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THE INNATE IMMUNE RESPONSE IN EISENIA FETIDA TO MICROBIAL CHALLENGES

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Dorothy Wrigley, Faculty Mentor (Biological Sciences)

The common earthworm, *Eisenia fetida*, exhibits a rudimentary immune system. The earthworm needs cellular and chemical responses against a constant microbial exposure from its natural environment. Some cellular and chemical responses are found in the coelomic fluid and have been shown to demonstrate anti-microbial characteristics. This project uses microscopy and modified staining techniques to differentiate and categorize the cellular components found in the coelomic fluid. Following a microbial challenge by *Klebsiella pneumoniae*, an inflammatory response was initiated. Six groups of earthworms were injected with 0.05 ml of 1.0×10^6 cfu /ml *K. pneumoniae* on day one and tested over a period of five days. A group of three worms was shocked each day for the next five days to cause the coelomic fluid and cells to pass through the body wall. The coelomic fluid was placed directly on glass slides, dried and stained with a modified Wright's stain using a wash buffer solution with a pH of 6.3. The stained cells were differentiated into four categories. Total cell counts were determined. The data indicated a marked proliferation in total cell counts in comparison to the control worms. This trend of increasing total cell counts continued over the five days. The percentages of the four types of coelomic cells from the differential remained constant. Cells were photographed and documented for comparisons. Additional studies are ongoing to determine how long the *Eisenia fetida* take to remove *Klebsiella pneumoniae* from the coelomic cavity.

Introduction

Inflammation is a chemical and cellular response to irritants like bacteria, viruses and foreign objects. In mammals, inflammation involves both innate and immune defenses. Innate responses are immediate nonspecific defenses like skin, chemicals in blood and cells that attack anything foreign to the body (Goldsby, 2000).

One aspect of this study focused on innate responses to inflammation, without the interactions of the adaptive immune system, by examining the responses in earthworms to bacteria. The earthworm model was for several reasons chosen for analysis. They have a simple system, in that they lack adaptive immunity (Fischer, 1977), and they display an innate immune response (Dhainaut, 2001). They are also successful in a highly contaminated world (Dhainaut, 2001). The earthworm lives a relatively long time and must have a functional defense system against microbes. Earthworms also have a well studied life cycle. *Eisenia fetida* is one earthworm species that is an easily maintained and can be used in continuing studies.This study focused mainly on the coelomic cells, which are also called coelomocytes (Dhainaut, 2001). Coelomocytes are found in the coelomic cavity of the earthworm. Earthworms have pores that connect the coelomic cavity to the exterior, through which cells are exuded following stress. Coelomocytes are similar in appearance and function to some human white blood cells (Dhainaut, 2001; Affar, 1998). The purpose of this experiment were to establish methods to quantify the innate responses in *Eisenia fetida.*

Methods

Earthworms were purchased from Carolina Biological Supply Co. They were maintained on non-soil earthworm matrix and fed Magic™ Worm food. Mature earthworms about 8 cm long were used. For each experiment earthworms were separated into groups of 3 worms and housed in glass jars with a sterile damp paper towel bedding. The bedding was kept damp with bottled spring water (Cub) and food was provided once a week.

Bacterial Inoculation: The gram negative bacterium, *Klebsiella pneumoniae*, was grown in nutrient broth (NB) overnight. The bacteria were washed in phosphate buffered saline (PBS) and then suspended in PBS to 10^6 cfu/ml. The worms were anesthetized in 5ml of 5% ethanol and PBS for 5-10 minutes (Affar, 1998) and injected in between the 4^{th} and 8^{th} segment behind the clitellum. Next, an amount of 0.05 ml of bacteria was inoculated into the experimental group of anesthetized worms. Then the control worms were inoculated with 0.05 ml of PBS. The experimental worms were separated into five jars, with each glass jar containing three bacterially inoculated worms. The control group was then separated into five jars, with each control jar containing three PBS inoculated worms.

Total Coelomic Cell Count: To extract the coelomic cells the worms had to be prepared. This was achieved by taking the three worms out of the jar and placing them in 5 ml of PBS in a Petri dish. The worms were shocked with a 9volt battery for 1-2 seconds. After 5 minutes the exuded coelomic cells were collected and centrifuge in micro-centrifuge tubes. The micro-centrifuge tubes were decanted and the coelomic cell pellets were pooled into one centrifuge tube and diluted with 0.5ml of PBS as a step to concentrate cells and to remove excess mucus. The cells were then washed and mixed in 0.5 ml of PBS. The cells were counted using a bright field microscope and a

hemacytometer. This procedure, of collecting and counting cells, was performed on the experimental and the control group for each day of the experiment.

Cell Differentiation: The procedure for staining cells, for the purpose of coelomic cell differentiation, was accomplished by utilizing a modified Wright's stain. The procedure for staining these exuded cells was done by making a smear of the exudate on a glass slide. The cells on the slide were air dried. Two or three drops of Wright's stain was used to make a film on the slide and allowed to dry for 60 seconds. The slide was rinsed with 0.02M phosphate buffer at pH of 6.3. The slide was blotted dry with a paper towel and then flooded with Wright's stain for another 60 seconds. The slide was rinsed using the same rinse buffer for a second time and immersed in the buffer for 5 minutes. The slide was dried and examined and the cell types determined.

The Neutral Red Uptake: The neutral red uptake procedure was done to assess some of the coelomocytes' characteristics. This procedure was done by using earthworm exudate from three worms given an electrical shock for 2-3 seconds with a nine volt battery. Then a 1:1 dilution of worm exudate and neutral red dye was made. The solution was mixed gently and then incubated for 15 minutes at room temperature. The solution was mixed gently again before placing a drop on a glass slide and a cover slip was placed over the drop. The slide was placed on a microscope using the 40x objective to search for red stained cells. One hundred cells were counted and the number of red cells indicated the percent of neutral red uptake.

K. pneumoniae Clearance: It was of interest to determine how long the earthworm would take to remove bacteria from the coelomic cavity; therefore, the following method was developed. The clearance of *Klebsiella pneumoniae* from earthworm coelomic cavity method also used the bacterial inoculation method. The only difference was the number of worms being exposed to the bacteria slightly different. Twelve worms were inoculated using a sublethal dose of 0.05ml of *K. pneumoniae* at concentration of 10^6 cfu/ml. These twelve worms were separated into glass jars, each containing three worms containing the non-soil earthworm matrix. There were four worms that were injected with 0.05ml of PBS to be used as the control group. They were also put into a glass jar with the non-soil earthworm matrix. On day one, three worms that had been injected with *K.pneumoniae* were wiped with an alcohol wipe and injected with 0.05ml of PBS. This was then extracted from the coelomic cavity of the worm and plated directly onto three urea agar plates. One control worm was injected with 0.05ml of PBS and plated onto the urea agar as a negative control, used to monitor aseptic technique. The procedure was then done on days two, four and seven. The positive growth was indicated by pink color found on the urea agar. This was also Gram stained as a secondary test; to make sure the growth on the urea agar were gram negative rods.

Results

Cell Differentiation: The types of cells found in the worm exudate were determined through the modified Wright's stain.(Figures 1-3). Cells were easily differentiated into four types: small agranular, lymphocyte-like cells; large agranular monocyte-like cells; and small and large granulocyte-like cells. Differential counts of the exudates cells indicate that there was little difference in the proportion of cells in the four cell types. (Figure 4 and 5). However, the proportion of large granular cells increased over the five day period.

Figure 1. This photo shows two large granular cells compared to one small lymphocyte-like cell that were Wright stained and photographed at 100x.

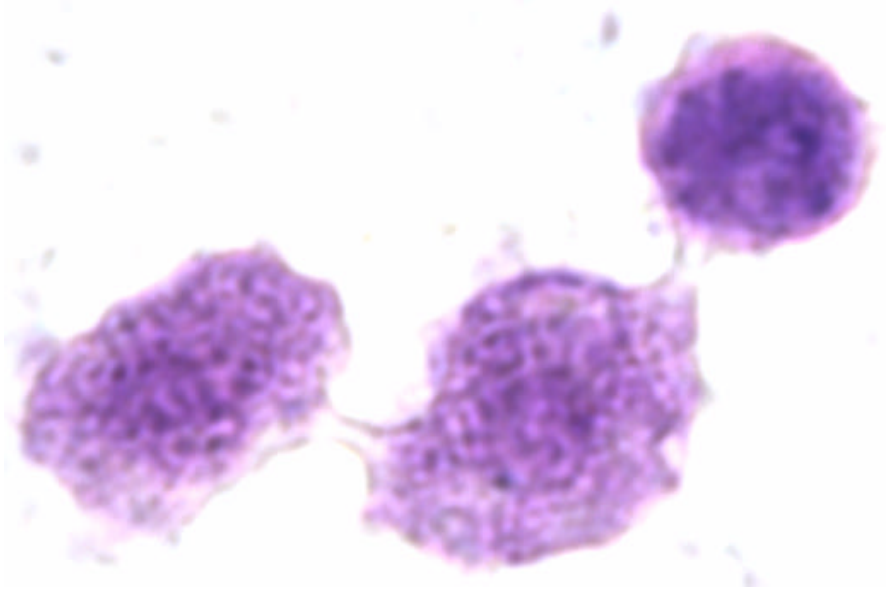


Figure 2. This shows one of the large monocyte like cells found in the cell counts, also Wright stained and photo taken at 100x.

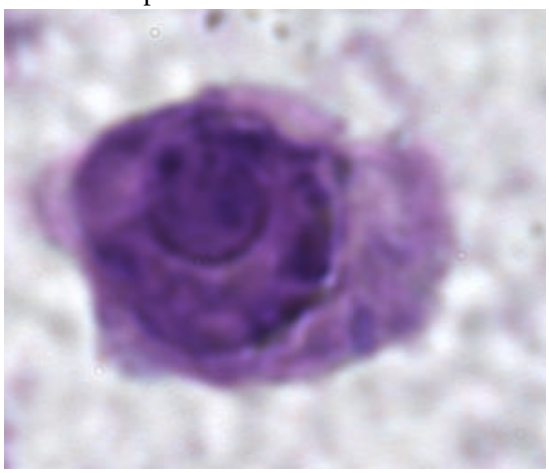


Figure 3. This photo compares one of the small granular cells to two of the lymphocyte-like cells. The small granular cell is the darker cell in the upper left corner.

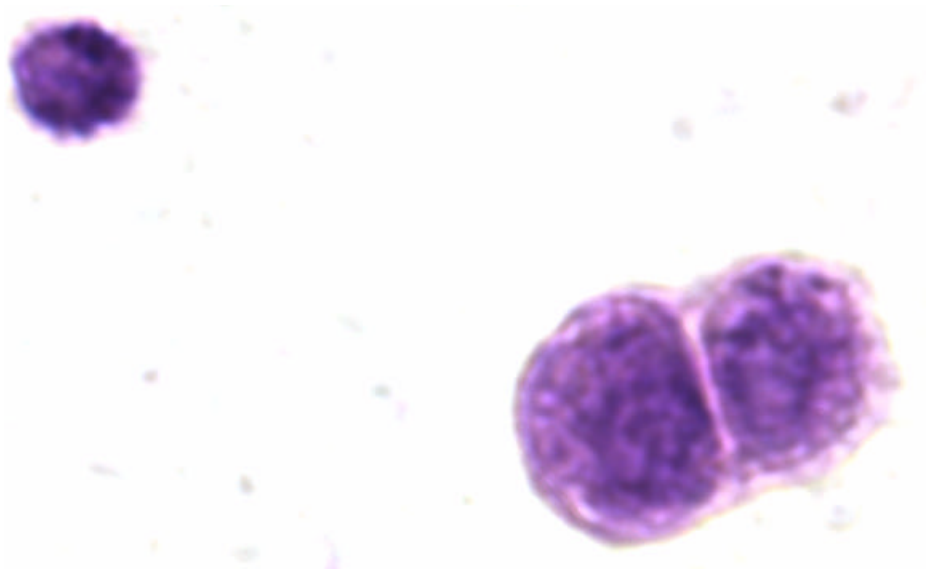


Figure 4. Comparison of the percentages of Agranular cells from earthworms that have been inoculated with *Klebsiella pneumoniae* and earthworms that were not inoculated, over a five day period.

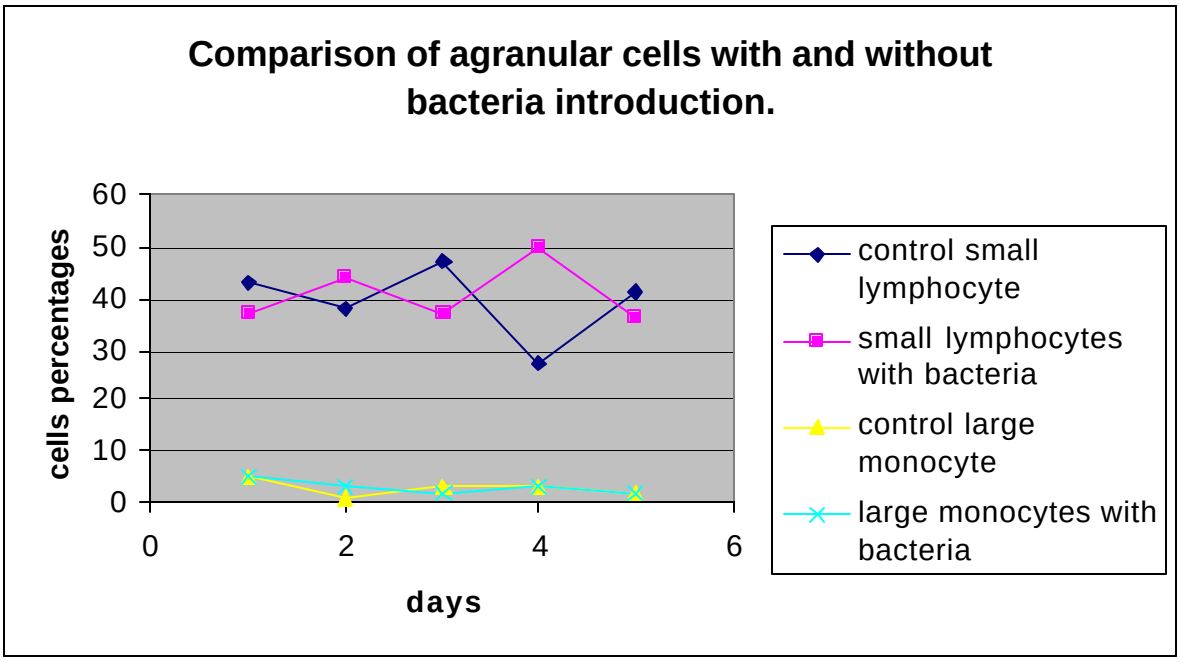
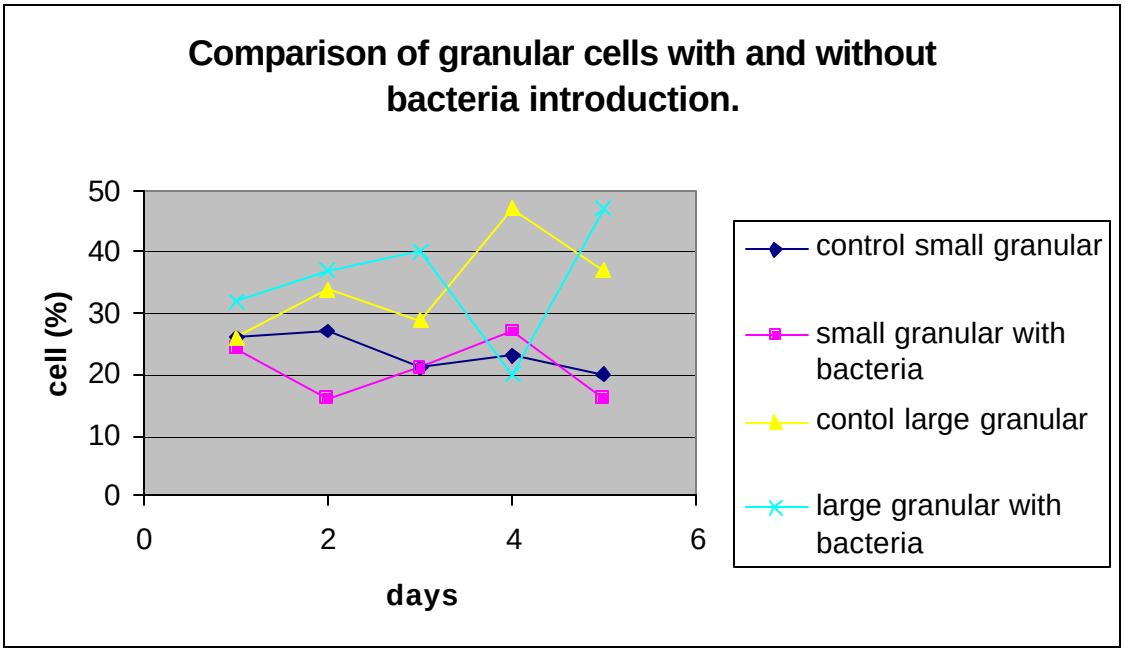


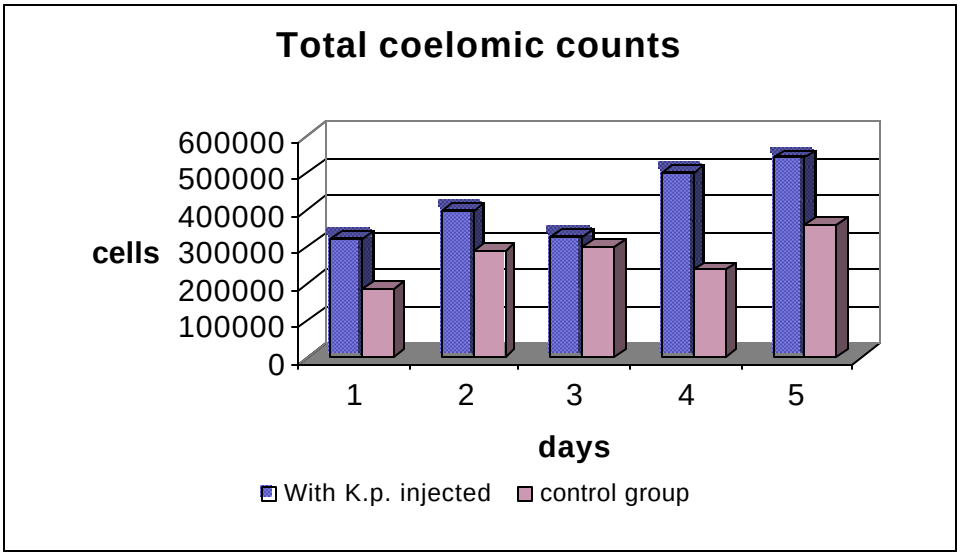
Figure 5. Comparison of the percentage of granular cells taken over a five day period. This graph also compares the percentages between the control group and the group of earthworms inoculated with Klebsiella pneumoniae.



Total Coelomic Cell Count: The number of coelomic cells from the *K. pneumoniae* inoculated worms and the control worms were followed for five days after inoculation. The data indicates that *K. pneumoniae* worms had more cells in the exudates

for four of the five days. This study demonstrated that *E. fetida* coelomocytes were more numerous following gram negative bacteria challenge. The increase in the total coelomic cell counts can be seen in Figure 6.

Figure 6. Total coelomic cell counts taken over a five day test period. This graph also compares the total cell counts between the control group and the group of earthworms inoculated with *Klebsiella pneumoniae*.



The Neutral Red Uptake: The procedure for neutral red uptake was developed as part of this study to observe endocytosis in the control group. Although the bacterially

challenged worms were not analyzed, the control group provided a baseline to work with in the future. Photos of the cells without and with neutral red were taken at 40x (Figure 7 and 8). The neutral red uptake percentage in normal worm coelomocytes was determined to be at 27%.

Figure 7. This is a normal wet mount, without neutral red, of coelomic fluid taken at 40x.

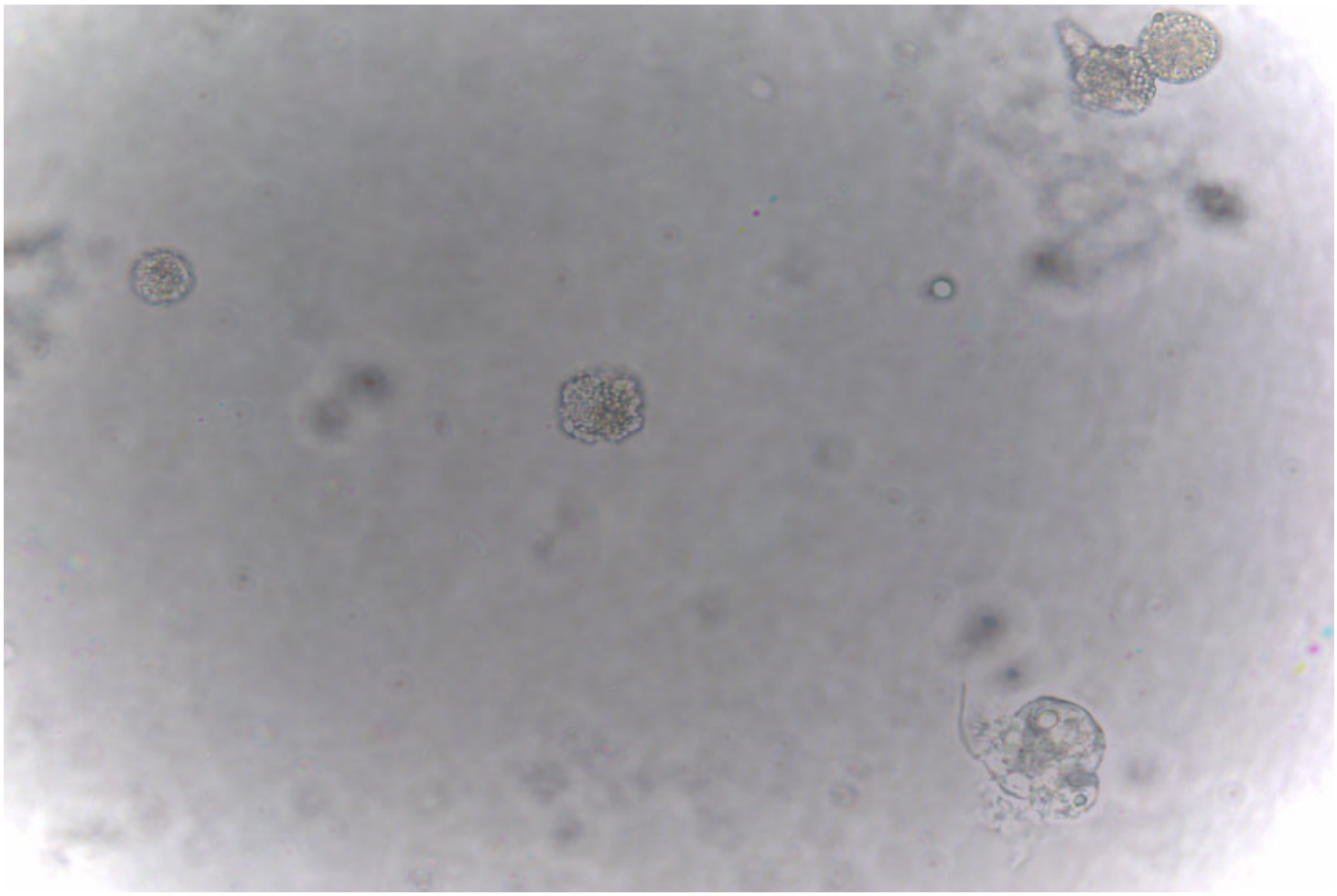
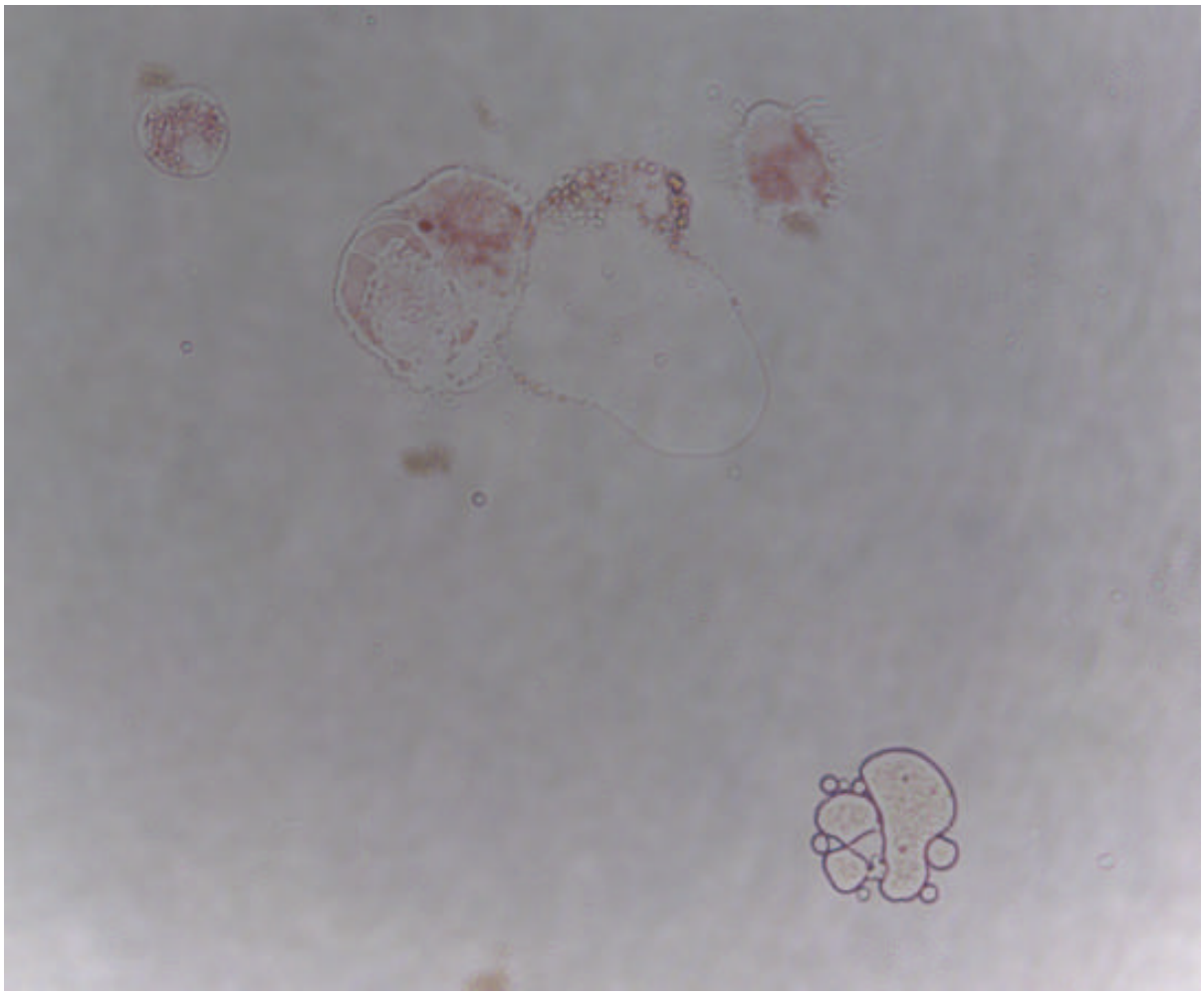


Figure 8. This is a wet mount of coelomic fluid showing coelomic cells with a positive neutral red uptake at 40x.



K. pneumoniae clearance: *K. pneumoniae* was isolated from all the inoculated worms and from the control worms. This indicates that somehow the bacteria contaminated the other cultures or that some reagent had become contaminated.(Table 1.)

Table 1. *Klebsiella pneumoniae* extracted from the coelomic cavity of experimental and control worms.

	Clearance of Klebsiella pneumoniae from coelomic cavity of earthworms.			
	Day1	**Day 2**	**Day 4**	**Day 7**
	1	1	0	1
	1	0	1	0
	1	0	1	0
total positive for K.p.	3	1	2	1
Negative control	0	1	1	1
Gram Stain	negative rods	negative rods	negative rods	negative rods

1 = growth of *K. pneumoniae,* 0= no growth

Discussion

The cells were first differentiated into four visible groups and are indicated in Figure1,2, and 3. The total cell counts were visibly increased in the group of worms that were inoculated with *Klebsiella pneumoniae* as seen in Figure 6. Figures 4 and 5 shows in the experimental and in the control groups, the concentration of the four cell types did not significantly change. The neutral red uptake was determined to be at 27% for normal coelomic cells. Also, clearance of *K. pneumoniae* from the worms was not conclusively shown due to contamination. The data indicate that *K. pneumoniae* does induce an increase in coelomic cell number and that this could be an innate response to this gram negative bacterium. The cell type did not differ much which indicates that all the cell types increased in number to the stimulus.

Conclusions

The purpose of this experiment was to establish methods to quantify the innate responses in *Eisenia fetida*. The data in this experiment indicates that there are four cell types that can be analyzed in the coelomic fluid. That the inoculation of *K. pneumoniae* increases the number of exudate cells but does not alter the cell types. Therefore, *Eisenia fetida* responds to bacterial challenges by increasing the number of coelomic cells.

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