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Distribution of ferritin receptors and coated pits on giant HeLa cells

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HeLa cells bind horse spleen ferritin when the two are incubated at 0°C. Since the majority of this bound ferritin is located in coated pits, we conclude that the ferritin binds to a specific receptor which takes part in an endocytic cycle. When substrate-attached and well-spread giant HeLa cells are briefly labelled at 0°C with ferritin, ferritin particles are found to be concentrated towards the cell periphery, where they exist largely outside coated pits. This peripheral concentration is a property of circulating (and not just newly synthesized) receptors because it is not affected by prior incubation of giant cells in cycloheximide. However, coated pits are themselves roughly uniformly distributed over the surface of these cells. These results provide evidence that the membrane internalised by coated pits on these cells is returned to the cell surface at the leading edge of the cell. Because of this separation of the sites of endocytosis and exocytosis, a flow of membrane must occur across the cell surface. This flow is composed of lipid plus receptors. The implications of this for capping and for cell spreading are discussed.

Key words: ferritin receptor/coated pit/giant HeLa cell/endocytic cycle/leading lamella

Introduction

Coated pits are characteristic structures found on the surfaces of most mammalian cells: they concentrate specific receptors onto a portion of the plasma membrane of the cell which the cell then endocytoses (Pearse and Bretscher, 1981). This enables the cell to bring into itself these specific receptors plus their associated ligands – a process called receptor-mediated endocytosis. Some receptors are largely located in coated pits, whereas others are not so effectively sequestered: the extent to which they are so localised depends on the cell type and the receptor. For example, at any given time the low density lipoprotein (LDL) receptor on human fibroblasts is largely (~70%) concentrated in coated pits, whereas only a small fraction (~4%) of the same receptor on A431 carcinoma cells is present in coated pits (Anderson *et al.*, 1977, 1981). Coated pits are roughly randomly distributed on the surface of cells, and the receptors, whether localised in coated pits or not, appear randomly distributed when viewed at low resolution.

Certain cells possess receptors which bind ferritin: in erythroblasts (Fawcett, 1965), macrophages (Lagunoff and Curran, 1972) and fibroblasts (Anderson *et al.*, 1977) these receptors are all localised in coated pits. Here we show that normal HeLa cells also possess ferritin receptors which are largely located in coated pits. On giant HeLa cells which are well-spread, these receptors are concentrated at the leading edge of the cells; the majority of these receptors are not located in coated pits. However, the distribution of coated pits on these cells is essentially random. These results imply that the endocytosed ferritin receptors, like those for LDL and transferrin (Bretscher, 1983), are returned to the cell surface at the leading edge of the cell.

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Results

The ferritin receptor on normal HeLa cells

Substrate-attached HeLa cells were labelled at 0°C with horse spleen ferritin. Thin sections of labelled cells were examined to locate any bound ferritin molecules. This revealed that each cell has ~5000–10 000 bound ferritin molecules and that the vast majority (>85%) are located in coated pits. The remainder are seen as occasional individual molecules, except when two cells touch each other: in this latter situation several ferritins may be seen lined up between the adjacent plasma membranes. The typical distribution, however, is shown in Figure 1. This observation suggests that HeLa cells possess a receptor which can bind native ferritin: the fact that it is largely associated with coated pits indicates that it is brought into the cell with other receptors and is, presumably, returned to the cell surface later.

Distribution of ferritin receptors on giant HeLa cells

Giant HeLa cells, growing on an araldite substrate, were labelled at 0°C with ferritin. The washed cells were fixed, then stained with crystal violet and well-spread cells marked so that they could be located when embedded. Thin sections were cut from the middle regions of six cells. Of these, two had rather few ferritins on them and were discarded. The remaining four were selected for further study, and a section through each of them is shown at low magnification in Figure 2(a–d).

To estimate the distribution of ferritin particles on these cells, each cross-section was divided into five segments, as indicated in Figure 2. The ferritin particles along the dorsal surface of the cell in each segment on several sections for each cell were counted. As these cells have some microvilli, only ferritin particles along the base of the plasma membrane (including those in coated pits) were scored. The number of ferritins in these segments, together with the approximate

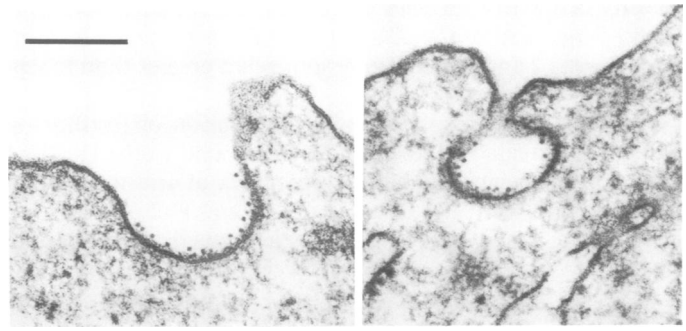

Fig. 1. Ferritin receptors located in coated pits. HeLa cells were labelled at 0°C with native ferritin, the cells washed, fixed and embedded. Examination of thin sections reveals that the majority of bound ferritin particles are associated with coated pits. Bar = 0.2 μm.

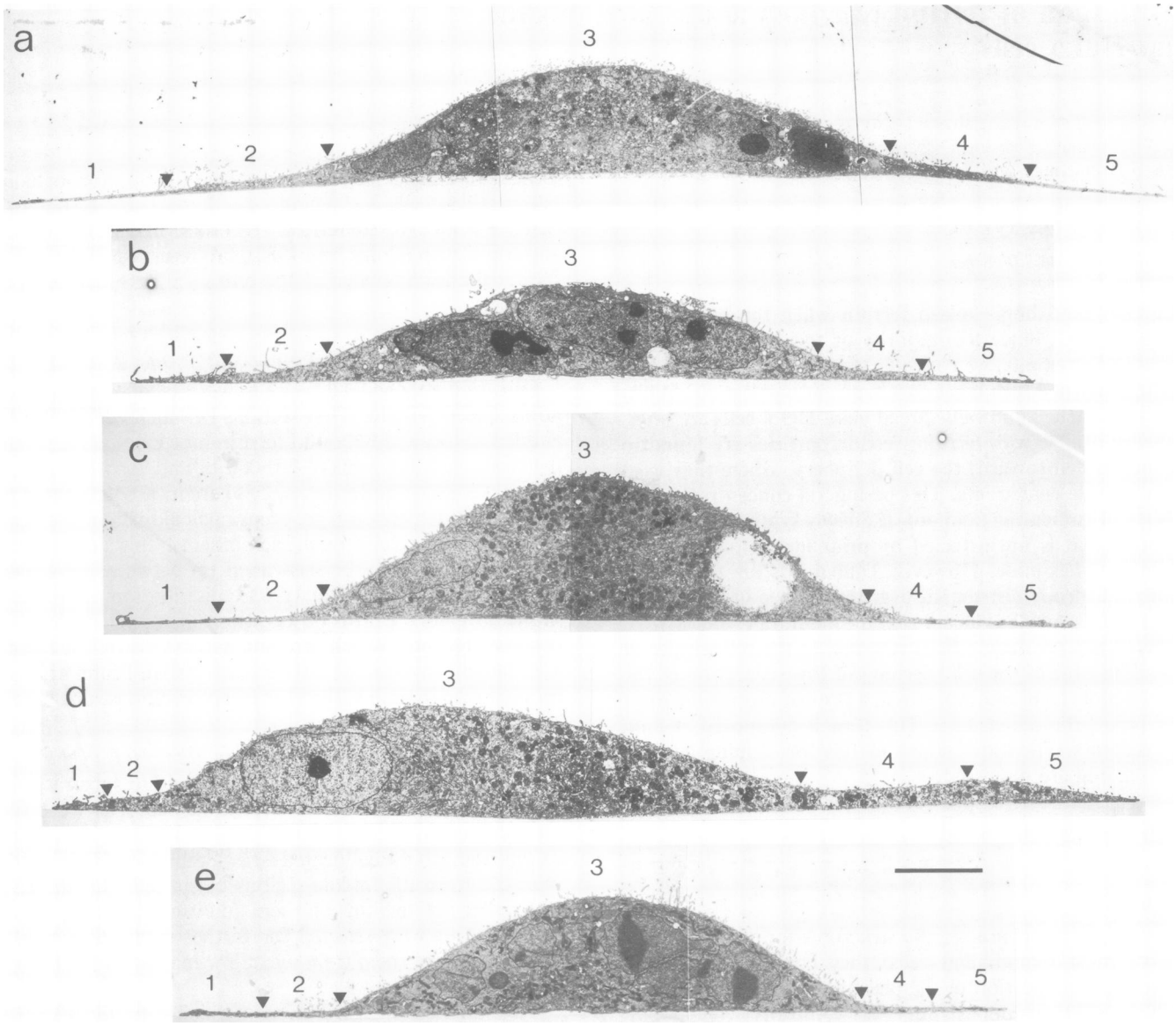

Fig. 2. Sections through giant HeLa cells. The cells shown are those whose distribution of ferritin particles and coated pits are described in Tables I and II. The arrow heads indicate the approximate borders of the segments referred to in Tables I and II. Cells a–d were labelled with ferritin at 0°C as described in Materials and methods, whilst cell e was preincubated with 100 μg/ml cycloheximide in growth medium at 37°C for 2 h prior to labelling at 0°C. Scale bar = 10 μm.

contour lengths of these segments (excluding microvilli), are presented in Table I. It will be seen that there is an increased density of ferritin particles on the leading lamellae (segments 1 and 5) on most of the cells, whereas the densities of ferritins in segments 2 and 4 are usually somewhat greater than in segment 3.

To examine if this increased concentration of ferritins on the end segments of these cells could be due to the localised insertion of newly synthesised receptors at the tips of the leading edges (c.f., Marcus, 1962), giant HeLa cells were grown for 2 h at 37°C in complete medium containing 100 μg/ml cycloheximide. They were chilled and labelled. Three cells were examined. Of these, two had a somewhat greater concentration of ferritins on at least one lamella; the third cell was studied in more detail and is cell e in Figure 2 and Table I. It will be seen that its ferritin distribution is similar to that in cells a–c. This indicates that the higher concentrations of ferritin observed on the leading edges of giant

Table I. Distribution of ferritin particles over the dorsal surface of giant HeLa cells

Cell	Number of sections scored	Total ferritins/μm plasma membrane in segment: 1	2	3	4	5
a	7	20	1.5	0.8	1.5	11
b	8	11	2.3	1.7	2.0	24
c	5	40	6.4	2.3	4.5	25
d	6	4.9	2.3	3.1	3.0	16
e	10	38	14	4.5	17	53

The total number of ferritin particles/segment (and the length of that segment in μm) in the number of sections indicated were:
cell a: 399(20), 30(20), 51(62), 26(17), 183(17).
cell b: 122(11.3), 26(11.3), 104(60), 26(12.8), 307(12.8).
cell c: 479(12.0), 77(12.0), 165(73), 50(11.1), 273(11.1).
cell d: 38(7.7), 18(7.7), 225(71.5), 62(20.4), 337(20.4).
cell e: 349(9.2), 125(9.2), 283(63), 148(8.6), 458(8.6).

HeLa cells is not due to the localised insertion of newly synthesised ferritin receptors, but rather is a property of the circulating receptor on these cells.

The variation in density of ferritins along the plasma membrane is sometimes much more striking than is indicated in Table I. The leading lamella of that cell having the greatest gradient of ferritins at one end which was observed (the left end of cell c) is shown in Figure 3. The lamella end has many ferritin particles on it, the density of ferritins decreasing as one moves from the end of the lamella towards the cell body.

There are three additional features of this distribution worth mentioning. (1) The two end lamellae of cell d are obviously not equally labelled. The short lamella (segment 1) has few excess ferritins over that seen in segments 2–4; this is especially striking when compared with the large numbers present on its larger lamella. A few other large cells, which are poorly spread, have been looked at qualitatively. They never have the large amounts of ferritin at their leading edges noted in Table I and in Figure 3. (2) The majority of ferritin particles seen over the cell body reside in coated pits; this is similar to what is found in normal HeLa cells. In striking contrast, the majority of ferritins seen on the ends of the lamellae are not located in coated pits. The receptors are free, presumably diffusing around in search of a forming coated pit. (3) In two other experiments, giant HeLa cells were likewise labelled with ferritin at 0°C and several cells from each experiment examined in thin sections. A cursory examination of the distributions of ferritin on these cells indicated that ferritin particles were not especially concentrated on their lamellae. However, it was also obvious that these cells were almost detached from the substrate, having large gaps (up to 1 μm in depth) between them and the substrate. Further growth of a parallel culture of these cells for a day left almost no giants attached to the substrate: they had all rounded up. These observations are consistent with the view that, when these cells stop spreading, the internalised membrane is no longer returned to the extending lamella.

Distribution of coated pits on giant HeLa cells

To obtain an approximate idea of the distribution of coated pits on these cells, the number of coated pits in the different segments of cells a–e were counted in a series of cell sections. Only coated pits open to the medium in the section studied were scored, and only every third section on a ribbon of sections looked at in order to prevent a coated pit being scored more than once. The results of this are shown in Table II. They indicate that, for a given cell, coated pits have roughly the same density in each of the five segments of that cell. As each coated pit straddles roughly four sections, and would be scored in two sections, these figures indicate that ~3% of the plasma membrane of the cell is taken up by coated pits (neglecting the area taken up by microvilli).

A further feature of coated pits on these cells is shown by a more detailed examination of the number of ferritin particles in coated pits in the different segments. Only one cell was studied in detail, cell a. The total number of ferritins in each coated pit, measured across several adjacent sections, was counted. This showed that the average number of ferritins in coated pits in segments 1 and 5 were 5 and 6, in segments 2 and 4 were 2 and 1 and was 0.2 in segment 3. This indicates that, on a given cell, coated pits do not have a fixed composition.

Table II. Distribution of coated pits over the dorsal surface of giant HeLa cells

Cell	Number of coated pits/10 μm plasma membrane in segment:				
	1	2	3	4	5
a	0.23	0.32	0.31	0.52	0.38
b	0.44	0.35	0.37	0.51	0.62
c	0.83	1.4	0.55	1.6	1.0
d	1.1	0.50	0.73	1.1	1.3
e	0.32	0.64	0.53	0.96	0.34

Every third section in a ribbon of sections was examined for coated pits open to the external medium. The number of coated pits scored in each segment (and the number of sections examined) were:
cell a: 8, 11, 33, 15, 11 (17 sections).
cell b: 10, 8, 45, 13, 16 (20 sections).
cell c: 9, 15, 36, 16, 10 (9 sections).
cell d: 11, 5, 68, 30, 35 (13 sections).
cell e: 5, 10, 57, 14, 5 (17 sections).

Discussion

HeLa cells possess ferritin receptors which participate in the endocytic cycle of the cells, apparently in a similar fashion to LDL (Goldstein *et al.*, 1979) or transferrin receptors (Bleil and Bretscher, 1982). They are highly concentrated in coated pits.

Giant HeLa cells which are well spread also bind ferritin, but the majority of the bound particles are found at the end of the leading lamella, their concentration declining towards the cell body. This observation is similar to that found by autoradiography for the LDL and transferrin receptors on these cells (Bretscher, 1983). In each case, the peripheral location of the receptors is not due to the localised insertion of newly synthesized receptors (c.f., Marcus, 1962) because prior incubation of the cells with cycloheximide does not affect their distribution. The simplest interpretation of this observation is that, as part of the endocytic cycle, the receptors are returned to the surface of a giant HeLa cell at the tip of the leading edge of the cell. From there, the receptors diffuse around until they are bound in a coated pit, when they are endocytosed. The reason one can see this so easily on a giant cell must be because, on average, the ferritin receptors are endocytosed before they have had time to diffuse to the middle of the cell. Thus, to diffuse from the leading edge to the middle of a cell whose radius is 75 μm would take ~1 h, if the diffusion coefficient of the receptors at 37°C was 5×10^{-9} cm²/s. This time should be compared with the expected residence-time of the receptor on the surface of normal HeLa cells (and presumably on giant HeLa cells), which must be about the same as the life-time of a coated pit, which is ~1 min. In other words, on average, the receptors would be expected to be endocytosed well before they could diffuse far from the leading edge, and would then be returned to the leading edge again during exocytosis.

The observation that coated pits are approximately randomly distributed on the dorsal surface of these cells means, presumably, that endocytosis occurs uniformly over the surface of the cell. This separation between the sites of exocytosis and endocytosis means that there must be a bulk flow of membrane [composed only of lipid plus receptors, but excluding many non-circulating membrane proteins (Bretscher, 1976, 1982; Bretscher *et al.*, 1980)] away from the leading edge towards the middle of the cell. It is this flow which presumably leads to the capping of erythrocytes attached to the dorsal surface of these cells (Marcus, 1962).

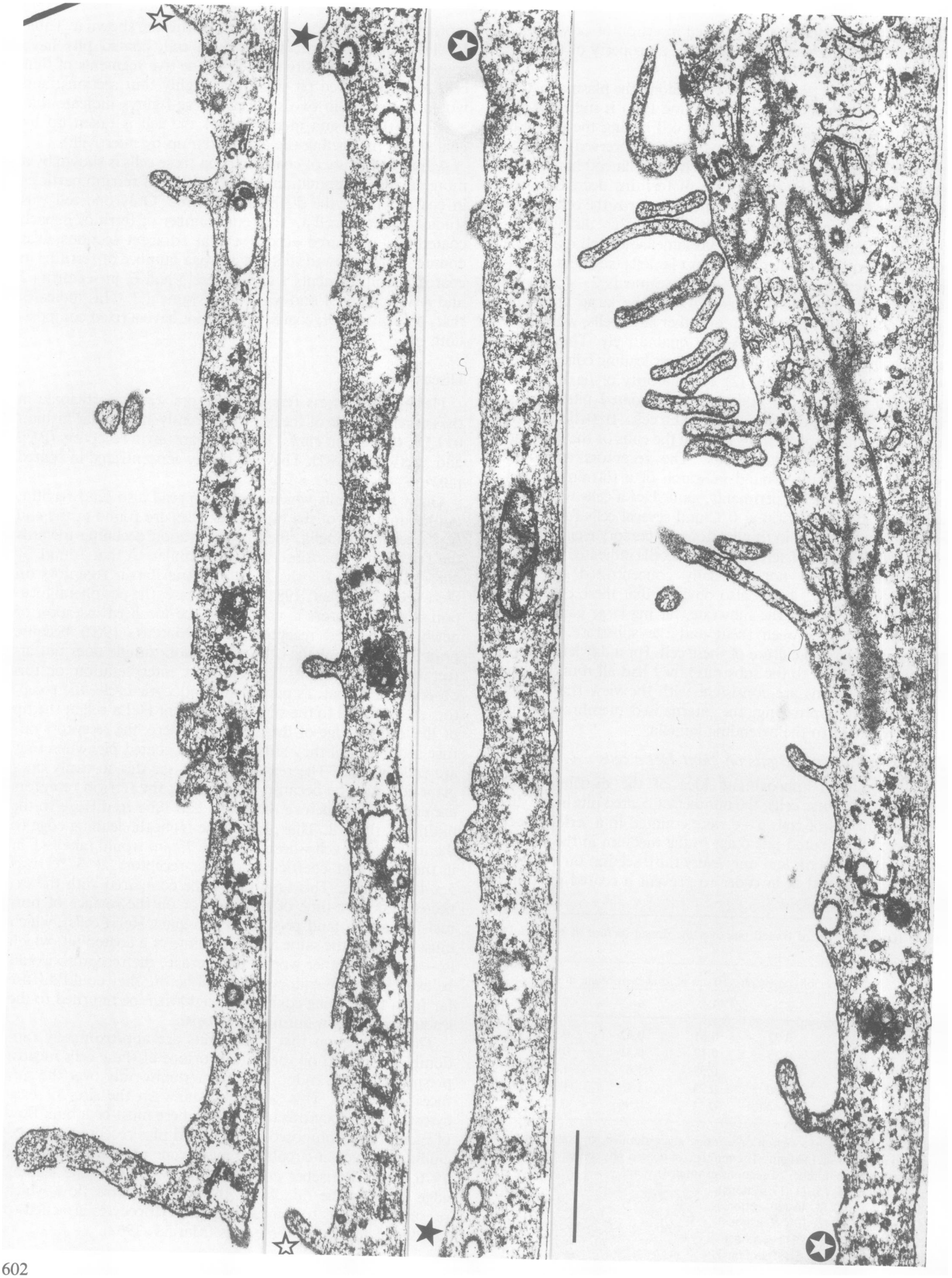

Fig. 3. A single leading edge of a giant HeLa cell, showing the distribution of ferritin particles along it. The lamella (the left hand edge of cell c in Figure 2, although a different section) is presented as four segments; these are related by the different stars. The numbers of ferritin particles along the dorsal plasma membrane in these four segments, starting at the leading edge, are: 77, 20, 6 and 10 (including those on microvilli and in coated pits). Ferritin particles between the cell and substrate should be ignored, as labelling medium here would probably not have been removed during the brief washes after labelling. Bar is 0.5 μm.

The main conclusion from these observations, as well as from those on the transferrin and LDL receptors on these cells (Bretscher, 1983), is that the endocytosed membrane is returned to the cell surface at the leading edge of the cell. This localised addition of circulating membrane is what would be expected if the cell extends itself, as it spreads outwards, by adding membrane to its leading edge. It is analogous to a scheme, originally proposed in outline by Abercrombie *et al.* (1970), for the extension of a motile fibroblast at its leading edge (Bretscher, 1982).

Materials and methods

The HeLa cell line used here was kindly provided by L.J.Tolmach of Washington University. Giant HeLa cells were induced by a lethal dose of X-irradiation (Puck and Marcus, 1956; Tolmach and Marcus, 1960), and then grown for 4 days in Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum, antibiotics and glutamine. The surviving cells were then trypsinized and replated in the same growth medium in dishes containing an araldite substrate ~2–4 mm deep, and grown for a further 2 days. Normal HeLa cells were likewise grown on an araldite base for 3 days.

To label the giant cells with ferritin, the cells were chilled to 0°C as quickly as possible and placed in Joklik's medium containing 0.02 M Hepes pH 7.3, 1 mg/ml bovine serum albumin and 5 mg/ml horse spleen ferritin (Calbiochem, 7 x recrystallized) at 0°C for 15 min. Normal HeLa cells were likewise labelled at 0°C for 1 h. Preparations were then washed three times at 0°C in labelling medium lacking ferritin, once in 0.15 M NaCl at 0°C and fixed in 2.5% glutaraldehyde (w/v) in 0.1 M sodium phosphate pH 7.2 at 22°C for 30 min. After washing with 0.15 M sodium cacodylate pH 7.2, the cells were further fixed with 2% OsO_4 in 0.15 M sodium cacodylate pH 7.2 at 0°C for 1 h. They were then washed with water and held in 1% uranyl acetate at 22°C for 1 h. Certain giant cells were then selected for study: they were stained with 0.1% crystal violet and well-spread cells marked with graphite in the araldite base so that they could later be found when embedded. The cells were then dehydrated in a graded series of alcohol, washed in propylene oxide and embedded. Thin sections were cut out of the middle region of marked cells or through the centre of a colony of normal cells, stained with uranyl acetate and lead citrate and examined.

Acknowledgements

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References

Abercrombie,M., Heaysman,J.E.M. and Pegrum,S.M. (1970) *Exp. Cell Res.*, **62**, 389-398.
Anderson,R.G.W., Brown,M.S. and Goldstein,J.L. (1977) *Cell*, **10**, 351-364.
Anderson,R.G.W., Brown,M.S. and Goldstein,J.L. (1981) *J. Cell Biol.*, **88**, 441-452.
Bleil,J.D. and Bretscher,M.S. (1982) *EMBO J.*, **1**, 351-355.
Bretscher,M.S. (1976) *Nature*, **260**, 21-23.
Bretscher,M.S. (1982) *Cold Spring Harbor Symp. Quant. Biol.*, **46**, 707-712.
Bretscher,M.S. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 454-458.
Bretscher,M.S., Thomson,J.N. and Pearse,B.M.F. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 4156-4159.
Fawcett,D.W. (1965) *J. Histochem. Cytochem.*, **13**, 75-91.
Goldstein,J.L., Anderson,R.G.W. and Brown,M.S. (1979) *Nature*, **279**, 679-685.
Lagunoff,D. and Curran,D.E. (1972) *Exp. Cell Res.*, **75**, 337-346.
Marcus,P.I. (1962) *Cold Spring Harbour Symp. Quant. Biol.*, **27**, 351-365.
Pearse,B.M.F. and Bretscher,M.S. (1981) *Annu. Rev. Biochem.*, **50**, 85-101.
Puck,T.T. and Marcus,P.I. (1956) *J. Exp. Med.*, **104**, 615-628.
Tolmach,L.J. and Marcus,P.I. (1960) *Exp. Cell Res.*, **20**, 350-360.