FLUORESCEINATED CATIONISED FERRITIN AS A MEMBRANE PROBE FOR ANIONIC SITES AT THE CELL SURFACE

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1. Introduction

Anionic sites on eukaryote cell membranes have been studied extensively and these surface components have been assigned many biological roles, e.g., cell to cell adhesion, cation transport [1]. The existance of these negative charges can be demonstrated using the technique of cell electrophoresis [2,3]. However, the most convenient method of detecting the anionic sites is by following their interaction with cationic polymers, e.g., polylysine [1], lysozyme [4], and cationised ferritin [5], using thin section electron microscopy techniques.

Cationised ferritin can be used at physiological pH, does not necessarily require prior fixation of the cells and can be detected as an individual molecule ultrastructurally due to the electron scattering power of the ferric iron present in large amounts in the 7.5 nm inner core [6].

2. Materials, methods and results

The cationised ferritin used in this study, obtained from Miles Laboratories, had been prepared by the method of Danon et al. [5] i.e., horse spleen ferritin was coupled with *N,N*-dimethyl 1,3-propanediamine via the carbodiimide activation of the apoferritin carboxyl groups. The substitution of free carboxyl groups of ferritin with amino groups changed the isoelectric point from about 4.6 to a value in excess of 8 [7].

In order to react with fluorescein isothiocyanate (FITC) proteins must have free amino groups. Since cationised ferritin has many introduced amino groups, it appeared likely that an FITC-labelled cationised ferritin preparation could be made easily. The general method of Rinderknecht [8] was used.

One millilitre of commercial cationised ferritin solution (10–12 mg/ml), 1 ml of 0.05 M Na_2CO_3–$NaHCO_3$ buffer, pH 8.5 and 1.5 mg of FITC on Celite (10%) were placed on a magnetic stirrer for 30 min at room temperature. With one batch of cationised ferritin it was necessary to use 0.05 M Tris–HCl buffer, pH 7.6, in place of the bicarbonate buffer as isoelectric precipitation of cationised ferritin occurred. The FITC-cationised ferritin was separated from the free fluorochrome using Sephadex G-25.

Using the standard value for the optical density of FITC at 492 nm given by Holborow and Johnson [9] it was estimated that the FITC-cationised ferritin preparations contained between 6–10 FITC moieties per cationised ferritin molecule.

Preparations of Rhodamine (TRITC) labelled cationised ferritin could be prepared in a similar way but it proved impossible to label ferritin with either FITC or TRITC under these conditions.

The interaction of FITC-cationised ferritin with two widely differing cell systems form the basis of this report.

2.1. *FITC-cationised ferritin*–Naegleria gruberi *interaction*

Interaction of FITC-cationised ferritin with the soil amoeba, *Naegleria gruberi*, forms part of a more generalised study of the cell surface properties of this protozoan organism [10].

The amoebae were cultured and harvested by the method given in [10]. They were then further puri-

fied by two passages through prepared Ficoll gradients.

The cells suspensions and solutes used in this section were all made up in low ionic strength *Naegleria* saline (0.1 mM KCl, 0.3 mM $CaCl_2$, 0.3 mM NaH_2PO_4 and 1.4 mM $NaHCO_3$adjusted to pH 7.4).

The amoebae were incubated with various amounts of ferritin, cationised ferritin, or FITC-cationised ferritin. The cell suspensions were then centrifuged and the optical density of the supernatant at 360 nm was measured. By comparison with standard solutions of ferritin, cationised ferritin and FITC-cationised ferritin the amount of protein bound by the cells could be calculated. It can be seen from table 1 that cationised ferritin and FITC-cationised ferritin are adsorbed to the same extent but ferritin is not adsorbed at all.

A cell preparation containing 10^6 amoebae adsorbed approximately 30 μg of cationised ferritin and the cell pellet was noticeably rust coloured.

Cells treated with FITC-cationised ferritin were observed using a Leitz Ortholux Incident Light Fluorescence Microscope. In the original experiments it was found that considerable amounts of FITC-cationised ferritin were not bound to the amoebae but to other material present in the preparation even though the cells had been washed extensively prior to viewing under the microscope. This problem was overcome by adding the total incubation mixture (cells and FITC-cationised ferritin) to the top of a prepared Ficoll gradient (0.4 ml of incubation mixture, 0.4 ml of 5% Ficoll, 0.4 ml of 7% Ficoll, 0.8 ml of 10% Ficoll, and 0.1 ml of 20% Ficoll). The tubes were spun at 1400 rev./min for 10 min. The cells were collected from the 10–20% Ficoll interface and viewed under the microscope. The background fluorescence in these samples was extremely low.

The FITC-cationised ferritin was not found over the whole cell surface but restricted to the posterior two-thirds or less (fig.1). As the amoebae warmed up and locomotion became more pronounced, concentration of the fluorescent label at the uroid, i.e., 'capping' [11] occurred rapidly (fig.2). Sometimes these caps were sloughed off.

Aggregated cell could be found and these were attached by the strongly fluorescent regions in the area of the uroids.

Amoebae fixed with 0.5% w/v glutaraldehyde (Taab), and washed with 1% glycine to block any unreacted aldehyde groups immediately before exposure to FITC-cationised ferritin exhibited general, not local surface fluorescence. This staining pattern was not altered as the cell warmed to ambient temperature.

2.2. *FITC-cationised ferritin–sheep erythrocyte interaction*

Phosphate buffered saline (pH 7.4) was used as a suspending medium in this section. Sheep blood in Alsever's solution (Gibco-Biocult) was washed three times and a suspension containing 2×10^7 erythrocytes/ml was prepared. These cells were then incubated with ferritin, cationised ferritin or FITC-cationised ferritin and the results of the experiment are shown in table 2.

Table 1
Adsorption of ferritin, cationised ferritin and FITC-cationised ferritin by *Naegleria* amoebae

μg Protein/0.5 ml incubation mixture	μg Protein bound/10^6 amoebae		
	Ferritin	Cationised ferritin	FITC-cationised ferritin
50	0	22	23
100	0	28	34
150	0	31	32

0.1 ml of 10^7 amoebae/ml + 0.4 ml of protein solution were incubated for 30 min at 0°C, the cell suspensions were then centrifuged and the optical density of the supernatants measured.

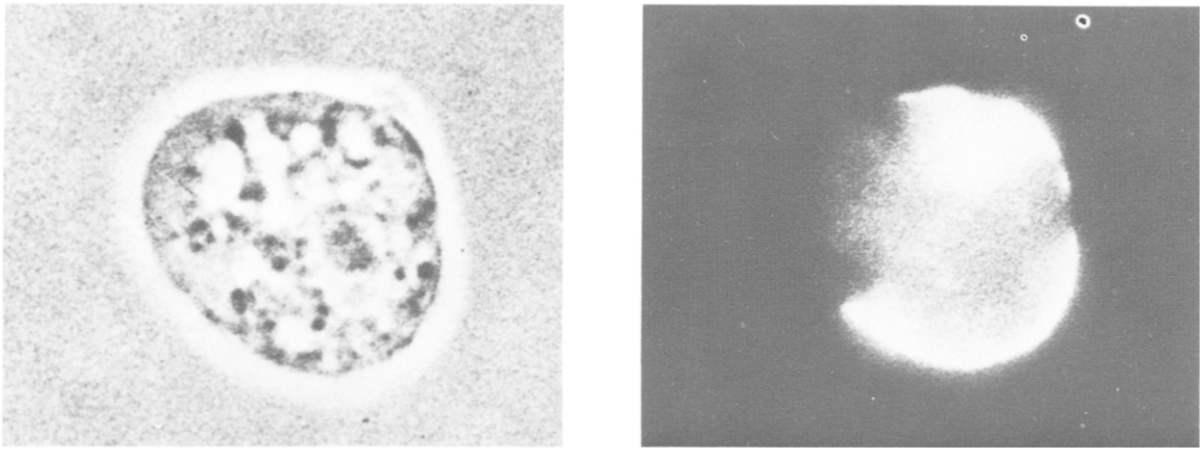

Fig.1. Paired phase contrast and fluorescence micrographs of a *Naegleria* amoebae to show the initial distribution of the FITC-cationised ferritin.

The sheep erythrocytes were smaller than the amoebae and it was estimated that the cell surface area of the two cell preparations used (10^6 amoebae and 10^7 erythrocytes) would be of the same order of magnitude.

It can be seen from table 2 that the same general behaviour of the erythrocytes towards the three proteins was observed as in table 1.

The cells treated the FITC-cationised ferritin were washed twice with phosphate buffered saline and observed under the fluorescence microscope. Most of the cells were strongly agglutinated and the fluorescence was found over the whole cell surface with no localised concentration (fig.3).

3. Discussion

The fluorescence observed on the surface of the erythrocytes after interaction with FITC-cationised ferritin agrees with the observations made from electron microscope studies on cationised ferritin treated erythrocytes [5] i.e., general surface staining with no redistribution. The experiments with unfixed

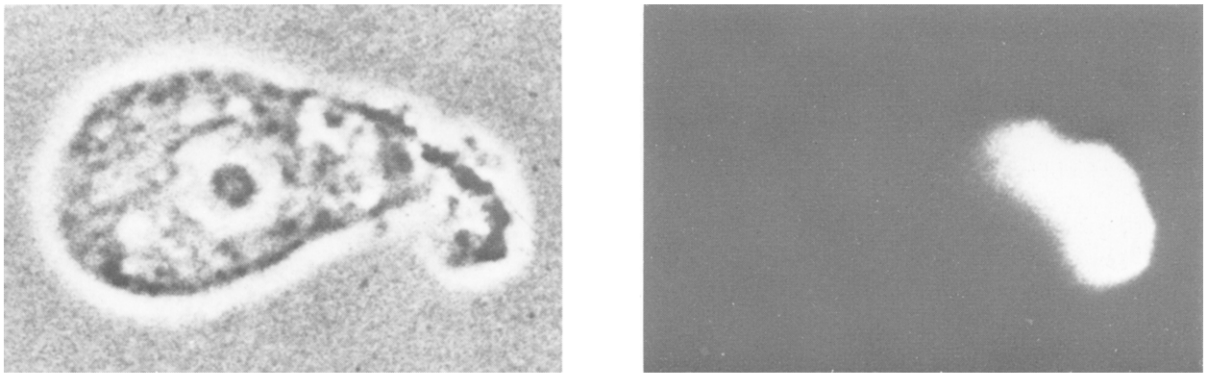

Fig.2. Paired phase contrast and fluorescence micrographs of a *Naegleria* amoebae to show the distribution of FITC-cationised ferritin after capping has occurred.

Table 2
Adsorption of ferritin, cationised ferritin and FITC-cationised ferritin by sheep erythrocytes

μg Protein/ml incubation mixture	μg Protein bound/10^7 erythrocytes		
	Ferritin	Cationised ferritin	FITC-cationised ferritin
50	0	12	11
100	2	13	12
200	0	13	13

1 ml of 2 × 10^7 sheep erythrocytes + 1 ml of protein solution were incubated for 30 min at 22°C, the cell suspensions were centrifuged and the optical density of the supernatants measured.

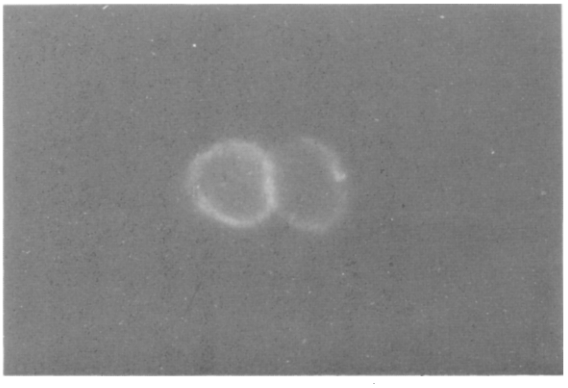

Fig.3. Fluorescence micrograph of two sheep erythrocytes to show the distribution of the FITC-cationised ferritin.

Naegleria amoebae showing redistribution of the label shows the potential mobility of membrane anionic sites. Redistribution of cationised ferritin on the surface of baby hamster kidney cells has been recently described in an ultrastructural study by Grinnel et al. [12].

At the present time 2 general types of fluorescent tagged ligands are used in cell surface studies:

(i) Antibodies – directed against specific antigens at the cell surface [11]

(ii) Lectins – directed against saccharide components present in cell membranes [13].

A third class of fluorescent probes, the FITC-labelled cationic proteins could provide an addtional valuable tool in the elucidation of the structure and dynamic behaviour of membranes.

From the experiments described here on the uptake of cationised ferritin by two very different cell types, the behaviour of this protein does not appear to be affected by the reaction of FITC with some of its positively charged amino groups. The number of positive charges available on the FITC-cationised ferritin must be decreased, but the overall response towards the cell surface remains the same (i.e., the proportion of positive charges removed by the sub stitution is probably small).

It is thought that anionic radicals covalently linked to the membrane matrix would be present as carboxyls, sulphonyls and phosphatyls [1]. Cationised ferritin would have to act as a multivalent ligand in order to produce the capping results seen in the *Naegleria* experiments. At neutral pH a large number of positively charged groups per molecule of cationised ferritin [5] should be available for interaction with anionic sites.

In the past frequent mention has been made of 'fixed negatively charged sites' on cell membranes. However, the capping of these peripheral anionic sites (as witnessed in the experiments using *Naegleria*) demonstrates that this concept cannot be applied to all cell types. Since the polyvalent cationised ferritin may act as a multivalent ligand altering the distribution of these sites to form patches or caps, caution should be shown in the use of cationised ferritin as a stain for showing distribution of anionic sites on unfixed cells.

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