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Role of Cytoskeletal Elements in Expression of Monocyte Urokinase Plasminogen Activator Receptor, Activation-Associated Antigen Mo3

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Peripheral blood monocytes exposed to bacterial products, phorbol esters, cyclic AMP, and cyclic AMP analogs express cell surface activation protein Mo3, which is the human urokinase plasminogen activator receptor (uPA-R). uPA-R is expressed by circulating monocytes from patients with multiple sclerosis (MS). We examined the role of cytoskeletal elements in the surface expression and subcellular distribution of uPA-R in nonactivated and lipopolysaccharide-activated monocytes and in monocytes from patients with MS. By using immunofluorescence techniques and confocal laser microscopy, we found that in unactivated monocytes, cytoplasmic uPA-R is found to one side of the nucleus, colocalizing with the Golgi. Upon activation with lipopolysaccharide, cytoplasmic Mo3–uPA-R becomes dispersed throughout the cytoplasm and projections concomitant with an increase in the monocyte perimeter (spreading). Cytoplasmic dispersion, as well as cell surface deposition, is dependent on microtubule integrity. Cell surface deposition of uPA-R upon activation is reduced by colchicine, which disrupts microtubules; however, once associated at the cell surface, uPA-R becomes associated with microfilaments via vinculin. Disruption of microfilaments with cytochalasin also alters surface expression of immunologically reactive uPA-R, as well as the distribution pattern. Monocytes from patients with MS display the uPA-R distribution pattern characteristic of an activated monocyte.

Monocytes and macrophages possess cell surface receptors important in recognition, phagocytosis, and cell killing. A cell surface receptor, Mo3, has been described by Todd and colleagues (9, 19) and identified as the urokinase plasminogen activator receptor (uPA-R) (12). Surface uPA-R is induced on peripheral blood monocytes (PBM) and U937 cells exposed to bacterial products, phorbol esters, cyclic AMP (cAMP), cAMP agonists, and protein kinase C (18). While increased surface expression is characteristic of stimulated (activated) monocytes-macrophages, cytosolic antigen is found in both activated and resting monocytes (20). Activation also results in an increase in cytoplasmic uPA-R. Similar cytoplasmic accumulation in producer cells has been shown for endothelial cell growth factor (8), lipocortin (7, 15), tumor necrosis factor (22), and interleukin-1 (11, 16).

In this study, we investigated the subcellular localization of uPA-R in human PBM and the role of the cytoskeleton in translocation of antigens to the cell surface. We demonstrated by immunofluorescence microscopy and confocal laser microscopy (CLM) that cytoplasmic uPA-R may be colocalized with the Golgi in nonactivated monocytes. Upon activation of monocytes with lipopolysaccharide (LPS), uPA-R is distributed through the cytoplasm by microtubules (MT) and colocalizes at the cell surface with microfilaments (MF). Monocytes from patients with multiple sclerosis (MS), in the absence of exogenously added activators, morphologically exhibited antigen distribution comparable to that in LPS-stimulated normal monocytes.

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MATERIALS AND METHODS

Reagents. Cytochalasin B (CB), phorbol myristate acetate, LPS, dibutyryl cAMP, colchicine, and γ- and β-lumicholchicine were purchased from Sigma Chemical Co., St. Louis, Mo. CB was dissolved in 0.1% dimethyl sulfoxide at 1 mg/ml and then diluted in culture medium before use. The following reagents were purchased from Gibco Laboratories, Grand Island, N.Y.: RPMI 1640, L-glutamine, and penicillin-streptomycin. All media and reagents were screened for endotoxin with the *Limulus*

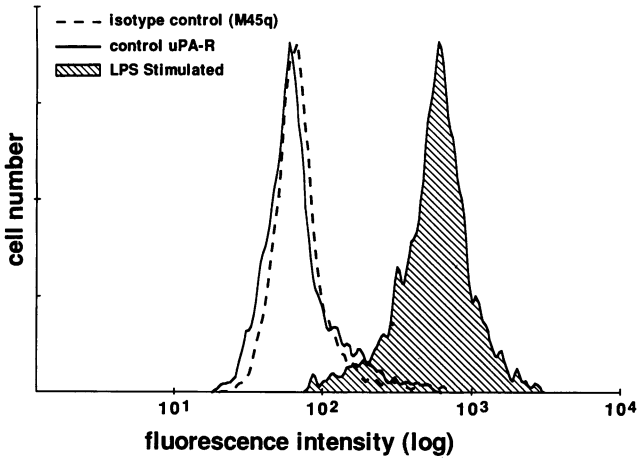

FIG. 1. Expression of uPA-R by PBM (10^6 cells) after exposure to the activating stimulus LPS (50 ng/ml). Cells were cultured in medium alone or with LPS. The cells were harvested and stained by indirect immunofluorescence for expression of uPA-R (cross-hatched) compared with a negative control IgG MAb (open histogram) as measured by flow cytometry.

TABLE 1. Activation of uPA-R with LPS as demonstrated with a fluorescence-activated cell sorter

Treatment	N[a]	Linear FI (mean ± SD)[b]	
		M45/9q	uPA-R
None	5	72 ± 25	290 ± 60
LPS	5	66 ± 10	682 ± 88[c]

[a] N, number of individual volunteers tested. No more than two were examined per experiment.
[b] Mean linear FI as defined by flow cytometric analysis as described in Materials and Methods. A minimum of 20,000 cells were counted per determination.
[c] Significantly different ($P < 0.01$) from values for untreated samples.

TABLE 2. Analysis of uPA-R average FI in activated and nonactivated monocytes by confocal laser cytometry

Monocyte treatment[a] (concn)	N[b]	Linear FI (mean ± SD)
None	56	703 ± 292
LPS (10 mg/ml)	50	1,698 ± 220[c]
cAMP (10^{-5} M)	50	1,003 ± 288[c]

[a] Treatment lasted 24 h.
[b] N, number of monocytes analyzed.
[c] Statistically significantly different from FI for untreated monocytes ($P < 0.01$).

amoebocyte lysate assay (Cape Cod Associates, Woods Hole, Mass.). Only endotoxin-free reagents were used.

Monoclonal antibodies (MAbs) and fluorochromes. Anti-Mo3 (Mo3f; immunoglobulin G2a [IgG2a]) recognizes human uPA-R (9, 18) and was kindly provided by Robert F. Todd, University of Michigan, Ann Arbor. Mo2 (CD14) and OKMI (CD11b) were purchased from Ortho Diagnostic Systems Inc., Raritan, N.J. Isotype-similar, nonspecific MAb MS IgG was purchased from Sigma. We also used another isotype-similar, nonspecific MAb, M45/g (IgG2), provided by Robert F. Todd, which was produced during the same immunization protocols

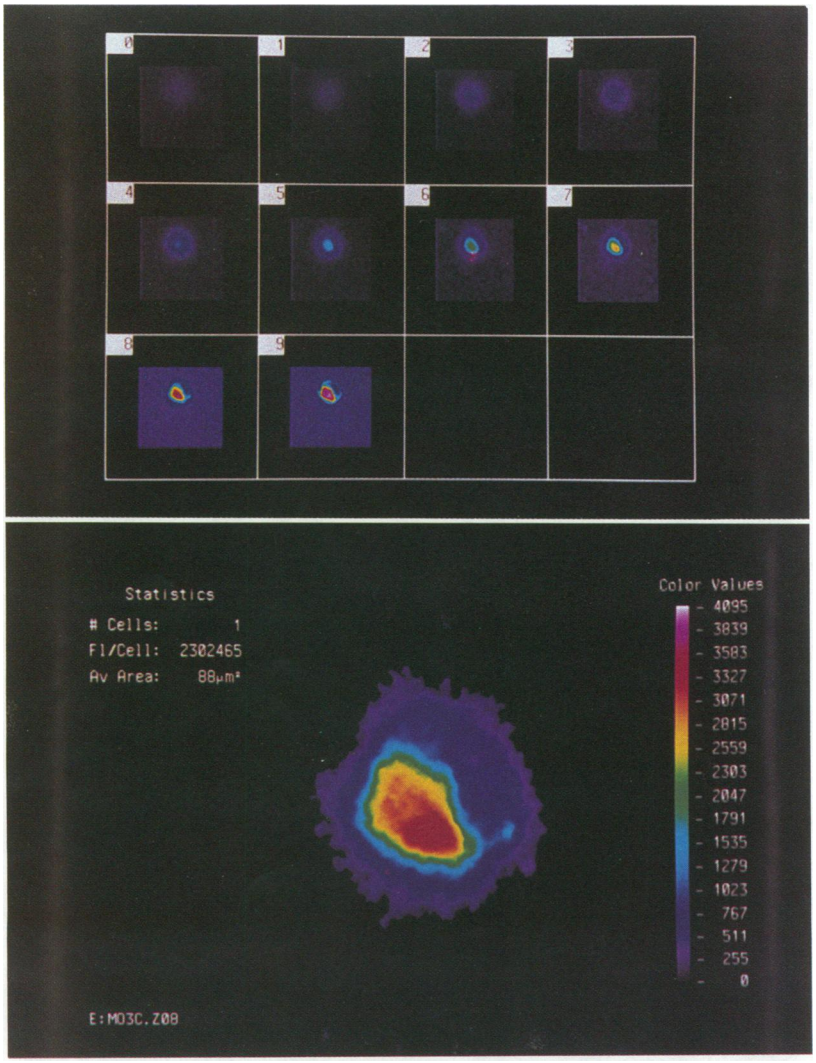

FIG. 2. Cytoplasmic distribution of uPA-R in unactivated monocytes. Cells were allowed to adhere overnight at 37°C, fixed, permeabilized with 0.01% Triton X-100, and then stained for uPA-R expression. CLM was performed to show cytoplasmic distribution (top) as indicated in Materials and Methods. An enlargement of frame 8 is shown in the bottom panel. The laser was set to make a cut at 1-µm intervals through the cell. FI is indicated by color representation as indicated on the side panel (bottom), where blue indicates little to no FI and white or pink indicates very high FI.

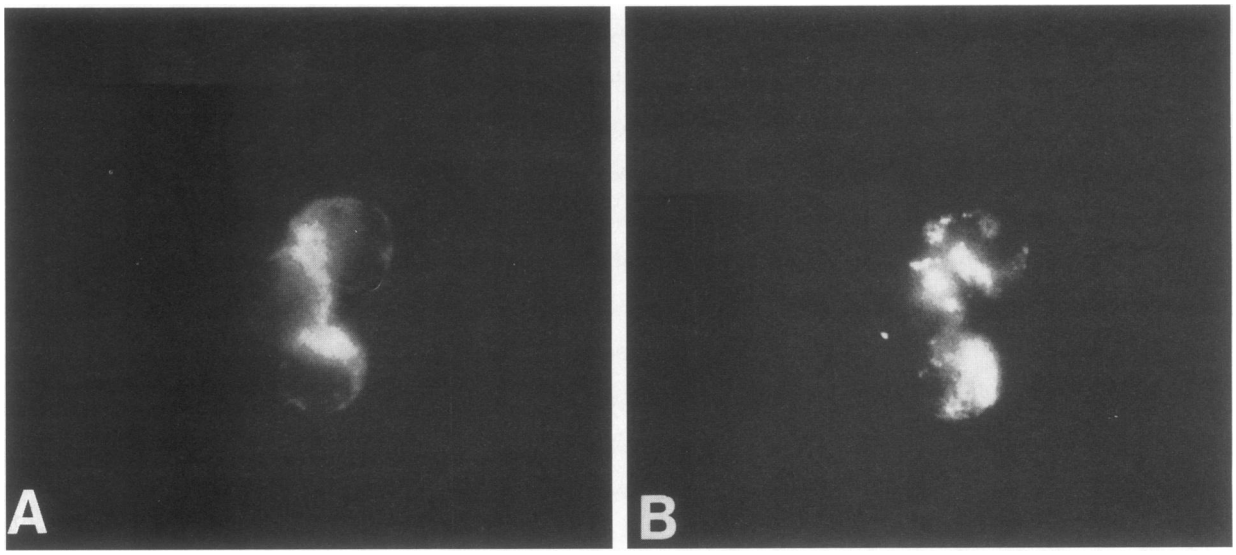

FIG. 3. Colocalization of uPA-R in unactivated monocytes with the Golgi. Cells were incubated for 4 h at 37°C, fixed, permeabilized, and then stained by indirect immunofluorescence (rhodamine) for uPA-R expression and for the Golgi with FITC-conjugated wheat germ agglutinin (Sigma). Panels: A, uPA-R; B, Golgi.

as Mo3f (13). Fluorescein isothiocyanate (FITC)-conjugated F(ab_2 anti-mouse antibody was purchased from Cappel Laboratories, Inc., Cochranville, Pa. BODIPY ceramide *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-3-indacene pentanoyl) was purchased from Molecular Probes, Eugene, Oreg. FITC-labeled phalloidin was purchased from Sigma.

Mononuclear cell isolation. PBM were obtained from defibrinated venous blood by centrifugation on Ficoll-Hypaque gradients (LSM Bionetics Laboratory Products, Litton Bionetics Inc., Kensington, Md.). The monolayer was washed and resuspended in RPMI 1640 (Gibco) at 4.5×10^7 cells per ml. Monocyte-enriched cell suspensions were prepared by adherence. Mononuclear cells (10^7/ml) were layered on petri dishes first coated with 2% gelatin and then treated with heat-inactivated autologous plasma and incubated for 2 h at 37°C. Adherent cells were removed with 5×10^{-4} M EDTA–phosphate-buffered saline and quantitated by assaying for the presence of nonspecific esterase (23). This technique routinely yields monocyte-enriched cell suspensions containing greater than 96% esterase-positive monocytes. There was no free-floating or adherent platelet contamination.

Cell culture. Isolated monocytes were plated on tissue

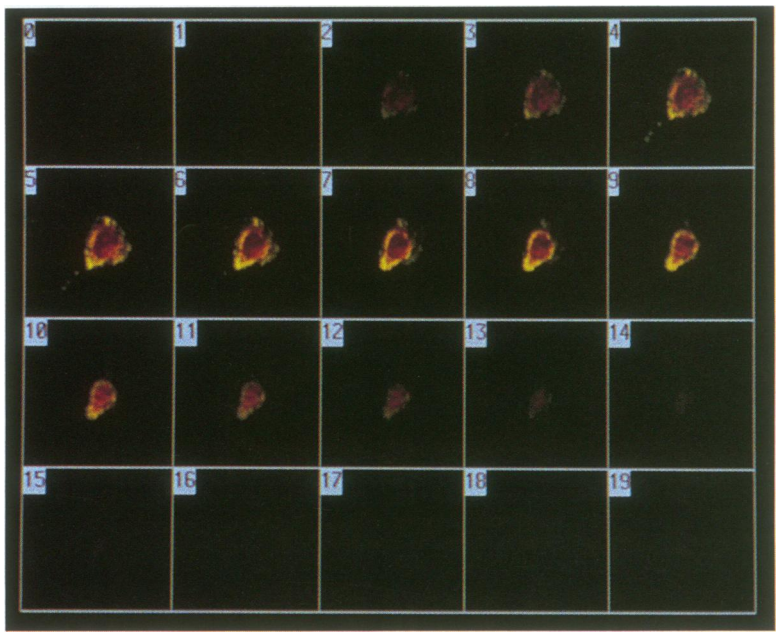

FIG. 4. Confocal laser cytometry was performed with 0.3-μm cuts. Areas of dual staining are represented by the yellow stain. Dual staining appears perinuclear and to one side of the nucleus in the cytoplasm.

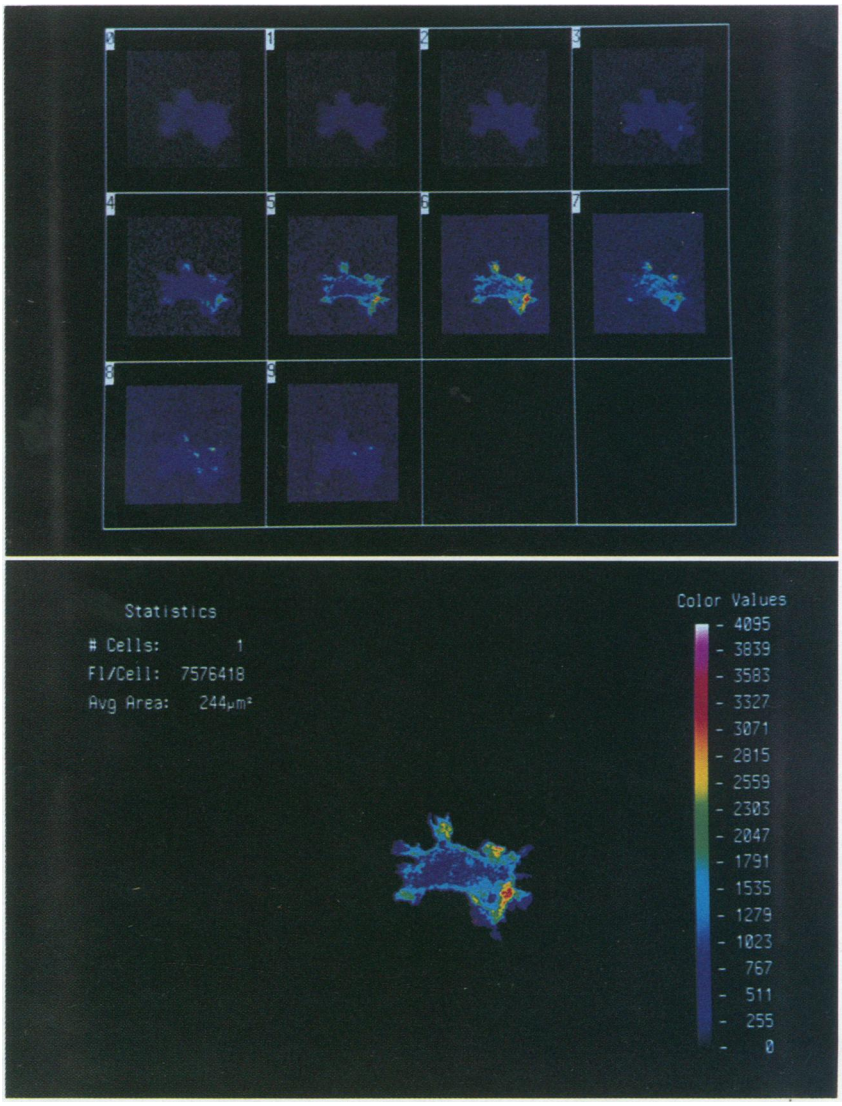

FIG. 5. Cytoplasmic distribution of uPA-R in LPS-activated monocytes. Cells were incubated for 24 h in LPS (10 mg/ml), fixed, permeabilized, and then stained for uPA-R by using indirect immunofluorescence techniques. CLM was performed as described for Fig. 2 (top). An enlargement of frame 6 is shown in the bottom panel. These results show that cytoplasmic uPA-R loses its polar distribution and becomes dispersed through the cytoplasm and into projections.

culture grade plastic petri dishes overnight at 37°C in an atmosphere of 5% CO_2 in air in RPMI 1640 plus 10% heat-inactivated autologous serum alone or in medium containing 10^{-5} M CB with or without 10^{-5} M dibutyryl cAMP, or 10^{-5} or 10^{-5} M dibutyryl cAMP only. For laser microscopy, cells were plated on glass coverslips.

Immunofluorescence and flow cytometric analysis. After isolation, cells were washed and resuspended at 5×10^6/ml in RPMI plus 10% heat-inactivated autologous serum for subsequent indirect immunofluorescent staining. For CLM, monocytes were allowed to adhere to coverslips. After incubation, cells were fixed in 3% paraformaldehyde and permeabilized with 0.01% Triton X-100. Cells were then incubated with 200 μl of MAb (plus 0.01% sodium azide) in antibody excess for 30 min at 37°C, washed, and then incubated with a saturating concentration of FITC-conjugated $F(ab)'_2$ sheep anti-mouse IgG (Cappel Laboratories). For fluorescence-activated cell sorter analysis, monocytes were stained live in suspension.

TABLE 3. Effects of LPS, cAMP, and inhibitors of cytoskeletal function on Mo3 surface distribution and macrophage spreading

Treatment (concn [M])	Avg FI ± SA (avg linear FI ± SD)[a]	Avg cell perimeter (μm) ± SD
None	626 ± 71 (290 ± 35)	42 ± 6
Lumicolchicine	405 ± 155 (310 ± 29)	51 ± 10
LPS	1,306 ± 419 (816 ± 31)	77 ± 13
cAMP	1,479 ± 324 (720 ± 60)	74 ± 10
Colchicine (10^{-5})	463 ± 135 (322 ± 40)	42 ± 10
CB (10−5)	527 ± 146 (220 ± 25)	36 ± 9
CB + LPS	587 ± 163	33 ± 10
CB + cAMP	(325 ± 31)	36 ± 8
Colchicine + LPS	454 ± 136 (375 ± 60)	44 ± 11
Colchicine 22 cAMP	501 ± 145 (485 ± 48)	58 ± 10
Lumicolchicine + LPS	1,210 ± 353	80 ± 23
Lumicolchicine + cAMP	1,384 ± 210 (689 ± 72)	74 ± 13

[a] Average FI was determined by laser cytometry (n = >50 cells), and linear FI was determined by fluorescence-activated cell sorter analysis.

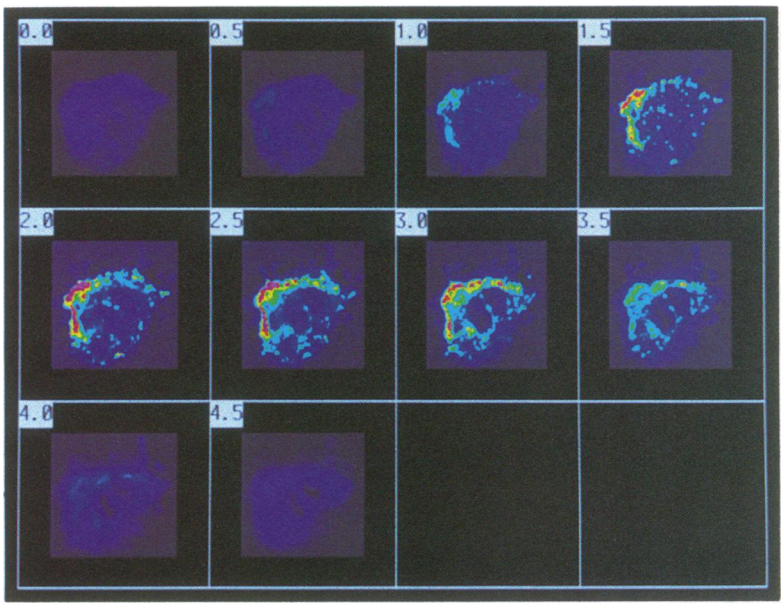

FIG. 6. Monocytes were incubated overnight with LPS (10 μg/ml) or with LPS plus colchicine and then stained with Mo3f for uPA-R expression. Confocal laser cytometry was performed with 0.5-μm cuts. Cytoplasmic uPA-R retained the nonactivated localization.

Cells were incubated for 30 min at 4°C in antibody excess. Staining with a second antibody was performed as described above. Stained cells were analyzed on a Becton Dickinson flow cytometer with a logarithmic amplifier. The channel number (log scale) representing the mean fluorescence intensity (FI) (major fluorescence peak) of 10,000 to 20,000 cells (minimum) exposed to an experimental MAb or an isotype-identical negative or positive control MAb was determined. Specific FI was determined from the mean channel number (adjusted to a linear scale) as modified by cells positively stained with an experimental MAb minus the mean channel number of cells stained with a negative control antibody. For immunofluorescence analysis of adherent monocytes, the larger cell scatter (log forward angle light scatter versus log 90°C light scatter) was preselected to exclude any contaminating lymphocytes.

CLM scanning analysis. Prepared monocytes were mounted in a gel mount (Biomeda, Foster City, Calif.) which contained phenylenediamine (Sigma), which reduced photo bleaching and was sealed with nail polish before analysis. The cells chosen for analysis were randomly spaced on the coverslips and not in contact with surrounding cells. Relative FI and distribution were analyzed with an ACAS 470 interactive laser cytometer (Meridian Instruments, Okemos, Mich.). The 5-W argon laser was set for a 200-mV output and excitation at 488 nm. A 2.0 optical density neutral-density filter was placed in front of the incident light. Fluorescence emission of FITC was measured through a 530 ± 30-nm band pass filter to a linear photomultiplier. The instrument was aligned daily in accordance with the manufacturer's specifications. Cells to be analyzed were selected by the operator and scanned at 1.0-μm increments through the glass coverslip with a 40× oil immersion lens. The photomultiplier tube was set to subtract any fluorescence due to nonspecific binding of the control antibody.

RESULTS

Human nonactivated PBM, when placed on plastic and/or a fibronectin matrix after overnight incubation, remained predominantly round with some minor spreading. Activation of PBM caused them to take on macrophage-like qualities with increased cell spreading. The amount of cytoplasmic spreading was, however, not often sufficient to view cytoskeletal structures and distribution of uPA-R by light microscopy. CLM allowed us to visualize the subcellular localization of uPA-R in both activated and nonactivated monocytes, as well as quantitate the amount of spreading.

Distribution of uPA-R monocyte activation antigen in resting and activated PBM. The relative FI and cellular distribution of fluorescence were determined by flow cytometric analysis (Fig. 1 and Table 1) and by laser cytometry (Table 2). Total relative linear FI increased with activation of monocytes with LPS (10 μg/ml) (Fig. 1 and Table 1) and with LPS and 10^{-5} M cAMP after 24 h of incubation (Table 2). In Fig. 2, a panel of optical sections from a representative CLM scan of an unactivated resting monocyte-macrophage shows the distribution of uPA-R. uPA-R was found in aggregates within the cytoplasm and was not found in the nucleus. Most of the fluorescence appeared to be distributed to one side of the nucleus. Figure 2 (bottom) shows an enlargement of one laser cut. Comparison of staining of unactivated monocytes with BODIPY ceramide (4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene), anti-Golgi MAb, and FITC-conjugated agglutinin suggests colocalization of uPA-R with the Golgi. Results in Fig. 3 show the Golgi stained with agglutinin (red) and Mo3f (green), and Fig. 4 shows results of analysis by confocal laser cytometry. Dual-staining areas are represented as yellow.

Activation of PBM with LPS (10 μg/ml) for 24 h resulted in induction of uPA-R and loss of polar distribution. There were cytoplasmic spreading and dispersion of uPA-R throughout the cytoplasm and into the monocyte projections (Fig. 5). Overnight incubation of monocytes without addition of LPS or cAMP produced little to no dispersion of uPA-R in the cytoplasm. A panel of optical scans are shown in Fig. 5 (top). The last panel is an enlargement of confocal scan 6 (Fig. 5(bottom). Depolymerization of MT at 0°C before fixation and staining (data not shown) or disruption of MT assembly in LPS-activated monocytes with colchicine but not lumicolchicine disrupted redistribution of the cytoplasmic antigen and

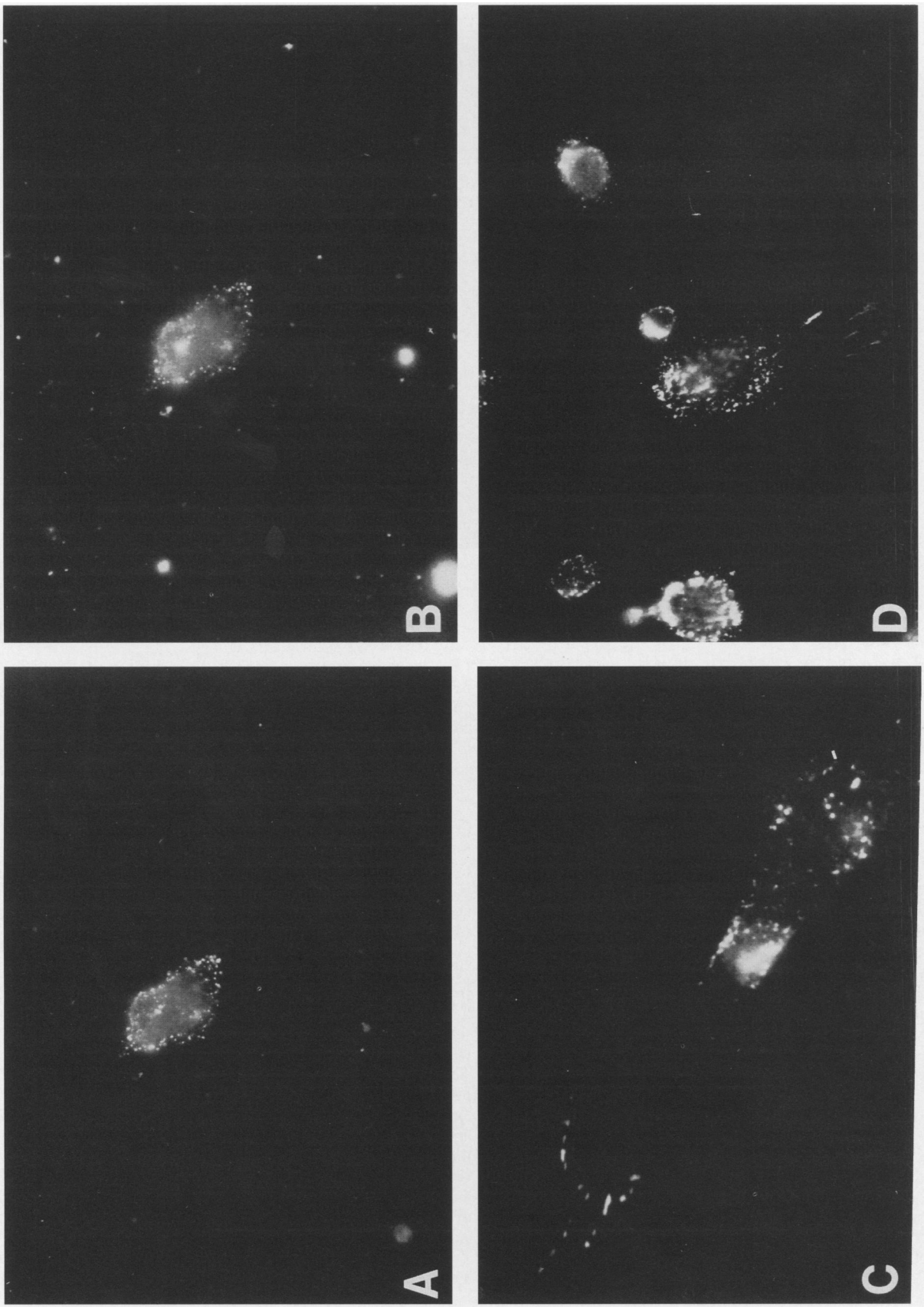

FIG. 7. Cells were incubated for 24 h with LPS, fixed, and stained for uPA-R (A) and then permeabilized and stained with an anti-vinculin MAb (B). Vinculin staining is shown in panel B, and uPA-R staining is shown in panel A. Strands of uPA-R are shown in panel C, and one monocyte is shown in panel D. This may indicate focal adhesion sites or places where uPA-R remained attached to the petri dish surface with detachment or movement of monocytes on the petri dish surface. Round cells had evenly dispersed surface expression (D).

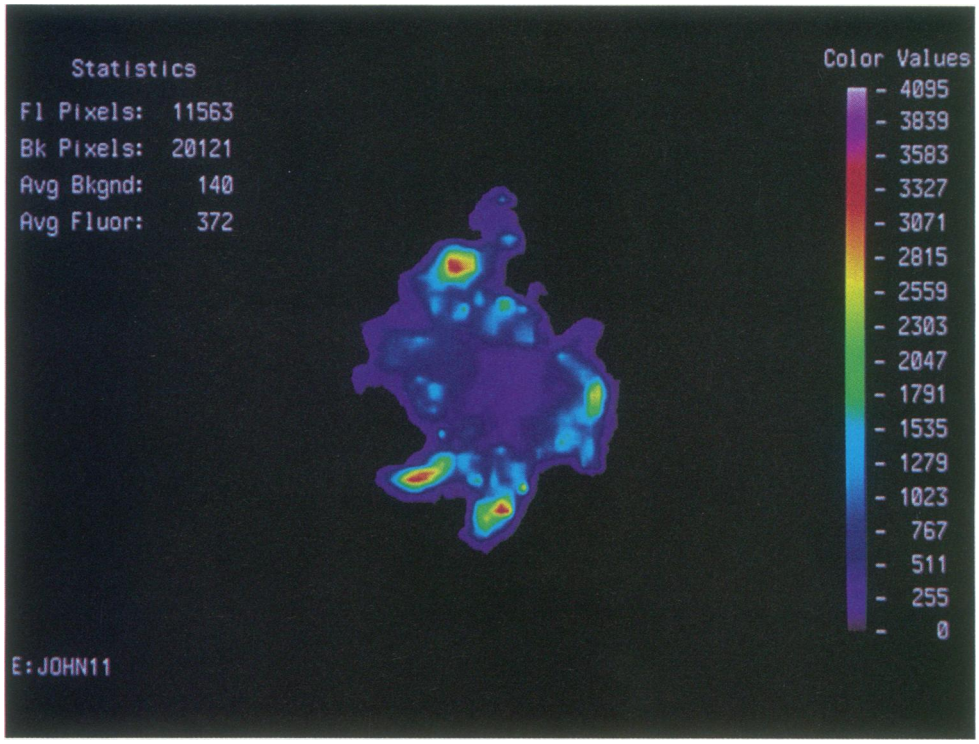

FIG. 8. Expression of cytoplasmic uPA-R in monocytes from a patient with chronic progressive disease. Cells were incubated overnight in medium alone, fixed, and permeabilized, and then CLM was performed (data not shown). An enlargement of frame 7 shows cytoplasmic distribution of uPA-R similar to that in activated monocytes shown in Fig. 4.

reduced surface uPA-R (Table 3). In these cells, uPA-R remained to one side of the nucleus and the cell surface antigen was not enhanced (Table 3 and Fig. 6). In addition, the pattern of indirect immunofluorescent staining of activated PBM with a MAb to β-tubulin suggests that uPA-R colocalized with MT in the cytoplasm (data not shown). When LPS-activated monocytes were stained with Mo3f prior to permeabilization with 0.05% saponin and then stained with phalloidin (*N*-7-nitrobenz-2oxa-1,3diazol-4yl), surface uPA-R appeared to colocalize with MF. Phalloidin did not stain cells that had not been permeabilized (data not shown). However, in fibroblasts, uPA-R has been shown to colocalize with vinculin (6). The pattern of immunofluorescence shown in Fig. 7 demonstrates that monocyte uPA-R and vinculin staining were in the same plane of focus (Fig. 7A). Some uPA-R lacked detectable colocalization. In many of the activated cells, uPA-R strands appeared to be offset along the cell projections, perhaps at focal adhesion sites (Fig. 7B and C). In more rounded cells, uPA-R took on an evenly dispersed appearance (Fig. 7D). Pharmacologic disruption of MF integrity with CB was found to inhibit macrophage spreading, as well as inhibit cAMP- or LPS-induced increases in surface uPA-R (Table 3). No effect on viability was seen following incubation with these agents.

Cellular distribution of uPA-R in monocytes from patients with MS. Circulating monocytes from patients with MS were incubated overnight in the absence of exogenous activators. We have previously shown that monocytes from these patients exhibit increased surface uPA-R and that further treatment of MS monocytes with LPS did not significantly alter surface uPA-R (3). When the surface distribution of antigen in unactivated MS monocytes was examined by CLM, MS monocytes displayed dispersed uPA-R in a pattern similar to that in LPS-activated control cells (Fig. 8). Addition of colchicine to MS cells causes a decrease in surface Mo3. However, this change in surface FI does not occur for 24 to 48 h. Cytoplasmic changes occur more rapidly (data not shown).

DISCUSSION

Previous studies have shown that uPA-R is constitutively produced in nonactivated monocytes and U937 cells (9, 19). Surface uPA-R is minimal in nonactivated monocytes; however, a significant concentration is found stored in the cytoplasm (19, 20). Upon activation, with LPS, phorbol myristate acetate, and cAMP, both surface uPA-R and cytoplasmic uPA-R are increased (18). We have shown, by using confocal laser cytometry, that cytoplasmic uPA-R in unactivated monocytes appears to localize to one side of the nucleus. Results from dual staining and CLM analysis indicate that this subcellular distribution of uPA-R is consistent with colocalization at the Golgi. Upon activation, cytoplasmic uPA-R becomes distributed throughout the cytoplasm into monocyte projections. That this was more readily viewable by CLM may be because human monocytes do not spread sufficiently to be viewed that easily by light microscopy. Cytoplasmic distribution in LPS-activated cells is MT dependent. Surface antigen becomes linked to the monocyte cytoskeleton via colocalization with vinculin. These data are consistent with observations by Hebert and Baker (6), who investigated the extracellular localization of uPA-R-bound urokinase plasminogen activator on the surface of fibroblasts.

Similar experiments have been used to show the cytoskeletal association of other proteins (7, 8, 11, 15, 16, 22). Interleukin-1β was found to associate with cytosolic microtubules in activated monocytes (11, 16). It is unclear what the relevance

of cytoplasmic uPA-R association with MT is. However, the finding that the surface antigen colocalizes with MF suggests that the mechanism of transport of uPA-R to the cell surface involves both MT and MF. This is supported by experiments showing that both CB and colchicine altered activation-induced increases in the surface antigen.

Cytoplasmic association of uPA-R may be important to a potential role in stimulating second-messenger systems. Human monocyte uPA-R is a glycosylphosphatidylinositol-anchored protein 19). Binding of uPA to its surface receptor induces a tyrosine phosphorylation reaction which is glucosylphosphatidylinositol dependent (4). Tyrosine phosphorylation may indicate that uPA binding induces a paracrine or autocrine system-like reaction. Similar activity has been shown for other glucosylphosphatidylinositol-linked surface proteins such as Ly-6 (17).

Activation results in an increase in both surface uPA-R and cytoplasmic uPA-R. It may be that rapid surface deposition of uPA-R is important in monocyte function. However, it is unclear whether the increase in surface expression of uPA-R seen upon activation is related to a translocation process of constitutive cytoplasmic uPA-R. uPA-R on stimulated monocytes may indicate that they are primed for rapid migration into inflamed or infected tissues (1, 6, 21). uPA-R has been found on the surface of circulating monocytes from patients with MS, a disease characterized by perivascular leukocyte infiltration into the central nervous system (3). Surface-bound urokinase plasminogen activator is thought to represent a highly efficient cell surface proteolysis system which functions in cell migration (adhesion), wound repair, and metastasis (1–3, 5, 21). uPA-R localization with high density at focal adhesion sites (1, 5) may provide a high enough concentration for cleavage of subendothelial matrix proteins (5, 14) and migration through the blood-brain barrier. These observations point to intriguing questions about the possible role of uPA-R in central nervous system inflammation in general and MS in particular and may provide alternative therapeutic approaches (10).

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