Mobility of Surface Proteins on Normal Rat Macrophages and on a "Macrophagelike" Rat Tumor

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ABSTRACT Peritoneal macrophages endocytosed their histocompatibility antigens (RT1), Fc receptors (FcR), and concanavalin A (Con A) receptors after cross-linking by ligands, but did not cap these membrane proteins. The 323N cell, a "macrophage like" tumor cell, under identical conditions capped its surface proteins. Experiments measuring fluorescence recovery after photobleaching showed that the mobile fraction of RT1 was significantly greater in 323N cells than in normal peritoneal macrophages. Presumably, the membrane proteins of 323N are not as tethered to the cytoskeleton, or, if so, are in a nexus that is not the same as that which occurs between membrane proteins of normal macrophages and the cytoskeleton. The mobility of RT1 on normal lymphocytes was also different from that of macrophages. These observations suggest that the movement of membrane molecules is determined by cell type and is regulated by the cytoskeleton which varies in structure and function from cell type to cell type.

Capping and mobility of integral membrane proteins are regulated by the cytoskeleton (3, 4, 6, 7, 17) and may be modulated by changes in membrane fluidity (1, 8, 16) and changes in cytoskeletal structure and function. We began this study to examine the lateral mobility of the same membrane molecules in macrophages, tumor cells resembling macrophages, and lymphocytes. Several investigators have described alterations of membrane protein mobility in transformed cells, with augmented or diminished lateral mobility in different transformed elements (1, 5, 9). Furthermore, in lymphocytes, capping and mobility of surface immunoglobulin and histocompatibility antigens (15–17) reveal different kinetics and diffusion coefficients.

In this study we found that the same integral membrane proteins, histocompatibility antigens (RT1), Fc receptors (FcR), and concanavalin A (Con A) receptors were rapidly endocytosed by peritoneal macrophages after cross-linking by ligands. In tumor cells resembling macrophages (323N), these membrane proteins were capped after binding ligands. Fluorescence recovery after photobleaching (FRAP) showed that the mobile fraction of RT1 was greater in the transformed cell. Colchicine augmented the number of tumor cells with caps but did not affect normal macrophages. Mobility of RT1 and Con A receptors in normal lymphocytes was different from that found in normal macrophages and similar to that of 323N cells.

MATERIALS AND METHODS

Cells

Resident macrophages were harvested from the peritoneal cavity of male Lewis rats by injecting 35–50 ml of Minimum Essential Medium (MEM) into the peritoneal cavity, massaging the abdomen, and removing the fluid. After centrifugation, cells were washed two times in MEM containing 10% newborn bovine serum (NBS) (Flow Laboratories, Inc., Rockville, Md.) and were suspended in that medium. Elicited macrophages were similarly harvested 3–6 d after the intraperitoneal injection of 10 ml of 10% proteose-peptone. 50–60% of resident peritoneal cells and 80–90% of elicited peritoneal cells were nonspecific esterase positive. After adherence to glass, >95% of cells in both groups were nonspecific esterase positive. Twice as many cells were recovered from the peritoneal cavities of rats that received proteose-peptone. These cells spread more readily than resident macrophages.

Lymphocytes were obtained by preparing cell suspensions from normal Lewis rat spleens. Lewis rats, from the breeding colony of the Research Institute of Scripps Clinic, were adult males and females, 12–16 wk old, maintained in a light- and temperature-controlled environment.

Tumor cells resembling macrophages were obtained from the spleens of rats

bearing 323N, a spontaneous tumor derived from the spleen of a male Lewis rat. The tumor line has been passaged in vivo for 24 months, and is fatal within 15–25 d after intraperitoneal transfer of 10^7 tumor cells, with terminal leukemia and splenomegaly up to 25 g. 323N cells display many of the properties of macrophages. They are irregular in shape, have an eccentric nucleus, a prominent Golgi complex zone, and the cytoplasm of some cells contains small azurophilic granules. The cells adhere to and spread on glass. 90–95% are nonspecific esterase positive (NaFl inhibitable); >80% rosette with IgG-coated sheep red blood cells; 30% rosette with IgE-coated sheep red blood cells; 10–15% phagocytosed IgG-coated sheep red blood cells or opsonized Zymosan particles. A detailed description of this tumor will be presented elsewhere.

Antibodies and Immunoglobulin Reagents

RT1 were detected with Brown Norway (BN) anti-Lewis alloantibody conjugated with florescein (Fl) prepared as previously described (16). In a direct immunofluorescence assay, the BN anti-Lewis alloantibody stained >95% of Lewis spleen cells, resident and elicited peritoneal macrophages, and 323N cells. Fl-labeled Lewis anti-BN alloantibody (control) stained <1% of these cells. Fl-Fab' fragments of BN anti-Lewis alloantibody were prepared as described previously (16). Other reagents were: (*a*) rabbit IgG and $F(ab')_2$ fragments of rabbit IgG, and rabbit antibody to rat γ chain (RARtG) (16); (*b*) aggregated rabbit IgG, prepared by heating a solution of 2.5 mg/ml of rabbit IgG in phosphate-buffered saline at 63°C for 20 min. This latter material was centrifuged at 2,000 *g* for 15 min, and the supernate was aliquoted and stored at −70°C. It was used within 2 wk; (*c*) rhodamine (Rh) and Fl $F(ab')_2$ goat anti-rabbit IgG (GARb) obtained from N. L. Cappel Laboratories, Inc. (Cochranville, Pa.) and checked for specificity by double diffusion in gel.

Redistribution of Surface Molecules: Cells in Suspension

RT1-2×10^6 resident or elicited peritoneal macrophages, 3×10^6 lymphocytes, and 2×10^6 323N cells in 100 μl of MEM-NBS, were stained with 25 μl of Fl-BN anti-Lewis antibody for 30 min. at 4°C, washed twice, and incubated with 20 μg of Fl-RARtG for 30 min. at 4°C. The cells were washed twice, resuspended in 0.5 ml of warm (37°C) MEM-NBS, and incubated at 37°C for 5–30 min. The time of incubation at 37°C influences the extent but not the pattern of redistribution. Redistribution was stopped by adding 200 μl of 4% paraformaldehyde (final concentration 1.4%). After 10 min, cells were washed, mounted on glass slides, and placed on coverslips. Similar results were obtained when cells were stained with Fl-BN anti-Lewis antibody alone.

FcR were detected by incubating cells with 25 μg of heat-aggregated rabbit IgG and then staining with Fl-GARb. With this method, >90% of all cells were fluorescent. When heated, $F(ab')_2$ was substituted for heat-aggregated IgG, <1% of the cells were fluorescent.

Con A Receptors

Con A receptors wre visualized by incubating cells with 50 μg/ml of Fl-Con A (Miles Laboratories, Elkhardt, Ind.) in MEM–1% NBS. All cells bound the Fl-Con A.

Adherent Cells

12-mm round glass cover slips (Bellco Glass Co., Vineland, N. J.) placed in 35-mm plastic petri dishes (BBL Microbiology Systems, Becton, Dickinson & Co., Cockeysville, Md.) were overlain with 323N cells at a concentration of 1.2×10^7/ml; resident peritoneal cells at a concentration of 1×10^7/ml; or elicited peritoneal cells at a concentration of 5×10^6/ml. After 1 h (peritoneal cells) or 2 h (323N cells), the nonadherent cells were removed by repeated rinsing of the plates with warm media. Experiments were performed 2.5 h after the initial plating of the cells. RT1, FcR, and Con A receptors were identified with the same reagents and at the same final concentrations as described for cells in suspension. After completion of staining and redistribution for 5 or 15 min, the cells were fixed with 2% paraformaldehyde, washed, and the cover slips were mounted with elvanol on glass slides.

Suspension and adherent cells were examined with a Zeiss fluorescence microscope equipped with Rh and Fl filters. 100–200 positively stained cells were observed to ascertain the pattern of staining. Stained cells were classified as "rings" when the entire cell circumference was outlined by fluorescent material in a thin or beaded line; capped, when less than one-third of the membrane was fluorescent; and patched-endocytosed when large fluorescent dots were present on the cell surface or inside the cells.

Colchicine Treatment

Adherent and suspension cells were treated with 1×10^{-5} M colchicine for 60 min before treatment with antibodies or Con A. Colchicinized cells were kept in colchicine-containing media for the entire experiment.

FRAP

The diffusion of membrane lipids and integral membrane proteins can be measured by FRAP (11, 12). An intense laser light pulse irreversibly bleaches a fluorophore attached to a lipid or protein probe. Fluorescence in a bleached region recovers due to the entry of fluorophore from adjacent parts of the membrane. The mobile fraction of fluorophore is determined by the magnitude of fluorescence recovery in the bleached area. Cells stained with Fl-Fab' anti-RT1 were used for FRAP experiments which were done as previously outlined (17), except that the bleaching pulse was 0.2 s. The percentage of fluorescence recovery was calculated as follows:

$$\% \text{ Recovery} = \frac{I_A(m) - I_A(0)}{I_B(0) - I_A(0)} \times 100$$

where $I_A(m)$ is the maximal fluorescence intensity at the end of recovery, $I_B(0)$ and $I_A(0)$ are, respectively, the fluorescence intensity immediately before and after bleaching. The fraction of fluorescence that returns to the bleached area represents the fraction of mobile fluorophore within the time-span of the experiments. Diffusion coefficients were calculated as previously described (17).

RESULTS

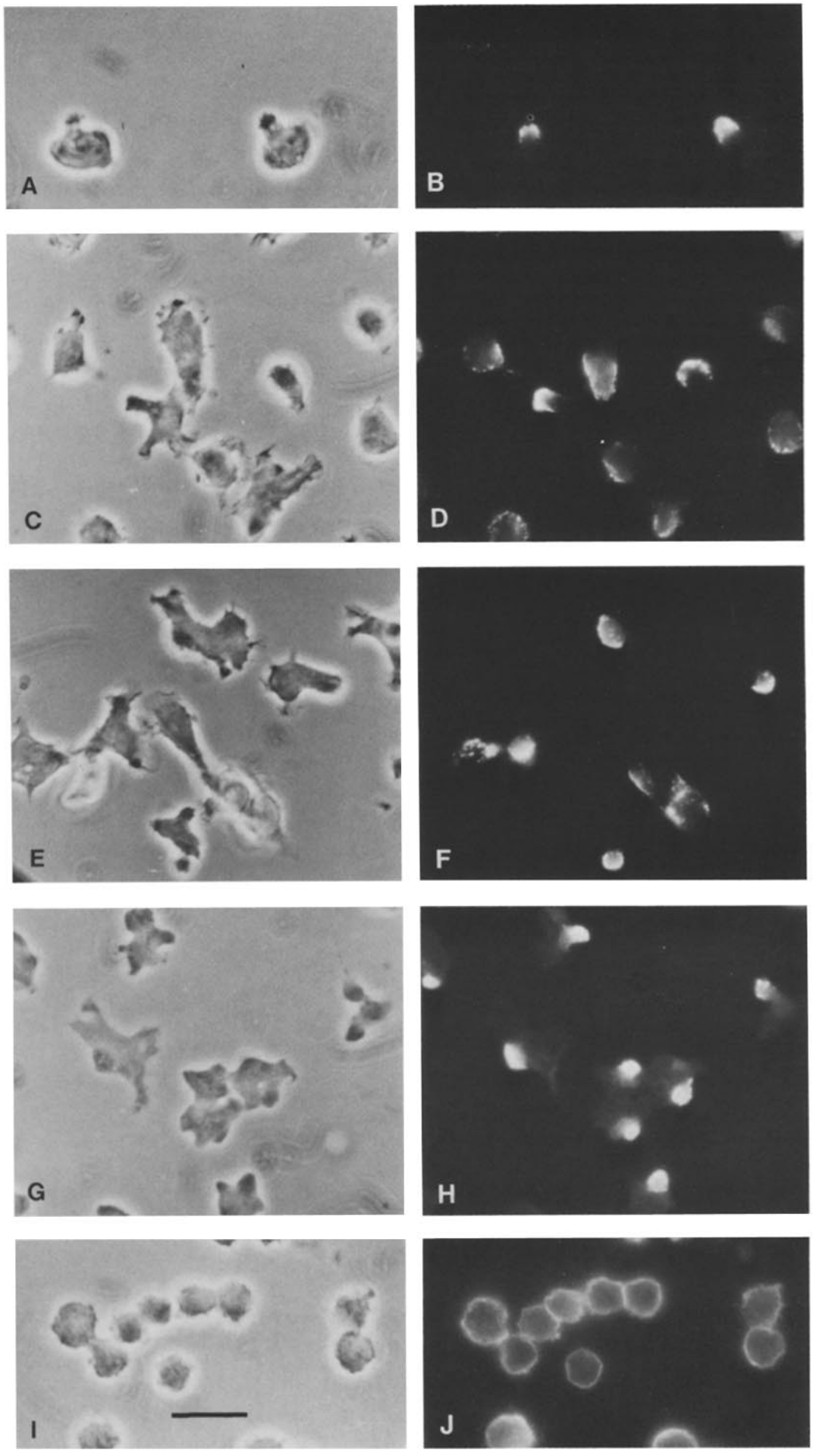
Redistribution of Surface Proteins: 323N Cells

323N cells in suspension were treated with ligands reacting with RT1 antigens, FcR, and Con A receptors and incubated at 37°C for 15 (RT1, FcR) or 30 min (Con A). These membrane proteins were readily redistributed into caps, which were localized to uropods (Fig. 1 *a* and *b*). The capping of these molecules was enhanced by colchicine treatment (Table I).

323N cells, adherent to glass cover slips, were reacted with the same reagents and under the same conditions of staining and redistribution. Within 5 min after incubation at 37°C, redistribution of RT1 was evident (Fig. 1 *c* and *d*). Some cells were outlined by patches and beads of fluorescent material and others showed caps. Within 15 min, most cells had capped (Fig. 1 *e* and *f*). Caps were seen on the membrane close to the nucleus or in pseudopods. Colchicine enhanced capping of adherent cells (Table I); cells capped more quickly and showed "tighter" caps which were present in uropodlike structures (Fig. 1 *g* and *h*).

When 323N or colchicinized 323N cells were stained at 4°C, washed, and fixed with paraformaldehyde, or else were fixed with paraformaldehyde at 37°C and then stained, they all displayed "ring" fluorescence (Fig. 1 *i* and *j*).

FIGURE 1 Distribution of surface proteins on 323N cells: *A*, suspension cells, phase-contrast; *B*, RT1 caps after 15 min at 37°C; *C*, adherent cells, phase-contrast; *D*, RT1 was redistributed into patches and caps by 5 min at 37°C; *E*, adherent cells, phase-contrast; *F*, RT1 was capped on most cells after 15 min at 37°C; *G*, adherent cells, colchicine-treated, phase-contrast; and *H*, RT1 caps on colchicinized cells at 15 min at 37°C. Note the uniform redistribution of RT1 on uropodlike structures: *I*, adherent cells, phase-contrast; and *J*, cells were labeled with Fl-Con A at 4°C, washed, and paraformaldehyde-fixed. Bar, 10 μm. × 1,300.


A
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Resident and Elicited Macrophages

The ligand-induced redistribution of surface proteins present on resident and elicited peritoneal macrophages in suspension was entirely different from that observed in 323N cells in suspension. In normal macrophages, patches of fluorescent material were present on the plasma membrane or within the cytoplasm and were occasionally concentrated at one pole of the cell (Fig. 2 *a* and *b*). Some of this material clearly outlined endocytic vesicles. These cells did not cap the bound fluorescent probe (Table I), did not form uropods, and did not display caps when exposed to colchicine.

TABLE I

Redistribution of Membrane Proteins on 323N, Cells Peritoneal Macrophages and Lymphocytes *

	RT1	RT1-colchicine	FcR	FcR-colchicine	Con A	Con A-colchicine
			% of capped cells‡			
323N cells in suspension	52 ± 3(6)§	86 ± 2(6)	40 ± 5 (3)	83 ± 8 (3)	29 (1)	48 (1)
323N cells adherent	67 ± 7(3)	92 ± (3)	78 ± 4 (3)	90 ± 4 (3)	39 (1)	87 (1)
Suspension			*% of cells with patching and endocytosis‖*			
Resident macrophage	96 ± 2(3)	99 ± 1(3)	95 ± 7 (3)	97 ± 5 (3)	100 (1)	100 (1)
Elicited macrophage	97 ± 1(3)	98 ± 2(3)	96 ± 6 (3)	99 ± 2 (3)	100 (1)	100 (1)
Adherent						
Resident macrophage	91 ± 1(3)	96 ± 2(3)	92 ± 3 (3)	93 ± 2 (3)	94 ± 3 (3)	95 ± 3 (3)
Elicited macrophage	98 ± 3(3)	98 ± 1(3)	100 (1)	100 (1)	100 (1)	100 (1)
			% of capped cells¶			
Lymphocytes	72 ± 3(4)	91 ± 2(3)	—	—	31 ± 2 (4)	60 ± 2 (3)

* The data show the redistribution of RT1 and FcR at 15 min and Con A at 30 min.
‡ Endocytosis by 323N cells is not apparent within the time-span of the experiments.
§ % ± S. E. M. In parentheses, number of separate experiments. In each experiment, 100–200 cells were recorded.
‖ There is no capping by resident or elicited macrophages.
¶ This is the percentage of capped cells and cells that have endocytosed their caps.

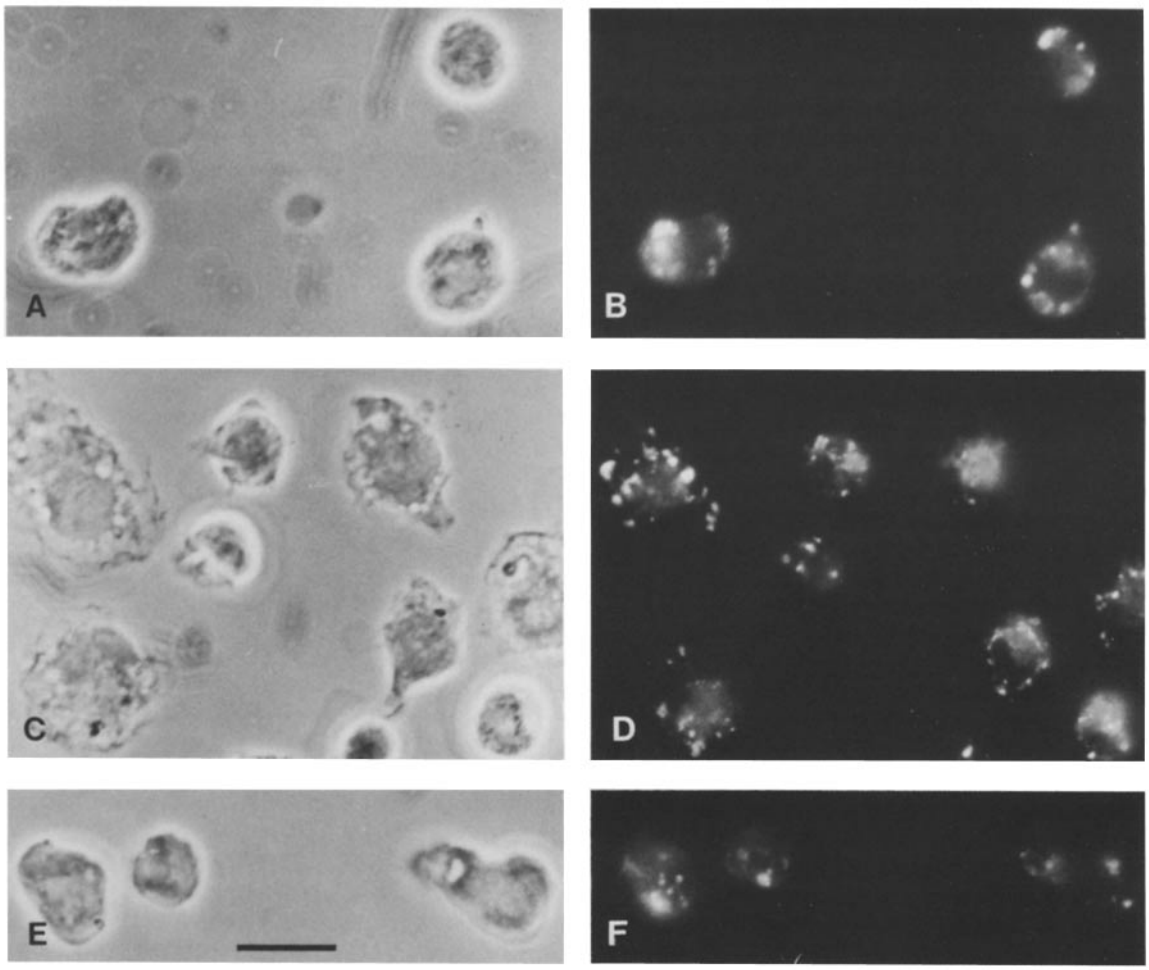

FIGURE 2 Distribution of surface proteins on macrophages: *A*, suspension cells, phase-contrast; *B*, RT1 was endocytosed at 15 min at 37°C; *C*, adherent cells, phase-contrast; *D*, FcR were endocytosed at 15 min at 37°C; *E*, adherent cells, colchicine-treated, phase-contrast; and *F*, RT1 was endocytosed by colchicinized cells for 15 min at 37°C. Bar, 10 μm. × 1,300.

In adherent resident and elicited peritoneal macrophages, patching and endocytosis of the fluorescent probes were observed (Fig. 2 *c* and *d*). No caps were detected. With colchicine treatment there was also no evidence of cap formation even though ameboid cells were seen (Fig. 2 *e* and *f*).

Experiments were performed on peritoneal macrophages to ascertain that the bound complex, consisting of RT1, rat anti-RT1 antibody and Fl-labeled RARtG was actually endocytosed and that the polar coalescence of Fl-labeled material was intracellular and was not present as a cap in the plasma membrane. We stained paraformaldehyde-treated and paraformaldehyde-treated, acetone-fixed preparations with rhodamine-labeled goat anti-rabbit IgG (Rh-GARb). If the Fl-labeled material had been endocytosed, no binding of the Rh labeled antibody would occur in paraformaldehyde-treated cells but would bind after penetrating paraformaldehyde-treated, acetone-fixed cells. These studies showed that after 15 min approximately one-half of the Fl-labeled dots were stained with the Rh-GARb in paraformaldehyde-fixed preparations. After acetone fixation, all of the Fl-labeled material was stained by Rh-GARb. When paraformaldehyde-fixed 323N cells were studied with the same procedure, all the Fl-labeled material was stained with Rh-GARb.

Lymphocytes

The pattern of redistribution of membrane proteins in lymphocytes was similar to that of 323N cells. Lymphocytes readily redistributed their RT1 antigens and Con A receptors into caps after treatment with ligands reacting with these proteins. The capping of these molecules was enhanced by colchicine treatment (Table I).

FRAP

FRAP experiments provide measurements of two parameters of membrane protein mobility: the fraction of the membrane protein which is free to diffuse in the plane of the plasma membrane (mobile fraction) and the rate at which the mobile fraction diffuses (diffusion coefficient).

FRAP experiments performed on cells labeled with Fl-Fab' antibody to RT1 showed that the mobile fraction of RT1 antigen in the membrane of 323N cells was significantly greater than that in resident or elicited peritoneal macrophages and was similar to that of spleen lymphocytes (Table II). The diffusion coefficients of mobile fluorophore were similar in all groups of cells (323N cells, 1.1×10^{-9} cm^2/sec; resident macrophages, 1.3×10^{-9} cm^2/sec; and lymphocytes, 1.5×10^{-9} cm^2/sec). Fl-Fab' anti-RT1 remains in the plane of the plasma membrane of 323N cells and normal macrophages during the time-span of the experiments, producing a thin, uniform, rim labeling of the cell. Immune complexes and Con A cross-link and immobilize their receptors; hence, FcR and Con A receptors were not suitable for FRAP studies.

DISCUSSION

These experiments showed that in cells of a transformed macrophage line, integral membrane proteins, such as RT1, FcR, and Con A receptors, were capped but not endocytosed; in contrast, in normal resident and elicited peritoneal macrophages, the same membrane proteins were endocytosed but not capped. Colchicine enhanced capping by the transformed macrophage, and the caps were located on a uropodlike structure or protuberance. A similar observation has recently been reported (13). While altering the morphology of the normal macrophage, colchicine had no effect on the pattern of membrane protein redistribution.

We can only speculate about the biological meaning of this observation. The differences recorded between transformed and normal macrophages might be due to changes that have occurred in the composition and anatomy of the cell membrane, i.e., the membrane may have altered lipid fluidity, or the RT1, FcR, and Con A receptor molecules may be positioned differently in the membranes of transformed cells. Several groups of investigators have described the altered lateral mobility of certain membrane molecules in transformed cells (1, 5, 10, 18). There may also be an altered relationship of surface molecules to the underlying cytoskeleton. If lateral mobility or immobility of integral membrane proteins is regulated by the cytoskeleton (3, 4, 6, 7, 17) and by the nexus between cytoskeleton and these molecules, then the nexus in transformed cells may be different from that operating in normal macrophages. Finally, the contractile proteins of the cytoskeleton and the biochemical processes involved in their polymerization and depolymerization may also be altered in the transformed cell.

In our study, the mobile portion of RT1, as measured by FRAP, was significantly greater in the transformed cell than the mobile portion of RT1 in normal macrophages, although the diffusion coefficients in each cell type for this molecule

TABLE II

Mobile Fraction of RT1 and Diffusion Coefficients in 323N Cells, Peritoneal Macrophages, and Lymphocytes *

	Recovery	P‡	Diffusion coefficient§
	%		cm^2/sec
323N cells	73 ± 1.4(24)‖		$1.1 \pm 0.1 \times 10^{-9}$
Resident macrophage	36 ± 1.3(27)	<0.001	$1.3 \pm 0.1 \times 10^{-9}$
Elicited macrophage	29 ± 2.4(15)	<0.001	$1.3 \pm 0.2 \times 10^{-9}$
Lymphocytes	77 ± 1.9(15)	NS	$1.5 \pm 0.2 \times 10^{-9}$

* Data shown are the mean percent of fluorescence recovery ± S.E.M. Cells labeled with Fl-Fab' anti-RT1 were used for FRAP experiments. The percent recovery of fluorescence in the bleached area was determined by the following formula:

$$\% \text{ Recovery} = \frac{I_A(m) - I_A(0)}{I_B(0) - I_A(0)} \times 100$$

Where $I_A(m)$ is the maximal fluorescence intensity at the end of recovery, $I_B(0)$ and $I_A(0)$ are, respectively, the fluorescence intensity immediately before and after bleaching. Experimental details are given in materials and methods.

‡ *P* values were calculated by Student's *t*-test and compare 323N cells with macrophages and lymphocytes.

§ Apparent diffusion coefficients were calculated from the values of *t*1/2 as previously outlined (17).

‖ In parentheses, number of bleached cells examined.

were similar. The implication of this is that RT1 is not so tethered to the cytoskeleton in transformed macrophages as it is in normal macrophages; however, the mobile portion of RT1 moves at the same rate in both cells.

The characteristics of capping and lateral diffusion of RT1 in lymphocytes were similar to those of the transformed macrophages and different from those observed in normal macrophages. It appears that normal macrophages are programmed for endocytosis when their membrane proteins are cross-linked, whereas the 323N cells and lymphocytes are programmed to cap their cross-linked membrane proteins. The difference in the capping-endocytosis behavior of these cells may be reflected in the measured difference in the mobile fraction of RT1.

An alternative explanation for the absence of cap formation by normal macrophages is that these cells do not form uropods which are the usual anatomic site for cap formation. We believe that uropod formation is not a strict prerequisite for cap formation by 323N cells because, as is shown on Fig. 1 *c–f*, caps are seen in the absence of classic uropods and, in the presence of trifluoperazine, adherent 323N cells round and form caps on round cells without forming a uropodlike structure (B. Woda. Unpublished observations).

We also compared adherent 323N cells and 323N cells in suspension, adherent macrophages and macrophages in suspension, and resident and elicited macrophages, with respect to capping and diffusion coefficients. There were no significant differences between any two pairs of cell types. It is noteworthy that the membrane proteins of both resident and elicited peritoneal cells exhibited similar patching-endocytosis and lateral diffusion. As measured by these parameters, they were indistinguishable from each other despite their biological differences.

Particularly interesting was the fact that identical patterns of membrane protein redistribution occurred in both adherent and suspension macrophages and 323N cells, even though the array of cytoskeletal proteins differs in rounded and adherent cells (14). This observation raises the possibility that there may be an allocation of functions in the cytoskeletal system; some portions are committed to structural functions; some portions are committed to movement of membrane molecules and endocytosis; and still other parts are engaged in moving organelles within the cell.

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