabetes mellitus (DM) has long been established, with the initial descriptions of AP patients presenting with DM after a bout of AP published in the 1940s and 50s. However, the potential mechanisms involved, particularly those components related to the immune system, have not been well defined. The *D*iabetes *RE*lated to *A*cute Pancreatitis and its *M*echanisms (DREAM) study is a multi-center clinical study designed to understand the frequency and phenotype of DM developing after AP. This article describes one objective of the DREAM study: To determine the immunologic mechanisms of DM after AP, including the contribution of β-cell autoimmunity. This component of the study will assess the presence of islet autoimmunity, as well as the magnitude and kinetics of the innate and adaptive immune response at enrollment and during longitudinal follow-up after one or more episodes of AP. Finally, DREAM will evaluate the relationship between immune features, DM development, and pancreatitis etiology and severity.

Keywords

T1D; autoimmune; immune system; RNAseq; autoantibody; diabetes

Background

Both innate and adaptive immunity have been implicated in the development of acute pancreatitis (AP). However, there remains relatively little clear mechanistic information about the precise roles and kinetics of any immune pathway, which has limited the discovery of novel immune therapies for AP. Each arm of the immune system exhibits hallmark cellular and cytokine changes in this disease. Early neutrophil infiltration is common in pancreatitis and can propagate further inflammation via neutrophil-extracellular traps (NETs).[1] Activation of the transcription factor NF-κB may occur early in the course of

AP, leading to the synthesis of pro-inflammatory mediators such as interleukin (IL)-6, IL-8, tumor necrosis factor-α (TNF-α), cyclooxygenase 2, and nitric oxide synthase.[2] Injured acinar cells and early infiltrating neutrophils induce macrophage migration in animal models,[3] where macrophage activation is an important determinant of pancreatitis severity. More recently, heterogeneous monocyte populations have been detected in human peripheral blood during AP.[4] Regarding the adaptive arm of the immune system, T cells have also been found in small numbers in pancreata inflamed from pancreatitis in both animals and humans.[5,6] Human studies have demonstrated a significant drop in peripheral lymphocytes during an episode of AP, with a greater drop in CD4+ T cells relative to CD8+ T cells.[7] Elevated serum levels of T helper (Th) 1 (IL-2 and interferon [IFN]-γ), Th2 (IL-4, IL-5), and other inflammatory (TNF-α) cytokines have been detected in AP.[8,9] The relationship between each of these measures and the development of a type 1 diabetes (T1D)-like disease after AP is of great interest.

Animal Models: Understanding the Role of the Immune System in AP and DM

Animal models have greatly contributed to our understanding of the relationship between the immune system and development of DM in the context of AP. One of the first in vivo experiments to highlight the interplay between AP and DM revealed that whereas Th2 cells produce mild insulitis in non-obese diabetic (NOD) neonates, they generate an intense pancreatitis and insulitis with IL-10-mediated islet cell necrosis, abscess formation, and DM in NOD.scid mice. These findings set the stage for future investigations into the AP-DM association.[10] In a rat model of cerulein-induced AP, islet cell integrity was maintained by the addition of bone marrow-derived mesenchymal stem cells (MSCs) in the presence of ascorbic acid (AA) and N-acetylcysteine, which effectively blocked IL-1ß, TNF-α, and nuclear factor kappa-B. The implication is that inflammatory cell damage from AP is not limited to the exocrine compartment, but also causes damage of the endocrine compartment – and specifically the islet cells – which can exacerbate AP severity.[11] In mice with pre-existing DM mimicked via either permanent (Ins2Akita) or conditional (EL-CreER/-/IRfl/fl or PACIRKO) loss of insulin signaling, the severity of AP in two models of AP induction (palmitoleic acid/ethanol, and cerulein) was far worse than in control mice,[12] providing a link between immune changes in the context of DM and inflammation induced by AP. Beyond these studies, there is little evidence of immune-mediated cytotoxicity linking AP and DM, though other AP-associated molecular signals may contribute to DM development.

There is evidence that modulating factors associated with the immune system can improve AP or DM outcomes in vivo. For example, L-serine supplementation can reduce acinar tissue damage during cerulein-induced AP in streptozotocin-treated diabetic mice, likely via reduced reactive oxygen species, endoplasmic reticulum (ER) stress, and apoptosis.[13] Notch inhibition was tested in cerulein-induced AP in mice; in this model an increase in cytokeratin 5+ (CK5+) progenitor cells gave rise to increased levels of beta cell differentiation and subsequent improved glucose homeostasis. Interestingly, in patients with acute necrotizing pancreatitis, notch levels were high and associated with CK5+ cell activation and the production of multiple duct-like structures.[14] Such studies imply that a

better understanding of the human immune response to AP and subsequent development of DM may yield therapeutic options for future clinical trials.

Autoantibodies Predict and Define T1D Development

The landmark discovery of islet cell specific autoantibodies[15] not only heralded T1D as an autoimmune disease, but also provided a marker for predicting and diagnosing this disease. Subsequently, the primary autoantigens associated with autoimmune T1D were identified and assays were developed and validated to detect autoantibodies to each of the following molecules: Insulin,[16] glutamic acid decarboxylase (GAD),[17] islet cell antigen 512 (ICA512) or insulinoma associated-2 (IA-2),[18,19] and zinc transporter 8 (ZnT8).[20] Use of these four autoantibody assays to predict T1D has been extensively reviewed elsewhere.[21–23] An important consequence of these studies is that reliable assays are now available, creating an opportunity to detect and characterize whether and how often autoimmune DM occurs after AP. In T1D, knowledge has been gained from extensive longitudinal studies performed in pediatric populations, both in high-risk populations (family history of T1D), and general population studies (school children). In sum, these published studies have provided consensus on the highest risk of DM development in the setting of a) multiple autoantibodies, b) longitudinally persistent autoantibodies, c) sequence-dependent appearance of autoantibody specificities, and d) autoantibody titer and/or affinity.[21]

Islet Autoimmunity Can Be Detected After AP

A limited body of evidence suggests that pancreatic damage, either transient or persistent, may induce signs of islet autoimmunity. A case report of a 57-year-old man with T2DM (documented GAD autoantibody negative and not requiring insulin) found that during a bout of AP, he developed positive GAD autoantibodies, which disappeared after resolution of AP.[24] Among 397 children with recurrent AP or chronic pancreatitis (CP), 24 (6%) were found to have DM. Not all were tested for islet autoantibodies, but among the 13 assessed, 5 (38%) were positive for islet autoantibodies. In this pediatric cohort, risk factors for DM included older age, hypertriglyceridemia, coexisting autoimmune disease, and pancreatic atrophy[25]; of note, the latter three reflect pathologies of T2DM, T1DM, and pancreatopathy, respectively. This suggests that inflammation in surrounding exocrine tissue may extend to nearby islets, causing damage that allows the immune system to react to islet antigens. However, it should be noted that spillover inflammation may not necessarily result in development of autoantibodies. For example, among 61 patients with CP, 10 had pancreas specimens studied by electron microscopy. All 10 showed signs of islet inflammation (islet cell lysis, islet fibrosis, and immune cell infiltrates), yet none of the 10 patients developed islet autoantibodies, including those carrying T1D-predisposing haplotypes (human leukocyte antigen [HLA] DR3 and/or DR4).[26] A few case reports have described autoimmune T1D developing after AP.[27,28] In a two-year prospective study of 152 patients with AP who did not have DM at baseline, one patient developed T1D.[29] Though infrequent, development of islet autoimmunity may be clinically significant. In a series of patients undergoing total pancreatectomy with islet autotransplantation (TPIAT) for intractable pain due to CP, 9 of 350 patients had positive GAD autoantibodies, only one of whom had preexisting DM with preserved C-peptide; after TPIAT, none of these 9 achieved

insulin independence (versus 33% of those without GAD autoantibodies) and 2 had islet graft failure and poor glycemic control.[30] In a similar group of 11 subjects post-TPIAT, 3 showed biopsy-confirmed insulitis, indicative of a T1D-like disease.[31] Autoantibodies have also been reported in subjects with recurrent AP[32]; while this study detected the presence of anti-insulin antibodies in subjects who had at some point been treated with insulin, 8% of individuals in a recently described cohort had multiple T1D-associated autoantibodies, suggesting the presence of islet autoimmunity in this setting. Analyses from the *DI*abetes *RE*lated to *A*cute pancreatitis and its *M*echanisms (DREAM) study are poised to provide a clear picture of the prevalence and incidence of islet autoimmunity associated with AP, and whether that impacts the future risk of developing DM, and investigate potential mechanisms.

STUDY DESIGN

Overall Study Design

The DREAM study is a multi-center observational cohort study, prospectively enrolling adults with a recent episode of AP, that aims to understand the frequency and phenotype of DM developing after AP. Targeted enrollment is 1000 individuals of all races, ethnicities, and sexes/genders following a qualifying episode of AP, defined using the revised Atlanta criteria.[33] Participants will be 18–75 years of age, and have no history of CP or other pancreatic diseases. Of this cohort, an estimated 800 participants without DM preceding AP will undergo longitudinal clinical and laboratory assessment of glycemic and autoantibody status to determine the cumulative incidence rates of DM and to provide information on the type of DM observed. Individuals will attend study visits and provide samples for longitudinal assessment of metabolic and immunologic parameters. Considerable clinical data relevant to understanding the immunology of AP-induced DM will be collected on all participants, including use of therapies that impact the immune system, vaccination history, and presence of pre-existing autoimmune disease(s). The primary immunological goal of the DREAM study is to deeply characterize the autoimmune and transcriptional changes that occur in the context of AP, and to relate these immunopathological features to endocrine, exocrine, clinical, and metabolic measures obtained at study visits in the context of DM development. Remaining biosamples will be stored in a central biorepository for future studies. For details on the protocol, biospecimens, or ascertainment of DM, please see the relevant articles within this issue.[34–36]

Immunological Assessments: Autoantibodies

The DREAM study design allows for islet cell autoantibodies to be measured in serum samples at all study visits, from the most proximal visit to AP diagnosis through a 5-year follow-up phase. We will measure autoantibodies against insulin, ZnT8, IA-2, and GAD65, using the same assays currently implemented by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)-funded Type 1 Diabetes TrialNet Pathway to Prevention study which follows individuals with islet autoimmunity to clinical T1D development (reviewed in[21]). Serum and plasma samples will be available to test additional autoantibodies in future research. There are also ongoing efforts to understand how newly

developed assays for the 4 commonly measured autoantibodies compare regarding ability to predict T1D onset, which can also be tested in the DREAM population.

An important goal of this work is the identification of existing autoantibody-positive individuals (if any) at baseline; some of these individuals will have pre-existing autoantibodies prior to their AP episode while others may rapidly develop autoantibodies during the early weeks after diagnosis. We will also be able to identify individuals who seroconvert after their AP diagnosis at follow-up visits, as well as any who develop transient autoantibody positivity.[24] All of these individuals will be followed to ascertain future DM and the relationship between islet autoimmunity and DM development after AP. Some individuals will also progress to T2D during study follow-up. We hypothesize based on previous studies[37–39] that most, but not all, individuals with T2D following AP will be negative for islet-specific autoantibodies. Nevertheless, further immune data from all participants with islet autoantibodies will be of great interest and provide pathophysiological insights.

Immunological Assessments: Whole Blood RNAseq

The goal of utilizing whole blood (wb) RNA-seq in DREAM is to identify differentially expressed genes or modules that change as AP resolves, or that occur contemporaneously with or appear prior to the development of DM. The wbRNA-seq measures transcriptional signatures on a genome scale, with many existing tools to annotate the ‘omics’ scale output and provide biological meaning. Importantly, this analytical method has been successfully deployed to identify mechanisms underlying disease pathogenesis and novel therapeutic targets in autoimmune and inflammatory diseases (reviewed in[40]). For example, wbRNAseq in systemic-onset juvenile idiopathic arthritis identified a strong IL-1 signature, which led to approval of IL-1 blockade for this disease (reviewed in[41]). Importantly, cell-type effects on gene expression can be de-convoluted using complete blood count (CBC) data, providing information about the drivers of an immune signature beyond a simple count of cells in blood.

In the DREAM study, wbRNAseq will provide a broad readout of immune and physiological status. Sequencing will be conducted on longitudinal samples collected at all visits throughout the study to assess the kinetics of resolution of inflammation after AP, and assess the relationship between the rate or nature of resolution and development of DM. After dimensionality reduction of the data, associations between transcriptional profiles and clinical features of DM and pancreatitis will be identified. Individuals with T1D and T2D will be used as comparators to understand the signatures of DM development in people with or without a history of AP. Additionally, we will use reference gene expression data from individuals without pre-existing AP obtained from public functional genomics data repositories including NCBI GEO (National Center for Biotechnology Information Gene Expression Omnibus).

Analytical Considerations for Planned Immune Assays

Early in the discovery of the various autoantibodies, workshops were formed in an attempt to standardize and harmonize assays.[42–44] These efforts have had a profound effect on

quality assurance of these measurements and the laboratories performing these assays continue to participate in worldwide workshops to maintain proficiency. In the DREAM study, statistical analyses of the relationship between autoantibody presence and titer with development of DM will parallel the primary outcome analyses described in detail in this issue.[34] Importantly, we will have the opportunity to utilize longitudinal, confirmatory measurements in the context of the DREAM study to reduce the frequency of false positive measurements.

Analysis of the wbRNAseq data will be more challenging, and methods in the field of transcriptome analysis are consistently improving. However, we plan to process the data as previously described.[45–49] Preliminary analysis and dimension reduction of the data will likely be conducted using pre-defined and functionally-annotated gene modules using methods such as those described in,[40,50] and/or by generation of new gene modules from within this dataset using Weighted Gene Correlation Network Analysis.[51] Modeling of these data will incorporate differing times from onset for enrollment samples, subsequent development of recurring pancreatitis, and temporary or chronic use of immunotherapies. In addition, models will incorporate features of AP and DM that are expected to influence or be influenced by the immune system, such as autoantibody status, or the etiology and severity of AP.

Biobanking for the Future: Peripheral Blood Mononuclear Cells

Since understanding the role of the immune system in the resolution of AP and the onset of DM is a novel and important focus of this study, we plan to collect and centrally process peripheral blood mononuclear cells (PBMC). The details of collection and shipment for central PBMC processing are described elsewhere in this issue.[35] Central processing of PBMC is critical to avoid batch effects in modern high-dimensional datasets, and to ensure that results of functional assays are not confounded by study site; this parallels the approach taken by the NIDDK Type 1 Diabetes TrialNet and the majority of studies conducted through the NIAID Immune Tolerance Network, among others. Peripheral blood mononuclear cells are typically the most important sample type to collect for immune research, as they enable analyses not just of immune cell frequency but also of their function, as well as providing material that can be sorted to specific immune cell subsets.

Much of our understanding of the cellular immunopathogenesis of T1D has come from PBMC samples. Therefore, it is optimal to use – and expand upon - similar assays to characterize DM following AP. We anticipate that PBMC from this study will be useful for flow cytometry or mass cytometry by time-of-flight (CyTOF) studies for phenotypic and functional bulk characterization of circulating immune cells.[52–54] Importantly, PBMC enable the measurement of antigen-specific T-cells specific for either endocrine or exocrine tissue, helping to define whether antigen specificity differs in the setting of the AP inflammatory insult compared to the well-described range of antigens detectable in typical T1D (recently reviewed in[55,56]). Understanding the presence and targets of antigen-specific cells represents a critical piece of information regarding whether and how the adaptive immune system drives AP-related DM.

Biobanking for the Future: Other Specimens

Other samples (serum, plasma, RNA, DNA, and blood stabilizer tubes) relevant to immunological assessments will be processed at the various clinical sites for interim storage followed by quarterly shipments to the biorepository. The sites will follow a study-specific manual of operations and receive training as necessary. These processes aim to ensure that samples will be highly useful for follow-up immunological and other assays in future.

The longitudinal biobanking and the variety of these additional samples provide opportunities for future studies applying state-of-the-art methodologies relevant to the understanding of immune activation. Relevant examples under consideration include: 1) Untargeted or targeted plasma proteomics, utilizing either traditional liquid chromatography with tandem mass spectrometry or more recently developed affinity-based methods.[57] Besides the biomarker discovery opportunity, the affinity-based options also provide validated multi-arrays targeting hundreds of inflammation proteins in small plasma volumes. 2) Discovery of neoantigens and characterization of novel autoantibodies that develop after AP in those who develop DM. These assays have identified novel antibodies in infectious and autoimmune settings[58,59] and may yield important information regarding the endocrine and exocrine attacks that occur during and after AP.

DISCUSSION

Strengths and Limitations

The relatively rare occurrence of DM after pancreatitis, and the inherent heterogeneity of both diseases, need to be overcome by creating a large consortium able to gather a sufficient number of participants. DREAM represents such a study, designed by experts across disciplines to pursue significant advancements in clinical understanding of DM and AP. The study will result in immunophenotyping and islet autoantibody data from a large prospective cohort, using standardized methods for biospecimen collection and assay conduct.

To ensure enrollment of a large cohort, flexibility is built into the study design; such flexibility is necessary to facilitate recruitment in the setting of variable disease severity, logistical difficulties of consent and specimen collection in patients who are ill, and participants' preference to enroll after clinical improvement. Therefore, the first visit (and the first blood sample) are subject to variability in the time of collection relative to presentation of AP. This necessary flexibility regarding timing of the first sample collection may be a limitation for understanding the role of the immune system at the time of pancreatitis and how it influences DM development.

Though sizeable, the study population, particularly the number expected to develop DM, does not guarantee sufficient power for genome-wide association studies (GWAS). Therefore, the DREAM study does not plan to execute GWAS. However, biospecimens for DNA extraction (PAXgene tubes) will be available to enable future studies of specific, hypothesized genetic components of AP and DM; HLA typing is a first planned assessment.

Additional Outcomes for Future Work

The immunological dataset obtained in the DREAM study offers the opportunity to evaluate other aspects of AP and DM, including: understanding the immunopathogenesis of any subtype of DM that may develop[60]; identifying immune factors associated with resolution or progression of pancreatitis; and defining the wb transcriptional signatures of different pancreatitis etiologies. It also establishes a biorepository of specimens (PBMC, DNA, and stool) for future translational research and mechanistic follow-up studies based on findings from the wbRNAseq data. This could include time course studies on mechanisms of disease progression, identification of novel biomarkers predictive of DM or other pancreatitis outcomes, or small mechanistic trials to identify novel therapeutic targets. Finally, mechanisms hypothesized from observations in the T1DAPC (Type 1 Diabetes in Acute Pancreatitis Consortium) cohort could subsequently be tested in animal models. Ultimately, our planned studies provide the framework for greater molecular assessments that can help conceptualize the types of mechanisms contributing to AP-induced T1D in patients, identify potential means to circumvent this interaction, and provide improved care for people caught in the exocrine-endocrine pancreatic storm. The DREAM study is expected to move our understanding of the immunology of AP forward in the coming years. In closing, the extensive immunological, metabolic, pancreatic imaging, and microbiome studies will yield cross-disciplinary findings in the context of DREAM, and provide samples and data for ancillary findings from this well-characterized cohort.

Conflict of Interest Disclosures:

J.A.H. consults for aTyr Pharma. FGST has received consulting fees from Sanofi, Eli Lilly, and AstraZeneca, and research support from Dompé. G.I.P. has <5% equity with Ariel Precision Medicine, has consulted with Nestle, and receives research funds from Abbvie. M.D.B. receives research support from Viacyte and Dexcom, and has participated on an advisory board with Insulet. C.S. participates on an advisory board for Vertex Pharmaceuticals. The rest of the authors declare no conflict of interest.

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