MACROPHAGE COLONY-STIMULATING FACTOR (rM-CSF) STIMULATES PINOCYTOSIS IN BONE MARROW-DERIVED MACROPHAGES

BY ESTHER L. RACOOSIN AND JOEL A. SWANSON

From the Department of Anatomy and Cellular Biology, Harvard Medical School, Boston, Massachusetts 02115

Polypeptide growth factors are involved in the regulation of cell proliferation (1). These factors bind to specific receptors on the cell surface, stimulating the formation of intracellular second messengers that elicit a variety of responses. Transient increases in intracellular pH (2) and calcium concentration (3) can occur soon after growth factor addition. Other early responses induced by epidermal growth factor (EGF) (4–9), platelet-derived growth factor (5, 10), insulin (7, 11, 12), and insulin-like growth factor-1 (4, 5, 7), include cell ruffling, stimulation of pinocytosis, and redistribution of cell surface receptors. Ultimately, binding of growth factors to responsive cells leads to later activities such as initiation of DNA synthesis and mitosis. A correlation between growth factor–stimulated pinocytosis and proliferation was seen in studies of EGF treatment of normal human fibroblasts (8, 13). However, it is not known how the initial signal elicited by mitogen binding leads to such distinct activities as pinocytosis and mitosis, or whether the coincidence of the two responses is significant.

We chose to study the regulation of growth factor–stimulated pinocytosis in macrophages because their growth factor, CSF-1, and its receptor have been characterized, and because solute and membrane movement within these cells are active and well-documented processes (reviewed in references 14, 15). Moreover, PMA treatment of resident, protease peptone–elicited, and thioglycollate-elicited macrophages stimulates cell spreading and pinocytosis (16). Addition of PMA to bone marrow–derived macrophages (BMM) stimulates cell ruffling and the formation of large, phase-bright cytoplasmic vesicles, termed macropinosomes (17). These organelles had been observed in previous investigations of growth factor–stimulated pinocytosis (18). The morphological changes in BMM correlated with increased flow of Lucifer Yellow-CH (LY) through the endocytic compartment, and added pinocytosis to the list of PMA-stimulated responses that mimic known mitogen-elicited responses. We therefore asked if the macrophage-specific growth factor, CSF-1, stimulated pinocytosis in a...
similar way. Here, we show that BMM treated with recombinant human macrophage CSF (rM-CSF), which is highly homologous to murine CSF-1 (19) and is capable of stimulating the proliferation of murine monocytic cells (20), rapidly exhibit stimulated pinocytosis. The stimulated influx of solute in response to rM-CSF appears to be qualitatively different than constitutive pinocytosis and may participate in regulation of membrane flow through the endocytic compartment.

Materials and Methods

Cells. BMM were obtained from C3H/HeJ female mice (The Jackson Laboratory, Bar Harbor, ME), 6–10 wk old, by the method of Celada et al. (21), with some modifications. These mice do not respond to bacterial LPS (22) and were extruded by inserting a 26-g needle into one cut end and flushing with a syringe containing cold DME (Gibco Laboratories, Grand Island, NY) + 10% heat-inactivated FCS (HIFCS; Gibco Laboratories) + 100 U/ml pen-strep (DME-10F; Gibco). Cells released from the marrow were washed twice with DME-10F, then resuspended in DME + 30% L cell conditioned medium + 20% HIFCS (complete bone marrow medium). 5 x 10⁶ bone marrow cells per 25 ml of complete bone marrow medium were plated into 100-mm Lab-Tek petri dishes (Nunc, Naperville, IL) and incubated at 37°C for 6 d in an atmosphere of 5% CO₂. On day 3 of culture, 10 ml of complete bone marrow medium was added to each dish. On day 6, adherent macrophages were washed briefly with ice-cold divalent cation-free PBS (PD: 137 mM NaCl, 3 mM KCl, 7 mM phosphate buffer, pH 7.4); then cells were left on ice for 10 min by an additional 10 ml PD. Next, cells were washed off the dish with a gentle stream of cold PD. Cells were counted and plated in PD at a density of 3–3.25 x 10⁵ cells/well in 24-well culture plates (Costar, Cambridge, MA). After 30 min, PD was aspirated from wells, replaced with 0.5 ml of DME-10F, and cultured overnight. Cells were used in experiments no more than 48 h after plating. For microscopy, cells were plated onto 22 mm x 22 mm glass coverslips at a density of 1.5 x 10⁵ cells per coverslip. Greater than 95% of these cells were macrophages, as judged by their ability to phagocytose oposmenized sheep red blood cells.

Quantitation of Lucifer Yellow Pinocytosis. Pinocytosis of LY (Potassium salt; Molecular Probes, Eugene, OR) was quantified by allowing BMM in 24-well dishes to incubate in PBS containing divalent cations + 10% heat-inactivated FCS (PBS-10F) containing LY, then washing away extracellular dye, lysing the cells, and measuring the fluorescence of the lysate. For experiments with one time point of LY uptake, cells were washed free of DME-10F, incubated with PBS-10F, and chilled on ice for 30–45 min. Next, the PBS-10F was replaced by 0.25 ml of a chilled solution of PBS-10F containing 0.5 mg/ml LY, ± 3,000 U/ml highly purified rM-CSF (gift of Cetus Corp., Emeryville, CA) (23). Cells were either warmed to 37°C or kept on ice for the duration of the experiment. For time course experiments, cells were not chilled before addition of LY-containing solutions but were kept at 37°C while solutions were added. Experiments were planned so that all incubations ended simultaneously.

To terminate LY pinocytosis, incubation solutions were rapidly shaken from tissue culture plates, then plates were immersed in 1 liter of ice-cold PD containing 1 mg/ml BSA (Sigma Chemical Co., St. Louis, MO). Plates were removed from the beaker and resubmerged several times. They were then passed to a second 1-liter beaker of ice-cold PD for a 5-min wash, and then to a final beaker of ice-cold PD for a brief wash. Plates were shaken and aspirated dry, then either reinculated in PBS-10F (for efflux experiments, see below), or received fresh 0.5 ml of PBS-10F containing 0.1% Triton X-100 and 0.02% NaN₃ in double-distilled H₂O. Cells were incubated in lysing buffer for 40–60 min at 37°C before measurement of LY fluorescence or protein content. For LY efflux experiments, cells were reinculated in 0.25 ml of chilled PBS-10F ± rM-CSF, then were either kept at 4°C or warmed to 37°C for 1 h. After this chase period, cells were washed in two 1-liter beakers of ice-cold PD, then drained and lysed in Triton-containing buffer as described above.

To determine the fluorescence of the cell lysates, 0.4 ml of the lysate was diluted up to
1.15 ml in a buffer containing 0.1% Triton X-100, 0.1 mg/ml BSA, and 0.02% NaN₃, and read in a spectrofluorometer (model Aminco 500C; SLM Instruments, Urbana, IL). Specimens were excited at 430 nm with a 4-nm bandpass and emission was measured at 540 nm with a 4-nm bandpass. Protein content of lysates was measured using a modified version of the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL); 50 μl of the lysate was combined with 1 ml of reaction mixture and incubated at 37°C for 60 min. Dilutions of 1 mg/ml BSA were used as protein standard. This method was used to determine that 10⁶ BMM contained 180 μg protein. The amount of intracellular LY in each well of the tissue culture dish was expressed as nanograms LY/milligram protein/well. Each condition was done in triplicate and expressed as the mean ± SD. The apparent fluorescence of cells exposed to LY at 4°C was considered as background and subtracted from other fluorescence values to determine the cell-associated fluorescence of LY.

**Microscopy.** To quantify pinocytic vesicles, BMM plated at ~1.5 × 10⁵ cells/22 mm × 22 mm coverslip were preincubated for 45 min in PBS-10F ± 5 μM nocodazole at 37°C, then medium was aspirated and replaced by PBS-10F ± nocodazole with or without 3,000 U/ml rM-CSF. Those cells that previously had been incubated in nocodazole continued to receive medium containing drug. After a 60-min incubation, cells were washed rapidly with three changes of warm PBS, then were fixed in PBS containing 2.5% glutaraldehyde and 4.5% sucrose. Fixation proceeded for 45 min at room temperature. Fixed cells were washed several times with PBS, then mounted in PBS for microscopic study, and photographed using Pan-X film.

**Results**

**rM-CSF Stimulates Pinocytosis in BMM.** rM-CSF stimulated both LY influx and accumulation in BMM (Fig. 1). Cells treated with 3,000 U/ml growth factor exhibited an initial rate of LY accumulation of 13.0 fl/cell/min, which was nearly three times greater than untreated cells (4.6 fl/cell/min). This initial rate slowed within 30 min but remained three times higher than the steady-state control rates (3.6 fl/cell/min vs. 1.2 fl/cell/min). LY accumulation represents total influx of LY minus total efflux of LY over a given amount of time. As described in a previous study (24), at early time points of accumulation, efflux is relatively slight; therefore, the initial rate of accumulation approximates the rate of influx. Cells incubated in LY for the first 45 min, then in LY + rM-CSF, exhibited a burst in LY accumulation

**Figure 1.** Accumulation of LY by unstimulated macrophages and by macrophages in 3,000 U/ml rM-CSF. Macrophages were incubated in PBS-10F containing LY ± 3,000 U/ml rM-CSF for the times indicated (○, -; ●, +). In addition, some cells were incubated 45 min in LY only, then received LY + 3,000 U/ml rM-CSF for the remaining indicated times (▲). All incubations ended simultaneously. At this time, cells were washed in cold PD, then lysed in Triton X-100, and the fluorescence and protein content of that lysate were measured. Similar results were obtained in three repeats of the experiment.
within 5 min of addition of growth factor. This accumulation rate was comparable
to that of cells incubated in LY + rM-CSF from the beginning of the time course,
indicating that at steady state, when all cellular compartments are labeled with LY,
the cell can still be stimulated to increase LY accumulation. This response may be
effected by increasing the size of incoming pinocytic vesicles containing LY, by
increasing the rate of pinosome formation, or by decreasing the rate at which LY is
recycled to the medium.

rM-CSF-stimulated LY accumulation was dose dependent (Fig. 2). Half-maximal
LY accumulation occurred at a concentration of 1,000 U/ml rM-CSF. This concentra-
tion was found by Tushinski et al. (25) to stimulate DNA synthesis and prolifera-
tion in cultured BMM. We also found that L cell-conditioned medium, a source
of murine CSF-1 (26), stimulated both pinocytosis and DNA synthesis in quiescent
BMM (data not shown). At 3,000 U/ml rM-CSF, LY accumulation was twofold higher
than in cells incubated without growth factor. This concentration of rM-CSF was
used in further studies.

**rM-CSF Stimulates Pinocytosis More Rapidly than Does PMA.** rM-CSF stimulated the
rate of LY accumulation by BMM in a dose-dependent fashion; this response was
also observed in previous experiments treating BMM (17) or peritoneal exudate mac-
rophages (24) with PMA. It was of interest to determine what other characteristics
of stimulated pinocytosis were shared between BMM treated with rM-CSF or PMA.

One parameter of the rM-CSF- or PMA-stimulated response that could be com-
pared was the initial burst of LY accumulation observed after addition or either agent.
As shown in Fig. 1, rM-CSF stimulated initially a curvilinear rate of LY accumula-
tion, indicating that the growth factor acted rapidly. To compare the onset of stimu-
lated pinocytosis by rM-CSF and PMA, BMM were preincubated with rM-CSF
or PMA for various time periods up to 60 min, then were incubated with LY in
the continued presence of the factor for an additional 20 min. As shown in Fig. 3,
longer preincubations in rM-CSF decreased the total LY accumulated during a 20-min pulse. There was a rapid decrease within 5 min of preincubation; by 30 min, the stimulation of accumulation decreased to a lower rate and was sustained. In contrast, the PMA response developed gradually and approached the level of accumulation in rM-CSF-treated cells. Cells preincubated with PBS-10F alone for various times showed no change in the amount of LY accumulation. These data indicate that although both rM-CSF and PMA stimulate LY accumulation, they elicit that response with different kinetics.

**Trypsin Pretreatment Abolishes rM-CSF-stimulated Pinocytosis.** We hypothesized that rM-CSF stimulates pinocytosis by binding to a cell surface receptor, while PMA causes this response by interacting intracellularly on its effector, protein kinase C. The different cellular targets of PMA and rM-CSF could result in the different initial responses to each factor. A prediction of this hypothesis is that while the rM-CSF receptor on the cell surface should be accessible to extrinsically added proteases, the phorbol ester receptor should be protected. Cells were incubated at 4°C with increasing concentrations of trypsin, then washed with 50% HIFCS and ice-cold PBS to inactivate and remove the enzyme. Next, cells were incubated for 45 min at 4°C in PBS alone, PBS containing 3,000 U/ml rM-CSF, or 30 ng/ml PMA. Cells were then washed and incubated with LY at 37°C for 30 min (Fig. 4). Non-trypsin-treated cells that were incubated with rM-CSF accumulated nearly two times more LY than cells that did not receive growth factor. However, when cells were preincubated with trypsin at concentrations of 0.5 mg/ml or higher, the rM-CSF stimulated response was abolished. In contrast, PMA stimulated LY accumulation regardless of trypsin concentration. Similarly, treatment with increasing concentrations of trypsin did not affect LY accumulation by control cells. Thus, while rM-CSF stimulation of pinocytosis was dependent upon the integrity of cell surface proteins, PMA-stimulated and constitutive pinocytosis were not.
**rM-CSF Stimulates Macropinocytosis.** Although the initiation of stimulated pinocytosis by PMA and rM-CSF differ in kinetics of onset, the two factors elicit similar changes in cell morphology. We observed previously by time-lapse video microscopy (17) that within 5–10 min of addition of PMA, BMM increased formation of both small and large phase-bright vesicles, or macropinosomes. Cells treated with 3,000 U/ml rM-CSF were observed to respond similarly when viewed by the same method (data not shown). The formation of large, phase-bright vesicles appeared immediately after addition of rM-CSF and continued throughout a 60-min observation period. In contrast, cells incubated in PBS-10F alone actively extended and retracted sheets of membrane but formed mostly small phase-bright pinosomes. We attempted to quantitate the number of phase-bright pinosomes present in the cytoplasm of untreated and rM-CSF-treated BMM. Cells were incubated 60 min in PBS-10F with or without rM-CSF, then were fixed and prepared for light microscopy (Fig. 5). Micrographs of cells were enlarged, and the number and size of phase-lucent vesicles present in cells were determined. Cells incubated in rM-CSF contained larger macropinosomes than cells incubated in PBS-10F alone; indicating that the volume of solute internalized by growth factor-treated cells was greater (data not shown).

**rM-CSF Stimulation of LY Accumulation in BMM is Dependent on the Integrity of Microtubules and Microfilaments.** Because a previous study showed that PMA-stimulated pinocytosis in thioglycollate-elicited macrophages was inhibited by microtubule depolymerizing agents (24), we asked if these drugs also affected rM-CSF-stimulated LY accumulation. A time course of LY accumulation was performed using cells that had been preincubated in PBS-10F ± 5 μM nocodazole for 45 min. Next, LY-containing medium was added with or without 3,000 U/ml rM-CSF; nocodazole-treated cells received LY-containing medium plus drug, and LY accumulation was quantitated for up to 90 min (Fig. 6). Nocodazole inhibited both unstimulated and stimulated pinocytosis. Initial rates of LY accumulation were reduced by 50% in both rM-CSF-treated and untreated cells. By 30 min, cells incubated in nocodazole were dramatically impaired in their ability to accumulate LY. The rates of LY ac-
Figure 5. rM-CSF-treated BMM exhibit more large, phase-bright cytoplasmic vesicles than untreated cells. Micrographs show untreated or rM-CSF-treated cells, cultured, fixed, and prepared for microscopy as described in Materials and Methods. (A) Unstimulated macrophages incubated 60 min in PBS-10F display relatively few phase-bright cytoplasmic vesicles, but do show ruffling activity at their cell margins. In contrast, macrophages incubated in 3,000 U/ml rM-CSF for 60 min (B) contain larger and more numerous phase-bright vesicles. Bar = 10 μM.
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FIGURE 6. Nocodazole inhibits the rate of LY accumulation in untreated and rM-CSF-treated cells. Cells were preincubated at 37°C in PBS-10F ± 5 μM nocodazole for 45 min. Next, wells were aspirated and cells received solutions containing LY ± rM-CSF (○, minus rM-CSF; ■, plus rM-CSF); those cells that had been preincubated with nocodazole received solutions containing 5 μM nocodazole, ± rM-CSF (○, minus rM-CSF; □, plus rM-CSF). BMM were incubated in these solutions for the indicated times.

cumulation in these cells decreased, while cells not treated with nocodazole continued to accumulate LY at rates similar to those shown in Fig. 1.

The effects of cytoskeleton-destabilizing drugs on the stimulated accumulation of LY in BMM were further studied by allowing cells treated with or without either PMA or rM-CSF to pinocytose LY in the presence of these drugs. After a preincubation in PBS-10F containing either no drug, nocodazole, colchicine, lumicolchicine, or cytochalasin D, cells were incubated for 90 min in PBS-10F containing LY plus the appropriate drug, with or without PMA or rM-CSF (Fig. 7). Treatment of cells with either nocodazole or colchicine lowered LY accumulation in untreated, PMA-treated, and rM-CSF-treated cells. In each case, inhibition of stimulated pinocytosis was more pronounced than that of unstimulated pinocytosis. Lumicolchicine, a congener of colchicine that does not depolymerize microtubules, did not inhibit

FIGURE 7. Cytoskeleton destabilizing drugs inhibit LY accumulation in PMA- and rM-CSF-treated cells. Macrophages were preincubated for 45 min at 37°C using the following treatments, representing PBS-10F +: (A) no drug; (B) 5 μM nocodazole; (C) 5 μM nocodazole; (D) 5 μM colchicine; (E) 5 μM lumicolchicine; (F) 10 μM cytochalasin D. Next, some cells (treatment C) were washed two times in warm PBS, followed by a 45-min incubation at 37°C in PBS-10F alone. After these preincubations, cells received solutions containing LY without drugs (treatments A, C) or containing the appropriate drug at the same concentration (treatments B, D, E, F). One set of solutions contained LY alone or with 30 ng/ml PMA (A), while the other contained LY alone or with 3,000 U/ml rM-CSF (B). Cells were incubated in the LY-containing solutions at 37°C for 90 min. Next, cells were washed and lysed and LY accumulation was quantitated as described. The experiment with PMA was done at a different time than the experiment with rM-CSF, but control values (i.e., LY accumulated by cells incubated in the absence of PMA or rM-CSF) were similar. Data shown are representative of three repeats of the experiment.
accumulation of LY in control, PMA-treated or rM-CSF-treated cells. If cells preincubated for 45 min in nocodazole were washed and allowed to recover in drug-free PBS-10F for 45 min, both unstimulated and stimulated levels of LY accumulation were restored. Interestingly, cytochalasin D, a drug that causes the disassembly of microfilaments, inhibited both PMA- and rM-CSF-stimulated, but not unstimulated pinocytosis. These experiments indicate that the integrity of cytoplasmic microtubules and microfilaments is essential for both PMA- and rM-CSF-stimulated solute accumulation.

_rM-CSF Stimulates Efflux of LY from Cells Pre-loaded with the Dye._ Studies of the effects of PMA treatment of BMM on intracellular transport of LY revealed that the phorbol ester stimulated the recycling of preloaded LY from cells (17). We investigated if rM-CSF had a similar effect. BMM were incubated with LY for 2 h, then excess LY was washed away and cells were reincubated for 1 h at 37°C or 4°C in PBS-10F with or without rM-CSF. Temperature-dependent loss of LY from BMM was calculated by measuring the amount of LY remaining in cells after the 1-h chase period (Fig. 8). A negligible amount of LY was lost from cells reincubated at 4°C. When cells were reincubated at 37°C in PBS-10F alone, 23% of the LY was recycled to the medium (compared with the amount that recycled during a 4°C chase). However, in the presence of rM-CSF, the amount of LY recycled at 37°C increased to 37%. Thus, rM-CSF not only stimulated LY influx, as shown in Fig. 1, but LY efflux as well. These two activities occur simultaneously in response to the mitogen, stimulating membrane and solute flow through the endocytic compartment. Net accumulation of LY, then, must not be due to decreased efflux but to a stimulation of LY influx, either by increasing the rate of pinosome formation or the size of the incoming pinosome.

Discussion

Many studies have shown that stimulation of pinocytosis is one of the early consequences of growth factor and peptide hormone binding to cultured cells (6, 7, 9-11). Haigler et al. (6) found that EGF elicited a dose- and time-dependent increase in the rate of pinocytosis of horseradish peroxidase in A431 cells, and that PMA could mimic this response. We show here that rM-CSF treatment of BMM resulted in

**Figure 8.** rM-CSF stimulates LY efflux from preloaded BMM. BMM were incubated in PBS-10F + LY at 37°C for 2 h. Next, the dishes containing the cells were washed through three 1-liter beakers of ice-cold PD, then were drained and placed on ice. Some cells were lysed immediately (●), to quantitate the amount of LY accumulated during the 2-h period. Other pulsed cells received cold PBS-10F and were incubated an additional hour on ice (□). Additional pulsed cells received cold PBS-10F ± 3,000 U/ml rM-CSF (●, minus rM-CSF, ▲, plus rM-CSF), then were warmed to 37°C for a 1-h chase period. Cells were then washed and lysed, and LY contained in the lysates was quantitated. This figure is representative of six experiments in which it was consistently seen that rM-CSF stimulated efflux over control.
a rapid and sustained stimulation of pinocytosis, similar to the PMA-stimulated response described earlier (17). This indicates that rM-CSF shares with other growth factors and tumor promoters the ability to elicit this early response. Here, in light of our results, we discuss how the cell might facilitate increased pinocytosis and the physiological significance of this response.

**The Binding of rM-CSF to its Receptor Rapidly Elicits Stimulated Pinocytosis.** The rapidity with which the rate of LY influx increased in response to rM-CSF addition suggested that a response was elicited shortly after the mitogen bound to its receptor. This inference is supported by our finding that cell ruffling and macropinosome formation increased immediately after addition of rM-CSF. However, we observed that a high initial stimulated rate of LY accumulation always decreased to a lower stimulated rate within 30 min. It may be that this consistent decrease in pinocytic rate was brought about by downregulation of the CSF-1 receptor. Guilbert and Stanley (27) found that following addition of \( ^{125}I \)-CSF-1 to BMM, cell surface receptors were downregulated with a \( t_{1/2} \) of 7 min, that replacement of receptors occurred slowly, and that after replacement the number of CSF-1 receptors at the cell surface remained low. It would be expected, then, that the stimulated rate of pinocytosis would rapidly decrease to control rates; in our experiments, however, stimulated steady-state rates of LY accumulation remained three times those of control. Perhaps low amounts of bound rM-CSF are sufficient for sustaining stimulated pinocytosis. Rapid downregulation of the CSF-1 receptor could explain our observation that preincubation of BMM with rM-CSF resulted in a rapid decrease in LY accumulation (Fig. 3), and also that the constant presence of excess CSF-1 was required to sustain stimulated pinocytosis (data not shown). Tushinski and Stanley (28) determined that CSF-1 must be present for at least 9 h for stimulation of DNA synthesis in BMM. We have determined that stimulation of pinocytosis by rM-CSF is sustained for at least 6 h (data not shown); thus it is possible that macropinocytosis is a necessary physiological precedent to DNA synthesis.

**Increased Solute Influx and Retention Depends on the Integrity of Microtubules and Microfilaments.** The formation and internalization of macropinosomes in response to rM-CSF correlated with the stimulation of LY accumulation, implying that these organelles facilitated greater solute influx. The addition of nocodazole or colchicine in the presence of rM-CSF inhibited this stimulation, but it was not clear where the inhibition occurred. When rM-CSF-treated cells that had been preincubated with nocodazole were examined by phase microscopy, a few macropinosomes were evident, although they were considerably reduced in number when compared with cells treated with rM-CSF alone (data not shown). In addition, the nocodazole-treated cells showed a very slow rate of LY accumulation (Fig. 6). These findings indicate that although the nocodazole-treated cells could internalize LY, their ability to retain the dye was impaired. Perhaps in these cells macropinosomes are formed but are rapidly recycled to the cell surface. In order for LY to be retained, macropinosomes may be required to translocate to and fuse with other elements of the endocytic pathway. A recent study has described the extension of lysosomes on microtubules towards the periphery of the cell, where pinosomes most often form (29). Indeed, in BMM, many tubular lysosomes are evident in the cell cytoplasm. If fusion of pinosomes with organelles of the endocytic pathway depends on the integrity of microtubules, depolymerization of microtubules in the presence of nocodazole might...
reduce the efficiency of this process. Undirected pinosomes formed in cells treated with microtubule-depolymerizing drugs might then be rapidly recycled to the cell surface resulting in little or no net LY accumulation.

Both rM-CSF- and PMA-stimulated pinocytosis, but not unstimulated pinocytosis, were also inhibited by cytochalasin D, implying that the integrity of microfilaments is essential for the formation of macropinosomes. Cytochalasin D has been shown to interfere with cell ruffling (30), a process that is thought to precede pinosome formation (18). By video microscopy, we have observed cell ruffles and membrane sheets at the periphery of macrophages folding back on themselves to form pinosomes containing trapped solute. However, after preincubation in cytochalasin D, these movements were no longer observed and only saltatory movement of intracellular organelles could be seen (data not shown). In these cells, formation of macropinosomes appears to be inhibited. The observation that constitutive pinocytosis in unstimulated cells was unaffected by cytochalasin D implies that it is a process independent of cell ruffling and macropinosome formation. Constitutive internalization of solutes may occur predominantly within clathrin-coated pits.

Increased Solute and Membrane Flow Could Stimulate Receptor Movement.

rM-CSF, like PMA, stimulated both influx and efflux of LY, representing a stimulation of membrane and solute flow through the endocytic compartment. What is the significance of this stimulated membrane flow in the functioning of the cell? Previous studies describing the effects of growth factors and peptide hormones on intracellular membrane and receptor movement could be models for the activities stimulated by rM-CSF binding to BMM.

Addition of insulin to cells stimulates a variety of responses relating to membrane movement. Gibbs et al. (11) studied fluid phase endocytosis in monolayers of 3T3-L1 adipocytes and found that insulin stimulated a twofold increase in the pinocytic rate within 5 min and also stimulated efflux of the pinocytic marker [14C]sucrose. In earlier experiments, Wardzala et al. (31) found that addition of insulin to isolated rat adipose cells resulted in the rapid recruitment of IGF-II receptors to the cell surface. Insulin also rapidly stimulated the recruitment of glucose transporter proteins and transferrin receptors from an intracellular membranous location (32, 33). Taken together, these data show that soon after its addition, insulin stimulates both pinocytosis of fluid-phase markers and exocytosis of those markers and cell surface receptors. These activities might therefore be directly related by a process where the stimulated internalization of transporter- or receptor-deficient macropinosomes increases membrane recycling and the movement of transporter-rich vesicular compartments to the cell surface.

The rapid rM-CSF-stimulated increase in LY influx, which occurs simultaneously with stimulated recycling of intracellular membranes containing LY, could be like the insulin-stimulated responses. Growth factors have been shown to stimulate both pinocytosis and the redistribution of internal receptor pools and solutes to the cell surface. It would be of interest to determine if rM-CSF treatment of BMM stimulates increased surface expression of cell-specific receptors, and whether such an activity can be correlated with rM-CSF-stimulated pinocytosis.

Summary

Incubation of murine bone marrow–derived macrophages (BMM) in medium con-
taining recombinant macrophage colony-stimulating factor (rM-CSF) stimulated influx, efflux, and the net accumulation of the fluid-phase pinocytotic marker, lucifer yellow (LY). Stimulation was dose dependent, occurred within 5 min of addition of the growth factor, and was sustained. Previous experiments had shown that BMM treated with PMA were stimulated to accumulate LY, but compared with rM-CSF-treated cells, the onset of stimulation in PMA-treated macrophages was slower. In further comparisons of rM-CSF- and PMA-stimulated LY accumulation, it was found that rM-CSF-stimulated pinocytosis could be abolished by pretreatment with 0.5 mg/ml trypsin, whereas neither unstimulated nor PMA-stimulated LY accumulation was affected by trypsin pretreatment. These findings indicate that the rM-CSF response was initiated at the cell surface, while the PMA response occurred via intracellular (or trypsin-resistant) receptors. However, once initiated, the pinocytic responses elicited by either agent were very similar. First, rM-CSF-treated cells, like PMA-treated cells, showed extensive ruffling and formation of large phase-bright pinosomes. Second, both rM-CSF- and PMA-stimulated LY accumulation could be inhibited by treatment of cells with the cytoskeleton destabilizing drugs nocodazole, colchicine, or cytochalasin D. Finally, rM-CSF, like PMA, was found to stimulate efflux of LY from cells preloaded with the dye. Thus, both rM-CSF and PMA stimulate the net rate of solute flow through the macrophage endocytic compartment.

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