DEVeLOPMENT OvF METHODS TO EVALUATE THE USE OF MEDI-551 TO TARGET CD19 TRANSMEMBRANE PROTEINS ON B-CELLS TO IMPROVE SYSTEMIC SCLEROSIS

Natalia Dohman

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DEVELOPMENT OF METHODS TO EVALUATE THE USE OF MEDI-551 TO TARGET CD19 TRANSMEMBRANE PROTEINS ON B-CELLS TO IMPROVE SYSTEMIC SCLEROSIS

by

Natalia Dohman

A Thesis Submitted in Partial Fulfillment
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The members of the Honors Thesis Committee appointed to examine the thesis of Natalia Dohman find it satisfactory and recommend that it be accepted.

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ABSTRACT

DEVELOPMENT OF METHODS TO EVALUATE THE USE OF ME-DI-551 TO TARGET CD19 TRANSMEMBRANE PROTEINS ON B-CELLS TO IMPROVE SYSTEMIC SCLEROSIS

Natalia Dohman

Director: Dr. Victor Huber, Ph.D.

Systemic sclerosis, a subcategory of scleroderma, is a rare autoimmune disease that affects the skin and, in some cases, the internal organs of those diagnosed with the disease. There is currently no cure for this disease, only treatments to slow its progression. It is important to note that each individual diagnosed with scleroderma experience differences in severity and symptoms of the disease and are treated based on their symptoms, not as a whole. Many factors of this disease still remain unknown, such as what causes the disease or stimulates the onset of it. Recent studies have helped determine different treatment options for those diagnosed with the disease. For example, studies have been conducted using MEDI-551, a monoclonal antibody that targets CD19 transmembrane proteins, as form of treatment that could further improve systemic sclerosis. Here we present an initial evaluation of antibody therapies on whole cells as a way to start testing a therapeutic like MEDI-551 for its efficacy in killing B cells through either direct or indirect interactions with the immune system. Our findings discussed in the context of the disease state and the future of treatment using monoclonal antibody therapies.

KEYWORDS: systemic sclerosis, CD19, MEDI-551
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CHAPTER ONE

Introduction

Systemic sclerosis is an autoimmune disease that affects the skin and, in some cases, internal organs of those diagnosed with the disease. There is currently no cure for this disease, only treatments to slow its progression. Recent studies have been conducted using MEDI-551, a monoclonal antibody that targets CD19 transmembrane proteins, as form of treatment that could further improve diffuse systemic sclerosis from the treatments currently in use. The main objective of this thesis is to provide knowledge on systemic sclerosis and to elaborate on a new treatment for the disease. In order to better understand how the new treatment works effectively, it is important to understand systemic sclerosis, its symptoms, and former treatments.
CHAPTER TWO
Systemic Sclerosis

Section One: Background
Systemic sclerosis is a division of the autoimmune disease scleroderma; scleroderma is an incurable autoimmune disease characterized by the involvement of the connective tissues. According to the Scleroderma Foundation, “It’s estimated that about 300,000 Americans have scleroderma. About one third of those people have the systemic form of scleroderma (Scleroderma Foundation, 2019).” Scleroderma can be categorized into two types: localized and systemic. Localized scleroderma is normally found to only affect a few areas of the skin and muscle, and it does not affect the internal organs. Systemic scleroderma, also known as systemic sclerosis, affects the skin and has the ability to affect internal organs. This type of scleroderma is more severe because when the organs are affected, they become hard and fibrous. When the organs become hard and fibrous, their ability to function is inhibited and declines (Scleroderma Foundation, 2019).

Systemic sclerosis can be further categorized into either limited or diffuse. Limited systemic sclerosis normally affects the hands and face, develops at a slower pace, and internal organs are less likely to be affected. Diffuse systemic sclerosis occurs at a more rapid rate compared to limited systemic sclerosis; therefore, the skin hardens faster, and the internal organs are more likely to be affected. The main organs targeted by diffuse systemic sclerosis are the lungs,
heart, kidneys, and gastrointestinal tract (Scleroderma Foundation, 2019). Those whose disease have lung or cardiac involvement have a three-year survival rate of 47-56% (Sierra-Sepúlveda et al., 2019). According to “Systemic Sclerosis Pathogenesis and Emerging Therapies, Beyond the Fibroblast” by Sierra-Sepúlveda et al., “It is the single connective tissue disease with the worst survival prognosis (Sierra-Sepúlveda et al., 2019).”

There are no definitive answers on what causes systemic sclerosis or the onset of it. The current hypothesis for the onset of systemic sclerosis is that exposure to an infectious or environmental agent or an event that triggers the immune system occurs in a genetically susceptible individual, triggering the onset of systemic sclerosis. It is known that the immune system is involved in this disease because various autoantibodies specific to systemic sclerosis are present. Another reason it is known the immune system is involved is because genetic studies have shown that there are gene polymorphisms that are related to the immune response; a few of these gene polymorphisms are located in the major histocompatibility complex, types I and II interferons, cytokines, and chemokines (Sierra-Sepúlveda et al., 2019).

Section Two: Symptoms

While there are no specific answers on what the initiating factors or the triggering events are, the pathogenesis of systemic sclerosis is known to involve the endothelium, epithelium, fibroblasts, and both innate and adaptive immune systems (Abraham et al., 2009). By focusing on common symptoms patients
have with systemic sclerosis, it helps to better determine the pathogenesis of the disease. Common symptoms to look for when diagnosing scleroderma are discoloration of the fingers and toes when exposed to colder temperatures, as well as swollen hands before the thickening and hardening of the skin. Other common symptoms in the beginning stages include open sores on the distal end of the fingers, painful calcium deposits under the skin, and telangiectasia, which is small clusters of enlarged blood vessels underneath the skin; telangiectasia causes discoloration to the skin and appears as red dots on affected areas ("Systemic Sclerosis," 2019).

The most common symptom of systemic sclerosis is fibrosis, which occurs due to the excess production of collagen and other extracellular matrix proteins in the connective tissues of various organs. Myofibroblasts are the main cells responsible for this excess production. These myofibroblasts have different origins such as endothelial or epithelial cells, which play an important role in injury repair. In individuals diagnosed with systemic sclerosis, there is evidence that shows the process of injury repair is altered. Of all the symptoms, fibrosis is the prominent symptom that leads to the deterioration of the organs’ functions (Sierra-Sepúlveda et al., 2019).

Symptoms that are specific to systemic sclerosis, as it has the ability to attack internally, are shortness of breath, hypertension, dysphagia, and heartburn. These symptoms occur when systemic sclerosis attacks those specific organs and impair their function. The most common organs that are affect are the esophagus, heart, lungs, and kidneys ("Systemic Sclerosis," 2019). These
symptoms can become so severe that they begin to physically impair those affected by the disease and interfere with daily life.

A study was conducted in 2007 by Saurez-Almazor, Kallen, Roundtree, and Mayes to determine how specific symptoms affected individuals diagnosed with systemic sclerosis. The study used qualitative measures such as focus groups and in-depth interviews with patients to evaluate their quality of life living with the disease. The results of this study concluded that symptoms such as pain and fatigue were the most imperative towards quality of life and daily activities. Those affected internally stated gastrointestinal symptoms caused the most problems in their daily lives. Overall, patients who suffer from systemic sclerosis have stated the emotional and physical distress it causes. They have also stated the worst aspect of the disease is the overall effect on their daily lives (Almazor et al., 2007).

Section Three: Current Treatments

While the aforementioned study has shown how systemic sclerosis can affect those diagnosed with the disease, it is important to note that the disease does not affect each individual the same. For this reason, current treatments are specialized for each individual's need and aim to help slow the progression of the current symptoms an individual is facing. One of the most common forms of treatment is nonselective immunosuppressive medications; these are prescribed when an individual is first diagnosed with the disease and are used to treat specific organ manifestations as they appear (Manno & Boin, 2010).
Another common form of treatment for systemic sclerosis is B-cell-targeted immunotherapy. Rituximab, a monoclonal antibody that targets CD20 antigens, is used most commonly for B-cell-targeted immunotherapy. According to “Immunotherapy of Systemic Sclerosis” by Manno and Boin, “Selective depletion of CD20⁺ B cells is mainly achieved through complement-mediated and antibody-dependent cellular cytotoxicity as well as the induction of B-cell apoptosis (867).” Multiple studies have been conducted and shown the safety of rituximab and the improvement it has with systemic sclerosis. Studies show that rituximab has improved skin fibrosis and the prevention of worsening lung fibrosis (Distler et al., 2015).

A clinical trial that used rituximab to treat individuals with systemic sclerosis was conducted by Giuggioli et al. in 2015. This study treated ten individuals with systemic sclerosis with four weekly infusions of rituximab and evaluated after a 6-month period and an additional follow-up after another year. The results were unanimous with improvement among skin involvement and lung fibrosis (Giuggioli et al., 2015). This study and others have demonstrated the therapeutic benefits and safety rituximab has in individuals with systemic sclerosis. While this monoclonal antibody was a huge improvement in helping those with systemic sclerosis, a newer, more effective monoclonal antibody is under review. This monoclonal antibody is MEDI-551.
CHAPTER THREE
MEDI-551

Section One: Background

MEDI-551 is currently being researched to be used for B cell depletion as an effective form of therapy for systemic sclerosis. It is an anti-CD19 monoclonal antibody used to deplete B cells through the process of enhanced antibody-dependent cell cytotoxicity (Sierra-Sepúlveda et al., 2019). Enhanced antibody-dependent cell cytotoxicity is the process by which “antibodies coat a target cell and recruit effector cells to induce target cell death via non-phagocytic mechanisms (Zahavi, AlDeghaither, O’Connell, & Weiner, 2018).”

MEDI-551 is an optimal antibody to study because it targets CD19, which has a “broader and more specific pattern” of B cell expression than CD20, a common target for current therapy of systemic sclerosis (Sierra-Sepúlveda et al., 2019). CD19 is expressed constantly on naïve, transitional, germinal center, and memory B cells; another reason is that CD19 is only expressed on B cells. MEDI-551 was created from the mouse anti-human mAb HB12b, used successfully in depleting B cells in mice carrying the CD19 gene, to target CD19; HB12b was engineered to form MEDI-551 by “enhancing its drug-like properties as well as its B-cell-depleting activity (Sierra-Sepúlveda et al., 2019).” When tested in vitro, MEDI-551 had strong ADCC activity and “potently” depleted B cells that expressed CD19. It was also shown that MEDI-551 can directly deplete plasma cells, which is a new approach for immunotherapy for systemic sclerosis (Sierra-
Sepúlveda et al., 2019). The duration of depleted B cells in mice treated with MEDI-551, in comparison to rituximab, is much longer (Gallagher et al., 2016).

According to “Systemic Sclerosis Pathogenesis and Emerging Therapies, Beyond the Fibroblast” by Sierra-Sepúlveda et al.:

B cells are the producers of the autoantibodies characteristic of this disease, but we also know that these cells infiltrate tissues and show increased activation marks such as CD19, CD21, costimulatory molecules, and B cell activating factor (BAFF). There is evidence in murine models that overexpression of CD19 induces the production of cutaneous fibrosis and that the absence of B cells is associated with decreased fibrosis (Sierra-Sepúlveda et al., 2019).

For this reason, MEDI-551, an anti-CD19 monoclonal antibody, is being studied to see if it would improve systemic sclerosis.

Section Two: Clinical Trials

Studies have been conducted in mice prior to human studies to determine the safety and efficacy of MEDI-551. In 2016, Gallagher et al. conducted an experiment administering MEDI-551 to mice with the CD19 gene. This experiment concludes that depletion of B cells was dose-dependent, and after one week of treatment, the mice showed that more than 90% of B cells in the spleen, bone marrow, and blood had been depleted (Gallagher et al., 2016). According to Gallagher et al. (2016), “Both intravenous and subcutaneous administration of [MEDI-551] showed comparable efficacy, and its
pharmacokinetic profile was similar to those of other human IgG1 mAbs in mice (Gallagher et al., 2016)."

In 2016, a clinical study was conducted by Schiopu et al. to investigate the "safety and tolerability, pharmacokinetics, and pharmacodynamics" of MEDI-551 in subjects with systemic sclerosis. This clinical study was a "multicenter, randomized, double-blind, placebo-controlled, single escalating dose" study. The study consisted of twenty-eight subjects, twenty-four received a single dose of MEDI-551 and four received a placebo dose intravenously. The results concluded that a single dose of MEDI-551 was tolerable and safe, that B cell depletion was dose dependent, and a possible clinical effect was observed on affected skin (Schiopu et al., 2016).

The aforementioned clinical study in the paragraph above was conducted after a successful study was conducted in 2015 by Agius et al. However, the 2015 study was conducted to determine the safety and tolerability of MEDI-551 in subjects with multiple sclerosis instead of systemic sclerosis. The study showed "promising safety and tolerability" for MEDI-551 in subjects with multiple sclerosis, and it showed the effectiveness of MEDI-551 in depleting B cells at a rapid rate (Agius et al., 2015). For this reason, MEDI-551 has been tested in other autoimmune diseases, such as systemic sclerosis.

With any form of immunotherapy, there are side effects that need to be determined and further studied. In Agius et al. (2015) study, the common side effects of MEDI-551 delivery were fever, common cold, increased blood pressure, and oral herpess (Agius et al., 2015). MEDI-551 was shown to deplete
plasma cells, which are an important source for well-known antibodies that fight infections (Williams & Ahmed, 1999). When MEDI-551 depletes the plasma cells, it also depletes these important antibodies which can lead to serious infection because the body is unable to fight infection off without those antibodies. With these risks known, comprehensive evaluations were conducted on the mice after receiving MEDI-551. No major adverse effects were observed in the study (Gallagher et al., 2016).

The results of clinical trials proved that MEDI-551 was effective in improving systemic sclerosis. When comparing Rodnan Skin Scores from the beginning of the trial to the end of the trial, there was a significant decrease; a decrease in the skin score means an improvement in the thickening of the skin and helps decrease clinical activity. Another major improvement measured within these studies was the improvement in fibrosis of the lungs. MEDI-551 has proven to be more effective than previous forms of treatment with systemic sclerosis. It has proven so by being able to more thoroughly deplete B cells through enhancing antibody-dependent cell cytotoxicity. In order to better understand the mechanism of MEDI-551, a mini experiment was conducted in Dr. Huber's lab.
CHAPTER FOUR
Mini-Experiment

Section One: Materials and Methods

In Dr. Huber’s lab, I conducted a mini experiment with the resources he had to better understand the antibody interaction within cells. This experiment was important because it allowed me to work first hand in a lab setting. This experiment also brought a new technique into Dr. Huber’s lab that had not been previously performed, which brought value into the lab. Cetuximab (a monoclonal antibody) and A549 cells (epithelial cells) were used in the mini experiment to represent MEDI-551 and B cells respectively.

Cetuximab was of value to use because the lab had it on hand, and it has been used previously in human clinical trials. It binds to the extracellular domain of the epidermal growth factor receptor (EGFR), which prevents the EGFR from binding to the ligand. By blocking this action, it downregulates the EGFR and can induce apoptosis and inhibit metastasis (Bou-Assaly & Mukherji, 2010). Figure 4 represents a visual diagram of how cetuximab interacts with the cells. Since Cetuximab can bind EGFR in a manner similar to how MEDI-551 binds to CD19, in this experiment, the EGFR:Cetuximab interaction will mimic the expected CD19:MEDI-551 interaction.

The experiment conducted was to demonstrate antibody binding to cells. The first step was to seed 100 μL of A549 cells into a 96-well flat-bottomed plate (4x12) and incubate for twenty-four hours at 37°C. A 96-well flat-bottomed plate
was determined the best option, as it provides enough cells to test for antibody binding; it also provides one column for negative controls as well as eleven columns of different concentrations of Cetuximab. It is important to test different concentrations of Cetuximab because it can determine the optimal dose for binding to the cells. Table 1 demonstrates what concentration of Cetuximab that was added to each well, which happens as a later step in the experiment.

Once the A549 cells were incubated for twenty-four hours, the media was pipetted off and the cells were washed twice. Then the cells were fixed with a fixative (80% acetone in PBS), and the cells were incubated for twenty minutes. The fixative was then removed, and the plates were placed back in the incubator for five minutes. After incubation, several steps were performed in order to prepare the wells for the addition of the primary antibody, Cetuximab. The first step is preparing the primary antibody dilutions following the serial dilution scheme, shown in Figure 1. The plates were washed three times after following Figure 1, and then the 100 μL of the prepared primary antibody dilution was added to the wells. The primary antibody dilution was added to each well following the amounts given in Table 1. The 100 μL of blocking buffer was added to the negative control wells, and the plate was incubated at room temperature for an hour.

After incubation, the wells are prepared for the addition of the secondary antibody, Goat-α-human biotinylated IgG. This secondary antibody was chosen because it attaches to the Fc region; it was also chosen because it was available and had been tested in previous experiments in the Huber lab. The first step was
to wash the plates, then add 100 μL of the secondary antibody diluted 1:1000 in blocking buffer to each well, and 100 μL of blocking buffer was added to the negative control wells. Next the plate was incubated at room temperature for an hour.

The next step is to add a conjugated enzyme. Avidin-HRP was chosen because of its ability to bind to the secondary antibody, Goat-α-human biotinylated IgG. The secondary antibody has biotin bound to the antibody, and the Avidin part of Avidin-HRP has the affinity to bind to biotin, which is why it is chosen as the conjugated enzyme in this experiment. When they bind, a reaction occurs between the HRP and the substrate to produce a yellow color, which can be seen in Figure 2. The color of the yellow produced determines the amount of binding; the more yellow in the well, the more binding. Figure 2 demonstrates a visual diagram of what is occurring inside each individual well. The Avidin-HRP is diluted down to a ratio of 20 μL of Avidin-HRP in 5mL blocking buffer. The plate is washed six times, and then 100 μL of the diluted Avidin-HRP is added to each well. Next, 100 μL of blocking buffer is added to the negative control wells. Then the plate is incubated at room temperature for forty-five minutes.

The next step is to develop the plate, so it is ready to be read in a plate reader. After incubation, the plate needs to be washed six times, and 100 μL of the substrate is added to each well, including the negative control wells. The substrate will bind to the HRP part of Avidin-HRP enzyme and react to produce a yellow color; the goal was to have the yellow lighten in color from columns one to eleven. This goal was projected because the wells in each column, from one to
eleven, contain a more diluted concentration of Cetuximab the closer it gets to column eleven; the dilution for each well can be seen in Table 1. Column twelve should appear to be clear as those are the negative control wells, which do not contain cetuximab. Figure 2 demonstrates what is occurring in each individual well. The plate is placed into a drawer after the substrate is added to prevent light from interfering with the substrate-enzyme reaction; the plate is left in the drawer for five to ten minutes or when the negative control wells begin to show a color change, which can be seen in Figure 3.

Section Two: Results

While the plate is developing in the drawer, the plate reader is turned on and allowed time to warm up. When the plate is ready, it is put into the plate reader and read at 490nm; Figure 3 is a visual representation of the plate that was being read. The plate is put back into the drawer to allow for more reaction to occur and read again; this step is repeated a few times until the optical density (OD) readings, which can be seen in Figure 5, until the negative control well OD readings begin to increase. Figure 3 shows the yellow color appears to lighten the closer it gets to column eleven as expected. Comparing Figure 5 to Table 1, it can be concluded that the optimal dilution of cetuximab is 1:40.
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**Table 1.** This table shows the dilution of cetuximab that was added to each individual well. From left to right, the concentration of cetuximab becomes more dilute, and the last column is used as a control. Column 3, a dilution of 1:40, was determined to be the optimal dilution of cetuximab for the experiment.
Figure 1. This figure shows the Serial Dilution Scheme used for the preparation of the primary antibody, cetuximab. These dilutions were then pipetted into the wells according to Table 1.
Figure 2. This figure is a visual representation of what each individual well contains. It demonstrates how the primary antibody attaches to the cell, then the secondary antibody attaches to the primary antibody. After this occurs, the conjugate enzyme (Avidin HRP) is added in order for the substrate to bind to it. When the substrate binds with the conjugate enzyme, it produces the yellow color shown in the wells. This yellow color can be seen in Figure 3. If the cetuximab has been diluted out of the solution, then there will be no primary antibody to bind to the EGFR, and thus no secondary antibody to interact with the avidin. In these situations, there will be no color change in the well.
Figure 3. This image displays the plate before it was read in the plate reader. The plate is labeled by rows (A-D) and columns (1-12). Follow Table 1 to see the dilutions of cetuximab (primary antibody) concentration that was added to each well. When comparing Figure 3 with Table 1, column three was determined to be the optimal dilution for this experiment. The optimal dilution would be 1:40.
Figure 4. This figure represents how cetuximab interacts with the cell. Cetuximab binds to the EGFR. The binding of cetuximab to the EGFR inhibits the binding of the EGFR to the ligand, which downregulates the EGFR and induces apoptosis.
**Figure 5.** This figure displays the optical density (OD) readings at 490nm for each dilution of cetuximab. The dilution of 1:40 (column three) has shown to be the optimal dilution. **Figure 5** are the results that were read from **Figure 3** in a plate reader set to 490nm. This dilution of cetuximab will allow for a consistent positive reading to be made.
Section Three: Discussion

This experiment provided great background and methods to better understand how MEDI-551 would work; it also provided an optimal dilution prior to beginning an experiment with MEDI-551. Cetuximab was used as the model antibody to look at the interactions with the cells and provided a template to observe these interactions. This experiment has also built towards Dr. Huber’s lab for looking at influenza in a new way.

After looking at the interactions with the antibody in the cell and determining that cetuximab binds to the cells, the next step would be to set up Dr. Huber’s lab to work on the next interaction of the experiment. The next step would be to add macrophage and determine the macrophage interaction with the fixed cells and cetuximab. With this step, it is also important to determine natural killer cell interaction, which can be done with a Promega kit for evaluating antibody-dependent cellular cytotoxicity (ADCC); this kit would allow us to determine whether MEDI-551 could induce a response from the natural killer cells that are responsible for ADCC. After assessing the macrophage and natural killer cell interactions, the results can be compared to how the experiment would work using MEDI-551.

An assay would be used to determine how MEDI-551 interacts with B cells, which would further our knowledge on the immunology of systemic sclerosis. The results of the assay would help determine which mechanism would be more beneficial for the disease: B cell destruction or removal. Based upon
knowledge from the literature, the projected outcome would that complete B cell depletion would be the best mechanism to better improve systemic sclerosis.
CHAPTER FIVE

Summary

In conclusion, MEDI-551 is a newer form of treatment for systemic sclerosis and has proven to be more effective compared to the previous treatments. MEDI-551 is able to better target B cells because CD19 transmembrane proteins are expressed on a broader range of B cells. Once MEDI-551 binds to CD19 transmembrane proteins, it activates ADCC and potently depletes B cells that express CD19. By depleting B cells, it improves the skin score and fibrosis of the lungs. MEDI-551 and its effects are still under study with individuals diagnosed with systemic sclerosis, but it has already proven to be effective in improving the disease.
Works Cited


