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Uptake and Destruction of ^{125}I-CSF-1 by Peritoneal Exudate Macrophages

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The binding and uptake of the colony-stimulating factor CSF-1 by peritoneal exudate macrophages (PEM) from lipopolysaccharide insensitive C3H/HeJ mice was examined at 2°C, and at 37°C. At 2°C, ^{125}I-CSF-1 was bound irreversibly to the cell surface. At 37°C, 90% of the cell surface associated ^{125}I-CSF-1 was rapidly internalized and subsequently degraded and the remaining 10% dissociated as intact ^{125}I-CSF-1. Thus classical equilibrium or steady state methods could not be used to quantitatively analyze ligand–cell interactions at either temperature, and alternative approaches were developed. At 2°C, the equilibrium constant ($K_d \leqslant 10^{-13}$M) was derived from estimates of the rate constants for the binding ($k_{on} \simeq 8 \times 10^5 M^{-1}s^{-1}$) and dissociation ($k_{off} \leqslant 2 \times 10^{-7}s^{-1}$) reactions. At 37°C, the processes of dissociation and internalization of bound ligand were kinetically competitive, and the data was formally treated as a system of competing first order reactions, yielding first order rate constants for dissociation, $k_{off} = 0.7$ min^{-1} ($t_{1/2} = 10$ min) and internalization, $k_{in} = 0.07$ min^{-1} ($t_{1/2} = 1$ min). Approximately 15 min after internalization, low-molecular weight ^{125}I-labeled degradation products began to appear in the medium. Release of this degraded ^{125}I-CSF-1 was kinetically first order over three half-lives ($K_d = 4.3 \times 10^{-2}$ min^{-1}, $t_{1/2} = 16$ min). Thus CSF-1 binds to a single class of receptors on PEM, is internalized with a single rate limiting step, and is rapidly destroyed without segregation into more slowly degrading intracellular compartments.

Abbreviations used: CSF, colony stimulating factor; CSF-1, the colony stimulating factor subclass, defined by subclass-specific radioimmuno- [5,30] and radioreceptor [8] assays, that specifically stimulates mononuclear phagocyte proliferation; PEM, peritoneal exudate macrophages; EGF, epidermal growth factor; NGF, nerve growth factor; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; FCS, fetal calf serum; PBS, phosphate-buffered saline; α-Hepes, Bicarbonate-free, Hepes buffered alpha medium; DTT, Dithiothreitol; DOG, deoxyglucose; HCG, human chorionic gonadotrophin; PDGF, platelet-derived growth factor; IL-2, interleukin-2.

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The colony stimulating factors are growth factors that stimulate cultured hemopoietic cells to form colonies containing granulocytes and macrophages [reviewed in ref. 1]. Colony-stimulating factor-1 (CSF-1) specifically regulates the survival, proliferation, and differentiation of cultured mononuclear phagocytes [reviewed in ref. 2]. Murine L cell CSF-1 has been purified and shown to be a ~70,000 M_r dimeric sialoglycoprotein comprised of two similar 14,000 M_r polypeptide chains that are substantially glycosylated and maintained in dimeric association by disulfide bonds [3,4]. The native molecule can be radioiodinated to high specific radioactivity without loss of biologic activity [5].

Preliminary studies have shown that macrophages bind and degrade ^{125}I-CSF-1 over the same CSF-1 concentration range required for stimulation of their survival and proliferation [6]. A specific, high affinity binding site for ^{125}I-CSF-1 has been demonstrated that appears to be restricted to mononuclear phagocytic cells [7–9]. At 37°, autoradiographic analysis indicates that CSF-1 is internalized and transported to a "phagolysosomal" compartment [10], where it appears to be degraded. Thus, the binding and uptake of CSF-1 by macrophages appears to conform to the general pattern of cell surface binding, internalization, and intracellular degradation observed in the interaction of other growth factors with their target cells.

The interaction of CSF-1 with macrophages at 37°C differs from the interaction of other growth factors with their target cells in that almost all of the CSF-1 bound by the cells is released as degraded ligand [11,12]. The irreversible nature of the interaction of CSF-1 with macrophages at 37°C may be related to the role of the CSF-1 receptor in the clearance of circulating CSF-1 (Bartocci, A., Mastrogiannis, D., and Stanley, E.R., manuscript in preparation) or to the local production of CSF-1 in areas of inflammation [13], where the growth factor must regulate macrophage survival and function in the presence of a variety of degradative enzymes. It could result from either rapid movement of the CSF-1 receptor complex to a "protected" site near but not at the cell surface, from multiple (high- and low-affinity) binding sites, or simply from a unique combination of binding, dissociation, and internalization rates involving a single class of sites. Because of the complexity of the interaction, traditional methods for distinguishing between these possibilities [quantitative analysis by equilibrium, eg, reference 14, or steady state, eg, reference 15 analyses] were not applicable, and an alternative approach involving kinetic analysis following a temperature shift was developed. The utilization of this approach demanded a preliminary analysis of how CSF-1 is associated with cells at 2°C. The results explain the irreversible nature of the interaction at 37°C entirely in terms of the binding of CSF-1 to a single class of high-affinity sites from which it either slowly dissociates or via which it is rapidly internalized to a single, short-lived intracellular compartment.

MATERIALS AND METHODS

Reagents

Purified L cell CSF-1 (~ 8×10^7 units (U)/mg protein) and purified ^{125}I-CSF-1 (~ 400,000 cpm/ng protein) were prepared as described elsewhere [11]. The

purity of the CSF-1 preparation was checked by SDS-PAGE under reducing and nonreducing conditions and by complexing with rabbit anti-CSF-1 antibody [3]. Rabbit antiserum to purified L cell CSF-1 was prepared as described [11]. Freshly iodinated ^{125}I-CSF-1 is fully biologically active but loses biologic activity with time [$t^{1/2} \sim 6$ weeks, 5]. As anti-CSF-1 antibody only binds biologically active CSF-1 [5,11], the concentration of biologically active ^{125}I-CSF-1 in ^{125}I-CSF-1 preparations was directly determined from the concentration of ^{125}I specifically bound by the anti-L cell CSF-1 antiserum [11].

The molar specific radioactivity (msr, cpm/mole) of the ^{125}I-CSF-1 preparations used was calculated according to the formula:

$$\text{msr} = (F_p \times B) - (A \times 4.4 \times 10^{-16})$$

where F_p is the fraction of ^{125}I bound by anti-CSF-1 antibody, B is the concentration of ^{125}I (cpm/ml) and A is the concentration of CSF-1 in U/ml determined by ^{131}I-CSF-1 radioimmunoassay [11] (1 U CSF-1 $\approx$ 0.44 fmol). The molar concentration of ^{125}I-CSF-1 in experimental solutions was determined substituting in the equation, [^{125}I-CSF-1] = ($F_s \times c$) − msr, where F_s is the fraction of ^{125}I bound by anti-CSF-1 antibody, and C is the concentration of ^{125}I (cpm/1).

Cells

A rapidly adhering population of starch-induced PEM ($2–5 \times 10^5$ per 35 mm tissue culture plate, 7) from C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME) were incubated overnight in 2 ml 10% FCS (Flow Labs, Inc., Rockville, MD) in α-medium (Kansas City Biological, Kansas City, MO) in the absence of CSF-1 prior to ^{125}I-CSF-1 binding studies.

^{125}I-CSF-1 Binding at 2°C

The cultured adherent cells were washed once with ice-cold PBS and either PBS or 10% FCS in bicarbonate-free, Hepes-buffered alpha-medium [α-Hepes, 7], added (1 ml/dish). The cells were immediately cooled on ice for 20 min, ^{125}I-CSF-1 (final concentration, 300 pM) was added, and the plates were incubated on ice (2°C) for 5 min to 15 hr. At the end of the 2°C incubation, each plate was washed with 10 ml ice-cold PBS prior to determination of the ^{125}I content (by solubilization with 0.1 M NaOH and counting the eluate in a gamma counter), or initiation of either ^{125}I-CSF-1 dissociation studies or temperature shift experiments. Net high affinity binding is the binding which can be blocked by a 2-hr preincubation with 2 nM CSF-1 [11].

Dissociation of Bound ^{125}I-CSF-1 at 2°C

^{125}I-CSF-1 was bound to PEM at 2°C as described above. Treatment of the cells with Pronase (Calbiochem, B grade), Trypsin (Sigma, type III), chymotrypsin (Sigma, Type I-S), Triton X-100 (Sigma), DTT (Sigma), and methyl amine (HCl salt, Sigma) was carried out on ice for 2 to 20 hr as described in the text. Dissociation of bound ^{125}I-CSF-1 at pH 4 was carried out in 1 ml of dissociation buffer, (0.1% (v/v) FCS in 100 mM acetic acid, 10 mM NaCl, pH 4.0).

2°C–37°C Temperature Shift Experiments

The PEM were incubated for 2 hr with 300 pM ^{125}I-CSF-1 at 2°C as described above. The dishes were washed five times with ice-cold PBS and transported in ice

to a 37°C warm room. The temperature shift was carried out by floating the plastic tissue culture dishes containing the cells (at 2°C) in a shallow 37°C water bath and immediately adding 1 ml of prewarmed 10% FCS in α-Hepes. At various times after the temperature shift, the medium above the cells was removed and placed on ice for analysis and 1 ml fresh, prewarmed medium was added to the cells for the next time interval. The total ^{125}I cpm and the fraction of the total cpm recognized by anti-CSF-1 antibody (F) were determined for each of the samples and used to calculate the amount of intact ^{125}I-CSF-1 (in cpm) in the sample (= Fxcpm). Results from different experiments were combined by normalizing the total amounts of both intact and degraded ^{125}I-CSF-1 released to the total amount of ^{125}I-CSF-1 present at 2°C in a given dish for all the time intervals up to 17 hr.

The effect of metabolic inhibitors and lysosomotropic amines on the dissociation and degradation of ^{125}I-CSF-1 bound to PEM was determined by temperature shift analysis. The PEM were preincubated at 37°C in 5 mM 2-deoxyglucose (Sigma) and 1 mM sodium azide, 10 mM methylamine (Sigma), or 75 mM chloroquin (Sigma) for 15 min prior to washing once with PBS, cooling on ice, and incubation with ^{125}I-CSF-1 at 2°C. Both the 2°C incubation and the subsequent temperature shift experiment were carried out in the presence of the appropriate inhibitor or amine. Binding and dissociation were carried out in 10% dialyzed FCS in PBS.

Rate Equation for ^{125}I-CSF-1 Dissociation

Both dissociation and internalization of cell surface ^{125}I-CSF-1 proceed concomitantly and competitively:

$$\underset{(D)}{\text{outside}} \xleftarrow{k_{off}} \underset{(S)}{\text{cell surface}} \xrightarrow{k_{in}} \underset{(I)}{\text{inside}}$$

where k_{off} is the rate constant for dissociation of intact ^{125}I-CSF-1, D the concentration of intact ^{125}I-CSF-1, S the concentration of cell surface ^{125}I-CSF-1, k_{in} the rate constant for internalization and I the concentration of internalized ^{125}I-CSF-1.

If both internalization and dissociation are first order processes, the decay rate of surface bound ^{125}I-CSF-1 will be first order [16] with a rate constant k_s. Thus:

$$S = S_o e^{-k_s t} \tag{1}$$

For competing first order reactions, the ratio of products equals the ratio of rate constants (equation 2) and the reactant decay constant equals the sum of product appearance constants (equation 3) [16]:

$$k_{in}/k_{off} = I/D = R \tag{2}$$

$$k_s = k_{in} + k_{off} \tag{3}$$

A value for R can be obtained from the ratio of degraded ^{125}I-CSF-1 to intact dissociated ^{125}I-CSF-1 at the end of the process (see Results). Equations (2) and (3) can be combined to give:

$$k_s = k_{off}(R + 1) \quad (4)$$

The concentration remaining at the cell surface (S), expressed as the difference between initial cell surface concentration and the concentration removed by internalization and dissociation, equals $S_o - I - D$.

This latter equation can be combined with equation (2) to give:

$$S = S_o - D(R + 1) \quad (5)$$

combination of equations (1), (4), and (5) and rearranging gives:

$$\ln\{S_o/[S_o - D(R + 1)]\} = k_{off}(R + 1)t \quad (6)$$

Since the molar concentration terms S_o and D are related to cpm/l by the same proportionality constant (1/msr) substitution of the corresponding cpm values in equation 6 does not change its value.

Autoradiography of ^{125}I-CSF-1 Bound to PEM

Autoradiography was carried out on whole dishes [7] and 1 μm sections. For the preparation of sections, cells on dishes were fixed for 30 min at 20°C in Karnovsky's fixative [17] and washed four times with 0.1 M cacodylate buffer pH 7.4. They were post-fixed for 75 min in 1% OsO_4 in 0.1 M cacodylate buffer, rapidly washed twice with 70% ethanol at 4°C, and stained with freshly prepared 1% paraphenylene diamine in 70% ethanol for 20 min at 4°C. The cells were dehydrated in cold graded ethanol solutions and embedded in epon [18]. One-micrometer sections were cut at right angles to the cell layers. They were mounted on slides previously coated with a film of 0.7% parlodion in amyl acetate for coating with a 1:3 dilution of Ilford L4 dipping emulsion containing glycerol [19]. The slides were exposed for 4 wk at 4°C, then developed with Kodak D19 developer for 2 min at 20°C. They were then rinsed in CO_2-free water for 15 sec, fixed for 1 min in 20% $Na_2S_2O_3$, 2.5% $K_2S_2O_5$, washed in CO_2 free water (three changes, 20 min), and air-dried. Photographs were taken at a final magnification of 320× on a Zeiss Axiomat microscope using Technical Pan film 2415.

RESULTS

Cell Surface State of CSF-1 Bound to Cells at 2°C; Measurement of Cell Surface CSF-1 in 37°C Experiments

Following binding to its specific site on PEM at 2°C, ^{125}I-CSF-1 dissociates at 2°C extremely slowly, if at all [$t^{1/2} > 6$ wk, 11]. The ^{125}I-CSF-1 which binds in the first 5 min dissociates at the same, very slow rate as ^{125}I-CSF-1 that binds at later (up to 24 hr) times (data not shown). Autoradiography of 1 μm sections of PEM after a 2-hr incubation with ^{125}I-CSF-1 at 2°C showed that greater than 90% of the ^{125}I grains were associated with the cell periphery. In contrast, cells which had been warmed to 37°C after the 2°C binding displayed fewer than 15% of the grains at the cell periphery (Table I).

TABLE I. Percentage of Plasma Membrane-Associated ^{125}I Autoradiographic Grains Compared to the Percent of ^{125}I Dissociated at 2°C, pH 4

Time incubated at 37°C (min)[a]	Percent plasma membrane associated grains[b]	Percent ^{125}I dissociated at pH 4[c]
0	93.2	97.8 ± 0.2
15	14.8	16.7 ± 1.8

[a]PEM incubated with ^{125}I-CSF at 2°C, washed and incubated at 37°C as described in the Methods.
[b]A grain was considered plasma membrane-associated if it occurred within two grain cross-sections of the edge of the cell. Data compiled from 26 sections (0 min at 37°C) and 31 sections (15 min at 37°C).
[c]Dissociation carried out at 2°C for 20 min as described in Materials and Methods. Means of three replicates ± standard deviation; 89.4 ± 1.7 percent of the pH 4 dissociated ^{125}I was bound by anti-CSF-1.

Since it could not be determined from the autoradiographs whether ^{125}I-CSF-1 bound at 2°C was at the outer surface of the plasma membrane or just inside the cell, biochemical approaches were used. The action of proteolytic enzymes has been employed to distinguish exposed plasma membrane proteins from those which are inaccessible and presumably inside the cell [20–23]. At 2°C, unoccupied ^{125}I-CSF-1 binding sites were found to be very sensitive to the action of both Pronase and trypsin. A 1-hr incubation with 0.005% of either enzyme in PBS at 2°C removed more than 80% of the ^{125}I-CSF-1 binding sites. However, following occupation of binding sites by ^{125}I-CSF-1 at 2°C, treatment with either 0.005% Pronase, 0.01% trypsin, 0.01% chymotrypsin, 2nM CSF-1, 10 μg/ml anti-CSF-1 antiserum, 100 mM DTT, 10 mM methylamine, or 20 mM glucose failed to cause ^{125}I-CSF-1 dissociation. Thus at 2°C and physiological pH, ^{125}I-CSF-1 is firmly bound to cells.

The irreversibility of the binding of ^{125}I-CSF-1 at 2°C precluded equilibrium or steady state approaches to the determination of the equilibrium constant. However, the on-rate constant for the reaction was estimated from an analysis of the initial rate of binding at 2°C over the first 10 min (data not shown, $k_{on} \simeq 8 \times 10^5\ M^{-1}\ s^{-1}$) and the off-rate constant has been previously estimated ($k_{off} \leqslant 2 \times 10^{-7}\ s^{-1}$) [11]. Thus the equilibrium constant at 2°C ($K_d = k_{off}/k_{on}$) was determined to be $\leqslant 10^{-13}$M.

The dissociation rates of cell surface bound polypeptide ligands such as EGF [24], asialoglycoproteins [25], insulin [25,26], and IL-2 [27] have been shown to dramatically increase following acidification of the extracellular medium. In the case of CSF-1, lowering of the pH of the medium above macrophages to 4, resulted in a rapid dissociation of 98% of the ^{125}I-CSF-1 bound at 4°C ($t^{1/2}$ approximately 1 min, Fig. 1). However, only 17% of cell-associated ^{125}I dissociated at pH 4 from parallel cultures which had been warmed to 37°C for 15 min (Table I). In addition, at either temperature, the percentage of ^{125}I autoradiographic grains near the cell periphery was closely correlated with the percentage of ^{125}I dissociated at pH 4. This data indicates that pH 4 dissociation at 2°C accurately measures cell surface bound ^{125}I-CSF-1 even after warming to 37°C. After 20 min at pH 4, cells maintained both viability (by trypan blue exclusion) and full capacity to rebind ^{125}I-CSF-1 at physiological pH (data not shown). Almost 90% of the ^{125}I-CSF-1 dissociated by pH 4 treatment at either temperature was recognized by anti-CSF-1 antibody (Table I).

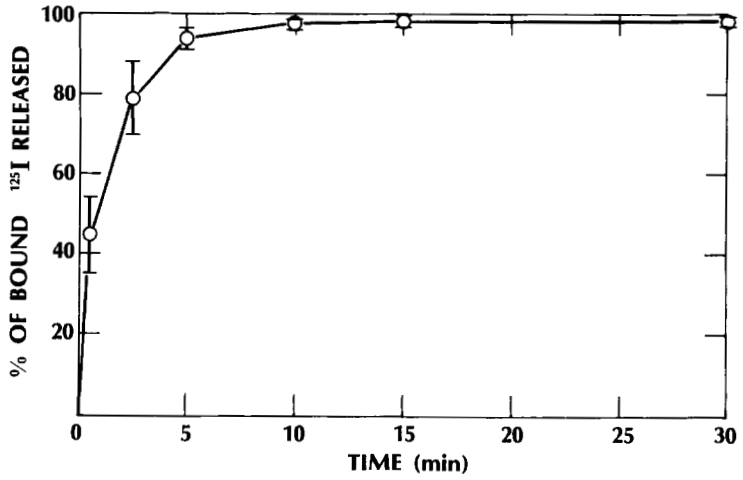

Fig. 1. Dissociation of ^{125}I from PEM at 2°C, pH 4. PEM were incubated with ^{125}I-CSF-1 (300 pM) at 2°C, washed with cold PBS, and the dissociation at pH 4 carried out as described in Materials and Methods. Ordinate: percent of original cell associated ^{125}I dissociated. Error bars represent standard deviations from the mean of three samples.

Analysis of the Dissociation and Internalization of Cell Surface CSF-1 Following a 2°C–37°C Temperature Shift

Dissociation of ^{125}I-CSF-1 bound to cells at 2°C was also induced by rapidly raising the temperature of the cells to 37°C (Fig. 2). In the 17 hr subsequent to the temperature shift, only 9% of the initial cell-associated ^{125}I that dissociated bound anti-CSF-1 antibody. The majority of this immunoreactive ^{125}I-CSF-1 dissociated within the first 10 min, representing the bulk of the ^{125}I released during this period. At times after 10 min, the proportion of ^{125}I released that did not bind anti-CSF-1 antibody increased dramatically (Fig. 2). The dissociation media harvested during the first 10 min and the media harvested during the following 110 min were separately pooled, dialyzed against water, lyophilized, redissolved in SDS-PAGE sample buffer, and subjected to SDS-PAGE on 7.5% gels (Fig. 3). Most of the ^{125}I from the 10–120 min dissociate passed through dialysis tubing (10,000 M_r cut-off) and was probably mono- and diiodotyrosine [28]. However, all of the non-dialyzable ^{125}I from both fractions migrated as a single sharp peak in exactly the same region of the gel as did the starting ^{125}I-CSF-1. This dissociated, high M_r ^{125}I-CSF-1 was fully biologically active and bound by anti-CSF-1 antibody (data not shown). Thus, ^{125}I-CSF-1 is reversibly bound to the cell surface at 37°C but very little is is able to dissociate. Furthermore, the high M_r material which dissociates does so and is not altered, indicating that degradation (which begins only after a lag period) does not occur at the cell surface immediately after the temperature shift.

The cell surface-bound ^{125}I-CSF-1 which did not dissociate at 37°C was rapidly internalized (Table I). Macrophage pinocytosis can be partially blocked with the metabolic inhibitors deoxyglucose and sodium azide [29]. Addition of these agents in 10% dialyzed FCS in PBS to the cells at 37°C for 15 min prior to ^{125}I-CSF-1 binding at 2°C does not reduce the extent of binding [7,11]. However, when such metabolically inhibited cells, after ^{125}I-CSF-1 binding at 2°C, were shifted to 37°C over 50% of the bound ^{125}I-CSF-1 dissociated as immunoreactive ^{125}I-CSF-1 within the first 15

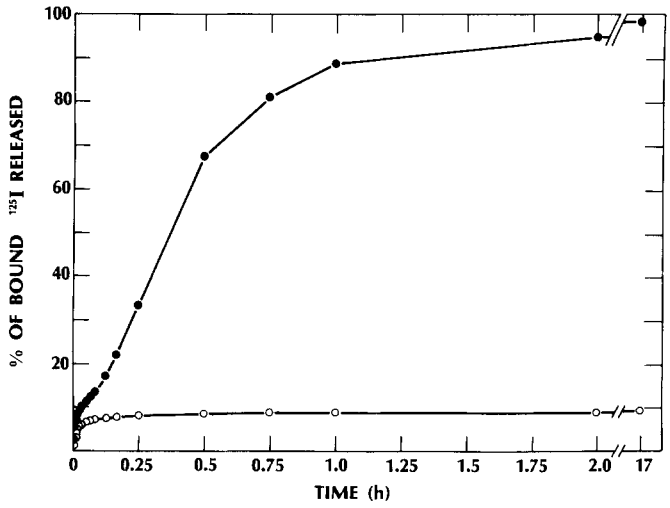

Fig. 2. Dissociation of ^{125}I from PEM at 37°C. PEM were incubated with 200 pM ^{125}I-CSF-1 for 2 hr at 2°C, washed with ice-cold PBS and shifted to 37°C as described in the Methods. Cumulative total ^{125}I (●) and immunoreactive CSF-1 (○) released at times after temperature shift and expressed as percent of the original cell-associated ^{125}I. Means of four replicates with an average relative standard deviation of 0.178 ± 0.095.

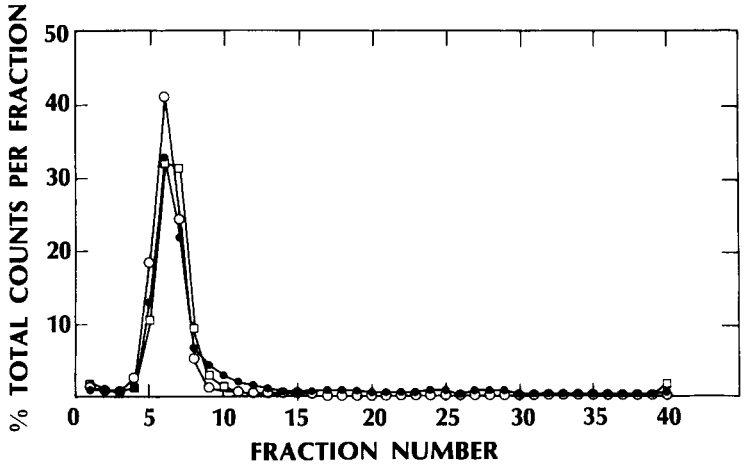

Fig. 3. SDS-PAGE analysis of the non-dialyzable ^{125}I dissociated at 37°C from PEM that had bound ^{125}I-CSF-1 at 2°C. Supernatants were collected serially 10 and 120 min after the temperature shift, dialyzed against PBS, lyophilized, redissolved in SDS sample buffer, and subjected to electrophoresis in a 7.5% SDS-PAGE slab gel. Parallel lanes containing the ^{125}I-CSF-1 applied to the cells (●), and the 10 min (○), and 120 min (□) samples, were excised, cut into 2 mm slices, and counted for ^{125}I. (Apparent M_r of ^{125}I-CSF-1 = 66,000; dye marker, fraction 40).

TABLE II. Effect of Metabolic Inhibitors and Lysosomotropic Amines on Dissociation of Cell Surface-Bound ^{125}I-CSF-1 and Degradation of Internalized ^{125}I-CSF*

37°C Incubation medium	Dissociation of cell surface ^{125}I-CSF-1[a] (%)	Degradation of internalized ^{125}I-CSF-1 ($t^{1/2}$ in min)[b]
Control	8.1	15.6
DOG + Azide	53	84
Methylamine	< 12	> 840
Chloroquin	16	260

*5×10^5 PEM were incubated with 25 pM ^{125} I-CSF-1 for 15 hr at 2°C, washed with cold PBS, and incubated at 37°C in duplicate in the indicated medium as described in Materials and Methods. Control medium was 10% dialyzed FCS in PBS. DOG, deoxyglucose.

[a]Percentage of the original cell-associated ^{125}I-CSF-1 dissociated during the first 15 min of the 37°C incubation as immunoreactive ^{125}I-CSF-1.

[b]Determined from the rate of appearance of non-immunoreactive ^{125}I in the cell supernatant.

min (Table II). This increase in dissociation observed following partial blocking of internalization indicates competition between the two processes for surface-bound ^{125}I-CSF-1. In the relevant case of unimolecular competition for a common reactant, the reactant decays with unimolecular kinetics having a rate constant equal to the sum of the individual, competing rate constants, and the ratio of products at all times is equal to the ratio of competing first order rate constants [16]. The ratio of reaction product concentrations (the ratio of ^{125}I-CSF-1 which is irreversibly committed to internalize to the ^{125}I-CSF-1 which dissociates) was estimated from the ratio of degraded to intact ^{125}I-CSF-1 at the end of the reaction (17 hr, Fig. 2). This ratio, R, is approximately 10. Rate constants for internalization ($k_{in} = Rk_{off} = 10 \times k_{off}$) decay of surface-bound ^{125}I-CSF-1 ($k_s = k_{in} + k_{off} = 11 \times k_{off}$) can both be calculated from the dissociation rate constant, k_{off}. The rate of dissociation of surface-bound ^{125}I-CSF-1 in this case should be first-order, but its progress reflects decay of surface-bound ^{125}I-CSF-1, ie, both dissociation and internalization rates. A value for k_{off} was accordingly estimated (see Materials and Methods for derivation) from the slope of a plot of $\ln \{S_o/[S_o - D(R+1)]\}/(R+1)$ versus time where S_o is the cpm of ^{125}I-CSF-1 originally bound at the cell surface, R is the ratio defined above, and D is the concentration (cpm/ml) of dissociated, intact ^{125}I-CSF-1 at a given time. This plot was linear over the first two half-lives of the decay of surface-bound ^{125}I-CSF-1 (slope = 7.2×10^{-2} min^{-1}, correlation coefficient = 0.97, data not shown). A simple linear plot for checking whether treatment of the data as a system of competing first order reactions is valid has been described [16]. For applicability, a plot of F_D (the ratio of any reactant or product to the total) versus $1-e^{k_s t}$ should be linear and intercept zero. In this case, k_s can be obtained from the above estimate of k_{off} ($k_s = 11 \times k_{off} = 0.8$ min^{-1}). Such a plot for the dissociation of intact ^{125}I-CSF-1 is indeed linear (correlation coefficient 0.991) and intercepts very near zero (Fig. 4). Thus, treatment of the data in terms of competing processes of internalization and dissociation appears valid and results in estimates for the dissociation rate constant ($k_{off} = 0.7$ min^{-1}, $t^{1/2} = 10$ min), and internalization rate constant ($k_{in} = 0.07$ min^{-1}, $t^{1/2} = 1$ min).

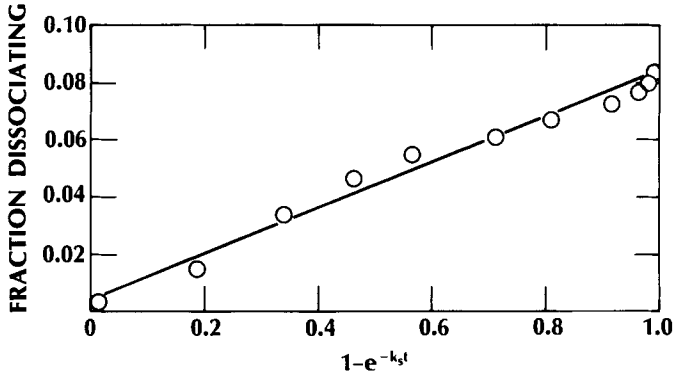

Fig. 4. Dissociation of ^{125}I-CSF-1 from cell surface receptors as part of a parallel first-order process. Ordinate: Fraction of initial cell surface-bound ^{125}I-CSF-1 which dissociates intact. Abscissa: An independently generated function representing the fraction of ^{125}I-CSF-1 disappearing from the cell surface. First-order rate constant for dissociation, k_{off} = slope = 0.077 min^{-1}; correlation coefficient for fit to straight line = 0.991.

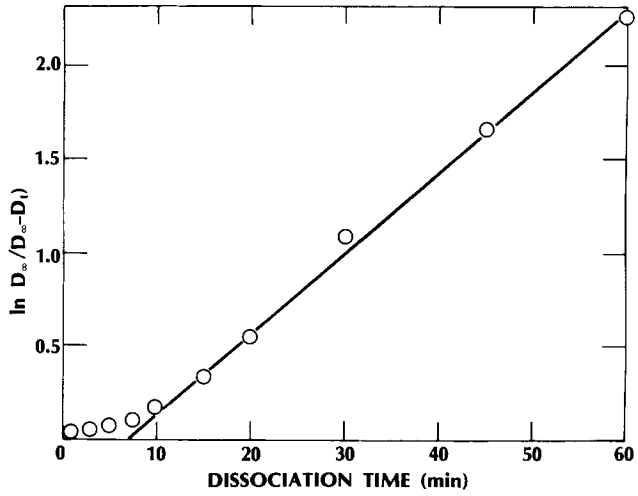

Fig. 5. Kinetics of ^{125}I-CSF-1 degradation at 37°C by PEM after a temperature shift from 2°C (data presented in Fig. 3). Ordinate: D_∞ = fraction of initial cell surface bound ^{125}I-CSF-1 degraded at the end of the reaction = 0.89, D_t = fraction of non-immunoreactive ^{125}I-CSF-1 at a given time. Abscissa: Incubation time at 37°C (t). Slope = First order rate constant for degradation = 4.28×10^{-2} min^{-1}; correlation coefficient for fit to straight line = 0.998. Data points are the means of replicate samples.

The Kinetics of CSF-1 Degradation at 37°C

Independent of this analysis, the data in Figure 2 show that cell-surface-bound ^{125}I-CSF-1 which did not rapidly dissociate when the temperature was shifted from 2°C to 37°C became irreversibly committed to destruction. Dialyzable ^{125}I-labeled material began to appear in the supernatant with a lag of 10-15 min after the temperature shift (Fig. 5). Taken together, this evidence indicates an intracellular degradation process. Further evidence for such a process is that other than intact ^{125}I-CSF-1, only dialyzable ^{125}I-labeled material and no intermediate sized labeled pep-

tides were evident in the supernatant at any time during degradation (Fig. 3). A possible mechanism for the intracellular degradation of endocytosed material is intralysosomal destruction [29]. Two lysosomotropic amines, chloroquin and methylamine, which elevate intralysosomal pH [30] markedly decreased the rate of intracellular ^{125}I-CSF-1 degradation (Table II), as did the combination of deoxyglucose and sodium azide, which has also been shown to increase intralysosomal pH [30]. These data are in accord with previously published autoradiographic analysis, which demonstrate that ^{125}I-CSF-1 enters a "phagolysosomal" compartment [10].

Together, both the fraction of internalized ^{125}I-CSF-1 degraded and the kinetics of the degradation process indicates that the internalized ^{125}I-CSF-1 is degraded in a single process. During the 17-hr incubation period following the shift to 37°C, only 9.4% of the ^{125}I-CSF-1 bound initially at 2°C is dissociated as intact ^{125}I-CSF-1 (Fig. 2). The remaining 90.6% was either degraded (89%) or remained on the dish with the cells (<2%). Autoradiographic examination of the monolayer after the 17-hr incubation at 37°C revealed that the residual ^{125}I was not associated specifically with the cells but appeared to be randomly distributed over both cells and tissue culture plastic (data not shown). Thus, it appears that all of the specifically bound ^{125}I-CSF-1 that failed to dissociate as intact ^{125}I-CSF-1 was internalized and degraded. After the initial 10- to 15-min lag, this specifically bound and internalized material was released with uniform first order kinetics, over three half-lives (correlation coefficient = 0.998, $t^{1/2}$ 16.2 min, Fig. 5). The linearity of the first order plot shown in Figure 5 indicates, up to the last meaningful data point (60 min = 89% of reaction), that there is one dominant, intracellular degradation process which appears to account for all of the specifically bound ^{125}I-CSF-1 that did not dissociate during the initial rapid ($t^{1/2}$ ~ 1 min) commitment event.

DISCUSSION

Analysis of the interaction of CSF-1 with macrophages at 37°C is complex, as rapid binding, rapid internalization, and relatively slow dissociation of the growth factor are involved. However, by reducing the number of simultaneous events, quantitative treatment of this interaction has been possible. Analysis of the dissociation, internalization, and degradation reactions was carried out with cells to which CSF-1 was already bound at the cell surface by preincubation at 2°C. Knowledge of the initial number of cell surface sites and their equivalence for either the dissociation or internalization reactions was essential to this analysis. Thus, prior to commencing experiments at 37°C, it was necessary to understand how CSF-1 was associated with cells following binding at 2°C.

At 2°C, ^{125}I-CSF-1 dissociates very slowly (if at all) from the complex, and the complex is impervious to treatment by proteases, sulfhydryl agents, excess CSF-1, and anti-CSF-1 antibody. This stability cannot be ascribed to ^{125}I substitution since native CSF-1 is also very tightly bound at low temperatures [7]. The irreversible binding, but especially the insensitivity to destructive or dissociating agents, argues for rapid movement into (upon association) and out (upon pH or temperature change) of a protected domain in the cell membrane. However, the presence of protected physical domains such as coated pits or deep membrane invaginations have to be precluded since the rate of ^{125}I-CSF-1 dissociation from the highly purified and solubilized CSF-1 receptor is also extremely slow (Yeung, Y.-G. and Stanley, E.R.,

unpublished observations). The stability of the complex thus appears to be an intrinsic property of either a monomeric or polymeric form or, alternatively, due to the ability of the complex to arrange molecules such as phospholipids or detergents protectively around it. Regardless of the nature of the stability, as CSF-1 remains irreversibly bound to cell surface receptors from 2°C to 10°C (Guilbert, L.J., unpublished observations), but not at 37°C (see Results), a temperature dependent transition occurs between 10°C and 37°C. The state of the complex can also be changed by proton addition. Thus at low temperatures, the complex exists in a stable conformation which requires either energy input or addition of protons for dissociation.

A similar thermodynamic state appears to exist for other polypeptide ligand–receptor complexes at the cell surface. NGF exists in a slowly dissociating, protease-resistant cell surface-bound state [31,32]. EGF, insulin, HCG, and PDGF bind at the cell surface in very high affinity states [20,33,34]. However, in contrast to the CSF-1 receptor complex, these high affinity, slowing dissociating complexes exist either as transition or "aged" forms at low frequency and are therefore difficult to examine biochemically.

Because of the irreversibility of binding at low temperature and the high ratio of uptake to destruction at 37°C, equilibrium binding methods could not be used to assess the homogeneity of binding sites [11,14,15]. However, the irreversibility of binding at 2°C, the protease resistance of bound CSF-1 and the demonstration of a single internally competing first order event involving association and internalization at 37°C are consistent with the existence of a single class of CSF-1 receptor complexes at the PEM surface. Furthermore, while the kinetics of association of CSF-1 with its receptor on PEM were not examined in detail, the association of ^{125}I-CSF-1 with receptors on bone marrow derived macrophages at 2°C [35], and the uniform sensitivity of the receptors to proteolysis, is predicted by the existence of a single class of unoccupied cell surface receptors on quiescent cells. Thus all the available data are consistent with the binding of CSF-1 to a single class of high affinity cell surface receptors on macrophages at both 2°C and 37°C.

The results of the present study indicate that the partial dissociation of bound ^{125}I-CSF-1 observed at 37°C is a result of the competitive processes of internalization and dissociation. Treatment of the data (Fig. 2) in terms of competing first order dissociation and internalization reactions was shown to be valid (Fig. 4) and yielded absolute values for the first order rate constants. Independently of the order of the reactions, the ratio of the rates of dissociation and internalization can be estimated from the fraction of original surface bound ^{125}I-CSF-1, which dissociates as intact ^{125}I-CSF-1 (the remainder irreversibly internalizes and is degraded) (Fig. 2). This ratio, like the ratio of rate constants, is 1:10, indicating a heavy bias towards the degradation pathway. Comparison of the half-life for CSF-1 internalization with the internalization half-lives for other receptor bound polypeptide ligands such as EGF [15], insulin [36], and transferrin [37] reveals that they are similar, in the range of 1–2 min. Thus, the comparatively small degree of dissociation of ^{125}I-CSF-1 at 37°C appears to be a consequence of a comparatively slower dissociation rate ($t^{1/2} \sim 10$ min) than the dissociation rates for these other ligands.

The final stage of CSF-1 macrophage interactions involves intracellular degradation probably in the lysosomal compartment (Results, this paper; 10). While polypeptide growth factors such as EGF and NGF are also degraded by receptor-mediated transfer to the lysosomal system, the existence of alternate, slowly decaying

internal compartments (cytoskeletal and nuclear) has been reported [37–39]. In contrast, all specifically bound ^{125}I-CSF-1 that does not initially dissociate from PEM is ultimately degraded (Fig. 2 and Results). Kinetic examination of the data indicates that all of the internalized material is degraded in a single first order process (the plot in Fig. 6 is still linear through the last data point which represents $\geqslant$ 89% of the internalized ^{125}I-CSF-1). Thus, within 15 min of the temperature shift to 37°C, it appears that all of the internalized ^{125}I-CSF-1 enters a uniform environment within which it becomes rapidly degraded ($t^{1/2}$ 16 min). After 15 min, there appears to be no sizable alternate internal compartment containing ^{125}I-CSF-1 that is more slowly degraded.

Thus the interaction of CSF-1 with PEM appears to be remarkably uniform. Since first-order rate constants have been estimated for most of the steps in the interaction, the average life time for CSF-1 in any one step (=1/k) can be calculated. A CSF-1 molecule spends an average of 78 sec on the cell surface, takes approximately 15 min to reach its site of degradation, and an average of 23 min being intracellularly degraded. The PEM, which resemble the majority of tissue mononuclear phagocytes in their relatively poor proliferative response to CSF-1 [40], are therefore highly efficient at destroying this growth factor.

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REFERENCES

1. Stanley ER: In Stewart WE II, Hadden JW (eds) "The Lymphokines." New Jersey: The Humana Press, 1981, pp 101–132.
2. Stanley ER, Guilbert LJ, Tushinski RJ, Bartelmew SH: J Cell Biochem 21:151, 1983.
3. Stanley ER, Heard PM: J Biol Chem 252:4305, 1977.
4. Das SK, Stanley ER: J Biol Chem 257:13679, 1982.
5. Stanley ER: Proc Natl Sci USA 76:2969, 1979 (erratum ibid 76:5411).
6. Tushinski RJ, Oliver IT, Guilbert LJ, Tynan PW, Warner JR, Stanley ER: Cell 28:71, 1982.
7. Guilbert LJ, Stanley ER: J Cell Biol 85:153, 1980.
8. Das SK, Stanley ER, Guilbert LJ, Forman LW: J Cell Physiol 104:359, 1980.
9. Byrne PV, Guilbert LJ, Stanley ER: J Cell Biol 91:841, 1981.
10. Chen BD-M, Kuhn C III, Lin H-S: J Cell Sci 70:147, 1984.
11. Stanley ER, Guilbert LJ: J Immunol Methods 42:253, 1981.
12. Stanley ER, Guilbert LJ: In R van Furth (ed) "Mononuclear Phagocytes—Functional Aspects." Boston, The Hague, London:Martinus Nijhoff, 1980, pp 417–433.
13. Cifone M, Mocarell, P, Defendi V: Exp Cell Research 96:96, 1975.
14. Scatchard G: Ann NY Acad Sci 52:660, 1949.
15. Knauer DJ, Wiley HS, Cunningham DD: J Biol Chem 259:5623, 1984.
16. Frost AA, Pearson RG: "Kinetics and Mechanism." New York: Wiley and Sons, 1962, pp. 160–162.
17. Karnovsky MJ: J Cell Biol 35:213, 1965.
18. Luft JH: J Biophys Biochem Cytol 4:409, 1961.
19. Kopriwa BM: J Histochem Cytochem 14:923, 1967.

20. King AC, Cuatrecasas P: J Biol Chem 257:3053, 1982.
21. Carpenter G, Cohen S: J Cell Biol 71:159, 1982.
22. Kono T, Barham FW: J Biol Chem 246:6210, 1971.
23. Cuatrecasas P: J Biol Chem 246:6522, 1971.
24. Haigler HT, Maxfield FR, Willingham MC, Pastan I: J Biol Chem 255:1239, 1980.
25. Dantry-Varsat A, Ciechanover A, Lodish HF: Proc Natl Acad Sci 80:2258, 1983.
26. Kohanski RA, Lane MD: J Biol Chem 258:7460, 1983.
27. Robb RJ, Lin Y: In Goldstein AL (ed): "Thymic Hormones and Lymphokines." New York: Plenum Press, 1984, pp 247–256.
28. Chen BD-M, Hsu S., Lin H-S: Biochim Biophys Act 72:366, 1982.
29. Steinman RM, Cohn ZA: J Cell Biol 55:186, 1972.
30. Ohkuma S, Poole B: Proc Natl Acad Sci 75:3327, 1978.
31. Sutter A, Riopelle RJ, Harris-Warrick RM, Shooter EM: J Biol Chem 254:5972, 1979.
32. Scheckter AL, Bothwell MA: Cell 24:867, 1981.
33. Huang JS, Huang SS, Kennedy B, Deuel TF: J Biol Chem 257:8130, 1982.
34. Corin RE, Donner DB: J Biol Chem 257:104, 1982.
35. Guilbert LJ, Stanley ER: J Biol Chem 261:4024, 1986.
36. Karin M, Mintz B: J Biol Chem 256:3245, 1981.
37. Miskimins WK, Shimizu N: J Cell Physiol 112:327, 1982.
38. Shooter EM, Yankner BA, Landreth GE, Sutter A: Prog Horm Res 37:417, 1981.
39. Gordon P, Carpentier J-L, Cohen S, Orci L: Proc Natl Acad Sci 75:5025, 1978.
40. Stanley ER, Chen D-M, Lin H-S: Nature 274:168, 1978.