Colony-stimulating factor-1 induces rapid behavioural responses in the mouse macrophage cell line, BAC1.2F5

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Summary
The cloned, SV40-immortalized mouse macrophage cell line, BAC1.2F5, resembles primary macrophages in its dependence on colony-stimulating factor-1 (CSF-1) for both viability and proliferation. Re-addition of CSF-1 stimulates rapid, transient behavioural changes in starved cells, which are rounded, with diffusely organized F-actin and few intracellular vesicles. Within 1 min, cells begin to spread, forming prominent, F-actin-rich ruffles. Small vesicles (0.5–1.0 μm), formed throughout extending lamellar processes, move centripetally and, after 3–5 min, fuse to form larger vesicles (2.0–4.0 μm), clustered around the nucleus. Immunofluorescence demonstrates that CSF-1, bound to cell-surface receptors, is internalized via these vesicles. Cell spreading and ruffling peak about 5 min after restimulation. Interference reflection microscopy indicates no corresponding change in the mode of cell–substratum adhesion: a single area of close adhesion underlies most of the cell and simply broadens during spreading. Analysis of cell aggregation kinetics shows no effect of CSF-1 on intercellular adhesiveness. Measurement of cell areas after starvation and restimulation demonstrates quantitatively the time-course and concentration-dependence of cell spreading. Mean area doubles within 5 min and, after a transient peak, decreases within 30 min to the value measured before starvation. This time-course corresponds to that of CSF-1 internalization and of the phosphorylation and subsequent degradation of CSF-1 receptors. The concentration-dependence of the spreading response resembles that of CSF-1-dependent survival and proliferation. The minimum detectable stimulation of spreading occurs at the concentration (22 pM) that supports survival without proliferation. Increasing stimulation of spreading occurs over the range of concentrations that elicit increasing proliferation.

Key words: macrophages, CSF-1, growth factor, cell spreading.

Introduction
Colony-stimulating factor-1 (CSF-1) stimulates the proliferation and differentiation of macrophages and their precursors (Stanley, 1986). Its cell surface receptor, a 165 000 M₀ protein tyrosine kinase expressed by cells of the monocyte-macrophage lineage, is the product of the proto-oncogene c-fms (Sherr et al. 1985). On binding of CSF-1, this receptor mediates the phosphorylation of several cellular proteins as well as autophosphorylation at tyrosine residues (Sherr et al. 1985; Yeung et al. 1987; Downing et al. 1988; Jubinsky et al. 1988). Uncontrolled cell proliferation can result from expression of CSF-1 and its receptor by the same cells (Baumbach et al. 1987; Roussel et al. 1987) or from expression in CSF-1-dependent cells of a modified and constitutively active receptor kinase, encoded by the v-fms oncogene of McDonough strain feline sarcoma virus (Wheeler et al. 1986).

As a model system in which to study the relation between early changes induced by growth factor binding to cells and long-term effects associated with the control of cell growth, we are using the simian virus 40 (SV40)-immortalized mouse macrophage cell line BAC1.2F5 (Morgan et al. 1987). BAC1.2F5 cells retain many characteristics of primary macrophages, including dependence on CSF-1 for both survival and proliferation in culture (Tushinski et al. 1982; Morgan et al. 1987). In
the absence of CSF-1, they cease to divide and eventually die. On re-addition of CSF-1, cells show a decrease in protein degradation and, after a 16 h lag, reinitiate DNA synthesis. Much earlier changes include the phosphorylation of cytoplasmic and membrane-associated proteins, including the CSF-1 receptor itself, the rapid, receptor-mediated internalization of CSF-1, degradation of both ligand and receptor, dramatic changes in cell shape and spreading and elevated transcription of the growth-associated genes, c-myc, c-fos, KC and JE (Morgan et al. 1987, Orlofsky & Stanley 1987; Downing et al. 1988; Jubinsky et al. 1988; Sengupta et al. 1988).

Since cell spreading is among the earliest observed responses to re-addition of CSF-1 to either BAC1.2F5 cells (Morgan et al. 1987) or to bone-marrow-derived macrophages (Tushinski et al. 1982), we have characterized this response in detail. Using phase-contrast microscopy, scanning electron microscopy, interference reflection microscopy (IRM), fluorescence cytochemistry and indirect immunofluorescence, we correlate the cell spreading and morphological changes rapidly induced by CSF-1 with the pattern of cell-substratum adhesion, the organization of the actin cytoskeleton and the internalization of cell-surface-bound CSF-1. In addition, by measuring cell areas during this response, we relate the time-course of CSF-1-induced cell spreading to that of the internalization of CSF-1 and the phosphorylation and subsequent degradation of its receptor. We also compare the concentration-dependence of the spreading response with the requirement for BAC1.2F5 cell survival and proliferation.

Materials and methods

Cell culture

Cells used in these experiments were a subclone of the cloned mouse macrophage cell line BAC1.2F5 (Morgan et al. 1987). These were maintained in growth medium consisting of α-MEM (Gibco Ltd, Paisley, Scotland) supplemented with 10 % (v/v) foetal calf serum (FCS; Imperial Laboratories), 1-32 ng/ml CSF-1 (Cetus Corporation), 2 mM-L-glutamine, 0-15 mM-L-asparagine, 15 mM-β-mercaptoethanol, 77-5 units ml⁻¹ streptomycin and 25 units ml⁻¹ penicillin. Cells were grown at 37°C in 100 mm tissue culture dishes (Becton Dickinson & Co.) kept in a humid atmosphere of 5 % CO₂. Growth medium was changed in 25 ml fragments derived from a rabbit antiserum raised against mouse CSF-1 (Stanley, 1985), diluted to 0-2 mg ml⁻¹ for use. Preimmune serum from the same goat was used in the controls. The secondary antibody consisted of TRITC-labelled F(ab')₂ fragments derived from a rabbit antiserum raised against goat IgG F(ab')₂ (Jackson ImmunoResearch Laboratories Inc.) and was diluted to 24 μg ml⁻¹. Both antibodies were diluted in PBS containing 0-2 % (w/v) BSA and 0-02 % (w/v) Na₂CO₃, then centrifuged at 10 000 g for 30 min at 4°C to remove aggregates. Labelled cultures were rinsed extensively in PBS containing 0-02 % (w/v) Na₂CO₃ and mounted in Uvinert for examination.

Scanning electron microscopy

Cultures were fixed for 15 min at 37°C in 3-7 % (v/v) paraformaldehyde in PBS and permeabilized for 10 min at 20°C in 0-1 % (v/v) Triton X-100 in PBS. Autofluorescence of residual aldehyde was quenched by rinsing cultures for 30 min in 50 mM-glycine in PBS. Immunolabelling was carried out using 50 μl of each antibody per coverslip and incubating for 45 min in a humid atmosphere at 37°C. The primary antibody was the immunoglobulin G (IgG) fraction of a goat antiserum raised against murine CSF-1 (Stanley, 1985), diluted to 0-2 mg ml⁻¹ for use. Preimmune serum from the same goat was used in the controls. The secondary antibody consisted of TRITC-labelled F(ab')₂ fragments derived from a rabbit antiserum raised against goat IgG F(ab')₂ (Jackson ImmunoResearch Laboratories Inc.) and was diluted to 24 μg ml⁻¹. Both antibodies were diluted in PBS containing 0-2 % (w/v) BSA and 0-02 % (w/v) Na₂CO₃, then centrifuged at 10 000 g for 30 min at 4°C to remove aggregates. Labelled cultures were rinsed extensively in PBS containing 0-02 % (w/v) Na₂CO₃ and mounted in Uvinert for examination.
Quantification of cell spreading

BAC1.2F5 cells were precultured 36 h on acid-washed glass coverslips at a uniform density of 2500 cells cm⁻², then starved of CSF-1 for 36 h. To reconstitute cultures, 1 ml of growth medium containing CSF-1 was added to the 3 ml of starvation medium covering cultures in each dish. To investigate the time-course of the spreading response, triplicate cultures were fixed for 5 min at 37°C in 0-5 % (v/v) glutaraldehyde in PBS at intervals after addition of CSF-1 at a final concentration of 1·32 nm. To investigate the concentration-dependence of the response, triplicate cultures were fixed 5 min after addition of CSF-1 at various concentrations. Fixed cultures were rinsed in distilled water and stained for 30 min at 37°C in 0-2 % (w/v) Coomassie Brilliant Blue in 7 % (v/v) acetic acid, 46-5 % (v/v) ethanol, 46-5 % (v/v) distilled water, then rinsed and mounted in Uvinert.

Stained cultures were viewed under bright-field illumination using a Leitz Laborlux 11 microscope fitted with a 40X plan objective and a drawing tube. In each of three replicate cultures, areas of 50 cells, sampled at random but excluding any touching their neighbours, were measured by digitizing their outlines at a resolution of 0-5 μm, using a MOP-1 digitizer (Kontron Analytical, Slough UK) interfaced to a microcomputer as described by Pizzey et al. (1984).

Analysis of cell aggregation

Dishes of near-confluent cultures of BAC1.2F5 cells (Becton Dickinson & Co.) were rinsed with Ca²⁺-free, Mg²⁺-free Hank's balanced salt solution and dissociated by incubating for 15 min at 37°C with 1 ml of 0-15 % (w/v) porcine crystalline trypsin (Sigma Chemical Company Ltd) in PBS. Trypsin was inactivated by addition of an equal volume of FCS and cells were collected by centrifugation at 350 g for 3 min at 4°C. Cells were allowed to recover for 30 h in bacteriological grade Petri dishes (Sterilin, Feltham, UK) in growth medium with or without 1·32 nm-CSF-1. After recovery, cells remained sufficiently weakly attached that more than 90 % were resuspended by gentle trituration for 3 min at 20°C. Resuspended cells were diluted to 5 X 10⁴ cells ml⁻¹ in 25 mm-Hepes/MBE containing 10 % (v/v) FCS, with or without 1·32 nm-CSF-1. Collision efficiency, a measure of cell-cell adhesiveness, was calculated by analysis of cell aggregation kinetics, using the method of Curtis (1969). Briefly, 1 ml of cell suspension was introduced into the gap between the inner and outer cylinders of a couette viscometer and, by rotation of the outer cylinder, a laminar shear gradient was set up in this gap. Cell aggregation was monitored by periodically examining 20-μl samples taken from the viscometer, which was kept at 37°C throughout. Pilot experiments indicated that, for measurement of collision efficiency in BAC1.2F5 cells, optimal aggregation kinetics were given by a starting concentration of 5 X 10⁴ cells ml⁻¹, a liquid shear rate of 10 s⁻¹ and sampling at 5-min intervals for 20 min. Collision efficiency was calculated under these conditions according to Curtis (1969). Cells were sized using a Coulter counter fitted with a channalyser (Coulter Electronics Ltd, Harpenden, UK) and cell viability was assessed by Trypan Blue dye exclusion.

Results

CSF-1 stimulates rapid, transient cell spreading, membrane ruffling and cytoskeletal reorganization

The rapid and reversible changes in cell shape that follow stimulation with CSF-1 are illustrated by phase-contrast and scanning electron micrographs (Figs 1 and 2). Cells cultured in the presence of CSF-1 (Figs 1A and 2A) vary from highly elongated, bipolar shapes to rounded (possibly mitotic) forms with or without several short flattened processes. Many contain vesicles of various types. In the absence of CSF-1 (Figs 1B, E and 2B), most cells become rounded, anchored only by fine retraction fibres, and lack intracellular vesicles. On re-addition of CSF-1, cell spreading begins within 1 min. Cells extend several ruffled, lamellar processes within which small (0·5 μm), phase-dark vesicles appear (Fig. 1F). Pre-existing processes of some more-extended cells retract at this stage (Fig. 1E, F). Between 1·5 and 3·5 min (Fig. 1G), small phase-lucent vesicles are formed and begin to move towards the nucleus where they coalesce to form larger vesicles (Fig. 1H, I). After 5–15 min, most cells have a roughly circular, ruffled lamella and prominent vesicles clustered around the nucleus (Figs 1C, H, I and 2C, D). Scanning electron microscopy shows that membrane ruffles, initially formed all over the upper cell surface, are progressively restricted to marginal and central areas (Fig. 2C, D).

Labelling with TRITC-phalloidin reveals major cytoskeletal reorganization during these changes (Fig. 3A–D). Labelled F-actin is uniformly distributed in cells cultured without CSF-1 (Fig. 3B) but is strongly concentrated into the membrane ruffles formed within 5 min of restimulation (Fig. 3C). After 1 h, cultures resemble those fixed before withdrawal of CSF-1 and no further change occurs upon extended incubation. Cells are heterogeneous in shape, many becoming elongated and polarized with ruffles and vesicles predominantly confined to one end (Figs 1A, D, 2A, E and 3A, D, E, H). In these cultures, TRITC-phalloidin labels chiefly the margins and remaining areas of ruffling as well as retraction fibres, where present, and occasional clusters of dots on the undersurfaces of cells. These dots do not correspond to any structures visualized with phase-contrast microscopy or to regions of closer cell-substratum contact indicated by IRM.

The effect of CSF-1 on cell-substratum adhesion was investigated by IRM (Fig. 3I–P). Comparison of phase-contrast and IRM images shows no change in the overall pattern of adhesion during the dramatic stimulation of spreading and ruffling activity on re-addition of CSF-1. Adhesion at all stages of starvation and restimulation seems to be mediated by a single area of close contact occupying most of the undersurface of each cell. No focal adhesions are seen and the darker margins of some cells observed by IRM after 30 min (Fig. 3K) probably represent very thin lamellar extensions, almost invisible by phase-contrast microscopy (Gingell, 1981).

Stimulation of cell spreading by CSF-1 is reversible and concentration-dependent

Fig. 4 shows quantitatively both the time-course and the concentration-dependence of cell spreading on re-addition of CSF-1 after 36 h of starvation. Preliminary experiments indicated that 36 h of culture without CSF-1 results in maximal contraction of cell area without loss of viability (data not shown). After starvation, mean cell area has decreased by 27 %. On restimulation with
1-32 nM-CSF-1 (3000 units ml$^{-1}$), it more than doubles within 5 min, then decreases sharply, returning after 15–30 min to its value before starvation. This value is stable up to 6 h after restimulation and does not differ significantly from the values measured for cells fixed without starvation at 0 or 360 min.

The concentration-dependence of the spreading response was determined 5 min after restimulation, the time-point at which spreading was maximal with 1-32 nM-CSF-1. The minimum concentration to stimulate cell spreading is 22 pM (50 units ml$^{-1}$), which results in a 38% increase in mean cell area. Stimulation increases with concentration above 132 pM (300 units ml$^{-1}$), with no saturation up to the concentration required for maximal proliferation.

**Stimulation of cell spreading coincides with rapid internalization of CSF-1**

Fig. 5 shows immunolocalization of CSF-1 in cells preincubated with CSF-1 at 0°C to saturate cell surface receptors. In cells fixed before warming, either permeabilized or unpermeabilized (data not shown), labelling is continuous, though uneven, all over the cell surface (Fig. 5A,E). Within 1 min of warming cells rapidly to 37°C, CSF-1 is detectable only after permeabilization and is concentrated into small, phase-lucent vesicles (0.5–1.0 μm), which appear throughout newly formed lamellae (Fig. 5B,F). Cells at this stage typically still exhibit retraction fibres as well as new lamellar extensions. After 5 min (Fig. 5C,G), annular, ruffled lamellae have replaced all retraction fibres and contain few vesicles. Most CSF-1 is concentrated into larger vesicles (2–4 μm) in the perinuclear area. Limiting membranes of these phase-lucent vesicles are intensely labelled with anti-CSF-1 in a punctate pattern. Phase-dark vesicles, more numerous than after 1 min, are not labelled with anti-CSF-1 antibodies. After 15 min (Fig. 5D,H), well-spread cells have started to retract and display both retraction fibres and flattened lamellae devoid of vesicles and of CSF-1. Around the nucleus are clustered vesicles of various sizes labelled to various extents with anti-CSF-1 antibodies, some still showing a punctate distribution.

**CSF-1 does not alter the adhesiveness of BAC1.2F5 cells**

Cell aggregation kinetics in couette viscometers were analysed to calculate collision efficiency, the percentage of cell–cell collisions that result in stable adhesion. This gives a reproducible measure of cell–cell adhesiveness (Curtis, 1969; Jones et al. 1985). Table 1 gives collision efficiencies measured in five replicate experiments com-
Table 1. Effect of CSF-1 on cell–cell adhesiveness, measured by collision efficiency (%)

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<th>Recovery, −CSF-1*</th>
<th>Recovery, +CSF-1*</th>
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<tr>
<td>Aggregation, −CSF-1</td>
<td>11.32 ± 0.82</td>
<td>9.83 ± 0.34</td>
</tr>
<tr>
<td>Aggregation, +CSF-1</td>
<td>11.76 ± 0.65</td>
<td>10.05 ± 0.06</td>
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* Collision efficiencies (%) are given as mean ± S.E.M. of five separate experiments. Cells dissociated with trypsin, then allowed to recover for 30 h in the presence or absence of 1·32 nm-CSF-1, were aggregated for 20 min in a couette viscometer in medium with or without 1·32 nm-CSF-1. Aggregation kinetics were determined by examination of 20-μl samples taken from the viscometer at 5-min intervals and analysed by the method of Curtis (1969).

Comparing intercellular adhesiveness of cells treated in four ways. Cells were allowed to recover from trypsin in the presence or absence of 1·32 nm-CSF-1, then aggregated in medium with or without 1·32 nm-CSF-1. Comparison using Student's t-test shows no significant differences between the four treatments at the level of P < 0.05.

Collision efficiencies measured in all experiments (10.74 ± 0.34 %, n = 20) considerably exceeded values (3·5–4·0 %) measured for freshly trypsinized cells, indicating an increase in adhesiveness during the 30 h recovery period. Thirty hours has previously been found ample for the maximal replacement of CSF-1 receptors following trypsin treatment (Stanley, unpublished observations). Growth kinetics of BAC1.2F5 cells maintained on bacteriological grade plastic (as used here during recovery from trypsin) do not differ noticeably from those of cells in tissue culture grade dishes (Morgan et al. 1987).

Discussion

Using scanning electron microscopy, phase-contrast and fluorescence microscopy with TRITC-phalloidin labelling, we have demonstrated in BAC1.2F5 macrophages stimulated with CSF-1, rapid, transient, coordinated
changes in cell shape, spreading, membrane ruffling activity and organization of the actin cytoskeleton. Within 1 min of stimulation, cells extended prominent, ruffled lamellae, which were intensely labelled with TRITC-phalloidin, suggesting a localized polymerization or concentration of F-actin at their protruding margins. In preliminary experiments, primary bone-marrow-derived macrophages showed similar behavioural responses to CSF-1 (P. W. Tynan, L. J. Guilbert & E. R. Stanley, unpublished observations). Similar phenomena also follow target cell interaction with PDGF, EGF, NGF, insulin and chemoattractant peptides (Chinkers et al. 1979; Connolly et al. 1979; Rao & Varani, 1982; Bockus & Stiles, 1984; Miyata et al. 1988).

The increased actin nucleation in neutrophils responding to chemoattractant peptides appears independent of cytoplasmic Ca$^{2+}$ fluxes (Carson et al. 1986). In view of functional similarities between neutrophils and macrophages, it is therefore of interest that the response of bone marrow macrophages to CSF-1 does not raise cytoplasmic Ca$^{2+}$ levels (Whetton et al. 1986).

During the dramatic stimulation of BAC1.2F5 cell spreading by CSF-1, observation by IRM showed no corresponding changes in the pattern of cell–substratum adhesion. Indeed, unlike fibroblasts and epithelial cells, BAC1.2F5 macrophages appear to lack specialized adhesions and to adhere instead by a single close contact, which underlies most of the cell and simply extends during spreading. To investigate the induction of macrophage spreading by CSF-1, it was of interest to determine whether CSF-1 increased cell-surface adhesiveness as well as cytoskeletal activity. Cell–cell adhesiveness is conveniently measured by analysis of cell aggregation kinetics in a couette viscometer (Curtis, 1969). In the laminar shear gradient within the viscometer, cell–cell collisions occur at a constant frequency and cell–cell adhesion is opposed by a constant shear force. The proportion of collisions resulting in adhesion of sufficient strength to resist this force is reflected by the formation of aggregates and measured as the collision efficiency. In the early stages of aggregation, when most collisions are between single cells, it indicates the strength of initial adhesion rather than the resistance of aggregates to dissociation after spreading over their mutual surface has stabilized adhesion between cells. In the experiments described here, a substantial increase in cell–cell adhesiveness occurred during 30 h of recovery after trypsinization, but this increase was largely insensitive to the presence or absence of CSF-1 during recovery or aggregation. Thus CSF-1 regulates cell spreading but not intercellular adhesiveness.

Early in the response of BAC1.2F5 cells to CSF-1, numerous, tiny vesicles (0.5–1.0 μm) appear within 1 min of stimulation and subsequently move towards the nucleus, where they fuse to form larger vesicles (2.0–4.0 μm). Immunofluorescence using antibodies to CSF-1 showed these vesicles to be the site of receptor-mediated internalization of CSF-1. The larger vesicles may correspond to a phagolysosomal compartment where CSF-1, and possibly its receptor, is degraded (Chen et al. 1984). These vesicles form and persist over the interval
Fig. 5. Internalization of cell-surface-bound CSF-1. Internalization of CSF-1 was synchronized by saturating cell surface receptors with pure L-cell CSF-1 at 0°C, then, after rinsing, rapidly warming cultures to 37°C. A–D. Fluorescence micrographs of Triton-extracted cells labelled by indirect immunofluorescence using antibodies against murine CSF-1. E–H. Corresponding phase-contrast micrographs. Bars, 10 μm. Cells were fixed: A, E, before warming; B, F, 1 min; C, G, 5 min; and D, H, 15 min, after temperature shift.

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during which CSF-1 receptor becomes tyrosine-phosphorylated and is degraded (Downing et al. 1988). In A212 cells, which display prolonged morphological responses to EGF (Chinkers et al. 1979), the EGF receptor, tyrosine-phosphorylated following ligand binding, persists for several hours on the cell surface (Sturani et al. 1988).

From measurements of cell area, we have determined the time-course of BAC1.2F5 cell spreading in response to growth-stimulatory concentrations of CSF-1. Cell area doubles within 5 min of stimulation but, after a transient peak, decreases rapidly and after 15 min does not differ significantly from the value measured in cultures fixed without starvation. The interval of stimulation thus corresponds closely with both the internalization of CSF-1 and the presence of tyrosine-phosphorylated receptor (Downing et al. 1988). The concentration-dependence of the spreading response was investigated at the time-point (5 min) of maximal stimulation. This did not show the sigmoidal relationship that might be expected if a critical proportional receptor occupancy simply triggered a full response. Instead, the concentration-dependent increase in stimulation appeared to be biphasic. A similar biphasic concentration-dependence of spreading has been found in BAC1.2F5 cells allowed to settle from suspension and spread for 6 h in the presence of CSF-1 (Boocock et al. 1987). These results showed considerable correspondence with CSF-1 requirements of BAC1.2F5 cell survival and proliferation (Morgan et al. 1987). The minimum concentration of CSF-1 that stimulates cell spreading (22 pm) supports survival without proliferation. Stimulation of spreading increases with CSF-1 concentration, above 132 pm, as does cell proliferation between 220 pm and 1-32 nm (Morgan et al. 1987). This pattern of response is unlikely to indicate the existence of two classes of CSF-1 receptor site. The pseudo-first-order kinetics of 125I-labelled CSF-1 binding to bone-marrow-derived macrophages at high ligand concentrations at 4°C, together with the first-order kinetics of its dissociation from receptors at 37°C, are consistent with the existence of a single class of cell surface receptors, and even the cryptic binding sites externalized during extended exposure to sub-saturating concentrations of CSF-1, are kinetically indistinguishable (Guilbert & Stanley, 1986).

CSF-1 brings about many changes in macrophages, starting with the phosphorylation of its receptor and other cellular proteins on tyrosine, and culminating in the stimulation of cell growth (Morgan et al. 1987; Yeung et al. 1987; Downing et al. 1988; Jubinsky et al. 1988; Sengupta et al. 1988). The time-scale of tyrosine phosphorylation of the receptor and other cellular proteins corresponds closely with that of the early spreading, ruffling and endocytic responses. Furthermore, the concentration-dependence of this set of responses resembles that of cell survival and growth. It is clearly of interest to investigate the relationship between these early, CSF-1-induced responses. This will be facilitated by the availability of a number of mutant clones derived from BAC1.2F5, which no longer require CSF-1 for survival and/or growth (Pollard, J. W., C. J. Morgan, C. Cheers & E. R. Stanley, unpublished data).

This work was performed under grants from the Cancer Research Campaign (UK), and grants CA26504, CA32551 and the Albert Einstein College of Medicine Cancer Center Core F30-CA13330 Grant.

References


**Effects of CSF-1 on macrophage behavior**


(Received 28 February 1989 – Accepted 19 April 1989)