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Recovery of the ciliate *Paramecium multimicronucleatum* following bacterial infection with *Holospora obtusa*

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Abstract

The macronucleus (Ma) of *Paramecium multimicronucleatum* can be experimentally infected with bacteria of the species *Holospora obtusa,* a macronuclear-specific parasite of *P. caudatum*. However, usually all bacteria disappear from the nucleus within 1–2 days after infection. The results of infecting several different stocks of *P. multimicronucleatum* with several different isolates of *H. obtusa* from *P. caudatum* were studied during some days after infection to investigate this disappearance. Using light, fluorescence and electron microscopy at different stages of bacterial disappearance, it was shown that what we call ''cleaning'' of the nucleus is a fast and active process. *P. multimicronucleatum* can be infected with the infectious forms of *H. obtusa* within 2 h, but the majority of the bacteria were lost from the Ma by 10–19 h. They were released from the host nucleus into the cytoplasm and then to the surrounding medium. At first, the infected Ma shortened and became almost rounded. Before leaving the Ma, the majority of the bacteria somehow assembled into groups and these aggregates, coated with material that appears to be nuclear chromatin, protruded into the cytoplasm and were finally separated from the Ma. Sometimes single bacteria were extruded in the same manner. Bacterial release from infected Ma can be stopped by low temperature and is delayed and reduced by nocodazole treatment suggesting that intra-macronuclear microtubules may be involved.

Keywords: Endocytobiosis; *Holospora obtusa*; Infection; Nocodazole; *Paramecium multimicronucleatum*

Introduction

Ciliates may be hosts for numerous microorganisms (mostly bacteria), which can occupy any cell compartment of the protists (Fokin 1993, 2004; Görtz 1983; Görtz and Schmidt 2005). Some of these bacteria are infectious and can invade new host cells either in nature or in laboratory experiments. The establishment of endocytobiosis between ciliates of the genus *Paramecium* and specific intranuclear *Holospora* bacteria was actively studied during the last two decades (Fokin 1993, 2004; Fokin et al. 1996, 2003; Fujishima et al. 1990, 1997; Görtz 1983, 1988; Kawai and Fujishima 2000; Skovorodkin et al. 2001). This process is connected with the ability of the infectious (I) forms of bacteria to reach their target nucleus, either a micro- (Mi) or a macronucleus (Ma), and survive in the nucleoplasm to produce

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numerous reproductive (R) forms (Fokin 2004; Görtz 1996). Typically, it takes bacteria about 1 h to get into either the Ma or the Mi (Fokin and Skovorodkin 1991a, b; Görtz 1986). Depending on the number of I-forms in the medium and the bacterium-ciliate strain combinations, this time may be reduced for *Holospora obtusa* to 10–15 min (Fujishima and Görtz 1983) or increased for *H. undulata* to 1.5–2 h (Fokin and Skovorodkin 1991b). Usually, about 100% of the ciliates are infected during this time, though sometimes the infection intensity may be lower (Fokin and Skovorodkin 1991a; Fujishima and Görtz 1983). In some combinations bacteria are able to enter their target nuclei, but after a short time they disappear from this compartment without development into R-forms. This is the case for *Paramecium multimicronucleatum* experimentally infected with *H. obtusa* (Fokin 1993; Fujishima and Fujita 1985), but no details are known about this failure of infection to persist.

Apparently, ciliates possess defensive mechanisms against bacterial infection (Fokin and Skovorodkin 1997; Görtz 1986), since the proportion of natural populations infected with *Holospora* (even for its specific host — *P. caudatum*) usually does not exceed 5% (Fokin 2004). Our investigations revealed that some clones and possibly even populations of the hosts were not susceptible to infection (Fokin et al. 2003; Fujishima and Fujita 1985).

In this study, we have tried to verify experimentally how cells of *P. multimicronucleatum* can avoid a permanent infection with *H. obtusa*.

Material and methods

The following *P. multimicronucleatum* cultures were used in this study: strain JYU-P.m., isolated from a small pond at the Yamaguchi University, Japan (1999); strain GMA-P.m., isolated from Ammersee, Germany (1993), and strain HH1-2, isolated from a sample collected in a pond near Honolulu, Oahu Island, Hawaii (2001) by I. N. Skovorodkin. Two laboratory stocks of *P. caudatum* infected with *H. obtusa* were used as a source of the I-forms for experimental infection: strain RWK98-2 H.o., isolated from a lake on the Kartesh peninsula, White Sea, Russia (1998) and RB-1, isolated in Katzenbachsee near Stuttgart and infected with *H. obtusa* originally isolated from a pond in Bensersiel, German North Sea coast, Germany (1993) by H.-D. Görtz.

Paramecia were cultivated at 18–20 °C in lettuce medium inoculated with *Enterobacter aerogenes*. Living cells were immobilized for observation with the help of a compression device (Skovorodkin 1990). Living and fixed cells were examined by Nomarski interference-contrast (DIC) with Zeiss Axioskop and Olympus BH2-RFL microscopes.

For investigation of morphology of the infected Ma and localization of the intracellular bacteria the cells were fixed and stained by the Feulgen method or stained with DAPI. To confirm the identity of bacteria, paramecia were prepared for in situ hybridization by a standard procedure (Fokin et al. 1996), and exposed simultaneously to a eubacteria-specific probe, 5′-GCTGCCTCCCGTAG-GAGT-3′, labelled with fluorescein isothiocyanate (FITC) and a probe specific to the alpha-subgroup of proteobacteria, 5′-GCGTTCGCTCTG-AGCCAG-3′, labelled with tetramethylrhodamine isothiocyanate (TRITC) (Amann 1990; Amann et al. 1991). For electron microscopical (EM) investigation infected cells were prepared according to a protocol used before (Fokin 1989).

Experimental infection was carried out using a homogenate prepared from infected cells according to Preer (1969). *P. multimicronucleatum* cells were infected by mixing equal volumes of a dense cell culture of host cells and the homogenate in a 3 ml depression slide, and maintained at 18–20 °C. In most cases, the mean number of bacteria that infected the Ma was more than 5 per Ma. To check the infection, living cells were observed by DIC at 2, 5, 8, 10, 12, 15, 19 and 24 h after mixing with homogenate. Simultaneously, samples of the same stocks were stained by the Feulgen method. Series of experiments were carried out using each of the three *P. multimicronucleatum* stocks. For the combination JYU-P.m. and *H. obtusa* from RWK98-2 H.o., the infection experiment was repeated three times. Electron microscopical observations, nocodazole treatment and low temperature experiments (see below) were performed for the same combination. Light microscopical observation (only) were carried out once for other combinations.

The size of infected Ma at the different investigated stages was determined by measurement of 20–25 Feulgen-stained cells using a standard procedure (Fokin et al. 1999), and compared with Ma of uninfected control cells maintained under the same conditions. A one-way-ANOVA was used to test differences between experimental and control data ($p < 0.005$). The morphology of living infected cells and the number of I-forms in the nuclei were recorded from at least 10 specimens at each step from 2 to 24 h. The percentage of infected cells in the same set of cells at each stage (2–24 h) was calculated as well.

Treatments of infected cells with nocodazole, a synthetic microtubule inhibitor (Janssen Pharmaceutica), or with low temperature (4 °C), were commenced after 2 h of experimental infection of the JYU-P.m. + *H. obtusa* from RWK98-2 H.o. combination and the treated infected cells were checked at the same intervals of time as untreated infected cells. A final nocodazole concentration of 5 µg/ml was used, according to