RECEPTOR-MEDIATED BINDING AND INTERNALIZATION OF COLONY-STIMULATING FACTOR (CSF-1) BY MOUSE PERITONEAL EXUDATE MACROPHAGES

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SUMMARY

Colony-stimulating factor (CSF-1) purified from L-cell-conditioned medium is a haemopoietic growth factor that specifically stimulates the proliferation and differentiation of mononuclear phagocytes. Using radioactively labelled CSF-1 ([¹²⁵]CSF-1), the presence of specific CSF-1 receptor has been identified in the cells of the mononuclear phagocytic series and their precursors only. To determine the fate of [¹²⁵]CSF-1 bound to peritoneal exudate macrophages (PEM) at 37°C, we have examined the distribution of radioactivity as a function of time by quantitative electron microscopic autoradiography. At 0°C, we have localized the initial step in the binding of [¹²⁵]CSF-1 to the plasma membrane and its invaginations of the mouse PEM. Approximately 16% of the macrophages were not labelled at this time point. When the temperature was raised to 37°C, the labelled CSF-1 was internalized progressively by the cells in a time-dependent fashion. The proportion of grains associated with the phagolysosome compartment increased progressively, reaching a plateau by 40 min after warming up, while the relative areas of the surface membrane and its invaginations decreased in invaginated membrane. At 37°C, incubation with unlabelled CSF-1 resulted in a 'down-regulation' of the subsequent [¹²⁵]CSF-1-binding activity by PEM in a time- and dose-dependent fashion. The restoration of CSF-1-binding activity after CSF-1 induced down-regulation was inhibited by cycloheximide, a potent protein synthesis inhibitor. These data provide direct evidence that at 37°C, saturable binding of CSF-1 to PEM is followed by internalization and cellular degradation of the ligand and possibly its receptor by phagolysosomes.

INTRODUCTION

Colony-stimulating factor (CSF-1) purified from L-cell-conditioned medium is a sialoglycoprotein of molecular weight 70000 composed of two disulphide-bonded subunits of similar size and charge (Stanley & Heard, 1977). It specifically stimulates macrophage colony formation by the committed stem cells for both granulocytes and macrophages and by various classes of more-differentiated mononuclear phagocyte colony-forming cells (Stanley, 1981; Stanley, Chen & Lin, 1978). Recent studies using radioactively labelled CSF-1 have shown that at 0°C [¹²⁵]CSF-1 interacts with

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target cells by direct and specific binding through membrane receptors (CSF-1 receptors). These target cells are restricted to cells of the mononuclear phagocyte system and to macrophage cell lines (Chen & Lin, 1982; Byrne, Guilbert & Stanley, 1981; Guilbert & Stanley, 1980). It is believed that these CSF-1 receptors mediate both CSF-1-induced cell proliferation and CSF-1 destruction by target cells at 37 °C (Chen, Hsu & Lin, 1982; Stanley & Guilbert, 1981). Tushinski et al. (1981) showed that CSF-1 is degraded by macrophages in a concentration-dependent manner and that the rate of degradation is saturable at CSF-1 concentrations that cause maximum proliferation.

Recent studies using quantitative electron microscopic (EM) autoradiography combined with 125I-labelled ligand provide strong evidence that several polypeptide hormones are rapidly internalized by the target cells after binding to their specific membrane receptors and become progressively associated with lysosomes or other intracellular organelles (Gorden, Carpentier, Cohen & Orci, 1978; Goldfine et al. 1978; Bergeson, Posner, Josetsberg & Sikstrom, 1978; Campbell et al. 1979). In the present study, we have used a similar EM autoradiographic technique to explore further the interactions of 125I-labelled-CSF-1 to PEM. Our results showed that after the initial binding, 125I-CSF-1–receptor complex is rapidly internalized by peritoneal exudate macrophages (PEM) and becomes progressively associated with phagolysosome. There is loss of [125I] CSF-1-binding activity accompanying this process, which can be restored after prolonged incubation at 37 °C in serum-containing medium.

**MATERIALS AND METHODS**

**Animals**

Male C3H/He mice (derived from C3H/HeJ) 8–12 weeks of age were obtained from our own SPF colony. All mice were fed standard laboratory chow and water ad libitum.

**Reagents**

We obtained foetal calf serum (FCS) and alpha-MEM from GIBCO, Grand Island, NY. We purchased carrier-free Na125I from Industrial Nuclear Co., St Louis, MO, chloroquine and cycloheximide were obtained from Sigma, St Louis, MO.

**Cells**

Peritoneal exudate cells were harvested by peritoneal lavage with 5 ml alpha-MEM 3 days after one intraperitoneal injection of 1.5 ml of thiglycollate medium (Difco Laboratories, Detroit, MI). All cells were washed once with alpha-MEM containing 10 % (w/v) FCS (alpha-10) and re-suspended in cold alpha-10 to reduce their adherence to the tubes (Falcon 2059). We obtained peritoneal exudate macrophages (PEM) by seeding 10⁶ peritoneal exudate cells in 1 ml of alpha-10 in 35 mm Falcon tissue-culture dishes for 15 min at 37 °C. We then removed the non-adherent cells by washing once with phosphate-buffered saline (PBS) (Guilbert & Stanley, 1980). For the [125I]CSF-1-binding study, we further cultured the adherent cells at 37 °C for 20 h in a humidified incubator continuously flushed with 10 % CO₂ in air. Over 95 % of these adherent cells could be identified as mononuclear phagocytes.

**Purification and iodination of CSF-1**

CSF-1 was purified from serum-free L-cell-conditioned medium by a five-step procedure as described by Stanley & Heard (1977) with slight modification. The specific activity of the purified CSF-1 was at least 5 × 10⁷ units/mg protein as determined by the standardization method of van den
Internalization of colony-stimulating factor

Engh (1974). To determine the purity we used sodium dodecyl sulphate/polyacrylamide disc gel electrophoresis (SDS/PAGE) at 9% and 12.5% acrylamide. Conjugation of biological activity and labelled protein was used as the index of purity (Stanley & Heard, 1977; Chen & Lin, 1982). Iodination of CSF-1 was carried out by a method described previously (Stanley & Guilbert, 1981). The specific activity of [125I]CSF-1 used in this study was 150 μCi/μg. The labelled CSF-1 was further purified by gel filtration on G-25 Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) at room temperature before the experiment. [125I]CSF-1 prepared in this manner retained its biological activity and also reacted with either rabbit or rat anti-CSF-1 antiserum (Stanley & Guilbert, 1981; Chen, Hsu & Lin, 1983). We used [125I]CSF-1 within 4 weeks of iodination since its biological activity decreases with time (t1/2 = approximately 6 weeks).

Direct [125I]CSF-1-binding assay

To measure [125I]CSF-1 binding, we washed cultured cells twice with cold PBS. We then added to each dish 0.9 ml of cold alpha-MEM containing 10% FCS and 20 mM-MOPS instead of the usual sodium bicarbonate (alpha-MOPS-10), pH 7.2. Labelled CSF-1 was added to a final volume of 1 ml. All binding assays were performed at 0 to 2°C. After incubation for 16 h, we removed unbound [125I]CSF-1 by washing the cells three times with cold PBS. The cells were then solubilized from the dishes for gamma counting with two washes of 0.75 ml of 50 mM-Tris-HCl, 0.5% SDS (pH 7.4). Non-specific binding, determined as the amount of radioactivity bound in the presence of a 200-fold excess of unlabelled CSF-1, was subtracted from the results to give specific binding. Non-specific binding was always less than 3% of the total binding.

Autoradiography

For EM autoradiographic studies, freshly obtained peritoneal exudate cells (10^7 cells in 2 ml alpha-MOPS-10) were incubated with 10^6 c.p.m. of [125I]CSF-1 (approx. 10 ng) for 2 h at 0°C. We stopped the reaction by layering the cell suspension over 5 ml of ice-cold FCS in a Falcon culture tube (no. 2059); the cells were then centrifuged (750 g, 10 min, 0°C) to remove the unbound [125I]CSF-1. The pellet was resuspended in 2 ml warm alpha-MOPS-10, mixed and then cultured in a 37°C warm room for various periods of time.

At intervals, we transferred 200 μl of cell suspension to microfuge tubes, chilled and rapidly pelleted the cells by centrifugation for 1 min at 1300 g. The pellets were fixed in 3% glutaraldehyde in 0.1 M-cacodylate buffer (pH 7.3) and stored in this fixative at 4°C until the end of the experiment. Fixation of the pellets was continued for an additional hour in glutaraldehyde; the pellets were then rinsed in cacodylate buffer, fixed with 1% OsO4 in cacodylate buffer, dehydrated in graded concentrations of acetone and embedded in Epoxy resin (Spurr, 1969). Thin sections (80 nm) were collected on copper grids and carbon-coated. Ilford L-4 nuclear track emulsion was applied by the technique of Caro, Van Tubergen & Kolb (1962). Autoradiographs were exposed in light-tight boxes at 4°C for 120 days; they were developed in Kodak Microdol-X developer for 6 min and fixed with Kodak Rapid Fix (Neeley & Combs, 1976). The emulsion was removed with 0.05 M-NaOH; the grids were then stained with uranyl acetate and lead citrate and examined with a JEOL 100 CX electron microscope.

Initially, we examined 150-200 consecutive cells for each period of time, and recorded the number of grains over each cell. Twenty-five consecutive cells containing four or more grains were then photographed and printed at a magnification of ×12,500 for detailed analysis. The average number of grains per cell varied among the cells photographed from 19 ± 2 at 1 min to 7.2 ± 0.5 at 1 h. The centre of each grain was marked with a pinhole as described previously (Campbell et al. 1979) and the underlying organelle or cellular compartment was recorded. The photomicrographs were then covered with a transparent overlay marked with the multipurpose test system of Weibel & Bolender (1973), which contained 50 lines. The ends of the lines provided 100 points, which we used to determine the volume density of the various compartments. We used the lines to measure the relative lengths of two plasma membrane compartments (to be defined below) by intercept counting. Approximately 600 intercepts/cell were counted.

Histochemistry

Ultrastructural histochemistry was carried out as an aid in the identification of organelles. Pellets of freshly harvested peritoneal exudate cells were stained with ruthenium red, as described by Luft
Acid phosphatase staining was carried out on pellets after fixation for 30 min at 3 °C in 3 % (v/v) glutaraldehyde in 0.1 M-cacodylate buffer. After three 15-min washes in 7 % (w/v) sucrose, the cells were incubated at 37 °C for 30 or 60 min in a substrate containing 11 mm-sodium β-glycerophosphate, 2.4 mm-lead nitrate and 5 % sucrose in 0.02 M-3-Tris maleate buffer (pH 5.2). After washing in three changes of 7 % sucrose, the pellets were fixed in 1 % OsO4 in 0.1 M-cacodylate buffer (pH 7.4) for 30 min, dehydrated with acetone and embedded in Epoxy resin. Control pellets were incubated in the same substrate lacking β-glycerophosphate. Cytocentrifuge preparations of the same cells were fixed for 10 min in cold acetone and stained for acid phosphatase, for light microscopy using a simultaneous azocoupling procedure with naphthol AS-BI phosphoric acid as substrate and fast garnet GBC diazonium salt as the coupling reagent (Burstone, 1961).

**SDS/polyacrylamide gel electrophoresis (SDS/PAGE) and gel autoradiography**

To characterize the intracellular radioactivity after [125I]CSF-1 binding and internalization, we carried out SDS/PAGE on slab gel (9 % and 12.5 % gel concentrations) according to the method of Laemmli (1970) described previously (Stanley & Heard, 1977), with slight modification. Briefly, 2×10⁶ cultured PEM were labelled with 200 000 c.p.m. of [125I]CSF-1 at 0 °C for 3 h. After labelling, cells were washed with cold PBS to remove unbound [125I]CSF-1 and were resuspended in 1 ml of warm alpha-MOPS-10. After various time periods at 37 °C, cells were washed extensively with cold PBS and were then lysed with 0.5 ml of sample buffer containing 12.5 % (v/v) glycerol, 1.25 % SDS, 0.001 % Bromophenol Blue, and 1 mm-phenylmethylsulphonyl fluoride in 0.06 M-Tris-HCl buffer (pH 6-8). Before electrophoresis, all samples and marker proteins (Pharmacia Fine Chemicals, Uppsala, Sweden) were boiled for 5 min. Electrophoresis was carried out at a constant voltage (100 V) for 3-5 h at 20 °C. For autoradiography, gels were fixed, stained for 2 h with 0.25 % Coomassie Blue, destained overnight and then dried on a slab gel dryer (Hoefer Scientific Instruments, San Francisco, CA). Autoradiography was performed by exposing the dried gel to X-ray film (Kodak X-Omat AR film) for 3-7 days at room temperature.

**RESULTS**

**Morphology and histochemistry**

The outlines of the PEM were irregular with numerous short projections of cytoplasm, some protruding radially, others folding back parallel to the cell surface. A zone of 1 to 3 μm beneath the plasma membrane was occupied largely by round or oval membrane-bound structures, which appeared to be empty vacuoles 0.3-1.2 μm in diameter. Ruthenium red, which stains glycocalyx but is excluded from intracellular structures, was bound to the membrane of these structures, indicating that they were invaginations of the plasma membrane rather than complete vacuoles.

Fig. 1. Macrophages stained with ruthenium red showing binding of the stain to the plasma membrane of empty subplasmalemmal structures (i), which appear as vacuoles but must be invaginations of the plasmalemma by virtue of their accessibility to ruthenium red. The more convoluted membranes of vacuoles (ii) containing flocculent material remain unstained. Counterstained with uranyl acetate. ×44800. Bar, 0.5 μm.

Fig. 2. A. Acid phosphatase stain showing irregularly shaped vacuoles (phagolysosomes) in the cytoplasm with flocculent lead deposits. ×11000. Bar, 1 μm. B. A higher magnification of the same preparation as in A, contrasting the homogeneous dense staining of lysosome granules (i) with the less concentrated flocculent lead deposits in a phagolysosome (ii). The invaginations of the plasma membrane (i) are unstained. ×30000. Bar, 0.5 μm. C. Acid phosphatase stained by the azo dye method. Lysosomal granules appear black while the less heavily stained phagolysosome is grey. ×45000. Bar, 0.5 μm.
(Fig. 1). The cells had the usual morphological features of macrophages: eccentric reniform nucleus, prominent cytocentre with centrioles, Golgi apparatus and lysosomes. In addition there were vacuoles of irregular shape and size varying from 0.5 μm to 5 μm in diameter distributed apparently randomly throughout the cytoplasm. These vacuoles contained a granular or finely fibrillar material of varied electron density, which we presumed to be materials that were originally present in thioglycollate medium and taken up by the cells. The membrane lining these vacuoles did not stain with ruthenium red in contrast to that of the empty vacuoles. In cytocentrifuge preparations these vacuoles stained for acid phosphatase with variable intensity, usually faintly (Fig. 2). Even when relatively strongly stained, the staining was distinctly weaker than the punctuate staining of the granular lysosomes. With the electron microscopic acid phosphatase reaction, focal deposits of lead were present in the contents of these vacuoles, generally less concentrated than the staining of the lysosomal granules (Fig. 2). We designated vacuoles of this type as phagolysosomes, based on acid phosphatase activity and presumed materials from thioglycollate medium.

**Autoradiography**

In analysing the autoradiographs, we defined the membrane rim compartments as two half-distances on either side of the membrane, taking the half-distance for 125I to...
be 85 nm (Goldfine et al. 1978; Salpeter, McHenry & Salpeter, 1978). The plasma membrane rim was subdivided into 'surface membrane', where the membrane contacted the extracellular space in the plane of the section, and 'membrane invagination' when the plane of section did not contain a site of contact with the extracellular space, i.e. where the section appeared to show a subplasmalemmal vacuole.

Since only approximately 10% of peritoneal exudate cells form colonies (Lin & Stewart, 1974), we initially recorded the number of grains per cell at low power to determine whether two distinct populations could be distinguished, i.e. cells that bound a large amount of CSF-1 corresponding to the colony-forming cells, and cells with few CSF-1 receptors corresponding to the remainder. Even at the earliest times, 1 and 3 min, only 16% of the macrophages were unlabelled; this number increased to 30% by 40 and 60 min. Moreover, the number of grains per cell was unimodally distributed and did not permit the recognition of two distinct populations (Fig. 3).

At 1–10 min the grains were distinctly localized to the cell periphery (Figs 4, 5). Grain counts (Table 1) showed that more than half the label was associated with the plasma membrane compartment including its invagination. As early as 3 min after warming, a few grains were already observed in the phagolysosome compartment. The proportion of grains in phagolysosomes increased progressively to a plateau by 40 min (Figs 6–8). Owing to the irregular shape and exceedingly convoluted membrane of the phagolysosomes, it was not possible to determine whether grains were associated with the membrane or contents of the phagolysosomes.

Table 2 shows the volume density of different cellular compartments for comparison with the grain counts. The distribution of volume densities differs markedly from the distribution of grains ($P < 0.01$), as shown by Chi-squared analysis at all time

Table 1. Distribution of grains from [125I]CSF-1 in various compartments of macrophages

<table>
<thead>
<tr>
<th>Membrane rim</th>
<th>Sur</th>
<th>Inv</th>
<th>Lys</th>
<th>Mit</th>
<th>Cyt</th>
<th>Pl</th>
<th>Pl rim</th>
<th>Nu + Nu rim</th>
<th>Total grains counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>15.3</td>
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<td>-</td>
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<td>1.6</td>
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<td>3.8</td>
<td>0.8</td>
<td>30.5</td>
<td>8.4</td>
<td>25.2</td>
<td>7.6</td>
<td>131</td>
</tr>
</tbody>
</table>

* Grain distribution analysis of [125I]CSF-1 in PEM. Autoradiographs were analysed in the electron microscope.

The centre of each grain was marked with a pinhole and the underlying organelle or cellular compartment was recorded. Data are percentages of total grains counted at each time point.

Sur, surface; Inv, invagination; Lys, lysosome Mit, mitochondria; Cyt, cytoplasm; Pl, phagolysosome; Nu, nucleus.
Figs 4-5
Fig. 6. Autoradiograph after 40-min incubation at 37 °C. Membrane invaginations are less numerous than earlier and grains are associated with various intracellular structures including phagolysosomes, lysosomes and the nucleus. ×8800. Bar, 1 μm.

Fig. 8. Autoradiographs showing grains associated with phagolysosomes (pl) or their limiting membranes at 37 °C for: a, 40 min; b, 60 min. ×13 000. Bar, 1 μm.

Fig. 4. Autoradiograph of a cell fixed after 2-h incubation with [125I]CSF-1 at 0 °C, washing and 3 min at 37 °C. Grains are limited to the periphery of the cell in the zone occupied by invaginations of the plasma membrane. ×8800. Bar, 1 μm.

Fig. 5. Autoradiograph of cells fixed after 1-min incubation at 37 °C. Grains are mainly located near the cell surface and over invaginations (i) of the membrane. ×13 000. Bar, 1 μm.
Fig. 7. Fraction of grains associated with plasma membrane (▲) (including its invaginations) and phagolysosomes (△). Data were obtained from Table 1 as % of total grains associated with cells.

Table 2. Volume densities of various compartments of macrophages

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Membrane rim</th>
<th>Sur</th>
<th>Inv</th>
<th>Lys</th>
<th>Mit</th>
<th>Cyt</th>
<th>Pl</th>
<th>Pl rim</th>
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</table>

The volume densities of the organelles were determined according to the method of Weibel & Bolender (1973). Abbreviations: see Table 1. Ext, extracellular.
### Table 3. Comparison of observed grain distribution with that predicted from volume densities

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<tr>
<th>Time</th>
<th>Membrane</th>
<th>Sur</th>
<th>Inv</th>
<th>Lys</th>
<th>Mit</th>
<th>Cyt</th>
<th>Pl</th>
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<th>Nu</th>
<th>Nu rim</th>
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<th>χ²</th>
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<td>1 min</td>
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<td>68</td>
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<td>9</td>
<td>128</td>
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<td>40</td>
<td>22</td>
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<tr>
<td></td>
<td>Observed</td>
<td>78</td>
<td>219</td>
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<td>2</td>
<td>92</td>
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<td>14</td>
<td>7</td>
<td>9</td>
<td>8</td>
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<tr>
<td></td>
<td>(Pr–Ob)²/Pr</td>
<td>13-6</td>
<td>335</td>
<td>1-6</td>
<td>5-5</td>
<td>9-8</td>
<td>13-4</td>
<td>16-9</td>
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<td>7-7</td>
<td>34-3</td>
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<td>2</td>
<td>38</td>
<td>7</td>
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<tr>
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*The number of grains predicted assuming that all cellular compartments contained the same concentration of label per unit volume.
Abbreviations: see Table 1. Ext, extracellular.
periods (Table 3). There was a decrease with time at 37°C in the volume density of the invaginated membrane rim compartment. In order to confirm the decrease in the invagination of the membrane, we used linear intercepts to measure the relative areas of the two membrane compartments. This measurement also shows the decrease in invaginated membrane with time (Fig. 9). Comparing their relative areas, the invaginated membrane was more heavily labelled \((P < 0.01)\) than the surface membrane at all times except 40 min (results not shown).

**Effect of lysomotropic agents on the degradation of cell-bound \(^{[125I]}\)CSF-1**

From the studies presented in the previous section, it appears that internalized \(^{[125I]}\)CSF-1 are most probably degraded in phagolysosomes. If this is true, one would expect a delay in the disappearance of cell-associated \(^{[125I]}\)CSF-1 when the cells are exposed to chloroquine, a potent lysomotropic agent. In this experiment, PEM were preincubated with \(^{[125I]}\)CSF-1 at 0 to 2°C for 16 h. We then removed the unbound \(^{[125I]}\)CSF-1 by washing the cells three times with cold PBS. Next, we resuspended cells in 1 ml of alpha-MOPS-10 in the presence and absence of chloroquine (1 mM) and raised the temperature to 37°C. At various periods of time, cell-associated radioactivities were determined as described previously. As shown in Fig. 10, warming cells to 37°C resulted in a rapid loss of cell-associated radioactivity by PEM.
Internalization of colony-stimulating factor

Fig. 10. Effect of chloroquine on the internalization and degradation of cell-bound \[^{125}\text{I}]\text{CSF-1}. PEM (10^6/dish) were labelled with \[^{125}\text{I}]\text{CSF-1} (100,000 c.p.m.) at 0 °C for 16 h. The cultures were then washed to remove unbound \[^{125}\text{I}]\text{CSF-1}. Cells were resuspended in 1 ml/dish of alpha-10 with or without chloroquine (1 mm). After 30 min of incubation at 0 °C, the temperature was raised to 37 °C. At the indicated time, the total cell-associated radioactivity was determined. Data are means from duplicate cultures. Initial bindings at 0 °C: 37,667 c.p.m.

Intracellular breakdown of internalized \[^{125}\text{I}]\text{CSF-1}

The gel electrophoresis pattern of internalized \[^{125}\text{I}]\text{CSF-1} by PEM was determined as described in Materials and Methods. We have shown previously that

with an approximate half-time of 30 min. However, in cultures containing 1 mm chloroquine, the loss of cell-associated ^{125}\text{I} was greatly reduced, with only a 25 % loss of the initially bound \[^{125}\text{I}]\text{CSF-1} after 80 min at 37 °C.
[\textsuperscript{125}I]CSF-1 molecules were internalized and degraded very rapidly ($t_1 = 30$ min) by PEM at 37°C with the subsequent release of [\textsuperscript{125}I]tyrosine into the culture medium (Chen et al. 1982). Although cell-associated radioactivity also decreased rapidly, the chemical nature of these intracellular radioactive components has not been characterized. Using SDS/PAGE and gel autoradiography, we found that the majority of cell-associated radioactivity comigrates with intact [\textsuperscript{125}I]CSF-1 although its intensity decreases with time. Gel autoradiography did not reveal a significant band(s) or activity of lower molecular weight polypeptides or molecules, even at the earliest time point (2 min, 37°C). A single band of relatively faint activity, however, was detected at later time points (Fig. 11).

\textit{Cycloheximide inhibits recovery of CSF-1 receptor activity following down-regulation}

The mechanism by which a specific receptor is rapidly removed by its ligand from the cell surface has been termed down-regulation and most probably involves the internalization and degradation of hormone–receptor complex as in the case of epidermal growth factor (EGF) and insulin receptors (King & Cuatrecasas, 1981). Fig. 12 shows that when PEM were incubated continuously with unlabelled CSF-1, their [\textsuperscript{125}I]CSF-1-binding activity dramatically decreased in a fashion dependent on time and CSF-1 concentration. Ten units of CSF-1/ml caused a 15% inhibition in

![Fig. 11. Gel electrophoresis and autoradiography of intracellular [\textsuperscript{125}I]CSF-1. Cultured PEM were labelled with [\textsuperscript{125}I]CSF-1 at 0°C for 3 h. After labelling, cells were washed to remove unbound [\textsuperscript{125}I]CSF-1 and were then resuspended in warm medium. After various times at 37°C, cells were washed, lysed and subjected to gel electrophoresis (12.5% SDS/PAGE) as described in detail in Materials and Methods. Arrow indicates intact [\textsuperscript{125}I]CSF-1. Numbers at the top are times (min) of incubation at 37°C. Numbers on the right are molecular weights (×10\textsuperscript{3}).]
Fig. 12. Down-regulation of $^{125}$I-CSF-1-binding activity induced by unlabelled CSF-1. PEM (10^5/dish) were incubated continuously with unlabelled CSF-1 ranging from 10 to 1000 units/ml at 37°C for various periods of time as indicated; (▼) 10 units; (●) 100 units; (○) 1000 units. After treatment, cells were washed with cold PBS. $^{125}$I-CSF-1-binding activity by the treated cells was then determined as described previously with 50000 c.p.m. of $^{125}$I-CSF-1 per dish. All data were corrected for non-specific binding.

Control cultures bound 15816 c.p.m.

1 h, while 100 units/ml and 1000 units/ml caused 45% and 95% inhibition, respectively, in the same period. With the lowest concentration of CSF-1, the $^{125}$I-CSF-1-binding activity restored to full capacity in approximately 8 h, presumably due to the internalization and degradation of the available CSF-1 present in the medium and to the reappearance of CSF-1 receptor. At higher concentrations, $^{125}$I-CSF-1-binding activity did not reappear in this period (Fig. 12). It is not clear at present whether the restoration of $^{125}$I-CSF-1-binding activity is due to the recycling of old receptors or
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Fig. 13. Restoration of $[^{125}\text{I}]$CSF-1-binding capacity by PEM in the presence and absence of cycloheximide. PEM ($10^6$/dish) were treated with 1000 units/ml unlabelled CSF-1 for 1 h at 37°C. After treatment, cells were washed extensively with cold PBS and resuspended in 1 ml of warm alpha-10 with or without cycloheximide (10 µg/ml). After various periods of time at 37°C, $[^{125}\text{I}]$CSF-1-binding to PEM was determined as described previously; (○) without cycloheximide; (●) with cycloheximide; (▲) control cultures without treatment. Data were means from duplicate cultures ± s.d. All data were corrected for non-specific binding.

the production of new ones. We investigated the effect of a potent protein synthesis inhibitor, cycloheximide, on the restoration of CSF-1-binding activity following CSF-1 induced down-regulation. As shown in Fig. 13, in the presence of cycloheximide, the recovery of $[^{125}\text{I}]$CSF-1-binding activity was completely blocked, suggesting that protein synthesis is required for receptor restoration.

DISCUSSION

In this study quantitative EM autoradiography was used to examine the interaction of bound $[^{125}\text{I}]$CSF-1 with intracellular organelles of PEM at 37°C. Our results confirm and complement earlier studies (Chen et al. 1982; Stanley & Guilbert, 1981), which indicated that $[^{125}\text{I}]$CSF-1, after binding to PEM, can enter the interior of its target cells and become associated predominately with phagolysosomes. This indicates that the breakdown of intact $[^{125}\text{I}]$CSF-1 is most probably carried out inside phagolysosomes, whose activities can be blocked by agents such as primary and tertiary amines (Fig. 10). It is believed that these agents inhibit lysosomal function by becoming sequestered into lysosomes (phagolysosomes), where they are trapped.
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within the organelles; this results in a pH shift such that acid hydrolases become inactive. They may also influence lysosome–phagosome fusion.

We attempted to identify the intermediate degraded materials of \([^{125}\text{I}]\text{CSF-1}\) after its internalization by PEM. We have used polyacrylamide gel electrophoresis and autoradiographic techniques to examine the intracellular radioactivity associated with PEM after they have been warmed up, and we were unable to detect a significant quantity of lower molecular weight polypeptides. The majority of the intracellular radioactivity recovered after cell lysis appeared to comigrate with intact \([^{125}\text{I}]\text{CSF-1}\) (Fig. 11). This means that the degradation of \([^{125}\text{I}]\text{CSF-1}\) proceeds rapidly to form small peptides and results in the immediate loss of \([^{125}\text{I}]\text{tyrosine}\) into the culture medium without any appreciable retention of intermediate fragments. Thus, it appears that the autoradiographic grains we obtained (see Figs 4–6) were most probably derived from intact labelled molecules rather than their fragments or free \([^{125}\text{I}]\text{tyrosine}\). However, it is equally possible that we failed to detect intermediate degraded products of \([^{125}\text{I}]\text{CSF-1}\) simply because the degraded fragments contain no \([^{125}\text{I}]\text{tyrosine}\) residue(s) and, therefore, cannot be detected. Thus, the question of whether the degradation of ligand–receptor complexes results in the production of an intracellular mitogen capable of directly interacting with the nucleus or other compartments has not been answered definitively.

Previous studies (Stanley et al. 1978; Lin & Stewart, 1974) have shown that about 10% of PEM are capable of proliferating extensively and forming exclusively macrophage colonies \(\text{in vitro}\) under the influence of CSF-1. These subpopulations of macrophages have an absolute requirement for CSF-1 for growth. van der Zeijst, Stewart & Schlessinger (1978) showed that when CSF-1 is removed from actively proliferating macrophages they leave the cell cycle and eventually die. Using conventional autoradiography, Guilbert & Stanley (1980) showed that over 98% of the PEM bind \([^{125}\text{I}]\text{CSF-1}\). Thus, not all CSF-1 receptor-bearing cells are capable of extensive proliferation when stimulated with CSF-1. In this study, EM autoradiography at 1 min showed that about 16% of the cells contained no grains (Fig. 3). The average grain number per cell at this time point was 5–94. It appears that this grain distribution does not follow a simple Poisson distribution as it should if one assumes that each cell has the same or a very similar number of receptors. This discrepancy suggests that some of the cells may not exhibit CSF-1 receptors and that the distribution of binding sites is heterogeneous among PEM. Others (Byrne et al. 1981), using light microscopic autoradiographic methods, have reported heterogeneity of grain distribution among other CSF-1 receptor-bearing cells. In our study, fresh mouse peritoneal exudate cells were used for EM autoradiography in contrast to the previous study in which cultured PEM were used for \([^{125}\text{I}]\text{CSF-1}\)-binding studies and autoradiography. Thus, the lower percentage of labelled cells in this study may be caused by locally produced CSF-1, which blocks or down-regulates available cell receptors. It is well known that colony-stimulating and macrophage growth activities are produced at inflammatory sites (Cifone, Mocarelli & Defendi, 1975; Adolphe, Fontagne, Pelletier & Giroud, 1975), and we have shown that prolonged culture of these cells after down-regulation by CSF-1 resulted in the full restoration of \([^{125}\text{I}]\text{CSF-1}\)-binding activity (Figs 12, 13).
It is pertinent to mention here that the loss of [\(^{125}\text{I}\)]CSF-1 receptors by down-regulation at 37 °C (Fig. 12) is not due to the direct occupancy of CSF-1 receptors by unlabelled CSF-1, since previous results have shown that at 37 °C: (1) bound CSF-1 readily dissociates from the cell membrane, if not internalized, within 15 min (Chen et al. 1982; Stanley & Guilbert, 1981); and (2) internalized [\(^{125}\text{I}\)]CSF-1 is rapidly destroyed by cellular degradation with a half-time of 30 min (Guilbert & Stanley, 1980; Chen et al. 1982). Data in Fig. 13 show that only about 6% of the total binding activity by PEM was recovered after 30 min of incubation at 37 °C. Alternatively, the heterogeneity of receptor numbers may simply be due to various cell cycle states. Further study is needed to differentiate between these two possibilities.

The results of this study are strikingly similar to those with EGF. Using similar EM autoradiographic techniques, Gorden et al. (1978) showed that [\(^{125}\text{I}\)]EGF is internalized progressively by human fibroblasts in a time- and temperature-dependent fashion. The internalized EGF was found to associate with lysosomal structures as a function of time. In this study, we found that 40–60 min after warming, the fraction of grains associated with phagolysosomes reached about 35% (Fig. 7), while the fraction of grains associated with plasma membranes (including their invaginations) dropped from 70% to approximately 20%. Unlike resident peritoneal macrophages, we found relatively few granular lysosomes in the cytoplasm of PEM. The loss of granular lysosomes is most probably due to the formation of much larger phagolysosomes from lysosomal fusion, caused by the phagocytosis of injected materials in the thioglycollate medium. The fine fibrillar material of varied electron density inside phagolysosomes was interpreted as components of thioglycollate medium (Fig. 1). It is noteworthy that a large area (1–3 \(\mu\)m) beneath the plasma membrane was occupied by empty vacuoles, which we found to be invaginations of the plasma membrane rather than true vacuoles (Fig. 1). These invaginations of the plasma membrane are more heavily labelled than the surface membrane. Furthermore, there is a decrease in invaginated membranes with time, suggesting that these membrane invaginations may be involved in the process of the internalization of [\(^{125}\text{I}\)]CSF-1.

This study provides evidence concerning the metabolic fate of internalized [\(^{125}\text{I}\)]CSF-1 by PEM, but provides no specific information on when and how the biochemical signals are generated in this sequence of events. Internalization of the ligand could provide a mechanism by which the level of CSF-1 in the body is regulated. In addition to its activity in cell proliferation, recent studies have demonstrated that CSF-1 may have other biological activities (Hamilton, Stanley, Burgess & Shadduck, 1980; Moore et al. 1980; Kurland et al. 1979; Wing et al. 1982). It appears that the pleiotropic effects of CSF-1 are mediated via the same membrane receptors. The mechanism of action by which CSF-1 and its receptors interact to initiate and maintain these complex programs leading to cell proliferation is largely unknown. Recent studies have shown that the specific receptors for several polypeptide hormones such as EGF, insulin and platelet-derived growth factor (Cohen, Carpenter & King, 1980; Kasuga, Karlsson & Kahn, 1982; Nishimura, Huang & Deuel, 1982) are protein kinases that autophosphorylate upon binding to their specific
ligands. Therefore, it would be of great interest to examine whether protein phosphorylation also occurs upon the binding of CSF-1 to its specific receptor on the PEM membrane. However, even if this is indeed the case, the biological significance of receptor-mediated internalization and cellular degradation is by no means fully understood.

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