THE REDEVELOPMENT OF HETEROPOLAR DOUBLETS AND MONSTER CELLS OF *OXYTRICHA FALLAX* AFTER CYSTMENT

ROBERT L. HAMMERSMITH
Department of Zoology, Indiana University, Bloomington, Indiana 47401, U.S.A.

SUMMARY

Heteropolar doublets of the hypotrichous ciliate *Oxytricha fallax* can encyst, as can monster cells containing multiple copies of at least part of the ciliature. The resulting cysts excyst as heteropolar doublets or monster cells, respectively. Transmission electron microscopy of resting cysts derived from monster cells and from singlets, however, indicates a complete loss of the ciliature — all kinetosomes, ciliary axonemes, and microtubules. Thus, the determination of each ciliature of a monster cell cannot be attributed to the persistence of any visible part of the ciliature.

Transmission electron microscopy of monster and singlet cysts suggests that the information for a ciliature may be related to the system of cyst wall grooves and possibly to differentiations in the inner cell membrane which separates the cyst cytoplasm from the cyst wall.

The redevelopment of heteropolar doublets upon excystment indicates that the anterior-posterior polarity is independently determined for each ciliature and is maintained through cystment. This polarity is not, however, reflected in a recognizable anterior-posterior polarity of any visible structure of the resting cyst.

The number of adoral zones of membranelles (AZMs) may increase or decrease in some excysting monster cells compared with that observed in the encysting cells. Possible explanations for these changes are offered in the Discussion.

INTRODUCTION

Singlet resting cysts of the hypotrichous ciliate *Oxytricha fallax* lack all organelles composing the ciliature — all kinetosomes, ciliary axonemes, and microtubules (Grimes, 1973a). Thus, the cyst represents a stage in the life-cycle when the ciliature is completely ‘dedifferentiated’. Yet upon excystment, ‘redifferentiation’, or redevelopment, of the ciliature occurs within a few hours. Furthermore, cysts derived from singlets (cells containing one ciliature) excyst as singlets, and cysts derived from homopolar doublets (cells containing 2 ciliatures) excyst as homopolar doublets (Grimes, 1973b). Thus, the cyst contains some mechanism for ‘remembering’ how many ciliatures were present prior to encystment.

Several questions arise regarding the encystment and excystment of cells containing more than one ciliature. First, is the polarity of each ciliature independently determined during excystment, or is the same polarity imposed on all ciliatures developing in an individual cyst? Secondly, does the cyst or its parts possess any visible anterior-
MATERIAL AND METHODS

The cell line (2AI8k) used in this study was isolated from a subculture of a line of *Oxytricha fallax* irradiated by a 15-W G.E. germicidal ultraviolet lamp at a distance of 40 cm for 4 min. Cell line 2AI8k undergoes abortive divisions resulting in the formation of heteropolar doublets and monster cells.

Line 2AI8k was grown with excess food at 27 °C in small Petri dishes for several days during which the heteropolar doublets and monster cells were produced. Desired cells were washed through 3 changes of Osterhout solution (Osterhout, 1906) and left in a fourth change to starve, which induces encystment within 4-5 h. Precystic cells were isolated for further examination (see below). They are recognizable by the accumulation of groups of dark crystalline material in the posterior portion of the cell, the anterior portion appearing pale and non-refractile. The groups of crystalline material are eventually extruded, the entire cell then appearing very pale and non-refractile. Formation of the resting cyst occurs within approximately 1 h after extrusion of the crystalline material.

Precystic cells were individually compressed in a microcompressor until slightly flattened, and then observed with a × 40 phase-contrast microscope to ascertain the number of complete and incomplete adoral zones of membranelles (AZMs). After the number and relative positions of the AZMs were recorded, the cell was returned to Osterhout solution in a culture slide and allowed to complete encystment.

Resting cysts were stored 1 week in Osterhout solution and then, in order to induce encystment, placed in fresh cerophyl medium (Sonneborn, 1970) inoculated 12 h earlier with *Klebsiella pneumoniae*. Excysted cells were placed in the microcompressor and the number of AZMs (complete and incomplete) again ascertained.

Only those late precystic heteropolar doublets which were fused along their mid-dorsal surfaces except for a small separation at each end (generally greater at one end than at the other), and which contained two and only two complete ventral surfaces facing in opposite directions (Figs. 2A–C, p. 566), were selected for the experiment.

Resting cysts were individually fixed for transmission electron microscopy (TEM) in 1.5 % (v/v) biological grade glutaraldehyde in 0.05 M phosphate buffer (pH 7.4) for 1 h, washed in 4 changes of 0.1 M phosphate buffer (pH 7.4) for a total of 1 h, and postfixed 30 min in 1 % (w/v) osmium tetroxide in 0.05 M phosphate buffer (pH 7.4). Cysts were dehydrated in an ethanol series, transferred through 2 changes of propylene oxide, and impregnated with a mixture of equal parts of propylene oxide and Epon 812 for 1 h. Cysts were individually embedded in a thin layer of Epon in a polystyrene Petri dish. Serial sections, made with a diamond knife and stained with uranyl acetate and lead citrate (Reynolds, 1963), were examined in a Philips EM 300 operated at 60 kV. Each section was carefully observed at a final magnification of 31,300 times for kinetosomes, ciliary axonemes, and microtubules. Approximately one third of the sections were photographed and observed a second time at a final magnification of approximately 190,000 times.

Cells were fixed for scanning electron microscopy (SEM) in 2 % (v/v) formaldehyde (1 pt) and 2 % (w/v) osmium tetroxide (2 pt) for 15 min. The cells were washed in distilled water for 30 min to 1 h, then placed individually or in small groups into a brass microchamber (Hammersmith, unpublished), covered with a Nitex-brass lid, and dehydrated in an ethanol series. The microchamber was transferred through an ethanol-amyl acetate series to absolute amyl acetate, placed in a critical point drying apparatus and flushed with liquid carbon dioxide for approximately 25 min (Anderson, 1951). After drying, the microchamber was opened and the cell(s) transferred by a glass needle to a metal specimen stub. Specimens were coated with palladium-gold and observed on an Etec Autoscan Electron Microscope.
RESULTS

Cortical morphology of vegetative cells

The cortical morphology of *O. fallax* has been previously described (Grimes, 1972). However, since familiarity with morphological features is necessary in order to understand this paper, a brief review is provided.

*O. fallax* in a non-dividing state is approximately 80 μm long and 30 μm wide. The dorsal surface is convex and the ventral slightly concave.

The ventral ciliature (Fig. 1) includes an adoral zone of membranelles (azm) which extends from the mid-ventral region of the cell to the left and anteriorly around the pole of the cell. To the right of the AZM are 2 undulating membranes (um) which end posteriorly in the cytostome or mouth (not shown in Fig. 1). Four groups of cirri complete the ventral ciliature. These consist of 8 frontal cirri (*fr*–8), 4 ventral cirri (*vr*–4), 6 anal cirri (*ar*–6), and a variable number of marginal cirri (mc), a row of which extends lengthwise on both the left and right edges of the ventral surface.

On the dorsal surface are five or six longitudinally arranged kinetics (Grimes & Adler, 1976), also known as dorsal bristle rows.

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**Fig. 1.** Camera lucida drawing of the ventral ciliature of a non-dividing cell. *azm*, adoral zone of membranelles; *um*, undulating membranes; *fr*–8, frontal cirri 1–8; *vr*–4, ventral cirri 1–4; *ar*–6, anal cirri 1–6; *mc*, marginal cirri.
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Formation of heteropolar doublets and monster cells

Chains resulting from abortive cell divisions are the source of all heteropolar doublets and monster cells. In a chain, there are 2 complete sets of ventral and dorsal structures in a tandem arrangement (tandem doublet) (Fig. 3). As a result of further divisional morphogenesis, tandem doublets may form normal divisional products from either the anterior or posterior ends or both. Occasionally, however, both the anterior and posterior divisional products fail to separate, resulting in 4 sets of ventral and dorsal structures arranged in tandem. Such cells invariably twist and distort, eventually forming a monster cell.

Tandem doublets may also form heteropolar doublets (doublets in which the ventral surfaces have opposite polarities, as compared to homopolar doublets which have identical polarities for both ciliatures). This begins to occur when the posterior component of a tandem doublet bends upon the anterior component so that the dorsal surfaces are touching. A heteropolar doublet is formed when the fusion region between the 2 components increases to include most of their dorsal surfaces (Fig. 2A–C).

Heteropolar doublets, like tandem doublets, may initiate divisional morphogenesis. However, the fission furrow remains incomplete, resulting in the formation of a monster cell containing an increased number of ventral and dorsal structures. Further divisional morphogenesis results in larger monster cells containing greater numbers of AZMs (Fig. 4). Monster cells containing as many as nine AZMs have been found.

After or during the formation of a monster cell, incomplete development or degradation of cortical structures occurs. This results in incomplete AZMs, complete loss of some AZMs, and/or the loss of other cortical structures.

Comparison between singlet and monster cyst ultrastructure

The basic ultrastructure of the singlet resting cyst of _O. fallax_ has been previously described (Grimes, 1973c) and therefore is not presented here.

TEM examination of complete serial sections from 2 cysts derived from encysting monster cells (372 sections of a monster cyst derived from a cell containing 5 AZMs and 392 sections of a monster cyst derived from a cell containing 6 AZMs) and 2 singlet cysts (254 sections each) revealed no kinetosomes, ciliary axonemes, or microtubules. The basic ultrastructure of the cyst wall, the appearance of the cytoplasm and autophagic vacuoles, and the clustering of mitochondria were the
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same in both singlet and monster cysts. Likewise, the 2 unit membranes of the vegetative cell surface were retained intact in both singlet and monster cysts. (In the cyst, the inner membrane separates the cytoplasm from the cyst wall; the outer, the cyst wall from the environment.) However, several differences between cysts derived from singlets and monster cells were observed. These differences involve the multiplicity of the cyst wall grooves and the number of their convergent points, the number of micronuclei, and the number and shape of the macronuclei. Singlet cysts appeared to contain fewer cyst wall grooves and fewer convergent points of those grooves than did monster cysts. It was not, however, technically possible to correlate the multiplicity of the grooves or the number of convergent points with the number of AZMs present in the precystic cell. Singlet cysts also contain one or two micronuclei, and one oval, centrally located macronucleus. Both monster cysts contained numerous micronuclei. One monster cyst contained a single convoluted macronucleus while the other had 2 juxtaposed convoluted macronuclei.

Comparison of the number of AZMs before encystment with the number after excystment

Cysts derived from monster cells excyst as monster cells (Figs. 5–8). However, the number of AZMs is changed in approximately one half of the excysted cells. Usually (10 of 23 cases) the number of AZMs decreases; however, occasionally (3 of 23 cases) the number of AZMs increases (Table 1). In 6 of 10 excysted monster cells with a reduced number of AZMs, more sets of marginal cirral rows and groups of anal cirri were found than are normally associated with the number of AZMs present. This indicates that only a partial reduction of some ventral ciliature(s) had occurred.

Table 2 presents data on homopolar doublets, heteropolar doublets, and singlets. Two of 23 homopolar doublets compressed during encystment also showed a reduced number of AZMs upon excystment. However, non-compressed homopolar doublets showed no reduction in the number of AZMs after cystment, even though the sample contained as many as 7 times the number of homopolar doublets as was observed in the compressed sample. This result is independent of whether the non-compressed homopolar doublets were derived from the 2A18k line or a non-irradiated cell line. Cysts derived from structurally normal singlets always excysted as singlets.

Fig. 5A, B. Phase micrographs of a precystic monster cell which possesses 3 AZMs (arrows). × 580.

Fig. 6. Phase micrograph of an excysting monster cell. This is the same monster cell as that shown prior to encystment in Fig. 5A, B. Note the presence of 3 AZMs (arrows) after excystment in approximately the same relative positions as observed in the cell prior to encystment. × 580.

Fig. 7A, phase micrograph of a precystic monster cell containing 5 AZMs (arrows a–e mark the AZMs). Those labelled b and e are not in focus. × 580. B, C, phase micrographs focused on AZMs labelled b and e respectively, in Fig. 7A. × 580.

Fig. 8A, B. Phase micrographs of an excysting monster cell, the same monster cell as is shown prior to encystment in Fig. 7A–C. Note that there are 5 AZMs present after excystment (arrows a–e). The letters assigned to the AZMs do not necessarily correspond to those in Fig. 7A–C. × 580.
Thus far, 10 heteropolar doublets meeting the required criteria have been encysted. However, only 8 of these cysts have been successfully excysted (Table 2). In all 8 cases, the excysting cell possessed 2 complete ventral ciliatures in a heteropolar arrangement.

Table 1. Number of AZMs per monster before encystment

<table>
<thead>
<tr>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of obs.</td>
<td>4</td>
<td>14</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Monster cells with identical nos. of AZMs after excystment</td>
<td>1</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Monster cells with fewer AZMs after excystment Reduced to:</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2 (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monster cells with more AZMs after excystment Increased to:</td>
<td>2</td>
<td>1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3 (2)</td>
<td>4</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

( ) the number of cases.

Table 2. Data on homopolar doublets, heteropolar doublets, and singlets

<table>
<thead>
<tr>
<th>Normal homopolar doublet (compressed)</th>
<th>Singlet (compressed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. encysted as dbl.</td>
<td>29</td>
</tr>
<tr>
<td>No. excysted as sing.</td>
<td>21</td>
</tr>
<tr>
<td>No. excysted as sing.</td>
<td>2</td>
</tr>
<tr>
<td>Non-viable</td>
<td>6</td>
</tr>
<tr>
<td>No. encysted as sing.</td>
<td>30</td>
</tr>
<tr>
<td>No. excysted as sing.</td>
<td>27</td>
</tr>
<tr>
<td>Non-viable</td>
<td>3</td>
</tr>
<tr>
<td>Normal homopolar doublet (not compressed)</td>
<td>Singlet (not compressed)</td>
</tr>
<tr>
<td>No. encysted as dbl.</td>
<td>154</td>
</tr>
<tr>
<td>No. excysted as sing.</td>
<td>148</td>
</tr>
<tr>
<td>Non-viable</td>
<td>6</td>
</tr>
<tr>
<td>No. encysted as sing.</td>
<td>110</td>
</tr>
<tr>
<td>No. excysted as sing.</td>
<td>104</td>
</tr>
<tr>
<td>Non-viable</td>
<td>6</td>
</tr>
<tr>
<td>2A18k homopolar doublet (not compressed)</td>
<td>2A18k heteropolar doublet</td>
</tr>
<tr>
<td>No. encysted as dbl.</td>
<td>68</td>
</tr>
<tr>
<td>No. excysted as sing.</td>
<td>66</td>
</tr>
<tr>
<td>Non-viable</td>
<td>2</td>
</tr>
<tr>
<td>No. encysted as sing.</td>
<td>8</td>
</tr>
<tr>
<td>No. excysted as heter. dbl.</td>
<td>8</td>
</tr>
<tr>
<td>Non-viable</td>
<td>2</td>
</tr>
</tbody>
</table>

DISCUSSION

Redevelopment of the monster phenotype and multiple AZMs occurs upon excystment of cysts derived from monsters, even though transmission electron microscopy of such cysts indicates a complete loss of all kinetosomes, ciliary axonemes, and microtubules. This suggests that determinative factor(s) for a ciliature are not attributable to the persistence of any visible part of the ciliature. In addition, the number of ciliatures that redevelop upon excystment of a monster cyst cannot be attributed to the nuclear genotype, since structurally normal singlet cells derived from monster cells and thus having the same genotype always encyst and excyst...
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as singlets. Thus, the redevelopment of the monster phenotype serves as a dramatic example of the phenomena described, and the conclusion arrived at, by Grimes (1973b) for the excystment of homopolar doublets. Based on the results from cutting experiments, Grimes (1973b) concluded that neither the quantity of the macro-nuclear material nor cell size determines the number of ciliatures in the excysting cell. He further concluded that the basis for the number of ciliatures was determined by some localized cytoplasmic differentiation which acts as a determinative region.

A comparable region of determination is associated with the ventral surface of Urostyla grandis, which Jerka-Dziadosz (1964) designates as the 'presumptive organization area'.

If the determinative factor(s) for a new ciliature are associated with the cell's ventral surface, then what physical basis might be involved in the retention of this information in the cyst? One conspicuous feature which is correlated with singlet and monster cysts is the multiplicity of the cyst wall grooves and their convergent points. The cyst wall grooves were observed with the electron microscope by Grimes (1973c) and identified with what Hashimoto (1964) had previously designated on the basis of optical microscopy as a 'fibrillar system'. Hashimoto reported that the position of one of the two convergent points found in singlet cysts corresponds to the anteriormost part of the oral primordium site in the excysting cell. Furthermore, this convergent point is also positioned on or near the site previously held by the posterior extremity of the original AZM in the encysting cell. Thus, a precise positional relationship between the pre- and postcystment AZMs exists and is apparently related to the organization of the cyst wall grooves. However, since the 2 vegetative cell surface membranes are apparently retained intact within the cyst, the system of cyst wall grooves and especially the sites of the convergence of these grooves might more plausibly be attributed to membrane differentiations, specifically to differentiations of the inner surface membrane which separates the cyst cytoplasm from the cyst wall grooves. This possibility is especially attractive in view of the mounting evidence implicating membrane differentiations in other developmental phenomena - e.g. trichocyst/mucocyst placement sites (Janish, 1972; Satir, Schooley & Satir, 1972a, 1973), cilia (Satir, Schooley & Satir, 1972b; Sattler & Stachelin, 1972), and cell junctions (Gilula & Satir, 1971; Kreutziger, 1968). Freeze-fracture studies of the inner surface membrane of singlet and monster cysts might provide information in this regard.

Whatever factor(s) may determine a ciliature, they or their final expression, however, are not unalterable, since the number of AZMs can increase or decrease during encystment of some monster cysts.

In approximately half of the excysted monster cells the number of AZMs was reduced. This reduction could be the result of one or more of the following: (a) an actual loss or disturbance before or during encystment of some determinative factor(s) necessary for the formation of a new ciliature upon excystment; (b) blockage in the initiation of a new ciliature during excystment; and/or (c) initiation of a new ciliature during excystment and then either an incomplete development or a partial resorption of that ciliature. The last possibility presumably can occur since 6 of 10 excysted
monster cells with a reduced number of AZMs also exhibit more sets of marginal cirral rows and group(s) of anal cirri than would normally be associated with the number of AZMs present. These supernumerary groups of cirri presumably arise during encystment in connexion with the formation of a new ciliature, since cirri of the ventral surface are thought to develop from the same primordium that forms the AZM. It should also be noted that 2 of 23 compressed homopolar doublets were reduced to singlets upon encystment. This presumably could occur if the compression caused a partial cell lysis which disturbed and/or caused a loss of some determinative factor(s) necessary for the formation of a new ciliature in the encysting cell. Partial cell lysis sometimes occurs with the use of a microcompressor. In most monster cells many of the separate ciliatures are positioned in very close proximity to one another. Such abnormally close positions of the ciliatures during encystment may result in interactions between the ciliatures or have inhibitory effects upon factor(s) which determine the formation of a new ciliature. Either of these 2 effects could result in a reduced number of AZMs after encystment.

The observation that a few monster cells contained more AZMs after encystment than prior to encystment suggests 3 (not mutually exclusive) possibilities. (1) An AZM consisting of only a few membranelles may have been overlooked during the examination of precystic cells, although this seems unlikely. (2) Some determinative factor(s) previously associated with 1 ciliature prior to encystment may give rise to 2 ciliatures during encystment. (3) Some determinative factor(s) necessary for the formation of a new ciliature in the encysting cell may have been present prior to encystment although lacking an associated AZM and possibly the complete ciliature. The latter possibility is supported by the observations that the capacity to form a new ciliature persists even after surgical removal of the AZM from precystic or morphostatic cells (Grimes, 1973b; Jerka-Dziadosz, 1964, 1972, 1974).

The redevelopment of heteropolar doublets upon encystment indicates that the anterior-posterior polarity is independently determined for each ciliature and is maintained through encystment. This polarity is not, however, reflected in a recognizable anterior-posterior polarity of any visible structure of the resting cyst.

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