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Effect of pH on Binding and Dissociation of Colony-Stimulating Factor (42639)

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Abstract. ^{125}I-labeled colony-stimulating factor (CSF) binds to granulocytic and monocytic cells in the bone marrow in an irreversible manner. Addition of a 1000-fold excess of unlabeled CSF does not displace the bound material. The present studies showed that brief exposures to pH 2.7–5.0 caused a marked release of the bound material. Such treatments were nontoxic to the marrow cells as judged by trypan blue dye exclusion, assay of colony-forming cells, and by analysis of rebinding of fresh ^{125}I-CSF to the acid-treated cells. The CSF released from marrow cells by low pH revealed two peaks of radioactivity on SDS–acrylamide gel. The first peak (67,500 Da) corresponded to native CSF; a second peak of 53,500 Da was observed. Despite this apparent mild degradation of CSF, the released material showed greater binding to marrow and greater precipitation by anti-CSF than the native ^{125}I-CSF. Further studies showed that acid treatment of marrow cells led to stabilization of the CSF receptors. Pretreatment at pH 4.0 led to retention of binding sites after conversion of marrow cultures to pH 7.5 and incubation at 22–37°C. In contrast, cells that were not exposed to low pH lost receptors rapidly at these temperatures. The extent of preservation of the binding sites was related to the duration of acid exposure. These studies indicate that CSF is retained on the cell surface after binding at 0°C and that the CSF can be eluted by acid conditions. Similar treatment of the cells prior to binding appears to stabilize the cells and prevent the receptor loss that ordinarily occurs at 22–37°C.

Colony stimulating factors (CSF) are glycoproteins that induce extensive proliferation and differentiation of bone marrow progenitor cells *in vitro* (1, 2). These materials have been purified to homogeneity from murine lung (3) and murine fibroblasts (L-cells) (4–6). The pure material has been radioiodinated without loss of biologic activity (7, 8), thereby providing the necessary labeled tracer for radioimmunoassays (7, 9) and for binding studies (8, 10). The radiolabeled CSF binds to granulocytic and monocytic precursor cells in the bone marrow (8), spleen (11), and fetal liver (12) as judged by autoradiographic studies (13).

Initial studies showed that radiolabeled CSF did not bind to marrow cells over intervals of several hours at 37°C. Uptake of ^{125}I-CSF was first observed after a 6-hr exposure and reached a maximum after 24 hr at 37°C (8). When marrow cells were held at 37°C for 24 hr prior to addition of CSF, there was generation of surface receptors so that binding could be assessed over short time intervals at 0°C (10). The inability to detect such receptors in the initial study was due to rapid internalization or degradation of these receptors at room temperature prior to initiating the binding studies. If cells are held at 0°C after harvest, there is preservation of receptors such that binding studies may be done immediately (14). These findings suggest that marrow cells have surface receptors for CSF; however, there appears to be a continual turnover, such that there may be a rapid loss *in vitro*.

Unlike other hormones, the binding of radiolabeled CSF is largely irreversible. Addition of a 1000-fold excess of unlabeled CSF prevents the binding when added with the tracer, but does not release ^{125}I-CSF after attachment to the cells (8). This indicates that CSF binding is irreversible or, alternatively, that internalization has occurred even at 0°C.

The present studies were undertaken to determine whether alterations in pH to acid conditions were capable of eluting CSF from the cell surface. The results indicate that intact ^{125}I-CSF can be eluted and that the mar-

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row cells remain viable after such treatment. A second goal was to determine whether low pH treatment of the cells might stabilize the CSF receptors. The results indicate further that acid treatment tends to preserve binding sites such that they remain on the cell surface following incubation at 37°C.

Materials and Methods. L-cell CSF was prepared by the growth of murine fibroblasts in serum-free CMRL 1066 medium (Grand Island Biological Co., Grand Island, NY) as described previously (5). A 10-liter pool of the resultant conditioned medium was concentrated 250-fold by ultrafiltration using an Amicon ultrafiltration cell and PM10 membrane. Highly purified CSF was obtained by an affinity chromatographic technique (6). The CSF was devoid of contaminating proteins as judged by electrophoretic migration of the radioiodinated material in SDS–acrylamide gels and had a specific activity of approximately $5–7 \times 10^7$ units/mg of protein (6).

The highly purified CSF was iodinated by the chloramine-T technique with addition of small quantities of dimethylsulfoxide (DMSO) as described previously (8). The reactants were separated from free ^{125}I on a column of Sephadex G-150. The CSF was stored at 4°C and was biologically active for 3 weeks. CSF iodinated at different times contained 2.5 to 5.5 mole of ^{125}I/mole of CSF.

Bone marrow cells were obtained from the femurs of CF_1 mice at 0°C. Cells were expelled by inserting a 23-gauge needle in the proximal end and forcibly rinsing out the contents with 2 ml of modified McCoys 5A medium supplemented with 15% fetal calf serum (Grand Island Biological Co.).

Cultures were prepared using 10^7 bone marrow cells in 1 ml of McCoy's 5A medium supplemented with 15% fetal calf serum and saturating doses of ^{125}I-CSF (300,000–400,000 cpm), which represented approximately 100 units or 2 ng of CSF. These cultures were incubated in 1 by 7.5-cm tubes at 0°C for 30 min. Triplicate samples were used for each experimental point. After incubation, culture tubes were counted for total radioactivity, centrifuged for 10 min, and washed once with 1 ml of serum-free McCoy's medium at 0°C to remove unbound CSF, and the supernatant was discarded. Experiments indicated that a single washing step removed virtually all unbound CSF. After washing, repeat counts were obtained to determine the level of cell-associated radioactivity. In control tubes, an 80-fold excess of unlabeled CSF was used in addition to labeled CSF. Specific counts were determined by subtracting values obtained in tubes containing the additional unlabeled CSF from those with ^{125}I-CSF alone (10).

To determine the effect of pH on radiolabeled CSF bound to marrow cells, the washed cells were treated with 1 ml of buffer at pH 3.9–10 for 5 min at 0°C. The cells were centrifuged and counted after removing the supernatant. Citrate buffer, 0.15 *M*, was used for exposing the cells to pH 3.9, 5, and 6. Serum-free McCoy's medium was used for pH 7.5 and for pH 10, 0.15 *M* glycine–sodium hydroxide was utilized. All buffers contained 0.3% polyethylene glycol 4000 (PEG) to prevent loss of CSF on glass surfaces or Millipore filters (15).

Radioactive material released from cells after binding of ^{125}I-CSF by exposure to low pH was compared with native ^{125}I-CSF. Tests included assessment of molecular weight, precipitation with anti-CSF (9), and evaluation of the ability to rebind to fresh marrow cells. This radioactive material was produced by incubating marrow cells (550×10^6) with 13×10^6 cpm of radiolabeled CSF. After 30 min at 0°C, the cells were centrifuged, washed, and exposed to pH 5.0 for 5 min. The radioactive material released in the supernatant was concentrated to 1.5 ml and was used for rebinding to fresh marrow cells. Precipitation of the released material and native ^{125}I-CSF was measured using anti-CSF serum (16). Radioactivity (12,000 cpm) from each source was incubated with dilutions of anti-CSF serum in phosphate buffer. After 72 hr at 4°C, sheep anti-rabbit IgG serum was added. Each tube was centrifuged and the radioactivity of the precipitates was determined. For molecular weight evaluation, the material released from the marrow cells was subjected to SDS gel electrophoresis using 10% acrylamide; another gel contained radiolabeled CSF as a control. The gels were cut into 2-mm sections and counted and the relative mobility of radioactivity was deter-

mined. The molecular weight was estimated by comparing the mobility of the released ^{125}I-CSF to that of standard marker proteins.

In vitro colony-forming assays (CFU-C) were performed as described (17). In brief, 10^5 CF_1 bone marrow cells were immobilized in 1 ml cultures of supplemented McCoy's medium in 0.3% Bacto-agar (Difco Laboratories) supplemented with 15% fetal calf serum and other additives as described previously. L-cell CSF (0.1 ml, approximately 200 units of CSF) was added to all cultures in 35-mm plastic petri dishes (Corning Glassware, Corning, NY). After gelling, the plates were incubated in a well-humidified 37°C incubator in the presence of 7.5% CO_2. Seven days later, colonies of 50 or more cells were scored using a dissecting microscope.

Results. The effect of different pH treatments on the release of cell-bound ^{125}I-CSF is shown in Fig. 1. Binding of ^{125}I-CSF for 30 min at 0°C yielded an average of 8507 cpm bound/10^7 cells. A 5-min exposure to pH 3.9 and 5.0 led to release of >90% of cell-bound radioactivity. Approximately 46% of the radioactivity was released at pH 6, whereas only 4–10% was dissociated at pH 7.5 and 10.0, respectively.

The effect of various pH exposures on cell viability was tested using fresh marrow cells.

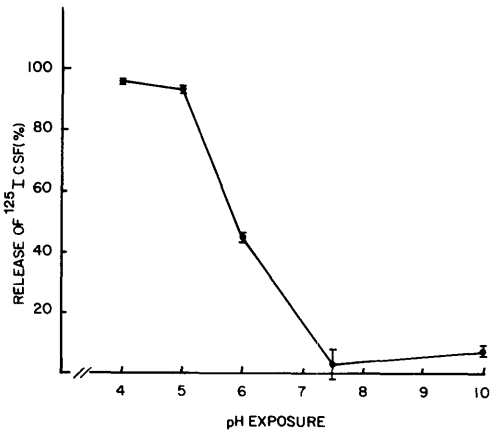

FIG. 1. Effect of pH treatment of the release of ^{125}I-CSF. Marrow cells were incubated for 30 min with ^{125}I-CSF and washed and cell-associated radioactivity was determined. Cultures were then exposed to various pH values for 5 min at 0°C. Supernatant radioactivity was determined after centrifugation. The initial cell bound radioactivity was considered 100% for each culture.

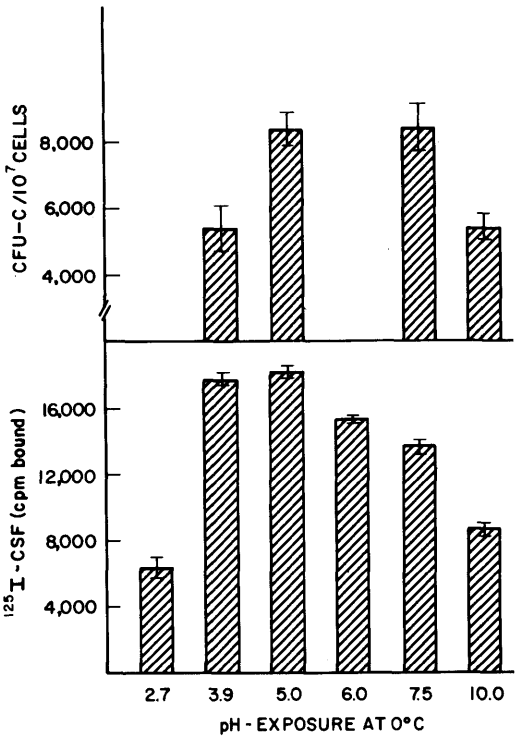

FIG. 2. Effect of pH on marrow cell viability. Marrow cells were exposed to different pH values for 5 min at 0°C. Cultures were adjusted to pH 7.5 for ^{125}I-CSF binding for 30 min at 0°C. The number of CFU-C were determined by *in vitro* colony assay. CFU-C were not done after pH 2.7 and 6.0 exposure. Values are means ± 1 SE.

After a 5-min exposure to pH 2.7–10.0, cells were readjusted to pH 7.5 and tested for trypan blue exclusion, *in vitro* colony growth, and the ability to bind ^{125}I-CSF. Greater than 90% cell viability was detected in all cultures by dye exclusion. The results of *in vitro* colony growth and ^{125}I-CSF binding are shown in Fig. 2. Colony-forming potential was unchanged after brief exposure to pH 5.0 and was reduced by approximately 40% after treatment at pH 3.9 and 10.0. Extremes of pH treatment, i.e., exposure at pH 2.7 and 10.0, also caused a modest decrease in CSF binding. In contrast, CSF binding appeared to be enhanced after incubation of the cells at pH 3.9 and 5.0.

Repetitive binding of ^{125}I-CSF to cells after exposure to acid pH was explored. The results are presented in Table I. Initially, 6838

TABLE I. REBINDING TO CELLS AFTER pH TREATMENT

Initial binding (cpm)	After dissociation		Second binding (cpm)
	pH	cpm	
6838 ± 237	3.9	284 ± 37	6506 ± 390
	5.0	435 ± 8	6477 ± 589
	6.0	3716 ± 118	8548 ± 156
	7.5	6616 ± 356	10687 ± 458
	10.0	6203 ± 128	9952 ± 525

Note. Marrow cells were incubated with 378,000 cpm of ^{125}I-CSF for 30 min at 0°C both before and after dissociation at various pH values. Numbers are means ± SE.

cpm of radiolabeled CSF were bound. The majority of the CSF was removed at pH 3.9 and 5.0 with virtually no dissociation at pH 7.5 and 10.0. Addition of fresh tracer yielded approximately 6500 cpm bound with a repeat 30-min exposure to ^{125}I-CSF. Higher total binding was seen with cells exposed to pH 6, 7.5, and 10.0. Since there was less dissociation from the cells, the higher amount of ^{125}I-CSF bound appeared to represent an additive effect as plateau binding is not ordinarily observed until 2–3 hr total exposure to radiolabeled CSF.

^{125}I-CSF that was released from a mass culture of marrow cells at low pH was tested for retention of its biologic properties. This released material had, essentially, twice the avidity of the native tracer for CSF receptors on marrow cells (Table II). Moreover, both the native tracer and that released from the marrow cells were precipitated by anti-CSF serum. It is of interest that the released material was precipitated to a greater extent than the native CSF. In further studies, both CSF were subjected to electrophoresis in SDS–10% acrylamide gel. The native CSF migrated with an apparent molecular weight of 69,000. The ^{125}I-CSF released from the cells yielded two peaks with apparent molecular weights of 67,500 and 53,500.

Studies were done to determine whether acid pH treatment stabilized the CSF receptors. Exposure of fresh marrow cells to pH 4.0 and 5.0 for 1 hr at room temperature led to preservation of CSF receptors whereas, those cells held at pH 6.0, 7.5, and 10.0 had marked loss of CSF binding (Fig. 3).

The effect of varying the duration of acid exposure is shown in Fig. 4. Those cells held at pH 7.5, 0°C throughout had no loss of binding activity. In contrast, conversion of these cultures to 37°C for 1 hr led to marked loss of binding sites. Exposure to pH 5.0 for 5–60 min was associated with incremental preservation of CSF receptors even with conversion of the cultures to 37°C.

Discussion. The binding of ^{125}I-CSF to responsive progenitor cells in the marrow differs somewhat from classic hormone-receptor interactions. When cells are held at reduced temperatures to retain surface receptors, the binding proceeds rapidly but is largely irreversible. Addition of up to a 1000-fold excess of unlabeled CSF fails to displace the bound material. This suggests either very high-affinity binding sites or, alternatively, that the CSF–receptor complex may internalize rapidly even at 0°C.

In previous studies, the association constant for CSF binding was estimated as 10^{-11} to 10^{-12} *M* for bone marrow (8, 14) and fetal liver cells (12) and 10^{-13} *M* for peritoneal macrophages (18). As compared to insulin and other growth factors, CSF appears to have the highest known affinity for binding to its receptor. Addition of 2-deoxy glucose (19) or sodium azide (20), agents that prevent internalization of hormone–receptor complexes, during the binding has not altered the ability to displace the ^{125}I-CSF with excess unlabeled CSF (R. K. Shadduck and A. Waheed, unpublished observations).

Previous observations have indicated that various hormones may be dissociated from

TABLE II. ACTIVITY OF NATIVE ^{125}I-CSF AND THAT RELEASED FROM MARROW CELLS AT pH 5.0

^{125}I-CSF	Specific cpm bound	Precipitated with anti-CSF	
		1/1000 dilution	1/40,000 dilution
Native tracer	5,000 ± 40	67%	36%
Released tracer (pH 5.0)	11,442 ± 809	87%	45%

Note. 136,000 cpm of native tracer or that released from marrow cells at pH 5.0 was bound to fresh marrow at 0°C for 30 min. Precipitation of tracer with anti-CSF was measured after 72 hr at 4°C.

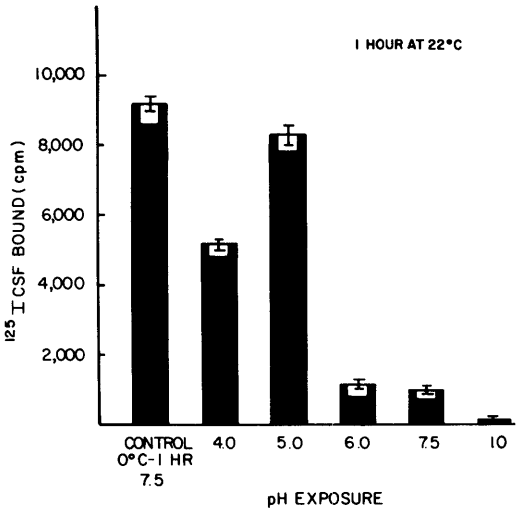

FIG. 3. Stability of CSF receptors after different pH exposures. Cultures of marrow cells were held at 22°C for 1 hr at the various pH values. They were converted to pH 7.5 and binding was measured after a 30-min exposure to ^{125}I-CSF at 0°C. Control cultures were held at 0°C, pH 7.5, throughout. Values are means ± 1 SE.

their receptors using low pH conditions. Insulin, asialoorosomucoid (19), and adrenocorticotropic hormone (ACTH) (21) can all be released from their receptors at pH 4.0–5.0. Guilbert has reported recently that exposure to pH 4.0 causes the release of CSF-1 from receptor sites on peritoneal macrophages (22, 23). The released CSF was undamaged by the low pH exposure as judged by behavior on SDS gel, retention of biologic activity, and precipitation by anti-CSF. The present study extends these findings by using bone marrow rather than peritoneal macrophages. The radiolabeled material was released from cells at 0°C at low pH within 5 min. With variation in pH from 3.9 to 5.0, about 90% of the radiolabeled material was dissociated. Approximately 60% was released at pH 6.0, whereas only 4–10% of the bound radioactivity was detected in the supernatant after incubation at pH 7.5 and 10.0. The released radiolabeled material, as noted recently (23), had similar properties to the native tracer. The released radioactivity bound with anti-CSF and bound to fresh marrow cells with even greater avidity than the native ^{125}I-CSF. Unlike previous findings with peritoneal macrophages (23), the released material resolved into two peaks on SDS–acrylamide gel with molecular weights of 67,500 and 53,500 Da. The finding of a secondary peak suggests mild degradation of the CSF; however, this did not appear to influence its biologic activity.

Exposure of marrow cells to pH 3.9 to 6.0 did not alter their viability as judged by trypan blue exclusion or by the ability of these cells to bind fresh ^{125}I-CSF after acid exposure. *In vitro* colony growth remained normal after exposure to pH 5.0. The release of ^{125}I-CSF without destruction of marrow cells at acid pH indicates that the CSF remains on the cell surface after binding with virtually no internalization of the tracer at 0°C. These observations are in accord with previous studies that have shown a very high association constant for CSF binding to its receptor (8, 14).

A problem in the study of CSF binding has been the relatively rapid loss of surface receptors at 22–37°C. This may result from internalization, shedding from the surface, or perhaps proteolytic digestion of the receptors. Our initial attempts to isolate such receptors were hampered by loss of cell binding activity after solubilizing marrow cells at physiologic pH values. The addition of var-

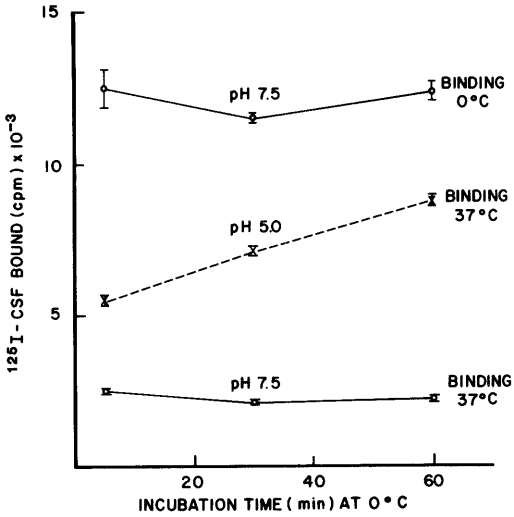

FIG. 4. Preservation of CSF receptors as a function of exposure time to acid pH. Marrow cells were exposed to pH 7.5 or 5.0 at 0°C for the times indicated. The pH of all cultures was adjusted to 7.5 for binding at 0 or 37°C for 30 min. Values are means ± 1 SE.

ious proteolytic inhibitors did not prevent receptor degradation. The present study also sought to determine whether exposure to low pH would preserve binding sites and, therefore, aid in the isolation of CSF receptors. Exposure to low pH for 60 min at 22°C was associated with preservation of CSF receptors, whereas those cells held at pH 6–10 had a marked loss of CSF binding. Increased exposure to pH 5.0 at 0°C from 5 to 60 min was associated with incremental preservation of receptors, even after conversion of the cells to 37°C.

Previous CSF-binding studies with marrow cells (8) or peritoneal macrophages (23) have shown that continued incubation of radiolabeled cells at 37°C is associated with loss of radioactivity into the medium. This radiolabeled material is degraded as judged by a molecular weight of less than 10,000 Da and an inability to bind to fresh marrow cells. This indicates that the CSF is internalized and degraded. In previous studies (24) incubation of cells with 5–50 m*M* ammonium chloride was associated with decreased CSF degradation. Since ammonium chloride and related amines are known to inhibit lysosomal function (25), this suggests that CSF or the CSF–receptor complex is internalized and, perhaps, degraded in the lysosomes. Recent studies (26) using quantitative electron microscopic autoradiography indicated similar findings with binding of radiolabeled CSF to peritoneal macrophages. At 0°C, the tracer was localized in the plasma membrane and its invaginations, whereas when the temperature was raised to 37°C, the CSF internalized progressively. Localization of radioactivity over the lysosomal compartment increased with time reaching a plateau within a 40-min exposure to 37°C. The present study does not indicate the fate of CSF or its receptor but does indicate that acid treatment of the cells does prevent loss of receptors at 37°C. This suggests that the surface receptors have been stabilized, perhaps, by interference with the transport mechanism. In recent studies, acid treatment of the cells has prevented the loss of binding sites that occurs with solubilization of the cells. Using this technique, it has been possible to isolate CSF receptors (27) using an immunoadsorbent column containing purified CSF. This suggests that acid treatment of the cells not only stabilized the surface receptor but may also interfere with either lysosomal degradation or other proteolytic processes.

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