Retention of folate receptors on the cytoskeleton of *Dictyostelium* during development

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A revised folate binding assay was employed to demonstrate the presence of folate receptors on vegetative *Dictyostelium discoideum* amoebae. These receptors have a dissociation constant of 300 nM and are present at 45000 per cell. We found a pool of receptors attached to the cytoskeleton of vegetative amoebae and these receptors (10000 sites/cell, K_d 480 nM) remain associated with the cytoskeleton through the first 12 h of development. We discuss the possible roles for these retained receptors in later *D.discoideum* development.

Folate receptor *Cytoskeleton* *Deaminofolate* *Distribution-free plot* Dictyostelium

1. INTRODUCTION

Dictyostelium discoideum is widely used as a model system to study chemoattractant-initiated, receptor-mediated, transduction events leading to amoeboid cell motility. Receptors of folate, the vegetative chemoattractant, and cAMP, the attractant for aggregation, are known to be present on the *D.discoideum* cell surface (reviewed in [1]), and the binding characteristics of cAMP to cell surface receptors are well established [2–4]. More recently, workers have attempted to define the characteristics of folate binding in this model organism. Early receptor binding work [5,6] conducted at low temperatures in the absence of a folate deaminase inhibitor was complicated by the deamination of folate to DAFA [7]. This problem has since been circumvented by using non-degraded ligands such as methotrexate and aminopterin [8], (but see [9]), and most recently by inhibiting the deaminase with 8-aza-guanine [10–12]. The author in [10] reported the presence of polyspecific DAFA/folate receptors and specific folate receptors and suggested that the latter were likely to be the chemotactic receptors by which *D.discoideum* amoebae detect their bacterial food source during the vegetative phase of growth [13]. Chemotactic responsiveness to folate is lost in amoebae after 2–4 h of development [13] with a corresponding reduction in receptor number [5,14], but a chemokinetic response is retained through aggregation (7–8 h) [8,15]. Authors in [16] suggested a renewed role for folate later in development (12 h) as assessed by increased actin accumulation in the cytoskeleton after folate stimulation of amoebae that had been allowed to develop in shaker culture. We have repeated this experiment using amoebae developed on a solid substratum and found that the folate response corresponds with the appearance of tips in the tight aggregate stage (14 h) of development (unpublished). This finding has led us to restudy folate binding in *D.discoideum* amoebae as well as cytoskeletons. We report here data on amoebal binding inconsistent with those found in [10] and we additionally find that folate receptors, present at 20–30% of the concentration of the vegetative surface receptors, are associated with the

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Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; FA, folic acid; DAFA, 2-deamino-2-hydroxy folic acid; HPLC, high-pressure liquid chromatography

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cytoskeleton and are retained through 12 h of development.

2. MATERIALS AND METHODS

2.1. *Cell growth and development*

The wild-type strain *D.discoideum* NC4 was cultured on *Klebsiella aerogenes* OXF1 and synchronous development induced on a solid substratum by previously published methods [17,18].

2.2. *DAFA synthesis, characterization and purification*

DAFA was synthesized as in [19] and purity checked by paper chromatography [5], thin-layer chromatography (TLC) and HPLC [20]. HPLC chromatography was conducted on a Partisil 10/25 SAX Whatman column using an Altrex pump model 100A and an Altrex 165 variable wavelength detector, with a flow rate of 1 ml/min of the solvent 50 mM phosphate buffer (Na/Na_2) at pH 7.0. Preparative paper chromatography of folates was conducted on 0.38 mm thick, Whatman 3MM chromatography paper and eluted by centrifugation [21] after detecting the desired bands with long wavelength UV illumination. Folate derivatives were analyzed by UV spectroscopy on a Unicam SP1800 scanning spectrophotometer.

2.3. *Isolation of cytoskeletons*

Cytoskeletons were prepared by the addition of 1/10 volume of 10% Triton X-100, 10 mM EGTA in 20 mM phosphate buffer (pH 6.1) to cells suspended at 1×10^8/ml in 20 mM phosphate buffer containing 0.6 mM 8-aza-guanine [22]. Cell lysis was complete within 2 min at room temperature and the cytoskeletons were used immediately in the binding assay.

2.4. *Folate binding to amoebae*

[^{3}H]Folate binding assays on whole cells were conducted by adding 50 μl of cells at 1×10^8/ml in 20 mM phosphate buffer (K/Na_2, pH 6.1) to 80 μl reaction mixture containing, at final reaction concentrations, 0.5 mM 8-aza-guanine, 30–1500 nM [^{3}H]folate, and in early assays, 0.1 mM DAFA in 20 mM phosphate buffer (pH 6.1). Cells were incubated in this reaction mixture for 2 min, then 100 μl aliquots were layered onto 150 μl of 1:1, AR20:AR200 silicone oil (Wackerchemie) in 400-μl long-form Eppendorf tubes and centrifuged at 8000 × *g* for 30 s. Cell pellets were recovered for scintillation counting by clipping the tips of the tubes directly into counting vials and adding Unisolve I scintillant (Koch-Light). Non-specific binding was determined by isotopic dilution of [^{3}H]folate with 0.1 mM unlabeled folate. Whole cell binding to developed aggregates was conducted as above. The aggregates were removed from the millipore filters by direct vortex mixing of the filter in phosphate buffer. Cell concentration was adjusted by counting on a hemocytometer and confirmed by protein assay (Bio-Rad kit) [23] of duplicate 50 μl cell samples.

2.5. *Folate binding to cytoskeletons*

For [^{3}H]folate binding to cytoskeletons a modification of the method in [22] was followed: 200 μl cytoskeletons, prepared as stated above, were added to 40 μl [^{3}H]folate at 30–1500 nM and allowed to incubate for 2 min. Then, duplicate 100 μl cytoskeleton samples were layered onto 150 μl of 2.6:1, mineral oil (Sigma, heavy oil):silicone fluid 550 (Dow Corning) in 400 μl Eppendorf tubes and centrifuged at 8000 × *g* for 1 min. For non-specific binding controls, the cytoskeleton preparative solution contained additionally 0.1 mM unlabeled folate.

2.6. *Data analysis*

Distribution-free plot (non-parametric) analysis of binding data employed the micro-computer program used in [24] modified in our laboratory to directly accommodate Scatchard binding data. Line fitting of all Scatchard plots was by least squares linear regression analysis.

3. RESULTS

3.1. *Identity and purity of DAFA*

Folate binding studies were initially conducted as suggested in [10]. To perform this assay, DAFA was chemically synthesized, since a commercial source was not available, and purified by recrystallization followed by preparative paper chromatography. The identity of the synthesized product was confirmed by UV spectroscopy (fig. 1) and appeared comparable to earlier reports [26,27]. Paper and TLC separations revealed R_f

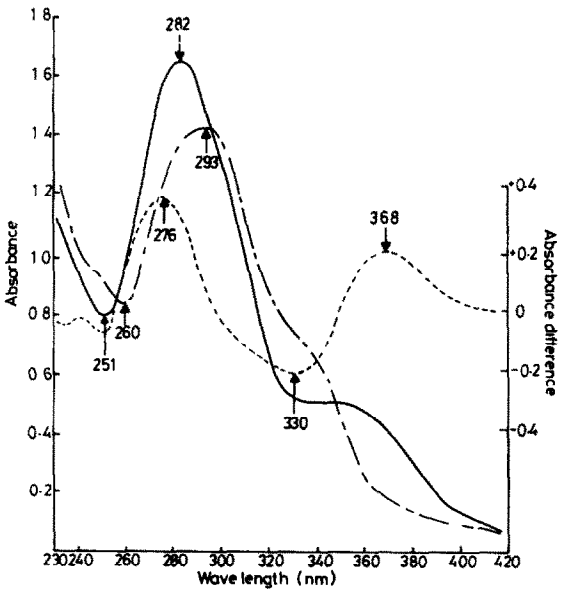

Fig.1. 1. UV absorption spectra of DAFA (-·-·-) and folic acid (——). Difference curve (– – –) obtained with folate in a sample cuvette and DAFA in the reference cuvette. Solvent = 10 mM PO_4 (K/K_2) buffer (pH 6.0). These results are representative examples of 15 spectra.

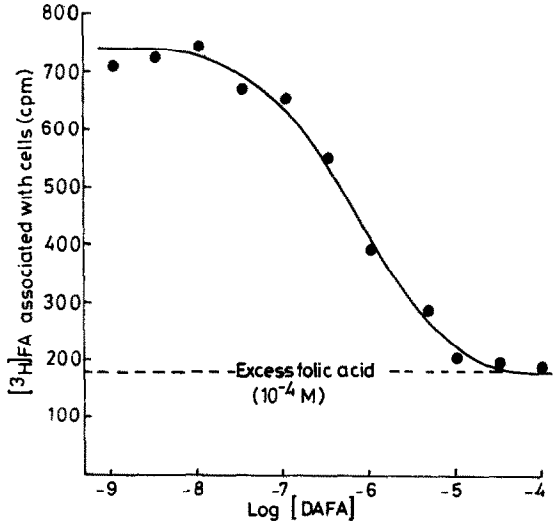

Fig.2. [^{3}H]Folate binding in competition with DAFA. Binding of [^{3}H]FA (●) was measured at variable concentrations of DAFA. Non-specific bound radioactivity (– – –) was determined with 0.1 mM unlabeled folate present (representative example of 14 experiments).

values for folic acid and DAFA of 0.57 and 0.73, respectively (cf. [5]). In testing the recrystallized DAFA product before final preparative chromatography, HPLC showed less than 0.1% contamination with folate, and retention times identical to those found in [20] with the same solvent and column (not shown). During the course of the study a gift of DAFA was obtained from Mr Vitrus Lau of the American Cyanamid Co., and comparison studies with UV spectroscopy and paper chromatography showed full identity.

3.2. *Folate binding to vegetative* D.discoideum *amoebae*

In [10] it was reported that the specific folate sites on *D.discoideum* were detectable only in the presence of excess DAFA. As shown in fig.2, we were consistently unable to show the presence of folate-specific sites. All folate binding appeared to be sensitive to competitive binding by DAFA. With this result, we chose to continue, using 8-azaguanine as the folate deaminase inhibitor but deleted DAFA from follow-up assays.

Folate binding data for vegetative amoebae (fig.3a) revealed 45–50000 binding sites per cell with a K_d of 300 nM which is similar to earlier results obtained in the absence of a folate deaminase inhibitor [5,6] and to the methotrexate binding studies [8].

3.3. *Folate binding to cytoskeletons*

Scatchard analysis of folate binding to cytoskeletons is shown in fig.3b. Data analysis by least squares linear regression showed 10750 sites and a K_d of 470 nM which agreed closely with the means of distribution-free plots from 8 experiments which showed 10730 sites and a K_d of 480 nM. Therefore, in comparison with the control binding to whole cells, which showed 35000 sites/cell and a K_d of 210 nM (under the same buffer conditions and silicone separating oil as for cytoskeletons, insert fig.3c), the isolated vegetative cytoskeletons retain nearly 30% of the sites detected on intact amoebae.

3.4. *Folate receptors through development*

Authors in [16] recently reported a renewed responsiveness in later development to folate as assayed by increased F-actin accumulation in the cytoskeleton. This result prompted us to look

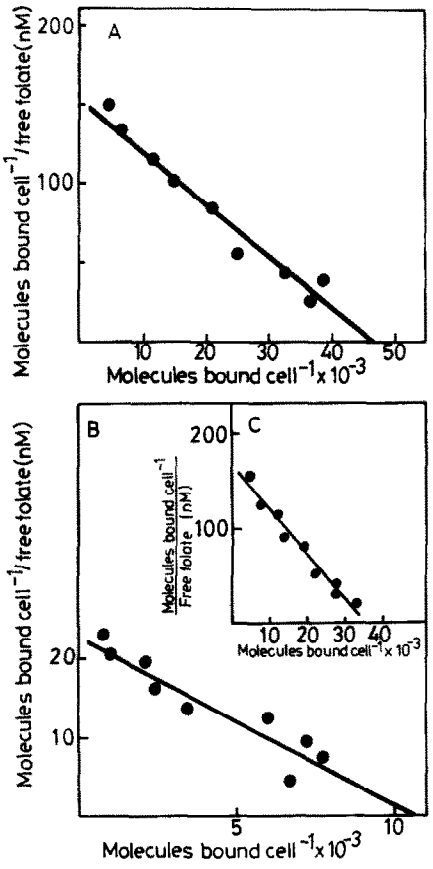

Fig.3. Scatchard plots of [^{3}H]folate binding to: (A) vegetative amoebae, mean of 3 experiments; (B) cytoskeletons from vegetative amoebae, mean of 8 experiments; (C) (inset) vegetative amoebae (control for B). Binding was assayed using the same buffer conditions and silicone oil as in B, mean of 3 experiments. Line fit was by least squares linear regression analysis.

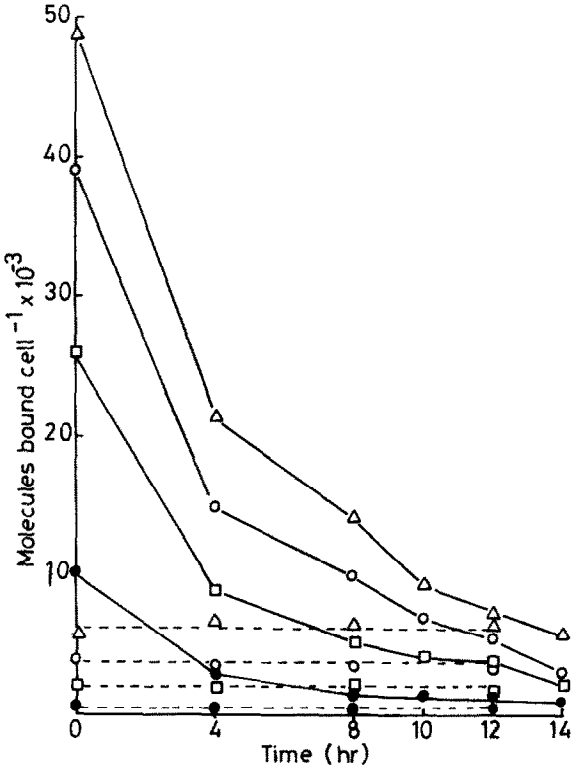

Fig.4. Developmental time course of [^{3}H]folate binding to whole cells (——) and cytoskeletons (– – –), measured at 1000 nM (△), 500 nM (○), 200 nM (□), and 50 nM (●). Data for whole cells, mean of 3 experiments; cytoskeletons, mean of 6 experiments.

again at the retention of folate receptors as *D.discoideum* proceeded through aggregation to the tipped aggregate (14 h) stage of development on a solid substratum. The results in fig.4 do not confirm the work in [6], where no change in site number or affinity throughout 8 h of development was reported, but show a rapid loss in receptors as the organism proceeds through development, as found by others [5,8], and see [14]. Controls using the mutant strain of *D.discoideum* (AX3) grown axenically showed the same pattern of receptor loss through 8 h of starvation, confirming that the reduction in binding is not an artifact due to phagocytosis of residual bacteria (not shown). We found no reappearance of folate receptors at the 12–14 h period. However, fig.4 does reveal the retention of a small pool of approximately 6000 receptors on the cell surface after the onset of starvation and development. A similar sized pool of folate receptors was also found to be present on the cytoskeletons and was shown to be retained through 12 h of development (fig.4).

4. DISCUSSION

We have detected 45000 folate receptors on vegetative *D.discoideum* amoeba with a dissociation constant (K_d) of 300 nM. It is unclear why the author in [10] was able to distinguish between polyspecific DAFA/folate sites and specific folate sites while we cannot. Two possibilities are that

our DAFA was contaminated with folate or that we were not inhibiting the folate deaminase. Neither possibility seems likely. Great care was taken to characterize and purify the DAFA extensively; and, control tests with 8-aza-guanine, as described in [10], showed complete inhibition of the folate deaminase (not shown).

We have demonstrated the presence of 10000 folate receptors per vegetative cytoskeleton and found that these receptors remain associated with the cytoskeleton throughout the first 12 h of development. In a brief abstract, the author in [25] reported finding methotrexate receptors attached to the cytoskeleton in *D.discoideum*, although no quantitative data were presented for comparison. The cytoskeletal folate receptors that we observed as remaining during development, may function, as previously suggested [8,15], in the chemokinesis mechanism which is retained during development after folate chemotactic capability is lost. Alternatively, these cytoskeletal receptors may play the primary role in chemotaxis throughout growth and may have a similar functional role in later development. It is then possible that the membrane surface receptors that are not associated with the cytoskeleton in vegetative amoebae may have no function in chemotaxis but may play another role, such as folate transport [6].

The reappearance of folate responsiveness as seen in the cytoskeletal actin accumulation studies [16] does not involve a reappearance of folate receptor sites later in development. The retention of sites on the cytoskeleton through development, however, suggests a continued role for folate. To explain the actin response to folate at the tipped aggregate stage of development, it will be necessary to search for other indicators of receptor coupling and signal transduction.

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