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Characterization of apoptotic mechanisms involved in R body induced death in *Paramecia tetrarurelia*

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Abstract. *Paramecium tetrarurelia* is a unicellular eukaryotic ciliate. Certain strains of paramecia have been shown to contain endosymbiotic bacteria. Furthermore, some of these endosymbiotic bacteria confer a killing ability on the host paramecia, known as killer paramecia, because the endosymbiont can be lethal to other paramecia, known as sensitive paramecia, if it is released into the environment. This death has been connected to a protein found within the *P. tetrarurelia* known as an R body. Interestingly, the R body-related-death resembles apoptosis in that it causes membrane blebbing, a characteristic of apoptosis. Furthermore, paramecia contain another marker of apoptosis, a mechanism to degrade DNA which is used in normal paramecia conjugation. Therefore, exposure to the R body and toxin may be activating some existing pathways in the paramecia, leading to apoptosis. To determine whether the mode of cell death induced by the R bodies is apoptotic, a flow cytometer was used to examine DNA fragmentation, a characteristic of apoptosis, using a Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) assay. Three groups of paramecia were examined with TUNEL: sensitive-alone, killer-alone, and killer mixed with sensitive. The results of the TUNEL assay were inconclusive because the only cell group showing positive TUNEL staining was the sensitive paramecia group. This could possibly be due to the DNA degradation that occurs during normal cell conjugation. Therefore, this study was the first to use flow cytometry in order to examine R body induced cell death and provides a clear basis for further research.

Introduction

*Paramecium tetrarurelia* is a unicellular eukaryotic ciliate that is found naturally in fresh water lakes, rivers and streams.

These organisms feed on bacteria and are utilized as a food source by other protists or small animals. In addition, certain strains of paramecia have been found to contain bacterial *Caedibacter taeniospiralis* endosymbionts that exist in a symbiotic relationship with the paramecium within the cytoplasm (Beier, 2004). These endosymbionts generally do not harm their host paramecium but can be toxic to other strains of paramecia that are not able to establish this symbiotic relationship.
relationship (sensitive strains) (Pond, 1989). Under some conditions, the endosymbiont is released from the host paramecium and taken up by a sensitive paramecium, after which the sensitive paramecium is killed. Therefore, the host paramecia that contain these bacterial endosymbionts are often referred to as ‘killer’ paramecia.

Using phase microscopy, investigators have shown that the killing trait is dependent upon whether or not the genome of the endosymbiont bacterium contains the genetic information encoding a specific coil-shaped protein complex known as an R body (Pond, 1989). Occasionally, the endosymbiont containing an R body may be released into the environment and eaten by another paramecium. As the endosymbiont is destroyed in the sensitive paramecium’s food vacuole, the R body is released and unwinds in the acidic environment of the food. When the R body unrolls, it punctures the food vacuole and the putative toxin is released into the cytoplasm, leading to death in that paramecium (Preer, 1974). However, at the present time, relatively little is known about the exact mechanism by which death occurs. Preliminary results in our lab suggest that the rebD gene may code for the killing toxin that is associated with R bodies produced by *C. taeniospiralis* (Riordan, 2006). This gene is part of the R body encoding reb locus, which consists of four genes: A, B, C, and D. Initial studies in our lab have shown that when the rebD gene is expressed in *E. coli* cells and these cells are then fed to sensitive paramecia, the paramecium dies (Heruth, personal communication).

Interestingly, the mechanism of cytotoxicity induced by the R body-containing bacteria in sensitive paramecia resembles apoptosis in some ways (Wiegel, 2004). For example, membrane blebbing is often observed. In addition, it is known that in many cases, a bacterial toxin (e.g. anthrax toxin) can trigger an apoptotic response in mammalian cells and lead to cell death (Drysdale, 2007). Apoptosis is a type of programmed cell death that is characterized by several observable morphological changes, including nuclear condensation and membrane blebbing (Springer, 1993). Apoptosis is typically the mechanism employed by a multicellular organism to remove damaged cells. Therefore, it is somewhat counterintuitive that this type of cell death would occur in a unicellular organism. However, the *P. tetraurelia* genome contains the genes DAD1 and PARP, homologs of which are known to be involved in apoptosis in multicellular eukaryotes. In addition, many ciliates have been shown to undergo DNA degradation during conjugation, a process of genetic exchange, by a mechanism that is similar to the DNA degradation that occurs during apoptosis. *P. tetraurelia* contain both a macronucleus and two micronuclei. The macronucleus is polyploid and is transcriptionally active during the vegetative life cycle of the organism, while the micronuclei are diploid and transcriptionally inactive (Preer, 1974). During conjugation, a new zygote nucleus is formed and the old macronucleus is destroyed. Taken together, all these factors suggest that there could be an apoptotic mechanism that exists in paramecia which may be involved during the R body induced death.

Therefore, insights into the molecular and genetic regulation of R body-induced killing of sensitive paramecia may provide a better mechanistic understanding of cell death as a whole, and specifically via apoptosis. The goal of this research project was, therefore, to work toward characterizing the mechanisms involved in the death of sensitive paramecia in response to exposure to a toxin produced by the symbiotic bacteria. The assay used to quantify apoptosis in this experiment used actual killer and sensitive paramecia, rather than isolated R bodies. While there is a method for the isolation of R bodies from *Caedibacter taeniospiralis* that maintain toxic activity, the problem with using this method of killing is that current R body isolation procedure only allows for small quantities of R bodies to be isolated (Wiegel, 2004). Use of killer and sensitive paramecia allowed for a higher number of cells to be tested, which was necessary for TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP-biotin Nick End Labeling), an assay for detecting apoptosis.

Sensitive paramecia were incubated both alone and in the presence of killer paramecia. There was also a control group that contained only killer paramecia. The hypothesis was that if the R body and toxin produced by the endosymbionts...
of the killer paramecia were in fact causing apoptosis in sensitive paramecia, then we would expect to see apoptosis in the group of mixed cell types but not the group containing only sensitive or only killer paramecia. TUNEL uses a transferase enzyme to incorporate labeled nucleotides into the 3’ ends of DNA (Springer, 1993). This can be used to detect the presence of nicked and therefore, degraded DNA, which would signify the process of apoptosis.

Materials and Methods

Growing medium preparation
Rye grass medium was used to grow the paramecia (Dilts, 1976). It was prepared by combining deionized water with rye grass in a ratio of 3.5g rye/1L water. The solution was boiled for 10 minutes and filtered with cheesecloth in order to remove excess rye grass. The pH of the solution was adjusted to 7.7 to 7.8 with dibasic sodium phosphate heptahydrate. Finally, the medium was autoclaved.

Inoculation and feeding procedures
The rye grass medium was inoculated with Klebsiella pneumoniae, which serves as a food source for the paramecia. After inoculation, the medium was left overnight at room temperature to allow for the bacterial cultures to grow. The medium was then adjusted to a pH of 6.8 to 7.2 with 1N HCl. The medium was tested by adding 2mL of media to a small culture of paramecia, which was allowed to grow for at least six hours at room temperature. If the paramecia were still alive after this time, the medium was used to feed the cultures by pipetting the appropriate amount into the existing culture. In order to maintain the endosymbionts within the killer paramecia, the paramecia could not be fed more than twice their culture size every four days.

Killing test
An equal amount of killer paramecia (K) and sensitive paramecia (S) were concentrated by centrifugation for 10 minutes at 9,500 rpm. The supernatant was removed and the cells were resuspended in one-fourth the original amount with Dryl’s solution (1 mM NaH₂PO₄, 1 mM Na₂HPO₄, 2 mM Sodium Citrate, 1.5 mM CaCl₂). These cells were then combined and incubated at room temperature for 6 hours. In addition, samples of sensitive paramecia alone and killer paramecia alone were prepared in the same way as the mixed paramecia.

TUNEL
Cell suspensions of sensitive-alone, killer-alone, and sensitive-killer-combined were prepared following combined paramecia killing test. TUNEL was performed using the Calbiochem Fluorescein FragEL DNA Fragmentation Detection Kit. Specifically, cells were pelleted by centrifugation at 5000rpm for 5min at 4°C and the supernatant was removed. The cells were resuspended in 4% formaldehyde/1X Phosphate Buffer Solution (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄) to a concentration of 1x10⁶/ml and incubated at room temperature for 10min. Cells were then centrifuged as before, the supernatant was removed, and the cells were resuspended in the same amount of 80% ethanol.

Cells were then immediately centrifuged as before and the supernatant was removed. The cells were resuspended in 200 μl 1x Tris Buffered Saline (20 mM Tris pH 7.6, 140 mM NaCl) and incubated for 10-15 minutes at room temperature. Cells were pelleted by centrifugation and the supernatant was removed. The cells were resuspended in 100 μl of 20 μg/ml proteinase K (prepared previously by making a 1:100 dilution of 2mg/ml proteinase K in 10mM Tris pH 8) and incubated at room temperature for no more than 5 min. Cells were then pelleted and the supernatant was removed. Cells were then resuspended in 100 μl of 1x TdT equilibration buffer (1:5 dilution of 5 X TdT Equilibration Buffer [from kit] with dH₂O) and incubated at room temperature for no more than 10-30 min. During this time, two reaction mixtures were prepared on ice. One mixture contained 57.0 μl Fluorescein-FragEL TdT Labeling Reaction Mix [from kit] and 3 μl TdT enzyme [from kit] per sample. The second mixture was made in the same way except that the TdT enzyme was replaced with dH₂O. Each cell
sample was then divided in half; 60 μl of the reaction mix with TdT was added to one of the samples and the reaction mix without the TdT was added to the other sample. These cells were then incubated at 37°C for 1-1.5 hours in the dark. Next, the cells were centrifuged for the same time as before and the supernatant was removed. Cells were resuspended in 200 μl 1xTBS and centrifuged. The supernatant was removed and the cells were resuspended in a final volume of 500 μl 1xTBS.

Cells were analyzed using flow cytometry. The cytometer used was Becton Dickinson FACSCalibur (P1: Detector FSC, Voltage E00, Ampl. Gain 1.00, Mode Lin; P2: Detector SSC, Voltage 285, Ampl Gain 1.00, Mode Lin; P3: Detector FL1, Voltage 375, Ampl Gain 2.00, Mode Log).

Results

Sensitive paramecia stain positively for TUNEL

This study was focused on establishing methods for performing apoptosis assays with paramecia. Rather than trying to use isolated R bodies, which are difficult to isolate in large quantities, the killer paramecia themselves were used in this study. Initially, killer paramecia were incubated with sensitive paramecia for six hours at room temperature, a period of time that has been shown to allow for cell death to occur. TUNEL was used to examine the cells for evidence of DNA degradation. This assay uses the enzyme terminal deoxynucleotidyl transferase (TdT) to add fluorescein labeled nucleotides to the ends of nicked DNA fragments produced during DNA degradation. The fluorescence was then measured using a flow cytometer. The control sensitive and killer groups were also assayed.

During the first TUNEL trial, the cells were incubated for 6 hours prior to the TUNEL assay. For each group of cells, the sample without the TdT enzyme was analyzed first (-TdT). This was done to establish baseline fluorescence and determine the amount of fluorescence present from non-specific staining. A marker region was then set that would include less than 5% of the -TdT cell population. The sample of cells with the TdT enzyme was then analyzed (+TdT). The marker region was kept on the histogram and the amount of cells present in the marker region for the +TdT sample was compared to the amount of cells present in the -TdT sample (% +TdT cells in marker region - % -TdT cells in marker region = specific staining). The specific staining is an estimate of the amount of fluorescence that is due to the presence of nicked DNA. The specific staining of the different cells groups could then be compared to determine whether one group contained more nicked DNA than another.

Flow cytometric analysis showed that there was no significant specific staining present in any of the cell groups (51S, 51K, or 51S+51K) after 6 hours. Additionally, the fluorescent peaks present in the histogram of either the 51K or the mix were small. However, it was also shown that the cell population that was present in the dot plot of the 51S group was not present in the 51S+51K mix (Figure 1A and 1B). The dot plot shows each individual cell and its location in the plot based on size (x-axis) and granularity (y-axis). Therefore, cells with the same characteristics should appear in the same area of the dot plot. If the 51S cells were present in the sample, these cells should still be visible in the dot plot in the same location that they were found when tested alone. Therefore, although unlikely, it was possible that the 51S cell population had been degraded completely by the end of the six-hour incubation. The reason that the 51K cells were not observed in this dot plot is most likely because of the increase in granularity of the cells due to the presence of the endosymbionts and therefore they may have been located on a different part of the dot plot because their granularity would have been found higher up on the y-axis.

In order to test for the possibility that the sensitive cells were completely degraded by six hours, the TUNEL procedure was repeated in similar conditions to the first trial. However, this time, a sample of cells was taken out immediately and then every two hours for six hours. This would allow for the detection of apoptosis if it is occurring at an earlier stage than the six-hour point. The results for the zero
hour time are not shown because there was a very small cell pellet for this group and a similar cell count to the later time samples could not be obtained. One possible explanation for this is that the initial centrifugation time used (5 min) was lower than what it should have been and some of the cells could have been lost in the supernatant.

Once again, the population of cells that was present in the 51S group was not present in the dotplot of the 51K cells and 51S+51K mix of cells for all time periods (results not shown). There was no significant specific staining present in any of the samples taken from the 51K or the 51S+51K cells (Figure 2 and Table 1). Similar to the first trial, there was significant staining observed for each of the 51S samples. Furthermore, it was shown that the 51S peak did change over time and with the addition of the TdT enzyme (Figure 2 and Table 1). Initially, there was no specific staining for the 51S group, similar to the 51K and the mixed group of cells. However, there was an increase in specific staining in the four hour group (2.228%) and the six hour group (30.04%; Table 1 and Figure 3).

Discussion

Previous studies have shown that the R body found in the endosymbiont *Caedibacter taeniospiralis* is associated with the toxin that kills sensitive paramecia (those that do not contain the endosymbiont). The purpose of this study was to extend these findings to determine whether or not apoptotic mechanisms were involved in the R body induced death. Originally, these studies were performed using R bodies isolated from the *Caedibacter* (Wiegel, 2004). However, although these assays were effective, this isolation procedure is time-consuming and requires large volumes of paramecia culture. Therefore, while using isolated R bodies may provide the best method for killing cells to obtain images with microscopy, it is not the best method of killing to use for certain apoptosis assays, such as TUNEL (the nicked ends of degraded DNA are labeled with a fluorescent nucleotide), which may require high concentrations of cells. However, TUNEL is one of the best measures of apoptosis because it is able to clearly distinguish apoptotic cells from necrotic cells. Therefore, initial apoptosis
Figure 2. Histogram results for second TUNEL trial. The sample taken after two hours is shown on the left and the sample taken after 6 hours is shown on the right. The top row is the 51S sample, the middle row is the 51K sample, and the bottom row is the mix of the 51S and 51K. The region labeled M1 is the marker region that was used to determine specific staining.
assays were performed using sensitive (51S) and killer (51K) paramecia allowing for large concentrations of cells to be used, which is necessary for analysis with the flow cytometer. The initial results from this study showed that there was no significant difference in specific staining between the control cells (sensitive or killers alone) and the mix of the two cell types (K + S). However, there was an increase in the specific staining of the sensitive cells alone as the incubation time increased. These results were unexpected because the 51S should not be going through apoptosis when not exposed to 51K. However, one possible explanation for this is that the observed increase in specific staining with time (from 2 to 6 hours) in the 51S paramecia was from autogamy, a sexual process involving self-fertilization. Previous studies have shown that conjugating ciliates, thus undergoing sexual reproduction, will stain positively for TUNEL (Ejercito, 2003). This is because of the degradation of the macronucleus which occurs during conjugation and autogamy. In addition, paramecia are known to undergo autogamy when in starvation conditions. Therefore, it is possible that the 51S cells were undergoing autogamy, which would lead to an increase in degraded macronuclei. This would also explain why there was an increase in fluorescence over the six hour time period.

However, the 51K and 51K+51S mix did not stain positively for TUNEL over the six-hour time period. Since these cells should also undergo conjugation under starvation conditions, these results were unexpected. There are several possible explanations for this, although further studies would be needed to confirm them. One possibility is that the culture of killer paramecia contained more bacteria than the culture of the sensitive paramecia. If this was the case, then despite resuspension in Dryl’s solution, there may have been some of the bacterial food source left in the culture. Therefore, it may be beneficial to repeat the study with increased washing and/or incubation in order to completely remove the food source. This would ensure that each culture of paramecia observed would be in the same starvation conditions. Therefore, although cells may be undergoing conjugation, any increase in DNA degradation from the control group to the mixed group could be attributed to the R body induced death.

The overall benefit to be derived from this type of research is that it will potentially provide new insights into the mechanisms involved in cell death and specifically, apoptosis. This knowledge will provide a basis for further investigations into the possible role of apoptosis in single celled organisms. In addition, the ability to study apoptotic pathways in a model system such as paramecia may provide insights that could be applied to other higher organisms. This research will also provide a better understanding of toxin-induced apoptosis. The ability of bacterial toxins to induce
apoptosis in different cell types presents several possible medical implications, including the possible treatment of cancerous cells.

Therefore, it is clear that further studies are needed to determine whether or not the R body induced death is occurring through apoptotic mechanisms. Although several initial studies have hinted at the possibility, this study was unable to confirm the presence of apoptotic mechanisms (Wiegel, 2004).

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Literature Cited


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