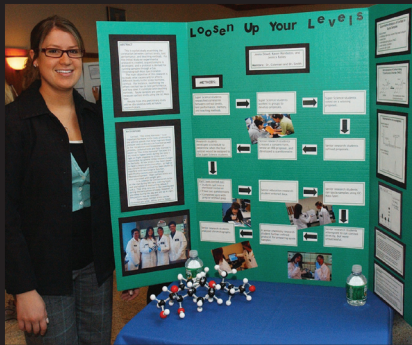
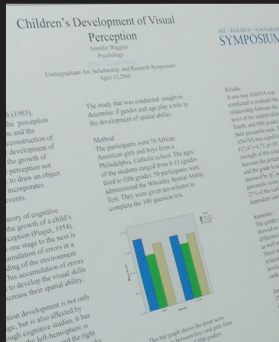
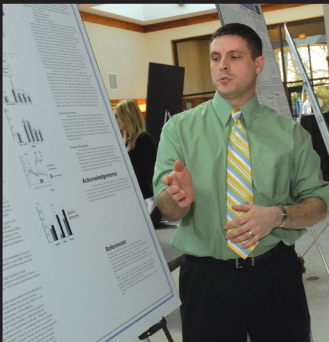


CABRINI COLLEGE

2009 JOURNAL OF UNDERGRADUATE RESEARCH

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Undergraduate Research

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Letter from the President

To the Cabrini College Community:

It is a pleasure to introduce Cabrini College's first Journal of Undergraduate Research and I extend my gratitude to the Journal, its editorial board and contributors for this opportunity to offer a letter of introduction for its first edition.

The merit of the undergraduate research experience begins in its effectiveness as a pedagogical tool: students demonstrate greater gains in learning, personal initiative and communication skills because of the opportunities for in-depth understanding presented by undergraduate research opportunities. Students who are able to contribute creative, original and intellectual concepts to a project become engaged in exciting learning environments and undergo rewarding study experiences that spark the awakening of intellectual curiosities. As they gather valuable, hands-on skills and build toward successful future careers, they develop strong, long-lasting relationships with faculty and student colleagues. Making new discoveries and producing research heightens the undergraduate experience throughout the many disciplines students study, and it invigorates their academic pursuits.

In the context of a Cabrinian education, undergraduate research melds seamlessly with the College's strategic agenda—transformational learning is at the heart of a successful and valuable undergraduate research experience. Moreover, undergraduate research is a value-added experience in that it immerses a student in a process augmenting critical thinking, leadership, and academic skills. Meaningful scholarship is a delightful consequence of this unique experience, as are academic seriousness and intellectual ambition. In short, the benefits of the undergraduate research experience are enduring and profound; it is an honor to be Cabrini's president at the time of this Journal's opening publication.

I commend the Journal of Undergraduate Research's contributing students for their dedication to outstanding scholarly achievements and for their "rigorous and distinctive efforts" in becoming public, civic-minded intellectuals. Your remarkable scholarly pursuits have resulted in your publication within the pages of the College's first Journal of Undergraduate Research. I applaud your accomplishments and wish each of you continued success in your academic efforts and future endeavors.

Sincerely,



Marie A. George, Ph.D.
President

Editorial Statement

The Cabrini College Journal of Undergraduate Research is dedicated to the discovery, promotion, and publication of outstanding work done annually by Cabrini undergraduates. The Journal's Editorial Board selects and cultivates the best work for inclusion. Drawn from the Undergraduate Arts, Research, & Scholarship Symposium—an annual event where students present and display their research to the College community—the Board seeks academically rigorous and distinctive efforts that demonstrate Cabrini students' evolution into public intellectuals with a firm grasp of the stakes and conventions of meaningful scholarship.

Articles are selected for publication based on their scholarly and rhetorical quality. They are from all disciplines, and exemplify one or more of the following accomplishments:

- an original research project
- unique contribution to the scholarship of the students field
- a new interpretation of an intellectually important problem, phenomenon, or text
- an interdisciplinary endeavor that suggests innovative approach to an altogether new subject for scholarly inquiry

The Board also considers for publication any work of artistic merit that demonstrates academic seriousness and intellectual ambition.

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Arts and Humanities

Lazarillo, Reflejo de la Sociedad Renacentista

Brittany Giampola

Preface

During her sophomore year at Cabrini College, Ms.

Brittany Giampola studied Spanish literature with me during the fall of 2007. She was captivated by the Renaissance literature of Spain, especially with the work commonly considered the precursor of the picaresque genre, the anonymous *Lazarillo de Tormes* that first appeared in Spain at the beginning of the 16th century. The figure of the *pícaro*, we learned, has its origins in the stories of beggars and scoundrels that were popular in Europe during the first part of the sixteenth century. The *pícaro* is a clever rogue who learns to survive in a harsh world. He travels from place to place, serving various masters and encountering socially diverse people. Through his experiences and his astute observations, he learns the lessons of life, motivated by the need to satisfy his hunger and the quest to survive. The picaresque novel narrates the many adventures of the *pícaro* while giving the reader an ample perspective of his society. Although the picaresque novel includes a variety of social classes, it generally focuses on the lower class and its beggars and indigent people. The *pícaro* is an antihero, a

victim of his cruel society that teaches him that he can depend on no one and that he must learn to care for himself through trickery and deception. From his first master, Lazarillo learns that he cannot trust anyone and how to take advantage of others to survive. From a cleric, he learns about the hypocrisy of the Church, and from the squire, he learns about irresponsibility. Survival is the only preoccupation of the rogue who is forced to live a life of deception where the ideals of the upper class and the Church, honor, beauty, chastity, lineage and courtesy, do not exist. *La vida de Lazarillo de Tormes y de sus fortunas y adversidades* was published anonymously in 1554. The originality of the *Lazarillo* is demonstrated by the fact that this work of fiction is written in the first person through the eyes of the young rogue beginning from childhood through adulthood. It is evident that the author was well educated as the style of the work demonstrates.

Brittany contributes to the study of Spanish literature with her ideas and viewpoint that the young Lazarillo is both a victim and a reflection of the sordid Renaissance society of Spain of the sixteenth century. Brittany presented her scholarly work at the 2008 Foreign Language and Culture Symposium at Holy Family University and at the 2008 Undergraduate Art, Scholarship, and

Research Symposium at Cabrini College.

Brittany is a current junior at Cabrini College. She is an Education and Spanish major.

Faculty Mentor – Dr. Cynthia Halpern, Professor of Spanish

Lazarillo, Reflejo de la Sociedad Renacentista

El personaje, Lazarillo de Tormes de la obra anónima del mismo nombre, es una víctima de la sociedad del Renacimiento. Por la lección principal del ciego Lazarillo aprende que no se puede depender de nadie para sobrevivir. Durante el tiempo que Lazarillo pasa con el ciego, el clérigo y el escudero, cada persona refuerza la lección principal y la realidad de la vida difícil de la sociedad renacentista. El ciego abusa al Lazarillo, enseñándole que no debe confiar en nadie, y que debe aprovecharse de otras personas para sobrevivir. Cuando Lazarillo sirve al clérigo casi muere de hambre, porque el ciego es tan ávaro que no quiere compartir su comida ni el vino con Lazarillo. El escudero demuestra un sentido fuerte del honor pero tiene un orgullo demasiado intenso. También, el escudero abandona a Lazarillo cuando Lazarillo empieza a confiar en él. Después de todos los sucesos, Lazarillo aprende la lección principal de la sociedad renacentista. Se debe cuidar de sí mismo y no se debe depender de

nadie.

El ciego es el primer amo de Lazarillo. El ciego adopta a Lazarillo como guía. Hay muchos incidentes de abuso cuando Lazarillo se queda con el ciego. El ciego solamente le da pequeñas raciones de comida a Lazarillo. Para sobrevivir, Lazarillo tiene que robar comida, dinero y vino del ciego. Cuando el ciego se da cuenta de que algunas cosas están desapareciendo, el ciego golpea a Lazarillo y le causa heridas enormes. Todas las heridas “tienen una lección” según al ciego. Cuando Lazarillo empieza a servir el ciego, el ciego decide enseñar a Lazarillo su primera lección. El ciego le dice que ponga su oreja contra una estatua de piedra de figura de un toro. Cuando Lazarillo lo hace, el ciego golpea la cabeza de Lazarillo contra la piedra fuertemente. Según el ciego, él dice “Necio, aprende, que el mozo del ciego un punto ha de saber más que el diablo” (Mujica, 111). Entonces, Lazarillo comienza a pensar “Verdad dice éste, que me cumple avivan el ojo y avisar, pues solo soy, y pensar cómo me sepa valer” (Mujica, 111). Lazarillo ya demuestra los pensamientos de la sociedad del Renacimiento. Todo el abuso que Lazarillo tiene que aguantar del ciego refuerza la lección principal de la sociedad y ayuda a transformarlo más en una persona de la sociedad renacentista.

Como el ciego solamente le da pequeñas raciones y manda que Lazarillo sobreviva de su propia cuenta, Lazarillo siente mucho resentimiento hacia el ciego. El resentimiento al ciego fuerza a Lazarillo ser más independiente y aprovecharse del ciego también. Lazarillo se aprovecha del ciego y cuando la gente le da monedas al ciego para rezar por ellos, Lazarillo cambia las monedas por otras de menos valor. Esta habilidad de aprovecharse y engañar a la gente refuerza la lección principal de la sociedad del Renacimiento. Al final de su época con el ciego, Lazarillo, ya astuto en la habilidad de engañar a los otros, guía al ciego contra un poste. Cuando el pobre ciego estaba en el suelo medio muerto del golpe contra el poste, Lazarillo lo deja contento de haberse tomado la venganza. Su próximo amo, el clérigo, repite la lección renacentista tanto como el ciego.

El clérigo es una figura de la Iglesia. Si alguien va a ayudar al pobre Lazarillo, éste sería un clérigo; pero, desafortunadamente para Lazarillo, el clérigo es mucho peor que el ciego. El clérigo no tiene ningún interés en el bienestar de Lazarillo y no le da de comer. Lazarillo tiene que robar pan para sobrevivir mientras el clérigo tiene una vida buena con bastante comida. La vida de Lazarillo con el clérigo le es difícil por el hambre y su experiencia

con el clérigo refuerza la lección principal de la sociedad renacentista. El clérigo, también, como el ciego, reza por otros y es hipócrita. El clérigo trabaja para la Iglesia pero le es difícil mostrar la caridad a Lazarillo. La caridad debe ser una característica fácil de encontrar en un hombre de Dios, pero el clérigo es más un hombre de la sociedad cruel. El clérigo enfatiza que uno debe comer lo que es suficiente, pero cuando el clérigo come, él come mucho y no se satisface fácilmente dejando casi nada para Lazarillo. Todo el tiempo que Lazarillo pasa con el clérigo, él aprende de la hipocresía del clérigo y entiende mejor las reglas de la sociedad para sobrevivir.

Otro amo de Lazarillo, el escudero, demuestra un honor fuerte, pero también demuestra mucho orgullo. Aunque Lazarillo es joven, él comprende que, de vez en cuando, una persona necesita dejar del orgullo para sobrevivir. Como el escudero no provee la comida irónicamente Lazarillo toma el papel de adulto para obtener comida para sí mismo y para el escudero. Lazarillo es solamente un niño, y él no debe tener la responsabilidad de obtener y proporcionar la comida. El encontrar comida era difícil, en el siglo XVI especialmente después de una ley que prohibía que los mendigos pidieran en el pueblo. Después de todo lo que Lazarillo y

el escudero tienen que aguantar juntos, el escudero abandona a Lazarillo. El escudero demuestra su falta de responsabilidad y poca conexión con Lazarillo. Por la irresponsabilidad y la falta de conexión entre el escudero y Lazarillo, el escudero refuerza la lección principal. Uno tiene que cuidar de sí mismo y no debe depender de nadie.

El ciego solamente usa a Lazarillo como guía. El abusa a Lazarillo, y le enseña a cómo aprovecharse de la gente. El ciego demuestra la primera lección de la sociedad del Renacimiento que no se debe depender de nadie al empujar la cabeza del joven Lazarillo contra una piedra porque lo obedece como su primer amo. El clérigo, como el ciego, le dan poco alimento a Lazarillo que siempre tiene hambre. El clérigo demuestra cómo ser hipócrita como el ciego. El escudero tiene un orgullo demasiado profundo y él no tiene ni quiere las responsabilidades ni para sí mismo ni para Lazarillo. El escudero abandona a Lazarillo cuando el niño quiere conectarse con él. Lazarillo es una víctima de la sociedad del Renacimiento cruel que insiste en que uno se cuide de sí mismo sin la ayuda ni la compasión de nadie.

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Philosophy of Language: The Linguistic Turn

Laura Dawidziuk

Preface

The so-called mind-body problem: the problem of the relation between the immaterial mind and the material brain (and body) is one of the great philosophical conundrums. Beginning in the 20th century, philosophers began to focus on language as the key to unlocking this mystery. During her time as a Cabrini College philosophy major, Ms. Laura Dawidziuk became more and more interested in the mind-body problem. In the Fall 2006 semester, this interest was the focus of her work in my “Contemporary Philosophy” course and in a simultaneous Independent Study in Philosophy of Language. This work culminated in the Capstone paper printed below in which Laura traces the development of the mind-body problem from its 18th century beginnings to the present-day debate between those who say that language and mind are due to brain structures we are born with and those who say that language and mind are forged through social interaction.

Laura has maintained her interest in this problem and is continuing her work at the University of Florida where she is pursuing a graduate degree in linguistics. In a recent letter, Laura said that she is also doing research in the area of second language acquisition and has begun to write

her master's thesis.

Faculty Mentor – Harvey Lape, Associate Professor of Philosophy

Philosophy of Language: The Linguistic Turn

Philosophical issues and topics range from business, ethics, morals, ontology, politics, virtues, and onward goes the list. Theories in philosophy infect and affect every aspect of life and reality, as we know it. Often times the philosopher is criticized for not having answers, which is a direct result of a skewed focus. The result of tackling philosophical problems is not always finding new answers, but uncovering new insights, which allow us to change the questions we ask. The mind-body problem is no different. Philosophers have approached this problem for centuries attempting to better understand and prove the existence of what occurs outside the body and inside the mind. In the early 20th century, philosophers began to change tactics in solving the mind-body problem. Instead of thinking in terms of thought, they approached language, hoping it would provide answers to their questions about the mind and the body. The so-called “linguistic turn” marked a new line of thought in philosophical history, but it did not answer any questions about the mind-body problem. However, it did result in a reshaping of the previously asked questions, which changed the way we understand language and cognition.

This paper intends to follow the history of philosophy through the linguistic turn, illustrating the effects linguistics has on the mind-body problem. Namely, that in turning towards linguistics for the answer, philosophers did not solve the mind-body problem, but stepped aside from it, allowing it to dissolve in its old terms, the terms of thought, and continue on new terms, the terms of language.

What our reality is changes how we act and how we ought to act within it. If, for example, we believe we create our realities, we will most certainly act differently than if we believe we are subjected to a predetermined reality ruled by other forces. We constantly negotiate our realities, choosing to believe or not believe the contents of our mind, hoping that it corresponds to what occurs outside of our minds. There are many times when we “swear” we hear things or “know” we saw things, but after a second experience, we realize we were wrong. Our senses then seem to be an unreliable source of certainty. How then are we to make sense and how can we be certain that what exists in our minds exists independently of our minds? How can we map our thoughts onto the world?

Prior to the linguistic turn, philosophers approached the mind-body problem in terms of thought. They sought to attach our thoughts to the world, hoping to prove the existence of what we sense. They asked if our

mind was blank, like Locke's empty canvas, or if we possessed innate knowledge (Locke, 1979). They also asked how much of our world is real; is only what we sense real if we sense it? What happens to it when we do not sense it? Where does our dog go when we turn our back on him to open his can of food? If the noumenal world does not exist, as Kant proposed, then what sense do we make of what occurs in the mind? Kant's solution was to dismiss anything that does not fall into one of his twelve categories.² To be an object is to adhere to one of the categories and to be perceived through an *a priori* judgment (Kant, 1998). In creating categories, Kant created rules to which reality must adhere, giving the world a sense of natural law and order. The instant problem, however, is locating the basis of all the rules, or the ultimate cause of the causal chain. We could perhaps uncover the rules for the first set of rules or the cause of the first effect, but then we would only have to look for the rules to those rules or the cause behind the cause, and so on infinitely. Kant attempted to give one last breath of life to the idea of man's pursuit for oneness, but seeking new answers to the questions surrounding the mind and body, philosophers of the early 20th century rebelled against any form of absolute idealism. They turned to language for answers, attempting to map language onto the world instead of mapping our thoughts onto the world.

Logical atomism marked the emergence of linguistics in

philosophy. The logical atomists sought to break down the complexities of ordinary language and uncover a pure form of language—the “atoms” of language—that maps onto the world in a one-to-one correspondence. The atoms (the universals and particulars of reality) could then be combined to denote relations between atoms, resulting in the molecules of language. The molecules, consisting of a combination of relations, result in facts through logical operators. Russell (2002) championed logical atomism; however, as he later admitted, it was not without faults.

The problems with logical atomism, and thus also with first order predicate logic and quantification theory, are within language. Because a statement can be broken down into atoms and rebuilt into molecules, does not gain proof of existence of independent objects, just as a true truth value in logic does not imply existence. To overcome this problem, Russell proposed that some statements denote, while others do not. For example, the phrase “average American” exists as a concept and we understand what it means, but the average American does not actually exist. Thus, logical atomism actually raised more questions concerning semantics than it answered in regards to reality.

If language does not accurately map onto the world in a one-to-one correspondence, then how does it map onto the world? Philosophers began to move from logical atomism to logical positivism. They moved the focus

from words to statements. They arrived at the idea that the sentence bears meaning, not the word, and, in doing so, they shut the door to logical atomism as a plausible theory. As expected, however, they encountered new problems. Now that statements are the bearers of meaning, from where does the meaning come? Philosophers concluded that the meaning of statements could only come from other statements, that statements are only verified by other statements, which immediately falls as infinite regress. The only way to avoid the infinite regress is to open the door to words again and attack the constructs of a statement. If words are the answer, then how do we arrive at words? Where do the words get their meaning?

After straying from logical atomism and moving to logical positivism, philosophers focused on the meaning of language and language acquisition. Spearheading the change in focus was the thought that, if language acquisition marked cognition, then uncovering how we arrive at language or how we acquire it would unlock the secrets of cognition. As the bridge between the mind and the body, a clearer understanding of cognition would result in a clearer understanding of the mind-body problem.

With language now a central focus in philosophy, theories of language began to develop and many different views emerged. Two

opposing views are those found in the latter works of Wittgenstein and those found in the early works of Chomsky. Wittgenstein took a radical approach to language, asserting that as we speak we constantly develop meaning. Chomsky, in stark opposition to Wittgenstein, posed the idea that language is hard-wired within the language faculty of the mind, taking shape through a triggering effect of stimuli as the body develops.

In the first chapter of *Rules and Representations*, Chomsky addresses the mind-body problem through the study of language.⁴ He states that language develops through a faculty of the mind. If we could locate this faculty, we could physiologically uncover the method of language acquisition and come to know how cognition is acquired. He presumes that language can be studied physiologically as a natural science. Chomsky also argues that language is too complex to simply be learned and so some of it must be innate. The innate aspects of language are contained within what he calls universal grammar, a pure form of language reminiscent of logical atomism (Chomsky, 1980).

Before Wittgenstein, philosophers generally accepted the idea that words were the bearers of meaning in language. Wittgenstein changed this view to one that is still held today, positing that it is the sentence that bears meaning in language. However, keep in mind that, at times, one word can be a sentence. Imperatives, for example, are single-word sentences

containing an understood but unstated subject and object, namely the speaker and the person spoken to, with only one verb command actually spoken and heard. More important are the corollaries of this concept.

Because the sentence bears meaning, Wittgenstein argued that language is in constant development; as we speak, we consistently toss words out into the audible world, hoping they convey what we want them to convey. When we utter words that have no relevance to the receiver of our words, we realize we misused those words, and thus we change the meaning of those words. Language exists as a public domain and cannot exist privately (Wittgenstein, 1996). Wittgenstein argued against private language theory, against the idea that one person can possess or develop his or her own language. This argument gives rise to counter arguments of “special circumstances.” Philosophers immediately point out those cases in which a man finds himself stranded on a desert island and somehow forgets all knowledge of language. He then must develop language on his own, for what reason, I have yet to determine, since living all alone on a desert island hardly creates a need for a language to use as a communication tool. However, if this man wants to make a language, he must rely solely on his own memory to keep track of what his utterances signify. Without a receiver of the communication, his utterances bear no meaning and stand no test. They are merely utterances and not language.

Language, then, cannot be private, it occurs outside the mind and between multiple communicators. In addition to negating the possibility of private language, Wittgenstein also supported his idea of public language by arguing against the idea of language as a system of rules.⁵ If the key to understanding language acquisition is to uncover and decipher a complex set of innate rules, then when we arrive at these rules, we will ask ourselves for the rules to these rules, and then the rules for those rules, in an infinite regress. Language cannot be systematized and located within a faculty of the mind. Language occurs outside the mind.⁶

Both views raise questions. If language exists as a faculty of the mind, then can we study the brain in an effort to locate this faculty and discover the connection between cognition and the body? Chomsky acknowledged that the mind has a highly differentiated structure and that it has very distinct subsystems, but this seems to go against his views, not support them. If knowledge of language is fundamentally different from the data gathered by our sensory organs, how can we study it in the same manner and expect the same results? Chomsky is quick to dismiss the indeterminacy theory of language as merely pointing out the obvious and both he and Wittgenstein show disdain for the museum theory.

The museum theory, as named by Chomsky (1980), poses that our mind holds images that map onto the world. The immediate response is to

question how blind people could learn to speak, which only demands a clarification of the meaning of the word image. A mental image, as science shows, has turned out to be not an actual picture in the mind, but a series and sequence of electrical brain impulses that result from a specific series of stimuli. Thus, the same brain wave patterns will appear in the brain of a blind person as do in a person with sight when each are asked to picture or imagine the same event, say a tennis match. Chomsky argues that the museum theory is too thin and merely glosses over language acquisition and use.

It could serve as a basis of cognition, to which humans add, creating a more complex language. As such, it would explain animal cognition; it gives plausibility to cognition without language. Animals certainly must have some sort of mental capacity that organizes their sensory world, they must have an interior mental language or images, which catalog their experiences. If a language faculty does exist, how can Chomsky be so sure that its purpose is not to attach symbols, like the words we acquire, to images? Wittgenstein argues against museum theory because it does not fit into his idea of language as public. If the mind makes any catalogues of words, they are merely short-term memory and cannot be viewed as an internal system of language. Whereas Wittgenstein dismissed museum theory entirely, Chomsky wanted more from it.

Chomsky and Wittgenstein did not agree in their views about the indeterminacy of language. Wittgenstein suggested that language is in constant development, even within our own languages—an idea that supports the theory of indeterminacy of language theory—while Chomsky quickly dismissed it as trivial, but he ought to pay closer attention to it. The indeterminacy theory points out that there is more to language than what occurs in the mind, giving way for any argument placing language outside the mind. Let us say that you and I are having a conversation. As we speak and communicate to each other, we are negotiating the meaning of our words; if I say a word you do not understand, I can only supply a meaning by locating and using words common to both of us. Then, the word I previously used gains meaning in your mind by being substituted with the string of explanation. Now, let us suppose that I use a word you do understand, but your definition is different from mine. We could continue through a lengthy discussion, positing different mental images, all the while assuming that we are in fact referring to the same image. The result is a miscommunication by fault of definition and a lack of certainty that when we use language to communicate what occurs in our minds we cause the same image to appear in the minds of our receivers. This point should not be so easily dismissed by Chomsky. If the meaning of language was hard wired, then how could such significant disparities in meaning

arise? Why is Chomsky not more concerned with what happens to language outside the mind?

Chomsky's view of an innate language assumes that discovering a physiologically readable language within the brain will result in an explanation of language (1980). Much as we will only uncover a series of chemical reactions when we study the functions of a cell or uncover a pattern of chemical composition when we study DNA, we will arrive at a series of electrical signals and a complex system of chemicals when we try to study the language of the mind. Furthermore, locating the language faculty of the mind will not provide clarity on the notion of causality, nor will it likely shed light on the concept of choice. Figuring out how we acquire words or give words meaning will not explain why we choose certain words over others or why we link them to objects and then form relations between the objects. It will not explicitly state the inner-workings of the mind or how we link and relate events, nor will it reveal how or why we, as humans, are capable of refusing some stimuli or ignoring them at will. It will not arrive at some sort of inner divinity or the key to an external divinity. In extension, it will not explain reality or anything beyond the mind; it will not solve the mind-body problem.

Language acquisition is obviously a complex process involving much more than a simple faculty of the mind. Even after auditory stimuli

begin to be categorized in our minds and speech patterns develop, a person might lack the mental capacities to convert mental “language” into audible language. If such a language never leaves the mind, its meaning can never be negotiated and, thus, never fully formed. What then of Chomsky’s language faculty? Where does the faculty begin and end, within understanding of language or within the communication of language? Wittgenstein would argue here that understanding and communication are indistinguishable and both occur through language use. Chomsky tries to say that use is a method of establishing and fine-tuning meaning, but that it is the structure of language that is innate, the universal grammar, which is already fully formed in the mind and through trigger responses develops into a specific language, such as English (1980). A correlation can be drawn here between Kant’s categories and Chomsky’s innate universal grammar. In the same way, that Kant’s categories fell into the problem of infinite regress, so too does Chomsky’s idea of a system of rules for language. Language is of the public domain, the only systematized aspect of it is that of prediction and result; we say words, predicting their meanings and expecting specific results. Depending on what those results are, we then say more words, again predicting their meanings and expecting specific results. It is almost the opposite of Chomsky’s infinite regress; it is an infinite progress. It renders rules of grammar, dictionaries,

and thesauruses as attempts to categorize and systematize the anarchy of vocabulary.

Some of language does map onto the world in a simplistic sense, fitting into the model of the earlier logical atomists and within Chomsky's universal grammar. However, not all of language can be mapped onto the world in a narrow and precise one-to-one correspondence. Some of it must be negotiated, especially when translations occur between languages or dialects, as posited in the indeterminacy of language theory. Some aspects of language are purely physiological and are learned, such as physically learning how to speak and developing speech patterns, while other aspects of language, such as the meaning of our words, are negotiated. This view provides for a middle ground theory somewhere between Wittgenstein's total negotiation of language and Chomsky's innate language. However, both theories pose ideas of cognition and contribute to the linguistic turn in philosophy, which affected various aspects of philosophy.

One significance of the linguistic turn within philosophy emerges in ontology. If Chomsky's view holds, then our innate language is a result of our reality. If something does not exist, we cannot have words for it. Conversely, only what we have words for exists, since language is hard-wired within our minds. Yet, Chomsky (1980) supports a Galilean effort to make any further advances in linguistics, positing that there must exist a

language outside the one hard-wired within our minds that, when found, would explain the language of our minds. If Wittgenstein's view holds, however, then through language, we create our realities. By speaking and negotiating the meanings of our words, we change the conceptualization of our surroundings. However, that is not to say that an independent reality does not exist, just that it undermines theory. Merely speaking of something creates its existence, as in the earlier reference to the notion of the average American. The average American certainly exists, but it is bound variably by the confines of our minds and its meaning is negotiated as we communicate what we think the average American is. In this creative sense, language can be viewed as a tool we use to navigate and negotiate our ways through the world, but it will not get us any closer to reality.

The extensions of linguistic theory within the history of philosophy changed the shape of the mind-body problem. Philosophers sought to discover the connections between language and cognition of the mind and the body in the hopes that doing so would result in a solution to the mind-body problem. Alternatively, it opened a whole line of new questions, while dissolving the old line of questions. Instead of questioning whether our thoughts match the world beyond our senses, we now are questioning if our meanings of words match the world beyond our senses and if

uncovering how we acquire them will reveal anything about the world. In the same way that we are uncertain about the thoughts of our minds proving that anything outside our thoughts exist, we must be uncertain that the language of our mind accurately corresponds to what we refer to outside our minds or that multiple communicators will have the same mental images when using the same words. Although the linguistic turn did not make gains for proving the existence of independent objects, it did help us clarify focus. The existence of purple unicorns, on Pluto or Earth, is not a real question and does not deserve our time. Instead of focusing on the existence of what occurs outside our minds, the linguistic turn caused philosophers to focus on communicating what occurs outside our minds and being sure that our perceptions match. In doing so they brought the private mind problem into the verbal public and as a result, metaphysics has somewhat dissolved into the realm of the unimportant.

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El propósito didáctico de *la Celestina* por Fernando de Rojas

Alyssa Kuhn

Preface

During her last year at Cabrini College, Ms. Alyssa Kuhn studied Spanish literature with me during the fall of 2007. She was intrigued by the complexity of the literature we read and found the Renaissance period to be of considerable interest to her. She especially enjoyed reading about the Spanish Inquisition, established in 1478 by the Catholic Kings, Ferdinand and Isabel, for the purpose of enforcing religious unity in Spain. Alyssa was also fascinated by the humanist movement in Spain that focused on the rational aspect of life and emphasized moderation and common sense. One of the first and most important humanist works in Spain is *La Celestina*, considered a precursor to both genres of the drama and the novel. The first edition appeared in 1499. The original version consisted of sixteen acts, and in 1502, a longer version of twenty-one acts appeared. The plot is quite simple. Calisto, a noble youth falls madly in love with a noble maiden upon seeing her in her orchard while pursuing his falcon. She rejects his advances and taking the advice of his servant Sempronio, he seeks the help of the old bawd, Celestina, to intervene on his behalf. She is skilled in the ways of love relying on witchcraft, concoctions of seduction, and tenacity. Through psychology and persuasiveness, she convinces Melibea that as a Christian

woman, she is the only person who can save the suffering young Calisto from despair and arranges their meeting. Despite her moral upbringing and common sense, Melibea succumbs to the wiles of Celestina and falls for Calisto and together they enjoy illicit love. At the end of the work, poetic justice reigns supreme. The servants of Calisto and Celestina together kill the old woman over money and they, too, sinners of avarice, die at the end. Calisto, also dies falling from a high ladder leading to Melibea's bed chamber, and Melibea, unable to live without him, jumps to her death from a tower leaving her father who laments the cruelty of the world and love.

Alyssa contributes to the study of Spanish literature calling *La Celestina* a piece of early Spanish didactic literature in which the author, Fernando de Rojas, a *converso*, highlights the power of poetic justice in order to condemn *el amor loco* or illicit love. Alyssa presented her scholarly paper at the 2008 Foreign Language and Culture Symposium at Holy Family University.

Alyssa graduated from Cabrini College in May 2008, and is currently applying for a teaching position. She is furthering her education with professional activities and is planning to apply to graduate school to obtain her master's degree in the field of education.

Faculty Mentor – Dr. Cynthia Halpern, Professor of Spanish

El propósito didáctico de *La Celestina* por Fernando de Rojas

Se considera *La Celestina*, una obra de Fernando de Rojas como precursora de la literatura moderna. En esta obra, el autor estudia el amor ilícito, la psicología de los personajes, sus acciones y sus intrigas. Hay una gran transformación de los personajes, de unos jóvenes inocentes a unos pecadores culpables. Rojas usa el didacticismo para demostrar a los lectores la manera apropiada de vivir por las acciones y por las palabras de los personajes de su obra. A través del uso del didacticismo y la justicia poética, Rojas desarrolla a sus personajes y la intención más importante de su obra.

El didacticismo es una gran parte de *La Celestina* Rojas usa el didacticismo para comunicar una moraleja a su audiencia. Este deseo literario de Rojas no es un secreto. Rojas incluye claramente su intención en el prefacio de su obra. Rojas escribe “la tragicomedia de Calisto y Melibea compuesta en reprehensión de los locos enamorados” (Mujica 60). El didacticismo es el propósito de primera importancia de Fernando de Rojas al escribir su novela dialogada.

Fernando de Rojas incluye el tema del amor loco en su obra porque tenía un papel importante en la última mitad del siglo XV y porque apoya su intención didáctica. En el primer acto de *La Celestina*, Calisto trata a Melibea como si fuera una diosa. Calisto exclama “yo Melibeo soy” (Mujica 62). Por su referencia a la diosa Melibea, él renuncia ser cristiano porque él

no sigue las reglas de la Iglesia al poner una mujer de carne y hueso en el lugar de Dios. Calisto rompe con uno de los mandamientos cristianos en adorar a Melibea como si fuera Dios. Con la ayuda de una alcahueta y los criados Sempronio y Pármeneo, Calisto continúa con su plan de seducir a Melibea. Por el amor loco, Calisto escoge el pecado en vez de pedirle la mano de Melibea a su padre y casarse con ella. Para alcanzar su deseos ilícitos, Calisto le pide consejos a su criado Sempronio. Sempronio avisa sobre los peligros de esta pasión y le dice a Calisto “destemplado está ese laúd” (Mujica 61) que prefigura el fallecimiento de Calisto. Sin embargo, Calisto no se rinde a los consejos de Sempronio y busca la ayuda de Celestina, una vieja alcahueta. Celestina decide ayudar a Calisto en su deseo de aprovecharse de la inocencia de Melibea porque así ella puede ganar mucho dinero. Aunque Sempronio le advierte del riesgo de sus malas intenciones, la avaricia de la alcahueta la controla.

La lección didáctica se desarrolla tanto por Melibea como por Calisto. En el comienzo, Melibea es una mujer inocente que no tiene interés en Calisto que la adora. Poco a poco, con la intervención y manipulación de Celestina, Melibea empieza a rendirse a los deseos ilícitos de Calisto.

Celestina decide tener acceso a Melibea por el aspecto religioso. Primero, Melibea rechaza sus intentos y le dice “... mi honra no dañes con tus palabras” (Mujica 77). Como Melibea es considerada una mujer honesta

y buena que quiere ayudar a los que sufren, Celestina se aprovecha de la bondad de la joven y, por la manipulación, convence a Melibea de la necesidad de ayudar al joven cristiano, Calisto. Celestina lo pinta como un hombre que sufre, diciéndole que solamente Melibea puede curarlo.

Celestina también trata de impresionar a Melibea de la buena fama de Calisto al compararlo con las figuras mitológicas. “Fuerzo y esfuerzo, no tuvo Hércules tanta” (Mujica 73). Celestina convence a Melibea de los atributos positivos de Calisto y de la enfermedad de Calisto que solamente ella puede curar. Melibea se pone de acuerdo porque es una mujer cristiana que tiene compasión y quiere ayudar a los que sufren. Es evidente que Melibea cree totalmente en las palabras de Celestina cuando ella refiere a Celestina como “aquella medianera de mi salud” (Mujica 75). Es en este tiempo, cuando Melibea se queda convencida de tenderse a su pasión por Calisto, que el amor loco entre Melibea y Calisto comienza a desarrollar.

Debido al poder de la manipulación y el amor loco, Rojas usa la justicia poética para ilustrar lo que puede pasar a las personas que no siguen las reglas de la Iglesia y que buscan la intervención de una alcahueta en asuntos de amor. Calisto y Melibea participan en un amor ilícito y luego se mueren por sus pecados. Es obvio que Calisto quiere evitar el casamiento y busca sólo la relación sexual con Melibea porque le falta la intención de casarse con ella. Melibea también acude a los deseos ilícitos de Calisto y se

olvida de sus orígenes cristianos. Melibea, tanto como Calisto participa, con ojos abiertos, en una relación condenada por la Iglesia.

Melibea se ve como una joven consentida y egoísta porque ella quiere participar en el amor ilícito con Calisto. Se arrepiente de no haber disfrutado más temprano con Calisto. Ella dice “¿Cómo no gocé más del gozo?”

(Mujica 81) Es evidente que Melibea no lamenta su decisión. Al contrario, Melibea desea tener más tiempo para gozar de su amor ilícito. Ella está tan afectada por la muerte de Calisto cuando él se cae de la escala que sube hacia la habitación de Melibea. Por esto Melibea se suicida porque el amor ilícito ha consumido su vida y no puede aguantar la pérdida de Calisto en su vida.

La depresión de Melibea se transmite a su padre Pleberio. Él no sabe ayudar a su hija y se presenta por el autor como un hombre desesperado. Melibea se arroja de la torre después de explicarle la causa de su deseo de suicidarse a su padre. Le dice que está “vencida” del amor de Calisto, y que perdió su virginidad y que quiere “seguirle, en todo.” Añade que quiere que “estén juntas las sepulturas” de ella y de Calisto para siempre. Después de la muerte de su hija, Pleberio con lágrimas grita en voz alta: “Por qué me dejaste, cuando yo te había de dejar?” (Mujica 86) En este momento, Pleberio comprende que el amor ilícito tiene la culpa de la muerte de su hija y dice que “su vida es trabajo sin provecho, dulce ponzoña, vana esperanza, falsa alegría, verdadero doler... Oh amor, amor! Que no pensé que tenías

fuerza ni poder de matar a sus sujetos!” (Mujica 85)

Por el didacticismo y la justicia poética, el suicidio, el amor ilícito y la manipulación de una alcahueta son actos en contra de la Iglesia. Rojas enseña que los que no siguen las reglas de la Iglesia pagarán las consecuencias. Debido al pecado del amor loco de Calisto y Melibea y la manipulación de la Celestina y los criados, todos mueren al final de la historia. Rojas pinta la imagen que lo malo del mundo no puede triunfar sobre lo bueno.

El amor loco, la psicología de los personajes y sus acciones e intrigas reflejan el propósito didáctico de Fernando de Rojas. Rojas fue un abogado converso de origen judío. Él era un hombre sumamente culto y con mucha influencia literaria. En *La Celestina* Rojas escogió sus palabras muy cuidadosamente porque él no quería escribir contra la Iglesia. Por la justicia poética, Roja enseña que el amor ilícito y loco no es bueno y que es un pecado que Dios va a castigar. *La Celestina* es un ejemplo temprano de la literatura didáctica de la literatura española del siglo XV.

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“Refuse to lose”: The Musical Leadership and Activist Legacy of Public Enemy

Andrew M. Madonia

Preface

Mr. Andrew Madonia explores the music and the personas of Public Enemy in the context of a tradition of philosophical reflections on the nature of freedom and justice in America. The work of Du Bois, Malcolm X, Dr. King, and others informs Andrew’s study of the dynamics of race and class in socially engaged rap—understood most powerfully as an act of political intervention and ideological challenge.

Faculty Mentor – Paul Wright, Assistant Professor of English

Introduction

The year is 1903. America, still mending its fratricidal wounds and, working to reunite, was adapting to one seemingly moral victory: the emancipation of slaves. This emancipation, despite its ostensible nationalism, and its restoring honor to the language of the U.S. Constitution, did not eradicate all forms of slavery. The whip was gone, but racism remained.

The South was rebounding and begrudgingly reforming, and the North was inflating with a surplus of migrating freed African Americans. With its cities and booming industries, the North was replete with

opportunity—if, however, you were white. In his recently published book, *The N Word*, Jabari Asim comments on the ominous climate for African Americans in the post-Civil War era. “Emancipated blacks,” he said, “could find little or no relief in the North, the South was out of the question, and the West was mostly off-limits as well. As large as the country had become...it still offered free Negroes few places to hide or to live in peace” (Asim, 2007, p. 38). The definition of slavery was reinterpreted, and it became expressed in new ways. Many ex-slaves were banned from certain public places, competed for jobs with white European immigrants, and consequently found themselves at an economic, social, and political impasse. The African American community was desperate for a leader.

A native of the state of Massachusetts, W. E. B. Du Bois experienced this racism firsthand. In *The Souls of Black Folk*, he assessed the condition of African Americans and the nihilism his collective race was conditioned to feel. “[T]he Negro,” he said, “...is born with a veil, and gifted with second sight in this American world—a world which yields him no true self-consciousness, but only lets him see himself through the revelation of the [eyes of white people]” (Du Bois, 2004, p. 7). Du Bois perceived and understood the psychological and physical expressions of racism, and knew its power of intention to indoctrinate African Americans

into a warped belief system of self-hatred and self-denigration. Du Bois, therefore, began a legacy of leadership for African Americans, and, through publication, continued a tradition of activism towards empowerment, pride, and self-love.

In 1965, in close collaboration with Alex Haley, Malcolm X published his autobiography. Though gentle in demeanor, Malcolm's words were often militant. "We can never win freedom and justice and equality," he said, "until we are doing something for ourselves!" (X, 1999, p. 225). As a leader, Malcolm emphasized and used the power of words. However, words, he knew, were not always expedient or effective. "*I am* for violence," he stressed, "if non-violence means we continue postponing a solution to the American black man's problem..." (X, 1999, p. 374). Throughout his autobiography, Malcolm eerily foreshadowed his own violent death. Sensing his imminent, even written, fate, he addressed the current and future leaders of the African American community. "The American black 'leader's' most critical problem," he said, "is lack of imagination!" (X, 1999, p. 353). Malcolm's positive contribution to the legacy of leaders of the African American community is immeasurable. Most contemporary leaders, however, are concerned more with integrating into the political landscape, leaving the ghetto, and less with their obligatory role in resolving the crises of their own people and culture. In

Race Matters Cornel West (2001) attacked these leaders.

One reason quality leadership is on the wane in black America is the gross deterioration of personal, familial, and communal relations among African Americans. These relations ...constitute a crucial basis for the development of a collective and critical consciousness ...with causes beyond that of one's self and family. (pp. 55–56)

During this time, in the urban streets of Bronx, NY, a youth culture of African Americans had formed, began to expand, and was expressing itself in graffiti, break dancing, and rapping. In addition to their creative resourcefulness, these expressions of impulse were inspired by an existential to rebelliously reform the oppressive nature of a racist society and, more broadly, of the American government. The culture became leaders known as “Hip-Hop,” and it produced a new legacy through popular art for the African American community.

This month's issue of *National Geographic* features an article entitled “Hip-Hop Planet,” written by James McBride. McBride, raised in the city, confesses his initial disdain for Hip-Hop music—music he now affectionately and respectfully calls “a universal expression of outrage.” In this article, McBride explores the essence of inspiration for, and tradition of, Hip-Hop music—better known as rapping.

“Ethnomusicologists,” he said, “trace hip-hop's roots to the dance, drum, and song of West Africa's *griots* [italics added], or storytellers, its

pairing of word and music the manifestation of the painful journey of slaves who survived the middle passage” (McBride, 2007, p. 106).

Hip-Hop music is the modern progeny of the marriage of slave spirituals with Jazz. “You can point to Jazz musicians,” said McBride, “such as Oscar Brown, Jr., Edgar ‘Eddie’ Jefferson, and Louis Armstrong...and easily find the foreshadowing of rap music in the verbal play of their work” (p. 106). The leaders produced by Hip-Hop upkeep an oral and musical tradition of social consciousness by necessity, and speak, through music, for their collective marginalized community. One leader was Public Enemy, whose “frontman,” Chuck D, presciently said: “[Hip-Hop] is the Mount Everest of battle.”

Music, by the 1980s, had become the universal tongue. With the mass production of compact discs and the upcoming television network MTV, musicians no longer had to travel for their music to be heard. A rising leader of the African American community, and while maintaining its ties with the ghetto, Public Enemy used the extensive influence of the media to transcend the ghetto and to achieve national recognition.

In her book, *Prophets of the Hood*, Imani Perry discusses the notion of “street credit” among the African American community. “The question is not ‘Are you black American?’” she writes, “but rather, ‘What kind of black American are you?’” (Perry, 2001, p. 21). Many musicians,

such as M. C. Hammer and Michael Jackson, wrote (and still write) songs solely “to go platinum.” They intentionally disconnect themselves from their community. Consequently, their ties with the street are severed, and any “street credit” and respect is lost. Public Enemy, however, knew its historical obligation to its people, and remained loyal to its deep ghetto roots. Songs like “Can’t Truss It,” “911 Is a Joke,” and “Prophets of Rage” serve as gory accounts of slavery, exposé of Capitalism, and as candid portraits of ghetto life. Public Enemy’s motive was clear: inspire change through music. Also, in 1990, the single “Fight the Power” musically brought the condition of Black America to the government’s doorstep.

“To engage in a serious discussion of race in America,” says West (2001), “we must begin not with the problems of black people but with the flaws of American society” (p. 6). Consumerism was on the rise, and with it raised prices of material necessities. America’s economy catered to the upper middle-class, outsourcing jobs and closing city factories, therefore, worsening the economic situation in poor urban communities. The crime rate soared by necessity, and white politicians harped on the supposed intrinsic criminality of African Americans. The gap between the races had widened. However, Public Enemy believed, like West, that the crises in contemporary Black America did not grow from the isolated seeds of racism; rather, they germinated from the foundations of the racism of the

government. In the opening verse of “Black Steel in the Hour of Chaos,”

Chuck D rhymes:

I got a letter from the government
 The other day
 I opened and read it
 It said they were suckers
 They wanted me for their army or whatever
 Picture me giving a damn—I said never!

Capitalism, Public Enemy felt, is racist by design, and works, with terrible success, to suppress minorities financially and even morally. The rising crime rate in urban communities began an epidemic of incarceration of African Americans. This biased lopsidedness of incarceration rates inspired “Hazy Shade of Criminal.” Chuck D confidently and angrily raps:

Some people accuse some people of crimes
 Some people get away wit’ losin’ my rhyme
 They don’t like where I’m comin’ from
 So they play dumb...
 But I’m tellin’ you what they do
 Play a fool
 While the real thief cools in a pool
 He who got the finger on the war button
 Talkin’ loud ain’t sayin’ nuttin
 TV got ‘em bigger than life
 All he needs is a knife
 Who’s the criminal?

“The knowledge accumulated in the collective memory,” states ethnographer Dan Ben-Amos, “becomes the subject of performance in society” (Ben-Amos, 1997, p. 632). Public Enemy assumed and embodied

the burden of the history of an entire race. Through contestable lyrics, Public Enemy empowered, and enabled pride in, every individual within its community. They voiced the soul-deep concerns of their race, which, for centuries, had been voiceless.

Oftentimes, a leader's impact and legacy is only fully felt years, perhaps even generations, after the campaign. This essay, therefore, is an ongoing examination of, and exploration into, the legacy of Public Enemy as a leader through Hip-Hop of the African American community. Public Enemy's deliberate reimagination of past leaders like Du Bois and Malcolm X, and how their unique leadership was applied to the context of music, has inspired many current Hip-Hop musicians like Jurassic 5, J-Live, and Jay-Z. Public Enemy adapted a legacy of African American leadership to the modern era. Moreover, today, with the 2008 Presidential election potentially featuring its first genuine and viable African American candidate, Chuck D's exhortation to "refuse to lose," in "Welcome to the Terrordome," today seems refreshingly influential.

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Social Sciences

Developing a Prediction Model for Academic Procrastination

Amanda Sizemore

Introduction

Amanda Sizemore is currently a graduate student in the Psychology program at Villanova University where heavy emphasis is given to research and quantitative methods. Amanda plans on continuing her graduate work through doctoral training. Villanova's program is considered an excellent resource for students considering future doctoral programs at prestigious institutions. While at Cabrini, Amanda distinguished herself through outstanding achievements as a dual major and demonstrated her talents in the research and quantitative realm. Amanda's research interests in procrastination culminated with presentation at The Eastern Psychological Association's regional meeting in Boston, March, 2007.

Faculty Mentor - Anthony Tomasco, Ph.D., Full Professor of Psychology

Procrastination is an all too common problem among college students, with approximately 70% struggling with the issue (Beck, Koons, & Milgram, 2000). It has been found that procrastination can lead to some health problems, lower grades and grade point averages (Beck et al., 2000), irritation, regret, despair, and self-blame (Haycock, McCarthy, & Skay, 1998). Many colleges and universities have added success seminars

to their curriculum for first-year students to assist them in their transition to college and future academic success. Techniques to manage and avoid procrastination are often discussed, as it is a behavior with which many students struggle. If procrastination and the factors and traits that lead to it are better understood, perhaps more helpful and innovative ways to prevent it could be implemented. With procrastination connected to so many negative outcomes, what might lead a majority of college students to partake in this behavior?

A likely reason is that procrastination might be used as a self-handicapping strategy, in which individuals put off doing work so that they are able to attribute their failures to the lack of available time, rather than to their own abilities. Students who have a tendency to self-handicap and procrastinate study less, delay more on exam preparation, and perform more poorly on exams. They seem aware that waiting until the last minute to prepare for an exam is a good excuse for failing (Beck et al., 2000).

A significant relationship was found between self-handicapping and self-esteem in which individuals who have a high, yet fragile, sense of self-esteem are more likely to use procrastination as a self-handicapping strategy (Beck et al., 2000). Ferarri (2001) suggested that chronic procrastinators might be unaware that they reduce their performance effort and attribute their poor performance to external factors to protect their

self-esteem. It is possible that procrastination is so widespread for college students because they are always expecting to be evaluated, making the fear of failure imminent, leading to procrastination as a self-handicapping strategy (Senecal et al., 1997). Chronic procrastinators are likely to report low self-confidence about their performance abilities and believe that they perform poorly on tasks (Ferarri, 2001).

Students rarely state reasons for procrastinating that are threatening to their self-esteem (Milgram, Marshevsky, & Sadeh, 1994). They are more likely to name nonthreatening reasons that refer to temporary, environmental circumstances. Excuses are designed to protect an individual from self-esteem threats. Multiple excuses maintain a positive self-image that might be adaptive for perceived well-being (Schouwenburg, 1992). The reasons that students give for their procrastinating behavior reflect mostly their efforts to protect their images and self-esteem (Milgram et al., 1994).

Solomon and Rothblum (1984) distinguish between two groups of procrastinators. The first group reports that procrastination is a result of the fear of failure. Students in this category tend to procrastinate because they cannot meet their own or others' expectations, or because of concerns about poor performance. The second group of procrastinators consists of students who report procrastinating because of aversiveness of the task.

Students are more likely to procrastinate on academic tasks that are unpleasant, boring, or difficult. This outcome can be easily explained because we tend to approach pleasant activities eagerly and negative activities with reluctance (Milgram et al., 1994). Aversiveness to the task, however, is rarely the only reason why students procrastinate, as they are also likely to cite other reasons such as a dislike in engaging in academic activities, lack of energy, difficulty making decisions, and problems with time management. One main difference between students who procrastinate because of aversiveness of the task and those who procrastinate because of fear of failure is that those who have a fear of failure also report high anxiety and low self-esteem (Solomon & Rothblum, 1984).

Students frequently describe their reasons for procrastinating as poor time management, which is more socially acceptable and relatively harmless when compared to reasons that imply a lack of ability (Milgram et al., 1994). Feelings associated with anxiety produce discomfort that might lead students to avoid tasks. Students who are low in self-esteem are more likely to procrastinate than students who are high in self-esteem. Students high in anxiety are also more likely to procrastinate than students low in anxiety (Owen & Newbegin, 1997).

Research also suggests that procrastination is more likely for

college students when writing term papers, studying for exams, and doing weekly readings, which indicates that students might view these tasks as most important and that these tasks have the biggest impact on their academic performance (Solomon & Rothblum, 1984). Students who describe themselves as high-trait procrastinators are significantly more likely to delay working on a boring or difficult activity when they expect to be evaluated. These students are also more likely to be slower in completing the task when they are expected to be evaluated (Senecal et al., 1997).

In their study, Owens and Newbegin (1997) found that the year level of students was related to procrastination such that, as the students aged, they were more likely to procrastinate. Solomon and Rothblum (1984) discovered a similar finding in which freshmen were found to procrastinate least, whereas seniors procrastinated more. This suggested that procrastination might be a learned behavior (Owens & Newbegin, 1997).

Many students who procrastinate might offer the explanation that they perform “well under pressure.” However, when a time limit was imposed on chronic procrastinators, they tended to reduce their effort. Chronic procrastinators were found to be less accurate in their performance when compared to nonprocrastinators (Ferarri, 2001). Ferarri

found that students with frequent procrastination tendencies might not effectively self-regulate a good combination of high performance speed and high quality to create accurate work when the cognitive demands are high. When chronic procrastinators were placed under stressful cognitive situations, they tend to perform poorly and not do well under pressure.

Chronic procrastinators might claim that their poor performance was due to external factors, such as task difficulty or luck, and not their actual effort (Ferarri, 2001). Ferarri found that, when working under pressure, chronic procrastinators might experience self-regulation failure of performance speed and accuracy. Chronic procrastinators tended to fail to regulate their performance skills effectively to get the best mix of speed and accuracy when they were under time constraints. Instead of doing well under pressure, chronic procrastinators demonstrated poor performances. Thus, Ferarri found that the popular notion of “working best under pressure” might not be so true for persons who are frequent procrastinators.

Self-efficacy pertains to a person’s judgment of how well he or she can perform certain tasks in specific situations (Haycock, McCarthy, & Skay, 1998). Haycock et al. found that a lack of self-efficacy could lead to procrastination because it contributed to behavioral avoidance. When people experienced weak efficacy in potentially threatening situations,

they also tended to experience increased anxiety. Individuals with higher anxiety were more likely to procrastinate. Procrastination might be used as an avoidance response related to anxiety or weak efficacy expectations. Haycock et al. found that the strength of efficacy was a good predictor of procrastination. Students who reported strong self-efficacy expectations tended to report less procrastination. Haycock et al. argued that strong efficacy expectations could lead to greater task initiation and persistence, and weak expectations could produce task avoidance and less persistence.

Solomon and Rothblum (1984) concluded that procrastination should be regarded as a behavioral, affective, and cognitive phenomenon. Procrastination, they found, was not simply a lack of study habits and organization of time, but it involved a complex interaction of behavioral, cognitive, and affective components.

In an attempt to understand better the behavior of procrastination, the present study examined the relationships among self-esteem, self-efficacy, internal control, task aversiveness, fear of failure, and procrastination as reported by respondents. In addition, this study sought to find how well these variables could predict procrastinatory behavior.

Method

Participants

A convenience sample of 173 undergraduate students from a small

liberal arts college participated in this study. A total of 131 females and 42 males served as respondents. Ages ranged from 17 to 31 ($M=19.60$). Fifty-six participants were first-year students, 64 were sophomores, 23 were juniors, and 22 were seniors.

Measures

Procrastination. Participants completed the Procrastination Assessment Scale (PASS), a self-report measure. The first section listed academic tasks involving writing a term paper, studying for examinations, keeping up with weekly reading assignments, performing administrative tasks, attending meetings, and performing academic tasks in general. Respondents were asked to complete three rating scales for each of the six tasks indicating the frequency with which they procrastinate on that task, whether their procrastination on the task was a problem, and whether they wanted to decrease their procrastination on the task. The second section of the PASS asked participants to think of the last time they procrastinated on writing a term paper and to indicate how much each of 26 reasons reflected why they procrastinated and were scored on levels of task aversion or laziness, and fear of failure.

Self-Esteem. The Rosenberg Self-Esteem scale was used to assess self-esteem. This self-report measure contained 10 items and was answered on a five-point Likert scale from 0 (*Strongly Disagree*) to 4

(Strongly Agree).

Academic Self-Efficacy. Participants completed the Student Academic Self-Efficacy Questionnaire, a self-report measure, which contained 46 items and was answered on a five-point Likert scale. Respondents indicated their confidence for each of the academic areas.

Internal Control. Participants completed the Internal Control Inventory (ICI), a self-report measure, in which they reported what their normal attitude, feeling, or behavior would be for each item. Respondents answered this 28-item measure on a Likert scale from 1 (*Rarely*) to 5 (*Usually*).

Procedure

The protocol was submitted to the Institutional Review Board (IRB), and APA guidelines for conducting psychological research were followed. The survey was then distributed at the beginning of several classes on a small college campus, including Freshman Seminar, Introduction to Psychology, Social Psychology, Health Psychology, and Introduction to Philosophy. Participants were instructed to complete the survey carefully and honestly. Participation was voluntary, and the survey was constructed in a way as to ensure anonymity.

Results

A stepwise multiple regression analysis was conducted to evaluate

how well self-esteem, academic self-efficacy, task aversiveness, fear of failure, academic year, and internal control predicted the total amount of procrastination reported. The data were examined for violations of homoscedasticity, linearity, and normality. Evidence for these violations was not apparent upon inspection of the scatterplot. Task aversiveness and academic self-efficacy were retained in the model as significant predictors, $F(2, 154) = 21.52, p < .01$ with a multiple correlation coefficient of .47 indicating that approximately 22% of the variance in Procrastination can be accounted for by the linear combination of task aversiveness and academic self-efficacy. Correlation coefficients, and Means and Standard Deviations are reported in Table 1 (See Appendix A). The retained regression model was as follows:

$$\text{Procrastination} = .215 \text{ task aversiveness} - .044 \text{ academic self-efficacy} + 18.336.$$

Discussion

In an attempt to investigate the relationship between self-reported self-esteem, academic self-efficacy, internal control, fear of failure, and task aversiveness and procrastination, a prediction model was for procrastination was created, with task aversiveness and academic self-efficacy as the best predictors, which is similar to findings in previous research. Self-esteem, however, did not correlate significantly with procrastination as prior research has suggested. The homogeneous nature

of the sample that was measured might have led to this finding due to little variance in self-esteem scores.

Students who reported higher scores in the variable of academic self-efficacy were less likely to procrastinate, suggesting that students with confidence in their ability to do their schoolwork are less likely to procrastinate, whereas students lower in academic self-efficacy tend to procrastinate more often because of their lack of confidence in their work. If students are confident in their abilities, they may be more motivated to initiate their assignments compared to those low in academic self-efficacy. A relationship might exist between low, academic self-efficacy and task aversiveness in which those with little confidence in their abilities to complete an assignment will have an aversion to it leading them to procrastinate and put off the assignment for as long as possible.

The sample used in this study might not be representative of the population, as a majority of the respondents was first-year students. A larger sample size might also be needed because of the amount of variables being tested. Further research should be done to account for more of the variance found in procrastination. Procrastination is a complex behavior, and other factors might be associated with it that were not examined in this study. Exploration into why self-esteem and fear of failure were not significantly related to procrastination should also be

considered.

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Appendix A

Table 1

The Bivariate Correlations of the Predictors with Procrastination with Means and Standard Deviations

	1	2	3	4	5	6	7
1. Procrastination	—						
2. Self-Esteem	-.06	—					
3. Academic Self-Efficacy	-.34**	.33**	—				
4. Fear of Failure	.03	-.29**	-.18*	—			
5. Task Aversiveness	.37**	-.01	-.12	.27**	—		
6. Internal Control	-.33**	.41**	.51**	-.27**	-.32**	—	
7. Academic Year	.01	.07	.04	-.04	.02	.20	—
<i>M</i>	18.19	29.10	160.76	30.35	32.06	98.19	2.05
<i>SD</i>	3.98	6.80	26.85	8.61	6.16	10.71	.99

Note. *indicates significance, $p < .05$; ** indicates significance, $p < .01$

Appendix B

This survey was compiled for Honors Research Practicum in Psychology. Your participation is voluntary and the completion of this survey indicates your willingness to participate. This protocol was constructed as to ensure all of your answers are anonymous. Please be sure to read each question carefully, and answer honestly. Your participation is greatly appreciated.

Gender: _____ Age: _____

Year: First-Year Sophomore Junior Senior

For each of the following activities, please rate the degree to which you delay or procrastinate. Rate each item on an A to E scale according to how often you wait until the last minute to do the activity. Then, indicate on an A to E scale the degree to which you feel procrastination on that task is a problem. Finally, indicate on an A to E scale the degree to which you would like to decrease your tendency to procrastinate on each task. Mark your answers by circling the appropriate letter below each question.

I. Writing a Term Paper

1. To what degree do you procrastinate on this task?

Never procrastinate A	Almost never B	Sometimes C	Nearly always D	Always procrastinate E
-----------------------------	----------------------	--------------------	-----------------------	------------------------------

2. To what degree is procrastination on this task a problem for you?

Not at all a problem A	Almost never B	Sometimes C	Nearly always D	Always a problem E
------------------------------	----------------------	--------------------	-----------------------	--------------------------

3. To what extent do you want to decrease your tendency to procrastinate on this task?

Do not want to decrease A	Almost never B	Somewhat C	Nearly always D	Definitely want to decrease E
---------------------------------	----------------------	-------------------	-----------------------	-------------------------------------

II. Studying for Exams

4. To what degree do you procrastinate on this task?

Never procrastinate A	Almost never B	Sometimes C	Nearly always D	Always procrastinate E
-----------------------------	----------------------	--------------------	-----------------------	------------------------------

5. To what degree is procrastination on this task a problem for you?

- | | | | | |
|------------------------------|----------------------|----------------|-----------------------|--------------------------|
| Not at all a
problem
A | Almost
never
B | Sometimes
C | Nearly
always
D | Always a
problem
E |
|------------------------------|----------------------|----------------|-----------------------|--------------------------|

6. To what extent do you want to decrease your tendency to procrastinate on this task?

- | | | | | |
|---------------------------------|----------------------|---------------|-----------------------|-------------------------------------|
| No not want
to decrease
A | Almost
never
B | Somewhat
C | Nearly
always
D | Definitely want
to decrease
E |
|---------------------------------|----------------------|---------------|-----------------------|-------------------------------------|

III. Keeping Up Weekly Reading Assignments

7. To what degree do you procrastinate on this task?

- | | | | | |
|-----------------------------|----------------------|----------------|-----------------------|------------------------------|
| Never
procrastinate
A | Almost
never
B | Sometimes
C | Nearly
always
D | Always
procrastinate
E |
|-----------------------------|----------------------|----------------|-----------------------|------------------------------|

8. To what degree is procrastination on this task a problem for you?

- | | | | | |
|------------------------------|----------------------|----------------|-----------------------|--------------------------|
| Not at all a
problem
A | Almost
never
B | Sometimes
C | Nearly
always
D | Always a
problem
E |
|------------------------------|----------------------|----------------|-----------------------|--------------------------|

9. To what extent do you want to decrease your tendency to procrastinate on this task?

- | | | | | |
|---------------------------------|----------------------|---------------|-----------------------|-------------------------------------|
| Do not want
to decrease
A | Almost
never
B | Somewhat
C | Nearly
always
D | Definitely want
to decrease
E |
|---------------------------------|----------------------|---------------|-----------------------|-------------------------------------|

IV. Academic Administrative Tasks: Filling Out Forms, Registering for Classes, Getting ID Card, etc.

10. To what degree do you procrastinate on this task?

- | | | | | |
|-----------------------------|----------------------|----------------|-----------------------|------------------------------|
| Never
procrastinate
A | Almost
never
B | Sometimes
C | Nearly
always
D | Always
procrastinate
E |
|-----------------------------|----------------------|----------------|-----------------------|------------------------------|

11. To what degree is procrastination on this task a problem for you?

- | | | | | |
|------------------------------|----------------------|----------------|-----------------------|--------------------------|
| Not at all a
problem
A | Almost
never
B | Sometimes
C | Nearly
always
D | Always a
problem
E |
|------------------------------|----------------------|----------------|-----------------------|--------------------------|

12. To what extent do you want to decrease your tendency to procrastinate on this task?

- | | | | | |
|---------------------------------|----------------------|---------------|-----------------------|-------------------------------------|
| Do not want
to decrease
A | Almost
never
B | Somewhat
C | Nearly
always
D | Definitely want
to decrease
E |
|---------------------------------|----------------------|---------------|-----------------------|-------------------------------------|

V. Attendance Tasks: Meeting with Your Advisor, Making an Appointment with a Professor, etc.

13. To what degree do you procrastinate on this task?

Never procrastinate	Almost never	Sometimes	Nearly always	Always procrastinate
A	B	C	D	E

14. To what degree is procrastination on this task a problem for you?

Not at all a problem	Almost never	Sometimes	Nearly always	Always a problem
A	B	C	D	E

15. To what extent do you want to decrease your tendency to procrastinate on this task?

Do not want to decrease	Almost never	Somewhat	Nearly always	Definitely want to decrease
A	B	C	D	E

VI. School Activities in General

16. To what degree do you procrastinate on this task?

Never procrastinate	Almost never	Sometimes	Nearly always	Always procrastinate
A	B	C	D	E

17. To what degree is procrastination on this task a problem for you?

Not at all a problem	Almost never	Sometimes	Nearly always	Always a problem
A	B	C	D	E

18. To what extent do you want to decrease your tendency to procrastinate on this task?

Do not want to decrease	Almost never	Somewhat	Nearly always	Definitely want to decrease
A	B	C	D	E

Think of the past time the following situation occurred. It's near the end of the semester. The term paper you were assigned at the beginning of the semester is due very soon. You have not begun work on this paper. There are reasons why you have been procrastinating on this task. Rate each of the following reasons on a five-point scale according to how much it reflects why you procrastinated at the time. Mark your answers by writing the letter A to E in the space to the left of each statement.

Use the scale:

Not at all
reflects why I
procrastinated

A

B

Somewhat
reflects why I
procrastinated

C

D

Definitely
reflects why I
procrastinated

E

- ___ 19. You were concerned the professor wouldn't like your work.
- ___ 20. You had a hard time knowing what to include and what not to include in your paper.
- ___ 21. You waited until a classmate did his/hers, so that he/she could give you some advice.
- ___ 22. You had too many other things to do.
- ___ 23. There's some information you needed to ask the professor, but you felt uncomfortable approaching him/her.
- ___ 24. You were worried you would get a bad grade.
- ___ 25. You resented having to do things assigned by others.
- ___ 26. You didn't think you know enough to write the paper.
- ___ 27. You really dislike writing term papers.
- ___ 28. You felt overwhelmed by the task.
- ___ 29. You had difficulty requesting information from other people.
- ___ 30. You looked forward to the excitement of doing this task at the last minute.
- ___ 31. You couldn't choose among all the topics.
- ___ 32. You were concerned that, if you did well, your classmates would resent you.
- ___ 33. You didn't trust yourself to do a good job.
- ___ 34. You didn't have enough energy to begin the task.

- ___ 35. You felt it just takes too long to write a term paper.
- ___ 36. You liked the challenge of waiting until the deadline.
- ___ 37. You knew that your classmates hadn't started the paper either.
- ___ 38. You resented people setting deadlines for you.
- ___ 39. You were concerned you wouldn't meet your own expectations.
- ___ 40. You were concerned that, if you got a good grade, people would have higher expectations for you in the future.
- ___ 41. You waited to see if the professor would give you some more information about the paper.
- ___ 42. You set very high standards for yourself and you worried that you wouldn't be able to meet those standards.
- ___ 43. You just felt too lazy to write a term paper.
- ___ 44. Your friends were pressuring you to do other things.

Please read each statement. For each item, decide what your normal or usual attitude, feeling, or behavior would be.

- A = Rarely (less than 10% of the time)
 B = Occasionally (about 30% of the time)
 C = Sometimes (about half the time)
 D = Frequently (about 70% of the time)
 E = Usually (more than 90% of the time)

Of course there are always situations in which this would not be the case, but think of what you would do or feel in most normal situations. Write the letter that describes your usual attitude or behavior in the space provided.

45. When faced with a problem, I try to forget it.
 (Rarely) A-----B-----C-----D-----E (Usually)

- 46. I need frequent encouragement from others for me to keep working at a difficult task. (Rarely) A-----B-----C-----D-----E (Usually)
- 47. I like jobs where I can make decisions and be responsible for my own work. (Rarely) A-----B-----C-----D-----E (Usually)
- 48. I change my opinion when someone I admire disagrees with me. (Rarely) A-----B-----C-----D-----E (Usually)
- 49. If I want something, I work hard to get it. (Rarely) A-----B-----C-----D-----E (Usually)
- 50. I prefer to learn the facts about something from someone else rather than have to dig them out for myself. (Rarely) A-----B-----C-----D-----E (Usually)
- 51. I will accept jobs that require me to supervise others. (Rarely)A-----B-----C-----D-----E (Usually)
- 52. I have a hard time saying “no” when someone tries to sell me something I don’t want. (Rarely) A-----B-----C-----D-----E (Usually)
- 53. I like to have a say in any decisions made by any group I’m in. (Rarely) A-----B-----C-----D-----E (Usually)
- 54. I consider the different sides of an issue before making any decisions. (Rarely) A-----B-----C-----D-----E (Usually)
- 55. What other people think has a great influence on my behavior. (Rarely) A-----B-----C-----D-----E (Usually)
- 56. Whenever something good happens to me I feel it is because I’ve earned it. (Rarely) A-----B-----C-----D-----E (Usually)
- 57. Enjoy being in a position of leadership. (Rarely) A-----B-----C-----D-----E (Usually)

58. I need someone else to praise my work before I am satisfied with what I've done.
(Rarely) A-----B-----C-----D-----E (Usually)
59. I am sure enough of my opinions to try to influence others.
(Rarely) A-----B-----C-----D-----E (Usually)
60. When something is going to affect me, I learn as much about it as I can.
(Rarely) A-----B-----C-----D-----E (Usually)
61. I decide to do things on the spur of the moment.
(Rarely) A-----B-----C-----D-----E (Usually)
62. For me, knowing I've done something well is more important than being praised by someone else.
(Rarely) A-----B-----C-----D-----E (Usually)
63. I let other peoples' demands keep me from doing things I want to do.
(Rarely) A-----B-----C-----D-----E (Usually)
64. I stick to my opinions when someone disagrees with me.
(Rarely) A-----B-----C-----D-----E (Usually)
65. I do what I feel like doing not what other people think I ought to do.
(Rarely) A-----B-----C-----D-----E (Usually)
66. I get discouraged when doing something that takes a long time to achieve results.
(Rarely) A-----B-----C-----D-----E (Usually)
67. When part of a group, I prefer to let other people make all the decisions.
(Rarely) A-----B-----C-----D-----E (Usually)
68. When I have a problem, I follow the advice of friends or relatives.
(Rarely) A-----B-----C-----D-----E (Usually)
69. I enjoy trying to do difficult tasks more than I enjoy trying to do easy tasks.
(Rarely) A-----B-----C-----D-----E (Usually)

70. I prefer situations where I can depend on someone else's ability rather than just my own.

(Rarely) A-----B-----C-----D-----E (Usually)

71. Having someone important tell me I did a good job is more important to me than feeling I've done a good job.

(Rarely) A-----B-----C-----D-----E (Usually)

72. When I'm involved in something, I try to find out all I can about what is going on, even when someone else is in charge.

(Rarely) A-----B-----C-----D-----E (Usually)

Below is a list of statements dealing with your general feelings about yourself. If you strongly disagree, circle A. If you disagree with the statement, circle B. If you neither agree nor disagree, circle C. If you agree, circle D. If you strongly agree, circle E.

Please use the following scale:

Strongly disagree	Disagree	Neither agree nor disagree	Agree	Strongly agree
A	B	C	D	E

73. On the whole, I am satisfied with myself.

(Disagree) A-----B-----C-----D-----E (Agree)

74. At times, I think I am no good at all.

(Disagree) A-----B-----C-----D-----E (Agree)

75. I feel that I have a number of good qualities.

(Disagree) A-----B-----C-----D-----E (Agree)

76. I am able to do things as well as most other people.

(Disagree) A-----B-----C-----D-----E (Agree)

77. I feel I do not have much to be proud of.

(Disagree) A-----B-----C-----D-----E (Agree)

78. I certainly feel useless at times.

(Disagree)A-----B-----C-----D-----E (Agree)

79. I feel that I'm a person of worth, at least on an equal plane with others.
(Disagree) A-----B-----C-----D-----E (Agree)

80. I wish I could have more respect for myself.
(Disagree) A-----B-----C-----D-----E (Agree)

81. All in all, I am inclined to feel that I am a failure.
(Disagree) A-----B-----C-----D-----E (Agree)

82. I take a positive attitude toward myself.
(Disagree) A-----B-----C-----D-----E (Agree)

How much confidence do you have about doing each of the behaviors listed below? Circle the letter that best represent your confidence.

Very little		Quite a lot
A	B C D	E

How much confidence do you have when...

83. Taking well-organized notes during a lecture?
(Little) A-----B-----C-----D-----E (A Lot)

84. Participating in a class discussion?
(Little) A-----B-----C-----D-----E (A Lot)

85. Answering a question in a large class?
(Little) A-----B-----C-----D-----E (A Lot)

86. Answering a question in a small class?
(Little) A-----B-----C-----D-----E (A Lot)

87. Taking "objective" tests (multiple-choice, T-F, matching)?
(Little) A-----B-----C-----D-----E (A Lot)

88. Taking essay tests? (Little) A-----B-----C-----D-----E (A Lot)

89. Writing a high quality term paper?
(Little) A-----B-----C-----D-----E (A Lot)

- 90. Listening carefully during a lecture on a difficult topic?
(Little) A-----B-----C-----D-----E (A Lot)
- 91. Tutoring another student? (Little) A-----B-----C-----D-----E (A Lot)
- 92. Explaining a concept to another student?
(Little) A-----B-----C-----D-----E (A Lot)
- 93. Asking a professor in class to review a concept you don't understand?
(Little) A-----B-----C-----D-----E (A Lot)
- 94. Earning good marks in most courses?
(Little) A-----B-----C-----D-----E (A Lot)
- 95. Studying enough to understand content thoroughly?
(Little) A-----B-----C-----D-----E(A Lot)
- 96. Making professors respect you?
(Little) A-----B-----C-----D-----E (A Lot)
- 97. Attending class regularly?(Little) A-----B-----C-----D-----E (A Lot)
- 98. Attending class consistently in a dull course?
(Little) A-----B-----C-----D-----E (A Lot)
- 99. Making a professor think you're paying attention in class?
(Little) A-----B-----C-----D-----E (A Lot)
- 100. Motivating yourself to do schoolwork?
(Little) A-----B-----C-----D-----E (A Lot)
- 101. Understanding most ideas you read in your texts?
(Little) A-----B-----C-----D-----E (A Lot)
- 102. Understanding most ideas presented in class?
(Little) A-----B-----C-----D-----E (A Lot)
- 103. Using a computer? (Little) A-----B-----C-----D-----E (A Lot)
- 104. Mastering new concepts in Reading and Writing?
(Little) A-----B-----C-----D-----E (A Lot)

105. Mastering new concepts in Calculus?
(Little) A-----B-----C-----D-----E (A Lot)
106. Mastering new concepts in Statistics or Finite Mathematics?
(Little) A-----B-----C-----D-----E (A Lot)
107. Mastering new concepts in World History?
(Little) A-----B-----C-----D-----E (A Lot)
108. Mastering new concepts in Biology, Physics, Chemistry, or Geology?
(Little) A-----B-----C-----D-----E (A Lot)
109. Mastering new concepts in American History?
(Little) A-----B-----C-----D-----E (A Lot)
110. Mastering new concepts in Psychology, Sociology, or Political Sciences?
(Little) A-----B-----C-----D-----E (A Lot)
111. Mastering new concepts in Foreign Languages?
(Little) A-----B-----C-----D-----E (A Lot)
112. Mastering new concepts in History, Philosophy, or English Literature?
(Little) A-----B-----C-----D-----E (A Lot)
113. Mastering new concepts in Music, Theatre, or Art?
(Little) A-----B-----C-----D-----E (A Lot)
114. Talking to a professor privately to get to know him or her?
(Little) A-----B-----C-----D-----E (A Lot)
115. Relating course content to material in other courses?
(Little) A-----B-----C-----D-----E (A Lot)
116. Challenging a professor's opinion in class?
(Little) A-----B-----C-----D-----E (A Lot)
117. Applying lecture content to a laboratory session?
(Little) A-----B-----C-----D-----E (A Lot)

- 118. Making good use of the library?
(Little) A-----B-----C-----D-----E (A Lot)
- 119. Getting good grades? (Little) A-----B-----C-----D-----E (A Lot)
- 120. Spreading out studying instead of cramming?
(Little) A-----B-----C-----D-----E (A Lot)
- 121. Understanding difficult passages in textbooks?
(Little) A-----B-----C-----D-----E (A Lot)
- 122. Mastering content in a course you're not interested in?
(Little) A-----B-----C-----D-----E (A Lot)
- 123. Playing an active role in group projects?
(Little) A-----B-----C-----D-----E (A Lot)
- 124. Finishing homework assignments by their deadlines?
(Little) A-----B-----C-----D-----E (A Lot)
- 125. Studying when there are other interesting things to do?
(Little) A-----B-----C-----D-----E (A Lot)
- 126. Completing your degree in a timely fashion?
(Little) A-----B-----C-----D-----E (A Lot)
- 127. Challenging another student's opinion in class?
(Little) A-----B-----C-----D-----E (A Lot)
- 128. Making presentations in class?
(Little) A-----B-----C-----D-----E (A Lot)

The Effect of Parental Involvement on an Adolescents' Self-Efficacy Due to the Parents' Involvement in Their Children's Athletic Participation

Kimberly Feeny

Preface

Ms. Kimberly Feeny is currently a graduate student in the Communications program at Villanova University. Among the courses she will complete is a course in quantitative research in communications. I understand from Kimberly that her research experiences at Cabrini have prepared her well for graduate study. As part of her undergraduate experiences, Kimberly had to complete a research study from start to finish—defining a problem of interest, creatively designing a research strategy, collecting relevant data, interpreting the data, and finally presenting her paper. Kim presented her research at the Eastern Psychological Association meeting last spring.

Faculty Mentor – Tony Tomasco, Full Professor of Psychology

Abstract

This research studied the effects of parent involvement on adolescent's self-efficacy due to their involvement in their child's athletic participation. Athlete's self-efficacy was measured using the Ralf Schwarzer and Matthias Jerusalem (1993, rev. 2000) 10 item self-efficacy test and was then followed by a questionnaire that measured the athlete's perception of their parents' involvement in their sport. A convenience sample of 30 female athletes, ages 12–18, from a competitive, traveling volleyball team based in Philadelphia were used as

participants. The research looked to confirm a hypothesis stating that, if the adolescent athletes perceive their parents' involvement level as overinvolved or underinvolved, their self-efficacy would be lower. The research also looked to confirming a second hypothesis stating that, if the adolescent athletes perceive their parents' involvement as appropriate, their self-efficacy would be high.

Introduction

This study examined the effect of parental involvement on adolescents' self-efficacy due to the adolescents' perspective of their parents' involvement in their athletic participation. Adolescents' self-efficacy is affected by the interaction with peers and family and by their involvement in activities, including school, social activities, and extracurricular activities, such as sports. Sport involvement among adolescents is a very common activity and one that provides a social environment along with competitiveness, hard work, and feelings of success and failure. These aspects, among others, make sport participation a factor in an adolescent's development of self-efficacy. Parents' involvement in their child's sports participation is crucial to this development as well. Parents might serve as motivators and supporters or they might place unwanted stress on their child through their involvement. This research hypothesis states that adolescent athletes' perception of their parents' involvement in their sports participation will negatively affect their self-efficacy if the involvement is seen as too

much or too little. The research in a second hypothesis states that the self-efficacy of adolescent athletes will be positively affected if they perceive that their parents' involvement is appropriate.

According to Kent and Todd (2003), adolescence is a time of many changes and transitions that usually occurs between the ages of 10 and 19 years. During adolescence, the individual's view of the self becomes more abstract and differentiated, resulting in a more complex identity formation. A major influence on the formation of identity is the desire to feel a part of a group. Therefore, family relationships, close friendships, and social acceptance are all factors that play a vital part in the adolescent's formation of identity, or construction of the self. Many children participate in sports during their adolescent years. This provides peer interaction and allows children to explore their identities through their peers and by themselves. Parental influence as expressed by fan presence, expectations, and involvement are also prominent variables in the relationship between sports participation and adolescent identity formation.

Sports participation by adolescents in the United States has grown to such a degree that it is a significant factor in the psychological development of a growing child (Bergin & Habusta, 2004). Researchers

and practitioners recognize that parents have a strong influence on children's sport experiences (Jambor, 1999). A primary way that parents influence a young athlete's sport experience is through involvement in their son's or daughter's sports participation. Parental involvement begins with socializing a child to play sports and continues by helping young athletes maintain their sport participation. Parental involvement entails the time, the energy, and the money that parents invest in their child's sport participation and includes things such as providing transportation, attending practices and games, providing instructional assistance, and purchasing equipment (Stein & Raedeke, 1999). According to Kidman, McKenzie, A., and McKenzie B. (1999), parents play an important role in determining their children's sport participation. "Parental support can have a positive influence on the experiences of children in sport, yet there are numerous reports of the negative influence that parents can exert on their children's sporting experiences as well" (Kidman et al., 1999, p. __). Through their involvement, parents can help create enjoyment by providing support and encouragement, or they can be a source of stress and anxiety by placing excessive pressure on a child (Stein et al., 1999).

Not surprisingly, parental involvement ranges from mothers and fathers who are minimally involved in their children's sport participation

to parents who are highly involved. According to Wells (2004), parent spectatorship is a unique category of sports fanship. The image of parent fans is one of driven, devoted, enthusiastic, and ambitious individuals who are oddly overly critical, yet supportive at the same time. Also, depending on the nature of the sport involvement and competition, the parent's "job" of invested, committed observer involves not only spectatorship, but also financial and shared symbolic investment, all of which serve to facilitate their roles as a fans and a parents. In sum, for the parents, watching their children participate in competitive sports can provide a source of pride, enjoyment, affiliation, and loyalty.

According to Kidman et al. (1999), negative parental behaviors at children's sporting events can lead to competitive stress, inhibit sport performance, and cause children to drop out of a sport. In contrast, parental encouragement and support can significantly enhance their children's experiences in competitive sports. Regarding appropriate behavior during children's sporting events, the least desirable behaviors were those that created pressure on the athletes. The greatest pressure was created when parents placed judgment on the athlete's self-worth. Excessive pressure also occurred when athletes' parents pushed them to excel or to train harder. The most supportive behaviors were identified as

those where parents encouraged competitive participation in the sport, especially by way of congratulations following a game or race. Parents must be aware that the athletes' perceptions of the parents' actions is important, not the parents' intentions of their actions. Indeed, the parents' intentions did not necessarily match their children's perceptions of the actions portrayed (Stein, 1999).

Often, parents and other spectators unwittingly create pressure on children by being "too involved" with the children's sport. "When the youth sport environment is characterized by intrusive spectator behaviors (e.g., shouting criticisms and insults, coaching from the sideline, and distracting attention), competitive stress may be increased" (Kidman et al., 1999). Similarly, excessive spectator verbalizations can lead to reduced motor performance by bombarding the young athlete with too much information. One of the consequences of excessive spectator comments, especially from those who attempt to tell children what to do, is that learning is inhibited because the children simply follow the instructions from the sideline, rather than decide for themselves or follow coaches' instructions. Research has suggested that learning does not occur when the decision-making process is removed. If children are given the opportunities to apply their own interpretations to the information or

instruction provided initially by their coaches, then more learning and retention of information take place. Furthermore, competitive sports are an already stressful context for children because parents, peers, and coaches scrutinize and evaluate their athletic ability. The additional burden of having a pushy parent is likely to aggravate this situation because the children will be anxious not only about their ability to perform to expectations, but also about how their parents might behave during the game or event, leading to embarrassment (Kidman et al., 1999).

According to Stein (1999), clarifying how parent involvement affects a child's sport experience requires more than just examining involvement level. Involvement level is neither inherently positive nor negative, but has a qualitative aspect that is subject to a particular athlete's evaluation. For example, two athletes might perceive their parents as highly involved. While one athlete views this involvement as the proper amount, the other athlete might perceive this involvement as too high. One athlete might feel that the high involvement places excessive pressure on him or her while the other might enjoy high level of parental involvement and perceive it as a source of encouragement and support. Low involvement levels likewise could be viewed as optimal or

too little. Some athletes might enjoy their parents' low involvement levels because it gives them a sense of independence from their parents, while others might prefer their parents to be more involved, and find it stressful and unsupportive when they are not.

Sports participation of adolescents affects their self-efficacy. Not only does it teach values such as competitiveness, hard work, teamwork, and ambition; sports also allow children to interact with peers, gain acceptance into a group, and form their self-concept. Through parent involvement, the adolescents' self-efficacy is also affected. Adolescent's either perceive their parents as over or under involved, resulting in lower self-efficacy, or they perceive their parents as appropriately involved and, as a result, have high or moderate self-efficacy. Through their encouragement or discouragement as spectators, their expectations of their children, and the involvement level their children perceive, parents positively or negatively affect their children's sport participation. Thus, they affect their identity formation and self-efficacy.

Method

Participants

Participants were chosen through the Outsiders Volleyball Club, which is located in Philadelphia, PA. The club consists of all female

players, ages 12 through 18. There were 30 female athletes asked to participate in this study during regular practice time between the months of March and April of 2006.

Instrumentation

Self-Efficacy Test. For the Ralf Schwarzer and Matthias Jerusalem 10 item self-efficacy test (1993, 2000), internal consistencies are between $\alpha = .75$ and $.90$. The scale is not only parsimonious and reliable, but it also has proven convergent and discriminate validity. In terms of reliability, in samples from 23 nations, Cronbach's alphas ranged from $.76$ to $.90$, with the majority in the high $.80$ s. The scale is unidimensional. Criterion-related validity is documented in numerous correlation studies where positive coefficients were found with favorable emotions, dispositional optimism, and work satisfaction. Negative coefficients were found with depression, anxiety, stress, burnout, and health complaints. The measure has been used internationally with success for two decades. It is suitable for a broad range of applications. It can be taken to predict adaptation after life changes, but it is also suitable as an indicator of quality of life at any point in time. See Appendix A for the specific items.

Parent Involvement Questionnaire. Questions include athletes' attitudes about their own competitiveness and confidence levels, along

with their feelings towards their parents’ presence at practices and games and their overall perception of their parents’ involvement level. See Appendix B for the full questionnaire.

Procedure

Participants were instructed to take the self-efficacy test at the start of practice. Following the distribution and collection of these tests, each participant was asked to fill out the questionnaire. The questionnaire and self-efficacy tests were grouped together, the self-efficacy tests were scored, and the responses from the questionnaire were entered into SPSS along with the self-efficacy scores. The results from the self-efficacy test were then correlated with the answers from the structured interview to see if athletes’ perceptions of their parents’ involvement affected their self-efficacy.

Table 1

Descriptive Statistics of Self-Efficacy Scores

Descriptive statistics	Self-efficacy scores
<i>M</i>	30.8
Median	31
<i>SD</i>	2.89

Effects of Parent Involvement 10

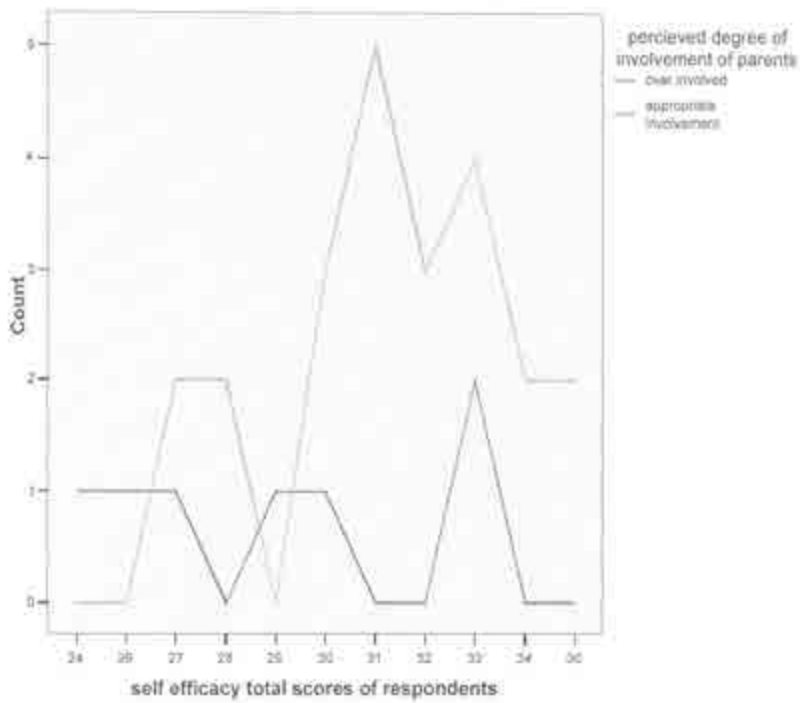


Figure 1. A clustered line graph displayed the involvement levels and self efficacy scores.

Table 2

Results From a One-Way ANOVA on the Relationship Between Self-Efficacy and Perceived Parent Involvement

	<i>df</i>	<i>F</i>	Sig.	Partial Eta Squared
Corrected	1	4.84	.04	.15
Model				
Intercept	1	2646.00	.000	.990
Involvement	1	4.84	.04	.15
Degree				

Table 3

Results From a One-Way ANOVA on the Relationship Between Perceived Parent Involvement and Perceived Embarrassment of Parents' Behavior at Sporting Events

	<i>df</i>	<i>F</i>	Sig.	Partial Eta Squared
Corrected	1	33.22	.000	.54
Model				
Intercept	1	325.25	.000	.92
Involvement	1	33.22	.000	.54
Degree				

Results

A one-way analysis of variance was conducted to evaluate the relationship between adolescents' self-efficacy and their perceptions of parental involvement in their sports participation. The independent variable, perceived degree of parent involvement, included three levels: overinvolvement, appropriate involvement, and underinvolvement. The dependent variable was adolescents' self-efficacy scores. The means and standard deviations for the self-efficacy scores are presented in Table 1 and the ANOVA scores are presented in Table 2 and 3. The results for the ANOVA were significant, $F(1, 28) = 4.84, p = .04$. The strength of the relationship between the perceived parental involvement and the self-efficacy score accounted for 15% of the variance of the dependent variable. A one-way analysis of variance was also conducted to see whether a relationship existed between perceived parental involvement and whether athletes felt embarrassed by their parents at games. The results for the ANOVA were significant, $F(1,28) = 33.2, p = .00$. The strength of the relationship between the perceived parental involvement and the perceived felt embarrassment accounted for 54% of the variance of the dependent variable.

Discussion

This research study supported the first hypothesis: If adolescent athletes perceive their parents' involvement level as overinvolved or underinvolved, their self-efficacy will be lower than those who perceive their parents' involvement level as appropriate. Athletes who stated that their parents were too involved in their sports participation scored significantly lower on the self-efficacy scale than those who said their parents' involvement level was appropriate. Overinvolvement by parents creates unwanted pressure and, as a result, might cause anxiety and a tense competitive environment. If parents are pushing their adolescent athletes too hard, it might result in a decrease in confidence levels and cause them to have feelings of inadequacy. Constant parental presence and inappropriate and embarrassing behavior at sports events also constitutes overinvolvement. Participants who reported that their parents were overinvolved also reported that their parents' behavior embarrassed them at games. The research supported this by showing a significant relationship between perceived overinvolvement and perceived embarrassment caused by parents. This indicates that a relationship exists not only between overinvolved parents and their adolescent athletes' lower self-efficacy scores, but also their adolescent athletes being embarrassed by their presence at games. Embarrassment might be caused

by excessive yelling, expressed anger when team loses, reprimands for play performance, questioning the coach, complaining, and even making negative comments about other players' performances. The embarrassment caused by this behavior might increase anxiousness in the athlete during sports participation as well as increase fear about how the parents might act.

This research study also supported the second hypothesis: athletes with high self-efficacy will perceive their parents' involvement as appropriate. Athletes who stated that their parents' level of involvement was appropriate scored significantly higher on the self-efficacy scale than those who said their parents' involvement level as overinvolved. Parents who restrain from adding additional pressure and expectations on their athletes and who do not behave inappropriately at sporting events allow their athletes more independence, while remaining supportive. The balance of interest, support, and involvement are crucial, and the line between this and added pressure and expectations is thin. The third level of involvement, underinvolvement, was not discussed because interestingly enough, no participants responded with perceived underinvolvement of their parents. This could be due to the wording of the questionnaire and the researcher might need to add a definition of

underinvolvement to the questionnaire (e.g., parents do not come to games and parents do not discuss the adolescents' sports experience with them). Participants' might also have not responded because of the sample of participants that was used in this study. The participants are members of a traveling, competitive volleyball club that is expensive and requires a sustained time commitment. Because of this expense and time commitment, parents are naturally more involved, for they must commit more financially and provide transportation because the practice and tournament locations are not local. In future research, it would be beneficial to expand the sample to other types of sports teams and to revise the questionnaire to see whether the number of participants who perceive underinvolvement increases. If no increase is found, the researcher should consider discarding this level of involvement as a variable.

Other limitations to the study do exist. The use of a questionnaire and the sample itself brings problems of internal and external validity into the research. The wording of the questionnaire might cause participants to feel as if they should answer in a way geared toward what they believe the experimenter wishes them to answer. Along with such experimenter effects, the absence of random sampling also threatens

internal validity. Concerns over external validity might result from generalizing beyond the limits of the sample. The sample, that was female and included members of the middle class in Philadelphia and its surrounding suburbs, is not a representative sample. In addition, these adolescent athletes are playing for a highly competitive, traveling team with which they tried out; therefore, they might introduce other variables affecting self-efficacy reports and perception of parental involvement. The cost to participate on this team is expensive, ranging from \$800 to \$1500; thus, the cost might also cause a change in parental involvement because of their financial investment in their children's sport participation; therefore, the change in parental involvement cannot be generalized. Nevertheless, overall, the results suggest that parents who foster a stress-free athletic experience for their adolescent athletes have children who have higher self-efficacy than parents who do not foster such an environment; therefore, both hypotheses are supported.

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Appendix A

Self-Efficacy Measurement

Authors: Ralf Schwarzer and Matthias Jerusalem (1993, rev. 2000)

Response format:

(1) not at all true, (2) barely true, (3) moderately true, (4) exactly true

1. I can always manage to solve difficult problems, if I try hard enough.
2. If someone opposes me, I can find the ways and means to get what I want.
3. I am certain that I can accomplish my goals.
4. I am confident that I could deal efficiently with unexpected events.
5. Thanks to my resourcefulness, I can handle unforeseen situations.
6. I can solve most problems, if I invest the necessary effort.
7. I can remain calm when facing difficulties because I can rely on my coping abilities.
8. When I am confronted with a problem, I can find several solutions.
9. If I am in trouble, I can think of a good solution.
10. I can handle whatever comes my way.

Appendix B

Perceived Parent Involvement Questionnaire

1. How many years have you been involved in sports?

2. Why did you get involved with sports? (Circle all that apply.)

Influence from friends

Influence from parents

For fun

To get in shape

To be competitive

Other _____

3. How competitive would you consider yourself? (Circle all that apply.)

I hate to lose.

Winning isn't important.

I give 100% all the time.

I consider myself very aggressive.

I associate winning with success.

Sometimes I try harder than others do.

I don't consider myself an aggressive or competitive person.

4. How much confidence do you feel you have in yourself? (Circle all that apply.)

I get upset when I make a mistake.

I realize that mistakes happen and I try to learn from them.

I don't get down if I mess up.

I like when people congratulate me or complement me.

Playing sports has given me more confidence.

Playing sports has given me less confidence.

I get embarrassed when people complement me or congratulate me.

I like when someone pushes me to do better.

I wish that people wouldn't push me to try harder or do better.

I feel confident in myself as an athlete.

I feel confident in myself as a person.

5. Do your parents usually watch you during practices?

Yes No

If Yes (Circle all that apply.):

I often wish they weren't there.

I am happy to have them there watching.

They congratulate me if I do something good afterwards.

They offer me positive advice on ways to improve.

They point out some mistakes that I have made.

When they point out mistakes that I have made, they criticize me or make fun of me in an angry/disappointed manner.

I feel more pressure when they are there.

I get nervous and often mess up, as a result I don't even notice that they are there.

They talk about the other players to me afterward in a negative manner.

If No (Circle all that apply.):

I often wish they were there.

I am happy that they are not there.

I get jealous when other players' parents are there.

I feel I would try harder if my parents were there.

I am glad they are not there because they would embarrass me.

I am glad they are not there because I would feel too much pressure.

6. Do your parents usually come to games or tournaments to watch you play?

Yes No

If Yes (Circle all that apply.):

I often wish they weren't there.

I am happy to have them there watching.

They congratulate me if I do something good afterwards.

They offer me positive advice on ways to improve.
They point out some mistakes that I have made.
When they point out mistakes that I have made, they criticize me or make fun of me in an angry/disappointed manner.
I feel more pressure when they are there.
I get nervous and often mess up, as a result I don't even notice that they are there.
They talk about the other players to me afterward in a negative manner.

If No (Circle all that apply.)

I often wish they were there.
I am happy that they are not there.
I get jealous when other players' parents are there.
I feel I would try harder if my parents were there.
I am glad they are not there because they would embarrass me.
I am glad they are not there because I would feel too much pressure.

7. How do your parents act when they are present at games?

They cheer.
They talk to other parents.
They make positive comments.
They make negative comments.
They are they quiet.
They yell loudly.
They get angry when the team loses.
They stay away from other parents.
They usually talk to the coach.
They compliment or congratulate you.
They talk about the other players when they mess up, criticize you and ask you to do better.
They _____.

Would you prefer your parents to come watch you play at...

Games/Tournaments Practices Both Neither

8. Do you feel that your parents' presence at practice or a game affects how you play?

Yes (negatively, play better) Yes (positively, play worse) No (play the same no matter)

(Circle all that apply.):

Does it create more pressure on you?

Does it encourage you?

Does it embarrass you?

Does it make you nervous?

Does it motivate you?

9. Are you embarrassed by your parent's presence or actions at any of your sporting events?

Yes No

10. How much do you think your parents are involved in your athletics?

Too much Not enough The right amount

11. How much do you want your parents to be involved in your athletics?

More Less No changes

Misguided Solutions: The Failure to Address the Root Causes of International Human Trafficking

William Elder, Jr.

Preface

For the past 2 years, Mr. William Elder has shown a passion for research and advocacy in the field of human trafficking. His work, “A New Perspective on Defeating International Human Trafficking,” marks the culmination of an independent study project that William began in the summer of 2006. Like many other researchers, through his work, he clearly and concisely outlines efforts on the part of the United States, the United Nations, and developing nations to deter this modern-day iteration of slavery. Departing from traditional research, William lays out a comprehensive plan aimed at stamping out his egregious practice.

Currently, William is in his second semester of graduate work. He is channeling his research skills and his passion for the study of human trafficking in the pursuit of an M. A. from the Master of Arts in History program at Villanova University. This too is a stepping-stone, for William’s ultimate goal is to earn his Doctor of Philosophy in history.

Faculty Mentor – Darryl Mace, Assistant Professor of History

Introduction

The phenomenon of international human trafficking is one of the most shocking human rights issues that plagues the world today. Essentially

modern-day slavery, international human trafficking sees human victims moved around the world while having the most atrocious human rights violations committed against them. Although it does not garner the attention it deserves from the international community, that fact makes the crime even more disturbing. No state is innocent of participating in trafficking activities.¹ The United States, however, is on the forefront of antitrafficking legislation and policy resultant from the Trafficking Victims Protection Act of 2000 and the State Department's annual Trafficking in Persons Report.

Despite already boasting the premier antitrafficking legislation and policies, the United States must improve its efforts to fight this new "peculiar institution." It is clear that the United States' solutions are ultimately ineffective, because they do not address the root causes of trafficking: the subordinate status of women and widespread government corruption. The United States needs to revise and better use its legislation and policy so that addressing these root issues becomes a top priority in fighting international human trafficking.

Definition and Overview of International Human Trafficking

The first step in investigating international human trafficking is finding a definition. The term, "human trafficking," describes a myriad of activities ranging from international smuggling, the international and internal

1. I frequently refer to the issue as "human trafficking" or simply "trafficking; however, it is important to remember that the focus of this paper is on the international level.

movement of persons for prostitution, sex tourism, forced labor, and migration. The United Nations (UN) defined “trafficking in persons” in its Protocol to Prevent, Suppress and Punish Trafficking in Persons, especially Women and Children, as:

The recruitment, transportation, transfer, harboring or receipt of persons, by means of threat or use of force or other forms of coercion, of abduction, of fraud, of deception, of abuse of power or of position of vulnerability or of the giving and receiving of payments or benefits to achieve the consent of a person having control over another person, for the purpose of exploitation. Exploitation shall include, at a minimum, the exploitation of the prostitution of others or other forms of sexual exploitation, forced labor or services, slavery or practices similar to slavery, servitude or the removal of organs.²

Many different experts and organizations offer their own definitions of human trafficking; however, the UN’s definition is beginning to be more widely accepted and used. Nevertheless, the major flaw with the UN’s definition is its length. A more concise version of the UN’s definition would be “the transportation of persons using deception, abduction, or coercion, for the purpose of controlling and exploiting them physically and psychologically.” Two extremely important points must be acknowledged concerning this definition of human trafficking.

First, the definition does not distinguish between voluntary and involuntary participation by the trafficked person. Many experts argue that when the person volunteers to participate in the transportation, she or he

2. U.S. Department of State, “Trafficking in Persons Report 2005” (U.S. Department of State, 2005), 10–12.

voids her or his claim to being a victim of trafficking. That criticism is ludicrous, for the deception and fraud used by recruiters clearly does not represent the reality of what will take place, and the person clearly does not volunteer to be physically and psychologically controlled and exploited.³ Furthermore, the experts using this criticism are the same experts who confuse the issue with other issues such as immigration and prostitution.

The definition's inclusion of the words "control" and "exploitation" are also key components because they effectively explain that human trafficking always contains elements of slavery. Cases of slavery exist in contemporary society without any element of human trafficking present being present; however, it is not possible to have an instance of human trafficking without some form of slavery being involved. Cases of contemporary slavery without any element of international trafficking occur within national borders in many areas of the world.⁴

The large variety in definitions of human trafficking causes enormous problems when trying to determine the extent of the problem. Some states consider all trafficking, internally and across international boundaries, while others take no position on trafficking and fail to maintain adequate statistics. Other states include data related to migrant smuggling and other migration

3. Kevin Bales, *Understanding Global Slavery: A Reader* (Berkeley: University of California Press, 2005), 131.

4. For invaluable insight on various forms of contemporary slavery, see, Jesse Sage and Liora Kasten, ed., *Enslaved: True Stories of Modern Day Slavery* (New York: Palgrave Macmillan, 2006).

movements in their human trafficking statistics, drastically influencing the final figures. Lastly, a large number of unreported, and thus undiscovered, trafficking cases exist that will never appear in any reports. Compounding this problem, many times rescued and freed trafficking victims are too embarrassed, ashamed, and afraid to report their case to authorities.⁵ These cases also might never be included in final estimates. The UN Office on Drugs and Crime (UNODC) has explained the complexity of calculating legitimate estimates of the number of trafficking victims. In its April 2006 report, “Trafficking in Persons: Global Patterns,” UNODC states, “that statistical goal may prove to be ultimately unachievable.”⁶

Despite the obvious setbacks, numerous organizations and individuals have attempted to quantify the problem. The United States government, for example, has reported that between 600,000 and 800,000 men, women and children are trafficked internationally every year.⁷ In her book, *Sex Trafficking: The Global Market in Women and Children*, Kathryn Farr of Portland State University used the estimate of four million people annually.⁸ These two estimates were clearly based on different definitions of

5. Michelle Anne Clark, “Trafficking in Persons and Human Security,” in *Human Insecurity in a Global World*, ed. Lincoln Chen, Sakiko Fukuda-Parr and Ellen Seidensticker (Cambridge: Harvard University Press, 2003), 89.

6. United Nations Office on Drugs and Crime, “Trafficking in Persons: Global Patterns” (UNODC, 2006), 45.

7. U.S. Department of State, “Trafficking in Persons Report 2005,” 6.

8. Kathryn Farr, *Sex Trafficking: The Global Market in Women and Children* (New York: Worth, 2005), 3.

human trafficking.

Thus, no universal consensus on the definition or scope of human trafficking can be agreed upon; therefore, there can be no consensus on how much profit criminals earn through their involvement. The UN and the International Organization for Migration have estimated that profits are over \$7 billion annually.⁹ Other reports have indicated that the number is much higher, evidenced by the approximately \$12 billion reported by *CQ Researcher* in, “Human Trafficking and Slavery: Are the World’s Nations Doing Enough to Stamp it Out?”¹⁰

In his book, *Disposable People: New Slavery in the Global Economy*, Kevin Bales approximated that slaves generate an annual profit of \$13 billion. Despite the inclusion of both internal trafficking and slavery in his study, Bales offered invaluable insight to the economics of the phenomenon. He explained that the indirect value is much greater than any direct value that might be calculated. For example, steel producers in Brazil use charcoal made by slaves. That steel is subsequently used in approximately one fourth of all Brazil’s exports. The free labor essentially cuts down on production costs, which then leads to greater profit earned from the sale of the steel.¹¹

9. Farr, *Sex Trafficking*, 20.

10. Mary H. Cooper, David Masci and William Triplett, “Human Trafficking and Slavery: Are the World’s Nations Doing Enough to Stamp it Out?” *CQ Researcher* 14.12 (2004), 275.

11. Kevin Bales, *Disposable People: New Slavery in the Global Economy*, Rev. ed. (Berkeley: University of California Press, 2004), 23.

Regardless of the overall annual revenue, factors exist in human trafficking that set it apart from trafficking drugs and arms. The perpetrators face less of a risk by participating in human trafficking than they do by trafficking drugs and arms. Human trafficking cases are extremely complex cases to investigate and prosecute because of the numerous international players involved. Either states do not have the resources to pursue cases or, although they do pursue them, the cases are later hampered by corruption. For the traffickers that means that human trafficking is a low risk and high profit crime.¹²

The human commodities involved in human trafficking also set the crime apart from the trafficking of drugs and arms. Profits obviously come from the selling of victims to their eventual holders; however, revenue continues to be earned when the victims are forced to repay the debt they accrued in transit. This debt-bondage system, somewhat resembling indentured servitude, allows the traffickers to charge the victims at highly inflated prices for their expenses used to bring them into the country. However, the victims never truly know how long this process will continue. Consequently, the traffickers and holders always have complete control over how long they force the victims to continue in the debt-bondage system.¹³

One last factor to consider is that the victims of human trafficking

12. Farr, *Sex Trafficking*, 22.

13. Farr, *Sex Trafficking*, 21, 32–33.

today are extremely inexpensive for their traffickers to obtain. Today, a healthy, 19-year-old man taken from the Ivory Coast for agricultural work would cost approximately \$50, whereas in 1850 the cost would have been \$1000 (close to \$40,000 in modern day's equivalency). Their low purchase cost, combined with the high profits they earn, results in the victims being treated as if they were disposable.¹⁴ The debt bondage system might continue for years or even generations, but it might also end abruptly if the victim becomes sick, disabled, or, in the case of forced prostitution, pregnant. Put simply, the traffickers and exploiters do not want to spend money on the health and welfare of their victims, because it is not cost-efficient.¹⁵

Clearly, the true scope of international human trafficking might never be ascertained. Nevertheless, what has been determined is that widespread violations of human rights have been committed against an enormous number of people. Even without concrete numbers on the subject, the international community must recognize and attack the root issues that allow human trafficking to exist.

The effects of globalization, the merging of states' economies and cultures through advances in communications and technology, have had a dramatic influence on the growth of international human trafficking. The

14. Bales, *Understanding Global Slavery*, 159–160. See also, Bales, *Disposable People*, 14.

15. Bales, *Disposable People*, 14–15.

flow of cultural traits, trade, and money have become free of the control previously held by individual states, and have emerged to be “supranational activities.”¹⁶ The rapid movement of capital, however, has been primarily among the developed world, and less so among the underdeveloped world (the leading sources of trafficked persons). While those less developed states have continued their existence in poverty and with rampant government corruption, the rest of the world has become technologically, financially, and politically sophisticated.¹⁷

Examining the Root Causes as They Exist in Russia, China, and Nigeria

Much of the world’s populations who live in dreadful social and economic conditions are vulnerable to traffickers. Compounding that, states with the highest numbers of trafficked persons typically exhibit cultural problems like the perceived inferiority of women and lack of women’s rights, as well as political turmoil in the form of corruption at all levels of government. Three states that best demonstrate those conditions are Russia, China, and Nigeria.

Russia

Before its collapse in December 1991, the Soviet government, for nearly 70 years, had total political and economic control over its country. Through this control, it provided guaranteed jobs and social welfare

16. Bales, *Understanding Global Slavery*, 112–113.

17. Clark, “Trafficking in Persons and Human Security,” 91.

programs for its people. The move to a market-style economy following the collapse of the Soviet Union shattered the lives of millions of Russians who depended on the social contract that the state provided. Those hit hardest by the change were women and children.¹⁸

Between 1990 and 1995, women lost 7 million jobs in Russia, compared to men who lost 2 million jobs. Even those with jobs have seen their wages significantly decrease, suggesting that women are devalued in Russian culture. In the early 1990's, women's salaries were approximately 70% of men's salaries, a percentage that decreased to 56% by the end of the decade. The troubles that have developed for Russian women, following the collapse of the Soviet Union, have been due to gender stereotypes and the transition of power to men among private businesses.¹⁹

Stereotypes concerning women's place in the workforce include not the only gender stereotypes present in Russian society. Donna Hughes, Professor at the University of Rhode Island and an expert on trafficking for sexual exploitation, has suggested that masochism exists in Russia. Masochism essentially justifies discrimination and violence against women—as the belief is that women desire such violence and harassment.

18. Sally Stoecker, "Human Trafficking: A New Challenge for Russia and the United States," in *Human Traffic and Transnational Crime: Eurasian and American Perspectives*, ed. Sally Stoecker and Louise Shelley (Lanham, MD: Rowman and Littlefield, 2005), 13–14.

19. Stoecker, "Human Trafficking," 15–16.

That stereotype has led to increased rates of violence against women.²⁰

The severe blow to the economic and social well-being of women in Russia also affects the children of Russia. The social welfare programs that helped protect children have also vanished, and the poor conditions their parents face everyday cause terrible strains on the family. A. I. Dolgova, a leading Russian criminologist, enumerates the problem:

Every year 500,000 children and teenagers lose a parent. Nearly 40 percent of all juvenile crime is committed in these families. . . . More than 24 thousand children have disappeared and are being searched for by the police. Another 27 thousand are becoming victims of crime.²¹

Conditions such as these create the perfect environment in which traffickers can operate. Women are desperate for work, money, and a purpose in society. Thousands of children live without any supervision and are looking to get into trouble or are already themselves victims of other crimes. These women and children are typically lured into a scheme that would take them to a foreign country where they would be guaranteed a job and a place to live. However, once their legal documents are handed over, they are at the mercy of their traffickers. They are physically and sexually abused, their families at home are threatened should they try to escape, and they are compelled to work in their destination country in forced labor.

20. Donna M. Hughes, "Trafficking for Sexual Exploitation: The Case of the Russian Federation" (International Organization for Migration, Migration Research Series, 2002), 11–12.

21. Stoecker, "Human Trafficking," 17.

The traffickers have become proficient at what they do, in large part because of the involvement of organized crime and the Russian Mafia. Following the collapse of the Soviet Union the mafia infiltrated all areas of society, including the government, and spread corruption throughout the country. Those involved with organized crime in Russia collaborate and cooperate with government authorities to make their trafficking schemes work. Officials in the Interior Ministry, Federal Security Service, Ministry of Foreign Affairs, and even smaller police agencies participate in the schemes at almost every stage of the process.²² Often, organized crime provides the cover for lower-level criminals who actually do the recruiting and trafficking. Through collaboration with government authorities, the cover provided by organized crime allows the traffickers to pose as employment, travel, and matchmaking agencies—all legal enterprises.²³

Russia is in a difficult situation because of its large number of problems. Involvement in international human trafficking has only compounded its problems. For example, the state's involvement creates a brain drain because a large number of educated women are trafficked out of the country. The trafficking of their children means countless potential leaders and scholars are lost. Steps to end gender stereotypes and

22. Farr, *Sex Trafficking*, 100.

23. Gilbert King, *Woman, Child for Sale: The New Slave Trade in the 21st Century* (New York: Chamberlain Bros., 2004), 206–207.

governmental corruption in Russia would certainly be steps in the right direction for the Russian government to undertake. The implementation of United States legislation and policy that addresses these root causes would serve as a catalyst for the implementation of such efforts in Russia.

China

Concerning the trafficking of their people, China's situation is somewhat different from Russia's. The source of this difference is China's population of 1.3 billion people. In Russia, the trafficking of its people out of the country hurts society because of the "brain drain" and an already negative birth rate. On the other hand, in theory, China can actually benefit from the trafficking of its people out of the country because of its already enormous population. As Shelley points out in her contribution to *Human Traffic and Transnational Crime: Eurasian and American Perspectives*, "The human trade for China is part of its overall economic growth; for Russia, it is a further drain on its resources."²⁴

Chinese women suffer from their low status in society just as Russian women do. The one-child per family policy illustrates the inferiority of women in China. The one-child policy was created in 1979 when China housed a quarter of the world's population in only 7% of the world's arable

24. Louise Shelley, "Russian and Chinese Trafficking: A Comparative Perspective," in *Human Traffic and Transnational Crime: Eurasian and American Perspectives*, ed. Sally Stoecker and Louise Shelley (Lanham, MD: Rowman and Littlefield, 2005), 64.

land. It consists of numerous regulations pertaining to the family, most notably the restricting of women to having only one child. Although there are exceptions to the policy, they tend further to portray women's subordinate status. For example, in rural areas, a second child might be approved, but typically only if the first child was a girl.²⁵ Many Chinese families prefer to have one boy instead of one girl; therefore, large numbers of girls are abandoned and left extremely vulnerable to sale directly to traffickers.²⁶ The disproportionate number of men in society also increases the demand for Chinese women for forced prostitution, not only in China, but also around the world. Chinese women, confronted with living in poverty and their inferior status, are extremely vulnerable to traffickers.

Organized crime groups, known as "triads," are also heavily entrenched into Chinese society. They developed during the Qing Dynasty in Imperial China because the booming population was not supplied with adequate work.²⁷ Initially formed as political secret societies, they evolved throughout history into organized crime groups with great influence and power in China and throughout the world. That influence includes countries throughout Asia and other destination countries where they send their

25. Therese Hesketh, Li Lu and Zhu Wei Xing, "The Effect of China's One-Child Family Policy after 25 Years," *New England Journal of Medicine* 353:11 (2005), 1171.

26. Mohamed Mattar, "Trafficking in Persons: The European versus the U.S. Approach," War Against Trafficking Alliance: Brussels. 11 May 2005.

27. Debra E. Soled, ed., *China: A Nation in Transition* (Washington: Congressional Quarterly, 1995), 24.

trafficking victims.²⁸

China's situation is unique in that it is a country that is experiencing incredible economic growth and is simultaneously moving toward become a more democratic society. When the population problem is considered, it makes the situation more complex. While economic growth and a move to a more democratic society would theoretically reduce the amount of potential trafficking victims, the population problem will most likely prevent the one-child per family policy from being abolished. Addressing, in United States legislation and policy, the root issue of the subordinate status of women will encourage the Chinese government to find alternative answers to their population problem. Furthermore, citing in the same legislation and policy the root cause of government corruption will push China to cease, or at least limit, their involvement with the triads.

Nigeria

Nigeria is the poorest state out of the three examined in this paper. Sixty percent of Nigeria's population lives below the poverty line, meaning that over 7 million Nigerians live in poverty. With a large population in poverty and a grim outlook on healthcare (life expectancy at birth is 47.08 years, and 3.6 million people live with HIV or AIDS), it is no wonder that

28. Farr, *Sex Trafficking*, 105–106.

Nigeria's population has become vulnerable to traffickers.²⁹

Unique to Nigeria's trafficking situation is the presence of African tradition that seemingly empowers women involved in the trafficking process.³⁰ Women play the role of "mama," or "madam," or "Mama Loa" ("the priestess"). In essence, one "mama" stays in Nigeria to receive the victims from the recruiters and prepares them for their trip to their destination country. The second "mama" stays in the destination country and essentially supervises the victims and their work.³¹ Therefore, it is a trade predominantly of women and children facilitated by women.

Explaining this phenomenon in Nigerian trafficking is difficult because of the historically complex status of women in their society. Throughout Nigeria's multifaceted history, women's rights have varied a great deal, depending on the region of the country in which they lived and the system of law they followed. The institution of marriage is a clear example of regional and legal variations in Nigeria. Marriages held generally in the northern region of the country, under customary or Islamic law, have been arranged and polygamous. Women in those marriages have restrictions

29. Figures based on and calculated from, Central Intelligence Agency. "CIA – The World Factbook – Nigeria." <https://www.cia.gov/cia/publications/factbook/geos/ni.html> (accessed on March 11, 2007).

30. Russian and Chinese women are also involved in trafficking operations in their respective countries (often times they are former victims themselves). The practice in Nigeria, however, has definitive cultural roots, whereas in Russia and China the practice is generally a continued form of exploitation that traffickers utilize over the women.

31. Farr, *Sex Trafficking*, 116.

placed on their right to move freely outside of their homes. The civil law followed in the south, however, has outlawed arranged marriages and requires all marriages to be registered voluntarily by both the husband and wife. Disparities such as this are prevalent throughout Nigeria's history in a number of different areas including property rights, divorce and custody law and labor rights.³²

Political corruption that aids international human trafficking in Nigeria is also complicated. Some studies have supported the argument that organized crime consists of members who are actually part of the political and economic elite in Nigeria, while others support the notion that organized crime involved in trafficking can be broken down into loosely organized crime groups or even smaller groups of just a few people.³³ In some areas of the world, it is reported that Nigerian trafficking activities are the least sophisticated and, instead of relying on collaboration with authorities, they rely mostly on brute force.³⁴ No matter which study is the most accurate, there is no doubt that government officials are involved in one form or another. Unfortunately, Nigeria's history of military coups has most likely ingrained the legacy of political corruption in contemporary Nigerian society. In *The Challenge of Third World Development*, Howard Handelman

32. Center for Reproductive Law and Policy, "Women of the World: Laws and Policies Affecting Their Reproductive Lives, Anglophone Africa" (CRLP, 1997), 83–84.

33. Farr, *Sex Trafficking*, 115–116.

34. King, *Woman, Child for Sale*, 215.

explained that constant gains and losses in the power of civilian political leaders only encourages those leaders to grab a hold of as much power as they can, no matter what the cost.³⁵

Nigeria is Africa's most populous country, and hosts over 250 different ethnic groups.³⁶ As a result, its history of varied women's rights and political corruption will be difficult legacies to overcome. However, that does not mean that it should be ignored. History and cultural tradition should be embraced, but simultaneously a push for countrywide standards must be a priority. The government must also take a stand and refuse to be the leading West African state in international crime. By combating corruption within its own ranks, the government will be able to reduce its participation in international crime and be a better, more positive role model for other West African states. Again, addressing in United States legislation and policy the root causes of trafficking will encourage Nigeria to take these kinds of steps.

Real Improvement Makes the Root Causes the Number One Priority

Throughout the last section, it has seemed that this writer's recommendations to attack the root causes of the absence of women's rights and the presence of rampant political corruption make perfect sense in the effort to defeat international human trafficking. Both root causes are present

35. Howard Handelman, *The Challenge of Third World Development*, 4th ed. (Upper Saddle River: Pearson Prentice Hall, 2006), 238.

36. U.S. Central Intelligence Agency, "CIA – The World Factbook – Nigeria." <http://www.cia.gov/cia/publications/factbook/geos/ni.html>.

in the examples of Russia, China, and Nigeria, and likely exist in every other major supplier of trafficked persons around the world. The U.S. Department of State's annual Trafficking in Victims Report, currently the most influential antitrafficking document, refuses to use those important root causes the way that they should. The report focuses on the "three P's" and "three R's" of trafficking, which are prosecution, protection, prevention, rescue, rehabilitation, and reintegration. Under the title of "The Human and Social Costs of Trafficking," the 2006 report acknowledged the existence of root causes, but acknowledges them more as results of trafficking than as the causes of trafficking.³⁷ Later, the Department of State seemingly acknowledged the issues as root causes, but then downplayed their significance by calling them only "worthwhile endeavors," which are not focused on in the report.³⁸

The Department of State uses the annual Trafficking in Persons Report to categorize states into four classifications; Tier 1, Tier 2, Tier 2 Watch List, and Tier 3. A state with a trafficking problem might be persuaded to enhance its efforts so that it would be portrayed in a better light by the United States in the report. That would be especially true for those states that are categorized as Tier 3, for they are subject to losing nonhumanitarian and nontrade-related assistance from the United States. The

37. U.S. Department of State, "Trafficking in Persons Report 2006," 10–14.

38. U.S. Department of State, "Trafficking in Persons Report 2006," 27.

problem is that the report makes the “three P’s” and “three R’s” the determining criteria in its categorizing of states into the different “tiers.”

Without a doubt, the “three P’s” and “three R’s” are very important in the fight against trafficking. The criminals participating in the trafficking must be prosecuted; victims must be protected, rehabilitated, and reintegrated into society; and preventive measures must be taken. Yet these actions alone will not end the human suffering and misery that is international human trafficking.

Referring traffickers for prosecution under law is definitely a start, but there is a major difference between prosecuting and convicting. Convicting means that the traffickers are found guilty of the crime and that they will be punished, something that incapacitates them and serves as a deterrent to others. Unfortunately, convictions are lacking. The 2006 Trafficking in Persons Report referred to only 53 prosecutions that took place in Russia between April 2005 and March 2006.³⁹ That is a significant increase from the 2005 report, which indicated that there had been only 11 prosecutions. Those prosecutions, however, were not very successful. The 2006 report only acknowledged 9 convictions out of the 53 prosecutions. Furthermore, the 2005 report on Russia explained, “Official corruption continues to facilitate and protect the operation of criminal trafficking

39. U.S. Department of State, “Trafficking in Persons Report 2006,” 210–211.

networks.”⁴⁰ The 2006 report indicated much the same message, explaining that corruption continued to be a “serious” problem for Russia. One specific example cited the resignation of several prosecutors in trafficking cases after it was discovered that they had received bribes.⁴¹

The information on Russia from the Department of State’s 2005 and 2006 Trafficking in Persons Report perfectly illustrate why it is wrong to make the “three P’s” and “three R’s” the number one priority in combating international human trafficking. The worst part of the problem is that, although the Department of State acknowledges that political corruption allows prosecutions to turn into unsuccessful convictions, focusing on that as root cause is to date not emphasized in the report. Without convictions, prosecution numbers are pointless. Therefore, addressing the root cause of political corruption should be one of the top priorities the Department of State uses in determining what category a state falls under in its annual report.

The deplorable state of women’s rights and the low status of women must also be heavily weighted in the criteria for categorizing countries. The 2005 Trafficking in Persons Report section on China discussed some of the prevention efforts the country has undertaken. It mentioned the efforts to use mass media to educate local leaders and citizens of the threat of trafficking,

40. U.S. Department of State, “Trafficking in Persons Report 2005,” 184–185.

41. U.S. Department of State, “Trafficking in Persons Report 2006,” 211.

and to distribute posters, pamphlets, and videos throughout the country to raise awareness of the dangers of trafficking.⁴² Those types of efforts might be extremely helpful in a smaller country or in a country that has only a minor trafficking problem. However, China is home to 1.3 billion people and is one of the leading source countries of international human trafficking. Even if every single Chinese citizen viewed a video on the dangers of human trafficking, it would not change the fact that their society undervalues women and young girls so much that they are extremely susceptible to trafficking. Making the root issue (the low status of women) a top priority in the categorization process will better serve the purpose of fighting international human trafficking.

Although the U.S. Department of State should modify its Trafficking in Persons Report to include the root issues when determining a country's placement in one of the tiers, the United States should also embrace the root issues in other forms of legislation and policy. One of the major provisions under the Trafficking Victims Protection Act of 2000 authorized the President to provide assistance to foreign countries in developing programs, projects, and activities "to meet the minimum standards for the elimination of trafficking."⁴³ At least 25 countries that have antitrafficking legislation that addresses the "three P's," and 10–15 countries are considering such

42. U.S. Department of State, "Trafficking in Persons Report 2005," 84.

43. Section 109(a), TVPA of 2000, H.R. 3244.

antitrafficking legislation.⁴⁴ The United States can help those countries to develop such legislation and can encourage them to make attacking the root issues (enhancing women’s rights and diminishing political corruption) a top priority.

Highlighting the root issues as top priorities in states’ new antitrafficking legislation will help minimize the “push-down pop-up” effect that Phil Marshall and Susan Thatun describe in their contribution to *Trafficking and Prostitution Reconsidered: New Perspectives on Migration, Sex Work, and Human Rights*. The “push-down pop-up” effect occurs when community-level antitrafficking efforts reduce the number of trafficked victims in that geographic area, but the number of victims from a surrounding area subsequently increases. This occurs because the trafficking industry is, as Marshall and Thatun describe, “a dynamic phenomenon and traffickers can quickly adjust to changing environments, in particular, but not only, by shifting the geographic focus of their activities.”⁴⁵ When more states in the global community adopt antitrafficking legislation that includes the phenomenon’s root issues as top priorities, it will be more difficult for traffickers simply to move the focus of their activities to a neighboring geographic area.

44. Mattar, “Trafficking in Persons: The European versus the U.S. Approach,” __.

45. Phil Marshall and Susan Thatun, “Miles Away: The Trouble with Prevention in the Greater Mekong Sub-Region,” in *Trafficking and Prostitution Reconsidered: New Perspectives on Migration, Sex Work, and Human Rights*, ed. Kamala Kempadoo, Jyoti Sanghera and Bandana Pattanaik (Boulder, CO: Paradigm, 2005), 44.

The issues of women's rights and political corruption have been present since the beginning of history. Today they play a major role in the growth of international human trafficking. Every major policy or report, however, fails to acknowledge that trafficking will never be defeated until these root issues are addressed. The United States is currently the leader in antitrafficking legislation and the emphasis placed on the "three P's" and "three R's" is critical. Nevertheless, any hope of long-term success will fail, unless a universal policy is implemented that makes improving the status and rights of women and ending political corruption top priorities in the fight against international human trafficking.

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Life and Physical Sciences

Detailed Growth Analysis and rRNA Gene Expression Patterns of the Halophile *Halosimplex carlsbadense*

Katie Lowther, Lara D'Alessandro, and Kathleen Grant

Preface

During her last year at Cabrini College, Ms. Katie Lowther worked diligently in my laboratory, conducting experiments to understand the basic biology of unique halophile (salt-loving) microbe called *Halosimplex carlsbadense*. *H. carlsbadense* was originally isolated by Russ Vreeland's laboratory of West Chester University from a 250-million-year-old salt crystal. Halophiles are sometimes referred to as extremophiles, a coin used to determine an entire host of microbes that thrive in harsh environmental conditions such as high salt, near the boiling point of water and in subfreezing temperatures near the polar ice caps.

The basic biology of this microbe is very ill defined as is the biology of most extremeophilic micro-organisms. Many of our current biotechnological innovations have come about by understanding the functions of genes of several extremeophiles. Katie has conducted a series of experiments to detail *H. carlsbadense*'s growth requirements and growth rate. Understanding the growth profile of this organism is an extremely important first step in attempting to find genes that are important for *H. carlsbadense* to lie dormant for millions of years.

Other students in my laboratory are currently conducting

experiments to identify such genes. Our hope is that discovery of such genes will give us insight into genes and gene functions involved in allowing other microbes to lie in dormant states for extended periods of time (for example, *Bacillus anthracis*, the microbe that is the causative agent for the deadly disease of anthrax). Katie presented her work at the 2007 National Conference on Undergraduate Research Symposium and is the first author for this article. She also presented her work at the spring 2005 Pennsylvania Academy of Science annual meeting and was featured on the front cover of *Cabrini Magazine* highlighting her undergraduate research accomplishments.

Katie is currently pursuing a Ph.D. at the University of Connecticut Health Center in the biomedical sciences with a concentration in cell biology. Her current thesis research is in the field of reproductive biology. She is interested in cell signaling events that regulate mouse egg maturation. Specifically, she is studying how meiotic arrest is maintained, as well as how luteinizing hormone overcomes the arrest to signal the oocyte to mature into a fertilized egg. I have no doubt Katie will accomplish all of her research goals in graduate school and continue to conduct research on the cutting edge of science.

Faculty Mentor – David Dunbar, Associate Professor of Biology

Abstract

Halosimplex carlsbadense is a halophilic archaeon that was isolated from a 250-million-year-old salt crystal from the Salado formation in New Mexico.¹ The rod-shaped, gram-negative, obligate aerobe lacks the ability to grow on complex media. Additionally, *H. carlsbadense* lacks the ability to grow on any organic compounds. This inability suggests that *H. carlsbadense* possesses novel catabolic pathways or lacks membrane transport enzymes necessary to use such substrates.¹

A detailed growth curve analysis was performed in order to gain more insight into its basic biology and to compare its growth characteristics to that of other halophilic microorganisms. *H. carlsbadense* was grown in a defined glycerol-acetate medium containing 25% NaCl with a pH of 7.4. The results indicate optimal growth characteristics similar to that of other members of the *Halobacteriaceae* family.² *H. carlsbadense* grows optimally between the temperatures of 38–42 degrees Celsius.

This organism is also unique because it contains three divergent 16S rRNA genes A, B, and C.¹ Genes A and B are 97% similar to each other. Gene C is 93.8% similar to gene A, and 92.2% similar to gene B. To investigate whether these three genes are expressed, a set of RT-PCR experiments were performed using *H. carlsbadense*'s optimal growth conditions as a reference. Oligonucleotide primers were used in a region of gene sequence in which all three genes could be differentiated. Of the 18 clones that were sequenced, 16 represented gene B and 2 represented gene A. Thus, under optimal growth conditions, gene B is preferentially expressed.

Current studies are aimed at determining the expression levels of the small subunit ribosome genes under altered or stressful conditions and at understanding what genes are involved in the transition between the different phases of growth.

Keywords: ribosomes, halophiles, archaea

Introduction

Halobacteriaceae reside in the domain Archaea and are obligate extreme halophiles. The family of Halobacteriaceae consists of 18 genera

and 49 validly described species.² Members of this family of halophiles grow anaerobically and thrive in a variety of hypersaline environments.³ At least 1.5 M NaCl is required for growth, in which 2.0–4.5 M NaCl promotes optimal growth. All members have high internal cation concentrations. Not only do Halobacteriaceae exhibit halophilicity, but also four genera have been described as aklaphila. Optimal growth of these organisms occurs between pH 9.5 and 10.0². Halophilic organisms have been isolated from various environments. For example, *Halorubrum lacusporfundi* was discovered in the sediment of a cold, hypersaline lake in Antarctica.⁴ *Haloferax volcanii* was isolated from the Dead Sea and *Halorabodus utahensis* was found in the Great Salt Lake.^{5, 6} *Natronomonas pharaonis* was discovered in a hypersaline soda lake in Egypt while *Halosimplex carlsbadense* was isolated from a Permian halite deposit salt crystal.^{7, 1} To better understand basic physiology characteristics of Halophiles, growth characteristics must be studied. Previous growth kinetics of several extremely Halophilic Archea members have been described.² Optimal growth temperatures range from 49 to 58°C, with generation times between 1.5 to 3 hours. It has also been indicated that the family Halobacteriaceae show similar growth kinetics.

H. carlsbadense is a halophilic archaeon that was isolated from a 250-million-year-old salt crystal from the Salado formation in New

Mexico.¹ It has been identified as a gram-negative obligate aerobe that produces primarily rod shaped cells and lacks the ability to grow on complex media, with the only carbon compound that supports the growth of this halophile being pyruvate. It is also incapable of growing on any organic compounds such as amino acids, methanol, nucleotides, formaldehyde, fatty acids, vitamins, proteins, or Krebs cycle intermediates, which would seem to suggest that either this organism possesses novel catabolic pathways or that it lacks the membrane transport enzymes that are needed to use such substrates.¹ This organism also contains three divergent 16S rRNA genes named A, B and C; more than any other organism known to date. Genes A and B differ primarily at the 3' prime end and are 97% similar to each other. Gene C is 93.8% similar to gene A, and 92.2% similar to gene B with ~7% divergence within one prokaryotic organism being the highest level reported so far.⁸ Because *H. carlsbadense* does not sporulate, but can lay dormant for long periods of time, it has been suggested that rRNA genes might be preferentially expressed during dormancy.³

The present study indicates that *H. carlsbadense* has a similar generation time to that of the characterized members of Halobacteriaceae. However, *H. carlsbadense* grows optimally between 38–42 degrees, unlike other members of the family where optimal growth is between 49 to

58°C. It has also been demonstrated that during exponential growth under optimal growth conditions, small subunit gene B is preferentially expressed. Whether small subunit genes A and/or C are preferentially expressed at suboptimal or altered growth conditions is an area under current investigation.

Materials and Methodology

Media Preparation

The following components were dissolved in 100 mLs of distilled water: 1.04 mLs glycerol, 1.25 grams Na Pyruvate, 2 grams KCl, 0.25 grams K_2HPO_4 , 0.05 grams $(NH_4)_2SO_4$, and 10 grams $MgSO_4$. The pH of the solution was brought to 7.4 with 10 M NaOH. After sterilizing the stock solution by autoclaving, 75 mL of sterile stock 25% NaCl solution was added to 100 mLs of the above described solution making the final solution. The NaCl used was from Morton solar water softener salt crystals that were 99.5% pure.

Growth Curve Analysis

Phase-contrast microscopy was performed to ensure culture purity using a Nikon Eclipse E400 phase contrast microscope (Figure 1). Baffled flasks were inoculated with 0.5 mL of a *H. carlsbadense* culture (strain 2-9-1) that was in exponential growth and incubated at various temperatures in a shaking incubator (model # 1400 from New Brunswick Scientific) at

225 rpm. Triplicate samples were inoculated per temperature. Optical Density readings at 600 nm were taken after inoculation and at regular time intervals using a Genesys UV spectrophotometer. When conducting OD readings, the spectrophotometer was blanked against sterile medium. 0.5 mL of the culture was removed from baffled flasks in the shaking incubator, placed in a sterile cuvette, and then measured by the spectrophotometer. If the culture had an OD reading over 1.2, a 1:4 dilution was performed and read again to give accurate readings. The generation time was calculated using the equation $G = t/n$ where $n = (\log_b - \log_B) / (\log 2)$. (b is the number of bacteria at the end of the time interval, B is the number of bacteria at the beginning of the time interval, t is time, and n is the number of generations).

Determination of the Expression of rRNA Genes A, B and C

Total RNA was extracted from *H. carlsbadense* using procedures outlined.⁹ Approximately 0.5 micrograms of total RNA was treated with 5 units of RNAase free DNAase, extracted with phenol/chloroform/isoamyl alcohol and ethanol precipitated. The RNA was reverse transcribed using 200 units of Superscript II reverse transcriptase (Gibco Pharmaceutical) and an oligonucleotide primer to position 1263–1286 (R) of all three ribosome genes. Reverse transcription reactions were done according to Gibco Pharmaceutical recommendations. PCR was performed on an

aliquot of the reaction using the oligonucleotide used for primer extension and a forward primer oligonucleotide complementary to position 1020–1040 of all three genes. The PCR conditions were: 95°C for 4 minutes (one cycle), then 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds (thirty cycles). The PCR products, approximately 275 base pairs, were cloned using the Invitrogen TOPO TA-Cloning kit for Sequencing. The Maine DNA Sequencing Laboratory sequenced the cloned inserts.

Results

The present study with *H. carlsbadense* indicates growth characteristics that differ somewhat from other members of the *Halobacteriaceae* family as recently reported.² *H. carlsbadense* grows optimally between the temperatures of 38–42°C with a generation time of roughly three hours (Figure 2). Growth below 38°C is significantly impaired with growth at room temperature almost nonexistent (Figure 2). The optimal growth temperature for other members of the genus *Haloarcula* is between 49 to 58°C.² Thus, growth analysis indicates that *H. carlsbadense* grows below the optimal growth temperatures of other *Haloarcula* bacteria. Growth differences could imply a difference in bacteria physiology of *H. carlsbadense* compared to that of other members of the family.

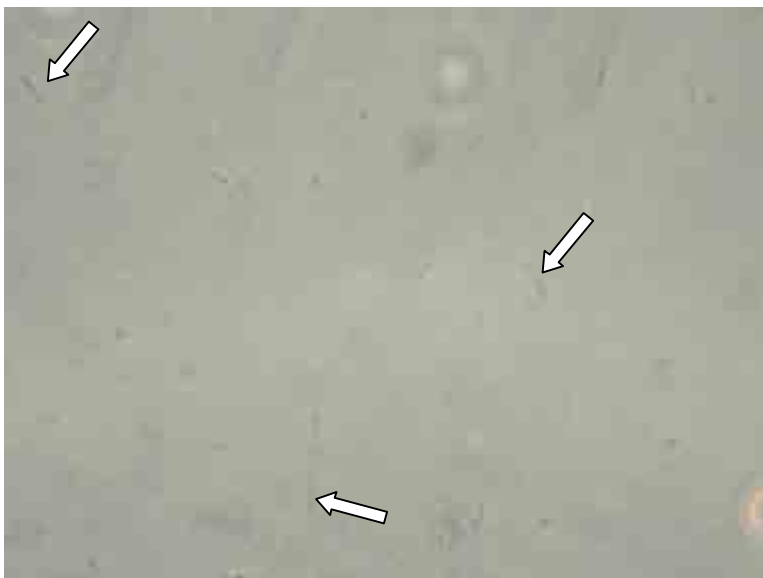


Figure 1. Phase-contrast microscopic image of *H. carlsbadense*.

In Figure 1, phase-contrast microscopy was performed to ensure culture purity. *H. carlsbadense* is a rod-shaped, gram-negative prokaryote. Arrows indicate the presence of *H. carlsbadense*.

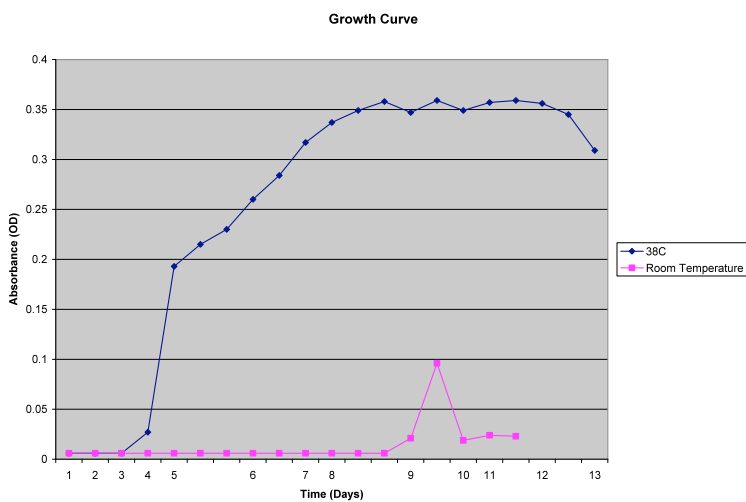


Figure 2. Growth characteristics of *H. carlsbadense* at two different temperatures.

Figure 2 shows the growth curve characteristics of *H. carlsbadense* at two different temperatures. Absorbance (OD 600) was measured at regular intervals over 13 days. At 23°C, the lag phase is much longer and the highest OD reading does not go above 0.1. Growth at 38°C shows a much shorter lag phase, followed by both a classic log phase and stationary phase. The OD reaches above 0.35. Between 12 and 13 days, death begins to occur due to nutrient deprivation and accumulation of toxic wastes. *H. carlsbadense* grows optimally at 38°C.

To determine which of the three small subunit ribosome genes are expressed under optimal growth conditions at 38°C, total RNA was isolated from cells in exponential phase followed by reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR was performed with primers that corresponded to a region in the genome in which all three genes had sequence differences. The amplified DNA products were then resolved on a 3% agarose gel (Figure 3). All RNA isolation methods lack ability to remove completely genomic DNA contamination from the RNA; therefore, treating the sample with DNase is a technique that helps reduce this contamination.¹⁰ The results indicate an approximately 275 base pair amplified DNA fragment from total RNA treated with DNase (Figure 3, lane 5). No product was observed with RNA treated with RNAase before RT-PCR, as expected (Figure 3, lane 3). In addition, the same size fragment was amplified from genomic DNA, as

expected (Figure 3, lane 1). However, amplification was seen when total RNA samples were not treated with DNase, indicating that DNase treatment is essential to remove residual genomic DNA contamination (Figure 3, lane 4). Amplified PCR products were cloned in bacterial plasmids using a TA-Cloning Kit by Invitrogen Corporation. After bacterial transformations using the procedure outlined with the Invitrogen TA-Cloning Kit, bacteria colonies containing plasmids with cloned inserts were grown in LB broth in the presence of the drug ampicillin. Plasmids from overnight cultures were then purified using standard procedures, digested with the restriction endonuclease EcoRI and resolved on a 2% agarose gel (data not shown). The Maine DNA Sequencing Laboratory sequenced 18 plasmids containing inserts. Sequencing results show that 16 inserts represented gene B and two inserts represented gene A. These results indicated that under optimal growth conditions, gene B is predominantly expressed.

Figure 3 shows the amplified DNA from a reverse transcriptase-PCR (RT-PCR) experiment was resolved on a 3% agarose gel. RNA was reverse-transcribed into single stranded DNA using the reverse primer and total RNA isolated from *H. carlsbadense*. The single-stranded DNA was made into double-stranded DNA and amplified by PCR using the reverse and forward primer. Lane 1: Genomic DNA (275 bp in size). Lane 2: Primers alone. Lane 3: Total RNA treated with RNase. Lane 4: Total

RNA without treatment with DNase. Lane 5: Total RNA treated with DNase.

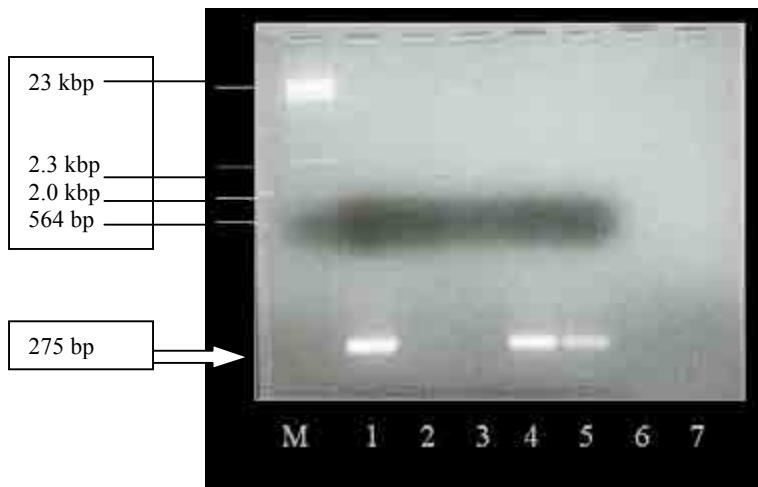


Figure 3. RT-PCR gel analysis.

Discussion

As a first step in understanding some of the basic biology of *H. carlsbadense* a detailed growth analysis over a wide range of temperatures was performed. Growth analysis concludes that the optimal temperature is between 38°C and 42°C. There are no apparent growth differences between these temperature ranges. In addition, growth at its optimal temperature, *H. carlsbadense* has a generation time of approximately 3 hours. The optimal growth temperature for other members of the genus *Haloarcula* is between 49 to 58°C². This indicates that *H. carlsbadense* has a somewhat lower optimal growth temperature as other members in the genus. *H. carlsbadense* does grow above 42°C but at a significantly

much slower rate (data not shown). In fact, at 55°C or higher, growth is nearly nonexistent. However, like other members in the genus, *H. carlsbadense* grows significantly slower below 35°C. In fact, growth is nearly nonexistent at room temperature (Figure 2). Growth did not occur at all above 60°C. The fact that *H. carlsbadense* is the only known microbe that cannot grow on any organic compounds, unlike other members of the Haloarcula genus, might explain why it has a different optimal temperature growth requirement.

In the present study, growth curves were important for identifying the temperature where optimal growth of *H. carlsbadense* occurs in order to conduct differential gene expression studies. As mentioned previously, *H. carlsbadense* can lay dormant for long periods. This organism does not sporulate; therefore, it has been proposed that specific genes are responsible for its ability to lay dormant.³ Future studies are aimed at identifying genes that are initiate dormancy by performing differential gene expression analysis on RNA isolated at the transition point between log and stationary phase. It would also be interesting to isolate RNA during stationary phase to identify the genes that allow it to remain in a dormant state.

The next step was to extract RNA from the cells and determine through RT-PCR whether all three small subunit ribosome genes were

actually being expressed under ideal growth conditions for *H. carlsbadense*. Plasmids were sent to the Maine Sequencing Facility for sequencing, and the analysis indicated that 16 samples represented Gene B and 2 samples represented Gene A. Although the results from RT-PCR did not show the expression of all three genes in the expected manner, the data would seem to suggest at least that Gene B is predominantly expressed under standard growth conditions. It remains to be determined whether Gene C is expressed or whether Gene C represents a pseudogene or a PCR artifact. Previous studies do indeed suggest that Gene C is a chimeric PCR artifact of the technique originally used to identify these genes and is in fact a composite of Genes A & B.⁸ This interpretation offers intriguing possibilities and might explain why Gene C expression by RT-PCR could not be demonstrated. It is also possible that *H. carlsbadense* would choose predominantly to express alternate small subunit ribosome genes under altered growth conditions. Because halophiles most likely needed to be able to adapt to changes in salinity, this possibility is not implausible. There is already the example of the organism *H. culirubrum*, which varies the expression of a unique operon when the salt concentration of the medium it is grown on changes.⁸

A possibility for future studies would be to test whether growth under altered or stressful conditions would induce higher levels of

expression of different small subunit ribosome genes. A sucrose gradient could also be performed to extract intact ribosomes and polysomes. The expression of one or all three genes in a polysome would indicate that not only are the genes involved in making a functional ribosome, but that they are also involved in active translation. If only one of the three genes is present in the polysome under optimal growth conditions, the experiment would be repeated using altered growth conditions. Despite the fact that *H. carlsbadense* is an extremely fastidious organism, it is capable of growing on only three different types of media: pyruvate alone, pyruvate plus glycerol, or glycerol plus acetate in a defined medium.¹ It is possible that *H. carlsbadense* expresses different rRNA genes to allow it to grow under different conditions.

It might also be reasonable to propose the idea that the other genes are expressed under varying salt concentrations. The ribosome is a highly organized organelle and the rRNA sequence is critical in the process of maintaining secondary and higher order structural features, which are essential for the ribosome to interact properly with other components of the translational apparatus.¹¹ The structure of the SSU needs to be retained for the LSU to properly cooperate with proteins and other RNA components.⁸ A typical cell would not be able to withstand the high salt concentrations that a halophile thrives in. This is because halophiles have adapted to these

conditions by accumulating high salt concentrations within the cell to maintain an osmotic balance. In addition, their intracellular enzymes have to be able to adapt to salt. In a typical cell, this high amount of salt would cause the denaturation of their proteins, but the proteins of a halophile contain an excess ratio of acidic to basic amino acids which are arranged in such a manner that the acidic residues are mainly presented to the surface which results in a negative net surface charge.¹² If a halophile were exposed to a lower salt concentration, this protein arrangement might not be as beneficial as it would be in a higher concentration. Therefore, it is not unreasonable to assume that perhaps a different rRNA gene would be expressed under altered environmental conditions. For example, another halophile, *H. culirubrum*, expresses a unique rRNA operon in accordance with variations in salt concentrations.⁸

Although a eukaryote, one could compare *H. carlsbadense* to the malaria parasite *Plasmodium berghei*, which has developed a switch that regulated the expression of its two divergent rRNA genes.¹³ This switch occurs between the different stages of the mosquito's development, with the A-type rRNA genes being expressed while the parasite lives in the liver and the blood, while the S-type rRNA genes are expressed in mature ookinates. During the transition between the blood stage and the mature ookinate, the S-type rRNA genes are detected only briefly due to a process

that has been termed “dilution.”¹³ It is possible that *H. carlsbadense* also posses a similar mechanism which would account for detection of low levels of rRNA gene A while rRNA gene B is predominantly being expressed. For these reasons, it is possible that grown under altered conditions, *H. carlsbadense* will express alternate small subunit ribosome genes. Future experiments also are aimed at understanding what genes are involved in the transition between lag phase and exponential phase growth and the transition between exponential phase and stationary phase growths to understand which genes might be involved in these critical growth phase transitions in *H. carlsbadense*.

Acknowledgements

The authors wish to express appreciation to Dr. Tami Mysliwicz, Associate Professor of Biology at Penn State Berks, for use of the phase contrast microscope. This microscope was used to ensure culture purity depicted in Figure 1. The authors also wish to acknowledge the Center for Teaching & Learning at Cabrini College specifically the director, Lisa Ratmansky, Coordinator of the Writing Center, and Tracie Kennedy for critically reviewing this manuscript. Lastly, the authors would like to thank Dr. Russ Vreeland, Full Professor at West Chester University who supplied the Halosimplex (strain 2-9-1) used in this study. This work was funded by a Department of Education Grant Award # P116Z050100 titled

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**The Effect of Proinflammatory Cytokines IL-1 beta, IL-2, GM-CSF,
and TNF-alpha on Phagocytosis and Cell Proliferation in
*Eisenia hortensis***

Laura Goodfield*, Katrina Hill*, Kathleen Grant, Nicole DeRogatis, and
Sheryl L. Fuller-Espie

*Authors contributed equally to the generation of this paper.

Preface

Ms. Laura Goodfield is double-majoring in a biology–
biotechnology concentration, and in Spanish, and will graduate in May
2009. She began her research in my laboratory during the summer of 2007
and has since been investigating the effects on invertebrate immune
systems. She received a student research grant in October 2007 from the
Pennsylvania Academy of Science to fund her research project. In
addition, she copresented her work with Katrina Hill at the 2008
Pennsylvania Academy of Science Annual Meeting in an oral presentation
titled, “The effect of pro-inflammatory cytokines IL-1 beta, IL-2, GM-
CSF, and TNF-alpha on phagocytosis and cell proliferation in *Eisenia*
hortensis” in Volume 81 of the *Journal of the Pennsylvania Academy of*
Science (p. 118). At Cabrini College’s Undergraduate Art and Research
Symposium, Laura presented her work in an oral presentation titled, “The
effects of pro-inflammatory cytokines on phagocytosis of coelomocytes
from *Eisenia hortensis*.” This fall Laura will continue her research in my
lab as part of her undergraduate senior thesis project investigating the

effects of pathogen-associated molecular patterns, namely flagellin and peptidoglycan, as well as IL-10 and gamma-interferon on phagocytosis in invertebrates, in addition to performing proliferation assays using enzyme-linked immunosorbent assays.

Ms. Katrina Hill is double-majoring in a biology–biotechnology concentration and mathematics, and she will graduate in May 2009. Since fall 2007, Katrina has conducted undergraduate research in my lab working on invertebrate immune responses to proinflammatory cytokines. She will continue to work in my lab 2008–2009 to obtain original data for her undergraduate thesis focusing on the effects of IL-10 and IL-12 on NK-like activity of earthworm coelomocytes. Katrina presented a portion of her research, which dealt with the effect of proinflammatory cytokines on cell proliferation in *Eisenia hortensis* at the 2008 Cabrini College Undergraduate Art, Research, and Scholarship Symposium. She also copresented with Laura Goodfield at the 2008 Pennsylvania Academy of Science Annual Meeting in an oral presentation titled “The effect of pro-inflammatory cytokines IL-1 beta, IL-2, GM-CSF, and TNF-alpha on phagocytosis and cell proliferation in *Eisenia hortensis*” in Volume 81 of the *Journal of the Pennsylvania Academy of Science* (p. 118). In the summers of 2006 and 2007, she completed internships in the Tissue Culture and Production Department and the Research and Development

Department at Intervet, Inc., a worldwide animal vaccine producer. In the summer of 2008, Katrina was selected to participate in the United States Department of Energy Global Change Education Program and perform mathematical biology research at the University of Notre Dame. She presented a poster outlining the leishmaniasis transmission mathematical model that she developed while at Notre Dame at the University of Michigan Undergraduate Summer Research Symposium.

Ms. Kathleen Grant graduated from Cabrini College in May 2007 with a B.S. in Biology–Pre-Medicine and a minor in Chemistry. She received the College’s 2006 Charles A. Mastronardi Service and Leadership Award for outstanding contributions to community service projects and civic engagement. Her undergraduate thesis title was *The Role of Interleukin-17, Interleukin-1, and TNF-alpha in the Pathogenesis of Rheumatoid Arthritis*. Kathleen participated in two undergraduate research projects as part of her undergraduate course work. In the summer of 2005, she worked with Dr. David Dunbar and student Katie Lowther investigating the growth characteristics of the extreme halophile, *Halosimplex carlsbadense*. The results of this project were presented in 2006 as a poster presentation at the Pennsylvania Academy of Science Annual Meeting. In the summer of 2007, she was part of a research project in my lab together with Laura Goodfield investigating innate immune

responses of *Eisenia hortensis*. She coauthored papers accepted as oral presentations at the 2008 at the Pennsylvania Academy of Science Annual Meeting and the Cabrini College 2nd Annual Undergraduate Art, Research, and Scholarship Symposium.

Kathleen is currently a student at Thomas Jefferson University where she is pursuing a master's degree in public health. In the fall, she will be participating in a clerkship within the Epidemiology Department of the Philadelphia Department of Public Health. She will be assisting in the department's KIDS Registry Survey Project, a Web database for the immunization records of children within the city of Philadelphia. Upon graduation from Jefferson, Kathleen hopes to pursue a Ph.D. in epidemiology. Kathleen is also currently employed by the University of Pennsylvania School of Medicine, where she works under Dr. Arupa Ganguly as a clinical lab technician within the Genetics Department.

For faculty mentor comments for Ms. Nicole DeRogatis, please refer to the paper titled, "The *in vitro* Effects of Human Proinflammatory Cytokines Granulocyte-Macrophage Colony-Stimulating Factor and Interleukin-1 beta on Natural Killer-like Responses in *Eisenia hortensis*" in this volume of the *Undergraduate Art, Research and Scholarship Journal*.

I have been especially fortunate to have had the opportunity to

work with these four exemplary young women over the past 12 months! Each of them has brought different strengths to the projects that we were investigating, and collectively we were a team that worked extremely well together. Through their commitment and passion for immunology, they have grown as scientists and will take the skills they have developed through their undergraduate research experiences to greater heights after leaving Cabrini College. Watch this space.....I'm certain we will be hearing a lot more from these talented young scholars in the research arena!

Faculty Mentor – Sheryl L. Fuller-Espie, Ph.D., DIC, Associate Professor of Biology

Abstract

This study was aimed at determining the influence of proinflammatory cytokines, including interleukin-1 beta (IL-1 beta), interleukin-2 (IL-2), tumor necrosis factor-alpha (TNF-alpha), and granulocyte-macrophage colony-stimulating factor (GM-CSF), on innate immunity in *Eisenia hortensis* (earthworm). Following overnight incubation with the cytokines, two different innate mechanisms were characterized using flow cytometry including phagocytosis and proliferation of earthworm leukocytes (coelomocytes), specifically the large coelomocytes known as the amoebocytes, using flow cytometry. The phagocytosis assay employed measuring uptake of *Escherichia coli* expressing green fluorescence protein in the presence or absence of proinflammatory cytokines. Proinflammatory cytokines stimulated statistically significant ($p \leq 0.05$) enhanced phagocytosis in 10.5–25% of cases for IL-1 beta, 18.4–50% for GM-CSF, 17.2–50% for IL-2, and 20.7–30% for TNF-alpha, depending on the cytokine concentration used. Cell proliferation was determined by measuring DNA content using propidium iodide. In contrast to the results obtained with phagocytosis, there was little to no effect on cell proliferation when coelomocytes were treated with the proinflammatory

cytokines; only 4.1% of cases exhibited increased proliferation in response to TNF-alpha, and no increased proliferation was observed in response to IL-1 beta, IL-2, or GM-CSF. Experiments are underway to investigate cytokine effects on NK-like responses.

Introduction

Earthworms and other invertebrates use innate immunity as their primary defense against the many contaminants and microbes that they contact in their habitats (Salzet, Tasiemski, & Cooper, 2006; Cooper, 2006). Innate immunity is characterized by nonspecific, nonclonal, and nonpre-emptive immunological responses, including phagocytosis and proliferation. Molecules such as lysenin, fetidin, eiseniapore, coelomic cytolytic factor (CCF) and Lumbricin I are produced and secreted as a part of innate immunity defenses in earthworms (Cooper, 2006).

Innate immunity heavily relies on pattern recognition receptors (PRRs), which are expressed on the surface of professional antigen presenting cells (APCs). These PRRs recognize and bind to pathogen associated molecular patterns (PAMPs), including lipopolysaccharide (LPS), and initiate cell-signaling pathways of innate immunity. One type of PRR is the evolutionarily conserved Toll/Toll-like receptor, which has the ability to recognize a broad spectrum of ligands (Janssens & Beyaert, 2003). The Toll receptor was discovered in *Drosophila* several decades ago during an investigation of dorsoventral patterning in embryonic

development (Janssens & Beyaert, 2003). When homologues of insect TOLL receptors were later found in mammals and other animals, they became known as Toll-like receptors (TLRs). Cytokines regularly stimulate TLR signaling pathways and are produced because of these signaling pathways. Two examples of TLR signaling pathways are (a) TLR2, which binds to peptidoglycan of both gram-positive and gram-negative bacteria, lipoteichoic acid of gram-positive bacteria, and zymosan of yeast; and (b) TLR4, which binds to LPS of gram-negative bacteria and certain viruses (Kurt-Jones, Mandell, Whitney, Padgett, Gosselin, et al., 2002).

Cooper (2006) states in his commentary that the discovery of Toll-like receptors in mammals and other animals “linked innate and adaptive immunity” and was responsible for “unifying aspects of evolution of immune competence.” Since then it has been shown that Toll and Toll-like receptor signaling is necessary for phagocytosis and synthesis of antimicrobial compounds to occur in vertebrates, insects, and the invertebrate *Caenorhabditis elegans*, but it is not known whether they are needed in order for these mechanisms to occur in earthworms (Cooper, 2006). Cooper suspects that the lack of study in earthworms concerning Toll-like receptors is the cause of this uncertainty.

In addition to the conservation of Toll-like receptors in a

significant number of invertebrates, the JAK/STAT signaling pathway, known to function in vertebrates in response to cytokines and growth factors, has also been observed in *Drosophila* (Cooper, 2006). Similarly, further examples of conservation of innate immunological elements indicate that some invertebrates including Annelida, such as *Nereis diversicolor* and *Eisenia fetida*, produce molecules with similar function to mammalian cytokines such as interleukin-1 alpha (IL-1 alpha), interleukin-1 beta (IL-1 beta), and tumor necrosis factor alpha (TNF-alpha) (Mandrioli, Malagoli, & Ottaviani, 2007). Additionally, human cytokines IL-1 beta, IL-2, and TNF-alpha may all bind to the same receptor found on molluscan immunocytes, which shows that there might be receptor-driven ligand evolution in invertebrates, as well as conservation of ligand receptors from invertebrates to vertebrates (Mandrioli et al., 2007). A study conducted by Hughes, Smith, Leung, and Stefano (1992) showed that invertebrates (in particular, bivalve molluscs) and vertebrates respond to recombinant human TNF-alpha and IL-1 in a similar manner. More interestingly, in invertebrate immune cells, mammalian cytokines actually initiate certain immune mechanisms such as phagocytosis, cytotoxicity, cell motility, and chemotaxis (Mandrioli et al., 2007).

In our study, we investigated the effects of recombinant human

cytokines IL-1 beta, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-2 (IL-2), and TNF-alpha on innate immune responses in *Eisenia hortensis*. There are three types of cells that participate in innate immunity of earthworms: the chloragocytes/eleocytes (involved in the nutrition, excretion, and production of cytotoxic compounds) (Engelmann, Molnar, Palinkas, Cooper, & Nemeth, 2004), large coelomocytes/hyaline amoebocytes (possessing phagocytic activity) and the small coelomocytes/granular amoebocytes (possessing NK-like activity) (Komiyama et. al., 1998). Our focus, however, was on large coelomocytes, which make up approximately 42% of the coelomic population (Adamowicz & Wojtaszek, 2001), and the effects of human cytokines on the large coelomocytes.

Mammalian IL-1 beta is produced by macrophages and induces the proliferation of macrophages and astrocytes, and their production of TNF-alpha and interleukin-6 (Mason, Suzuki, Chaplin & Matsushima, 2001). TNF- alpha is produced by mammalian macrophages and NK cells, and stimulates local inflammation, endothelial activation, expression of cellular adhesion molecules and cytokines, and cell death (Chandrasekharan et al., 2007). Mammalian TNF-alpha is also associated with both apoptosis and cell proliferation in various cells lines (Baxter, Kuo, Jupp, Vandenabeele, & MacEwan, 1999). GM-CSF inhibits the

growth of T cells, is involved in the differentiation of hemopoietic cells into granulocytes and macrophages, activates macrophages (Ponomarev, Shriver, Maresz, Pedras-Vasconcelos, Verthelyi, et al., 2007), and enhances neutrophil response to bacterial and yeast cell wall components (Kurt-Jones et al., 2002). Finally, IL-2 promotes growth of T cells and NK cells (Mostbock et al., 2008). These four cytokines induce signal transduction pathways, which are explained below.

TNF-alpha, IL-1 beta, IL-2, and GM-CSF, all play a significant role in signal transduction pathways. TNF-alpha and IL-1 beta can initiate the nuclear factor-kappaB (NF-kB) signaling pathway when they bind to TLRs or the IL-1 receptor (Kurt-Jones et al., 2002). NF-kB acts as a transcription factor that regulates expression of apoptotic, inflammatory, and immunity-related genes (Hongxiu, Masayuki, Marzenna, Yun, & Xin, 2006). In some cells, this results in the production and secretion of TNF-alpha and IL-1beta (Kurt-Jones et al., 2002). The classical pathway of NF-kB involves the binding of a signaling molecule to a receptor, for example, such as TNF-alpha binding to a TNF receptor 1 (TNF-R1). TRAF2 then binds to the death domain protein TRADD and the complex is recruited to the receptor (Takeuchi, Rothe, & Goeddel, 1996). The receptor-interacting protein (RIP) is also recruited in this process (Hongxiu et al., 2006). The beta subunit of the Ikb kinase (IKK) complex is activated and

subsequently phosphorylates I κ B proteins at the N terminus (Hayden & Ghosh, 2004). In the alternative pathway, IKK α becomes activated and phosphorylates I κ B proteins. The I κ Bs are ubiquitinated and degraded by ubiquitin ligase or are processed by a proteasome. The NF- κ B dimers are then able to proceed into the nucleus where they bind to certain portions of the promoter region of the target genes (Hayden & Ghosh, 2004).

Classical TLR4 signaling results in the NF- κ B pathway and involves the binding of a ligand to TLR4. The IL-1 receptor, or the IL-18 receptor, then draws MyD88 and IRAK to these receptors, leading to the phosphorylation of IRAK and the activation of TRAF6 (Haynes et al., 2001). NF- κ B is then able to enter the nucleus where it functions as a transcription factor. Only certain TLR signaling pathways, such as the classical TLR4 signaling pathway, are MyD88-dependent (Haynes et al., 2001).

In contrast to the NF- κ B signaling pathway initiated by TNF- α and IL-1 β , GM-CSF and IL-2 stimulate the JAK-STAT pathway, which relies on Janus kinases (JAK) and signal transducers and activators of transcription (STAT) (Arbouzova & Zeidler, 2006). This pathway involves the binding of a ligand to a transmembrane receptor, which activates the Janus kinases so that they can in turn phosphorylate

themselves (Arbouzova & Zeidler, 2006). This creates binding sites for the SH2 portion of cytoplasmic STATs, which are phosphorylated and dimerized after binding. They then move into the nucleus and bind to a portion of the promoter region of pathway-associated genes to initiate transcription of genes that regulate the apoptosis and MAP kinase signaling mechanisms, thus affecting the cell cycle and immune responses (Arbouzova & Zeidler, 2006; Aalinkeel et al., 2008). It has been shown that the JAK-STAT pathway components are molecularly and functionally conserved from the invertebrate *Drosophila* to humans to a high degree (Arbouzova & Zeidler, 2006).

There was a significant amount of conservation of the signaling pathways and signaling molecules among invertebrates and vertebrates; therefore, we hypothesized that large coelomocytes of *E. hortensis* would respond to human proinflammatory cytokines IL-1 beta, IL-2, GM-CSF, and TNF-alpha resulting in enhanced phagocytosis and induction of cell proliferation.

Materials and Methods

Reagents

All reagents were purchased from Fisher Scientific unless otherwise noted. Phosphate buffer saline (PBS) was purchased from Invitrogen.

Cell Culture

All cell culture reagents were purchased from Invitrogen unless otherwise noted. Dulbecco's Modified Eagle Medium (DMEM) was supplemented with 10% fetal calf serum, 100 mcg/mL ampicillin (Shelton Scientific), 10 mcg/mL kanamycin (Shelton Scientific), 10 mcg/mL tetracycline, 5 mcg/mL chloramphenicol (Fluka Biochemika), 1x penicillin/streptomycin/ amphotericin B, 1x nonessential amino acids and 1x L-glutamine (Invitrogen) to comprise Super DMEM (SDMEM).

Earthworm Husbandry

E. hortensis (European nightcrawlers) were purchased from Vermitechnology, Orange Lake, FL and kept at room temperature in the dark on autoclaved shredded paper moistened with water and Single Grain Rice Cereal or Rice with Bananas Cereal (Gerber) until use.

Extrusion of Coelomocytes

Prior to experimentation, earthworms were chosen based on their color and activity; earthworms with healthy deep coloration, lacking yellow appearance, and with high activity were placed overnight on paper towels pretreated with 2.5 mcg/mL Fungizone (Fisher Scientific) to reduce the level of fecal material and other surface contaminants. For proliferation assays involving longer *in vitro* culture times, povidone iodine (CVS pharmacy[®]) at 10% was also included on paper towels to

further reduce contamination.

To collect coelomocytes, earthworms were extruded according to Engelmann, Molnar, Palinkas and Cooper (2004). Briefly, earthworms were placed in a 100 mm Petri dish containing 3 mL Extrusion Buffer (71.2 mM NaCl, 5% v/v ethanol, 50.4 mM guaiacol-glyceryl-ether, 5 mM EGTA, pH 7.3). The coelomocytes were then transferred to 1 mL Accumax (Innovative Cell Technology) for a 5-minute incubation period at room temperature. Then the cells were washed with 5 mL Lubricus Balanced Salt Solution (LBSS, 71.5 mM NaCl, 0.3 mM NaH₂PO₄, 4.2 mM NaHCO₃, 4.8 mM KCl, 0.4 mM KH₂PO₄, 1.1 mM MgSO₄ x 7 H₂O, pH 7.3.), prior to centrifugation at 150x g for 5 minutes at 4°C. Enumeration of coelomocytes was determined using a hemocytometer.

Cytokines

All cytokines were reconstituted and stored according to vendors' recommendations. Table 1 illustrates the vendor, purity, endotoxin level, activity, and suggested concentration for biological effect (Human Cytokine/Growth Factor/Chemokines Sample Spec sheets, 2007) of each cytokine according to vendors' specifications in addition to the concentrations used for the phagocytosis assays. Expecting cross-species differences in affinity, concentrations were used above suggested ranges, and dose responses were tested to find the concentrations with the

strongest reactions. Concentration doses were optimized to maximize the effects in the phagocytosis and proliferation assays as detailed in Table 1.

Bacteria

E. coli HB101 transformed with pGLO (BioRad) and expressing green fluorescent protein (GFP) were grown on tryptic soy agar containing 100 mcg/mL ampicillin and 0.2% arabinose at 37°C for 24 hr. Cells were chemically fixed using 4% paraformaldehyde and washed three times with PBS. Centrifugation was carried out at 3273 x g for 5 minutes at 4°C, then the cells were resuspended in FACS flow (BD Bioscience) and enumerated using a hemocytometer. These cells hereafter are referred to as *E. coli*-GFP.

Table 1

Specifications of Cytokines

Cytokine	Supplier Catalog #	Purity	Endotoxin Level	Activity	Manufacturer's Recommended Conc.	Phagocytosis Conc. Low (Lo); High (Hi)	Proliferation Assay Conc.
Recombinant Human IL-1beta	R & D Systems/ 201-LB	> 97%	< 1.0 EU/ mcg	ED ₅₀ < 12 pg/mL	0.1 to 10.0 ng/mL	20; 40 ng/mL	40 ng/mL
Recombinant Human GM-CSF	ProSpec-TanyTechno Gene/ CYT-221A	> 98%	< 1.0 EU/ mcg	ED ₅₀ < 0.1 ng/mL	0.05 to 0.5 ng/mL	2; 4 ng/mL	2 ng/mL (assay 1), 8 ng/mL (assay 2)
Recombinant Human IL-2	ProSpec-TanyTechno Gene/ CYT-209	> 98%	< 1.0 EU/ mcg	ED ₅₀ < 0.0645 ng/mL	0.1 to 1.0 ng/mL	12.5; 25 ng/mL	25 ng/mL
Recombinant Human TNF-alpha	ProSpec-TanyTechno Gene/ CYT-223	> 98%	< 1.0 EU/ mcg	ED ₅₀ < 0.05 ng/mL	0.05 to 20.0 ng/mL	2.5; 5 ng/mL	5 ng/mL

Phagocytosis Assay

Coelomocytes were pretreated with cytokine at the concentrations specified in Table 1 at 20°C, 5% CO₂, for 21 hours. Following cytokine treatment, phagocytosis assays were performed using SDMEM and included 20,000–50,000 coelomocytes/well (depending on yield) with an m.o.i. of 1000 bacteria: 1 effector cell per well in triplicates. To control for nonspecific binding of *E. coli* to the external surface of coelomocytes, cytochalasin B was included in the assay at 5 mcM, 30 minutes before the addition of *E.coli*-GFP. Table 2 illustrates the varied incubation time with *E. coli* among assays at a constant temperature of 30°C.

Table 2

Incubation Time With E. coli in Phagocytosis Assays

Assay 1	Assay 2	Assay 3	Assay 4	Synergy Assay 1	Synergy Assay 2	Synergy Assay 3
3 hours	2 hours	1.5 hours	1 hour	2 hours	1 hour	1 hour

Note. Coelomocytes from *E. hortensis* were incubated with *E.coli* in the phagocytosis assays for various amounts of time depending on the assay.

Following *E. coli*-GFP uptake, trypan blue was used at a final concentration of 0.02% for 30 minutes at room temperature in the dark, for quenching purposes to reduce background fluorescence (Mosiman, Patterson, Canterero & Goolsby, 1997). The cells were centrifuged at 340 g for 5 minutes at 4°C, washed with PBS, and then resuspended in FACS Flow (BD Bioscience) for analysis on a FACSCalibur flow cytometer.

Proliferation Assay

All assays were performed using SDMEM and assay 2 was supplemented with 0.36 mg/mL Nystatin (MP Biomedicals) to reduce fungal contamination and permit longer incubation times. All assays included 50,000 coelomocytes per relevant well in duplicate. Extrusion was performed as mentioned above, and after the coelomocytes were added to the appropriate wells, they were incubated in the presence or absence of cytokines at the concentrations specified in Table 1 for a specified incubation time at 20°C, 5% CO₂. In one assay, coelomocytes from each earthworm were incubated for 24 hours or 48 hours with GM-CSF. In a separate assay, coelomocytes were incubated for 48 hours at 20°C, 5% CO₂ with IL-1 beta, IL-2, GM-CSF, or TNF-alpha. Control coelomocytes were incubated in the absence of the cytokines under the same conditions.

After incubation with cytokines, the coelomocytes were washed twice with PBS by centrifugation at 150 x g, 4°C for 5 minutes, and then resuspended in 200 mcL SDMEM. The cells were then chemically fixed by slowly adding 1.8 mL ice-cold 70% ethanol while gently vortexing. Samples were stored at 4°C for 1 to 2 days until analyzed. The cells were pelleted by centrifugation before adding 0.2 mL propidium iodide (PI) cocktail (0.1% NP₄₀, 200 mcg/mL RNase A, 200 mcg/mL PI) and

incubating at 37°C for 15 minutes. The cells were then placed on ice and analyzed on a FACSCalibur flow cytometer (BD Biosciences).

Flow Cytometry

For the phagocytosis assay, fluorescence was measured using a FACSCalibur flow cytometer and Cell Quest Software (BD Biosciences) with the following specifications: Forward Scatter (FSC) E00 Linear, Side Scatter (SSC) 332v Linear, FL-1 312v Log. Cells with granularity and size corresponding to the large coelomocyte population were analyzed further using WinList 5.0 (Verity Software House, Inc.) and Cell Quest Software. Percent specific phagocytosis was determined by subtracting the auto-fluorescent background of the control from the sample fluorescence measured by the FL-1-detector.

Proliferation was determined by selecting the relevant coelomocyte populations (R1), then gating on the PI-positive (FL-2) cells with the appropriate width and area to omit doublets and debris from the analysis (R2). Regions corresponding to G₀/G₁ (R3), S (R4), and G₂/M (R5), were then set for quantitation of cells in different stages of the cell cycle.

Statistical Analysis

All data were collected and analyzed using Cell Quest software and WinList 5.0. All graphs and data analysis were created and processed using Microsoft Excel. Only statistically relevant results with $p \leq 0.05$ as

defined by the researcher's *t* test were reported when comparing untreated with cytokine-treated samples. All histograms and dot plots were created using WinList 5.0.

Results

Phagocytosis Assay

Figure 1 illustrates a typical profile of earthworm coelomocytes measuring forward scatter (FSC) versus side scatter (SSC) properties. Three characteristic populations were observed and three regions (R1, R2, and R3) were drawn around the subpopulation corresponding to the chloragocytes, large coelomocytes, and small coelomocytes, respectively.

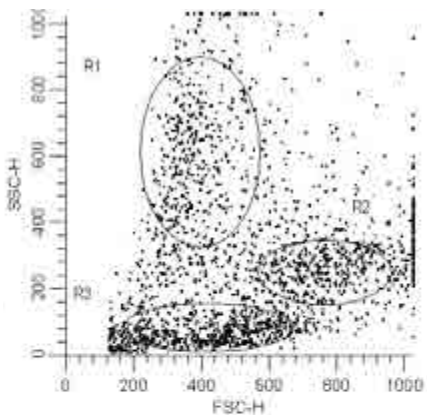


Figure 1. Typical scatter profile of earthworm coelomocytes using flow cytometry. A two-parameter dot plot measuring forward scatter (FSC) versus side scatter (SSC) of a typical earthworm coelomocyte population after extrusion and incubation overnight in SDMEM medium is shown. Three distinct populations are indicated; region 1 (R1) represents the chloragocytes, region 2 (R2) represents the large coelomocytes, and region 3 (R3) represents the small coelomocytes.

Percent specific phagocytosis was determined for each earthworm treated with IL-1 beta, GM-CSF, IL-2, and TNF-alpha. Dot plots were based on forward scatter (FSC) versus fluorescence (FL-1) and gated on the R2 subpopulation illustrated in Figure 1. Each dot plot was divided into four quadrants (upper left, upper right, lower left, and lower right) with the lower right corresponding to coelomocytes that did not phagocytose fluorescent *E. coli*, and the upper right depicting those coelomocytes that did. Figure 2A shows the large coelomocytes incubated in the absence of *E. coli*-GFP illustrating the level of background autofluorescence observed. Figure 2B shows the large coelomocytes incubated with *E. coli*-GFP together with cytochalasin B, an inhibitor of phagocytosis. This control was important to exclude the possibility of nonspecific binding of *E. coli*-GFP to the cell surface of the large coelomocytes and provided background values that were later subtracted from the experimental values to obtain percentage specific phagocytosis. Percent specific phagocytosis was calculated by averaging the percent gated events of triplicate samples in the upper right quadrants and subtracting the autofluorescent background (Figure 2B) from the samples treated with only *E. coli*-GFP (Figure 2C), or treated with *E. coli*-GFP plus cytokine (Figure 2D).

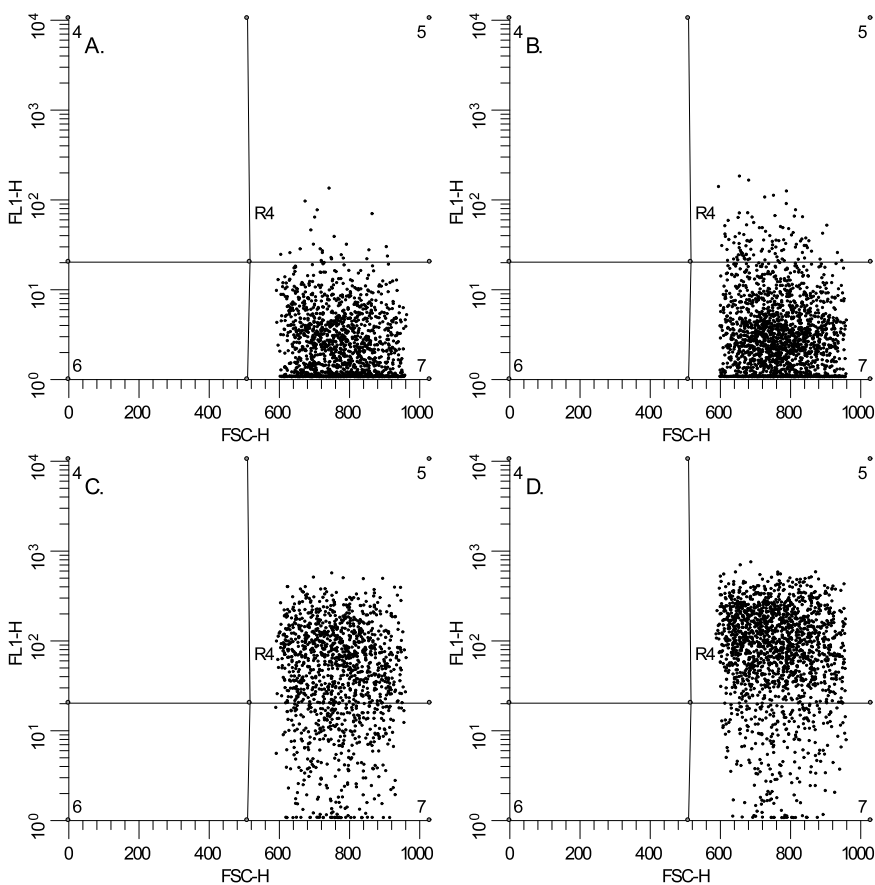


Figure 2. Characteristic profile of large coelomocytes before and after phagocytosis and cytokine treatment. Two dimensional dot plots measuring forward scatter (FSC) on the x axis versus green fluorescence (FL-1) on the y axis were generated after gating on the R2 subpopulation (large coelomocytes) indicated in Figure 1. (A) represents large coelomocytes in the absence of both *E. coli*-GFP and cytokines illustrating the level of auto-fluorescent background; (B) represents the large coelomocytes in the presence of *E. coli*-GFP plus cytochalasin B, an antibiotic which interferes with microfilament activity; (C) represents the large coelomocytes in the presence of *E. coli*-GFP only; and (D) represents the large coelomocytes in the presence of *E. coli*-GFP plus TNF-alpha hi.

Four worms out of 38 responded to IL-1beta at the “hi concentration” (as described in Materials and Methods section), and 1

worm out of 4 responded at the “lo concentration” with a statistically significant increase in phagocytosis (Figure 3).

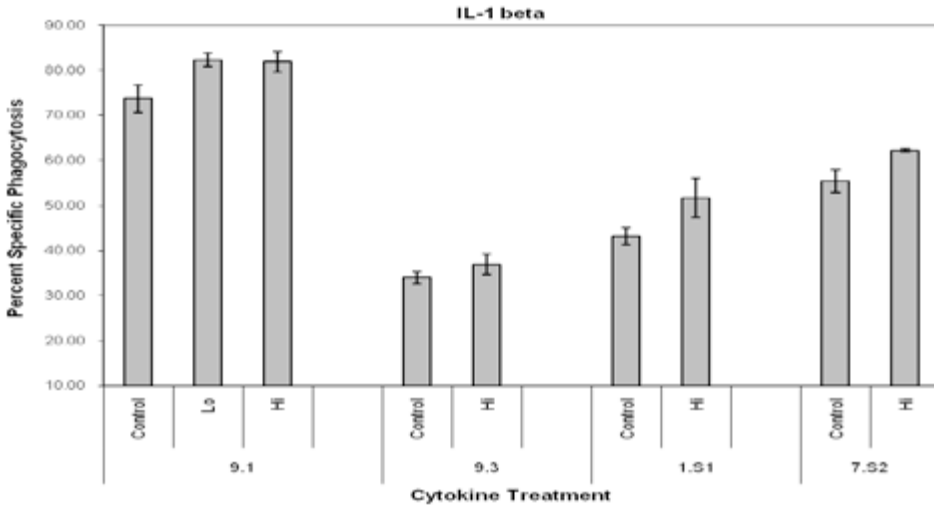


Figure 3. Effects of IL-1 beta on phagocytosis in *Eisenia hortensis* large coelomocytes. Percent specific phagocytosis is shown for large coelomocytes incubated with *E. coli*-GFP in the absence (control) or presence of cytokine at the indicated concentration. (See Methods for concentrations of IL-1 beta). Percent specific phagocytosis represents an increase in relative fluorescence intensity for FL1 minus the background autofluorescence. Error bars represent +/- standard deviation. Only test samples with $p \leq 0.05$ are illustrated.

Two worms out of 4 responded to GM-CSF at the “hi concentration,” and 7 worms out of 38 responded at the “lo concentration” with a statistically significant increase in phagocytosis (Figure 4). Five worms out of 29 responded to IL-2 at the “hi concentration,” and 2 worms out of 4 responded at the “lo concentration” with a statistically significant increase in phagocytosis (Figure 5).

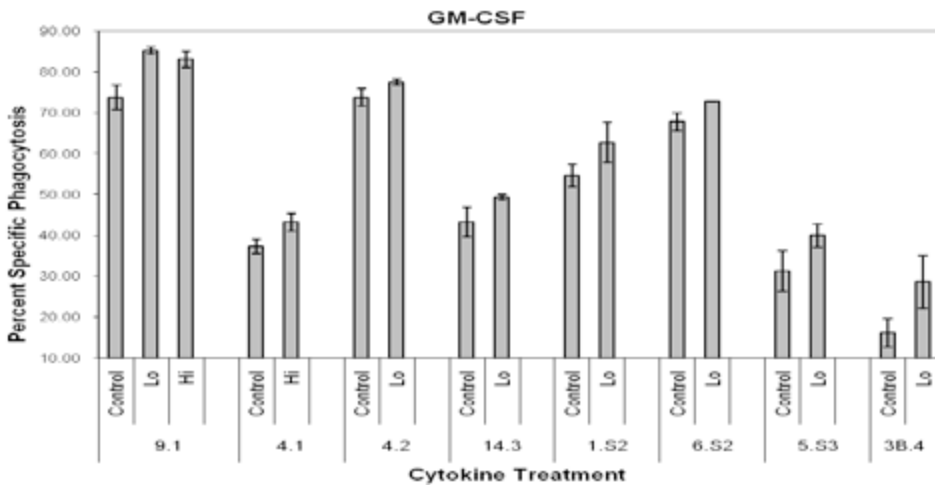


Figure 4. Effects of GM-CSF on phagocytosis in *Eisenia hortensis* large coelomocytes. Percent specific phagocytosis is shown for large coelomocytes incubated with *E. coli*-GFP in the absence (control) or presence of cytokine at the indicated concentration. (See Methods for concentrations of GM-CSF). Percent specific phagocytosis represents an increase in relative fluorescence intensity for FL1 minus the background autofluorescence. Error bars represent +/- standard deviation. Only test samples with $p \leq 0.05$ are illustrated.

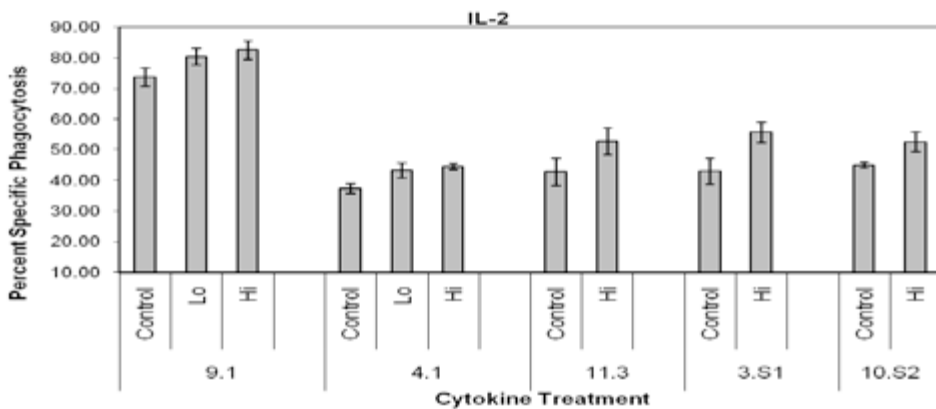


Figure 5. Effects of IL-2 on phagocytosis in *Eisenia hortensis* large coelomocytes. Percent specific phagocytosis is shown for large coelomocytes incubated with *E. coli*-GFP in the absence (control) or presence of cytokine at the indicated concentration. (See Methods for concentrations of IL-2). Percent specific phagocytosis represents an increase in relative fluorescence intensity for FL1 minus the background auto-fluorescence. Error bars represent +/- standard deviation. Only test samples with $p \leq 0.05$ are illustrated.

Finally, 6 worms out of 29 responded to TNF-alpha at the “hi concentration,” and 3 out of 10 responded at the “lo concentration” with a statistically significant increase in phagocytosis (Figure 6).

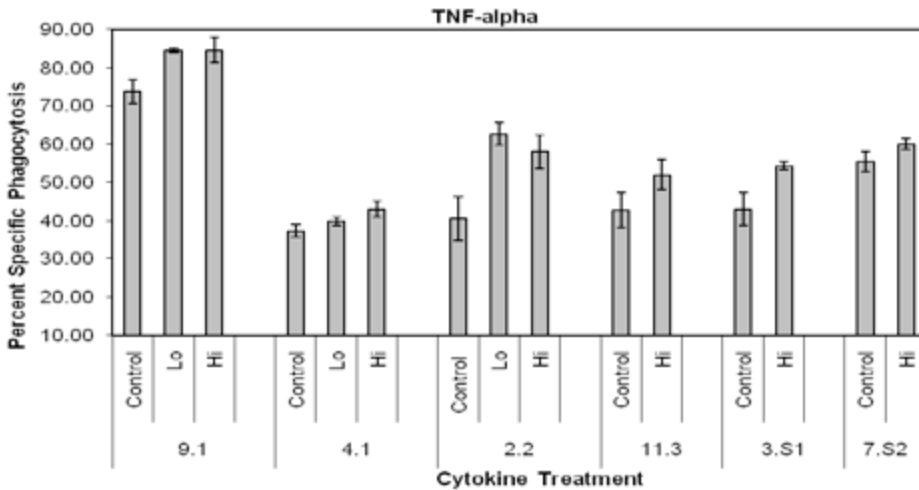


Figure 6. Effects of TNF-alpha on phagocytosis in *Eisenia hortensis* large coelomocytes. Percent specific phagocytosis is shown for large coelomocytes incubated with *E. coli*-GFP in the absence (control) or presence of cytokine at the indicated concentration. (See Methods for concentrations of TNF-alpha). Percent specific phagocytosis represents an increase in relative fluorescence intensity for FL1 minus the background auto-fluorescence. Error bars represent +/- standard deviation. Only test samples with $p \leq 0.05$ are illustrated.

Table 3 summarizes the statistically significant responses obtained for phagocytosis assays. These results show an overall increase in phagocytosis in 10.5 – 50% of cases.

Table 3

Percent Response of Earthworms for Each Cytokine

Number of worms exhibiting statistically significant response to cytokine compared to untreated controls	Cytokine tested and concentration	Percentage of Response
4/38	IL-1 beta hi (40 ng/mL)	10.5%
1/4	IL-1 beta lo (20 ng/mL)	25.0%
2/4	GM-CSF hi (4 ng/mL)	50.0%
7/38	GM-CSF lo (2 ng/mL)	18.4%
5/29	IL-2 hi (25 ng/mL)	17.2%
2/4	IL-2 lo (12.5 ng/mL)	50.0%
6/29	TNF-alpha hi (5 ng/mL)	20.7%
3/10	TNF-alpha lo (2.5 ng/mL)	30.0%

After incubation of the coelomocytes with the cytokines and *E. coli*, the samples were analyzed on a FACSCaliber flowcytometer and the responses by each earthworm to the cytokines were determined. The left column of the table displays the ratio of earthworms exhibiting statistically significant responses to the total number of earthworms incubated with that cytokine at the specified concentration. The middle column shows the cytokine being tested and its concentration. The right column displays the percentage corresponding to the ratio in the left column.

Proliferation Assay

Cell cycle analysis was performed to investigate the effects of the cytokines on cell proliferation by measuring the amount of DNA per cell. Propidium iodide is a highly sensitive indication of cell division because of its high binding affinity to double stranded nucleic acids.

RNase-A treated coelomocyte populations ensured that only DNA was included in the final flow cytometric analysis of nucleic acid content using the FL-2 detector. Furthermore, gates and regions were used to eliminate aggregates and debris (Figure 7, left column), to exclude doublets (Figure 7, middle column), and to divide histograms according to relative DNA amounts, that is, G₀/G₁ (R3), S (R4), and G₂/M (R5) (Figure 7, right column).

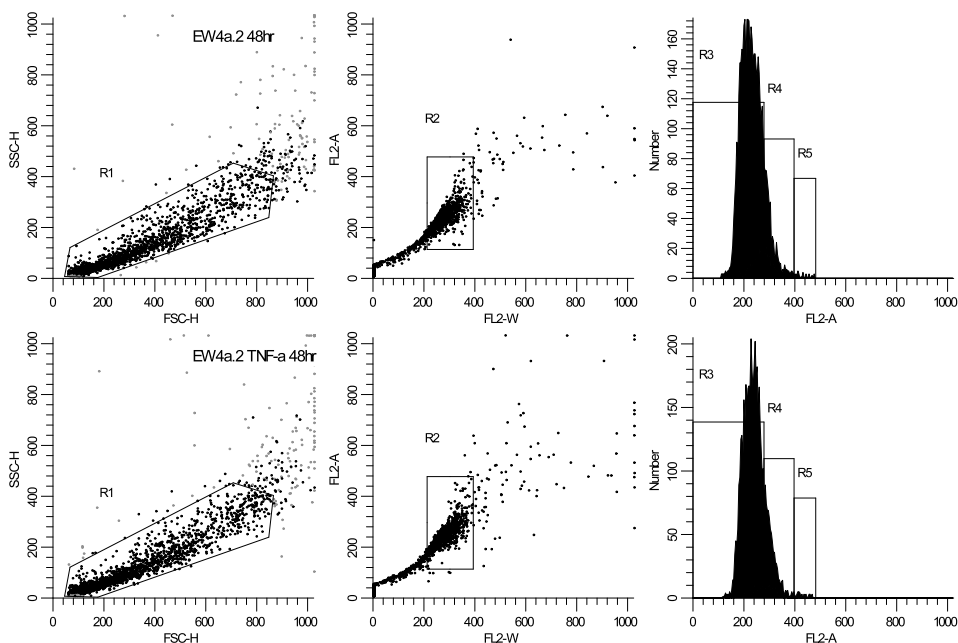


Figure 7. Multigraph of enhanced proliferation by TNF-alpha in *Eisenia hortensis* coelomocyte population in proliferation assay 2. These histograms and dot plots were generated on WinList 5.0 using the flow cytometric data collected for earthworm 4a after a 48 hr incubation at 20°C, 5% CO₂ in the absence and presence of TNF-alpha (5 ng/mL). A dot plot was generated measuring forward scatter (FSC) versus side scatter (SSC) and a region (R1) was drawn to encompass cells with similar sizes and granularities to those of small and large coelomocytes (left column). A dot plot based on FL-2-width versus FL-2-area was then gated on the R1 subpopulation (middle column). Another region was drawn on this dot plot to encompass events approximately the same fluorescence peak width and area as single cells to exclude doublets and debris from the final analysis. Finally, a single parameter histogram based on FL-2-area (FL2-A) was generated and gated on R2. Three additive regions were created, R3, R4, and R5 (right column). R3 included cells with FL2 areas associated with cells normally in G₀/G₁, R4 included cells with FL-2 areas associated with cells normally in S, and R5 included cells with FL2 areas associated with cells normally in G₂/M.

Out of 12 earthworms tested in the first assay, none of the coelomocyte populations showed a statistically significant increase in

proliferation in the presence of GM-CSF compared to control. In the second proliferation assay, 12 additional earthworms were used, expanding the 48-hour cytokine treatment to include IL-1 beta, IL-2, GM-CSF, and TNF-alpha. In this assay, only one earthworm, (Earthworm 4a), exhibited enhanced proliferation in the presence of TNF-alpha compared to the control (Figure 7).

After the 48-hour incubation in the absence of TNF-alpha, coelomocytes of Earthworm 4a showed an 81.99% increase in G₀/G₁, 17.76% in S, and 0.25% in G₂/M. In contrast, when these coelomocytes were treated with TNF-alpha, a stimulatory effect was observed with an increase in S and G₂/M to 22.84% and 0.53%, respectively (Figure 7, right column). This difference is statistically significant.

Discussion

Phagocytosis Assay

Our results show that the proinflammatory cytokines, IL-1 beta, IL-2, TNF-alpha, and GM-CSF significantly increased the levels of phagocytosis of the large coelomocytes in 10.5–25% of cases for IL-1 beta, 18.4–50% for GM-CSF, 17.2–50% for IL-2, and 20.7–30% for TNF-alpha, depending on the cytokine concentration used.

In addition, we tested the effects of GM-CSF together with IL-1 beta on phagocytosis and preliminary evidence (data not shown) suggests

a synergistic effect as seen by enhanced phagocytosis. These results need to include a larger number of respondents to verify synergistic effects of proinflammatory cytokines on phagocytosis. Future studies may include employing a wider variety of stimuli and combinations.

As previously mentioned, innate immune cells can utilize PRRs to recognize chemical entities that are common to different classes of pathogens. Perhaps the enhancement of phagocytosis observed in our experiments is due to the binding of cytokines to receptors, which stimulates the upregulation of PRRs resulting in higher levels of bacteria being phagocytosed due to elevated levels of ligand receptors. It would be interesting to carry out a microarray analysis, or generate a cDNA library to observe the effects of human proinflammatory cytokines on the coelomocytes at the molecular level.

Proliferation Assay

Proliferation assay 1 was performed to determine whether the effects of GM-CSF on cell proliferation were more pronounced after incubation for 24 or 48 hr. The samples in assay 2 were incubated with cytokine for 48 hours to provide sufficient time for any effects to be observed. Only 1 of the 24 worms from both assays exhibited cell proliferation that was statistically significant; therefore, our results suggest that IL-1 beta, TNF-alpha, IL-2, and GM-CSF do not enhance cell

proliferation significantly in *E. hortensis* coelomocytes under the conditions used in this study.

It is possible that a much longer incubation time or increased incubation temperature is needed to see an effect. Problems with fungal and bacterial contamination, despite the inclusion of a large number of antimicrobials in the culture medium, restricted our incubation period to a maximum of 48 hours. It would be worthwhile to explore different combinations of antimicrobials to determine which would be more effective in reducing contaminants. In addition, plans include using isolation techniques to enrich the different coelomocyte subpopulations, and this might lead to conditions warranting longer incubation periods that could reveal proliferation responses to these cytokines.

Future research could investigate the signaling pathways associated with phagocytosis and proliferation that are induced by cytokines or invertebrate monokines, for example TLR and MyD88 signaling for phagocytosis of prokaryotic cells (Shin et al., 2008). In the same way, further insight into the evolution of immunity from invertebrates to vertebrates could be achieved. It would also be desirable to understand better the mechanisms underlying phagocytic and cell cycling processes and their physiologic functions, and the effects of cytokines on apoptosis.

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The Identification of Downstream Signaling Partners to PTHrP in the Mammary Gland

Allison Superneau and Ashley Mayer

Preface

Serving as Ms. Allison Superneau's undergraduate research advisor, I have had the privilege of getting to know her work quite well over the last several years. As such, I can speak directly to the strong commitment she has made to her studies and the high quality of her research.

About a year and a half ago, Allison took the initiative to get involved in one of my ongoing research projects involving understanding more about genes that play a role in mouse mammary gland development. Our goal is to identify several candidate genes whose aberrant expression leads to abnormal mammary gland development in mice as a tool to understand human counterpart genes potentially involved in abnormal human mammary gland development, such as breast cancer. Previous studies in mice have shown that the parathyroid hormone-related protein (PTHrP) is necessary for normal mammary gland development. Overexpression of PTHrP in the mouse leads to severe defects in mammary gland development during puberty, a period of intense estrogen induced growth. It is our hypothesis that PTHrP regulates pubertal growth

of the mammary gland by modulating the expression and activation of growth factors in response to estrogen.

To learn more about PTHrP's role in mammary development, Allison conducted differential gene expression experiments using adolescent wild-type and PTHrP-overexpressing mice to determine PTHrP effects on the expression levels of other genes. She has identified several candidate genes that are expressed at higher levels in transgenic mice. Allison has identified one gene in particular, a transcription factor called *rtfl* that is expressed at a much higher level in transgenic mice than in wild-type mice. She confirmed her results using a real-time PCR approach. Allison is currently conducting experiments to explore further the effect of PTHrP on the expression of *rtfl* in the mammary gland and its role in normal mammary gland development. That Allison is capable of designing and conducting her own research with my supervision is indeed one aim all faculty who engage with their students in undergraduate research share.

To say that Allison has made tremendous strides in her project over the past year is to understate the case. Allison not only mastered high-level technical skills, but also has become a teacher of these skills to other undergraduates. Her lab work required her to clone genes by reverse-transcriptase PCR, perform automated DNA sequencing and master real-

time PCR. To accomplish her research goals, she took the initiative to master both automated DNA sequencing and Real-time PCR with very little guidance from me. As a result of all these efforts, Allison presented her mammary gland work at the 2007 National Conference on Undergraduate Research (NCUR) Symposium, and was first author of a paper published in the *Journal of the Proceedings of the NCUR*. Allison also presented her work at the inaugural Pennsylvania Capitol Poster on the Hill event this past October 2007 in Harrisburg as well as presented her work at December 2007 American Society for Cell Biology annual meeting in Washington, DC.

As you can see, Allison has already contributed significantly to an important project in gene research. She has already published a paper on her work and plans on publishing at least one more as she is progressing very rapidly in understanding the roles of other genes in mouse mammary gland development. Allison's plans are to enroll in Medical School. I have the utmost confidence that Allison is going to be a great medical doctor some day.

Faculty Mentor – David Dunbar, Associate Professor of Biology, Cabrini College

Faculty Advisor – Dr. Maureen Dunbar, Pennsylvania State University – Berks

Abstract

Although mammary development is well defined structurally, not much is currently known regarding the molecules that are involved in regulating its development. It is important to identify the genes involved in mammary development, as these genes may also play a role in the development of breast cancer. Previous studies have shown that the parathyroid hormone-related protein (PTHrP) is necessary for normal mammary gland development. Overexpression of PTHrP in the mouse leads to severe defects in mammary development during puberty, a period of intense estrogen-induced growth. It is our hypothesis that PTHrP regulates pubertal growth of the mammary gland by modulating the expression and activation of growth factors in response to estrogen. In an attempt to learn more about PTHrP's role in mammary development, we set out to examine the differential gene expression in adolescent wild-type and PTHrP-overexpressing mice. For these experiments, RNA was isolated from both wild-type mice and transgenic mice and reverse transcribed into cDNA. The cDNAs were then amplified using arbitrary primers from the GeneFishing™ kit. This kit allows the comparison of differentially expressed genes in two different samples. Using this technique, several cDNAs have been identified that appear to be differentially expressed in mammary glands from wild-type and PTHrP-overexpressing mice. Of particular interest is a cDNA that has been identified as the transcription factor Rtf1. This transcription factor has been shown to be expressed in the mammary gland during puberty and appears to play an important role in its development. Experiments are currently ongoing to explore further the effect of PTHrP on the expression of Rtf1 in the mammary gland. These results should help to elucidate the role of PTHrP in regulating estrogen-induced growth in the mammary gland. Keywords: PTHrP, mammary development, epithelial-mesenchymal interacti

Introduction

The mammary gland is the key defining organ of mammals. Its function in female mammals is in the production and secretion of milk during lactation. The mature mammary gland is composed of a series of epithelial ducts that are embedded in a cushion of fat, called the mammary

fat pad. Although much is known concerning the structural features of mammary development, little is currently known regarding the molecules that are necessary for its development. Understanding how the mammary gland develops, as well as the genes and molecules that are involved, not only allows a greater insight into the development of the mammary gland but also into the intricacies of breast cancer.

Mammary gland development in mice takes place in three distinct phases: embryonic, pubertal, and pregnancy and lactation. Development begins on embryonic day 10 (E10) when five pairs of mammary buds are formed.^{6,7} The buds are made up of a group of epithelial cells that are surrounded by a dense mammary mesenchyme.⁶ By E16 the rudimentary mammary ductal tree is generated by the elongation and initial branching morphogenesis of the mammary epithelium into the mammary fat pad precursor.^{6,7} By the time of birth, the mammary gland consists of a primary duct with 15–20 secondary ducts. Little to no further development occurs until the time of puberty, which in mice is at around 3–4 weeks of age. Under the influence of estrogen and progesterone, the distal ends of the epithelial ducts form specialized structures called terminal end buds (TEBs), which serve as the sites of active cellular proliferation (Figure 1). Ductal growth continues during puberty until the epithelial ducts reach the border of the fat pad at which time the TEBs deteriorate.^{3,6,9,15} The

mammary gland does not complete its development until pregnancy and lactation, when the epithelial ducts form specialized structures called lobuloalveolar structures that are the sites of milk production during lactation. When the pups are weaned and lactation ceases, the lobuloalveolar structures die as result of massive apoptosis, a process known as involution. At the end of this stage of development, the ductal system will resemble its appearance before pregnancy and will remain this way until pregnancy occurs again.^{3, 6, 9, 15}

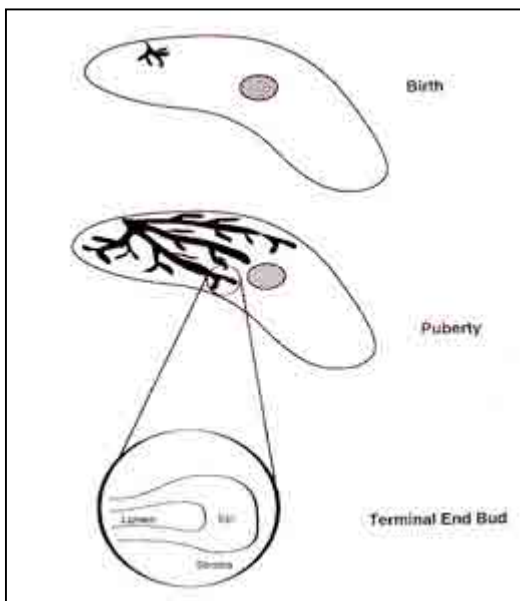


Figure 1. Schematic diagram illustrating mouse mammary development during puberty.

Mammary development relies on the sequential and reciprocal exchange of soluble factors between the epithelial cells and the surrounding mesenchymal and stromal cells.⁸ In fact, at all stages of its

life cycle, mammary development is regulated by systemic hormones as well as local epithelial-mesenchymal and stromal interactions. The effects of hormones on mammary development are in part modulated by the production of paracrine growth factors. One such growth factor is parathyroid hormone-related protein, or PTHrP.¹⁴

PTHrP is a normal product of mammary epithelial cells, and several previous studies have implicated a critical role for this protein in mammary development.^{4,5} Previous studies have demonstrated that PTHrP signaling is absolutely necessary for embryonic mammary development. If the PTHrP gene is removed from mice, the mammary epithelial buds form, but never develop into the rudimentary ductal tree.^{2,7,14} Overexpression of PTHrP in mammary epithelial cells results in severe impairments in ductal development during puberty.^{5,6} Not only was ductal proliferation inhibited in these mice, the degree of ductal branching was also significantly reduced. These two components led to ductal outgrowth into the fat pad being delayed, which resulted in the appearance of a simpler duct system in transgenic mice as opposed to wild-type mice. It is thought that PTHrP continues to function in the signal interactions after birth as a way to help regulate further ductal branching morphogenesis by regulating the proliferation of the terminal end buds.^{4,5}

During all stages of its development, PTHrP is expressed

predominantly in the epithelial cells of the mammary bud. While its receptor, the type I PTH/PTHrP receptor (PPR1), was found to be expressed in the mesenchymal cells and continued its expression in the stromal cells after the initial round of ductal branching in the fat pad.^{4, 5, 14} These results suggest that PTHrP is acting as a paracrine factor made by the mammary epithelial cells which signals through PPR1 on the neighboring stromal cells to regulate ductal growth during puberty.¹⁴ Therefore, the hypothesis is that PTHrP regulates pubertal growth of the mammary gland by modulating the expression and activation of growth factors in the mammary gland during puberty.

The purpose of the present study is to determine the molecules that may act in concert with PTHrP to regulate ductal development during puberty. To this end, the differential gene expression in mammary glands from adolescent wild-type and PTHrP-overexpressing mice was examined. Here the identification of several genes whose products might act downstream of PTHrP in regulating ductal proliferation of the mammary gland during puberty is presented.

Materials and Methods

RNA Isolation

For RNA isolation, the #5 inguinal mammary glands were harvested from 5-week-old, virgin, wild-type and PTHrP-overexpressing

(K14-PTHrP) mice and immediately flash frozen on dry ice. Total RNA was isolated using the RNeasy Lipid Tissue RNA kit from Qiagen (cat. #74804). To ensure complete removal of genomic DNA, an on-column DNase digestion was performed using the RNase-free DNase set from Qiagen (cat. #79254). RNA quality was assessed by agarose gel electrophoresis.

Differential Gene Expression Analysis

Differential gene expression analysis was performed using the GeneFishing™ DEG 101 and 102, and 103 and 104 Premix Kits according to the protocols outlined in the kit (SEEGENE Pharmaceuticals cat.# K1021 and K1022). Reverse transcription reactions were performed on 3 micrograms of total RNA isolated from either wild-type or PTHrP transgenic mice using M-MLV reverse transcriptase and the appropriate primer to make cDNA specifically from poly A containing mRNA. The cDNA was used in PCR reactions using the designated primer combinations as specified in the kit. The PCR conditions were as follows: 94°C for 5 minutes (one cycle), 50°C for 3 minutes (one cycle), then 94°C for 40 seconds, 65°C for 40 seconds, 72°C for 40 seconds (thirty cycles). This was followed by 72°C for 5 minutes using a Biorad I Cycler PCR machine.

Molecular Cloning

The PCR products from differential gene expression analysis were resolved and gel purified from 2% agarose gels using the QIAquick Gel Extraction kit (Qiagen cat.# 28704). Purified PCR fragments were cloned using the Invitrogen TOPO TA-Cloning Kit for Sequencing. Bacterial transformations were conducted using chemically competent *E. coli* according to the manufacturer's directions. Transformed bacteria were spread onto LB plates containing 50 micrograms/mL ampicillin and incubated overnight at 37°C. Ampicillin-resistant colonies were grown in 2 mLs of Magnificent Broth liquid growth media (MacConnell Research) in the presence of 50 micrograms/mL ampicillin overnight at 37°C in a shaking incubator. Plasmids were isolated from overnight grown cultures and purified using the MacConnell Research mini-Prep 96 machine according to the manufacturer's directions. Verification of plasmids containing PCR inserts was performed by plasmid EcoR1 digests and 2% agarose gel electrophoresis.

DNA Sequence Analysis

DNA sequencing reactions on selected plasmids containing inserts were conducted using the SequiTherm EXCEL II DNA Sequencing Kit-LC by Epicenter Biotechnologies using an IRD700-labeled M13 forward primer and an IRD800-labeled M13 reverse primer. The PCR conditions

were 95°C for 2 minutes (one cycle), then 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 45 seconds (thirty cycles) followed by 72°C for 5 minutes using a Biorad I Cycler PCR machine. DNA sequence data analysis was performed using a LICOR 4300 Automated DNA Sequencer.

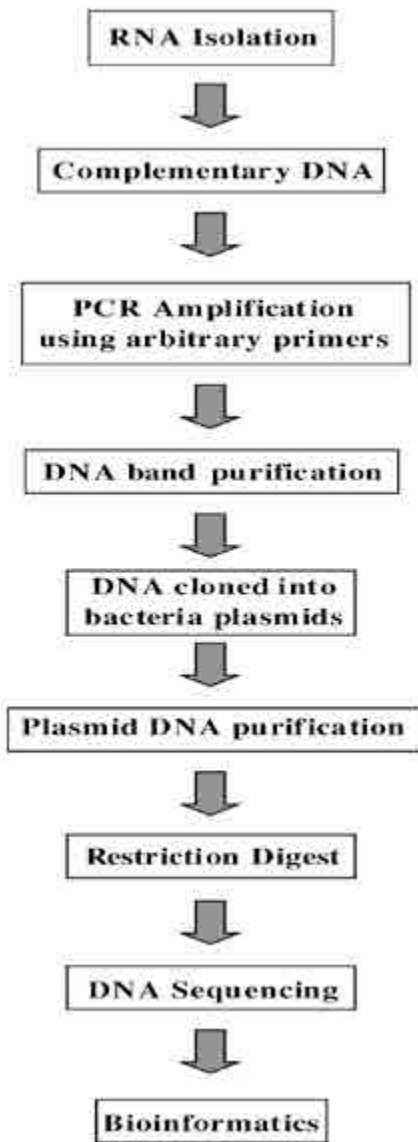


Figure 2. Flow chart of methods.

Results

As a first step to determine which gene(s) potentially affect gross mammary gland development in PTHrP-overexpressing mice, we performed the GeneFishingTM method of differential display developed by See-Gen Inc. This approach utilizes a series of random oligonucleotide primers, used in succession, to identify differentially expressed genes in multiple samples (Figure 2). For these experiments, total RNA from mammary glands of 5-week-old, wild-type and PTHrP-overexpressing mice was isolated using the Lipid Tissue RNeasy kit from Qiagen. This kit was found to be absolutely necessary to purify intact, high quality RNA for these experiments, as the mammary gland has a very high percentage of fat tissue. To ensure RNA quality, 5 micrograms of total RNA from both sources was resolved on a 1% agarose gel (Figure 3). Approximately 80% of cellular total RNA is ribosomal-RNA; thus, intact ribosomal RNA is indicative of RNA integrity of other RNA classes as well (such as mature RNA). Figure 3 demonstrates the presence of intact large subunit ribosomal (LSR) RNA and small subunit ribosomal (SSR) RNA. This analysis was also performed to determine whether there was any contaminating genomic DNA in the RNA preparation, as this could interfere with differential gene expression analysis resulting in false positives. As shown in Figure 3, no high molecular weight genomic DNA

was present in the RNA samples.

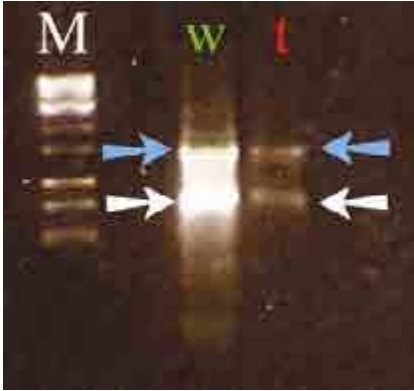


Figure 3. Total RNA isolated from the mammary glands of both wild-type and PTHrP-transgenic mice.

In Figure 3, approximately 5 micrograms of total RNA was resolved on a 1% agarose gel and stained with ethidium bromide. Lane 1: DNA molecular weight marker. Lane 2: Total RNA from wildtype mice mammary glands. Lane 3: Total RNA from PTHrP-transgenic mice mammary glands. The blue arrows refer to large subunit ribosomal RNA, while the white arrows refer to the small subunit ribosomal RNA.

Using the GeneFishingTM method, total RNA was used as a template for reverse transcription PCR to generate complementary DNA (cDNA). The cDNAs were then amplified by PCR using random oligonucleotide primers producing different sized double-stranded PCR products (see Figure 2). Each double-stranded PCR products represents a gene that is expressed in the different samples. The PCR products were

then resolved on 2% agarose gels to reveal differences in gene products in wild-type and PTHrP-transgenic mice. The results in Figure 4 show a representative gel of the differential gene expression analysis of mammary gland RNA from wild-type and PTHrP-overexpressing mice. Note that, for most primer sets, the intensity of the amplified products were nearly identical in wild-type and transgenic samples, indicating that the amount of PCR-amplified DNA in both wild-type and PTHrP-transgenic wells was equivalent. This suggests that the amount of starting material in each sample was equivalent. However, Figure 4 also shows several PCR products that appear either more intense in the wild-type sample (primer 22 and 35 sets) or more intense in the transgenic sample (primer 21 and 36 sets). Overall, several bands were identified that might represent differentially expressed gene products.

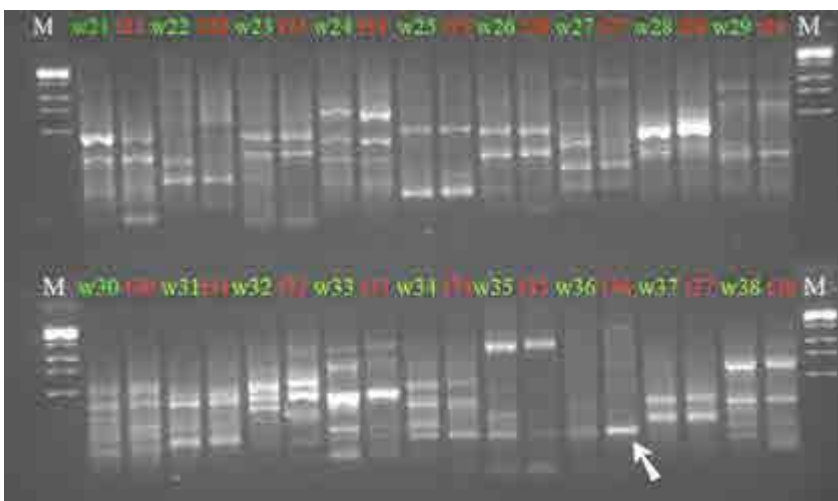


Figure 4. Amplified DNA from wild-type and PTHrP-transgenic mice.

Figure 4 shows a 2% agarose gel stained with SYBR Safe DNA gel stain showing PCR amplified DNA fragments using random primers 21–38. This step was performed using the GeneFishingTM technique. The amplified DNA is from wild-type mice (labeled in green) and PTHrP-transgenic mice (labeled in red). Lanes marked “M” are DNA molecular weight markers. The DNA fragment indicated by the arrow in the T36 lane was gel purified, cloned, and sequenced.

In theory, the GeneFishingTM technique should identify genes whose expression differs by only a small percent. However, because of the potential for a high number of false positives, it was initially decided to focus only on those bands that were markedly different in either wild-type or PTHrP-overexpressing samples (Figure 4). Using these criteria, one specific potentially differentially expressed gene was identified using primer 36. This band from transgenic mice (lane t36) was gel-purified, cloned into an appropriate DNA sequencing vector and transformed into *E. coli* using established procedures outlined in the Materials and Methods section. Determination of plasmids containing inserts was conducted by resolving EcoR1-digested plasmids and resolving the digested plasmids on a 2% agarose gel (Figure 5). Figure 5 indicates that four of four plasmids contained inserts of approximately 250 base pairs in length. Recombinant plasmids shown to contain PCR-inserts were used in sequencing reactions

using a LICOR automated DNA sequencer. Genbank searches were then conducted to determine matches of sequenced inserts to genes contained within the mouse genome. All four inserts showed a perfect match to the mouse transcription factor *rtfl* gene. This was followed by an extensive literature search using Pubmed and other on-line tools to gather an understanding of what is currently known about the *rtfl* gene and the *rtfl* protein in both mice and other organisms with a similar homolog to *Rtfl*.



Figure 5. EcoRI restriction digests of T36 selected clones.

In Figure 5, note that selected plasmids were digested with EcoRI and resolved on a 2% agarose gel stained with SYBR Safe DNA gel stain.

Lane 1: undigested plasmid DNA. Lanes 2–5: Plasmid DNA from t36

digested with EcoRI. Lane 6: DNA molecular weight marker. Note that digested clones in lanes 2–5 contained inserts of approximately 250 base pairs in size. All four of these plasmids contained inserts, so each one was sequenced and found to be identical to the *Rtf1* gene.

Discussion

Several previous studies have implicated a role for parathyroid hormone related protein (PTHrP) in the regulation of ductal proliferation and branching during pubertal mammary development.⁵ However, because PTHrP was identified out of its biological context (i.e., because of its role in Humeral Hypercalcemia Malignancy), little is currently known regarding the molecules that might act downstream of PTHrP in the developing mammary gland. To identify potential downstream effectors to PTHrP's actions in the mammary gland, a differential display technique to analyze global changes in gene expression in wild-type and PTHrP-overexpressing mice was employed. Here the identification of several genes whose products might act with PTHrP in regulating mammary development is reported.

One particular gene of interest that was identified using the GeneFishingTM technique is the *rtf1* gene. *Rtf1* was initially identified as a novel gene important for TATA site selection by the TATA box-binding protein in the yeast *Saccharomyces cerevisiae*.¹³ Later studies indicate that

the *rtf1* protein in yeast is part of a larger complex of proteins called the Paf1-RNA polymerase II complex that is required for the expression of genes known to function in the yeast cell cycle such as G(1) cyclin CLN1.^{1,10} Other studies in yeast indicate that the Paf1-RNA II complex plays a role in transcription elongation by RNA polymerase II.^{11,12} These results collectively argue strongly that the Rtf1 protein serves as a crucial transcription factor in yeast cells involved in the regulation of the yeast cell cycle.

Very little is currently known regarding the function of *rtf1* in higher eukaryotes. However, recent studies in *Drosophila* have suggested that it plays a role in development by regulating notch signaling. Although nothing is currently known regarding *rtf1* in the mammary gland, it is interesting to speculate that it might also function in regulating development. Paf1 has also been implicated in Wnt signaling. Previous studies have also demonstrated a role of PTHrP in Wnt signaling. Because of the implicated role for *rtf1* in the regulation of both the notch and the Wnt signaling pathways, we are excited about the potential for *rtf1* as a signaling partner to PTHrP in the mammary gland. PTHrP regulates the epidermal and mesenchymal expression of Lef1 and B-catenin, suggesting that the PTHrP and Wnt signaling pathways converge at some point during the signaling cascade.⁷

Conclusion

In conclusion, a nonradioactive method of differential display to identify potential downstream signaling partners to PTHrP in the mammary gland has been utilized. Using this approach, one gene known as *rtfl*, has been identified as a potential to signaling partner to PTHrP in the mammary gland. Most interestingly, the differential display suggests that this gene is overexpressed in PTHrP-transgenic mammary glands as compared to wild-type. Future experiments are planned utilizing real-time PCR to confirm the differential expression of this gene. The identification of downstream signaling partners to PTHrP will lead to a better understanding of the role of PTHrP in mammary development.

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**The *in vitro* Effects of Human Proinflammatory Cytokines
Granulocyte-Macrophage Colony-Stimulating Factor and
Interleukin-1 beta on Natural Killer-like Responses in
*Eisenia hortensis***

Nicole DeRogatis and Sheryl L. Fuller-Espie

Preface

While an undergraduate in the Science Department at Cabrini College, Ms. Nicole DeRogatis wanted to focus her interests on immunology, an area of science she particularly enjoys. She chose to write her senior thesis using original research that she conducted in my research laboratory and that focused on innate immunity in invertebrates. Specifically, Nicole worked on a research project investigating the *in vitro* effects of recombinant human cytokines interleukin-1beta and granulocyte-macrophage colony-stimulating factor (GM-CSF) on natural killer-like (NK-like) responses of coelomocytes from the earthworm *Eisenia hortensis*. The purpose of the research was to determine whether the cytokines could enhance or inhibit cytotoxic responses between coelomocyte effector cells and a human tumor cell line (K562), which served as the target cell population. Interestingly, many invertebrate immune responses are analogous to human and other mammalian responses. The fact that human cytokines can affect invertebrate immune responses supports the idea that functional homologues of the vertebrate

immune system might exist in invertebrates. Perhaps some degree of conservation among cytokine receptors is present through evolution. Continuing these investigations might identify conserved sequences in receptors or possibly primitive invertebrate molecules involved in signal transduction. Comparative immunology is important and proves to be useful in further understanding cytokine activities among mammals. Nicole has also coauthored research that investigated the effects of interleukin-1 beta, GM-CSF, interleukin-2 and tumor necrosis factor alpha on phagocytosis in *E. hortensis* (for more details, please refer to the paper by Goodfield et al. in this journal), and she conducted preliminary research studying the effect of pathogen-associated molecular patterns (PAMPs) on phagocytosis during the summer of 2008 in my lab.

During Ms. DeRogatis's senior year at Cabrini College, she received the W. W. Smith Scholarship and graduated with a B.S. in the Biology–Biological Studies Concentration. She was also a speaker and presented a poster at the annual Undergraduate Art, Research, and Scholarship Symposium at Cabrini College. Her understanding of the immune system and hands-on research experience helped qualify her for a research position at the Abramson Pediatric Research Center at the Children's Hospital of Philadelphia (CHOP) where she now works with Long Zheng, M.D., Ph.D. Dr. Zheng is the principal investigator of the

coagulation lab at CHOP and Assistant Professor of Pathology and Laboratory Medicine at the University of Pennsylvania School of Medicine. The research in his lab investigates the pathogenesis of diseases such as thrombotic thrombocytopenic purpura (TTP) that involves the formation of small blood clots in blood vessels and hemolytic uremic syndrome (HUS) that is associated with acute renal failure and is most often caused by Shiga toxin producing *Escherichia coli*. Autoimmunity occurs in HUS where auto-antibodies against complement proteins are produced. This may result in reduced levels of C3b, a complement protein deposited on the surface of infected cells, in turn, promoting their clearing from the body. The structure and biosynthesis of ADAMTS13 (a protease that cleaves a large protein involved in platelet adhesion during the coagulation process) is a major focus of the lab. In the absence of ADAMTS13 due to genetic defect or inhibition of its expression owed to other factors, the large protein (known as Von Willebrand factor) is not cleaved and thrombosis occurs.

I am grateful for Nicole's enthusiasm, positive attitude, and unswerving commitment while working in my lab. I am confident that she will continue to develop as a highly qualified scientist and will accomplish her goal of completing a graduate-level degree in the biomedical sciences. I wish her great success.

Faculty Mentor – Sheryl L. Fuller-Espie, Ph.D., DIC, Associate Professor of Biology

Abstract

This research focused on the *in vitro* effects of recombinant human proinflammatory cytokines interleukin-1 β (IL-1 β) and granulocyte-macrophage colony-stimulating factor (GM-CSF) on natural killer (NK)-like responses of leukocytes (coelomocytes) in the earthworm *Eisenia hortensis*. The K562 human erythroleukemic tumor cell line served as targets for the earthworm effector cells. NK-like activity lies amongst small coelomocytes and requires cell-to-cell contact with targets. The LIVE/DEAD[®] cell-mediated cytotoxicity kit was used to distinguish targets from effectors during flow cytometric analysis. Following overnight incubation of earthworm cells with both cytokines, the green fluorescent dye 3,3',-dioctadecyloxycarbocyanine (DiO) was first used to label the plasma membrane of the targets. Second, the red fluorescent membrane impermeable dye propidium iodide (PI) was added. Populations labeled with DiO and PI that were susceptible to NK-like killing exhibited compromised plasma membranes and, therefore, were double positively labeled for subsequent flow cytometric analysis. Effector/target ratios of 5:1 and 2.5:1 were employed. After factoring in spontaneous PI uptake in controls, specific cytotoxicity was calculated. Preliminary data for 12 earthworms exhibited 4 statistically significant inhibitory effects ($p \leq 0.05$), of which 3 pertained to IL-1 beta and 1 for GM-CSF. Further investigation into the inhibitory response, phosphorylation events, and signal transduction in *Eisenia hortensis* are also planned. It is necessary to repeat these experiments examining dose-response curves to determine if effects correlate with concentration. Studying cytokine-like responses in invertebrates may identify conserved molecules and shed light on more complex cytokine activities amongst vertebrates.

Introduction

This research investigated the *in vitro* effects of recombinant human cytokines interleukin-1 β (IL-1 beta) and granulocyte-macrophage colony-stimulating factor (GM-CSF) on natural killer (NK)-like responses

of earthworm coelomocytes (leukocytes). The purpose of the research was to determine whether exposure to these proinflammatory cytokines would influence an NK-like response against the K562 human erythroleukemic tumor cell line. K562 is highly sensitive for NK activity allowing for nonspecific killing and thus serves as an ideal target for the earthworm effector cells used in this study. Cell-to-cell contact between the small coelomocytes and the target cells is required to carry out NK-like activity (Salzet, Tasiemski, & Cooper, 2006). It is believed that the cell-mediated killing occurs through some sort of lectin-like binding to glycoproteins on the surface of tumor cells (Cossarizza et al., 1996). We proposed that the addition of these recombinant human cytokines would significantly affect the level of cytotoxicity. Using an invertebrate model to study cytokine-like responses might shed light on more complex invertebrate immunological activities. In addition, this might allow for possible identification of conserved molecules that might be involved in vertebrate cytokine activities.

Earthworms possess only an innate immune system that includes both humoral and cell-mediated immunity. Three main cell types have been identified in their coelomic cavity: large coelomocytes (12–15 μ m) which are involved in phagocytosis, small coelomocytes (8–11 μ m) involved in NK-like activity, and chloragocytes associated with a humoral

response (Cooper, Kauschke, & Cossarizza, 2002). Through the use of mouse antihuman monoclonal antibodies, Cossarizza et al. (1996) established crossreactivity in earthworms with the following human cell surface molecules: Small coelomocytes were positive for CD11a, CD45RA, CD45RO, CDw49b, CD53, Thy-1 and β_2 -microglobulin, while large coelomocytes were negative for all of these markers. CD11a is the alpha chain of LFA-1 molecule expressed on the surface of leukocytes. This molecule binds to ICAM-1 (an intercellular adhesion molecule) and facilitates the entry of leukocytes into infected tissues, stimulating NK activity against target cells (Cruse & Lewis, 2004, p. 36). CD45RA and CD45RO are isoforms of CD45 (leukocyte common antigen), which is a glycoprotein expressed on leukocytes and possess tyrosine phosphatase activity within the intracellular domain (Cruse & Lewis, 2004, p.48). Thy-1 is also a glycoprotein found on the surface of fibroblasts. Although small coelomocytes were positive for β_2 -microglobulin (which is noncovalently linked to human MHC class I and class I-like molecules) both coelomocyte populations proved to be negative for MHC class I and II markers (Cossarizza et al., 1996).

Chloragocytes are large granular cells that produce humoral components including antimicrobial and lytic molecules such as eiseniapore (perforin-like molecule), fetidin, CCF-1, lysenin and lumbicin-

I (nonlytic) (Engelmann, Cooper, & Nemeth, 2005). Fetidins are found on the external surface of the earthworm and are thought to serve as a physical barrier for protection (Cooper et al., 2002). They are also involved in hemolytic and antibacterial activity, as well as agglutination (Komiyama, Kauschke, & Cooper, 2002). Eiseniapore binds to sphingomyelin or galactosylceramide on a target cell forming pores and therefore, facilitating entry of fetidin and lysenin to carry out cell lysis (Komiyama et al., 2002). The prophenoloxidase cascade (an analog of complement in vertebrates) is activated by a cytokine-like molecule similar to mammalian TNF-alpha known as coelomic cytolytic factor-1 (CCF-1) upon binding to pattern recognition molecules (PRM's) (Field, Kurtz, Cooper, & Michiels, 2004). Although there is no gene or protein sequence homology between CCF-1 and TNF-alpha, they are thought to be functionally homologous through similar N,N'- diacetylchitobiose lectin-like activity. As with eiseniapore, CCF-1 is also cytolytic to different mammalian tumor cell lines (Salzet et al., 2006). Another similarity between vertebrates and invertebrates lies in the Toll like receptors (TLR's) where recognition of PRM's such as LPS, peptidoglycan and teichoic acid generate antimicrobial proteins and cytokines. In both humans and *Drosophila melanogaster*, signal transduction pathways closely resemble each other (Flo & Aderem, 2005).

The fact that human cytokines can manipulate an invertebrate immune response suggests a presence of cytokine-like receptors might exist. Two situations have been proposed where it is thought that ligands influenced the evolution of receptors with the idea that one ligand binds to different receptors. The other possibility is that the development of the ligand is dependent on the receptor and different ligands bind to one receptor (Mandrioli, Malagoli, & Ottaviani, 2007). Particular emphasis is placed on the hematopoietic/cytokine receptor superfamily (class I) and interferon receptor superfamily (class II) due to their significant homology. Class I receptors consist of an extracellular WSxWS sequence and four conserved cysteine amino acids located at the N-terminal end (Bazan, 1990). Class II cytokine receptors include the interferon receptors and tissue factor receptors. These contain the four, cysteine amino acids, but not the WSxWS box. Signal transduction for class I and II receptors takes place through JAK/STAT pathways (Cruse & Lewis, 2004, p. 285).

Research in protein biochemistry indicates a folding domain of approximately 200 amino acids in class I receptors with significant homology to an approximately 100 amino acid domain of class II receptors. The class I receptors share a 90-amino-acid sequence homology with fibronectin type III domains. Fibronectin is a glycoprotein that is involved in contact inhibition, inflammation, and promoting adhesion

(Cruse & Lewis, 2004, p. 34). The seven β -strands that fold into a tertiary pattern are found in shared receptor and fibronectin domains, which the author proposes are the conserved binding sites for cytokines. It is believed that there is an evolutionary link between regulatory molecules of the immune system and adhesion molecules (Bazan, 1990). It is interesting that small coelomocytes are positive for human CD markers involved in binding to adhesion molecules. Similarities found in the binding domains of receptors support the idea of conserved relationships between receptors and not the ligands. Our research, in addition to the research of others, may provide evidence of the conservation of immune system functions through evolution and that functional homologues of the vertebrate immune system might exist in invertebrates. Amino acid sequences in the structure of mammalian cytokine receptors reveal specific conserved sequences through evolution supporting the possibility of cytokine-like molecules and receptors among invertebrates. It has been shown that NK killing activity increases 20–100 times upon *in vitro* exposure to IFN-alpha, IFN-beta, or IL-12 (Janeway, Travers, Walport, & Shlomchik, 2005, p. 90). This being the case in the immune system of humans, we questioned whether proinflammatory cytokines GM-CSF and IL-1beta would do the same in invertebrates.

Materials and Methods

Animal Husbandry

Earthworms were purchased from Vermitechnology Inc. The earthworms were stored at 20°C in small dark plastic boxes where they were kept on moist autoclaved shredded paper and fed Gerber baby cereal. The cages were changed twice weekly to help minimize contamination from fungal spores.

Cell Culturing of K562 Target Cells

The K562 cells (Sigma Aldrich) were cultured in Super Dulbecco's Modified Eagle Medium (SDMEM). This medium contained Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 100µg/mL ampicillin (Shelton Scientific), 10µg/mL kanamycin (Shelton Scientific), 10µg/mL tetracycline hydrochloride (Fisher Scientific), 5µg/mL chloramphenicol (Fluka Biochemica), 1x penicillin/streptomycin/amphotericin B, 1x nonessential amino acids, 1x L-glutamine (Gibco) and 10% fetal calf serum. Cells were cultured in T25 or T75 culture flasks and kept in a 5% CO₂ incubator at 37°C.

Collecting Coelomocytes (Extrusion)

Twelve earthworms were numbered 1–12 and set up in two 6 well plates containing 2.5µg/mL fungizone (MP Biomedicals) soaked filter paper approximately 12–14 hours prior to the assay. The fungizone was

used to inhibit the growth of fungal hyphae. Earthworms and 3mL of extrusion buffer were placed into numbered Petri dishes where they rapidly shed their coelomocytes. The extrusion buffer was made up of 71.2mM NaCl, 5% v/v ethanol, 50.4mM guaiacol-glycerol-ether, 5mM EGTA, pH 7.3. 1mL of Accumax™ (Innovative Cell Technology), an enzyme solution containing protease, collagenase, and DNase to dissociate cell aggregates. It was added to corresponding 15mL conical test tubes and kept on ice. The extruded coelomocytes were then collected from each plate and placed into their corresponding numbered test tube where they were kept at room temperature for 5 minutes for enzyme exposure. The test tubes were then placed back on ice where 5mL of PBS was added. The solution was then pipetted up and down which helped to remove chloragocytes that were not included in the final analysis. The test tubes were then centrifuged at 800 rpm (340g), 4°C for 5 minutes. Following centrifugation, the supernatant was decanted and the cells were resuspended in 1mL of SDMEM. The coelomocytes were then enumerated using a hemocytometer and assessed for quality.

Treatment of Coelomocytes With Cytokines

Using SDMEM, the coelomocyte concentration was adjusted to 1×10^6 cells/mL. Each well received 5×10^4 coelomocytes (50 μ L) and 50 μ L cytokine was then added to 96-well, round bottom plates for overnight

incubation at 20°C in 5% CO₂ in a total volume of 100µL/well. IL-1β (R & D Systems) was used at a final concentration of 40 ng/mL and GM-CSF (Pro Spec-Tany TechnoGene) was used at a final concentration of 8 ng/mL. Duplicate control tests for untreated coelomocytes and for coelomocytes plus PI were included; the latter to examine cell viability. Cytokine specifications are shown in Table 1.

Table 1

Cytokine Specifications

Cytokine	Supplier	Stock Conc.	Final Conc.	Endotoxin Level	Activity(ED ₅₀)	Purity
Recombinant Human GM-CSF	ProSpec-TanyTechnoGene	10ng/µL	8ng/mL	< 1.0 EU/µg	< 100pg/mL	> 98%
Recombinant Human IL-1-beta	R&D Systems	10ng/µL	40ng/mL	< 1.0 EU/µg	< 12pg/mL	> 97%

Triplicates were run for coelomocytes pretreated with cytokines plus PI and then incubated in the presence of K562 targets. K562 targets and PI were diluted with SDMEM prior to use and then K562 targets (1x10⁴), PI and/or SDMEM were added to the appropriate wells to bring the final volume of each well to 200µL.

Cell-Mediated Cytotoxicity Assay

The LIVE/DEAD[®] cell-mediated cytotoxicity kit (Molecular Probes) in combination with flow cytometry was used to label the K562 target cells and measure coelomocyte NK-like activity according to the

manufacturer's protocol.

K562 targets were labeled with 30 μ M 3,3'-diiodo-5-(6-diethylaminoethyl)-2-methylbenzofuran diiodide [DiOC₁₈(3) or DiO] and resuspended in VP-SFM. Then 1x10⁴ or 2x10⁴ targets were added to 96-well, V-shaped bottom plates containing 5x10⁴ effector cells at E:T ratios of 5:1 or 2.5:1 respectively, for cytotoxicity assays. Finally, propidium iodide (PI) was added at 75 μ M to each well and the plates were centrifuged briefly at 1200rpm for 0.5 minutes prior to incubation at 30°C in 5% CO₂ for 1–2 hours in a total volume of 200 μ L/well.

Flow Cytometric Analysis of Cell-Mediated Cytotoxicity

A FACSCalibur flow cytometer (BD Biosciences) employing Cell Quest Software™ was used to collect uncompensated data. A minimum of 5,000 events was acquired for each of the samples according to specific flow cytometry instrument settings (Table 2).

Table 2

Flow Cytometry Specifications

Parameter	Amplification (gain)	Scale
Forward scatter (FSC)	E-1 (3.33)	Linear
Side scatter (SSC)	362 (1.00)	Linear
FL-1 (green)	475V	Logarithmic
FL-3 (red)	398V	Logarithmic

Final analysis utilized WinList™ Interactive N-Color Compensation™ software (Verity Software House, Inc.). Flow cytometry controls included (a) unlabeled targets (Figure 1), (b) DiO-labeled targets (green control) for FL-1/FL-3 compensation adjustment (Figure 1), (c) unlabeled targets incubated with PI plus 1% saponin (red control) for FL-3/FL-1 compensation adjustment (Figure 1), (d) DiO-labeled targets incubated with PI for spontaneous uptake (Figure 2), and (e) DiO-labeled targets incubated with PI and 1% saponin for maximum uptake (Figure 2). The total volume in each control well was 200µL, with the exception of those containing saponin that contained a total volume of 222µL/well.

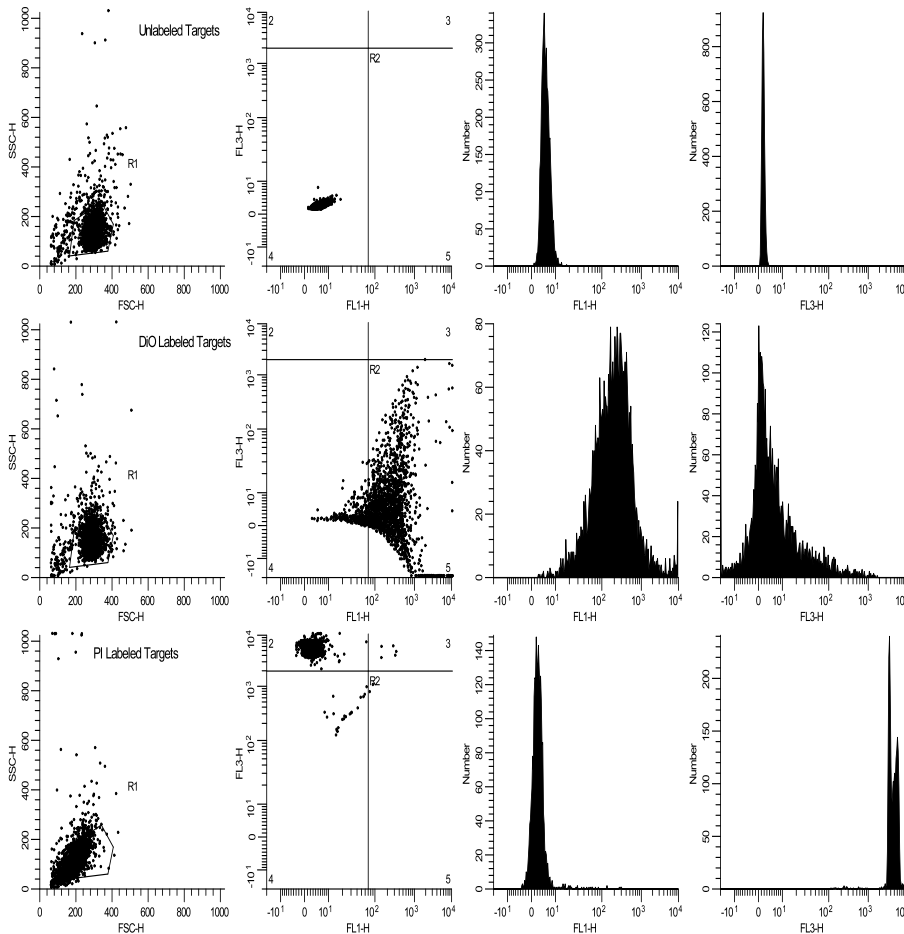


Figure 1. Flow cytometric analysis of controls. In the first row unlabeled targets in a FSC vs. SSC histogram show a region (R1) drawn around the cells. The three histograms that follow in this row are all gated on R1. Cells are located in the lower left quadrant of the FL-1 vs. FL-3 dot plot. FL-1 and FL-3 single parameter histograms are respectively DiO and PI negative. The second row shows DiO-labeled targets appearing in the lower right quadrant of the FL-1 vs. FL-3 dot plot. The FL-1 histogram shows cells are DiO positive, while the FL-3 histogram shows them PI negative. The third row displays PI labeled targets plus saponin. Here a shift in the population is seen probably due to compacted cells after saponin treatment. These cells are present in the upper left quadrant of the FL-1 vs. FL-3 dot plot. Here, the FL-1 histogram is DiO negative and the FL-3 histogram is PI positive. After examining background fluorescence, quadrant markers were placed appropriately. The x-axis threshold was set at 70 and ensured only DiO-labeled targets

exhibited fluorescence above this setting. The y-axis threshold was set at 2000 for FL-3 because the level of PI staining did not exceed this setting. Only target cells with compromised membranes had fluorescence intensity levels above this setting.

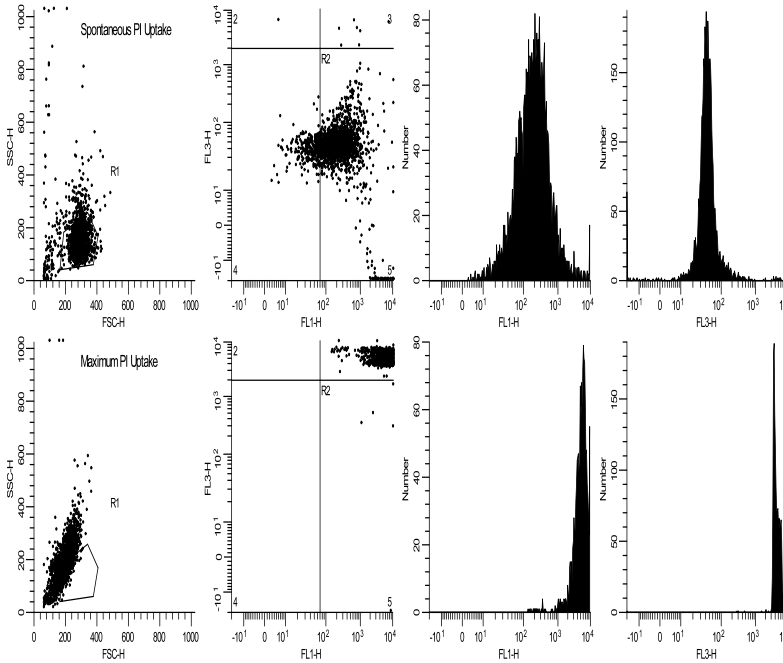


Figure 2. Flow cytometric analysis of controls. Spontaneous PI uptake is seen in the first row. As before, the three histograms were all gated on R1. The FL-1 vs. FL-3 shows most of the population in R5 and very little spontaneous uptake. The second row shows maximum PI uptake plus saponin and shows the cells are DiO and PI positive.

For analysis, several regions (R1-R5) had to be set. First, cells were analyzed in a forward versus side scatter dot plot where a region (R1) was drawn around the area corresponding to K562 target cells. Second, a single parameter histogram for FL-1 was created (green fluorescence) and gated on R1. Next, a single parameter FL-3 histogram was created (red fluorescence) and gated on R1. Finally, a two-parameter dot plot of FL-1 vs. FL-3 (also gated on R1) was created and consisted of

four quadrants corresponding to regions (R2-R5) (Figures 1 & 2). After examining background fluorescence for FL-1 and FL-3, the quadrant markers were set at 70 on the *x* axis and 2000 on the *y* axis. The *x*-axis threshold was set at 70 because earthworm coelomocyte gated for analysis exhibited some autofluorescence but the levels of FL-1 fluorescence did not exceed this threshold. Only DiO-labeled K562 targets exhibited fluorescence above this setting (Figures 1 & 2). The *y*-axis threshold was set at 2000 for FL-3 because the level of fluorescence for earthworm cells alone did not exceed this setting. Only target cells with compromised membranes had fluorescence intensity levels above the setting (Figure 2). R2 refers to the upper left quadrant and includes effector cells whose membranes were compromised and therefore, are positively labeled for PI but not for DiO. R3 refers to the upper right quadrant and represents DiO-labeled target cells whose membranes were disrupted and, therefore, are double positively labeled with DiO and PI. R4 represents the lower left quadrant and includes undamaged effector cells that are negative for both PI and DiO. R5 refers to the lower right quadrant and includes intact target cells that are DiO-labeled only. The quadrant of interest representing dead target cells caused by NK-like activity of earthworm coelomocytes are those events occurring in R3. Importantly, this region consists of only DiO-labeled target cells and does not include effector cells. The controls

were analyzed using four different histograms. In Figure 1, the first row shows the unlabeled targets in a FSC versus SSC histogram where a region (R1) is drawn around the cells. The three histograms that follow in this row are all gated on R1. These cells appear in the lower left quadrant of the FL-1 versus FL-3 dot plot. The FL-1 and FL-3 single parameter histograms are respectively DiO and PI negative. The second row shows DiO-labeled targets appearing in the lower right quadrant of the FL-1 versus FL-3 dot plot. The FL-1 histogram shows the cells are DiO positive, while the FL-3 histogram shows they are PI negative. The third row displays PI-labeled targets (treated with saponin) with the cells present in the upper left quadrant of the FL-1 versus FL-3 dot plot. Here, the FL-1 histogram is DiO negative and the FL-3 histogram is PI positive. Figure 2 shows spontaneous PI uptake in the first row. As before, the three histograms were all gated on R1. The FL-3 histogram shows the amount of spontaneous uptake is very little and most of the cells are DiO positive. The second row shows maximum PI uptake (treated with saponin) and shows the cells are DiO positive and of course PI positive. When saponin was used in two controls (red control and maximum PI uptake) the cells shifted and appeared smaller, probably due to their membranes shrinking and compacting close to each other. To analyze auto-fluorescence a FSC versus SSC histogram was created involving earthworm cells only, as well

as histograms for FL-1 and FL-3, which was important for setting the compensation (data not shown).

Statistical Analysis

Statistical significance between the control and experimental groups was determined using Student's *t* test in which *p* values ≤ 0.05 were obtained 4/24 times. Specific cytotoxicity was determined by subtracting spontaneous cell death from the total cell death determined using the following equation:

$$\% \text{ Specific Cytotoxicity} = \frac{\frac{R3}{R3+R5_{+EFFECTORS}} - \frac{R3}{R3+R5_{-EFFECTORS}}}{100} \times 100$$

Statistics were acquired with the use of a Microsoft Excel spreadsheet.

First, the average of R3/R3+R5 for spontaneous PI uptake was determined. Next, for each of the 12 earthworms the average of triplicate samples for R3/R3+R5 for no cytokine treatment, IL-1beta, and GM-CSF were calculated. From this information, the average, variance and standard deviation for specific cytotoxicity was calculated. Finally, the *t* test compared no cytokine treatment to either IL-1beta or GM-CSF treatments.

Results

The assays employed two different effector to target (E:T) ratios of 5:1 and 2.5:1. List mode data was collected in CellQuest and analyzed

using WinList 5.0 software that also included N-Color Compensation software. Determination of percentage specific cytotoxicity involved analysis of cells in R1 of FSC vs. SSC histograms that included earthworm cells with targets. Three FL-1 vs. FL-3 dot plots were created pertaining to no cytokine, IL-1beta, and GM-CSF treatments. In EW # 3.2 (E:T of 5:1) inhibitory effects for both cytokines are observed with a p value ≤ 0.05 for IL-1beta (Figure 3).

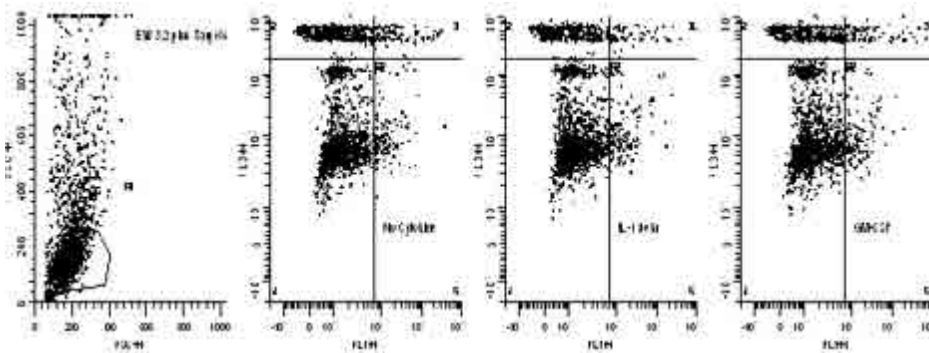


Figure 3. Earthworm 3.2. FSC vs. SSC histograms were created including earthworm cells with targets. FL-1 vs. FL-3 dot plots were created pertaining to no cytokine treatment, IL-1beta, and GM-CSF. Inhibitory effects for both cytokines are observed with a p value ≤ 0.05 for IL-1 beta. For EW# 11.2 (E:T of 5:1) inhibitory effects ($p \leq 0.05$) are shown in response to both cytokines (Figure 4).

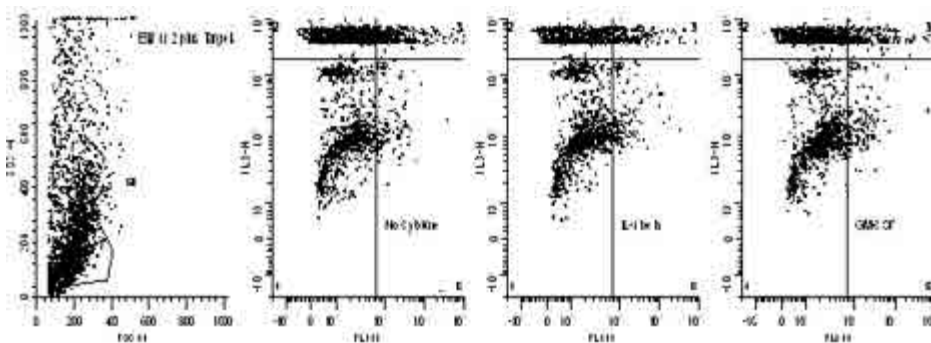


Figure 4. Earthworm 11.2. FSC vs. SSC histograms were created including earthworm

cells with targets. FL-1 vs. FL-3 dot plots were created pertaining to no cytokine treatment, IL-1beta, and GM-CSF. Inhibitory effects ($p \leq 0.05$) in response to both cytokines are displayed. In EW# 4.3 (E:T of 2.5:1) again, both cytokines displayed an inhibitory effect with a p value ≤ 0.05 for IL-1beta (Figure 5).

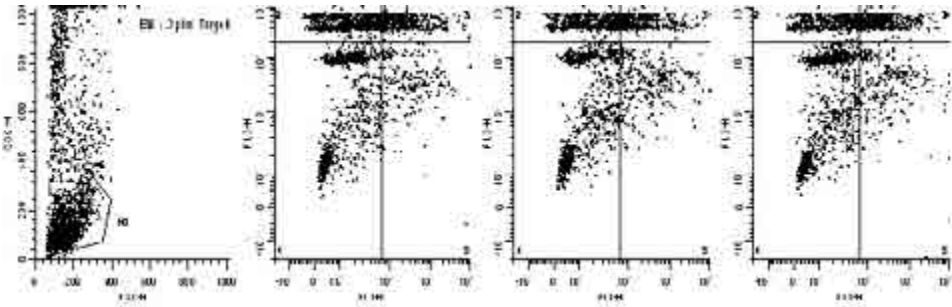


Figure 5. Earthworm 4.3. FSC vs. SSC histograms were created including earthworm cells with targets. FL-1 vs. FL-3 dot plots were created pertaining to no cytokine treatment, IL-1beta, and GM-CSF. EW# 4.3 shows inhibitory effects with both cytokines however, only IL-1beta proved to be statistically significant ($p \leq 0.05$).

Preliminary data for 12 earthworms tested showed 5 stimulatory responses with IL-1beta and 6 with GM-CSF. In contrast, 7 inhibitory responses were seen with IL-1beta and 6 with GM-CSF (Table 3). Although the earthworms displayed inhibitory and stimulatory effects in response to the cytokines, only the inhibitory responses proved to be statistically significant ($p \leq 0.05$). Of these, 3 p values ≤ 0.05 were obtained for IL-1beta (EW# 4.3, 3.2 and 11.2) and 1 for GM-CSF (EW# 11.2), which are displayed as bar graphs with error bars showing standard deviation in Figure 6.

Discussion

In cell-mediated cytotoxicity, more of a stimulatory effect ($n = 7$)

took place when the number of targets was increased from 10,000 to 20,000 targets. More of an inhibitory effect was seen ($n = 8$) with 10, 000 targets, of which three of the four p values of ≤ 0.05 were derived. Studies conducted by Goodfield, Hill, Grant, DeRogatis, and Fuller-Espie (manuscript submitted for publication) in the same lab utilized GM-CSF, IL-1beta, interleukin-2 (IL-2) and tumor necrosis factor-alpha (TNF-alpha) to examine effects on phagocytosis using *Escherichia coli*. The HB101 strain of *E.coli* used was transformed with the pGLO plasmid. The plasmid codes for green fluorescent protein (GFP) shares a promoter with a gene for metabolizing the sugar arabinose. When the promoter is induced in the presence of arabinose, the GFP gene is also transcribed and expressed. The quantity of GFP expressing *E. coli* was measured by flow cytometry and was proportional to the amount of *E. coli* phagocytosed by large coelomocytes. The results showed all cytokines, particularly GM-CSF exhibited a stimulatory effect on phagocytosis. We were initially expecting the same stimulatory effect to take place with cytotoxicity.

Table 3
Summary of Cytokine Effects on Cytotoxicity for 12 Earthworms

EW#	IL-1 beta	GM-CSF
2.2	inhibitory	stimulatory
3.2	Inhibitory $p \leq 0.05$	inhibitory

EW#	IL-1 beta	GM-CSF
5.2	stimulatory	stimulatory
7.2	stimulatory	inhibitory
10.2	inhibitory	inhibitory
11.2	Inhibitory $p \leq 0.05$	Inhibitory $p \leq 0.05$
1.3	inhibitory	inhibitory
2.3	stimulatory	stimulatory
4.3	inhibitory $p \leq 0.05$	inhibitory
8.3	stimulatory	stimulatory
9.3	inhibitory	stimulatory
12.3	stimulatory	stimulatory

Note. Results show 5 stimulatory responses observed with IL-1beta and 6 with GM-CSF. Seven inhibitory responses were observed with IL-1beta and 6 with GM-CSF. Of these, 3 statistically significant ($p \leq 0.05$) were obtained for IL-1beta (EW# 4.3, 3.2 and 11.2) and 1 for GM-CSF (EW# 11.2).

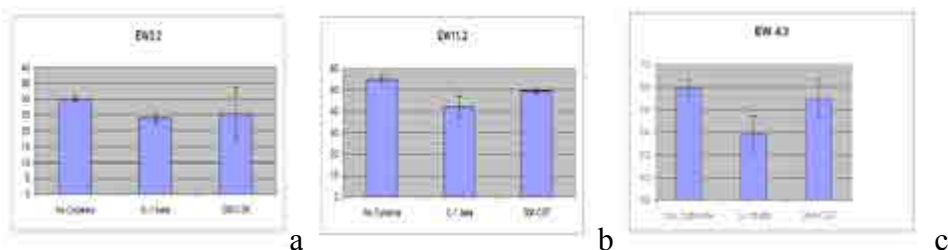


Figure 6. Bar graphs displaying statistically significant cytotoxic responses. All three bar graphs display error bars showing standard deviation. Earthworm 3.2 displayed an inhibitory effect with both cytokines of which $p \leq 0.05$ was obtained for IL-1beta (a). Earthworm 11.2 showed an inhibitory effect with both cytokines of which $p \leq 0.05$ was obtained for both GM-CSF and IL-1beta (b). Earthworm 4.3 also displayed inhibitory effects for both cytokines, of which $p \leq 0.05$ for IL-1beta (c).

Other research done by Goodfield et al. investigated the effects of IL-1beta, IL-2, GM-CSF, and TNF-alpha on cell proliferation (S. L. Fuller-Espie, personal communication). Propidium iodide (PI) was used in these assays to evaluate the different stages of the cell cycle. PI is a fluorochrome that binds with high affinity to double stranded nucleic acids after the cell membrane has been compromised. Ribonuclease was added to the samples to degrade RNA, so the amount of PI binding is proportional to the DNA content. In contrast to the phagocytosis assay, flow cytometric analysis revealed the cytokines had no effect on cell proliferation when compared to controls.

It is unclear why an inhibitory response was mostly observed. Perhaps the cytokines are down-regulating NK-like activity in a similar fashion to that seen in humans when CD4 T cells are differentiating into T_{H1} and T_{H2} cells. During this process, different cytokines are present and therefore, different cells are activated to direct the immune response. A closer look into the inhibitory response, phosphorylation events, and signal transduction in *E. hortensis* are also planned. In addition, repeating these experiments examining dose-response curves will allow us to determine if these effects correlate with concentration. Perhaps experiments involving inhibitory cytokines such as IL-10 or NK activating cytokines such as IL-12, IFN-alpha, and IFN-beta can be used to examine

their effects. In one study, IL-2 stimulated human lymphocyte controls showed a decreased level of cytotoxicity against K562 when compared to coelomocyte cytotoxic activity. According to Komiyama et al. (2002), calcium has been associated with small coelomocytes and NK-like activity against K562. To investigate this, ionomycin (a calcium ionophore) and was added to the experiments. The results showed an inhibitory affect amongst lymphocytes and coelomocytes in the presence of ionomycin. However, coelomocytes activity was not inhibited to the same extent as the lymphocyte control group (Komiyama et al., 2002).

Interestingly, a relationship has been established between protozoan pheromones and mammalian IL-2. IL-2 is capable of binding to protozoan cell surfaces and vice versa; pheromones from a marine protozoa *Euplotes raikovi* are capable of binding to IL-2 receptors on mammalian cells (Scapigliati et al., 2006). Again, this points to a conservation of signaling molecule or possibly a cytokine-like molecule. A study done involving *Tilapia nilotica* and *Ictalurus punctatus* investigated FasL-mediated cytotoxicity. The authors found nonspecific cytotoxic cells (NCC) purified from both fish do not express FasL or FasR, but are able to initiate apoptosis in targets cells expressing FasR by a soluble FasL (Bishop, Taylor, Jaso-Friedmann, & Evans, 2002). Cytotoxicity was measured by a ⁵¹chromium release assay and showed

anti-FasL mAb's inhibited cytotoxic activity of the NCC's. Killing of IM-9 targets was partially inhibited while killing of HL-60 targets was totally inhibited by anti-FasL mAb. Perhaps earthworm small coelomocytes also possess a soluble FasL to which cytokines can bind thereby inhibiting cytotoxicity against K562 (Bishop et al., 2002).

The various antimicrobial molecules found in the coelomic fluid are part of the remarkable innate immune system earthworms use to protect themselves from their environment and pathogens. Their ability to inhibit bacterial growth with the use of these molecules may lead to new antibiotic treatments that might be used in human treatments (Ros, 2002).

Cytokines are of great importance because of their numerous functions in the immune system. Recombinant cytokines are used in medicine today for research and a wide range of therapeutic purposes. Further investigation into the interactions between human cytokines and invertebrate immune system cells is forthcoming. Using invertebrates to study immune system mechanisms not only provides understanding of evolutionary developments, but also serves as a simple model for innate immunity. As previously mentioned, Bazan (1990) stated that an evolutionary link might exist between regulatory molecules of the immune system and adhesion molecules; hence, it is interesting that small coelomocytes are positive for human CD markers involved in binding to

adhesion molecules.

Understanding how cytokines developed through evolution and whether they are related to invertebrate primitive molecules would be helpful for understanding mammalian cytokine mechanisms. Because cytokines direct immune responses and control which cells will be activated under different circumstances, they are of extreme importance in medicine today. Investigation into cytokine mechanisms and cell signaling pathways may provide knowledge that is more extensive concerning the human innate and adaptive immune systems. Research might help improve present therapies and treatments for a wide range of immunological disorders including autoimmune diseases and cancer. Exploration of the structure of cell surface receptors specific for different cytokines and the families they belong to could offer additional information of how they have developed. Whether there is a link between regulatory molecules of the immune system such as cytokines and adhesion molecules is not clear. It is interesting, however, that such considerable similarities lie in the amino acid sequences between class I and class II cytokine receptors and fibronectin receptor domains. From an evolutionary standpoint, it is in itself amazing that an earthworm coelomocyte is capable of cytotoxic activity against a human cancer cell. Different research has already established that human recombinant cytokines can influence and

invertebrate immune response. This evidence supports the proposed idea of some degree of conservation amongst cytokines through evolution.

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Investigation of the Effects of 7, 12 Dimethylbenz[a]anthracene (DMBA) on Cell-mediated Cytotoxicity in *Eisenia fetida*: An Invertebrate Model to Study Innate Immune Responses

Mukti Patel and Sheryl L. Fuller-Espie

Preface

Ms. Mukti Patel conducted a 12-month research project in my laboratory from June 2006 to May 2007, culminating in the publication of a peer-reviewed article in the *European Journal of Soil Biology* (2007). The article was entitled “Development of a flow cytometric, non-radioactive cytotoxicity assay in *Eisenia fetida*: An in vitro system designed to analyze immunosuppression of natural killer-like coelomocytes in response to 7,12 dimethylbenz[a]anthracene (DMBA).” Her research was funded through an undergraduate research grant from the Pennsylvania Academy of Science awarded to her in September 2006 and entitled “Investigation of Immunosuppressive Effect of Environmental Pollutants in *Eisenia fetida*: An Invertebrate Bioindicator Model to Study Innate Immune Responses.” Her research was also funded through a student stipend provided through a Department of Education Student Undergraduate Research Experience (SURE) grant.

During her senior year, Ms. Patel presented her research at three venues: (a) the 83rd Annual Meeting of the Pennsylvania Academy of Science, Pittsburgh, PA; (b) the 16th Annual Infection and Immunity Forum, Drexel University School of Medicine, Philadelphia, PA; and (c) the inaugural Undergraduate Art, Research and Scholarship Symposium, Cabrini College,

Radnor, PA. Mukti's research was the foundation for her senior capstone course, a yearlong, seniors-only course where students write an undergraduate thesis, present a poster at a conference, and participate in an oral defense. It was indeed a great pleasure to serve as Mukti's faculty advisor for her senior thesis and I wish that I could have many more students like Mukti in my research lab. She has a level of maturity, thoughtfulness, determination, and commitment that not many undergraduates possess. She graduated in December 2007 and is seeking employment as an entry-level research scientist to obtain more research experience before applying to graduate school.

Faculty Mentor – Sherry Fuller-Espie, Associate Professor of Biology

Abstract

Eisenia fetida is an excellent invertebrate model to study conserved innate immune responses, and is an excellent bioindicator of contaminated soil. Polycyclic aromatic hydrocarbons (PAHs) are major pollutants found in the environment due to incomplete combustion of complex hydrocarbons. In our research, we examined immunosuppressive effects of 7, 12-dimethylbenz[a]anthracene (DMBA), a PAH, on *E. fetida* coelomocytes by measuring cytotoxicity responses. Coelomocytes (effectors) were harvested and exposed to DMBA *in vitro*, and then incubated with a K562 target cells, a human erythroleukemic cell line, at various effector-target ratios to test for NK-like cytotoxic activity. Cytotoxicity was measured using a nonradioactive flow cytometric assay. To differentiate targets (K562) from effectors (NK-like coelomocytes), the targets were labeled with 3,3'-dioctadecyloxycarbocyanine (DiOC₁₈(3) or DiO), a green fluorescent dye that stains the plasma membrane. The targets were then incubated with effector cells together with the membrane impermeable red dye propidium iodide (PI) that binds to DNA of cells with an injured plasma membrane. Double-labeled cells exhibiting coincident green membranes (DiO) and red-nuclear (PI) staining were identified as targets killed by NK-like effector cells. Intact targets and dead effectors were single-labeled, exhibiting only green or red fluorescence, respectively. Fluorescence was detected and measured using flow cytometry, and data

was analyzed using WinList's Interactive N-Color Compensation System of WinList5.0 software. Specific cytotoxicity was determined by gating on the DiO-positive population (targets), and determining the percentage of green target cells exhibiting both DiO and PI fluorescence in the gated population. Between 25–60% of earthworms tested demonstrated a significant decrease in the ability of their coelomocytes to kill targets following DMBA exposure in all assays performed compared to control. These results indicate that earthworm coelomocytes are sensitive to *in vitro* DMBA exposure in a dose-dependent manner resulting in inhibition of NK-like cytotoxicity.

Introduction

Scientists grow more concerned by the day about preserving biological diversity. The sample species of an ecosystem are known as bioindicators when that species can provide quick, yet reliable, information when studied. *Eisenia fetida* (earthworms) belong to the phylum Annelida in the class of Oligochaeta. This organism is an excellent invertebrate model to study conserved innate immune response. The natural habitat of earthworms is in terrestrial soil, and they have a relatively long life cycle; hence, they have potent antimicrobial molecules that help them survive against pathogens. In addition, as we share an intricate ecological relationship, it is essential to understand the survival strategies of earthworms. Furthermore, *E. fetida* is defined as an excellent bioindicator of contaminated soil and lends itself well to undergraduate research projects for a number of reasons. They are important in soil fertility, water infiltration, aeration, and major secondary decomposers.

Moreover, isolation of cells from the coelomic cavity is easy using extrusion buffer that contains 5% ethanol and induces external release of leukocytes from the dorsal pores (Engelmann, Palinkas, Cooper, & Nemeth, 2004). The purpose of this paper is to examine the immunosuppressive effects of polycyclic aromatic hydrocarbon (PAH) on *Eisenia fetida* using analysis of NK-like activity as an indicator.

Earthworms lack an adaptive immune system; they are limited to innate immune responses to eliminate microbes and prevent infection. Innate immune responses in *E. fetida* have been well studied and characterized. Although very little is known about innate immunity in earthworms, it has been proposed that it is regulated by both cellular and humoral elements. Their immunobiological responses include phagocytosis, agglutination, encapsulation, mitogenesis, inflammation and lysis, and destruction of targets (either allogeneic or xenogeneic) *in vivo* and *in vitro* (Cooper & Roch, 2003). In *E. fetida*, these defense mechanisms can be studied using coelomocytes obtained from the coelomic cavity, which are similar to vertebrate eukaryotic leukocytes structurally, functionally and cytochemically. According to Cooper and Roch (2003), several types of coelomocytes are produced by *E. fetida*. Using flow-cytometry and antibody staining approach, two types of coelomocytes, small coelomocytes (SC) and large coelomocytes (LC),

were identified (Cossarizza et. al., 1996; Cossarizza, Pinti, Troiano & Cooper, 2005). They also suggested that SC are dense and involved with natural killer (NK)-like activity and LC are involved with phagocytic activity. They are both negative for MHC class I and class II markers; however, SC, but not LC are positive for CD11a, CD45RA, CD45R0, CDw49b, CD54, β_2 -microglobulin and Thy-1(CD90). Besides these two types of coelomocytes, a third type, called chloragogen or chloragocytes, is responsible for synthesizing and releasing effector lytic molecules. Roch (1996) proposed that leukocyte cell-mediated activities in *E. fetida* involve engulfment and cell-to-cell recognition including cytotoxicity while humoral activities involve production and secretion of lytic components such as lysenin, fetidin, CCF-1, lumbricin, lytic factor, eiseniapore, hemolysin and serine protease inhibitor (as cited in Cooper, Kauschke & Cossarizza, 2002). Coelomic fluid and coelomocytes of earthworms, collectively, mediate recognition and immune responses. Earthworm coelomocytes exhibit cytotoxic activity against tumor cells, which is analogous to cytotoxic activity of NK cells in vertebrates.

The examination of the immunosuppressive effects of PAH on *E. fetida* was established using analysis of cell-mediated cytotoxicity as an indicator. We used the LIVE/DEAD[®] Cell-Mediated Cytotoxicity Kit together with flow cytometry to study cytotoxicity in *E. fetida*. This assay

employs two fluorescent dyes to discriminate between effector and target cells, and between live and dead target cells. The first dye, 3,3'-dioctadecyloxycarbocyanine (DiOC₁₈(3) or DiO) is a green fluorescent dye which is used to label the plasma membranes of cell line used as the target population. The second dye, propidium iodide (PI) is a membrane impermeable red fluorescent dye that binds to double-stranded nucleic acid in membrane-compromised cells. In our case, we used K562, an erythroleukemic cell line as targets stained with DiO. Earthworm coelomocytes were used as effectors. When targets and effectors are incubated together in the presence of PI, cytotoxicity occurs and the membrane of the targets is damaged, permitting the uptake of PI and subsequent display of red fluorescence. It is important to note that some effectors might also take up PI if their membranes are damaged; however, only targets prelabeled with DiO will exhibit green fluorescence and therein lies the ability to differentiate between effector and target cells. Intact target cells unaffected by effectors are single positive (and exhibit only green fluorescence) while targets killed by effectors bearing disrupted membranes will be double positive (and exhibit green as well as red fluorescence). Effectors bearing disrupted membranes will exhibit red fluorescence. To test the immunosuppressive effects, coelomocytes were exposed to the xenobiotic reagent 7,12 dimethylbenz[a]anthracene

(DMBA), a PAH, *in vitro* for a predetermined time. After DMBA was washed out, the DMBA free effectors were then incubated with DiO labeled K562 target cells in the presence of PI. PI uptake by DiO labeled K562 target cells was used as an indicator of cytotoxicity. DMBA has been well studied for its inhibiting activity on humoral immunity as well as cellular immunity in mammals. It depresses cellular immunity by preventing activation of NK-like cells; however, the mechanism of this DMBA-induced immunosuppression is not well characterized (Archuleta, Schieven, Ledbetter, Deanin & Burchiel, 1993; Burchiel, Thompson & Davis, 1991). The effector to target (E:T) ratio varied from 1:1 to 6:1 depending upon the yield of coelomocytes harvested from each earthworm. The number of targets remained constant in every assay at 1×10^4 targets per well. Therefore, the number of DMBA-treated effector cells ranged from 1×10^4 to 6×10^4 per well. Although *in vivo* studies of DMBA-induced inhibition of phagocytosis and H_2O_2 activity have been reported in *E. fetida* (Komiya *et. al*, 2003), to our knowledge direct *in vitro* assay aimed at studying NK-like cytotoxicity have not been reported until now.

Material and Methods

Reagents

VP-SFM (serum free medium) and Dulbecco's Modified Eagle

Medium (DMEM) were supplemented with 4mmol/L L-glutamine, 1x nonessential amino acids, 100 U/ml streptomycin, 100 U/ml penicillin, and 100 U/ml amphotericin B (Invitrogen). DMEM also contained 10% Fetal Calf Serum (FCS) (complete DMEM). Extrusion buffer contains 71.2 mM NaCl, 5 % v/v ethanol, 50.4 mM guaiacol-glyceryl-ether, 5 mM EGTA pH: 7.3. The Lubricus Balanced Salt Solution (LBSS buffer) contained 71.5 mM NaCl, 4.8 mM KCl, 1.1 mM MgSO₄ × 7H₂O, 0.4 mM KH₂PO₄, 0.3 mM NaH₂PO₄, 4.2 mM NaHCO₃, pH: 7.3. A human erythroleukemic tumor cell line, K562, (Sigma-Aldrich) was cultured in complete DMEM. 7, 12-dimethylbenz[a]anthracene (DMBA) and dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich Corporation. DMBA was dissolved in DMSO and stored at 100 mM stock solution at –80°C. This stock solution was diluted in VP-SFM to achieve desired concentration of DMBA. Live/Dead Cell-Mediated Cytotoxicity Kit was purchased from Invitrogen. Accomax was purchased from Innovative Cell Technologies.

Collection of Earthworms

Eisenia fetida were purchased from Carolina Biologicals. They were maintained in moist paper pulp in plastic cages, which were kept in dark condition at 20°C. The earthworms were fed Magic Worm Food (Worm Man's Worm Farm) or Gerber baby dry cereal (rice and oatmeal)

as needed. Earthworm bedding was changed weekly.

Harvesting Coelomocytes

To allow for defecation and to reduce contamination, the earthworms were acclimated for 2–3 days prior to experimental procedure. Then, the earthworms were wiped down one at a time with sterile cotton wool dampened in LBSS buffer. To harvest coelomocytes, each earthworm was exposed to 1 ml of ice-cold extrusion buffer until it stopped wiggling and solution became turbid. The solution was then incubated with 1 ml of Accumax for 5 minutes at room temperature followed by dilution with 5 ml of ice-cold LBSS buffer. Then, the solution centrifuged at 600 rpm (118g), 4°C for 5 minutes. Cells were washed twice with 3 ml of LBSS, suspended in 1 ml VP-SFM and counted using a haemocytometer. After the harvesting procedure, earthworms were returned to their original bedding for recovery.

Exposure of Coelomocytes to DMBA

7, 12-dimethylbenz[a]anthracene (DMBA) at concentrations ranging from 0 μM (control) to 500 μM was added to adjusted numbers of coelomocytes in 96 well plates, and incubated for a predetermined time, ranging from 30 minutes to 16 hours, at predetermined temperatures, ranging from 9°C to 25°C. The dose response assay control was carried out using coelomocytes without *in vitro* DMBA treatment. Coelomocytes

were washed three times before their treatment with target, K562 target cells. All tests were performed in duplicate. Effectors to target ratios varied from 1:1 to 6:1.

Cell-Mediated Cytotoxicity assay:

Live/Dead cell-mediated cytotoxicity kit was used to label K562 cells and to measure NK-like cytotoxicity using the flow cytometer as described in the manufacturer's protocol. K562, a human erythroleukemic cell line was used as the target cell population. K562 were washed twice with phosphate buffer saline (PBS) solution before staining with DiO. 10 μl of the DiO stock solution was used to label 1×10^6 cells/100 μl , and incubated for 20 minutes at 37°C. Prior to use in each assay, the stained K562 target cells were washed twice with PBS and resuspended in VP-SFM at a concentration of 1×10^6 cells /ml. Unlabeled and DiO labeled targets were run on the flow cytometer to confirm adequate fluorescent intensity at or greater than the second decade. K562 target cells were combined with effectors cells to achieve desired effectors: targets ratio. Propidium iodide (PI) at 75 μM was added to each well and the plates were centrifuged briefly at 118g for 0.5 minutes prior to incubation at 25°C, 5% CO₂ for predetermined assay times.

Flow Cytometric Analysis of Cell-Mediated Cytotoxicity

After incubation of DiO labeled target cells with effector cells for

predetermined time (1–3 hrs.), uncompensated data were acquired using a FACSCalibur Flow Cytometer employing Cell Quest Software™. The instrument settings for acquisition were as follows: (a) forward scatter was linear with voltage set at E-1 and amplification of 3.33; (b) side scatter was linear with voltage set at 362 and amplification of 1; (c) FL1 was log with voltage set at 374 and no amplification; (d) FL2 was log with voltage set at 381 and no amplification; and (f) FL3 was log with voltage set at 378 and no amplification. Coelomocytes population was defined using forward and side scattered properties. The number of events acquired depended upon the ratios used for each assay. A minimum of 5000 events were acquired for each sample, and stored as Listmode data format. The Listmode data was analyzed using WinList5.0 software (Verity Software House, Inc.) employing WinList's Interactive N-Color Compensation™ System. The K562 target population was gated for analysis purposes that are illustrated in Figure 1. In a forward versus side scatter dot plot, a region (R1) was drawn around the cell populations and gated on a single parameter green fluorescence (FL1) histogram to determine the relative green fluorescent intensity of membrane-stained K562 target cells. Regions (R2, R3, R4, R5) were drawn on a two parameter, FL1 (DiO) versus FL3 (PI) dot plot, using green control and red control, respectively. Because only the DiO-labeled targets were of interest, a region (R6) in a

single parameter FL1 histogram (gated on R1) was generated to include only DiO-positive events. Finally, the FL1 versus FL3 dot plot was gated on R6 and events in R3 (upper right, double positive) and R5 (lower right, DiO-positive) were used to calculate the percentage specific cytotoxicity. The percentage specific cytotoxicity was determined using the following formula: percentage specific cytotoxicity = (total cell deaths – spontaneous cell death/100)* 100

Total cell deaths = (Double dye positive cells (R3=green and red) + effectors

(Total green dye positive cells (R3+R5) + effectors

Spontaneous cell deaths = (Double dye positive cells (R3=green and red) – effectors

(Total green dye positive cells (R3+R5) – effectors

Dose response assay controls included (a) effector cells alone incubated with PI (to assess cell viability), (b) target cells alone, (c) DiO labeled target (K562) cells for FL1/FL3 compensation adjustment, (d) unlabeled target cells incubated with PI and 1% Saponin for FL3/FL1 compensation adjustment, (e) DiO labeled target cells with PI for spontaneous cell death, (f) DiO labeled target cells with PI and 1% saponin for maximum cell death. In the case of EF 8 and Ef 9, where DMBA was not washed from the effectors prior to the addition of targets, an additional control of DiO-labeled K562 targets incubated with DMBA and PI in the absence of effectors was also carried out to assess effects of DMBA on cell viability.

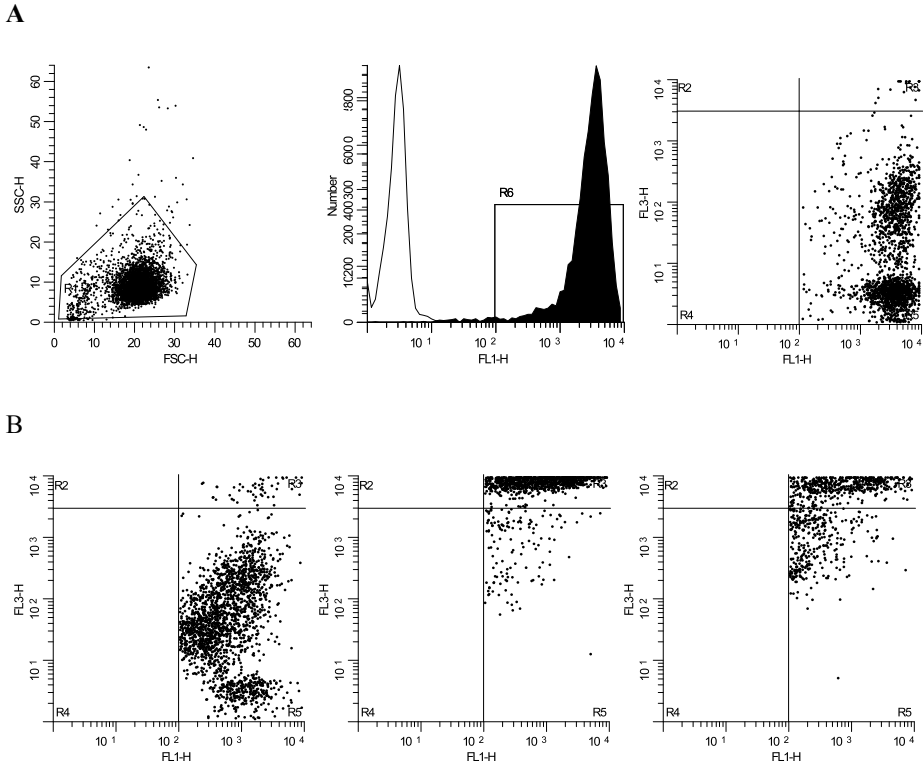


Figure 1. Flow cytometry analysis of cell-mediated cytotoxicity of K562 using WinList5.0 software. A: Histograms representing analysis of DiO-labeled K562 target cells. Left panel: Region 1 (R1) is drawn around cell population in the forward scattered versus side scattered histogram. Middle panel: a single parameter—green fluorescent (FL1, DiO) histogram showing target cells population from R1. Region (R6) is drawn around DiO positive population showing high intensity of green fluorescence in the FL1 histogram. Right panel: R6 is applied as a gate to a two parameters, FL1 (green fluorescence, DiO) versus FL2 (Red fluorescence, PI) dot plot to measure background FL3 signal after application of compensation. B: Analysis of Ef2 using FL1 (DiO) versus FL3 (PI) dot plot gated on R6. Left panel: spontaneous PI uptake control (background) by DiO-labeled targets in the absence of effectors. Middle panel: Percentage specific cytotoxicity of DiO-labeled targets after exposure to effectors without DMBA treatment. Right panel: Percentage specific cytotoxicity of DiO-labeled targets after exposure to effectors treated with 100 μ M DMBA.

Statistical Analysis

The student *t* test was used to establish significant differences between the experimental and control groups as explained in Trochim (2006). The *p* value < 0.05 was considered statistically significant as denoted by asterisks.

Results

Cytotoxicity of K562 by DMBA-exposed coelomocytes at 9°C for 16 hrs

Using an *in vitro* system, immunosuppressive effects of DMBA were tested by culturing freshly isolated coelomocytes in DMBA with concentration of 0 μ M, 4 μ M, 40 μ M and 100 μ M at 9°C for 16 hrs. Controls for these experiments were established as mentioned earlier. Figure 2A shows the cytotoxicity results using these experimental conditions.

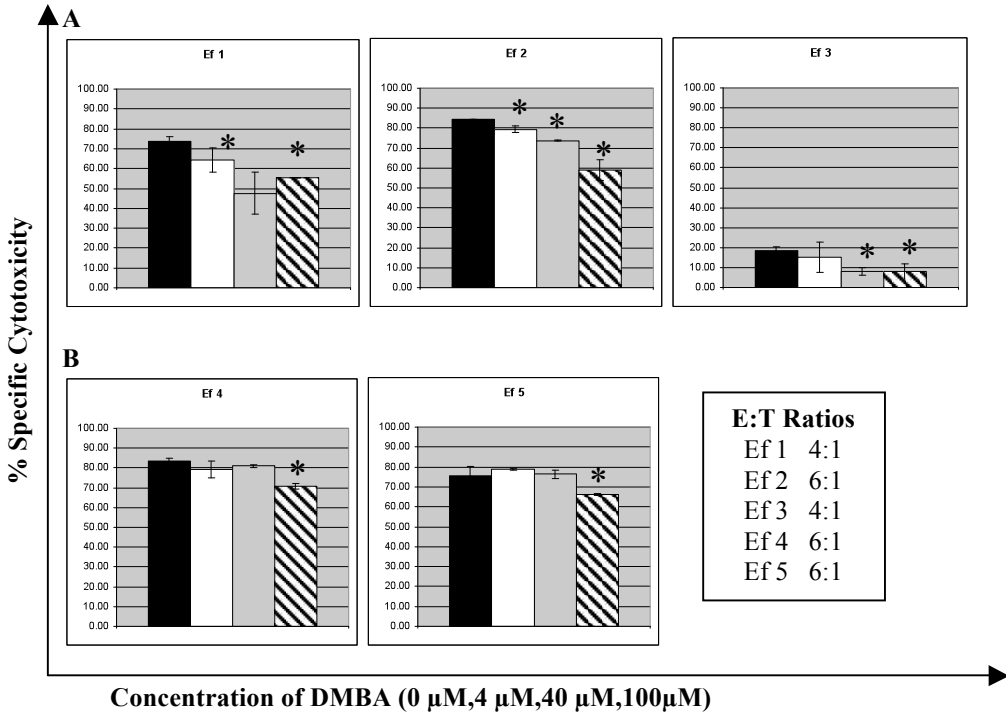


Figure 2. Percent specific cytotoxicity of K562 targets following *in vitro* exposure of earthworm coelomocytes to DMBA at 9°C for 16 hours. Percent of specific cytotoxicity of K562 targets by coelomocytes from Ef 1, Ef 2 and Ef 3 (A) and Ef 4 and Ef 5 (B) pretreated with 0 μM (untreated control) (black), 4 μM (white), 40 μM (gray) and 100 μM (hatched) DMBA for 16 hours at 9°C. Assay time was 3 hours. Statistical significance: * corresponds to *P* values less than 0.05. Error bars reflect minimum and maximum of duplicate samples.

In one assay, three (Ef1, Ef2 and Ef3) out of seven earthworms tested demonstrated a dose dependent decrease in percentage specific cytotoxicity of K562. In another assay, two out of four earthworms tested exhibited an inhibitive response (Ef4 and EF5; Figure 2b). The E:T ratio for Ef1 and Ef3 was 4:1, and for Ef2, Ef4 and Ef5 was 6:1. The results were statistically significant in every case with *p* value less than 0.05.

Results of nonresponders are not shown. These results encouraged us to test the effects of temperature on the immunosuppression of natural killer-like coelomocytes in response to DMBA.

Cytotoxicity of K562 by DMBA Exposed Coelomocytes at 25°C for 0.5 to 1 Hour

The next experiment involved a one-hour assay using K562 target cells and coelomocytes that were incubated for one hour with 0 μM , 10 μM and 100 μM DMBA at 25°C. The E:T ratio for this assay was 2:1. Two out of four earthworms tested showed a decrease in percentage specific cytotoxicity that was statistically significant (Ef6 and Ef7) (Figure 3A). Results of nonresponders are not shown.

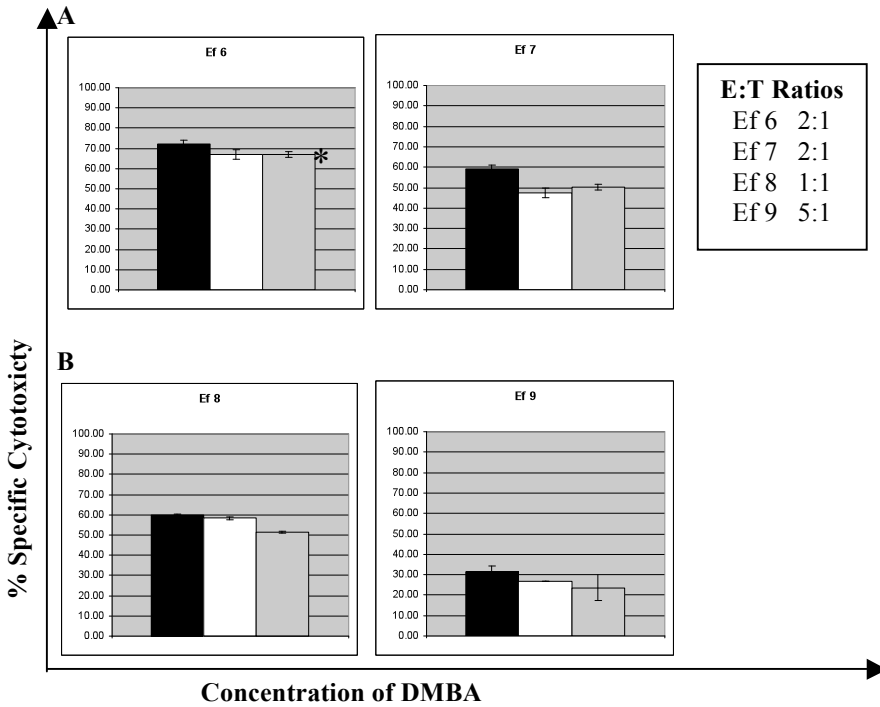


Figure 3. Percent specific cytotoxicity of K562 targets following *in vitro* exposure of earthworm coelomocytes to DMBA at 25°C for 0.5 to 1 hr. (A) percentage specific cytotoxicity of earthworm coelomocytes from Ef 6 and Ef 7 pretreated with 0μM (untreated control)(black), 10 μM (white), and 100 μM (gray) DMBA for 1 hr. at 25°C, to K562 targets. Assay time was 1 hr. (B) % specific cytotoxicity of earthworm coelomocytes from Ef 8 and Ef 9 pretreated with 0 μM (untreated control)(black), 200 μM (white), and 500μM (gray) DMBA for 30 min. at 25°C to K562 targets. Assay time was 30 min. Statistical significance: * corresponds to *P* values less than 0.05. Error bars reflect minimum and maximum of duplicate samples.

Similar results were obtained when 200 μM and 500 μM DMBA were used for 0.5-hour exposure period. Two out of four earthworms (Ef 8 and Ef 9) exhibited immunosuppressive effects of DMBA at E:T ratios of 1:1 and 5:1, respectively (Figure 3B). The decrease in percentage specific cytotoxicity between the untreated control and DMBA treated group was statistically significant as determined by student *t* test. An important difference in the 0.5-hour assay was that the DMBA was not washed from the effector population prior to the addition of K562. Hence, additional control was included to test the effects of DMBA on K562 at these higher concentrations in the absence of effector as described in the methods. The results indicated that for these additional controls there was no difference in PI uptake in K562 exposed to DMBA compared to K562 not exposed to DMBA (data not shown).

In conclusion, inhibition of cell-mediated cytotoxicity was observed in a dose-dependent manner when earthworm coelomocytes were exposed *in vitro* to DMBA (up to 500 μM). This inhibition of NK-like activity was true for all of the periods and temperatures employed ranging from 0.5 to 16 hours and temperatures ranging from 9°C to 25°C.

Discussion

We have demonstrated that a nonradioactive flow cytometric cytotoxicity assay can be used reproducibly to test earthworm NK-like

activity using the tumor cell line K562 as the target population. We were able to establish *in vitro* culture after a series of experiments using varying incubation periods (0.5 to 16 hrs) and temperatures ranging from 9°C to 25°C. On one occasion, a careful microscopic assessment was done to examine the relative health of the cells. Figure 4 illustrates the cells before and after culture. Relative similarities in the number of intact and healthy cells are noticeable in this figure.

In a separate assay, we also tested for cell viability of coelomocytes by including a PI control. Effectors were incubated with PI in the absence of target cells without appropriate range of DMBA concentrations; there was no obvious effect on cell viability observed. We attempted to eliminate dead cell prior to assay by separating coelomocytes using Percoll gradients as mentioned in Hamed, Kauschke & Cooper (2002). However, Percoll gradients were not successful owing to the very low yield obtained. As a result of these experiments, we were able to measure inhibition of cell-mediated function using this *in vitro* model.

We believe that we are the first team to demonstrate the *in vitro* effects of DMBA on NK-like activity in *Eisenia fetida*. Previous studies performed using *in vivo* models involved exposure of intact earthworms to environmental pollutants in soil. If we were to establish this experiment, we would have to establish the control, so that we can compare the

cytotoxicity potential of extruded coelomocytes pre-DMBA exposure and post-DMBA exposure. Given the relatively low rate of response, under the condition explained in this paper, including controls of this nature would be very laborious, time-consuming and costly, perhaps requiring as many as 16 earthworms per assay. In addition, the success rate of extrusion ranged average of 20%–30% and number of extruded coelomocytes for any given worm also varied considerably from 1.1×10^5 cells per earthworm to 1.1×10^6 cells per earthworm. Therefore, if we had elected to do an *in vivo* assay, many of the earthworms extruding prior to exposure to DMBA would not have extruded post-DMBA treatment, further emphasizing the importance of developing an *in vitro* assay to analyze NK-like cytotoxicity in *E. fetida*.

Moreover, we anticipated that we might need to do an *in vivo* assay involving DMBA contaminated soil exposure. This would necessitate having to freeze the coelomocytes of the earthworms prior to DMBA exposure so that they could be tested on the same day as the coelomocytes exposed to DMBA. A preliminary experiment was carried out to assess the relative viability of coelomocytes before and after cryogenic preservation. The result indicated increased cell deaths because of freezing in a DMSO based freezing medium averaging 25% to 50% (data not shown). The viability test was done using trypan blue assay. This experiment should be

repeated to validate increased cell death associated with freezing.

Different methods were used to harvest coelomocytes. These methods included ethanol extrusion, electric extrusion and ultrasonic extrusion as mentioned in Hendawi (2004) and in Homa, Olchawa, Stürzenbaum, Morgan & Plytycs, (2005). Ultrasonic treatment caused hemorrhaging around the clitellum, whereas ethanol extrusion failed to give desired number of cells per worm. When both ethanol extrusion and electric extrusion was used together, it gave an average of 8.3×10^5 cells per earthworm. It was interesting that we did not see any obvious correlation between weight of the earthworms and the extruded cells of these earthworms. However, a decreased disturbance of the worm prior to extrusion positively correlated with higher yield. In conclusion, the results of these experiments suggest that *in vitro* exposure of earthworm coelomocytes to DMBA, a PAH, has inhibitory effects on NK-like activity in *Eisenia fetida*.

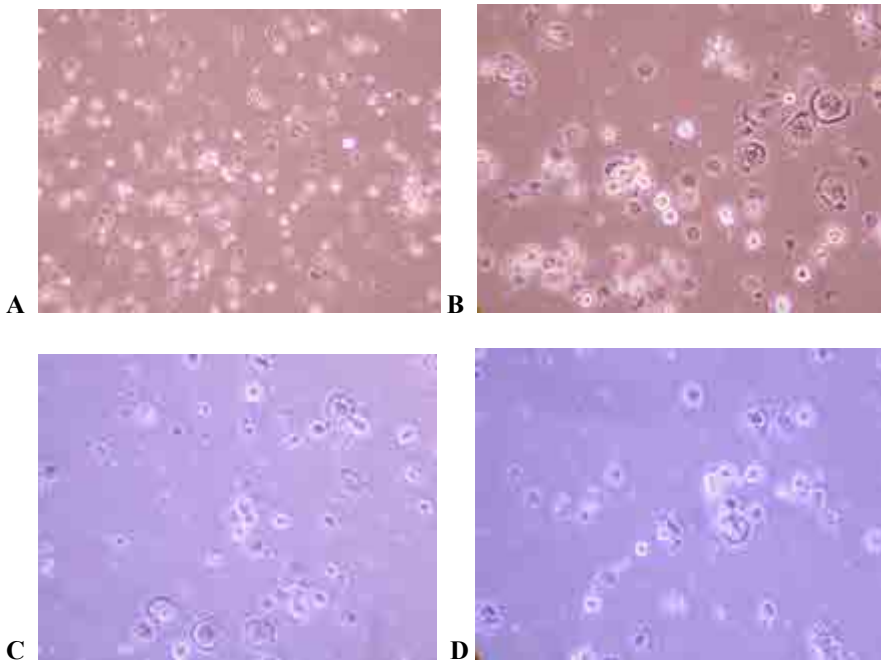


Figure 4. Effector cell viability test using Trypan blue assay. A: earthworm effector cells at 100X magnification. B: the same effector cell population at 400X magnification. C: same cells as shown in A and B, but with Trypan blue in different fields at 400X magnification.

Future Direction

The result of the study described and shown in this paper encourages us to assess the effects different xenobiotics or heavy metals in *E. fetida*. We also want to look at cell-to-cell interactions, and how it is causing effector cells to kill K562 tumor cell line by exploring signal cascade and adhesion molecules in *E. fetida*. There is a need for identification of cell surface markers unique to NK-like cells and generation of monoclonal antibody specific for these markers to facilitate more detailed analyses. If a protocol for isolating NK-like cells from other earthworm cells is established, then studies related to signal transduction and exocytic pathways could be carried out using cell sorting or magnetic bead techniques. Furthermore, cytotoxicity studies would be much easier if a NK-like cell line could be immortalized using transformation procedures amenable for earthworm coelomocytes. Finally, transfection protocols for earthworm coelomocytes need to be developed in order to study specific cell surface ligands needed for cell-to-cell interaction. One of the biggest barriers to understanding cell-mediated immune responses is the lack of genomic information for *E. fetida*. There is a need to sequence the genome of the earthworm and compare it to homologous molecules involved in innate immunity in more developed models.

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