CILIA REGENERATION IN TETRAHYMENA AND ITS INHIBITION BY COLCHICINE

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ABSTRACT

The cilia of Tetrahymena were amputated by the use of a procedure in which the cells remained viable and regenerated cilia. Deciliated cells were nonmotile, and cilia regeneration was assessed by scoring the percentage of motile cells at intervals following deciliation. After a 30-min lag, the deciliated cells rapidly recovered motility until more than 90% of the cells were motile at 70 min after amputation. Cycloheximide inhibited both protein synthesis and cilia regeneration. This indicated that cilia formation in Tetrahymena was dependent on protein synthesis after amputation. Conversely, colchicine was found to inhibit cilia regeneration without affecting either RNA or protein synthesis. This observation suggested the action of colchicine to be an interference with the assembly of ciliary subunit proteins. The finding that colchicine binds to microtubule protein subunits isolated from cilia and flagella (13) supports this possibility. The potential of the colchicine-blocked cilia-regenerating system in Tetrahymena for studying the assembly of microtubule protein subunits during cilia formation and for isolating ciliary precursor proteins is discussed.

INTRODUCTION

The elongation of ciliary buds in cultured fibroblasts is inhibited by the colchicine derivative colcemid (16). It is possible that this drug inhibits ciliogenesis by binding to the ciliary microtubule precursors, thus preventing their assembly. This suggestion is reinforced by the finding that colchicine binds in vitro to subunit proteins isolated from ciliary and flagellar microtubules (13), and that it causes the disruption of microtubules of other organelles in vivo by a mechanism which also seems dependent on an affinity for microtubule subunits (1, 2, 7, 17-19).

Systems of regenerating cilia and flagella in the protozoans have been shown to offer many advantages for the investigation of various aspects of the synthesis and assembly of ciliary and flagellar microtubules (3, 11, 12). It was of interest to determine the effect of colchicine on the regeneration of cilia in the protozoan Tetrahymena. If inhibition of cilia regeneration in the presence of colchicine occurred, and if this inhibition could be shown to result from the binding of colchicine to ciliary microtubule precursor proteins, the use of tritiated colchicine (1) in this system would permit the isolation of ciliary microtubule subunits from regenerating cells. One could then proceed with the biochemical characterization, investigation of turnover rates, and the study of the in vitro assembly of these precursor proteins.

The present report will show that both colchicine and the protein synthesis inhibitor, cycloheximide, inhibit the regeneration of Tetrahymena cilia, but that only colchicine has this effect without altering either protein or RNA synthesis. These results are consistent with the hypothesis that colchicine is interfering with cilia elongation in Tetrahymena by preventing the assembly of ciliary microtubule subunits.

1 Abbreviations: RNA, ribonucleic acid; EDTA-2 Na, disodium salt of ethylenediamine tetraacetic acid; TCA, trichloroacetic acid.
FIGURE 1 Cilia amputation in Tetrahymena pyriformis (W). Fig. 1 a. A typical ciliated cell before amputation or after complete cilia regeneration; Fig. 1 b. A completely deciliated cell characteristic of most cells after amputation and before regeneration. Fig. 1 c. Cell after amputation, with oral cilia still attached but immotile. These cilia will drop off before regeneration. Cells were fixed for 3 days in 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.0) at 4°C. Photomicrography with Zeiss 40 X Apo oil (phase) objective with mercury lamp and dark-field illumination. (Zeiss Ultra condenser, Carl Zeiss, Inc., New York, N.Y.). X 700.

MATERIALS AND METHODS

Culture Conditions

Tetrahymena pyriformis (W) was grown in defined medium (6). 125 ml of medium in a 1000 ml Roux flask were inoculated with 2.5 ml of a 2-day-old culture, and the organisms were allowed to grow for 2 days at 25°C. The cell density at this time was 5-7 X 10^5 cells/ml.

Cilia Amputation Procedure

The cells were harvested at room temperature (23°C) by centrifugation at 160 g for 3 min, and were concentrated and resuspended in their original growth medium (final concentration, 2 X 10^6 cells/ml). The concentrated cells were used immediately for the deciliation procedure which was carried out at 2°C. At zero time, 2.5 ml of the concentrated cells were added to 5.0 ml of medium A (10 mM EDTA-2 Na; 50 mM sodium acetate, pH 6.0) in a 50 ml conical centrifuge tube and mixed by swirling. At 30 sec, 2.5 ml of cold distilled water was added, followed by the addition at 90 sec of 0.25 ml of 0.2 M CaCl2. After the addition of calcium chloride, the suspension was mixed by inverting the tube several times. At 3 min and 30 sec, the suspension of cells was subjected to 2-4 shearings with a 10 ml glass syringe fitted with an 18 gauge needle. Immediately after deciliation (about 4 min), one volume of the suspension was pipetted into 20 volumes of recovery solution (fresh defined growth medium or 0.01 M potassium phosphate buffer, pH 7.0) at 25°C. The deciliated cells were then incubated at 25°C with gentle agitation, and cilia regeneration was assayed as described below.

Assay for Cilia Regeneration

Since deciliated cells are nonmotile, regeneration could be assessed by determining the percent of motile cells at various times after the amputation procedure. A 0.8 X 32 mm micro-hematocrit tube (Scientific Products Co., Evanston, Ill.) was filled by capillarity with the incubated cell suspension. The percent of moving cells was scored by placing the tube under heavy mineral oil, and observing the cells in the tube with a binocular dissecting microscope (40 X magnification). Only those cells with definite movements were scored as motile. At least 150 cells were observed in each sample.

Determination of In Vivo RNA and Protein Synthesis

The in vivo incorporation of 14C-amino acids and 14C-uridine into total Tetrahymena protein and RNA, respectively, was assayed by the filter disc method (8).

Biochemicals

Cycloheximide (Acti-dione) was obtained from The Upjohn Co., Kalamazoo, Mich., and from Nutritional Biochemical Corporation, Cleveland, Ohio. Colchicine was also obtained from Nutritional Biochemicals. Most of the work in this report was carried out with one lot number of colchicine. However, different lot numbers were shown to have somewhat varying potencies for the inhibition of cilia regeneration. It was necessary, therefore, to run a new dose-response curve for the inhibition of cilia regeneration for each new lot of colchicine. Colcemid was
RESULTS

The Cilia-regenerating System

The deciliation procedure: The continuity of the ciliary membrane with the cell membrane in Tetrahymena creates difficulties in designing a procedure for the removal of cilia which maintains both the viability of the cells and their ability to regenerate cilia. Detachment of all cilia usually causes lysis or, in nonlysing cells, failure to regenerate new cilia. These problems are circumvented by modifying the cilia isolation procedure of Watson et al. (20), omitting ethanol from the deciliation solution, lowering the pH, and adding a mechanical shearing step to remove immobilized cilia. This modified procedure results in deciliated cells which, although fragile, maintain their normal pyriform shape and regenerate cilia (Fig. 1a–c).

Phase microscope observations of deciliated cells indicate that the majority of the cells have lost all of their somatic and oral cilia (Fig. 1b). A few cells usually retain some oral cilia after the amputation procedure (Fig. 1c), but these cilia drop off after the cells have been in the recovery solution for about 10 min. Electron microscope observations of cells after cilia amputation and during regenera-
Figure 3 Effect of colchicine and colcemid concentration on cilia regeneration in *Tetrahymena*. Recovery of motility was assayed at 90-min postamputation. Circles, colcemid; triangles, colchicine.

Cilia regeneration: In Fig. 2 it can be seen that the first cells have recovered their motility by 30–35 min after amputation; this recovery is coincident with the appearance of ciliary shafts. The population then rapidly regains motility until 60–70 min after deciliation when more than 90% of the cells are motile. Observations of single cells during the recovery period indicate that the oral cilia grow out first and elongate synchronously, while the somatic cilia grow out later and elongate asynchronously over the cell surface.

Since the cells are motile when only part of their ciliary complement is regenerated, an assay based on the percentage of cells moving at any time is not completely representative of either the number or the length of the cilia on each organism. Phase and electron microscopic observations show this discrepancy. While the full complement of mature cilia is not present until almost 100 min after amputation, the assay shows the population to be fully motile by 60–70 min postamputation. However, the kinetics of recovery are reproducible and afford a quick and convenient method for assessing the over-all cilia formation in a population.

The Effect of Colchicine on Cilia Regeneration

Inhibition of elongation: The recovery of deciliated cells is completely inhibited when 4 mg/ml colchicine or 0.5 mg/ml colcemid is included in the recovery medium (Fig. 3). This inhibition of recovery is not due to an inhibition of motility since phase microscope observations indicate the absence of ciliary shaft regeneration in the presence of colchicine or colcemid.

The concentration of colchicine required for the inhibition of cilia regeneration is high but is found not to immobilize normal ciliated cells. Furthermore, this concentration has no observable effect on either RNA or protein synthesis in

**Figure 4** Effect of colchicine on uridine-\(^{14}\)C incorporation into *Tetrahymena* RNA. Uridine-\(^{2}\)\(^{14}\)C (specific activity, 48 mc/mole; concentration, 5 \(\mu\)C/ml cells) was added at zero time and the colchicine (4 \(\mu\)g/ml) at 20 min (arrow). Circles, control; triangles, colchicine.

*Tetrahymena* during the time course of the experiment (Figs. 4 and 5), or on the oxygen consumption of control and colchicine-treated cells. There is no effect on the recovery of motility when a concentration of sucrose, equimolar to the colchicine concentration, is added to the recovery solution. Deciliated cells maintain their normal pyriform shape and the contractile vacuole functions in the presence of colchicine.

Colchicine acts rapidly in inhibiting cilia regeneration. Addition of the drug to the recovery solution when cells are just beginning to recover their motility (40 min postamputation) completely inhibits recovery within 10 min (Fig. 6).

**Recovery from Colchicine Inhibition:** Fig. 7 shows the recovery kinetics of deciliated cells which have been diluted into fresh recovery medium after varying lengths of time in 4 \(\mu\)g/ml colchicine. After the removal of colchicine, motility is regained by normal recovery kinetics following the usual 30–40 min lag; this recovery is independent of the length of time (at least until 250 min) in colchicine.

In the presence of 4 \(\mu\)g/ml colchicine, deciliated cells will remain without cilia for about 3–5 hr and then begin recovery in the continued presence of colchicine. The cells will eventually regain full motility, although the recovery kinetics are slower than normal. Higher concentrations of colchicine (8–12 \(\mu\)g/ml) will extend the duration of the inhibitory period. Many vacuoles with yellow crystals can be observed in the cells coincident with the recovery. If cells recovering from the colchicine treatment are placed in fresh colchicine, they will continue to recover. This recovery occurs even if the recovering cells are redeciliated and placed in fresh colchicine. Thus, the cells may not only be able to concentrate the colchicine in vacuoles, but they may also become resistant to colchicine after exposure to it. Studies on the up-
take of colchicine-$^3$H by *Tetrahymena* have helped to illuminate this problem. The results indicate that at about the same time the cells are overcoming colchicine inhibition, the concentration of colchicine-$^3$H which has been taken up begins to decrease. It is relevant to point out here that no such spontaneous recovery from colchicine inhibition of elongation occurs when similar experiments are carried out with the flagella-regenerating system in *Chlamydomonas*; the cells remain without flagella as long as colchicine is present. Therefore, the overcoming of colchicine inhibition in *Tetrahymena* is probably a unique phenomenon related to the feeding and excretory mechanisms of this organism and will require further studies with colchicine-$^3$H.

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*The Effect of Cycloheximide on Cilia Regeneration*

Since colchicine inhibited cilia regeneration without affecting protein synthesis, it was of interest to determine the effect of an inhibitor of protein synthesis on cilia regeneration. In Fig. 8 it can be seen that 10 µg/ml cycloheximide, a concentration sufficient to inhibit amino acid incorporation into TCA precipitable protein of *Tetrahymena* (Fig. 9), prevented deciliated cells from recovering motility in the phosphate buffer recovery solution.

**DISCUSSION**

The cilia-regenerating system described in this report offers several advantages for investigating problems concerned with the synthesis and assembly of ciliary proteins: (a) *Tetrahymena* can be
grown in large quantities in a completely defined medium (6), (b) its division can be easily synchronized (9), and (c) methods are available for isolating the cilia (20) and fractionating them into component parts (4).

Results obtained with the use of this system indicate cilia regeneration to be dependent on protein synthesized after amputation. Thus, cycloheximide inhibits both protein synthesis and cilia regeneration. This result does not permit us to make an unequivocal statement about the amount of ciliary precursor pool in the cells, since the assay for cilia regeneration only measures the recovery of motility of deciliated cells. Therefore, a small amount of ciliary growth, insufficient to impart motility to the cycloheximide-inhibited cells, would not be apparent. Although phase and dark field microscope observation of cycloheximide-inhibited cells shows the absence of ciliary stubs, a more accurate assessment of the treated cells could be made with the use of the Protargol staining procedure for ciliated protozoa (21) and with electron microscopic observations. Studies on flagellar regeneration in Chlamydomonas have suggested the existence of a flagellar precursor pool which varies in amount in different mutant strains.3

Colchicine inhibits cilia regeneration apparently without affecting RNA or protein synthesis, and it is suggested that colchicine acts in this system by interfering with the assembly of ciliary microtubule subunits. This possibility is strengthened by studies on the mode of action of colchicine carried out in Taylor's laboratory (1, 2, 13, 17). He found that a concentration of colchicine sufficient to inhibit mitosis had little effect on RNA and protein synthesis and, by analyzing the kinetics of colchicine-3H uptake and binding, he was able to hypothesize that colchicine was blocking mitosis by binding to the subunits composing the mitotic tubules (17). This hypothesis was subsequently verified by the demonstration that colchicine will bind quite specifically to microtubule subunits isolated from various sources, including the mitotic spindle and flagellar axonemes (1, 2, 13).

The results presented on the reversibility of colchicine inhibition show that even after prolonged blocking of regeneration with colchicine, the rate of recovery of motility in fresh medium is

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**Figure 6** Effect of time of addition of colchicine on cilia regeneration in *Tetrahymena*. Colchicine (4 mg/ml) was added at zero time (open squares), 10 min (open circles), 20 min (closed triangles), 30 min (open triangles) and 40 min (closed squares); closed circles, control.
FIGURE 7 Recovery of motility in deciliated cells after removal of colchicine. Colchicine (4 mg/ml) was added at zero time and the cells were removed from colchicine at 20 min (open triangles), 30 min (open circles) and 50 min (closed triangles); closed circles, control.

If the cells are synthesizing ciliary protein during the block, and if the amount of precursor is the rate-limiting step in recovery of motility, the rate should be faster and/or the lag period shorter. The fact that the rate is not faster after release from colchicine inhibition suggests that the assembly step in ciliary elongation may be rate-limiting. That this is probably so has been shown in experiments (to be reported) with the Chlamydomonas flagella-regenerating system. These results indicate that the amount of flagellar elongation in cycloheximide but not the rate can be increased by treatment of deflagellated cells with colchicine prior to washing and placing the cells in cycloheximide.

A precautionary note should be added to the interpretation of the inhibitor results presented in this report. First, although the data show that cycloheximide will almost totally inhibit amino acid incorporation into the TCA-precipitable protein of Tetrahymena, they do not exclude the possibility that cycloheximide is inhibiting the synthesis of some nonciliary protein required for regeneration. However, a forthcoming paper reporting results obtained with the Chlamydomonas flagella-regenerating system treats this topic more thoroughly and indicates that this inhibition of nonciliary protein synthesis probably is not the case. Second, although the amino acid incorporation data indicate that colchicine is not blocking protein synthesis, an inhibition of about 1% would not be apparent with the accuracy of the procedure. This amount could be critical since the cilia comprise about 1% of the total protein, and it is conceivable that colchicine specifically inhibits ciliary protein synthesis. Some of the experiments described below will be necessary to define this latter problem more thoroughly.

The colchicine-3H binding assay for microtubule subunit proteins described by Borisy and Taylor (1) provides a method for directly determining whether or not the colchicine inhibi-
**Figure 8** Effect of cycloheximide on the incorporation of amino acid-\(^{14}\)C into TCA-precipitable protein of *Tetrahymena*. Amino acid mixture-\(^{14}\)C (concentration, 3 μCi/ml) was added at zero time and cycloheximide at 15 min (arrow). Closed circles, no cycloheximide; triangles, 1 μg/ml; open circles, 10 μg/ml.
FIGURE 9 Effect of cycloheximide concentration on cilia regeneration in *Tetrahymena*. Cycloheximide in varying concentrations was added to the cells at the time of amputation; the recovery of motility in phosphate buffer recovery medium was assessed at 90 min postamputation.

The isolation of cilia regeneration described in this report is caused by the binding of colchicine to the ciliary microtubule subunits. We are currently using this assay (a) to analyze the kinetics of colchicine-3H uptake and binding in *Tetrahymena*, (b) to determine if there is an increase in colchicine-binding protein in regenerating cells over that in non-regenerating cells, as is suggested by the cycloheximide inhibition studies, and (c) to isolate ciliary microtubule precursor proteins from colchicine-3H blocked cilia-regenerating systems.

There have been several studies on the chemistry of microtubules in recent years (2, 5, 10, 13-15), but most of these investigations have been carried out on subunits isolated from assembled tubules (see, however, reference 2). It would be important to isolate the precursor proteins before their time of assembly and to compare their biochemical characteristics with those of subunits isolated from assembled tubules. The isolation of these precursor proteins would permit one to attack such problems as the turnover rate of the ciliary microtubule precursors, the time of attachment of the guanine nucleotide to the subunits, and the possible role of the nucleotides in the polymerization of subunits (14).

Finally, observations on ultrastructural changes in *Tetrahymena* during (a) normal regeneration, (b) the period during colchicine inhibition when cells remain without cilia for a few hours, and (c) the period when cells are overcoming the colchicine inhibition, are feasible with this cilia-regenerating system. These problems are currently under investigation and offer a new approach for the study of ciliogenesis.

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