Observing the action of phagocytosis in rumen protozoa through the utilization of fluorescent latex beads

Katlyn N. Barr
Honors Research Thesis
Research Advisor: Jeffrey L. Firkins
Department of Animal Sciences
The Ohio State University
2010

Abstract

Protozoa comprise 10 to 50% of the rumen microbial biomass and contribute to fiber digestion and pH stability of the rumen, but they promote breakdown of protein from ingested feed or bacterial cells, which decreases efficiency of feed conversion into animal product. Protozoa take in feed by phagocytosis. We hypothesized that carboxylate-modified blue fluorescent latex beads could be used to assess rates of feed and bacteria uptake by protozoal cultures. We conducted an experiment to determine the optimum bead concentration to add to cultures and to assess differences between fed and unfed cells. Treatments were: 1.05×10^7 and 1.05×10^8 beads/ mL. We added 58 µL of each treatment to samples of fed and unfed 10-mL cultures and then incubated the samples for 1 min, 20 min, 1 hr and 24 hr. We fixed the samples with formalin and observed a 40-µL sample under a fluorescence microscope. The number of cells that consumed beads was counted out of 100 random cells. We discovered that the cells were in fact taking up the beads and that the number of cells that contained beads increased at a decreasing rate until a plateau as the incubation times increased. Through statistical analysis, we determined that saturation was reached by 3 hours, and the incubation time at which bead uptake was at one-half saturation was approximately 20 minutes. The total bead uptake was higher for the higher bead concentration and increased when cells were fed. Therefore, feeding triggers uptake of feed and carboxylated beads that mimic bacterial size and cell wall chemistry. With this valuable information, we can proceed with additional research on the phagosome-lysosome digestion process that drives protozoal nutrient acquisition and growth.

Introduction

Protozoa survive in an anaerobic environment in the rumen where the pH is buffered between the ranges of 5.7 to 7.3 (Rode, 2009). Bicarbonate from the saliva helps buffer the feed contents during chewing and rumination and the temperature of the rumen is kept between a range of 36 and 41 degrees Celsius. Within this type of environment, protozoa proliferate and contribute vastly to the overall health of the animal. Anaerobic fermentation by the protozoa and bacteria is an integral part of fiber and starch digestion in the rumen. The protozoa break down starch and cellulose in the feed in order to release volatile fatty acids (VFA) to be absorbed through the rumen wall as the main energy source of the animal. These VFA's provide 50-70% of the overall energy requirements, and microbial cells provide the animal with 60-70% of its protein once it passes out of the rumen (Russell, 2002). Protozoa feed in the rumen of an animal by pushing food through their ciliated, funnel-like mouth. While seeking out the digested feed of the animal, protozoa will eat what they want and simply excrete the rest as waste. Along the way, however, protozoa also ingest bacteria and other protozoa in addition to the normal feed. Therefore, the protozoa are unnecessarily wasting protein for the animal. In order to reduce nitrogen emissions, the Dijkstra model was developed to improve the efficiency of the nutrients used by the animal. Also, this model was used to try and decrease nitrogen excretion in the urine (Kebreab, 2002). However, this model is developed with the assumption that there is little or no protozoal outflow of the rumen and that the increased nitrogen recycling in the rumen occurs because of increased autolysis of protozoa (Dijkstra et al., 1994). Work at OSU contradicts this, however, and my research is integral to providing support for this hypothesis. Also, this model predicts that the passage rate of protozoa out of the rumen is half of the passage rate of solid feed, but OSU work suggests that the rate of passage of the feed and protozoa are the same (Sylvester et al., 2005). These assumptions

have carried over into other areas of study including experiments on *Isotrichid* behavior (Dijkstra et al., 1998). However, we feel that there is a significant amount of protozoal outflow that affects the overall nutritional efficiency of the rumen. By studying how protozoa ingest bacteria through active predation or less active 'grazing', we can attempt to improve the efficiency of rumen digestion and reduce nitrogen emissions.

Research Questions

1) Will the cells take up the fluorescent latex beads?
2) Will there be a difference in bead uptake in fed versus unfed cells?
3) Is this a reliable method for studying the protozoa phagosome-lysosome complex?
4) What incubation time will be the most useful for observing bead uptake?
5) Will we be able to utilize protozoa bead uptake for additional research in phagocytosis, chemotaxis, and cell growth of the protozoa?

Materials and Methods

For this experiment, we used 10-mL culture tubes of the predominant protozoan species *Entodinium caudatum.* Carboxylate-modified blue fluorescent latex beads were used to mimic the size of bacteria (0.5 µm determined by past experimentation). In order to determine the optimum concentration to feed protozoa, we tested two different concentrations: 1.05×10^7 beads/mL (bead treatment #1) and1.05×10^8 beads/mL (bead treatment #2). In order to determine if there was any difference between fed and unfed cells, we fed half of the original culture tubes the full 0.12 mL of substrate and left the other half of the tubes unfed. Sixteen 10-mL glass tubes were labeled accordingly and set aside. First, 2 mL of fed culture was put into four 10-mL glass tubes and labeled for Treatment 1 and the respective

incubation time (1 min, 20 min, 1 hr, or 24 hr). Then, 2 mL of fed culture was put into four more 10-mL glass tubes and labeled for Treatment 2 and the respective incubation time. This was then repeated for the Unfed culture. 58 µl of 1.05 x 10^7 beads/mL was added to each tube labeled fed Treatment 1 and unfed Treatment 1. Then, 58 µl of 1.05 x 10^8 beads/mL was added to each tube labeled fed treatment 2 and Unfed treatment 2. All tubes were vortexed to help prevent bead clumping and each tube was placed in a dark incubator for their respective incubation times. After the respective time was reached for each tube, 40 µl of 50% formalin was added. The samples were then transferred into 2 mL plastic eppendorf tubes and centrifuged for 2 min in the eppendorf centrifuge. The supernatant was discarded and the pellet was resuspended in 200 µl of distilled water. 40 µl of the final sample was placed onto a slide with a coverslip and was observed under a fluorescence microscope. I observed the number of cells out of 100 random cells that consumed beads and took pictures of distinctive cells. After the counts were taken, the experiment was then repeated to observe the consistency of results. Data were analyzed using a non-linear regression where the % of cells with bead uptake= $A(1-e^{-kt})$, where A= asymptotic bead uptake, k= rate of uptake and t=time (min). The treatment differences between A and the k were compared using PROC GLM (SAS).

Results

When viewing the 40 µl sample under the fluorescence microscope, we observed that the cells did have bead uptake (figures 1a,b, and c). By utilizing beads, we can avoid the dilemma of phagocytosis and digestion occurring simultaneously and we should be able to assess the rate of phagosomal bead uptake more clearly. After conducting this experiment, we conclude that the optimum bead concentration to feed the cultures would be bead treatment #2 (1.05 x 10^8 beads/mL) because it gives us higher percentages of bead uptake by the cells. The optimum bead incubation time was observed at around 20

min because this time was when uptake was at ½ of the maximum. We do not want to use incubation points any higher than 20 min because the cells have already reached their saturation point with the fluorescent beads. However, we do not want to use incubation points below 20 min because it would increase the occurrence of scientific error associated with low counts. This is because it is harder to be precise when adding formalin after 1 min as opposed to a 20 min incubation period but also because there would be more counting error with a short time. The bead concentration affected the asymptote (**A**), or the maximum percentage of bead uptake, and the feeding directly affected the rate of bead uptake (**k**). The fed cells ultimately displayed higher rates of bead uptake than the unfed cells in this experiment. This could be because of the protozoa more actively predating when fed versus when they are deprived of nutrients. Figures 2a. and 2b. highlight how results were consistent between repetition 1 and 2 when conducting the experiment a second time.

Bead Uptake Kinetics.

In terms of the rate of uptake (**k**), we did not observe a feeding x bead concentration interaction ($P > 0.10$). In terms of feeding treatment (fed versus unfed), we observed significant differences ($P<0.05$) over time, with the fed treatment displaying a higher proportion of bead uptake over time. Feeding *Entodinium caudatum* increased the rate of uptake over time. In terms of the asymptotic bead uptake (**A**), we did not observe a feeding x bead concentration interaction ($P > 0.10$). In terms of the feeding regime, we did not observe significant differences ($P>0.10$) over time. However, a significant difference ($P<0.05$) for bead concentration was observed in the asymptotic bead uptake.

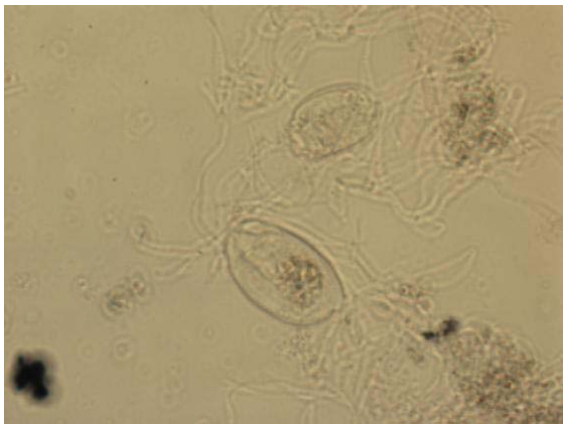

Figure 1a. Fluorescent microspheres inside of *Entodinium caudatum after a 24-h incubation, observed under white light.*

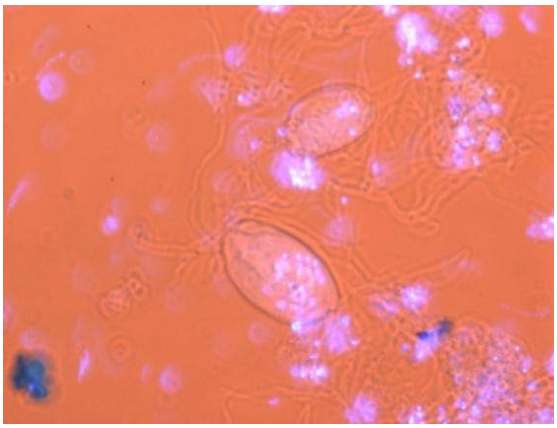

Figure 1b. Fluorescent microspheres inside of *Entodinium caudatum after a 24-h incubation, observed under a combination of white light and UV light. Arrows are pointing to beads.*

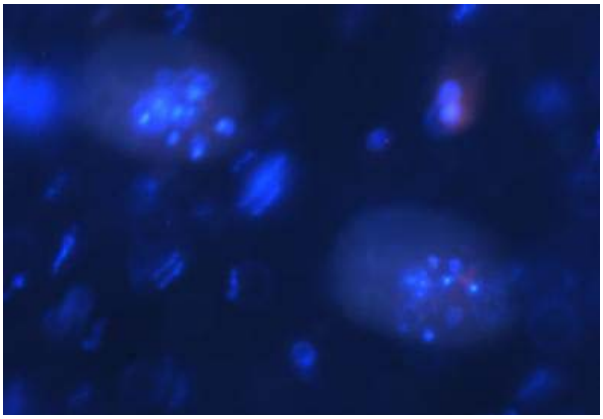

Figure 1c. Fluorescent microspheres uptake of *Entodinium caudatum after 20 min of incubation, observed under UV light. Arrows are pointing to beads.*

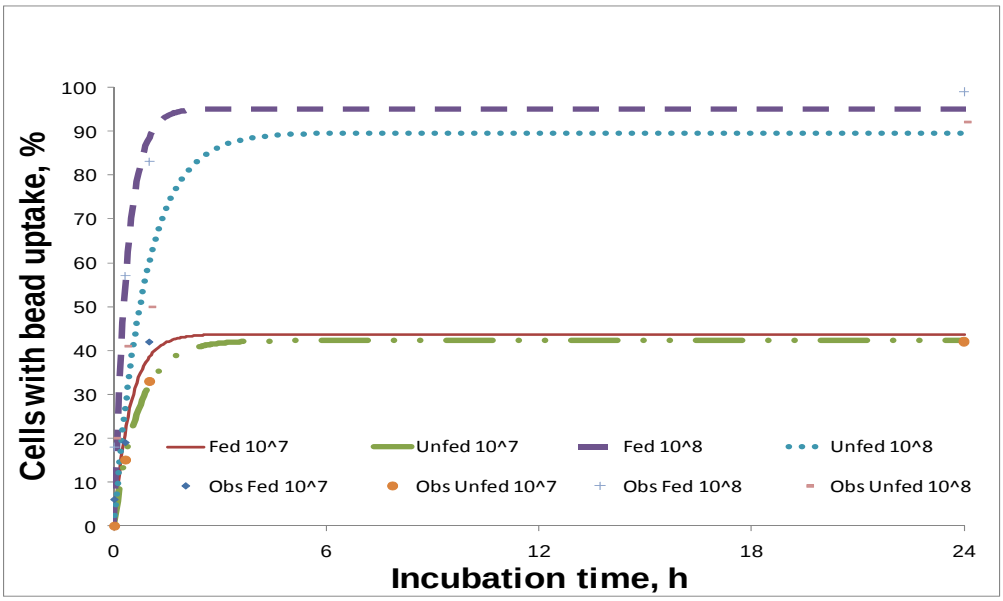

Figure 2a. *Entodinium caudatum* bead uptake (%) with a fitted monoexponential regression line are shown in Experiment 1.

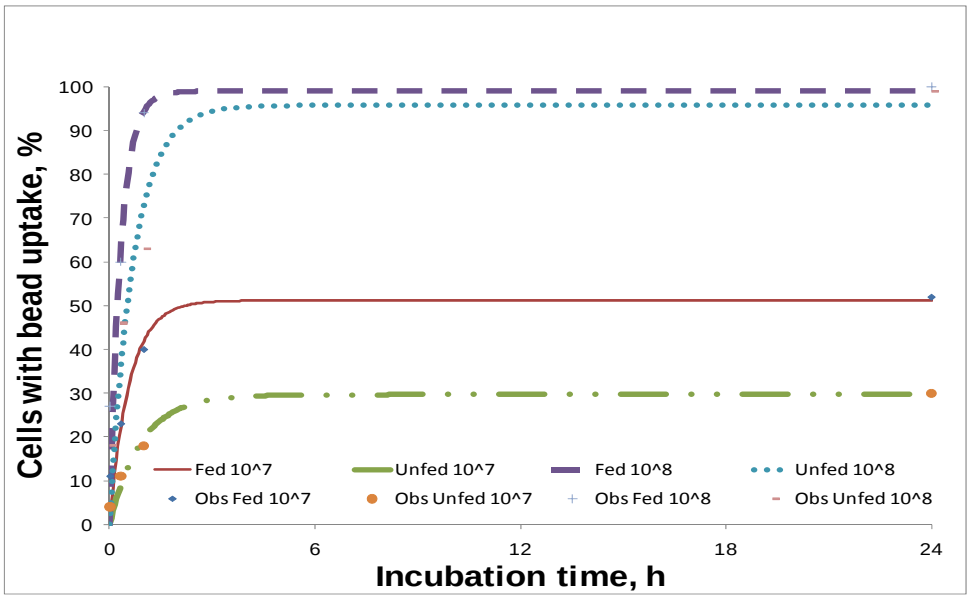

Figure 2b. *Entodinium caudatum* bead uptake (%) with a fitted monoexponential regression line are shown in Experiment 2.

Discussion

Now that we know what bead concentration and incubation time would be optimum for evaluating fluorescent bead uptake, we can continue to evaluate how this method will allow us to observe the action of the protozoa phagosome. With the results of this experiment, we can move on to future experiments involving the fluorescent latex beads. First, we wanted to make sure that our hand counting method is dependable by determining that the fluorescent beads are in fact within the cell. We could possibly accomplish this by using a technique such as flow cytommetry or by flushing the cells so the residual fluorescent latex beads outside of the cell are washed out of the sample. Next, we wanted to determine whether the beads within the cell are in fact within a phagosome. My results set the conditions for a future experiment, to use two different colored fluorescent carboxylate-modified beads to make sure that the rates of uptake between the two colors do not differ and to use paramagnetic carboxylate-modified latex blue fluorescent microspheres in order to try and isolate the phagosomes if the beads are actually inside of them (Arora et al., 2000). It is well-known that phosphatidyl inositol 3-P kinase plays a vital role in many mechanisms and its signaling pathway can be initiated by the chemotaxis of protozoa towards nutrients. We believe that PI3K is important to the phagocytosis process. In *Dyctostelium*, PI3K aids in localization and directional sensing of the overall nutrient gradient (Takeda, 2007). In some ciliated protozoa such as *Tetrahymena* species that are not in the rumen, PIK activation follows the authophosphorylation of the protein tyrosine receptor (Christensen, 2003). The Leondaritis et al. (2005) paper highlights how the compound wortmannin, which specifically inhibits PI3K, inhibits phagosome formation and lysosomal enzyme secretion in *Tetrahymena pyfriformis*. I have participated in research with Hector Diaz that shows how wortmannin has an apparent effect on the phagosomal maturation process. Also, insulin has been known to act similar to a growth factor in the *Tetrahymena* species (Christensen, 2003). This is important because the

internal PI3K cell pathway is conserved while the external signaling is not. Since wortmannin is a readily available resource, we are able to use this to initiate the external cell response that helps initiate the PI3K signaling pathway. Also, it would be interesting to see if a feeding x bead provision interaction exists. Does the beads pre-loading increase intracellular volume and decrease feeding behavior or is the signal simply based on a chemical signal? By continuing these bead experiments, we can better study the phagosome-lysosome mechanism and the PI3K pathway that is believed to be associated with it. The more we understand about how the protozoa function, the more we understand about the overall nutrition of the animal and how we can improve its efficiency to decrease the nitrogen output into the environment.

Works Cited

Arora, P.D., Manolson, M.F., Downey, G.P., Sodek, J., and Christopher, A. G. 2000. A novel model system for characterization of phagosomal maturation, acidification, and intracellular collagen degradation in fibroblasts. Journal of Biological Chemistry, 275 (45). 35432-35441.

Christensen, S. T., D.F. Guerra, A. Awan, D. N. Wheatley, and P. Satir. 2003. Insulin receptor-like proteins in Tetrahymena thermophila ciliary membranes. Current Biology, 13: R50-R52.

Dijkstra, J. 1994. Simulation of the dynamics of protozoa in the rumen. British Journal of Nutrition, 72: 679-699.

Dijkstra, J., J. France, and S. Tamminga. 1998. Quantification of the recycling of microbial nitrogen in the rumen using a mechanistic model of rumen fermentation processes. Journal of Agricultural Science, 130: 81-94.

Kebreab, K., J. France, J.A.N. Mills, R. Allison, and J. Dijkstra. 2002. A dynamic model of N metabolism in the lactating dairy cow and an assessment of impact of N excretion on the environment. Journal of Animal Science, 80: 248-259.

Leondaritis, G., A. Tiedtke, and D. Galanopoulou. 2005. D-3 phosphoinositides of the ciliate Tetrahymena: Characterization and study of their regulatory role in lysosomal enzyme secretion. Biochimica et Biophysica Acta (BBA)- Molecular Cell Research, 1745: 330-341.

Russel, J.B. 2002. Rumen microbiology and its role in ruminant nutrition. Ithaca, N.Y.

Rode, L. Maintaining a Healty Rumen- An Overview. Retrieved November 4th, 2009, from Research Centre, Agriculture & Agri-Food website: http://www.wcds.afns.ualberta.ca/Proceedings/2000/Chapter10.htm

Sylvester, J.T., S.K.R. Karnati, Z. Yu, C.J. Newbold, and J.L. Firkins. 2005. Evaluation of a real-time PCR assay for measuring the ruminal pool and duodenal flow of protozoal nitrogen. Journal of Dairy Science. 88: 2083-2095.

Takeda, K., Sasaki, A.T., Ha, H., Seung, H.A, and R. A. Firtel. 2007. Role of phosphatidylinositol 3-kinases in chemotaxis in *Dictostelium*. Journal of Biological Chemistry, 282(16): 11874-11884.