

ORIGINAL ARTICLE

Phagocytosis and uptake of *Holospora* bacteria in *Paramecium caudatum* require terminal N-acetylglucosamine and NANA residues of the host surface glycoconjugates

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Summary

Ciliates often form symbiotic associations with bacteria, phagocytosis serving as a gate for the infection. Lectin-glycoconjugate interactions are known to mediate host-endobiont interplay in many symbioses. Here, ingestion of symbiotic *Holospora* bacteria and food microorganisms by *Paramecium caudatum* was studied under treatments affecting terminal N-acetyl-D-glucosamine (GlcNAc) and N-acetylneuraminic acid (NANA) residues of the glycoconjugates of the ciliate surface, which demonstrated positive labeling with Wheat germ agglutinin (WGA) specific for these oligosaccharides. WGA treatment prevented ingestion of *Holospora* and hampered phagocytosis of food bacteria and yeast in ciliates, prolonged treatment being deleterious for the cells. The presence of N-acetyl-D-glucosamine and mannose in the culture medium did not affect the course of the ingestion. Removal of the terminal GlcNAc residues from the surface glycoconjugates with β -N-acetyl-D-glucosaminidase led to intensive vesiculation of the cytoplasm concomitant with a delay in the ingestion of food, while administration of mannosidase did not have this effect. The data obtained suggest that surface glycoconjugates containing terminal GlcNAc and/or NANA residues are indispensable for phagocytosis in *P. caudatum*.

Key words: ciliate, phagocytosis, symbiotic bacteria, surface glycoconjugates, WGA lectin, β -N-acetyl-D-glucosaminidase

Introduction

Symbiotic systems formed by *Paramecium caudatum* and bacteria belonging to the genus *Holospora* (Alphaproteobacteria, Holosporales) are a perfect

model for studying fine mechanisms of interactions between symbiotic partners (Ossipov et al., 1975; Fokin and Görtz, 2009; Schrallhammer and Potekhin, 2020). Stages of the infection process are well documented (Görtz and Wieman, 1989; Fujishima,

2009). In brief, the ciliate takes up the infection form (IF) of *Holospira* in the digestive vacuole (DV) in the course of phagocytosis. Upon the fusion of the digestive vacuole with acidosomes and acidification of the DV contents, the IF leaves the digestive vacuole exploiting host cell actin (Fujishima, 2009; Sabaneyeva et al., 2009) and reaches the host cell nucleus. Interestingly, *Holospira* bacteria demonstrate nuclear specificity, *H. obtusa* invading the macronucleus (MAC), and *H. undulata* – the micronucleus (MIC) of the host (Fokin and Görtz, 2009). Once inside the nucleus, the IF starts to divide, producing reproductive forms (RFs), which later differentiate into IFs. The newly produced IFs are released into the environment in the residual body formed after the host cell division, while the RFs remain in the daughter nuclei. Thus, RFs serve for vertical transmission of the endosymbiont, while the IFs – for the horizontal one (Schrallhammer and Potekhin, 2020).

Although some details of molecular mechanisms of this process have been revealed (Abamo et al., 2008; Iwatani et al., 2005; Sabaneyeva et al., 2005; Fujishima, 2009; Sabaneyeva et al., 2009), many issues remain unresolved. Among them are the factors which make some ciliate strains more susceptible to the infection with *Holospira* bacteria, than the others, as shown in a number of previous studies (Rautian et al., 1990; Rautian et al., 1993; Skoblo et al., 1996), or, conversely, which cause host cell resistance (Skovorodkin and Fokin, 1990; Fokin, 2004; Weiler et al., 2020).

Since lectin-glycoconjugate interactions are often involved in host-endobiont interactions (Sharon, 1987; Hooper and Gordon, 2001; Frayssé et al., 2003; Sharon and Lis, 2004; Varki, 2017; Lin et al., 2020; Aminov and Aminova, 2024), we wondered if this might be the case in *Paramecium-Holospira* symbiosis. Wheat germ agglutinin (WGA), which specifically binds N-acetyl-D-glucosamine (GlcNAc) and, with a lower affinity, N-acetylneuraminic acid (NANA) residues of the glycoconjugates, has been reported to label surface antigens on the cortex of *P. caudatum* (Lüthe and Plattner, 1986; Pape et al., 1988; Allen et al., 1988; Ramoino, 1997; Locatelli et al., 1998; Ramoino et al., 2001). Conspicuous labeling has been detected in the buccal cavity, at the trichocyst tips, along the cilia, at the cytoproct, and the food vacuoles, with maximal intensity in the buccal cavity. At the same time, Concanavalin A, which is primarily specific for mannose, has been shown to label nuclear

envelope and trichocyst tips, being never detected in the buccal cavity (Lüthe and Plattner, 1986). These observations made us suggest that surface antigens with terminal GlcNAc and/or NANA and not mannose might be involved in phagocytosis in *P. caudatum*. Involvement of WGA-positive structures in food perception has been also proposed in *Tetrahymena pyriformis* (Dürichen et al., 2016).

Although paramecia are filter feeders, and are believed to lack any food selection (Fok and Allen, 1988), observations on antibacterial resistance in certain *P. caudatum* strains makes one presume the existence of some forms of food selection in this species (Fokin and Skovorodkin, 1991; Fokin et al., 2003). Based on this assumption, we hypothesized that the presence of surface antigens with terminal GlcNAc and/or NANA residues might contribute to the uptake of *Holospira* bacteria by *P. caudatum*. The present exploratory investigation was undertaken to find out whether any disturbance of terminal GlcNAc and NANA residues of the surface glycoconjugates (by blocking with WGA lectin or by removing with specific enzymes) would affect the uptake of *Holospira* bacteria and ingestion of food microorganisms, such as *Klebsiella aerogenes* and *Saccharomyces cerevisiae* in *P. caudatum*.

Material and methods

CELL CULTURES

The strains used in the study are listed in Table 1. All strains were deposited in the RC CCM Culture Collection of Microorganisms (Research Center for Cultivation of Microorganisms, Research Park of Saint-Petersburg State University). In all experiments, only the strains of *P. caudatum* previously shown to be susceptible to infection with *Holospira* bacteria were used, while the strains stably infected with *Holospira* served as a source of IFs. The strains were maintained in the lettuce infusion inoculated with food bacteria, *K. aerogenes*. Fresh culture medium was added every 2–3 days. Before phagocytosis assays, the cells were not fed for at least 2 days.

LABELING CILIATES WITH WHEAT GERM AGGLUTININ (WGA)

Peroxidase conjugated lectin from *Triticum vulgaris* (Wheat germ agglutinin, WGA, Sigma-

Table 1. List of *Paramecium caudatum* strains used in the study.

Strain	Endosymbiont	Year	Origin	Collector
P03-6	–	2003	Russia, Saint-Petersburg, Pavlovsk	E. V. Sabaneyeva
NR09-4-3	–	2009	Russia, St.Petersburg, New Ropsha	E.V. Sabaneyeva
CKV8-74	–	2011	Czech Republic, Karlovy Vary	N.A. Lebedeva
VL18-5	–	2016	Russia, Vladimir	N.A. Lebedeva
R130i	<i>H. obtusa</i>	2001	Russia, Saint-Petersburg, Ropsha	M.S. Rautian
R130i -6	<i>H. obtusa</i>	2001	Russia, Saint-Petersburg, Ropsha	M.S. Rautian
UB1-2	<i>H. undulata</i>	2015	Russia, St. Petersburg region, natural landmark Brandovka	N.A. Lebedeva

Aldrich, Germany) was added to living cells at final concentration 25 µg/mL. 10 min later the cells were fixed in 1% paraformaldehyde (PFA) in PBS for 10 min and washed in PBS. After washing the buffer was removed and 3,3'-diaminobenzidine (DAB, Sigma-Aldrich) solution (0.05% DAB – 0.02% H₂O₂ in 0.01M PBS, pH 7.2) was added for 10 min to detect peroxidase. Then the DAB solution was removed, the cells were washed in PBS, transferred onto a slide and covered with a coverslip.

Alternatively, TRITC conjugated WGA (TRITC-labeled Wheat germ agglutinin, TRITC-WGA, Sigma-Aldrich, USA) was added to living ciliates at 25 µg/mL for 15 min. Then the cells were fixed in 1% PFA solution in PBS for 10 min and washed in PBS to remove unbound lectin. The cells were either directly transferred onto a slide and covered with a coverslip or were mounted in Mowiol4-88 (Sigma-Aldrich Co. LLC, USA), containing 0.1% p-phenylenediamine (PPD, Sigma-Aldrich Co. LLC, USA) and 300nM DAPI 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich Co. LLC, USA).

PREPARATION OF *HOLOSPORA* IFs FOR PHAGOCYTOSIS ASSAYS

Cells of the infected donor strains (R-130i or its subclone R130i-6, both bearing *H. obtusa*, or Ub1-2 carrying *H. undulata*) were concentrated by centrifugation and crushed by passing through a syringe with a fine needle. The concentration of IFs was calculated using a hemocytometer (Goryaev's chamber) by the formula $N=(V*n)/4*10^{-6}$, where V stands for the cell homogenate volume, and n – for the average number of IFs in a big square

of the hemocytometer. In the experiments with *H. obtusa*, concentration of IFs in the homogenate was usually not fewer than $2,75 * 10^4$ /mL; in some experiments the concentration was not estimated. In the experiments with *H. undulata* the IFs were added at a ratio 500 IFs per 1 ciliate, bringing multiplicity of infection (MOI) value to 500.

THE GENERAL SCHEME OF PHAGOCYTOSIS ASSAYS IN *P. CAUDATUM* IN THE PRESENCE OF CHEMICAL COMPOUNDS

Equal volumes of *P. caudatum* cell culture or equal number of ciliates (50) were placed in two wells of a three-spot depression slide. A chemical compound (WGA-TRITC, a monosaccharide or an enzyme) was added to experimental cells, while the corresponding volume of distilled water was added to control ciliates. 5-10 min later IFs of *Holospira* or food microorganisms (*K. aerogenes* supplemented with carmine particles or *S. cerevisiae*) were added to each of the wells. The wells were placed in a wet chamber and kept at room temperature. The exposition to the chemical and the food microorganism varied depending on the chemical used and the microorganism presented as food. Living cells were checked for the presence of ingested microorganisms 15-30 min after their addition and onwards. Since this investigation is exploratory, differences in the protocols were used to detect any differences in the effect of the chemical compounds caused by changes in concentrations of the chemicals and prolongation of the treatment. Lowering concentration of chemicals was needed to allow longer treatment time required for ingestion of *Holospira* IFs in control cells. Higher concentrati-

ons of *K. aerogenes* and *S. cerevisiae* compared to that of *Holospira* IFs required less time for ingestion in control ciliates. Each treatment with each kind of food was carried out at least three times. Detailed protocols for each of the treatments is provided below.

PHAGOCYTOSIS ASSAYS IN *P. CAUDATUM* IN THE PRESENCE OF WGA

Equal volumes of *P. caudatum* cell culture (strain P03-6) were placed in two wells of a three-spot depression slide. WGA-TRITC was added at concentrations 24–50 µg/mL to experimental cells, while the equal volume of distilled water was added to control ciliates. Lowering WGA-TRITC concentration was used to alleviate the detrimental effect of WGA treatment. 10 min later 0.5 mL of the homogenate containing IFs of *H. obtusa* was added to each well. The wells were placed in a wet chamber and kept at room temperature in the dark to prevent fading. 20 min later the process of ingestion was checked in living cells (about 15–20 in the experiment and in the control) using DIC and epifluorescence.

In order to check if the effect of treatment with WGA-TRITC was specific for ingestion of *H. obtusa* IFs, two control sets of experiments were carried out. First, lettuce infusion inoculated with *K. aerogenes* and supplemented with a needle tip of carmine particles was added to both wells. After 15 min, living cells (about 15–20 in the experiment and in control) were checked for the presence of food vacuoles. In another set of experiments, the yeast *S. cerevisiae* was added in the lettuce infusion as food particles.

A different protocol was followed in the experiments with *H. undulata*. The cell homogenate was prepared and the IF concentration was calculated as described above. 50 recipient cells (strains CKV8-74 and VL18-5) were placed in a microwell, both in experiment and in control. WGA at concentrations 5–25 µg/mL was added to the experimental well, the same volume of distilled water was used in control. A drop of fresh culture medium inoculated with food bacteria was added to stimulate phagocytosis. The IFs of *H. undulata* were added to each of the wells 5–7 min later. The process of infection was checked 30 min after the start of the experiment using DIC and epifluorescence. Alternatively, *K. aerogenes* supplemented with carmine particles was used under the same conditions as *H. undulata*.

PHAGOCYTOSIS ASSAYS IN *P. CAUDATUM* IN THE PRESENCE OF MONOSACCHARIDES β -N-ACETYL-D-GLUCOSAMINE AND D(+)-MANNOSE

These experiments were carried out to check if monosaccharides would compete with terminal monosaccharide residues of the surface glyco-conjugates for binding microorganisms in the course of phagocytosis. In this series of experiments only IFs of *Holospira* were presented as food to *P. caudatum*.

The homogenate containing IFs of *H. obtusa* was divided evenly between experimental and control wells. The recipient cells (strain NR09-4) were added to the obtained homogenate diluted with fresh culture medium inoculated with food bacteria at a ratio of 5:4:1. The IF concentration was not checked. β -N-acetyl-D-glucosamine (Sigma-Aldrich Co. LCC, USA) at concentrations of 1–2 mg/mL was added immediately to experimental cells, substituted with the equal volume of distilled water in control.

The procedure using IFs of *H. undulata* was as described above. β -N-acetyl-D-glucosamine (Sigma-Aldrich Co. LCC, USA) was added to experimental cells at concentrations of 1–2 mg/mL. Equal volume of distilled water was added to control cells. 5–7 min later the IFs of *H. undulata* were added to experimental and control cells in the same proportion as described above. The ingestion of IFs was checked in living cells starting 30 min after the addition of the IFs.

Experiments in the presence of D(+)-mannose (Sigma-Aldrich, Slovakia) were carried out as described above, the monosaccharide concentration being 2 mg/mL.

PHAGOCYTOSIS ASSAYS IN *P. CAUDATUM* IN THE PRESENCE OF ENZYMES β -N-ACETYL-D-GLUCOSAMINIDASE AND α 1-2,3 MANNOSIDASE

Experimental feeding of ciliates with *H. obtusa* IFs was performed as described above. Immediately after addition of IFs, β -N-acetyl-D-glucosaminidase (Sigma, USA) was added at a final concentration 4 µg/mL to the experimental cells, the equal volume of distilled water was added to the control cells. Ingestion of IFs was checked in living cells at 40 min–1 h, 3 h, 4.5 h and 24 h after the start of the experiment. Alternatively, ciliates were fed with *K. aerogenes* supplemented with carmine particles.

Experimental feeding of *P. caudatum* with the IFs

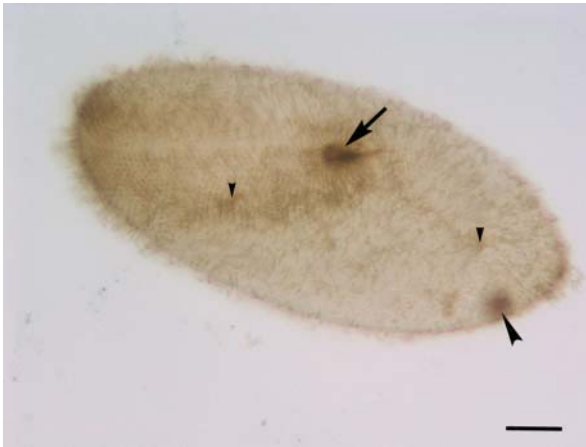


Fig. 1. *Paramecium caudatum* in the stationary growth phase labeled with WGA-HRP, bright field. Note the intensive labeling of the cortex and the cilia. Black arrow – buccal cavity; large arrowhead – cytoproct; small arrowheads – area of the pores of the contractile vacuoles. Scale bar: 20 μ m.

of *H. undulata* was carried out as described before. 50 recipient cells were placed in each microwell, and the IFs of *H. undulata* were added at a ratio 500 IFs per 1 ciliate. β -N-acetyl-D-glucosaminidase (concentration range 4–14 μ g/mL) was added to the experimental cells 5 min before the addition of the IFs, the equal volume of distilled water was added to control cells. The final volume of the culture medium was brought to 500 μ L. Living cells were checked for the presence of ingested IFs 30 min after the addition of the IFs, then every hour, each time starting with the control cells until first IF was detected in MIC. Alternatively, lettuce infusion containing *K. aerogenes* and carmine particles was used for feeding ciliates. The cells were observed 1h and 3h after the start of the experiment.

In another set of experiments, α 1-2,3 mannosidase (New England BioLabs, USA) was administered to experimental cells at concentrations varying from 3.2U/mL to 64U/mL 5–7 min before feeding ciliates with *H. undulata* IFs. The ciliates were checked for the presence of ingested IFs starting 30 min after addition of IFs.

MICROSCOPY AND IMAGE PROCESSING

Living cells were observed using a compression device for immobilization of the ciliates (Skovodkin, 1990) and a Leica DM6000B microscope (Leica Microsystems, GmbH, Wetzlar, Germany) with differential interference contrast or phase con-

trast. In experimental infections, control cells were checked before the experimental ones at each time point. WGA-peroxidase stained cells and carmine-fed cells were examined in the bright field mode. In the case WGA-TRITC was applied to living cells, the cells were examined with epifluorescence using an excitation BP filter cube N2.1 (515–560 nm). The images were obtained with a Leica DFC500 digital camera.

Mowiol4-88-mounted slides were analyzed with a Leica confocal scanning microscope TCS SPE (Leica Microsystems GmbH (Germany)) at the Core Facility Center for Microscopy and Microanalysis of St. Petersburg State University, Russia, using a 532 nm laser for TRITC and 405 nm laser for DAPI excitation.

All images were processed using Fiji software (National Institute of Health, USA).

Results

SURFACE ANTIGENS OF *P. CAUDATUM* CONTAIN TERMINAL N-ACETYLGLUCOSAMINE AND/OR N-ACETYLNEURAMIC ACID RESIDUES

To confirm the presence of terminal N-acetylglucosamine and N-acetylneuramic acid residues in the surface glycoconjugates of the ciliate strains used in our study we treated *P. caudatum* cells with peroxidase conjugated WGA and TRITC-WGA. Indeed, the cells stained with peroxidase conjugated WGA demonstrated labeling throughout the entire surface, on the cell cortex and the cilia, most intensive in the region of buccal cavity (Fig. 1). More intensive labeling was sometimes observed in the cytoproct region or was associated with the pores of the contractile vacuoles. Application of fluorescently labeled WGA combined with confocal laser scanning microscopy produced similar results, indicating that WGA applied to living ciliates (the cells were fixed after lectin treatment) normally did not penetrate the pellicle (Fig. 2). In some cells, a few phagosomes located close to the cytostome, were labeled, but this was rather an exception (Fig. 2, B).

TREATMENT WITH WGA BLOCKS THE UPTAKE OF *HOLOSPORA* BACTERIA AND INTERFERES WITH PHAGOCYTOSIS OF FOOD MICROORGANISMS IN *P. CAUDATUM*

Treatment with WGA prevented uptake of IFs of *H. obtusa* and *H. undulata* in all experiments ir-

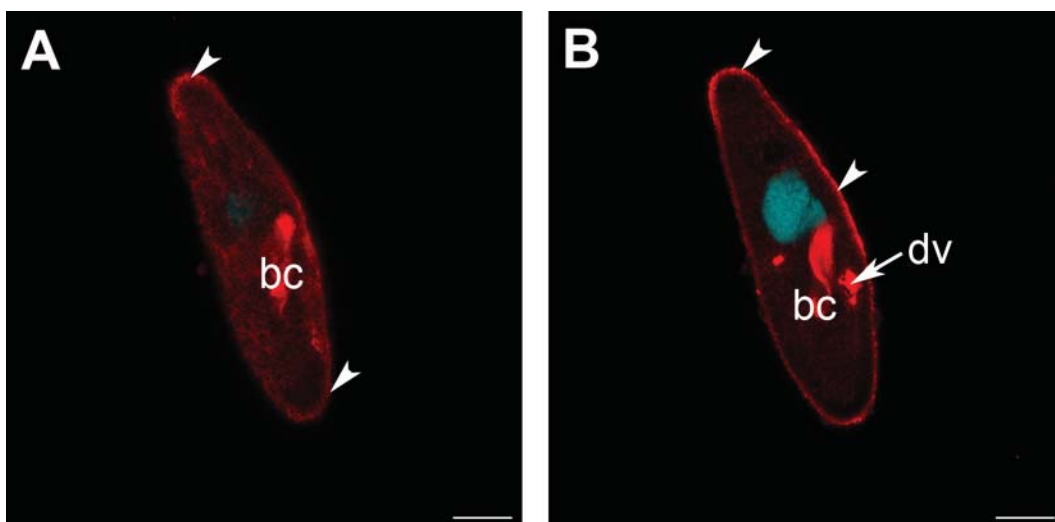


Fig. 2. *Paramecium caudatum* labeled with TRITC-WGA. CLSM, each image is a merge of 3 optical slices. A – Cell surface; B – middle part of the cell. Abbreviations: bc – buccal cavity, dv – digestive vacuole, arrowheads – cell cortex. Scale bar: 30 μ m.

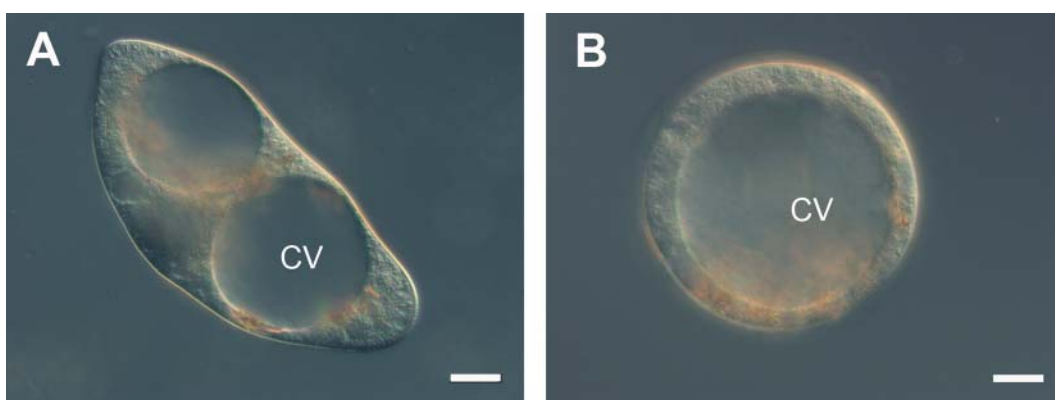


Fig. 3. *Paramecium caudatum* after a prolonged treatment with WGA (25 μ g/mL, 2.5 h.), DIC. A – Swollen reservoirs of both contractile vacuoles; B – a rounded cell with a single contractile vacuole left. Abbreviation: CV – contractile vacuole. Scale bar: 20 μ m.

respective of the differences in the protocols used for *H. obtusa* and *H. undulata*. IFs were never observed in the digestive vacuoles of lectin-treated ciliates. WGA appeared to be highly toxic for the ciliates, as prolonged treatments (up to 40 min) of the living cells with TRITC-WGA used in preliminary experiments at a concentration range from 24 to 50 μ g/mL led to a drastic increase of the contractile vacuole (CV) central bladders and to total disappearance of the CV canals (Fig. 3). The ciliates stopped moving, and in 3h after the start of the experiment all of the cells died. Thus, in the experiments using *H. undulata* smaller concentrations of WGA (5–25 μ g/mL) were applied to ensure ciliate survival. However, this change of the protocol did not

change the final outcome of the experiment, and though swelling of the CV was delayed, the IFs of *H. undulata* were not ingested.

Since we did not detect the IFs of *Holospira* bacteria in WGA-treated cells, phagocytosis assays using food bacteria, *K. aerogenes*, carmine particles and *S. cerevisiae* were performed to check if the effect of the treatment with WGA was specific for *Holospira* IFs. Phagocytotic capacity of the ciliates was perceptibly reduced with all food particles offered irrespective of WGA concentrations used (Fig. 4). Lower WGA concentrations slowed down the deleterious effect of WGA, however, phagocytosis was affected even at concentrations as small as 5 μ g/mL.

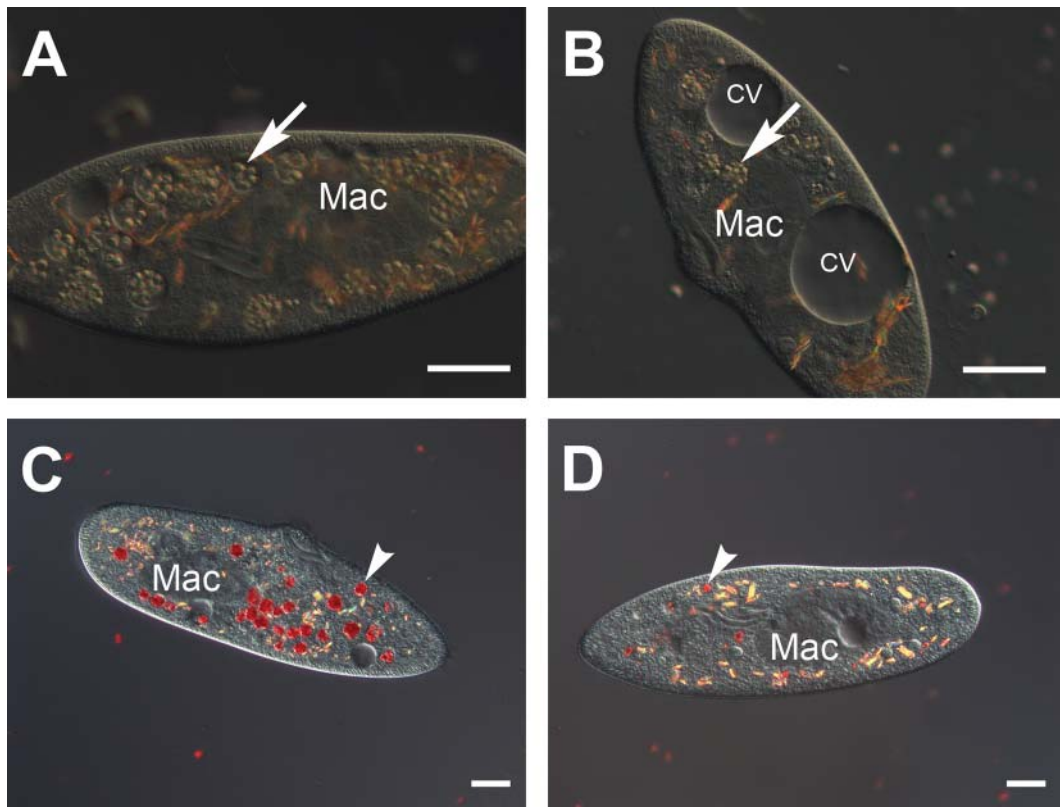


Fig. 4. *Paramecium caudatum* fed with *Saccharomyces cerevisiae* (A, B) and *Klebsiella aerogenes* combined with carmine particles (C, D), DIC. A, C – Control; B, D – under WGA treatment; B – WGA (25 µg/mL, 1 h), D – WGA (5 µg/mL, 1 h). Abbreviations: Mac – macronucleus, CV – contractile vacuole, arrow – digestive vacuole containing yeast, arrowhead – digestive vacuole with *K. aerogenes* and carmine. Scale bar: 20 µm.

MONOSACCHARIDES N-ACETYL-D-GLUCOSAMINE AND D(+)-MANNOSE DO NOT BLOCK INGESTION OF *HOLOSPORA* IN *P. CAUDATUM*

Feeding *P. caudatum* with *Holospira* bacteria in the presence of N-acetyl-D-glucosamine (GlcNAc) was conceived to test if the monosaccharide dissolved in the culture medium would compete with the terminal GlcNAc and/or NANA residues of the surface glycoconjugates for binding IFs and affect uptake of *Holospira* by the ciliates. In both series of experiments (using IFs of *H. obtusa* and *H. undulata*), addition of GlcNAc (1–2mg/mL) to the culture medium did not impair the ingestion of IFs. The IFs of *H. obtusa* were registered in digestive vacuoles both in control and experimental cells by the 40th min after the start of the experiment, and were detected in the MAC on the next day. The IFs of *H. undulata* were also observed in DV within the 1st h after the addition of the IFs to the recipient cells, but were found in the MIC in the experiment and in the control as early as 2h after the beginning of the experiment (Fig. 5).

In another set of experiments, D(+)-mannose was used to check if addition of this monosaccharide would compete with putative glycoconjugates bearing terminal mannose residues for binding IFs of *Holospira* and prevent their uptake. The presence of D(+)-mannose (2mg/mL) in the culture medium did not affect the course of ingestion of both endosymbiotic species.

PHAGOCYTOSIS ASSAYS IN *P. CAUDATUM* IN THE PRESENCE OF β -N-ACETYL-D-GLUCOSAMINIDASE AND α 1-2,3 MANNOSIDASE

In the phagocytosis assays using *H. obtusa*, in control ciliates the IFs could be seen in the digestive vacuoles 40 min after the start of the experiment, and in 3 h they invaded the host MAC (Fig. 6). All ciliates treated with β -N-acetyl-D-glucosaminidase (0.32U) for 1 h demonstrated intensive vesiculation of their cytoplasm (Fig. 6), however, no IFs of *H. obtusa* were registered in the vesicles by that time point. In the course of incubation with the enzyme the velocity of the ciliates decreased, and many of them

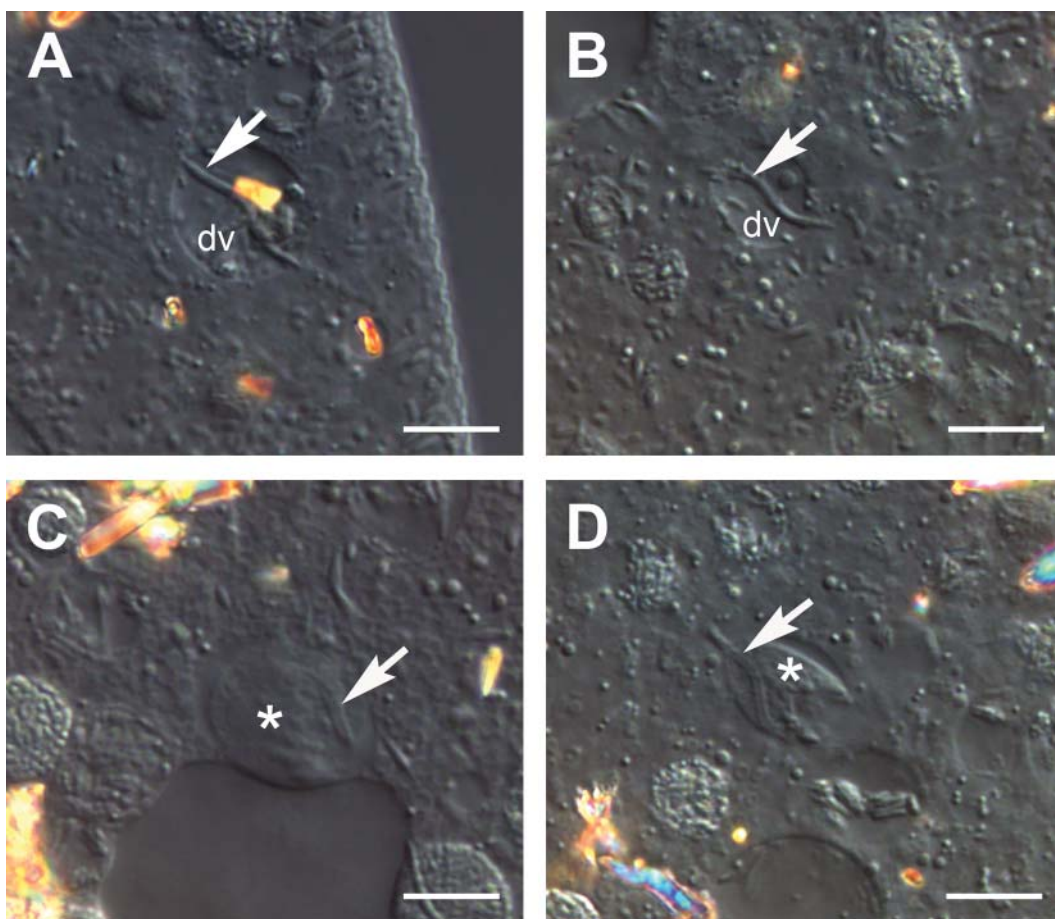


Fig. 5. *Paramecium caudatum* fed with *Holospora undulata* in the absence (A, C) and the presence of N-acetyl-D-glucosamine (B, D), DIC. Abbreviation: asterisk – micronucleus, dv – digestive vacuole, white arrow – IF of *H. undulata*. Scale bar: 10 µm.

dropped to the bottom of the well. In 4.5 h after the start of the experiment the number of living cells significantly reduced, however, in surviving cells the process of infection seemed to be much more intensive, than in the control, the IFs getting into the DVs in packs instead of singles (Fig. 6, D). In 24 h after the start of the experiment, both experimental and control ciliates contained IFs in the MAC.

Vesiculation of the cytoplasm was also registered in all ciliates after the treatment with β -N-acetyl-D-glucosaminidase applied at 4 µg/mL in the experiments with *H. undulata* (Fig. 7). IFs of *H. undulata* never got inside the ciliate during cytoplasm vesiculation. First IFs in the digestive vacuoles were detected only 2–3 h after addition of the enzyme, when the number of the vesicles in the cytoplasm perceptibly reduced, while in the control the IFs got into the DVs within the 1st h of the experiment. The MIC invasion was also delayed, being 4–5 h in the experiment versus 2 h in the control (Fig. 7).

WGA-TRITC labeling of the cells treated with β -N-acetyl-D-glucosaminidase was performed so as to confirm the enzyme efficiency. Indeed, treatment with β -N-acetyl-D-glucosaminidase led to a decrease in fluorescence intensity of the cells showing cytoplasm vesiculation compared to the control cells (Fig. 8), although fluorescence was retained in the buccal cavity and at the cell poles. The cells recovered after cytoplasm vesiculation (4 h after the enzyme treatment) demonstrated intensive fluorescence of the cilia, cell cortex and the buccal cavity, arguing for restoration of the receptors bearing terminal N-acetyl-D-glucosamines (Fig. 8). It should be noted, that the cells that were stained with WGA after vesiculation of the cytoplasm had finished often demonstrated fluorescence focuses under the cell cortex.

The vesicles formed in the ciliate cytoplasm under the treatment with N-acetyl-D-glucosaminidase never contained any IFs of *Holospora*, suggesting that

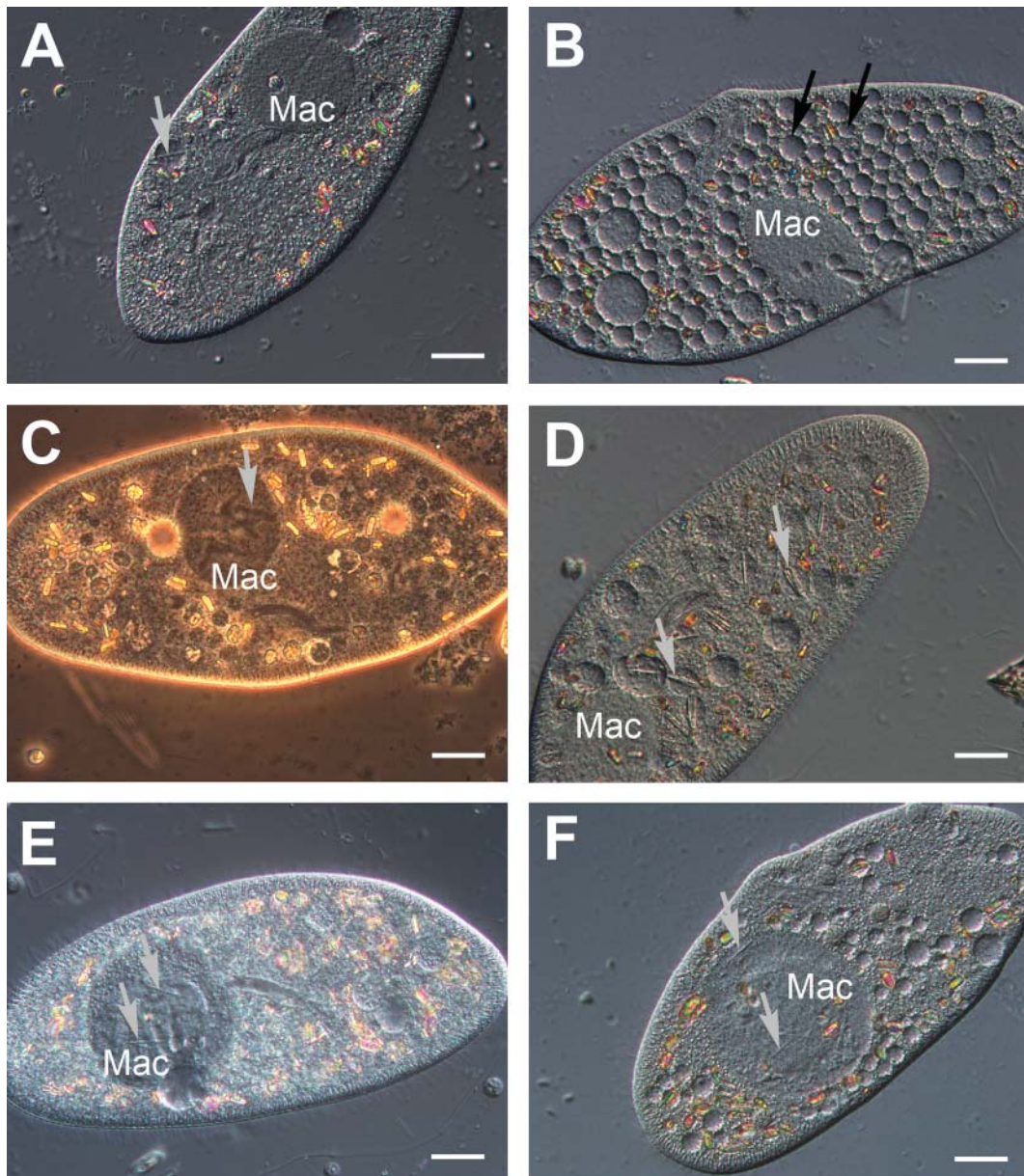


Fig. 6. *Paramecium caudatum* fed with *Holospira obtusa* in the absence (A, C, E) and the presence of β -N-acetyl-D-glucosaminidase (B, D, F); A, B, D, E, F – DIC, C – Phase contrast. A – 40 min after the start of the experiment; B – 1 h; C – 3 h; D – 4,5 h; E, F – 24 h. *Abbreviation:* Mac – macronucleus, white arrows – IFs of *H. obtusa*, black arrows - vesicles in the cytoplasm. Scale bar: 20 μ m.

their formation was not caused by phagocytosis. In order to verify this assumption, the ciliates were fed with *K. aerogenes* and carmine particles concomitantly with N-acetyl-D-glucosaminidase treatment. In 30 min after the start of the experiment, only carmine-stained vesicles were registered in the cytoplasm of the control cells, while in the experimental cells, numerous unstained vesicles of various size appeared evenly distributed in the cytoplasm alongside with a few vesicles loaded with

armine (Fig. 9). 3 h after addition of the enzyme nearly all unstained vesicles disappeared, and the cells became overloaded with carmine-stained phagosomes (Fig. 9, C).

It should be noted, that in another set of experiments with the fixed number of ciliates (50) subjected to the enzyme treatment no carmine containing vesicles were observed 30 min after the start of the experiment. First unstained vesicles appeared as early as in 5-15 min after addition of the enzyme.

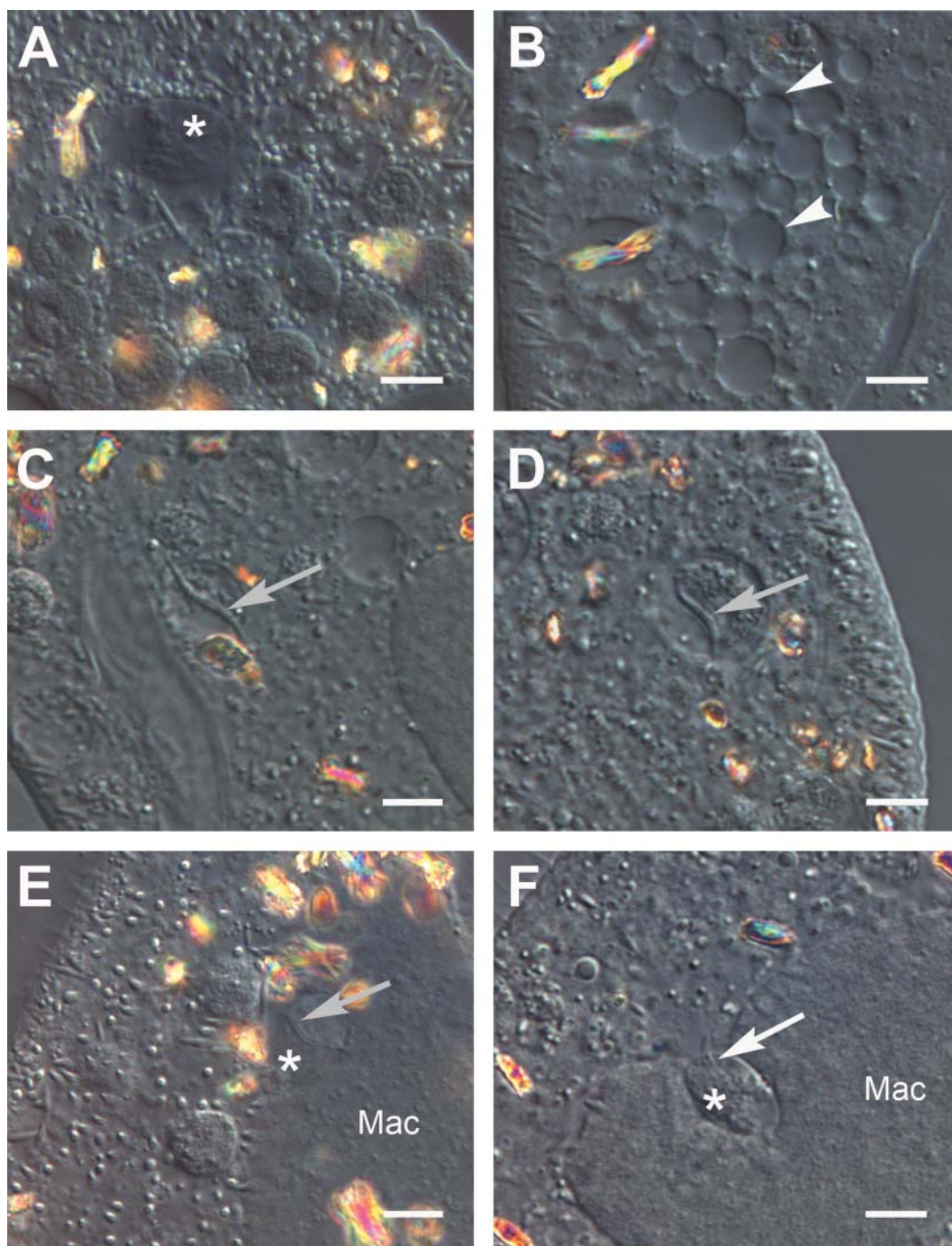


Fig. 7. *Paramecium caudatum* fed with *Holospora undulata* in the absence (A, C, E) and in the presence of β -N-acetyl-D-glucosaminidase (B, D, F), DIC. A – 0 h, B – 30 min, C – 1 h, D – 3 h, E – 2 h, F – 4 h. Abbreviation: Mac – macronucleus, asterisk – micronucleus, arrowheads – vesiculated cytoplasm, white arrow – IF of *H. undulata*. Scale bar: 10 μ m.

The size of the vesicles differed depending on the duration of the enzyme treatment. Small vesicles (less than 8 μ m in diameter) prevailed during the 1st h, larger vesicles (8–20 μ m) forming later, by the 2nd and the 3d h. In some cases, large vesicles were detected in the area of the contractile vacuole.

Additionally, *P. caudatum* were fed with IFs of *H. undulata* in the presence of α 1-2,3 mannosidase to check whether administration of the enzyme affecting mannose-bearing glycoconjugates would cause the same effect as the cleavage of terminal GlcNAc and NANA residues. In our experiments,

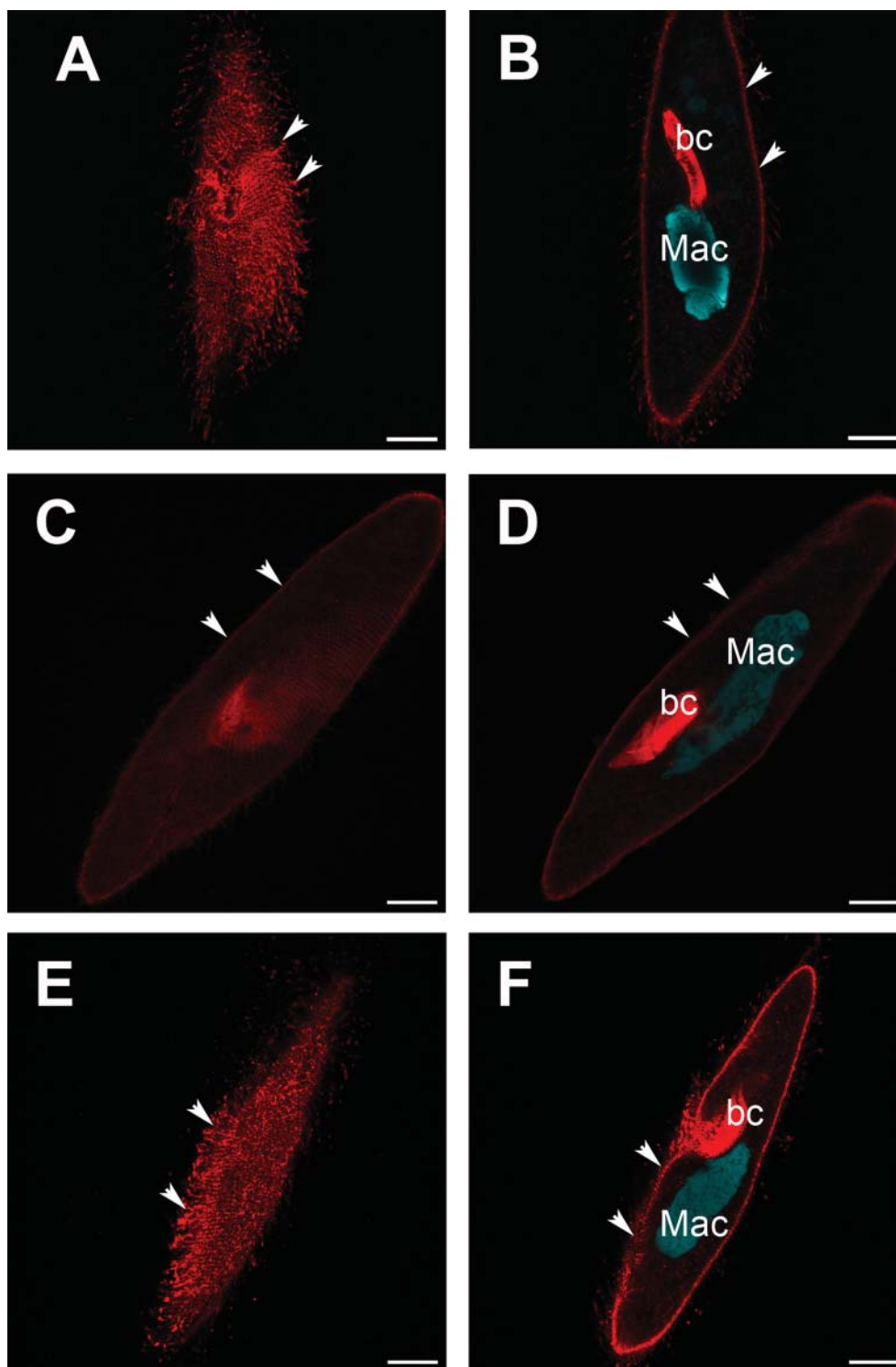


Fig. 8. *Paramecium caudatum* labeled with WGA-TRITC before (A, B), in the course of (C, D) and after treatment with β -N-acetyl-D-glucosaminidase (E, F). CLSM, each image is a merge of 3 optical slices. A, B – 0 min; C, D – 20 min, start of vesiculation; E, F – after the end of vesiculation. A, C, E – cell surface; B, D, F – middle part of the body. *Abbreviations:* bc – buccal cavity, Mac – macronucleus, arrowheads - cell cortex. Scale bar: 20 μ m.

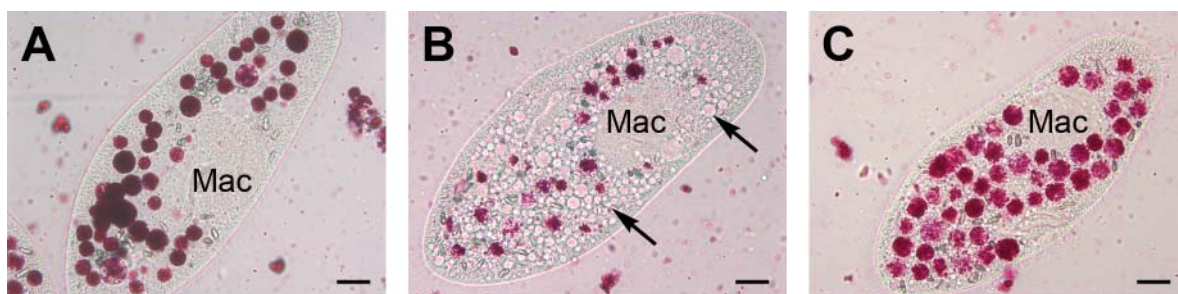


Fig. 9. Carmine fed *Paramecium caudatum* before (A) and after (B, C) treatment with β -N-acetyl-D-glucosaminidase, Bright field microscopy. B – 1h after treatment, note the presence of numerous vesicles lacking carmine; C – 3 h after the treatment, the cytoplasm looks overloaded with carmine-positive vacuoles.

Abbreviation: Mac – macronucleus, arrows – vesicles lacking carmine. Scale bar: 15 μ m.

α 1-2,3 mannosidase at a concentration range from 3.2U/mL to 64U/mL did not have any perceptible impact on the course of infection

Discussion

THE CELL SURFACE OF *PARAMECIUM CAUDATUM* BEARS GLYCOCONJUGATES WITH TERMINAL N-ACETYLGLUCOSAMINE AND N-ACETYLNEURAMINIC ACID RESIDUES

Intensive labeling of the cortex and the buccal cavity observed in *P. caudatum* after incubation with peroxidase or TRITC conjugated WGA lectin known to be specific for terminal GlcNAc and NANA residues is in good agreement with the data reported by other researchers in *P. aurelia* (Lütke and Plattner, 1986, Pape et al., 1988, Allen et al., 1988; Ramoino, 1997; Locatelli et al., 1998; Ramoino et al., 2001) and argues for the presence of terminal GlcNAc and NANA residues on the glycoconjugates of *P. caudatum*. Fluorescent digestive vacuoles and fluorescence focuses under the cell cortex occasionally occurring in some cells are concordant with the conclusions concerning recycling of the surface antigens in *Paramecium* (Flötenmeyer et al., 1999) and suggest possible involvement of the glycoconjugates with terminal GlcNAc and NANA in phagocytosis.

WGA LECTIN BINDING INHIBITS THE UPTAKE OF *HOLOSPORA* INFECTIOUS FORMS AND PHAGOCYTOSIS IN *P. CAUDATUM*

Treatment with WGA was performed to find out if blocking surface receptors of the ciliate, which are abundant in the buccal cavity, would affect the process of infection with *Holospira* bacteria. Indeed,

administration of WGA prevented ciliates from ingesting the IFs. However, control phagocytosis assays carried out using *S. cerevisiae*, *K. aerogenes*, and carmine particles as food showed that the effect was not specific for *Holospira* IFs, as WGA treatment proved to inhibit phagocytosis in *P. caudatum* in general. The insignificant number of food vacuoles registered in the experiments with the yeast could be caused by a delay in the lectin action or by existence of a threshold in a number of receptors to be bound for an effect to be seen. Inhibition of phagocytosis in *P. caudatum* by WGA lectin argues for direct involvement of receptors bearing terminal GlcNAc and NANA in phagocytosis and is concordant with the previous report on suppression of phagocytosis and membrane recycling in *P. aurelia* by alcian blue known to bind glucosaminoglycans and sialylated glycoconjugates (Kaneshiro et al., 1992). At the same time, swelling of the CV reservoir registered upon prolonged incubation with WGA, which is, apparently, a result of plugging the CV pore by the lectin, is in good agreement with previous studies showing intensive WGA labeling of the CV pores in *P. aurelia* (Lütke and Plattner, 1986) and some of our images, and implies a severe stress for the cell. Besides that, the cytotoxic effect of WGA, which has been described in literature in mammalian cells (Ohba et al. 2003), may result from activation of various signaling pathways, yet unknown, that may also contribute to inhibition of phagocytosis in *P. caudatum*.

Since many bacterial adhesins are lectins (Ofek, Sharon, 1990), we supposed that specific monosaccharides added to the cell culture would compete with the terminal sugar residues on the ciliate surface receptors for binding putative bacterial lectins and would block the uptake of *Holospira* IFs in *P. caudatum*. However, this conjecture proved to be

wrong, as administration of N-acetyl-D-glucosamine did not affect phagocytosis in our experiments. Taking into account the affinity of WGA towards sialylated glycoproteins (Itakura et al., 2017), it is NANA residues of the ciliate surface glycoconjugates that might hold the key to the early steps of infection with *Holospira* bacteria.

TERMINAL RESIDUES OF N-ACETYLGLUCOSAMINE OF THE CELL SURFACE GLYCOCONJUGATES ARE INVOLVED IN PHAGOCYTOSIS IN *PARAMECIUM CAUDATUM*

Treatments of ciliates with β -N-acetyl-D-glucosaminidase specifically cleaving terminal GlcNAc from the surface glycoconjugates of the ciliates always caused strong vesiculation of their cytoplasm. Since the numerously formed vesicles never contained *Holospira* IFs, food bacteria, or carmine, they were likely to be formed not in the course of phagocytotic activity and, thus, they cannot be considered DVs. Noteworthy, the surface of the vesiculated cells demonstrated weaker WGA-TRITC fluorescence (Fig. 8, C, D) compared to that of the control cells not treated with the enzyme (Fig. 8, A, B), implying that the enzymatic treatment removed a significant part of the terminal GlcNAc residues from the ciliate surface. The lag in phagosome formation in the cells treated with β -N-acetyl-D-glucosaminidase registered in our experiments implies that the presence of the terminal GlcNAc residues on *Paramecium* surface glycoconjugates might be a prerequisite for phagocytosis in general, including the uptake of *K. aerogenes*, carmine particles, *S. cerevisiae* and IFs of *Holospira*. Likewise, WGA-labeled structures have been suggested to be possibly involved in food perception in *T. pyriformis* (Dürichen et al., 2016). At later time points the cells recovered their ability to uptake food bacteria and the IFs of *H. undulata* and *H. obtusa*. Interestingly, by that time vesiculation of the cytoplasm decreased, while the fluorescence observed after the treatment with WGA-TRITC became more conspicuous. The recovery of fluorescence might be an indirect evidence of the recovery of the surface glycoconjugates with the terminal GlcNAc residues. Taken together, these data suggest that the terminal GlcNAc residues of the surface glycoconjugates in *P. caudatum* are indispensable for phagocytosis in this ciliate species.

A slight discrepancy between the data obtained in our two different sets of phagocytosis assays under treatment with β -N-acetyl-D-glucosaminidase

(presence of insignificant number of DVs in one set of experiments and the total absence of DVs in the second one) might be explained by differences in the substrate concentration. Although in all experiments the enzyme concentration was the same, the number of the ciliates taken in the two sets of phagocytosis assays differed greatly. According to classical Michaelis-Menten model of enzyme kinetics, at too high substrate concentrations the active sites of the enzyme become saturated, which leads to the reaction rate plateau, and sometimes, even to a decrease in the reaction rate (Sahin et al., 2023). The amount of the enzyme per ciliate in the experiments where only 50 cells (low substrate concentration) were subjected to the enzyme treatment must have been adequate for the total inhibition of phagocytosis, while for greater numbers of ciliates (high substrate concentration) the enzyme concentration appeared insufficient.

In our experiments, administration of α 1-2,3 mannosidase did not affect the course of infection with *H. undulata*, which does not conform to the results obtained previously in experimental infections with *H. obtusa* (Fujishima et al., 1991). In this study, mannosidase treatment prevented invasion of the IFs into the MAC. This contradiction may have arisen either due to the differences in the experimental protocol, or because of peculiarities of the ciliate strains and *Holospira* isolates used in the two studies.

THE NATURE OF CYTOPLASMIC VESICLES FORMED UNDER β -N-ACETYL-D-GLUCOSAMINIDASE TREATMENT

The nature of vesicles formed in the course of vesiculation of the cytoplasm caused by administration of β -N-acetyl-D-glucosaminidase is not quite clear. Several mechanisms of their origin, not necessarily mutually exclusive, might be proposed. These vesicles may be formed in the course of endocytosis of defective receptors from the cell surface, compensatory exocytosis, and disintegration of the contractile vacuoles. These processes may be complementary and take place nearly simultaneously. Since the numerous vesicles in the cytoplasm appear practically immediately after addition of the enzyme, defective receptors lacking terminal GlcNAc residues are presumably removed from the surface by means of endocytosis. Endocytosis in *Paramecium* is known to take place all over the cell surface via parasomal sacs and at the buccal cavity via nascent food vacuoles (Flö-

tenmeyer et al., 1999). Removal of the defective receptors might trigger compensatory exocytosis aimed at recovery of the receptors with terminal GlcNAc residues on the cell surface. It has been shown that the recovery of the surface antigens takes place in the same place, as endocytosis, i.e., in parasomal sacs (Flötenmeyer et al., 1999). In general, the vesicles appearing after the enzyme treatment were more or less evenly distributed in the cytoplasm of the ciliate, however, in some cells the vesicles tended to concentrate in the vicinity of the contractile vacuole. Thus, the structure of the contractile vacuole could be perturbed by the treatment with β -N-acetyl-D-glucosaminidase. Noteworthy, Rab11 protein known to be a marker of the recycling endosome in higher eukaryotes has been shown to participate in the transport of proteins to the plasma membrane in *Dictyostelium* (Harris et al., 2001) and *Trypanosoma cruzi* (Niyogi et al., 2014), which made this authors suggest that the contractile vacuole in Protista was an evolutionary precursor of the recycling endosomes in higher eukaryotes. We can hypothesize that the vesicles observed upon the enzyme treatment might originate from the contractile vacuole spongione, implying that the contractile vacuole mediates trafficking of the surface antigens.

Besides that, it is possible that the excessive glycoconjugates recovered on the surface of the ciliate at later time points upon the enzyme treatment are endocytosed in their turn. Fluorescent signal after WGA-TRITC treatment observed not only on the cell surface, but also in the cytoplasm support this possibility, especially that the signal in the cytoplasm was registered only in the cells where vesiculation of the cytoplasm had already finished. Taking into account that endocytosis of excessive glycoconjugates takes place also at the buccal cavity, increase in the number of *H. obtusa* IFs detected in *P. caudatum* treated with β -N-acetyl-D-glucosaminidase after vesiculation had finished (Fig. 6, F) supports this suggestion. It is noteworthy, that a certain limit of the number of the IFs which can invade the host nucleus under experimental infection (35 for *H. obtusa*) has been reported in an older study (Fokin and Skovorodkin, 1991). This observation implies the existence of a factor preventing the uptake of extra IFs once the limit is reached. We hypothesized that it is changes in the surface glycoconjugates, that might serve as this factor. The recently published data on alteration of the surface antigen expression in *P. caudatum* under

H. obtusa infection (Fujishima, 2025) speak in favor of our hypothesis.

To summarize, the results obtained in our study demonstrate that the presence of intact terminal GlcNAc and NANA residues on the surface glycoconjugates of *P. caudatum* are a prerequisite for phagocytosis in this ciliate species in general, and the uptake of *Holospira* IF in particular. Apparently, any disturbance of the terminal GlcNAc residues of the surface glycoconjugates, be that WGA binding or enzyme cleavage, serves as an emergency stop signal for phagocytosis. Both WGA binding and removal of GlcNAc with β -N-acetyl-D-glucosaminidase prevented phagocytosis and ingestion of *Holospira* IF. However, the interaction between the ciliate and the IF of *Holospira* are not specifically mediated by terminal GlcNAc residues. The key to the host ciliate might lie in its sialylated glycoconjugates, as WGA also shows extensive binding to sialylated glycoproteins (Itakura et al., 2017).

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