ACTA
PROTOZOOLOGICA

Acta Protozool. (2013) 52: 317–323
http://www.eko.uj.edu.pl/ap
doi:10.4467/16890027AP.13.029.1321

Short communication

Modulation of Phagocytosis in *Tetrahymena thermophila* by Histamine and the Antihistamine Diphenhydramine

Nikhil BUDUMA[1], Jenny BALABANIAN[1], Pranati DALVI[1], Soo-Khee CHIA[1], Ashreet DHALIWAL[1], Dany ELIYA[1], John BOOTHBY[1], Shannon BROS-SEEMANN[1], Ruthann KIBLER[1], Sami KHURI[2] and Sally VEREGGE[1]

[1] Departments of Biological Sciences and [2] Computer Science, San Jose State University, San Jose, California, United States of America

Abstract. Histamine is a significant biological compound because of its role in mediating the human allergic and inflammatory pathways. Antihistamines are often administered to control the irritating symptoms of allergic reactions. We evaluated phagocytosis in the protist *Tetrahymena thermophila* for use in screening for potential therapeutic compounds that mimic histamine and antihistamines. *Tetrahymena* has been frequently used as an experimental model to study compounds for biological effects or to study biological processes. Histamine and the antihistamine diphenhydramine were administered at concentrations ranging from 10^{-6} μM to 1000 μM, and the corresponding changes in phagocytosis were detected by flow cytometry. Treatment with histamine had no measurable effect on phagocytosis while diphenhydramine decreased phagocytic levels at concentrations above 50 μM. In a competition experiment between histamine and diphenhydramine, histamine did not reverse the dosage-dependent decrease in phagocytosis elicited by diphenhydramine. BLAST searches revealed no significant homologs of the human histamine receptors in *T. thermophila*. These results suggest that *T. thermophila* has a receptor for diphenhydramine that is linked to the phagocytic process, but not a histamine receptor. Further study is necessary to elucidate the nature of this previously uninvestigated receptor. The experimental protocol developed as a part of this study may serve as an inexpensive, high throughput, flow cytometric method to screen natural and synthetic compounds for pharmacologically significant properties.

Key words: *Tetrahymena thermophila*, phagocytosis, flow cytometry, histamine, diphenhydramine, drug screening.

INTRODUCTION

Histamine is a physiologically important molecule because it initiates the allergic and local inflammatory responses in humans (Campbell *et al*. 2008). In order to mitigate the irritating symptoms of allergic responses, physicians often prescribe antihistamines to antagonize the effects of histamine. One such compound is diphenhydramine, the active ingredient of the over-the-counter drug Benadryl. Developing a bioassay to swiftly screen compounds for their potential to act as histamines or antihistamines could serve as an effective tool for drug discovery.

Tetrahymena thermophila has been widely acknowledged as a promising experimental model for understanding biological processes and for biotechnological

Address for correspondence: J. Boothby, Department of Biological Sciences, San Jose State University, San Jose, California, USA; E-mail: john.boothby@sjsu.edu

applications because of its widespread availability, low cost to maintain, short generation time, and fully sequenced genome (Orias 1998). In particular, the work of Renaud *et al.*, suggest that *T. thermophila* may be a useful model to screen for clinically significant bioactive compounds (Renaud *et al.* 1996). Previous studies with *T. thermophila* have characterized opiate receptor proteins which appear to closely resemble their analogs in vertebrates (Quionones and Renaud 1987, De Jesus and Renaud 1989). More recent work has used *T. thermophila* as a cellular model to investigate the mechanism of action of the anti-inflammatory marine natural product, Pseudopterosin A (Moya and Jacobs 2006).

Most previous studies investigating the modulation of phagocytosis by therapeutic or potentially therapeutic compounds in the genus *Tetrahymena*, however, utilize the species *Tetrahymena pyriformis*. These investigations revealed that *T. pyriformis* responds to many bioactive compounds via changes in phagocytosis (Csaba and Darvas 1992, Csaba and Lantos 1973, Kovacs *et al.* 2002, Kovacs *et al.* 1996, Stefanidou *et al.* 1990, Chiesa *et al.* 1993). Because the genome of *T. thermophila* has been fully sequenced (Orias 1998), we propose that *T. thermophila* may be a more attractive option than *T. pyriformis* for use in drug discovery. Thus, characterizing similar protein receptors and pathways in *T. thermophila* may facilitate the development of drug screening protocols that can take advantage of the growing knowledge of its genome.

In 1973, Csaba and Lantos noted that exposing *T. pyriformis* to histamine resulted in an increase in phagocytic activity. Immunocytofluorimetry revealed that *T. pyriformis* harbors two subtypes of histamine receptors (H1 and H2) that are responsible for mediating the symptoms of allergic reactions in humans (Csaba *et al.* 1978, Kovacs *et al.* 1981). It was shown using FITC-labeled antibodies that these receptors were present on the cilia of the cell body, while binding sites were absent on the interciliar membrane regions and the cilia of the oral groove (Kovacs and Csaba 1980). The increase in phagocytic activity elicited by histamine was counteracted via administration of the H1 antagonist phenindamine (Csaba *et al.* 1978, Kovacs *et al.* 1981). Furthermore, it was shown that concanavalin-A was able to reverse the histamine-associated increase in phagocytosis in *T. pyriformis*, suggesting that the binding site contains simple sugars and glycosamine oligomers (Kovacs *et al.* 1981, Csaba *et al.* 1983). The H2 receptor subtype was found to be linked to glycogen synthesis and not the phagocytic pathway in *T. pyriformis* (Darvas and Csaba 1981). Because of its presence in *T. pyriformis*, it is plausible that the closely related *T. thermophila* also has a receptor for histamine that is linked to the phagocytic pathway.

This study investigated the effects of histamine and diphenhydramine on phagocytosis in *T. thermophila* in order to characterize their corresponding receptor systems. The methodology and results from this study may be used to generate high-throughput flow cytometric screening protocols for compounds that mimic the effects of histamine or diphenhydramine.

MATERIALS AND METHODS

Cell preparation and phagocytic activity evaluation

Wild type *Tetrahymena thermophila* was provided by Dr. Eduardo Orias (University of California, Santa Barbara, CA, USA). Stock cultures were maintained in a sterile medium of 2% axenic proteose peptone with 0.003% Fe-EDTA (Difco Laboratories, Detroit, MI). *Tetrahymena* cultures were sub-cultured every two weeks by pipetting 30 µL of the previous culture into 3 or 9 mL of sterile media. Cells were harvested and expanded in preparation for experimentation by pipetting 500 µL of the culture into 50 mL of sterile media in a flat bottom flask. *Tetrahymena* were then incubated at 30°C for 24 hours, and then at room temperature (RT)/23°C (depending on the experiment) for another 24 hours. The expansion cultures were checked under a microscope for healthy morphology and motility and diluted to contain a final concentration of 250,000 cells per mL (*Tet*-Prep) (Moya and Jacobs 2006). *Tet*-Prep cells were then evaluated for ability to phagocytize fluorescent, 2.19 µm Nile Red beads (approximately 1,375,000 beads, from Spherotech, Inc., Lake Forest, IL). The proportion of *Tetrahymena* cells containing fluorescent beads (percent phagocytosis) was determined for each experimental condition.

Effect of time at room temperature and 30°C on phagocytosis

To determine if temperature affected phagocytosis in *T. thermophila*, the percent phagocytosis was determined at two temperatures (23°C and 30°C) over a 15 minute period. Two sets of eight tubes with 450 µL of *Tet*-Prep were prepared (Time 0, 1, 2, 3, 4, 5, 10 and 15 min.). In each set, 100 µL volume of Nile Red beads (approximately 1,375,000 beads) was pipetted into each tube. After a specified amount of time (0, 1, 2, 3, 4, 5, 10, or 15 min.), 450 µL of 10% formalin was added to fix the contents of each tube for flow cytometry analysis. One set of tubes was incubated at RT/23°C, and the second set at 30°C (controlled by a water bath). Differences between temperature regimes (RT vs. 30°C) over time (0, 1, 2, 3, 4, 5, 10 and 15 min.) were evaluated with a Repeated Measures Analysis of Variance (Quinn and Keough 2003). Percentage Phagocytosis was transformed with the angular transform. Levene's test for equality of variance and Mauchly's Test of Sphericity indicated that the assumptions for the Repeated Measures ANOVA were met ($p > 0.05$).

Effect of histamine and diphenhydramine on phagocytosis

The effects of histamine or diphenhydramine on phagocytosis were evaluated by the percent phagocytosis in cultures of *T. thermophila* in increasing concentrations of histamine or diphenhydramine. At the start of the experiment, 450 µL of *Tet*-prep were added to triplicate sets of seven test tubes and allowed to equilibrate in a 30°C water bath. Six concentrations of histamine hydrochloride or diphenhydramine (Sigma-Aldrich, St. Louis, MO) diluted in axenic proteose peptone such that, when 10 µL of each preparation was combined with the *Tet*-prep, final concentrations of 10^{-6} µM, 10^{-4} µM, 10^{-2} µM, 1 µM, 100 µM, or 1000 µM were produced for each replicate. In addition, 10 µL of a placebo solution (axenic proteose peptone) was pipetted into the seventh tube of each replicate to act as a control. Five seconds later, 100 µL of 2.19 µm Nile Red beads was pipetted into each tube. At two, five and ten minutes after the addition of the beads, 450 µL of 10% formalin was added to fix the cells in each tube for flow cytometry analysis. Because some of the treatments were conducted on different days, the proportions of *T. thermophila* phagocytizing the red beads were adjusted by subtracting the average values of the control (placebo) for a particular day from each of the treatment values obtained on that same day. Because the values were proportions, all data were transformed with the angular transform; because some of the proportions were negative, a small positive number was added to each value prior to transformation. A Repeated Measures ANOVA (Quinn and Keough 2003) with time (2, 5, and 10 min.) as the repeated measure and histamine concentration as the between-subjects effect, was used to evaluate the effect of histamine or diphenhydramine on phagocytosis. Levene's test for equality of variance and Mauchly's Test of Sphericity indicated that the assumptions for the Repeated Measures ANOVA were met ($p > 0.05$). If the histamine or diphenhydramine concentration effect was significant, a Waller-Duncan *a posteriori* test was used to determine where differences lay among the treatments.

Competition between histamine and diphenhydramine

This experiment was conducted to determine if varying concentrations of histamine could affect phagocytosis in *T. thermophile* when 10^2 µM of diphenhydramine was present. Tubes with 450 µL of *Tet prep* were incubated in a 30°C water bath. The experimental design was analogous to the preceding experiments with four exceptions: 1) the experiment was only conducted at the 10 minute time interval, 2) there were 9 replicates, 3) each tube had a final concentration of diphenhydramine of 10^2 µM, and 4) the final concentrations of histamine were 10, 100, and 1000 µM. The adjustment and transform of values was the same as for the two preceding experiments. A one-way Analysis of Variance (Zar 2010) was used to determine if there were differences in proportion phagocytized among histamine concentration levels treatment when diphenhydramine was present in a concentration of 10^2 µM. A Levene's test of equality of variance indicated that the assumptions for the ANOVA were met ($p > 0.05$).

BLAST analysis of histamine receptors in *Tetrahymena thermophila*

In order to determine the presence of human histamine receptor homologs in *T. thermophila*, BLAST searches (www.ncbi.nlm.nih.gov) were performed to identify protein sequences with significant alignments with the H1 (GenBank: AAN01269.1) and H2 (GenBank: EAW61369.1) receptors. BLASTp was used with the human sequences as query sequences to match identified or putative genes in the *T. thermophila* genome. Scores were assigned using the BLOSUM62 substitution matrix in order to quantify the quality of identified matches based on sequence similarity and the length of homologous peptide tracts.

Flow cytometry bioassay (data acquisition and analysis)

Data was acquired for 500 *T. thermophila* events using a Becton-Dickinson FACSCalibur flow cytometer equipped with a 488 nm argon laser and a 635 nm red diode laser. Logarithmic amplification was used for the forward and side scatter detectors to account for the size difference between cells and fluorescent beads. The BD Biosciences CellQuest™ program was used for data analysis. *Tetrahymena* were identified and gated on the forward scatter (FSC) versus side scatter (SSC) plot. Cells were then observed on the fluorescence/SSC plots. These data were used to determine the percent of *T. thermophila* that had phagocytized the Nile Red beads. A zero control of *Tetrahymena* treated with beads post-fixation was run through the flow cytometer at the start of each experiment to distinguish attached beads from ingested beads. To ensure quality, only experimental results with control tubes demonstrating ≤ 10% attached beads were considered.

RESULTS

Phagocytosis at room temperature and 30°C vs. time

Phagocytosis in *T. thermophila* was temperature dependent, occurring at a higher rate at 30°C. The Repeated Measures ANOVA demonstrated that mean phagocytosis was significantly greater ($p = 0.009$) in tubes incubated at 30°C than in tubes incubated at room temperature (Table 1). Tests of within subjects indicated that, in either temperature regime, phagocystosis increased over time ($p < 0.001$) and that there was no interaction between time and temperature regimes ($p = 0.488$). Phagocytosis in *T. thermophila* modeled a hyperbolic curve when plotted versus time elapsed in minutes with the maximal value at approximately 10 minutes after the start of the experiment (Fig. 1). Subsequent experiments were performed at 30°C and included a 10-minute incubation.

Effects of histamine on phagocytosis

The Repeated Measures ANOVA indicated that histamine did not modulate phagocytosis in *T. thermophila* within ten minutes at concentrations ranging between 10^{-6} to 1000 µM (Table 2). The analysis showed that phagocytosis increased with time ($p = 0.006$) but the

Table 1. Repeated Measures ANOVA on the effects of temperature on percentage of phagocytosis in *T. thermophila*. The dependent variable was the angular transform of the proportion phagocytized. The independent variable was temperature regime (23°C and 30°C) and the repeated measure was time (0, 1, 2, 3, 4, 5, 10 and 15 min.).

Between Subjects Source	SS	df	MS	F	p
Intercept	31.356	1	31.356	2921.354	< 0.001
Temperature regimes (RT and 30°C)	0.157	1	0.157	14.592	0.009
Error	0.064	6	0.11		

Table 2. Repeated Measures ANOVA on the effects of histamine concentration on the proportion of phagocytosis by *T. thermophila*. The dependent variable was the angular transform of the proportion phagocytized plus 0.37. The independent variable was histamine concentration (10^{-12} μM, 10^{-10} μM, 10^{-8} μM, 10^{-6} μM, 10^{-4} μM and 10^{-3} μM) and the repeated measure was time (2, 5, and 10 min.).

Between Subjects Source	SS	df	MS	F	p
Intercept	23.776	1	23.776	1810.213	< 0.001
Histamine concentration	0.138	5	0.028	2.094	0.137
Error	0.158	12	0.013		

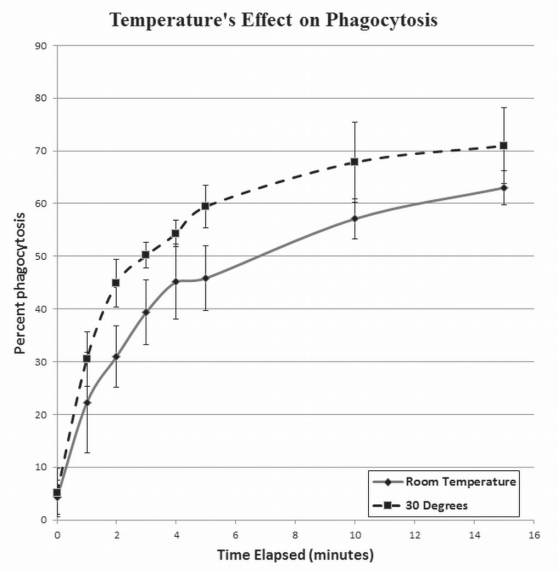

Fig 1. Results of a One Way Repeated Measures ANOVA addressing phagocytosis by *Tetrahymena thermophila* at two temperatures, room temperature and 30°C, over time. Error bars represent plus or minus 1 standard deviation.

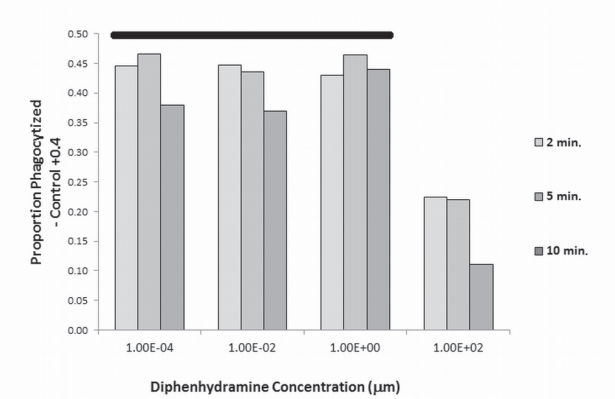

Fig 2. Results of One Way Repeated Measures ANOVA illustrating the proportion of *Tetrahymena thermophila* phagocytizied beads at varying concentrations of diphenhydramine (10^{-4} μM, 10^{-2} μM, 1 μM and 10^{2} μM). Values represent mean proportion phagocytized – control proportion + 0.4. Data were transformed (angular) for analysis. The solid horizontal bar indicates treatment levels that were not significantly different according to a Waller-Duncan a posteriori test.

time*treatment interaction was not significant (p = 0.176) indicating that all treatments responded over time in a similar manner. However, there were no significant differences in phagocytosis over the range of histamine concentrations (p = 0.137). The difference between the highest mean proportion and the lowest mean proportion was 0.076. The test was capable of resolving a difference in proportion as small as 0.18 with power at 80% (to resolve a difference in proportions as small as 0.10 at 80% power, a sample size of 6 would be required).

Effect of diphenhydramine on phagocytosis

The antihistamine diphenhydramine was found to suppress phagocytosis at a high concentration (Fig. 2). The Repeated Measures ANOVA indicated that the proportion phagocytized increased over time (p = 0.003)

but the treatments responded in a similar fashion over time (time*treatment, $p = 0.059$). There were highly significant differences in proportion of phagocytosis among the diphenhydramine concentrations ($p < 0.001$) (Table 3). The Waller-Duncan test indicated that the proportion phagocytized at a diphenhydramine concentration of 10^{-4} μM was significantly lower ($p < 0.001$) than all other concentration levels but there were no detectable differences ($p > 0.050$) among the remaining concentration levels.

Competition between histamine and diphenhydramine

Histamine, in concentrations from 10 μM to 1000 μM, failed to reverse the decrease in phagocytosis elicited by 100 μM diphenhydramine. The One Way ANOVA showed that there were no significant differences ($p = 0.067$) among treatment levels (Table 4). At 80% power, this test was capable of resolving a difference in proportion between treatments as low as 0.011.

BLAST analysis of human histamine receptor homolog genes in *Tetrahymena thermophila*

BLAST search for the homologs of the human histamine receptor H1 (AAN01269.1) returned as top hit the hypothetical TTHERM_00283580 [*Tetrahymena thermophila*] with e-value of 0.008. Only 12% of the residues of the human histamine were aligned with the hypothetical protein from *T. thermophila*. Similarly, the top match for the human histamine receptor H2 (EAW61369.1) returned the Major Facilitator Superfamily protein [*T. thermophila* with accession number XP_001020403] with e-value of 7e-04 as top hit. This time 57% of the residues of the human histamine were aligned with the hypothetical protein from *T. thermophila*.

DISCUSSION

Tetrahymena thermophila is a free-living fresh water ciliate for which the complete genetic sequence has become available (*Tetrahymena* genome project). A number of physiological, biochemical, and pharmacological response similarities including chemotaxis, and phagocytosis are shared between this microbe and cells of the mammalian immune system (such as neutrophils, macrophages, and mast cells). Common features between this free living phagocyte and mammalian phagocytes include signaling mechanisms involved with G-protein activation (Rosner *et al.* 2003, Renaud *et al.* 1996), protein kinases, and components of the inositol phospholipid pathway (Kovacs *et al.* 1996). Specific receptor recognition and uptake of particles are processes in mammalian phagocytes that are key elements of the immunological response in higher organisms; and are similar to the behavior and food uptake of many unicellular organisms (Baumert *et al.* 1998, Batz and Wunderlich 1976).

Time course studies presented here indicated that percent phagocytosis at 30°C increases with time, ta-

Table 3. Repeated Measures ANOVA on the effects of diphenhydramine concentration on the proportion of phagocytosis in *T. thermophila*. The dependent variable was the angular transform of the proportion phagocytized. The independent variable was diphenhydramine concentration (10^{-4} μM, 10^{-2} μM, 1 μM and 10^{2} μM) and the repeated measure was time (2, 5, and 10 min.).

Between Subjects Source	SS	df	MS	F	p
Intercept	15.555	1	15.555	6522.662	< 0.001
Diphenhydramine concentration	0.517	3	0.172	72.224	< 0.001
Error	0.024	10	0.002		

Table 4. One-Way ANOVA on the effects of varying concentrations of histamine with diphenhydramine concentration of 10^{2} μM on the proportion of phagocytosis in *T. thermophila*. The dependent variable was the angular transform of the proportion phagocytized. The independent variable was histamine concentration (10 μM, 10^{2} μM 10^{3} μM).

Source	SS	df	MS	F	p
Treatment	0.028	2	0.014	3.029	0.067
Error	0.112	24	0.005		

pering off at about 15 minutes at approximately 70% with low experimental variability between experimental replicates. The 10-minute time point was selected for the investigation of histamine and diphenhydramine because it is the first time point at which the curve begins to approach its maximal value. In addition, Stefanidou *et al.* (1999) reported that, at room temperature, defecation begins to occur by 20 minutes and after this time point, the formation of food vacuoles is so fast that they are not countable.

As would be expected, phagocytosis is temperature dependent, with higher temperatures eliciting higher rates of phagocytosis. Because a small difference in temperature resulted in a significant difference in percent phagocytosis, we determined that techniques using phagocytosis in *T. thermophila* as an investigative model should take extra precautions to control the temperature. We minimized changes in temperature in the sample tubes by maintaining the experiment in a 30°C water bath.

Our investigations of the effects of histamine and diphenhydramine on phagocytosis contradicted the studies by Csaba, Lantos, Darvas, and Kovacs (Csaba and Lantos 1973, Csaba *et al.* 1978, Kovacs *et al.* 1981, Kovacs and Csaba 1980, Csaba *et al.* 1983, Darvas and Csaba 1981) that found phagocytosis-linked receptors for histamine in *T. pyriformis*. We found no evidence for a phagocytosis-linked receptor for histamine in *T. thermophila*; that is we saw no direct effect of histamine on phagocytosis and no effect of histamine on diphenhydramine modulation of phagocytosis. This difference between our results and those of Csaba and Lantos (1973) may be due to a difference in experimental methods, however our BLAST analyses also suggest that *T. thermophila* does not possess histamine receptors. Our results demonstrated that the antihistamine diphenhydramine decreases phagocytosis in *T. thermophila*. Since histamine was unable to antagonize the suppression of phagocytosis elicited by diphenhydramine, histamine receptors are unlikely to mediate the modulation of phagocytosis by diphenhydramine.

Diphenhydramine is known to be a promiscuous molecule, affecting a number of processes in the body in addition to acting as an antihistamine. For example, the structure of diphenhydramine was used to design the selective serotonin reuptake inhibitor fluoxetine after it was found that diphenhydramine could be used to mitigate the symptoms of major depression (Wong *et al.* 1995). Diphenhydramine is also used as a cholinolytic to reduce the severity of muscle tremors in Parkinson's disease, often more effective than L-dopa administration (Carlson *et al.* 2000). Further work elucidating the mechanism of action of diphenhydramine in *T. thermophila* may lead to the characterization of clinically significant drug targets. Because the pathway is linked to phagocytosis, characterization of such targets may also lead to the development of cheap, high throughput flow cytometric protocols that can screen natural and synthetic compounds for specific therapeutic properties.

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Received on 9th April, 2013; revised on 26th May, 2013; accepted on 26th April, 2013