ISOLATION AND CHARACTERIZATION OF MACRONUCLEI OF PARAMECINUM CAUDATUM INFECTED WITH THE MACRONUCLEUS-SPECIFIC BACTERIUM HOLOSPORA OBTUSA

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SUMMARY
Macronuclei from Paramecium caudatum infected with Holospora obtusa may be isolated on sucrose step gradients. Macronuclei containing primarily infectious forms can be separated from those bearing predominantly reproductive forms. RNA polymerase activity in infected macronuclei is greater by a factor of 5 than that in uninfected macronuclei. Proteinase activity is also significantly higher.

INTRODUCTION
Endosymbiotic associations between bacteria and eukaryotes are commonly reported (Taylor, 1983). The prokaryotic cell (the cytobiont (Taylor, 1979) or endocytobiont (Schwemmler, 1979)) may live non-specifically within the intracellular habitat provided by the host, but some endosymbiotic bacteria are specialized to invade specific cellular organelles. Three species of the bacterial genus Holospora invade exclusively the nuclei of the ciliate Paramecium caudatum.

Like other ciliates P. caudatum contains two different nuclei, the generative, transcriptionally inactive micronucleus and the vegetative, transcriptionally active macronucleus (for a review see Nanney, 1980). Both types of nuclei can be infected by some species of the genus Holospora. Whereas Holospora elegans and H. undulata can only be found in the micronucleus of P. caudatum, H. obtusa is restricted to the macronucleus (for a review see Görtz, 1983). In all Holospora species two morphologically different forms can be distinguished. The long infectious form is taken up from the surrounding medium into a food vacuole and then transported into the nucleus that is specific to the species. Having reached the nucleus the infectious form differentiates by multiple fissions into the smaller reproductive form, which multiplies by binary fission. From the reproductive form the infectious form can then be developed and released into the medium (Ossipov & Podlipaev, 1977; Görtz & Dieckmann, 1980).

Normally, the endonuclear bacteria do not destroy the P. caudatum cell. They

Key words: Paramecium, macronucleus isolation, endonuclear bacteria, RNA synthesis, proteinase activity.
live in the nuclear microenvironment in a more or less permanent state, even providing the host with a certain resistance against a second infection by another *Holospora* species (Ossipov, Skoblo & Rautian, 1975; Görzt & Dieckmann, 1977). The persistent association indicates that a well-balanced equilibrium exists between the cytobiont and its host. We have isolated the bacteria within their habitat, the infected nucleus, in order to study more closely the physiological interactions.

Isolation of macronuclei by centrifugation through continuous or step gradients of sucrose is a well-established procedure for many ciliates (for a review see Cummings, 1977). In the present investigation these methods are modified for the mass isolation of macronuclei from *P. caudatum* infected with *H. obtusa*. Experiments on RNA synthesis and proteinase activity of the isolated infected macronuclei indicate that these nuclei are well suited for *in vitro* studies and provide a system that is not as complex as the intact cell, but has retained functional characteristics connected with the infection.

**MATERIALS AND METHODS**

**Cells and culture conditions**

*Paramecium caudatum* strain 27 a G 3, mating type V (syngen 3) obtained from Dr Koichi Hiwatashi, Sendai University, Japan was grown at 25°C in 2-l Fernbach flasks in 11 medium prepared according to Görzt & Dieckmann (1980). Infection of the cultures with the macronucleus-specific bacterium *H. obtusa* was performed as described by Görzt & Fujishima (1983) for *H. elegans*. To obtain macronuclei bearing predominantly the long, infectious form of *H. obtusa*, cultures were infected 8–10 days before isolation, whereas cultures infected 7 days before isolation yield macronuclei bearing predominantly the short reproductive form.

**Isolation of macronuclei and endonuclear bacteria**

*Paramecium* cells from 41 (cell density 700–1000 cells/ml) were filtered through eight layers of gauze, renewed each time after 11 of fluid had passed through, and subsequently concentrated by gentle centrifugation in an oil-test centrifuge (Hettich Roto, Silenta, 1 min at 200 g).

The following steps were performed at 4°C. The packed cells were resuspended in 0-25 m-

The following steps were performed at 4°C. The packed cells were resuspended in 0-25 m-TSCM buffer (10 mM-Tris, pH 7-9, 0-25 m-sucrose, 3 mM-CaCl₂, 8 mM-MgCl₂), sedimented once more in the oil-test centrifuge and the pellet was resuspended in 10 ml TSCM buffer containing 0-2% Nonidet P40 (Shell Company, FRG) 0-1 mM-phenylmethanesulphonyl fluoride (PMSF) and 0-1% (w/v) spermidine. The cell suspension was stirred in an ice-bath with a slowly moving magnetic bar for 5 min. Subsequently, cell lysis was performed by passing the suspension five to ten times (depending on the grade of infection) through a 20 ml pipette. When cell lysis was complete as checked in the phase-contrast microscope, the lysate was diluted with addition of 0-25 m-

Macronuclei were sedimented to the bottom of the tube by centrifugation for 10 min at 700 g in the swing-out rotor of a Christ Junior II KS centrifuge. The macronuclear sediment was resuspended in 0-5 ml of 1-6 m-TSCM and 9-5 ml 2-2 m-TSCM (TSCM containing 1-6 m-sucrose, 0-1% spermidine and 0-1 mM-PMSF), layered over a cushion of 20 ml 1-6 m-TSCM (TSCM containing 1-6 m-sucrose, 0-1% spermidine and 0-1 mM-PMSF). Macronuclei were sedimented to the bottom of the tube by centrifugation for 10 min at 700 g in the HB4 rotor of a Sorvall centrifuge. Macronuclei were either sedimented to the bottom of the tube (non-infected or bearing predominantly the short, reproductive form of *H. obtusa*) or formed a band at the top of the 2-2 m-TSCM cushion (bearing predominantly the long, infectious form of *H. obtusa*). Isolated macronuclei were resuspended in TGMED buffer (50 mM-Tris-HCl, pH 7-9, 30% glycerol, 5 mM-MgCl₂, 0-1 mM-EDTA, 0-1 mM-PMSF and 0-5 mM-dithiothreitol) and either used immediately or frozen in liquid N₂ and stored at −70°C. *H. obtusa* cells
Holospora-infected macronuclei in Paramecium

were purified by homogenization of isolated infected macronuclei in 0.05 M-TGEDA (50 mM-Tris-HCl, pH 7.9, 30% glycerol, 0.1 mM-EDTA, 0.5 mM-dithiothreitol, 50 mM-ammonium sulphate) and subsequent sedimentation of the macronuclear chromatin (5 min at 2000 g). The bacteria remaining in the supernatant were obtained by centrifugation at 10,000 g for 10 min.

RNA synthesis in isolated macronuclei

RNA synthesis in isolated macronuclei was measured by incubation of 1×10^5 to 4×10^5 macronuclei at 25°C in a total volume of 155 µl containing 50 mM-Tris- HCl, pH 7.9, 5% glycerol, 0.5 mM-dithiothreitol, 2 mM-MgCl_2, 50 mM-(NH_4)_2SO_4, 2 mM-ATP, 1 mM each of CTP and GTP, 0.01 mM-UTP and 2.5 µCi of [^35]UTP (35 Ci/mol, purchased from New England Nuclear, Boston, U.S.A.).

The reaction was stopped either by adding 2 ml ice-cold carrier RNA (50 µg/ml in 2 M NaCl) or by the addition of 0.5 ml TSSE (50 mM-Tris-HCl, pH 7.9, 1% sodium dodecyl sulphate (SDS), 5 mM-EDTA, 0.1 M NaCl) and subsequent incubation for 1 min in boiling water. Samples were precipitated with ice-cold 0.5 M-trichloracetic acid (TCA), filtered through glass-fibre filters (type 85/90, Macherey-Nagel, FRG) and washed with 0.01 M TCA. The radioactivity on the dried filters was determined by liquid scintillation spectrophotometry. Each value represents the average of four parallel determinations.

Assay of proteolytic enzyme activities in isolated macronuclei

The activities of proteolytic enzymes in infected and non-infected macronuclei were determined using Azocoll (Calbiochem-Behring Corp., La Jolla, U.S.A.; an insoluble protein—dye conjugate) as substrate. Essentially according to Chavira, Burnett & Hageman (1984), assays were performed as follows: 10 mg of Azocoll (prewashed in 50 mM-Tris-HCl, pH 7.9, 1 mM-CaCl_2) were suspended in 1 ml of the same buffer. The reactions were started by addition of 2×10^5 infected or non-infected macronuclei suspended in 50 µl TGMED buffer from which PMSF was omitted. Incubation was carried out at 25°C under vigorous agitation in a shaker. Reactions were stopped by immersion of the test tubes in ice and subsequent sedimentation of the remaining insoluble Azocoll in the cold. The absorbance of the supernatants was read against buffer at 520 nm using a Zeiss PMQII spectrophotometer. Each value represents the average of two determinations.

RESULTS

Isolation of infected and non-infected macronuclei

The isolation of pure macronuclei in *P. caudatum* is facilitated by the fact that this nucleus is by far the largest organelle within the cell. On the other hand, the large macronuclear size precludes simple cell lysis using an homogenizer because of damage to the macronuclei. Therefore, cells were lysed by gentle stirring of the cell suspension with a magnetic bar in the presence of the non-ionic detergent Nonidet P40. The cells were subsequently passed through a 10 ml pipette until lysis had been achieved as checked in the phase-contrast microscope. Addition of 0.1% spermidine to the lysis buffer (Gorovsky, Yao, Keevert & Pleger, 1975) leads in the case of the ciliate *Tetrahymena* to an increase in the yield of isolated macronuclei by a factor of 3, without affecting their transcriptional activity (Freiburg, unpublished). Consequently, spermidine was provided during lysis of the *Paramecium* cells.

Immediately after the rupture of the cells the lysate was diluted to decrease the concentration of the detergent, layered over a cushion of 1.6 M sucrose and centrifuged. This step separates the macronuclei very efficiently from most of the cellular...
debris – discharged trichocysts, the food bacteria and the micronuclei. The latter can be further purified to homogeneity by metrizamide isopycnic centrifugation according to Shiomi, Higashinakagawa, Saiga & Mita (1980; data not shown).

Infection of macronuclei with the macronucleus-specific bacterium *H. obtusa*

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**Fig. 1.** *P. caudatum*; unfixed, slightly pressed, phase-contrast. ×430. **A.** Non-infected; **B.** macronucleus infected with *H. obtusa*. Note that the infected macronucleus (mac) shows a significant increase in size compared to the non-infected cell.

**Fig. 2.** Photomicrographs of isolated macronuclei and endonuclear bacteria. **A.** Centrifuge tube after centrifugation at 16,300 g for 20 min. Macronuclei bearing predominantly the infectious form of *H. obtusa* band at the top of the 2-2 M-sucrose cushion (arrow). **B.** Isolated macronuclei derived from the 16,300 g sediment bearing predominantly the reproductive form of *H. obtusa*; bacteria are visible only after lysis of the macronuclei; differential interference contrast. ×400. **C.** Isolated macronucleus derived from the top of the 2-2 M-sucrose cushion bearing predominantly the infectious form of *H. obtusa*; differential interference contrast. ×770. **D.** Long infectious and short reproductive forms of *H. obtusa* purified from isolated macronuclei as indicated in Materials and Methods; differential interference contrast. ×1600.
leads, depending on the extent of infection, to a more or less drastic swelling of the nuclei (Fig. 1B). Consequently, the macronuclei bearing predominantly the infectious form of *H. obtusa* band at the top of the 2.2 M-sucrose cushion when centrifuged as indicated in Materials and Methods (Fig. 2A,C). Macronuclei bearing
predominantly the reproductive form sediment at the bottom. As can be seen from Fig. 2b at this stage of purification macronuclei are completely free from cytoplasmic contaminants and food bacteria, and show a shape similar to that found within the intact cell. In some preparations the macronuclear sediment contains water-insoluble crystals released from the *Paramecium* cells during homogenization. The overall yield of isolated macronuclei at this last stage of purification is 90–100% in the case of the non-infected nuclei and, due to their higher fragility lower for infected nuclei, ranging from 50 to 80%.

**RNA synthesis in infected and non-infected macronuclei**

Since the macronucleus of *P. caudatum* is the organellar target for the infection, we are interested in detecting any organelle-specific *in vitro* response of the host to

![Graph](image_url)

**Fig. 3.** Incorporation of [³H]UMP into RNA in isolated macronuclei as a function of time. Tests were carried out as described in Materials and Methods with 1·5×10⁵ non-infected (○—○) or infected (▲—▲) macronuclei per test.
Holospora-infected macronuclei in Paramecium

Table 1. RNA synthesis in infected macronuclei in vitro and distribution of \(^{3}H\)UMP incorporation among macronuclear chromatin and endonuclear bacteria

<table>
<thead>
<tr>
<th>[(^{3}H)UMP incorporation (c.p.m.)]</th>
<th>Infected macronuclei 87 536</th>
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<tr>
<td>2000 g sediment (macronuclear chromatin) 76 361</td>
<td></td>
</tr>
<tr>
<td>10 000 g sediment (H. obtusa; 8·6 (\times) 10(^7) cells) 392</td>
<td></td>
</tr>
<tr>
<td>10 000 g supernatant 2130</td>
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Isolated macronuclei (bearing predominantly the reproductive form of H. obtusa) were incubated with nucleoside triphosphates as indicated in Materials and Methods. After 30 min the reaction was stopped by chilling the test tubes in an ice-bath and increasing the concentration of the non-labelled UTP to 1 mM. The \(^{3}H\)UMP incorporation into RNA was either determined directly from the total incubation cocktail containing 7·5 \(\times\) 10\(^5\) macronuclei, or an equal number of macronuclei were homogenized by vigorous pipetting, and macronuclear chromatin and endonuclear bacteria were separated by centrifugation at 2000 g for 5 min. Subsequently, bacteria were harvested from the supernatant by centrifugation at 10 000 g for 10 min. Each probe was lysed by addition of TSSE buffer and processed as described in Materials and Methods.

H. obtusa. Therefore, an in vitro transcription system has been used to study whether RNA synthesis in the macronucleus is affected by the presence of bacteria. As can be seen from Fig. 3, RNA synthesis is stimulated very drastically, by a factor of approximately 5, in the macronuclei harbouring bacteria compared to the non-infected nucleus. Each of the infected macronuclei contained approximately 100–250 bacteria of the reproductive form and approximately 20–30 of the infectious form.

To test whether the increase in the \(^{3}H\)UMP accumulation in the infected macronuclei is due to RNA synthesis by the bacteria, the host nucleus or both, macronuclei were homogenized after 30 min incubation in an in vitro transcription cocktail containing nucleoside triphosphates. Macronuclear fragments including the chromatin were then separated from the bacteria by centrifugation. As one can see from Table 1, radioactivity was associated solely with the macronuclear chromatin, whereas the endonuclear bacteria were not labelled. This result indicates that the increase in RNA synthesis in the isolated infected macronuclei is due to an increase in the RNA polymerase activity of Paramecium.

Proteinase activity in infected and non-infected macronuclei

Endonuclear symbionts infecting the micro- and macronucleus of ciliates cannot affect only the shape and size of the host nucleus, but in addition can cause changes in the fine structure of the chromatin (see Görtz, 1983). This finding leads to the possibility that the bacteria may digest the host chromatin, or structural elements of the macronucleus like microtubules or microfilaments. For that reason it seemed promising to compare the proteinase activities associated with infected and non-infected isolated macronuclei.

To measure proteinase activities equal numbers of macronuclei were incubated
Fig. 4. Time-dependent hydrolysis of Azocoll after addition of $1.5 \times 10^5$ non-infected (○) or infected (△) macronuclei per test. Tests were carried out as described in Materials and Methods. Each value represents the average of two independent experiments.

When the peptide bonds of the collagen are hydrolysed by proteinases present in the macronuclear preparations, the dye is released into the suspending buffer and its absorbance at 520 nm can be measured. As can be seen from Fig. 4, the amount of the dye released into the suspending buffer during the incubation is linear as a function of time for both types of macronuclei, but is significantly higher in the case of symbiont-bearing macronuclei. This observation indicates that the infection of the macronucleus is correlated with a drastic increase in the proteinase activity associated with this isolated organelle.

**Discussion**

The macronucleus of *P. caudatum* can be regarded as a microenvironment for the cytobiotic bacterium *H. obtusa*. The mass isolation of infected macronuclei, and
Hoospora-infected macronuclei in Paramecium

their separation from food bacteria, provide the opportunity to study in vitro some of the specific interactions of the endonuclear bacteria with their eukaryotic host. The procedure for the isolation of infected macronuclei described here yields, after a few stages of centrifugation, macronuclei that are free from cytoplasmic contaminants and from the food bacteria that often cosediment with intact food vacuoles. In addition, macronuclei harbouring predominantly the long, infectious form of *H. obtusa* can be separated from those containing predominantly the short, reproductive form. Starting with isolated infected macronuclei the nucleus-specific bacteria can be isolated very easily without contamination by food bacteria or mitochondria.

To test whether the isolated macronuclei posses characteristics connected with the infection we first investigated whether differences between the infected and non-infected macronuclei could be detected in vitro. Both types of nuclei retain the ability to synthesize RNA in vitro in a way similar to other systems using isolated macronuclei of ciliates (Zaug & Cech, 1980; Eckert & Kaffenberger, 1980; Kaffenberger & Eckert, 1980; Freiburg, 1981). The finding that [³H]UMP incorporated into RNA is exclusively associated with the host chromatin and not with the endonuclear bacteria clearly shows that the isolated macronuclei respond to the bacterial infection in the in vitro transcription system with an increase in RNA synthesis. Further experiments are required to elucidate whether this increase in RNA synthesis found in vitro is the consequence of a degradation of the nuclear RNA by the bacteria in vitro, or whether it reflects a drastic stimulation of the physiological activity of the *Paramecium* as a response to the invading bacteria.

Microscopical observations have suggested that endonuclear bacteria may digest the host chromatin (see Görtz, 1983). This speculation is supported by the finding that infected macronuclei express a significantly higher proteinase activity; but it cannot yet be decided whether the proteinases have been produced by the bacteria, or by the nuclei in an attempt of the host to eliminate the invaded bacteria. It is found that *Paramecium* can very rapidly lose all its endonuclear bacteria under certain circumstances (Görtz & Dieckmann; personal communication).

Several attempts to measure the RNA synthesis of the endonuclear bacteria have failed. The bacteria, whether purified or studied in isolated infected macronuclei, using different synthetic media and adding either nucleotides or nucleosides, have not been demonstrated to incorporate significant amounts of labelled precursors into RNA.

For this reason, and because the isolated infected macronuclei retain characteristic functional peculiarities that differentiate the infected from the non-infected state, they offer a promising system to analyse further the interactions between the cytophont *H. obtusa* and its host *P. caudatum*.

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REFERENCES


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