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Metabolic and cytoskeletal modulation of transferrin receptor mobility in mitogen-activated human lymphocytes

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SUMMARY

The transferrin receptors which appear on mitogen-activated human peripheral blood lymphocytes were found by the use of immunofluorescence techniques to display temperature-dependent patching and capping reactions upon binding of transferrin. Lateral mobility of ligand-occupied membrane sites was accompanied by both shedding and endocytosis of receptor–transferrin complexes. In the presence of sodium azide or the microfilament inhibitor cytochalasin B, cap formation and shedding were markedly inhibited. In contrast, endocytosis of patched receptor–ligand complexes was inhibited by azide and microtubule inhibitors, including colchicine, vinblastine and vincristine. Co-capping experiments performed to elucidate further the alterations in membrane configuration involved in these reactions failed to reveal any topographical relationship between transferrin receptors and lectin-binding sites in these cells. These studies indicate that temperature-dependent mobility of transferrin receptors upon mitogen-activated peripheral blood lymphocytes is dependent upon the integrity of the cytoskeletal system and metabolic function of the cell.

INTRODUCTION

Membrane structures binding transferrin, a circulating carrier protein for ferric iron and other metallic cations (Morgan, 1974), have been detected on several different human cell types, including reticulocytes (Jandl & Katz, 1963), placental syncytiotrophoblast (Galbraith *et al.*, 1980c; Galbraith, Galbraith & Faulk, 1980a), lymphoblastoid and other transformed cells (Galbraith, Galbraith & Faulk, 1980b; Larrick & Cresswell, 1979) and mitogen-activated peripheral blood lymphocytes (Galbraith *et al.*, 1980d; Phillips, 1976). Their biological role remains uncertain, but it has been suggested that membrane-binding of transferrin results, among other processes, in the delivery of appropriate metallic cations to the metabolically active cell (Morgan, 1974). For example, in studies of the cellular fate of membrane-bound transferrin, clear evidence has been obtained in haem-synthesizing immature erythrocytes to support the concept that ligand receptor complexes can undergo internalization or endocytosis *in vitro* (Hemmaplardh & Morgan, 1977). This indicates in turn that receptor sites may be mobile within the membrane and consequently affected by the integrity of the cytoskeletal system (Hemmaplardh, Kailis & Morgan, 1974), but studies addressing this possibility directly have not yet been performed. In the present investigation, we have examined the membrane mobility of transferrin receptors by means of an immunohistolo-

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gical ligand-binding method using as target cells human peripheral blood lymphocytes, metabolically activated by exposure to mitogenic lectins. The interactions demonstrated between membrane structures binding lectin or transferrin, homologous ligands and the cytoskeletal system form the subject of this report.

MATERIALS AND METHODS

Cells and cultures. Human mononuclear cells (MNC) were obtained from normal healthy donors by Ficoll–Isopaque centrifugation of heparinized blood. The cell preparations obtained consisted of lymphocytes and a variable proportion (5–30%) of monocytes. Mitogen activation of MNC by phytohaemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM) was performed in the absence of human transferrin in medium containing 15% foetal calf serum as previously described (Galbraith *et al.*, 1980d). DNA synthesis associated with blast transformation occurring during activation was assessed by measurement of ^{3}H-thymidine incorporation (Galbraith *et al.*, 1980d). Transferrin-binding studies were performed within 1 hr of harvesting cells at 66 hr of culture.

Immunohistology. Viable cells in suspension in 0·15 M phosphate-buffered saline (PBS), pH 7·4, were examined for membrane-binding of human transferrin using immunofluorescence methods as detailed elsewhere (Galbraith *et al.*, 1980b, 1980d). In brief, cells were first incubated in V-bottomed microtitre plates (Falcon Plastics, Oxnard, California) at a concentration of 5×10^5 cells/well for 30 min at 4°C with human transferrin in the form of purified apotransferrin solution, 2·5 mg/ml PBS (Sigma Chemicals, St Louis, Missouri) or normal human serum (NHS). After washing three times in 0·15 M PBS, pH 7·4, the cells were serially reacted at 4°C for 30 min with an Ig fraction of a rabbit antiserum to human transferrin (Dakopatts AS, Denmark) and a fluorescein isothiocyanate (FITC) labelled sheep antiserum to rabbit Ig (Wellcome Laboratories, Research Triangle Park, North Carolina) with intervening washes, and finally mounted in 50% glycerol in PBS, pH 7·4. The FITC conjugate had a fluorescein/protein ratio of 2 μg/1 mg, and the optimal dilutions of both antisera were determined by end-point titration (Galbraith *et al.*, 1980a). Cells were examined for bound transferrin by fluorescence under a Leitz Orthoplan microscope fitted for epi-illumination with a Ploempak II and appropriate filter systems for FITC and tetramethylrhodamine isothiocyanate (RITC) fluorescence and a 100 W mercury (HBO 100) light source. All cell preparations were coded and examined by two independent observers. At least 200 cells in each preparation were examined and the percentage and pattern of fluorescent cells recorded.

Membrane mobility studies. The effect of temperature on the membrane distribution of bound transferrin was examined by the use of a modified 'cold–hot' technique (Galbraith *et al.*, 1980b). This involved performance of the transferrin-binding reaction and its immunohistological detection under varying conditions of temperature and times of incubation. In certain experiments, to avoid the possibility of confusion in interpretation due to continued appearance of receptor sites during testing, cells were treated with cycloheximide, an inhibitor of protein synthesis which we have shown to inhibit totally the expression of membrane receptors in mitogen-activated lymphocytes (submitted for publication). Cells were incubated with this inhibitor (25 μg/ml) for 30 min at 37°C, washed and then examined for temperature-dependent mobility of binding structures. The influence of cytoskeletal inhibitory substances and 'metabolic poisons' on the mobility of bound transferrin was examined by incubation of the cells with each agent for different time periods at 4° and 37°C prior to the temperature-dependent mobility studies described above. The following agents were used: the metabolic agent sodium azide (5–30 mM), the microtubule inhibitors, colchicine, vincristine and vinblastine (all at 10 μM–10 mM) and the microfilament inhibitor cytochalasin B (20–200 μM). All pharmacological agents were obtained from Sigma Chemicals. Cytochalasin B was dissolved in dimethyl sulphoxide (DMSO) at 10 mg/ml and subsequently diluted to working concentrations in PBS, pH 7·4. The other agents were dissolved in PBS. A wide dosage range was employed in each case, the upper limit being the highest sublethal dose consistent with >90% cell viability as assessed by trypan blue exclusion. Each experiment was performed at least three times with PHA-, PWM- and Con A-activated cells.

In order to determine any association between membrane mobility of transferrin-binding sites and lectin receptors, lymphocytes activated by culture with Con A labelled with RITC (Vector Laboratories, Burlingame, California) were subjected to temperature-dependent transferrin mobility studies as described above and examined simultaneously for Con A-RITC and the FITC fluorescence of bound transferrin.

Control experiments. Controls for unwanted fluorescence (UF) included substitution of PBS (conjugate control) and normal rabbit Ig (serum control) for the first antiserum layer. When UF was observed, it was abolished by immunoabsorption of the FITC-conjugate with NHS in solid phase (Galbraith, Galbraith & Faulk, 1978). The specificity of the antisera, the purity of the apotransferrin solution and the specificity of the transferrin-binding reaction were confirmed as previously described in detail (Galbraith *et al.*, 1980a, 1980b, 1980d). Finally, in mobility experiments (see Results) involving cytochalasin B in solution in DMSO, a solvent control was performed in which cells were treated with appropriate dilutions of DMSO alone prior to mobility studies.

RESULTS

All serum and conjugate controls were negative throughout, and binding of transferrin was detected in less than 5% of MNC cultured in the absence of mitogen. However, under optimal culture conditions derived from dose–response experiments performed as previously described (Galbraith *et al.*, 1980d), binding of transferrin in both NHS and purified solution was detected by membrane immunofluorescence upon greater than 80% of cells cultured with PHA, Con A, Con A-RITC, or PWM. When the transferrin-binding reaction was performed at 4°C throughout, a random distribution of fine fluorescent granules over the entire cell membrane was observed (Fig. 1).

Temperature-dependent mobility of receptor sites

In initial experiments, cells reacted with transferrin source, rabbit anti-transferrin, and FITC sheep anti-rabbit Ig at 4°C were subjected to a further period of incubation at 37°C for varying time periods prior to fixation in glycerol. Several differences in the fluorescence patterns were observed when cells were compared with similar cells treated at 4°C throughout. Thus, after 5–10 min of incubation at 37°C, aggregation or 'patching' of fluorescent granules was detected on greater than 90% of positive cells (Fig. 2). After 10–60 min of incubation, patching was still present in 50–70% of positive cells and occasional polar 'capping' of bound transferrin was detected (Fig. 2), but the

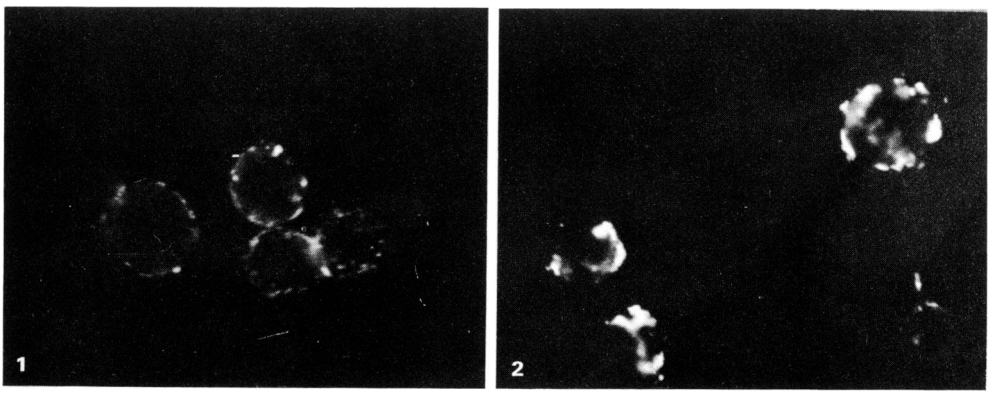

Fig. 1. Membrane fluorescence of bound transferrin on PHA-activated human lymphocytes. Cells were serially reacted at 4°C with normal human serum, rabbit anti-transferrin and sheep anti-rabbit Ig FITC. Note random granular pattern of fluorescence.

Fig. 2. Patching of bound transferrin on PHA-activated lymphocytes. Cells treated as in Fig. 1, then incubated at 37°C for 10 min. Note aggregation of fluorescent granules and rare cap formation.

proportion of cells displaying the latter pattern never increased above 5%, and large fluorescent granules were seen in the suspending medium. However, when incubation at 37°C was prolonged over 10–15 min, an increasing number of cells was detected in which the fine random distribution of granules had reappeared. Since the latter finding appeared to represent reattachment of shed ligand due to spontaneous continued appearance of membrane receptor sites in a random distribution, parallel experiments were performed in which cells were treated to induce blockade of receptor expression by incubation at 37°C for 30 min in the presence of cycloheximide (25 μg/ml) prior to performance of the binding reaction. These studies showed that incubation of treated cells at 37°C resulted in a gradual decrease in the number of patched and capped cells from approximately 80% at 10 min to between 15 and 30% at 30 min. This proportion remained constant even when the incubation step was expanded to 4 hr, and no cells demonstrating the secondary appearance of a random distribution of fluorescence were seen. These findings suggested that, during incubation of the cells at 37°C, the transferrin–receptor complexes moved laterally within the cell membrane to form patches and caps, some of which at least were shed into the medium. Moreover, in the absence of cycloheximide, this process was accompanied by the reappearance of transferrin receptors on the membrane.

In the second group of experiments, comparison was made of the effects of exposure of cells to 37°C during one, two or all three of the reaction layers of the immunohistological procedure. Thus, in order to determine whether the binding of transferrin could itself result in mobility of the membrane receptor, mitogen-stimulated cells were incubated at 37°C with transferrin source for varying time periods, again in the presence of cycloheximide. The cells were then cooled to 4°C, and the remaining two reaction layers performed at this temperature. The results of these studies were similar to those obtained in the first 'cold–hot' experiments, indicating that binding of transferrin could alone initiate mobility and disposal of its receptor (Table 1). Performance of two of the reaction layers at 37°C resulted in a further decrease in the proportion of patched cells, and when the entire three-layer procedure was performed at 37°C, membrane-bound transferrin was detected on very few cells. Concurrent with this progressive decrease in membrane-bound transferrin, increasing amounts of large fluorescent granules were visible in the suspending medium (Table 1). Further reaction of these cells with transferrin and antisera at 4°C did not result in detectable transferrin-binding. In the absence of cycloheximide, these procedures at 37°C resulted in a small residual number (0–10%) of positive cells which increased to 30–50% after further incubation of cells at 4°C with transferrin and antisera, indicating binding of freshly added ligand to newly developed receptor sites. The length of incubation steps necessary to cause loss of membrane-binding sites

Table 1. Temperature dependence of bound transferrin (TF) distribution. Representative experiment, PHA-activated MNC*

Temp. of incubation			Per cent positive cells			
TF	RαTF	SαR FITC	Random	Patched	Capped	Free granules
C	C	C	82	0	0	0
C	C	H	28	40	4	±
C	H	H	34	25	3	+
H	C	C	30	38	4	±
H	H	C	32	24	3	+
H	H	H	0	8	1	++

C = incubation at 4°C; H = incubation at 37°C. RαTF = rabbit anti-human transferrin; SαR FITC = sheep anti-rabbit Ig FITC.

* Cells were incubated with cycloheximide (25 μg/ml) for 30 min at 37°C and washed prior to transferrin-binding in order to induce blockade of new receptor expression.

showed variations both with different mitogens and individual cell donors, but was always between 10 and 30 min.

Internalization of transferrin–receptor complexes

Since lateral movement of receptor sites is frequently followed by endocytosis of ligand–receptor complexes in addition to shedding, experiments were performed in order to determine if cellular internalization of bound transferrin occurred during the 'cold–hot' procedures performed. For this purpose, cells treated with cycloheximide and reacted as described above were examined in parallel as suspensions and after cytocentrifugation and fixation in acetone at −20°C for 10 min. The results are shown in Table 2. As anticipated, no cytoplasmic fluorescence compatible with endocytosis of transferrin complexed to receptors could be discerned in cells maintained throughout at 4°C (CCC) whether examined as suspensions or after cytocentrifugation (Table 2; Expts Ia, b). Similarly, when the primary reaction was carried out at 37°C (HHH), examination of suspensions revealed the expected low proportion of positive cells, and even after secondary reaction with all three layers at 4°C, $< 10\%$ of cells demonstrated membrane fluorescence (Expt IIa and see above). In contrast, however, upon examination of such cells after cytocentrifugation, and with no further manipulation (Expt IIb), fluorescent granules of similar size to membrane patches were observed in the cytoplasm in approximately 20% of cells, indicating that transferrin had been endocytosed in association with its receptor. Subsequently, to investigate the possibility that this procedure resulted in underestimation of the proportion of cells which had endocytosed receptor sites during exposure at 37°C, such cytocentrifuged cells were secondarily reacted at 4°C with either second antibody layer (FITC conjugate) alone (Expt IIc), or first and second antibody layers sequentially (Expt IId), or ligand followed by both antibody layers (Expt IIe). These procedures resulted in a progressive increase in the percentage of cells containing cytoplasmic granules, from a mean of 20% with no further treatment to 30% with conjugate alone, and to $> 50\%$ when either two or all three of the reaction layers were repeated. These findings indicated that endocytosis of transferrin–receptor complexes occurred during incubation of the cells at 37°C, and further suggested that this process could be accomplished even when specific antibody was bound to the complex.

Effect of cytoskeletal and metabolic inhibitors

None of the inhibitors tested affected the binding of transferrin by activated lymphocytes at 4°C either in terms of the intensity of fluorescence or the proportion of cells positive, nor did the presence of DMSO (the cytochalasin B solvent) in appropriate dilutions modulate the binding or

Table 2. Temperature dependence of distribution of transferrin. Representative experiment, PHA-activated MNC

Experiment	Primary reaction layers			Suspension/ cytocentrifuge	Secondary reaction layers			Per cent positive	Fluorescence pattern
	1	2	3		I	II	III		
Ia	C	C	C	Susp.	—	—	—	80	M
Ib	C	C	C	Cyto.	—	—	—	80	M
IIa	H	H	H	Susp.	C	C	C	< 10	M
IIb	H	H	H	Cyto.	—	—	—	20	Cy
IIc	H	H	H	Cyto.	—	—	C	30	Cy
IId	H	H	H	Cyto.	—	C	C	> 50	Cy
IIe	H	H	H	Cyto.	C	C	C	> 50	Cy

Primary reaction layers: 1 = transferrin source; 2 = rabbit anti-transferrin; 3 = sheep anti-rabbit Ig FITC. C = incubation at 4°C; H = incubation at 37°C.
Fluorescence pattern: M = membrane; Cy = cytoplasmic.

mobility of transferrin. Serum and conjugate controls in studies of the effect of all agents upon temperature-dependent mobility were uniformly negative.

Both sodium azide and cytochalasin B clearly inhibited cap formation and shedding of bound transferrin (Table 3). This effect was concentration-dependent, and cells from different donors showed different behaviour at the same inhibitor dose. However, at the optimum concentrations (30 mM sodium azide, 100–200 μM cytochalasin B) approximately 50% of cells displayed fluorescence when the transferrin-binding reactions were performed at 37°C, whereas less than 10% of cells were positive in their absence. All the positive cells displayed patching of fluorescence and no caps were seen. In contrast, at all sublethal doses employed, none of the microtubule inhibitors (colchicine, vinblastine, vincristine) affected the fluorescence pattern of bound transferrin at 37°C. Furthermore, when cells were treated simultaneously with cytochalasin B and colchicine, the results obtained were identical to the effect of cytochalasin B alone. The results of one representative experiment are shown in Table 3.

In subsequent experiments, cells in suspension were treated in identical fashion (incubation with inhibitor followed by the three-layer transferrin-binding reaction at 37°C) and used to make cytocentrifuge preparations. These were examined directly, then subjected again to indirect immunofluorescence of transferrin in order to determine the effect of these inhibitors on endocytosis of bound ligand. The results obtained contrasted with the action of the agents on lateral membrane mobility. Thus, in preparations of cells treated with colchicine, vincristine or vinblastine, or sodium azide, the proportion of cells containing fluorescent cytoplasmic granules was less than 10%, whereas in untreated cells or those exposed to cytochalasin B, greater than 50% of cells were found to contain cytoplasmic granules after further reaction with antiserum to transferrin and FITC conjugate. These results suggest that cytochalasin B inhibited lateral membrane mobility of receptor–ligand complexes, microtubule inhibitors prevented internalization of patched complexes and azide inhibited both events. Treatment of cells with mixtures of cytochalasin B and colchicine resulted in simultaneous inhibition of lateral mobility and internalization.

Relationship with mitogen-binding structures

The double-label immunofluorescence studies of cells activated by Con A-RITC permitted simul-

Table 3. Effect of metabolic and cytoskeletal inhibitors on membrane mobility of bound transferrin in activated lymphocytes. Representative experiment, PHA-stimulated cells

Temperature of binding reaction (°C)	Reagent	Activity	Dose	Per cent positive cells		
				Patched	Capped	Random
4	PBS	—	150 mM	0	0	84
37	PBS	—	150 mM	7	2	0*
37	Sodium azide	Metabolic poison	5 mM	9	0	0
			10 mM	33	0	0
			15 mM	40	0	0
			30 mM	49	0	0
37	Cytochalasin B	MF inhibitor	20 μM	11	0	0
			100 μM	42	0	0
			200 μM	60	0	0
37	Colchicine	MT inhibitor	10 μM–10 mM	<10	0–5	0
37	Vinblastine	MT inhibitor	10 μM–10 mM	<10	0–5	0
37	Vincristine	MT inhibitor	10 μM–10 mM	<10	0–5	0
37	Cytochalasin B +colchicine		200 μM 100 μM	46	0	0

* Many large fluorescent granules in medium.

MF = microfilament; MT = microtubule.

taneous examination of the Con A and transferrin receptor sites. At the optimum stimulation dose of Con A-RITC, activated cells harvested from 37°C culture at 72 hr and fixed either immediately or after varying times of incubation at 22° or 4°C showed similar patterns of membrane RITC fluorescence. Thus, in each case, approximately 70% of cells displayed patching of fluorescent granules and 30% of cells were capped. In contrast, the pattern of transferrin-binding was dependent on temperature. Thus, when the binding reaction was performed on these cells at 4°C throughout, the fine granular fluorescence of transferrin contrasted markedly with the patch-and-cap formation of the Con A-RITC. when incubations with transferrin source and first antibody layer were performed at 4°C ('cold–cold–hot') the patching and rare cap formation of bound transferrin were found to be clearly distinct geographically on the cell membranes from the red fluorescence of Con A-RITC. Finally, when the entire procedure was performed at 37°C, very little transferrin fluorescence was observed, as described above, whereas the Con A-RITC pattern remained unchanged.

DISCUSSION

Our results demonstrate membrane mobility of bound transferrin, and hence of the transferrin receptor site, in human MNC activated by mitogenic lectins. The sequence of events resulting in this phenomenon appears to be similar to that found to occur during lateral movement of other integral components of cell membranes, such as the surface immunoglobulin of B lymphocytes (Raff & De Petris, 1973). Thus, at low temperatures the receptors are randomly distributed throughout the membrane, and warming of the cells results in aggregation or patching of receptors. These patches can then coalesce at one pole of the cell to form a 'cap' (Raff & De Petris, 1973). It is generally accepted that the ultimate fate of such a cap is either to be shed from the cell or to be internalized (Unanue & Karnovsky, 1973). In the present study, the finding of large fluorescent granules in the medium of the cells in suspension, and of intracellular granules in cytocentrifuged cells subjected to the temperature-dependent techniques described in this report is suggestive of both cap-shedding and internalization respectively in association with membrane mobility of transferrin receptor sites.

It has been clearly demonstrated that aggregation of membrane receptors can be initiated at physiological temperatures as a result of cross-linking by ligand or antisera used in its detection (Unanue, Perkins & Karnovsky, 1972). However, patching of receptors for apparently monovalent ligands such as insulin appears to be dependent upon receptor occupancy by the ligand, and is followed by internalization of the aggregated receptor–ligand complexes (Maxfield *et al.*, 1978). The results of the present study suggest that a similar sequence of events occurs when transferrin binds to its receptor sites on activated lymphocytes. Thus, when cells are harvested from culture at 37°C in the absence of human transferrin, receptor sites are present in a random distribution reflected by the fine granular fluorescence of transferrin subsequently bound at 4°C. Moreover, when similar cells are incubated with transferrin source at 37°C prior to reaction with antisera at 4°C (hot–cold–cold), marked aggregation of receptor sites is demonstrated, indicating that this event is initiated by receptor occupancy by ligand. This effect is enhanced by performance of the entire procedure at 37°C (hot–hot–hot), presumably as a result of cross-linking of the bound ligand by divalent antisera. Finally, the examination of these cells in cytocentrifuge preparations reveals clear evidence of endocytosis of patched receptor–ligand and receptor–ligand–antisera complexes. When such preparations are reacted either with transferrin and antisera, or with antisera alone, no differences in the percentage of cells containing fluorescent intracellular granules is found ($> 50\%$), providing further evidence that receptor sites are not aggregated or internalized unless occupied by ligand.

The mechanisms responsible for mobility of integral components within the lipid bilayer of the cell membrane are poorly understood. Extensive investigations of the mobility of certain components, including lectin receptors and membrane immunoglobulins of B lymphocytes, have implicated a role for the cytoskeletal system in this particular aspect of cell behaviour (De Petris, 1975; Nicolson & Poste, 1976; Sundqvist & Ehrnst, 1976). It is also accepted that cap formation is an active process requiring intact metabolic function in a viable cell (Unanue & Karnovsky, 1973).

Low temperatures will inhibit both metabolic and cytoskeletal function. In the present study, reaction of cells at low temperatures and treatment of cells with sodium azide at sublethal dosages clearly inhibited cap formation and disposal of bound transferrin. Similar results were obtained following treatment with the microfilament inhibitor cytochalasin B. In contrast, disruption of microtubular function by colchicine, vincristine and vinblastine did not appear to affect lateral membrane mobility of the transferrin receptor. These results are consistent with previous studies of other membrane components such as lymphocyte Con A receptors, immunoglobulin and θ antigen (De Petris, 1975; Nicolson & Poste, 1976). Certain of these studies have also yielded information suggesting a synergistic action of cytochalasin B and colchicine upon the lateral mobility of membrane components (De Petris, 1974): in the present investigation, we were unable to detect such an effect. Thus, lateral mobility of transferrin receptors in mitogen-activated lymphocytes appears to be dependent upon temperature, metabolic integrity and a particular cellular phenomenon (possibly microfilament function) which is inhibited by cytochalasin B. In contrast, internalization of receptor–ligand complexes appears to depend upon microtubular and metabolic function. These results are similar to those reported by Hemmaplardh *et al.* (1974), who demonstrated reduced 'uptake' of radiolabelled transferrin by rabbit reticulocytes in the presence of microtubule inhibitors. Studies of cells activated by fluorochrome-labelled mitogen revealed no spatial or kinetic relationship between transferrin and lectin receptors.

It is interesting to compare the data reported here with our results in previous studies of the membrane mobility of the transferrin receptor sites of human transformed and malignant cells (Galbraith *et al.*, 1980b). In those investigations, we were unable to detect mobility of bound transferrin in thirteen of sixteen cell lines of lymphoblastoid and malignant origin, and some patching but very rare cap formation in the other three cell lines. Changes in receptor mobility in association with cellular transformation is well recognized (Nicolson, 1976). In the context of membrane transferrin receptors, this difference between mitogen-activated normal cells and cell lines previously described suggests a possible modification of receptor activity or role in the abnormal cell.

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