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Fluorescein-Conjugated Folate as an Indicator of Specific Folate Binding to *Paramecium*[1]

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ABSTRACT. Normal *Paramecium tetraurelia* cells stained with fluorescein-conjugated folate show intense fluorescence that can be reduced to near background autofluorescence with excess K_2-folate, but not with excess KCl. Mutant d4-534, which is not attracted to folate and does not specifically bind ^{3}H-folate, shows reduced fluorescence when stained. This method of monitoring specific folate binding to cells can be adapted to a microscale for rapid screening of clones since cells are routinely fixed and stained in microtiter wells.

PARAMECIA detect and are attracted to folate anion relative to chloride at pH 7 (5). The cells show a characteristic change in membrane potential in folate relative to chloride, as in other attractants (6). It is believed that this change in potential with resultant change in ciliary beating causes the cells to change swimming to accumulate in folate (6). It is likely that attractants bind to specific sites on the cell membrane as part of the mechanism by which the cells change potential. Specific binding of folate to the cells has been demonstrated with radiolabeled folate and correlated with chemoresponse through the use of mutants and an inhibitor of folate attraction (4). The ^{3}H-folate is useful,

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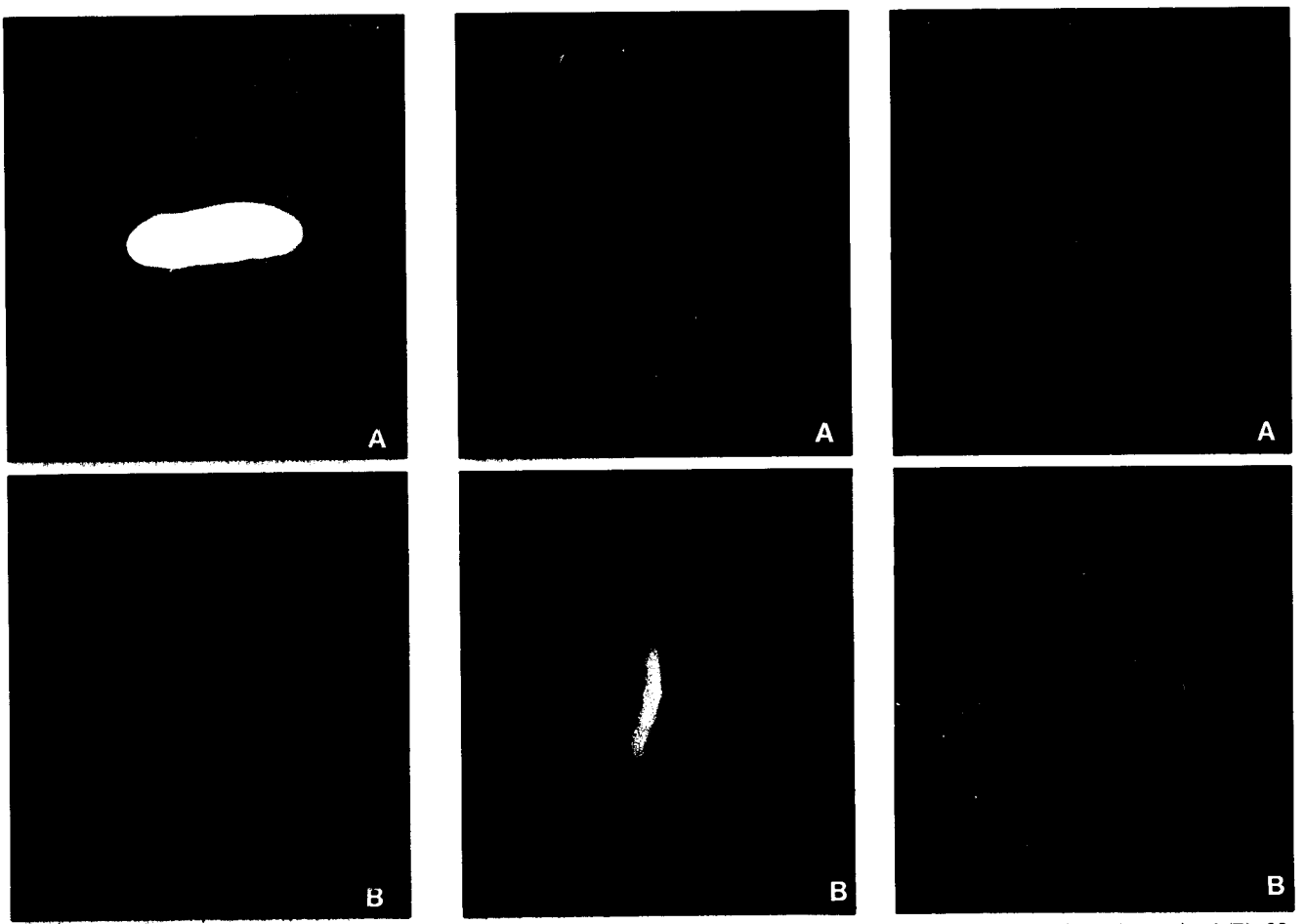

Fig. 1. Fluorescence micrographs of normal cells stained with a 100× dilution of fluorescein-conjugated folate (A) and unstained (B), 30 sec exposure. ×1400.

Fig. 2. Fluorescence micrographs of normal cells stained with 25× dilution of fluorescein-conjugated folate with 50 mM K_2-folate (A) and 50 mM KCl (B) as diluent, 30 sec exposure. ×1100.

Fig. 3. Fluorescence micrograph of mutant d4-534 cells stained with a 100× dilution of fluorescein-conjugated folate (A) and unstained (B), 30 sec exposure. ×1400. Print processing identical to that in Fig. 1. Cells fixed and stained at same time as cells in Fig. 1.

but cannot be used conveniently as a quick screen of clones of cells for binding capacity. For this purpose, a fluorescein-labeled folate was synthesized as described for the folate analog amethopterin (2) and a method of fixing and staining clones of cells developed. The resultant staining is specific for folate and can be used to distinguish mutant and normal cells by fluorescence microscopy.

MATERIALS AND METHODS

Cell culture. Cells used were *Paramecium tetraurelia* (formerly *P. aurelia* syngen 4) stock 51-s (sensitive to killer) or mutants derived from 51-s. Cells were grown in Cerophyl (rye grass) medium (7.5% Cerophyl extract with 1.1 g/liter Na_2HPO_4 and 0.2 g/liter NaH_2PO_4 buffering) with supplements of 0.34 g/liter proteose peptone and 0.8 g/liter Tris-HCl (tris(hydroxymethyl) aminomethane HCl). Cerophyl medium was inoculated with *Klebsiella pneumoniae* 24 h before use.

Dye synthesis and staining procedure. Fluorescein-conjugated folate was synthesized using a modified protocol of Gapski et al. (3) for the synthesis of FITC-amethopterin; 600 mg fluorescein isothiocyanate was reacted with 1.57 g 1,5-diaminopentane in 8 ml dimethyl sulfoxide (DMSO) for 8 h in the dark. The fluorescein-diaminopentane intermediate was separated on a DEAE cellulose column eluted with 0.3 M NH_4HCO_3 pH 7.8, lyophilized, dissolved in a minimal amount of 0.1 M NH_4OH, precipitated with HCl on ice, and redissolved in 20 ml DMSO; 441 mg folate and excess (1 g) 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were added and stirred in the dark for 1 h. Final product was separated by DEAE cellulose chromatography using a 0.1 M–0.5 M NH_4HCO_3 gradient. The eluent was lyophilized and redissolved in 0.1 M NH_4OH. A small amount of 99% glycerol was added to the solution, which was loaded on to preparative tube gels as described by Cooper (1). Gels were run at 3–4 mA/gel for 30 min and 6–8 mA/gel until the colored product had moved about ¾ through the gel. The gels were then removed and cut. The section of gel that was both yellow-orange and fluorescent was extracted with 0.1 M Tris-HCl and stored frozen. Absorbance spectra of the dye agree with that of Gapski et al. (3) and absorbance at 495 nm indicated that the stored dye was at a concentration of 0.2 mM (using the millimolar extinction coefficient in reference 3). Approximately 0.2 ml of cells in flat-bottom microtiter wells were kept at 4°C for 1 h before an equal volume of a 37% formaldehyde 0.5% NaCl solution was added to each well. The cells were incubated in formaldehyde for 24 h before the supernatant was gently aspirated with a pasteur pipet. The wells

TABLE I. *Blind test of fluorescence of normal and mutant cells.*

Slide #	Strain	Ave. score[a]	SD
1	Normal (51-s)	9.8	0.4
4		7.8	0.8
6		9.2	1.2
9		6.3	1.5
11		8.2[b]	1.5
15		10	0
3	Mutant d4-534	2.2	1.5
7		3.5	0.6
8		2.2	1.2
12		6.7[b]	1.4
2	Autofluorescence	0.8	0.4
5	(51-s)	1.0	0.6
10		2.5	3.3
14		1.4	1.0

[a] Normal and mutant average scores are significantly different by Mann-Whitney *U* test, as are normal compared to autofluorescence scores. Mutant and normal autofluorescences are not significantly different (data not shown).

n = six scorers who had not previously seen the slides or cells or who did not work directly with the fluorescence project. Slides are from two independent preparations of cells of all three strains or conditions.

[b] Dye at 50× dilution instead of usual 100×.

TABLE II. *Blind tests using microtiter plates.*

Well #	Strain	Ave. score[a]
1	Normal	6.0
2		5.6
4		5.4
6		6.5
7		6.8
5	Mutant	2.6
8		1.6
9		2.6
11		2.2
3	Autofluorescence	1.0
10	(normal)	1.6

[a] Normal and mutant average scores are significantly different by Mann-Whitney *U* test. Score is average from six scorers. (See legend, Table I.)

were washed with buffer and aspirated twice. Dye at indicated dilutions with buffer (1 mM Tris, 1 mM $Ca(OH)_2$, 1 mM citric acid, pH 7) was then added to each well. Usually 25×, 50×, and 100× standard dilutions were used. After 10 min, the dye was aspirated and the wells washed twice with buffer. Cells with no dye were routinely fixed and washed for a measure of autofluorescence.

Fluorescence microscopy. Samples of stained cells were removed from microtiter wells with pasteur pipets to glass slides for examination with Zeiss Ultraphot with epifluorescence (495 excitation and 510 nm emission filters). Either 25× oil immersion or 10× dry objectives were used. Fluorescence was documented with Ektachrome 400 ASA slide film with 30–60 sec exposure. In all cases cells were photographed without prior selection in order not to prejudice the results. Cells being compared within each figure were fixed, stained, and photographed on the same day. Figures represent two different preparations. In an alternative method, a stereo microscope was fitted with 500 nm short pass and 505 nm long pass excitation and emission filters (Corion). Wells were observed intact under 10–30× total magnification.

RESULTS AND DISCUSSION

Normal *Paramecium tetraurelia* cells were fixed to microtiter wells with formaldehyde, washed, and stained with dilutions of fluorescein-conjugated folate. Normal cells observed with the Zeiss Ultraphot show intense fluorescence relative to unstained cells (Fig. 1) in dilutions of dye as high as 100× (2 μM by absorbance estimate of dye concentration). Staining was specific for folate even at 25× dilution (the lowest dilution used with the Ultraphot) as evidenced by the severe reduction of fluorescence of cells incubated with 50 mM K_2-folate instead of buffer without folate (Fig. 2a). Dye diluted with 50 mM KCl showed no decrease in fluorescence (Fig. 2b).

Mutant d4-534 is not attracted to folate (2) and does not bind folate in a specific, saturable manner (4). Mutant d4-534 shows little fluorescence after staining with 100× diluted fluorescein-folate (Fig. 3, compare to Fig. 1). The fluorescence from the mutant can be distinguished from fluorescence of normal cells in blind tests in which color slides of cells from two separate experiments were presented in random order and scorers were asked to rank fluorescence intensity on a scale of 0–10 (Table I). Unstained normal and mutant cells could not be distinguished (Table I, Figs. 1, 3). It was not always possible to distinguish mutant from normal cells at lower dilutions of the dye.

Cells photographed under 10× dry objective displayed differences between normal and mutants (data not shown); however, this difference could not always be discriminated reliably as determined by blind tests. Similar blind tests were conducted with entire wells of normal and d4-534 cells stained with undiluted dye and unstained cells as control. Scorers using a dissecting microscope with cut-off filters, again, could reliably distinguish mutant from normal cells (Table II). It is not clear why strains of cells in microtiter wells can be more easily distinguished than cells under a 10× dry objective under epifluorescence. It may be that under conditions of low light intensity with a 10× objective or under a dissecting microscope, an entire well with all its cells stained with undiluted dye presents a sample size and intensity just sufficient for the scorer to judge more accurately the level of fluorescence.

It is assumed that fluorescent folate is binding to the cross-linked surface rather than to internal sites. This assumption is based on the work with the folate-binding mutant, which has lost saturable folate surface binding (4). It would be unlikely that one mutation would so dramatically decrease internal fluorescent-folate binding to the many possible high affinity binding sites, such as dihydrofolate reductase, and yet not affect the growth or health of the mutant cells. Detergent-extracted normal and mutant cells are currently being examined for fluorescence to address more directly the question of dye localization.

Bleaching of cells can be detected during long exposure (>60 sec) during photography or microscopic observation; *N*-propyl gallate did not significantly diminish the bleaching. Consequently, exposures of cells for photography were made for 30 sec or less when possible and, within an experiment, always made for same exposure times in order to make comparisons between strains and conditions. Thirty seconds is now our standard exposure time for all fluorescence photography.

A peculiarity of the fluorescein-conjugated folate is that stained cells increase in fluorescence with time. Cells are more intensely fluorescent when stained and stored at 4°C in buffer without dye in the dark for several days prior to observation. The reason for this increase is not yet known, but may be due to quenching by residual formaldehyde, which decreases as formaldehyde continues to evaporate from the well.

The specific staining of cells with fluorescein-conjugated folate will prove to be useful as a rapid indicator of folate binding for

selection of mutant clones or potentially for cell separation based on folate-binding capacity.

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