An Investigation Into the Oral Tolerance

In a Type 1 Diabetes Mouse Model

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Abstract

Type 1 Diabetes (T1D) affects millions of Americans and around 16% of those affected are under the age of 20. It is an autoimmune disorder in which the individual's own immune system attacks the insulin producing beta cells of the body. This study focuses on preventing the onset of T1D by modifying certain strains of *Lactococcus lactis* to secrete either pro-insulin, IL-10, or both pro-insulin and IL-10. There are three different strains of bacteria that will be used throughout the study and we are measuring the levels at which these strains secrete either pro-insulin, IL-10, or both pro-insulin and IL-10. It was hypothesized that the levels of secretion would increase oral tolerance and the results support the hypothesis. However, the levels of the *L.lactis* secreting either pro-insulin or IL-10 were higher than the *L.lactis* engineered to secrete both pro-insulin and IL-10. The levels of secretion in the gut by *L.lactis* can help prevent the onset of T1D by inducing tolerogenic dendritic cells, which play a role in helping create an immune tolerance.

Introduction

Type 1 diabetes (T1D) is an autoimmune disease in which an individual's immune system attacks its own beta cells in the islets of Langerhans, a specific cluster of cells in the pancreas. These beta cells create insulin to be released by the pancreas so without these cells, the body does not have a mechanism for producing insulin. Researchers have been experimenting with a modified bacterium known as *L.lactis* for a possible cure for T1D. *L.lactis* can be modified to secrete pro-insulin as well as IL-10 and when present in the gut, this bacterium increases the rate at which these two proteins are secreted. In studies relevant to my research, experimenters have proven that the engineered *L.lactis* is successful in NOD mice. IL-10 and pro-insulin are both particularly important in the body's regulation of glucose and vital in preventing the pathogenesis of T1D. One study found that "the presence of exogenous IL-10 in the gut induced IL-10 secretion by certain cells. There was also an increase in the measurement of IL-10 titers in the plasma" (Frossard, Steidler, & Eigenmann, 2007). This conclusion supported the idea that modified *L.lactis* can effectively begin the process of immune tolerance.

Aim and Hypothesis

My research will be expanding on the effects of the treatment of modified *L.lactis*. I have one main study aim. It is focusing on *L.lactis* secretion of proteins. As of right now, it is known that the bacterium can be modified to secrete insulin and IL-10. However, the levels at which the proteins of interest are secreted are still unknown, so I will attempt to answer this question. We predict that the *L.lactis* strains will successfully secrete levels of pro-insulin and IL-10 that will initiate tolerance to prevent the onset of T1D. Overall, the goal of the therapy is to induce tolerogenic dendritic cells in the gastrointestinal tract because they play an important role in stimulating and sustaining immune tolerance (Li & Shi, 2014). This immune tolerance will help prevent and reverse type 1 diabetes.

Methods

Our delivery vector is a GRAS organism, *Lactococcus lactis,* that has been genetically modified to secrete pro-insulin, IL-10, or pro-insulin and IL-10. NOD mice were orally gavaged 5x/week with the various strains of *L.lactis.* To confirm that the strains were secreting the proper proteins, we performed ELISA for IL-10 and/or proinsulin for each strain. The bacteria was diluted 1/1000 in GM17T(E) media overnight and allowed to reach stationary phase (16 hours later). From that overnight culture, we diluted an aliquot of bacteria 1/25 in BM9T media and allowed them to grow and secrete for 3 hours at 30 °C. The bacteria were spun down and supernatants were collected for ELISA.

1. Mercodia Proinsulin (PINS) ELISA

   This ELISA required a blank of zero, a coated plate, enzyme conjugate made of mouse monoclonal antibodies, enzyme conjugate buffer, assay buffer, substrate TBM, wash buffer, and stop solution in order to be run. A plate map was created for the ELISA that consisted of a blank, 4 standard measurements, and 2 different strains of *L.lactis* that included one with pro-insulin alone and one with both pro-insulin and IL-10. Each strain had three dilutions that were included in the plate: neat, diluted 1:100, and diluted 1:1000. Also, every well within the plate map is duplicated to ensure accuracy. When beginning the ELISA, we first prepared the enzyme conjugate and wash buffer solution. Then, 50 µL of the appropriate controls and samples were pipetted into the appropriate place, according to the plate map. Assay buffer is added to each well and then the plate was incubated on a plate shaker (700-900 rpm) for one hour at room temperature (18-25 °C). After the incubation period, the plate was washed 6 times with 700 µL of wash buffer using an automatic plate washer. Then, 100 µL of the enzyme conjugate that was prepared earlier in the process was added to each well and the plate was incubated on the plate shaker for one hour at the same rpm and temperature as before. The plate was washed using the automatic plate washer as described before, 200 µL of substrate TMB

was added to each well, and then incubated for 15 minutes on the bench at room temperature. Lastly, 50 µL of stop solution was added to each well.

a. Data Collection: The optical density was read at 450 nm and it was read using the spectrophotometer called SpectraMax M5.
b. Data Analysis: The average absorbance for the duplicated wells was plotted on the standard curve and the diluted samples (1:100 and 1:1000) were multiplied by their respective dilution factors. The SoftMax Pro 7.0 was used to analyze the results.

2. Mouse IL-10 ELISA

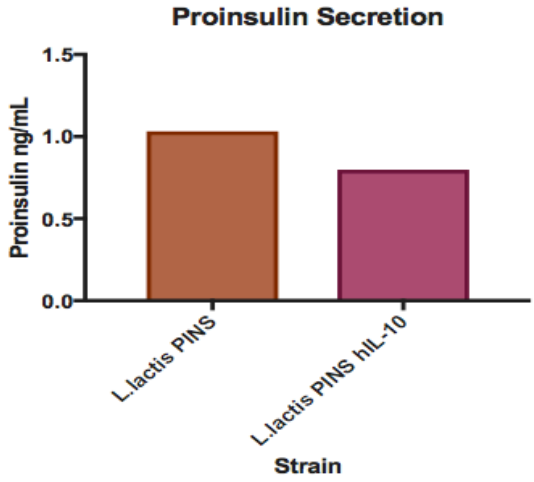
This ELISA required the same starting materials as the pro-insulin ELISA, however, the steps and measurements are different. The plate map was created to have 8 standard measurements, including a blank of 0, and 2 different strains of *L.lactis* (one with IL-10 alone and one with both pro-insulin and IL-10). These 2 strains were placed into wells in three dilutions: neat, diluted 1:100, and diluted 1:1000. The wells were also duplicated in order to check for accuracy. In order to begin the procedure, the wells needed to be coated with the capture antibody (100 µL per well) and left to incubate overnight at a temperature of 4 °C. The next day, the plate was washed 3 times with the wash buffer, 200 µL of assay diluent was placed in each well, and the plate was incubated for an hour. After the hour, the plate was washed 3 times again with the wash buffer and 100 µL of each standard and sample was placed into the appropriate wells, according to the plate map. The plate was sealed and incubated for 2 hours at room temperature. After the 2 hours, it was washed again, but 5 washes instead of 3, and 100 µL of working detector was added to each well before incubating again for 1 hour. The plate was washed again (7 washes), 100 µL of substrate solution was added to each well, and then incubated for 30 minutes in the dark. Finally, 50 µL of stop solution was added to each well.

a. Data Collection: The optical density was read at 450 nm and it was read using the spectrophotometer called SpectraMax M5.

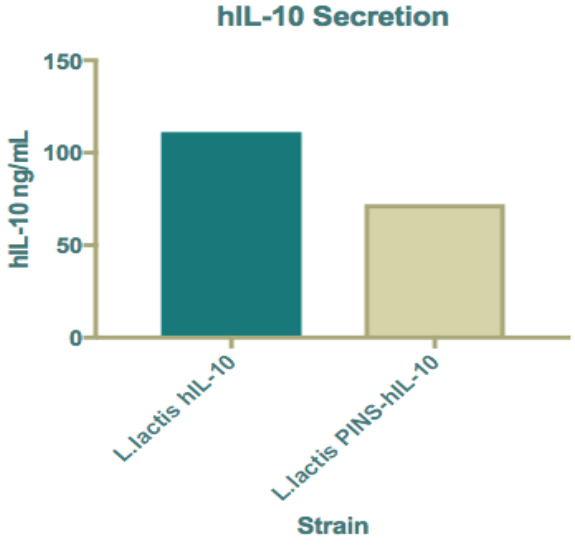
b. Data Analysis: The average absorbance for the duplicated wells was plotted on the standard curve and the diluted samples (1:100 and 1:1000) were multiplied by their respective dilution factors. The SoftMax Pro 7.0 was used to analyze the results.

Results

We hypothesized that the strains of *L.lactis* bacteria will secrete IL-10 and pro-insulin at the levels needed to induce tolerogenic dendritic cells and stimulate oral tolerance of type 1 diabetes. The hypothesis was proven to be correct and the results of the study are shown below. In both graph 1 and graph 2, it was proven that solo secretions work more effectively than when both pro-insulin and IL-10 are made to secrete together. In graph 1, *L.lactis* was modified to secrete pro-insulin alone and secreted about .20 ng/mL more pro-insulin than the *L.lactis* that was modified to secrete both pro-insulin and IL-10. In graph 2, the *L.lactis* that was engineered to secrete IL-10 alone secreted about 25 ng/mL more IL-10 than the *L.lactis* that was engineered to secrete both pro-insulin and IL-10. It is clear that *L.lactis* secreting IL-10 is more efficient than both the *L.lactis* secreting pro-insulin and the *L.lactis* secreting both pro-insulin and IL-10.



Graph 1: Pro-insulin Secretion



Graph 2: IL-10 Secretion

Discussion and Conclusions

Type 1 diabetes is an autoimmune disorder so the main goal of this study was to find a way to induce tolerogenic dendritic cells because these are the cells that help maintain immune tolerance. Overall, the results have proven that using *L.lactis* to secrete pro-insulin and IL-10 can induce oral tolerance to diabetic auto antigens in order to prevent people from developing type 1 diabetes. The *L.lactis* engineered to secrete either pro-insulin or IL-10 was more effective than the *L.lactis* that secreted both pro-insulin and IL-10. If this treatment were transferred to humans, an immunosuppressant would need to be considered in order to make sure that the individual's immune system would not negatively react to the treatments used to prevent the onset of type 1 diabetes. Future research should focus on mechanistic studies in order to determine the exact mechanisms of prevention because this knowledge will allow researchers to create a more specific target treatment.

References

Frossard, C. P., Steidler, L., & Eigenmann, P. A. (2007). Oral administration of an IL-10–secreting Lactococcus lactis strain prevents food-induced IgE sensitization. *Journal of Allergy and Clinical Immunology,119*(4), 952-959. doi:10.1016/j.jaci.2006.12.615

Robert, S., & Steidler, L. (2014). Recombinant Lactococcus lactis can make the difference in antigen-specific immune tolerance induction, the Type 1 Diabetes case. *Microbial Cell Factories, 13*(Suppl 1). doi:10.1186/1475-2859-13-s1-s11

Takiishi, T., Cook, D. P., Korf, H., Sebastiani, G., Mancarella, F., Cunha, J., . . . Mathieu, C. (2016). Reversal of Diabetes in NOD Mice by Clinical-Grade Proinsulin and IL-10–Secreting Lactococcus lactis in Combination With Low-Dose Anti-CD3 Depends on the Induction of Foxp3-Positive T Cells. *Diabetes, 66*(2), 448-459. doi:10.2337/db15-1625