Experimental Proposal for Identifying the Role of Erythroid-Like Transcription Factor-2 in *Caenorhabditis elegans*

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Abstract

Initially it was believed that when ELT-2 was knocked out in nematodes this caused the nematodes to become sick from all pathogens, even ones that were not previously pathogenic. However, an experiment conducted later suggested that in older cultures of bacteria the ELT-2 knocked out nematodes do not become sick from pathogens that were previously nonpathogenic. In order to clarify the role of ELT-2 in the immune system of nematodes this research proposal was developed. To prepare the nematode stocks, nematodes will be separated into two groups, ones with ELT-2 knocked out and ones with active ELT-2 transcription factors. Then, cultures of bacteria (Salmonella enterica, Pseudomonas aeruginosa, and Enterococcus faecalis) will be made with one of three varied lengths of incubation (24 hours, 48 hours, or 72). Then nematodes will be exposed to bacterial cultures nematodes in order to test their survival rate. Statistical analysis will first involve comparing the length of survival of ELT-2 knocked out nematodes to nematodes with active ELT-2 that were grown in the presence of the same bacteria. Second, analysis will involve comparing length of survival of nematodes to how long the bacteria were incubated. This experiment is designed to further understand the role of ELT-2 in the immune function of nematodes. Because of the similarity between ELT-2 in nematodes and transcription factors found in humans this experiment will indirectly help contribute to the greater knowledge of transcription factors in many organisms, including humans.

Introduction

In order to better understand scientific mechanisms further experimentation is always necessary to clarify any discrepancies between results from previous experimentation. This research paper focuses on a proposal for a future experiment designed to study a transcription factor found in *Caenorhabditis elegans* called ELT-2. Initially, it was believed that when the transcription factor was knocked out in *C. elegans* it caused the worm to get sick from multiple bacteria including bacteria that was not normally pathogenic (Kerry, 2006). However, further testing found contradicting data that in older cultures of pathogens knocking out ELT-2 did not cause a decrease in nematode survival rates (Gates, 2008).

Kerry's (2006) experiment involved knocking out ELT-2 in nematodes and exposing them to the pathogens *Pseudomonas aeruginosa, Enterococcus faecalis, and Salmonella enterica*. The results supported the idea that ELT-2 is required for an immune response to these pathogens. However, Gates (2008) more recent experimentation suggests that ELT-2 may not play a role in immunity. This later experimentation did not find that ELT-2 knocked out nematodes had decreased survival in comparison to nematodes with active ELT-2 transcription factors. This proposal was developed to pinpoint why these researchers found a difference in results.

Problem Statement

To rid this area under study of inconsistencies it is necessary to re-do the experiment with the previous results in mind and to focus only on ELT-2. Science is based on a series of proving and disproving theories and theorems. It is only through scientific trial and error that scientist are able to come as close as possible to the true mechanisms of science.

Purpose of Study, Research Questions and Hypotheses

The main purpose of this proposed experiment is to establish whether there is any validity to Gates (2008) results, which suggest that ELT-2 knocked out nematodes will not have a decreased survival rate in comparison to wild-type nematodes. It was previously believed that knocking out ELT-2 in nematodes caused the organism to be susceptible to any type of bacteria (Kerry, 2006). These results suggest that ELT-2 plays a role in the innate immune system of *C. elegans*. In light of the results from a follow-up experiment (Gates, 2008) the role of ELT-2 is still unclear. Gates (2008) experimentation suggested that ELT-2's function in the immune system of *C. elegans* was not true in older cultures of bacteria that nematodes were exposed to. This experiment was developed to address the discrepancy between the original and follow-up experiment by closely monitoring and manipulating the incubation length/age of bacteria cultures that nematodes will be exposed to.

The role of the transcription factor ELT-2 must be understand in order to reveal its importance in nematodes, and in general, science as well. In the process of trying to decipher the role of ELT-2 the purpose of this experiment is also to reveal the affect of incubation length on the pathogenicity of bacterial cultures

This experiment will address several issues relating to ELT-2 in nematodes and incubation time of bacterial cultures.

- The first research question this experiment will address is how does knocking out ELT-2 in nematodes affect the nematodes susceptibility to specific types of bacteria and other pathogens.
- The second question that will be addressed is how does the growth phase of the bacteria, older vs. fresher cultures, affect the bacteria's ability to affect the survival ability of nematodes.

- And the third question to be addressed is based on the consequences of the experiment; what role does ELT-2 seem to take in the immune system of nematodes.

Hypotheses

1. The first hypothesis for this experiment is that if ELT-2 is knocked out in nematodes the genetically altered organism will have a decreased survival rate when compared to nematodes with active ELT-2
2. The second hypothesis is that if ELT-2 is knocked out in nematodes then the nematodes will be unable to create an immune response in reaction to a pathogen.
3. The third hypothesis is that if *Salmonella enterica, Pseudomonas aeruginosa,* and *Enterococcus faecalis* are incubated for 72 hours they will have a decreased level of pathogenicity than cultures incubated for 48 or 24 hours.

Based on the suggestions of Gates (2008) the hypotheses for this experiment is that that the initial findings by Kerry (2006) are reliable representations of ELT-2 role in immune response. Considering from that fact that the initial experiment was not conducted with bacterial growth phases in mind it is possible that ELT-2 still plays a role in immunity. One main difference between Kerry (2006) and Gates (2008) experiment is how the different types of bacteria were cultured. Because of time constraints Gate (2008) made all the bacterial cultures at the same time and stored them in an incubator for later use. Kerry (2006) made cultures as needed, so the pathogen cultures were fresher than the cultures used by Gates (2008). When the bacterial cultures are fresher it is accepted as true that the bacteria's pathogenicity is stronger than when allowed to incubate and grow for a long period of time. Therefore it is hypothesized that ELT-2 indeed does play a role in the immune system by encouraging transcription of DNA that codes for immunology effector cells. When wild-type nematodes are exposed to bacteria we expect ELT-2 to help the infected nematodes produce cells that help fight off an infection and therefore increase the survival length of the nematodes.

Furthermore, it is hypothesized that because of the viable but non-culturable (VBNC) state older cultures of bacteria will show a decrease level of pathogenicity is allowed to incubate for long periods of time. This means that nematodes with and without ELT-2 knocked out will not get sick from "older" cultures of bacteria because the bacteria has gone into a sort of dormant stage referred to as the viable but non-culturable state. In addition, we expect that in fresh cultures of bacteria, nematodes with ELT-2 knocked out will have decreased survival from bacterial exposure than nematodes with active ELT-2 exposed to fresh cultures of bacteria.

Significance of Research

Even though ELT-2 is a transcription factor found only in nematodes, the mechanisms of how a transcription factor is the same in nematodes as it is in humans (Riddle, Blumenthal, Meyer, Priess, 1998). Because of this fact, the second reason

this research is significant is because the knowledge that can be gained from studying ELT-2 in *C. elegans* adds to the bigger picture of understanding transcription factors in general. Transcription factors play an important role in regulation transcription of DNA. The amount and kind of transcription factors that are active affect what pieces of DNA are transcribed, then translated, to become proteins (Mitchell and Tjian, 1989). Proteins can have 3 vital roles in the cell as enzymes, in cell signaling and structural proteins (Smith and Bender, 1997). Therefore both directly and indirectly, the three roles proteins can have in a cell also affect how an organism's immune system will respond to an infection. Also consequently, the number and type of transcription factors that are active can directly affect the immune system's ability to fight off an infection. By better understanding how transcription factors work scientist can learn more about the immune system and even learn how to manipulate them to fight infections better in both nematodes and humans as well.

In this experiment the model organism being used is a nematode from the species *C. elegans*. Using a nematode to study transcription factors allows a scientist to study basic genetic and immune mechanisms in a relative multifaceted organism without endangering the life of an actual human being. Although the nematode is extremely simple in structure it is sophisticated in other ways including adaptation to almost every type of ecological niche and having a mature form of sexual reproduction that ranges from fatal maternal birth to hermaphroditic reproduction (Wood, 1988). This sophistication increases *C. elegans* useful to science in many ways by being an ideal model organism (Patterson, 2008). Furthermore, nematodes are inexpensive to obtain and use as well as easy to breed and can be frozen until needed later. Being multicellular yet easy to work with makes nematodes an ideal candidate for immunology research.

Lastly, this experiment is important because it deals with 3 types of bacteria that humans can come into contact with on a day-to-day basis; *Pseudomonas aeruginosa, Enterococcus faecalis, and Salmonella enterica*. *Pseudomonas aeruginosa* is a common bacterium that can be found in soil, water, skin flora and many other environments. It is an opportunistic pathogen meaning that it can infect an individual whose immune system is not working at full capacity (Todar, 2008). This ability to cause disease in humans and animals will emphasize the importance of understanding the pathogenicity of *P. aeruginosa*. By knowing when *P. aeruginosa* is more likely to cause a disease people can better protect themselves from infection. *Enterococcus faecalis* is a commensal bacterium, which means that it can infect an organism without affecting the host and is found in the gastrointestinal tracts of human and other mammals (Paulsen et al., 2003). Since *E. faecalis* is a bacteria normally found in humans and other mammals people may come into contact with it is useful to know this bacterium's ability to cause any life-threatening disease. And *Salmonella enterica* is a bacterium that usually causes infection through food (Swanson et al., 2007). *S. enterica* is a bacterium that people can easily come into contact with if they are not informed about proper food handling. This experiment will reveal the pathology of this bacterium and therefore help people know about how to handle food to avoid diseases from *S. enterica*.

Summary of Theoretical Framework

The rationale for this experiment is based mainly on the findings of Kerry, TeKippe, Gaddis and Aballay (2006) and Gates (2008). Their experimental reports and references previously established the usefulness of studying innate immunity in simpler organisms like *C. elegans* (Mylonakis and Aballay, 2005). However to understand the basis for these previous experiments, as well as this one, it is necessary to understand three theories, innate immune response, viable but non-culturable (VBNC) state, and the danger theory.

Innate Immune Response

Innate immunity is the earliest phase of defense to an infection in which a variety of mechanisms respond in a way that will allow an organism to fight off a pathogen (Charles, Travers, Walport, & Shlomchik, 2001). Usually this response occurs within the first 4 hours and than after that the mechanisms are no longer considered innate immune responses and instead the body produces a response called an adaptive immune response. Nematodes do not have an adaptive immune response (Kerry, 2006) and therefore the innate immune response in *C. elegans* usually lasts longer than 4 hours since it is the main mode of defense for nematodes. The adaptive immune system is capable of distinguishing specific types of pathogens from each other while the innate immune response can only distinguish bacteria, protozoa, viruses and fungi from each other.

The innate immune response has three stages; infection by pathogen, recognition of pathogen by effector cells and than removal of the infectious agent. Infectious agents can infect a nematode 5 different ways; gut colonization, persistent infection, invasion of cuticle, biofilm formation and toxin mediated killing (Gravato-Nobre & Hodkin, 2005).

Viable But Non-Culturable State

Viable but non-culturable (VBNC) state refers to a stage in bacteria in which it fails to grow on typical media on which they would normally grow into colonies but are alive and capable of metabolic activity (Oliver, 2000). During this state a bacterium has a low metabolic rate but upon revival is again culturable. This VBNC state is the main basis for the hypothesis. It is proposed that in cultures made and incubated for longer periods of time, the bacteria will enter this state and will show a decrease level of pathogenicity. In fresh cultures the bacteria will still be in a culturable state and therefore cause ELT-2 knocked nematodes to die faster than nematodes with active ELT-2 transcription factors.

Danger Theory

The danger theory suggests that the mechanism that causes a cell to die determines whether an immune response is initiated (Todryk, Melcher, Dalgleish, and Vile, 1999). It is believed that if the mechanism that causes a cell to die is non-self (a foreign pathogen) than the body will initiate an immune response. The opposite is found to be true too; if the mechanism that causes a cell to die is self,

a biological agent normally found in the body, than the body will not initiate an immune response.

In this experiment *C. elegans* will be exposed to foreign pathogens, specifically 3 different types of bacteria. This will force the infected nematodes to initiate an immune response, which will be measured by recording the days of survival.

Assumptions, Delimitations and Scope

Assumptions

- The first assumption for this proposed experiment is that nematodes only have an innate immune response and do not possess an adaptive immune response.
- The second assumption for this proposed experiment is that the "viable but non-culturable state" theory is true for the bacteria *Pseudomonas aeruginosa, Enterococcus faecalis, and Salmonella enterica*
- The third assumption for this proposed experiment is that survival assays are an accurate measurement of nematode survivability

The first assumption for this experiment is that *C. elegans* only possess an innate immune system (Kurz & Ewbank, 2003) and not an adaptive immune system. Possessing both systems would complicate this study and make it hard to understand the nematodes reaction to being exposed to bacteria. The second assumption is that the VBNC state is true for the bacteria *Pseudomonas aeruginosa, Enterococcus faecalis, and Salmonella enterica* all of which are being used to form bacterial cultures. It is hypothesized that these bacteria will enter the VBNC state after being incubated for longer than 24 hours, which puts the bacteria in a dormant stage and therefore decreases that bacteria's ability to cause an infection. The third assumption for this experiment is that survival assays are an accurate way to test nematode survivability. The goals of establishing the role of ELT-2 and understanding the affect of incubation length on bacterial cultures can only be met if survival assays give an adequate representation of nematodes ability to survive after being exposed to bacteria.

Delimitations

This study will not cover other ELT transcription factors found in the immune system of *C. elegans*. This experiment limits its research to the transcription factor ELT-2. Furthermore this study is limited to studying nematodes from the species *C. elegans* (N2, wild-type) and does not cover the immune system of nematodes of other genetic strains.

Scope

Only one transcription factor will be studied in this experiment, ELT-2 transcription and its role in the immune system. In addition, the coverage of this study will only be on the model organism for this experiment, which will be of the species *C. elegans*. The study will consist of nematode stocks from the University of Minnesota Caenorhabditis Genetic Center and will take place over the span of 4 months in the biology laboratories of St. Mary's College of MD.

Definitions Related to Research

This section will define and explain important key terms related to this experiment.

Survival Assays

In general an assay is a process for measuring the activity of biological agent or process in an organism (Kerry, 2006). In this case the activity of interest is the survival of a nematode when faced with a bacterial or fungal pathogen. *C. elegans* survival assays will be conducted by transferring nematodes from RNAi plates onto each of the different types of bacterial plates. Every 24 hours the nematodes will be scored as alive, dead, or missing.

Nematode Survival

To establish a nematode's ability to survive it is necessary to define what constitutes as surviving and what constitutes as dying. A nematode is considered dead upon failure to respond to being touch by a pick and if the nematode responds to being touched than it is considered alive.

Nematode

Nematodes, also known as roundworms, are members of the phylum Nematoda. The ones used in this experiment are from the genus and species *Caenorhabditis elegans*. This particular species of nematode is free-living, transparent and lives in temperate soil environments (Culetto & Sattelle, 2000). Their structure system consists of an alimentary canal, which extends from the mouth to the anus, and a digestive, nervous excretory and reproductive system. They do not have a circulatory or respiratory system. Their bodies are long and narrow with their muscles lying right underneath the surface (White, Southgate, Thomson, Brenner, 1986)

RNAi Nematode

RNAi nematodes will be used to refer to the nematodes that have been genetically altered so that their ELT-2s are knocked out.

Transcription Factors

A transcription factor is a protein that binds to DNA sequences and controls the transcription rate of genetic information from DNA to mRNA (Latchman, 1997). Transcription factors can function alone or with the help of other proteins, forming a protein complex that either promotes or blocks the formation of RNA polymerase. The transcription factor of interest in this experiment is ELT-2. ELT-2 is a transcription factor for initiating and maintaining differentiation of the intestine (Maduro & Rothman, 2002). In addition, although it is solely expressed in the intestine, it also controls the endoderm development of an animal throughout a nematode's embryonic stage.

Erythroid-Like Transcription Factors

Refers to a family of factors found in C. *elegans* that are similar to a group of transcription factors found in vertebrates called GATA transcription factors (Shim, Bonner, & Blumenthal, 1995).

Knock Out

This refers to a process where a particular gene, DNA or, in this case, transcription factor, is no longer translated and therefore makes the gene or transcription factor inactive. This is done by a system called RNAi. RNAi refers to RNA Interference. It is a system within cells that helps control which genes are active and inactive. In this experiment RNAi will be used in order to knockout ELT-2. RNAi pathways are initiated by the enzyme Dicer which starts by cutting long double-stranded RNA molecules into short fragments of nucleotides. One of the two strands is incorporated into a complex called the RNA-induced silencing complex (RISC). This degradation of RNA molecules prevents the original DNA from which the RNA was transcribed from from being translated into what it is coded for.

Older Cultures

In general cultures are a process that cells or, in this case, bacteria, are grown under controlled conditions. In this experiment "older cultures" refers to cultures of bacteria that were allowed to incubate longer than 24 hours.

Fresher Cultures

"Fresh cultures" refer to bacterial cultures that were incubated for 24 hours or less.

Incubation

Incubation is a biological process for maintaining and growing a bacterial culture at a particular temperature. It is through incubation that cultured bacteria are able to grow into colonies in a controlled environment. This process is done in a machine called an incubator that allows an experimenter to control the temperature, humidity and air composition that a bacterial culture needs to grow.

Pathogen

A pathogen is a biological agent that can cause disease in an organism. In this experiment there are 3 bacterial pathogens of interest. In general a bacterium is a domain of unicellular microorganisms (NCBI database) and can be variously shaped as spheres, rods or spirals. They can survive in many environments ranging from extremely cold temperatures to high elevations. Three types of bacterium will be used in this experiment; *Salmonella enterica, Pseudomonas aeruginosa,* and *Enterococcus faecalis.*

Salmonella enterica is a rod shaped, flagellated, aerobic, Gram-negative bacterium. *Pseudomonas aeruginosa* is a rod shaped, single polar flagellum, aerobic, Gram-negative bacterium. *Enterococcus faecalis* is a spherical, non-motile, Gram-positive bacterium.

Literature Review

The following sections will analyze relevant literature to develop a relationship between the literature and the conceptual framework.

Innate Immunity in Caenorhabditis Elegans

Using *C. elegans* as a model organism has become a recently new idea to science (Adams, 2008). Before using *C. elegans* as a model organism can be truly useful to science, scientist must first develop experiments where the main goal is to understand the basic mechanisms of the immune system in roundworms. Patterson (2008) explored the inner workings of *C. elegans* by looking at its interaction with the bacterial pathogens *Burkholderia thailandensisi* and *defensin-like peptides.* His initially objective was to enhance the understanding of the human disease Meliodosis, which is caused by a pathogen closely related to *Burkholderia thailandensisi* called *Burkholderia pseudomallei*.

In Patterson's (2008) experiment *C. elegans* were exposed to *Burkholderia thailandensis* and then genes associated with the worm's immune system were measured to see which genes were being expressed when an infection occurred. In addition to measuring genes, CSαβ-type (defensin-like) peptides from the worm were compared to putative protein sequences from the human genome. Defensin-like peptides are proteins that help fight against bacteria, fungi and viruses (Yang et al., 1999) and therefore is another good indicator of an immune response. Patterson (2008) found that the defensin-like peptides shared some similarities to putative protein sequences from the human genome. Furthermore his results showed that *C. elegans* did indeed produce an immune response to *B. thailandensis.* This was shown through the up-regulation of lys-1and down-regulation of abf-2. The gene lys-1 is responsible for coding for lysozomes, which are useful for digesting pathogens (Mallo et al., 2002) and abf-2 codes for an antimicrobial peptide, which works like an antimicrobial (Kato et al., 2002).

Summary and Implications

The innate immune responses that *C. elegans* possess to a wide variety of pathogens has already been well documented and this experiment was a strong contribution to supporting this idea. Through the results of this study it is evident that even though *C. elegans* are simplistic in nature they still possess the genes and peptides to produce an effective response to an infection. Despite successful experiments like this one, scientist still do not know or understand a lot to about the genome of *C. elegans* nor the specifics of their immune system. This experiment has at least documented that *C. elegans* do indeed respond to pathogens through manipulation of its own genome. This experimentation has also shown some of the specific genes and proteins that are produced in response to a infection. Now this proposed experiment will take the next step to better understanding the role of certain genes in the immune system of *C. elegans* by looking at the transcription factor ELT-2.

Viable But Non-Culturable State Theory

Resuscitation of Enterococcal Cells

The VBNS state is a condition that was identified by Xu et al. (1982) and since then has gained a lot of attention. The viable but non-culturable state theory's significance lies in the fact that it describes the capabilities of an organism that are both potentially harmful and potential beneficial. Bacteria, both pathogenic and nonpathogenic, play a part in the day-to-day lives of humans, which drives scientist to understand the workings of bacteria as well as learn how to use them.

According to Lleo et al. (2001), and many other researchers, the VBNC state is a survival strategy used by bacteria when under environmental stress. Environmental stress can be caused by extreme temperatures, high or low elevations, abnormal air composition, moisture content, and many other unique environmental conditions. These experimenters sought to mimic what happens when fecal enterococci are released in nutrient-poor environments and than ingested by humans or animals, which allows the bacteria to revitalize. Enterococci can cause urinary tract infections, bacteremia, bacterial endocarditis and meningitis. It has a high level of antibiotic resistance and therefore understanding its pathology is important to knowing how to defend against it.

In order to study the pathology of fecal enterococci, different enterococcal species in the VBNC state were placed in a lake water microcosm, an artificial ecosystem, and were monitored for their rate of resuscitation. First enterococcal cells where induced into the VBNC state. Then these cells were stained with the Live/Dead kit to determine how many/which cells were dead or alive. For cells that were still alive but had low colony levels mRNA detection was used to determine if the cells were still viable. For microcosms whose colony reached lower than 0 or 1, resuscitation experimentations were conducted. These cells were plated and incubated and checked daily for colony formation.

The results showed that even though the enterococcal cells were in the VBNC state they still produced mRNA. Production of mRNA but lack of colonies signifies that the cells are non-culturable but still viable (Sheridan et al., 1998). In order to test if the cells in the VBNC state are capable of resuscitation they were played and incubated. They found that the cells of all three strains of enterococcal cells were actually capable of resuscitation from the VBNC state.

VBNC Aeromonas Hydrophila and Virulence in Goldfish

In 2000 Rahman, Suzuki and Kawai conducted an experiment similar to the one being proposed in this paper. These experimenters sought to study the virulence of *Aeromonas hydrophila* in goldfish. Goldfish were exposed to *A. hydrophila* cultured at varied lengths; 1-day, 28-day, and VBNC cells. Mortality of goldfish was monitored over the span of 15 days. To confirm the death of the fish from the bacteria, the bacteria from the goldfish's kidney was isolated and cultured and identified. They found that goldfish exposed to *A. hydrophila* incubated for 1 day had a significant higher mortality rate than bacteria incubated for 28 days. Furthermore they found

that goldfish exposed to *A. hydrophila* incubated for 1-day as well as 28-days both had a higher mortality than goldfish exposed to bacteria in the VBNC state.

Summary and Implications

In Lleo et al. (2001) the researchers were able to successfully induce enterococcal cells into the VBNC state as well as resuscitate them back into being culturable. Based on these findings it is now known that VBNC state occurs in *Enterococcus faecalis*. This experiment will try to replicate this state in *Salmonella enterica, Pseudomonas aeruginosa* as well as *Enterococcus faecalis*. If these states are inducible in all 3 bacteria it will then be determined if this state affects the pathogenicity of these bacteria to nematodes.

Futhermore, Rahman, Suzuki and Kawai (2000) experimentation supports the idea that bacteria entering the VBNC state can affect the pathogenicity of bacteria. It is hypothesized that the longer bacteria is incubated for the more likely it will enter the VBNC state. As it enters this VBNC state the ability of the bacteria to infect an organism will decrease. The end result will be a decrease in mortality.

Danger Theory

The danger theory refers to the way the immune system responds to an infection. It proposes that the immune system works be distinguishing between self and non-self. According to the danger theory if the cause of cellular death is from non-self (foreign pathogen) then the host will produce an immune response to fight off the cause of death. Although there has been some controversy about the danger theory being valid (Vance, 2000) it is still seen as the best explanation of the immune system.

The Danger Project is a group of researchers that seek to further explore this phenomenon and have done extensive studies to better understand the immune system. A study conducted by Aicklein and Greensmith (2007) sought to study the danger theory in a unique way by using two immune inspired algorithms. The immune inspired algorithms they looked at were the Artificial Immune System (AIS) and the Intrusion Detection Systems (IDS). These researchers sought to develop these algorithms in a way that would allow them to use them in order to detect a problem in an organism by taking concepts of the innate immune response and using it as the basis for the algorithms. The goal for their research was to discover the missing link between AID and IDS and this goal was reached by qualitatively comparing the two algorithms. The research and analysis showed that it is possible to develop a newer and better AID algorithm based on the principles of the Danger Theory.

Summary and Implications

There is some doubt about the validity of the danger theory. It is thought to be too simplistic in nature and does not explain all capacities of the immune system. However this study has shown that the danger theory is a valid foundation for

explaining the abilities of the immune system and that it is capable of explaining responses to infection that it was not even originally meant to explain.

Research Design & Methods

The following sections of Chapter 3 will concentrate on the methodology for how this experiment will be conducted. The topics that will be addressed are subjects of experiment, apparatus, design, procedures, data analysis strategies, strategies for minimizing bias and error, and ethical considerations.

Methodology

Subjects

Nematodes will be obtained from the University of Minnesota Caenorhabditis Genetic Center and maintained as published by Brenner (1973). Stock plates of N2 (wild-type) *C. elegans* will be briefly kept as dauers at 15°C on 10 centimeter SB plates (0.25% Bacto-peptone (BD Bioscience, Sparks, MD), 2.0% agar, 51.3mM NaCl, 1mM $MgSO_4$, 1mM $CaCl_2$, 25 mM KPO_4, pH 6.0, 12.9 μM cholesterol) with 200μl of OP50 *E. coli*. The worms will be grown at 25°C for all of the experiments.

Apparatus

First, an incubator will be used to encourage bacterial growth. Later, to separate nematodes from stock solution, a centrifuge will be used. GraphPad Prism software will be used to run statistical analysis at the end of the experiment.

Design

There are three independent variables in this experiment. The first independent variable is whether the nematode has been genetically altered to have ELT-2 silenced or is a wild-type nematode with active ELT-2. The second independent variable is the incubation time of the bacterial cultures with three levels, 24, 48 or 72 hours. And the third independent variable is the type of bacterial culture the nematode is exposed to. The dependent variable is the number of days the nematodes survive after being exposed to different bacterial cultures. The experimental group that contains nematodes that are wild-type serve as controls for corresponding genetically altered nematodes that were exposed to bacteria cultures incubated for the same amount of time.

Procedures

Bacterial strains. Bacterial strains will be stored as glycerol stocks in a freezer at -80°C. To make cultures each type of bacteria will be grown in Luria-Bertani (LB) broth and on agar plates, except *E. faecalis,* which will be grown in brain-heart infusion (BHI) medium. All pathogens will be grown at 37°C.

Six centimeter SK plates (0.35% Bacto-peptone (BD Bioscience, Sparks, MD), 2.0% agar, 51.3mM NaCl, 1mM $MgSO_4$, 1mM $CaCl_2$, 25 mM KPO_4, pH 6.0, 12.9 μM cholesterol) will be inoculated with 100 μL of the pathogen, which will be spread on the plate to ensure an even lawn. Then the plates will be put in a 37°C incubator. To compare older cultures to fresher cultures, plates will be incubated according to assigned incubation group; 24 hours, 48 hours, and 72 hours.

Forming transgenic nematodes. For this study, N2 (wild-type) worms will be transferred from their stock plates onto 15cm SB plates and will be incubated overnight at 25°C. Then the worms will be washed off the plates and put into a 15mL conical vial using 2mL of M9 buffer (22mM KH_2PO_4, 42.3 mM Na_2HPO_4, 85.6 mM NaCl, 1mM $MgSO_4$). After letting the worms settle to the bottom some of the supernatant will be removed. After resuspending the solution containing the nematodes, 200μL of the solution will be pipetted onto the RNAi plate (SB media containing 1mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and 50μg/mL ampicillin) containing either the blank vector control or ELT-2. These RNAi bacteria will be cultured from freezer stocks and grown in LB broth for 24 hours.

Survival assays. *C. elegans* survival assays will be conducted by transferring nematodes from RNAi plates or from original nematode stock onto each of the bacterial cultures. Every 24 hours the nematodes will be recorded as alive, dead, or missing. After recording their status the live nematodes will be put onto new bacterial cultures from the same incubation time group. *C. elegans* that do not move after being poked gently with a pick will be considered dead. *C. elegans* that respond to being poked by a pick will be scored as alive. Three independent trials will be run for each bacterial pathogens used.

Data Analysis Strategies

The data from each trial will be analyzed using GraphPad Prism® software. Statistical analysis will be performed for each trial using the Kaplan and Meier product limit method to generate survival curves (GraphPad, 2007). Then Log-rank (Mantel-Cox) tests will be performed on the combined data from each pathogen to determine if there is a difference between the survival rates of the RNAi knockdown treatments and the vector control treatments. The p-value will measure the likelihood of randomly selecting subjects whose survival curves that are different than will be actually observed. P-values below 0.05 are statistically significant or in other words that the result is not due to chance alone.

A 3-way ANOVA will also be used to compare the percentage of nematodes surviving in each experimental group to each other. Using the three previously mentioned independent variables as the three variables needed to conduct a 3-way ANOVA.

Strategies for Minimizing Bias and Error

To avoid bias and error three independent trials will be ran for each bacterial pathogen used. Furthermore to avoid counting offspring of nematodes as original nematodes the live nematodes will be transferred onto new plates for a total of four days while they are laying eggs. Another strategy that will be implemented is also an important part of the experiment itself. Instead of plating all the bacterial pathogens at the same time each trial will involve plating and incubating a specific bacterial pathogen at 24-hour intervals, going up to 72 hours. For example *Salmonella enterica* will be cultured first. After 24 hours plates that are intended for this category will then be exposed to ELT-2 knocked out nematodes or a vector control nematode. After 48 hours plates that are intended for this category will then be exposed to their corresponding treatment. This would be conducted up until 72 hours for each bacterial pathogen and each trial.

Ethical Considerations

Although nematodes are microscopic and sometimes parasitic organisms they are still animals and therefore subjected to the animal laws established by the government for using animals in research. According to animal laws one main rule to follow is to provide a humane environment. For the nematodes that are not being used currently, they will be kept in a freezer, which slows down their metabolic activity, but does not kill them. Inevitably all nematodes used in this experiment will die either from exposure to a pathogen or from it's short life span (2-4 weeks). The advantage of studying the immune system *C. elegans* is seen as beneficial enough to the science of Immunobiology that it outweighs the loss of *C. elegans*'s life and therefore this experiment will not violate the laws of the Animal Welfare Act.

Expected Findings and Future Research

Expected Findings

Since this is only a research proposal there are no real findings yet. Instead there are only the expected findings, which are the same as the hypotheses. They are listed below:

1. The first hypothesis for this experiment is that if ELT-2 is knocked out in nematodes the genetically altered organism will have a decreased survival rate when compared to nematodes with active ELT-2
2. The second hypothesis is that if ELT-2 is knocked out in nematodes then the nematodes will be unable to create an immune response in reaction to a pathogen.
3. The third hypothesis is that if *Salmonella enterica, Pseudomonas aeruginosa,* and *Enterococcus faecalis* are incubated for 72 hours they will have a decreased level of pathogencity than cultures incubated for 48 or 24 hours.

Future Research

Once this experiment is conducted then further experimentation can be done to further understand the immune system of *C. elegans* as well as the pathogenicity of specific types of bacteria. One potential topic for research would be to study the pathogenicity of other types of bacteria besides the 3 that will be tested in this experiment. Knowing the changes in the pathogenicity of bacteria has implications in the medical world as well as in experimentation. Everyday humans come into contact with bacteria, some lethal and some beneficial. Knowing the capabilities of these bacteria can help people better prepare themselves against infection as well as demonstrate to doctors the medicinal use of certain bacteria.

Another potential topic for future research could be studying other transcription factors in the immune system of *C. elegans*. ELT-2 belongs to a family of other factors, specifically the Erythroid-Like Transcription Family. Although some information is known about these other transcription factors, little is known about them in the context of the immune system of *C. elegans*. A similar experiment to this one could be conducted that focuses on the role of all ELT factors in the immune system of *C. elegans*.

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