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CYTOKINE REGULATION OF COMPLEMENT RECEPTOR-MEDIATED INGESTION BY MOUSE PERITONEAL MACROPHAGES

M-CSF and IL-4 Activate Phagocytosis by a Common Mechanism Requiring Autostimulation by IFN-β[1]

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Macrophage CSF (M-CSF, CSF-1) and IL-4 are two cytokines known to have effects on mature monocytic phagocytes in vitro. In this report we show that M-CSF and IL-4 activate resident mouse peritoneal macrophages to ingest particles via their C3b and C3bi receptors, which are not capable of mediating ingestion in resting cells. IgG-mediated ingestion was also increased by IL-4 and M-CSF. IL-1, IL-2, TNF-α, and IFN-γ were not able to stimulate C receptor-mediated ingestion. Stimulation by IL-4 and M-CSF is dependent upon high cell density and greater than 24-h exposure to the cytokine. Interestingly, antibody to IFN-α/β and mAb to IFN-β inhibited the enhanced ingestion caused by both M-CSF and IL-4. However, neither IFN-α nor IFN-β alone stimulated C receptor-mediated ingestion. M-CSF did not affect the ligand-independent distribution of CR3 on the macrophage surface. We conclude that two apparently unrelated cytokines, M-CSF and IL-4, both enhance macrophage phagocytosis of C and IgG-coated targets via a common pathway in which autocrine stimulation with IFN-α/β is necessary but not sufficient.

In the absence of an external stimulus, mouse peritoneal macrophages will bind, but not ingest, particles opsonized with the third component of C. Introduction of an inflammatory signal in vivo before harvesting the cells, however, will render these macrophages competent for ingestion via their C receptors (1). Several in vitro methods have been described to mimic this in vivo activation of C receptors. Griffin and Griffin reported that a T cell-derived factor caused ingestion of C opsonized SRBC when added to cultures of unstimulated resident peritoneal macrophages (2, 3). Also, phorbol esters and fibronectin stimulate C-mediated phagocytosis in human monocytes (4, 5). These substances all cause modulation of C receptor function within minutes. This study describes two cytokines that required much longer treatments to render C receptors competent for phagocytosis and that may be relevant to macrophage function at inflammatory sites in vivo.

M-CSF[3] (CSF-1) is a hemopoietic growth factor required for the proliferation, differentiation, and viability of mononuclear phagocytes. It is a glycosylated homodimer whose molecular mass ranges from 45 to 75 kDa. The single class high affinity receptor for M-CSF is the product of the *c-fms* proto-oncogene, which belongs to the family of tyrosine kinases that includes the PDGF receptor. The receptor is present in low amounts on monocyte precursors found in bone marrow, and increases in number as the cells differentiate (6–9).

In addition to its role in hemopoiesis, M-CSF has been shown to stimulate a variety of functions in mature monocytes, bone marrow-derived macrophages, and tissue macrophages. Some of these functions include production of cytokines (IL-1, IFN-α/β, and TNF) (10–13); enhancement of FcR expression (14); macropinocytosis (15), enhancement of tumor cell killing (16, 17); induction of viral resistance; secretion of superoxide and H_2O_2 (18), prostaglandins, plasminogen activator, and ferritin; enhancement of *Candida albicans* killing; and enhancement of the phagocytosis of *Listeria monocytogenes.*

IL-4 (B cell growth factor-1, B cell-stimulating factor-1, mast cell growth factor-2, and T cell growth factor-2) has in recent years been shown to have effects on monocytes and macrophages as well as lymphocytes and mast cells. Several groups have demonstrated that IL-4 increases the expression of MHC class II Ags on murine thioglycollate-elicited peritoneal macrophages (21), human peripheral blood monocytes (22, 23) and murine bone marrow-derived macrophages (24). In addition, IL-4 increases production of C2 in monocytes (25), up-regulates the expression of CR3 and p150/95 on monocytes (26), and increases the FcR-dependent binding of IgG-immune complexes to bone marrow-derived macrophages (21). Interestingly, IL-4 inhibits macrophage synthesis of IL-1 (22) and TNF (25), and monocyte production of superoxide products (26).

Our study has examined the role of several purified

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[3] Abbreviations used in the text: M-CSF, monocyte/macrophage CSF; E, sheep E; EIgG: sheep E opsonized with IgG antibody; EC3b: sheep E opsonized with complement component 3b; EC3bi: sheep E opsonized with complement component 3bi; LCM, L cell conditioned medium; VBS, Veronal-buffered saline; DME, Dulbecco's modified Eagle's medium; P.I., phagocytic index.

and recombinant cytokines on the phagocytic competence of macrophage C receptors, a highly differentiated function in these cells. We show that IL-4 and M-CSF both activate phagocytosis of C3b and C3bi opsonized targets by resident murine peritoneal macrophages. Stimulation in vitro is dependent upon cell density but independent of cell morphology. Kinetic studies with M-CSF show that exposure for at least 32 h is required to reach maximal phagocytosis; long exposures to IL-4 are also needed for enhancement. IgG-mediated ingestion also is increased after these long incubations with CSF-1 and IL-4. Apparently, enhancement of ingestion via all these receptors is dependent on IFN-α/β produced endogenously in culture by the macrophages. Thus two cytokines that interact with macrophages through distinct receptors and have no obvious functional similarity both stimulate macrophage differentiation into phagocytically active cells via a common pathway.

MATERIALS AND METHODS

Media. DME was obtained from GIBCO Laboratories, Grand Island, NY. Heat-inactivated FCS was purchased from HyClone Laboratories (Logan, UT). α-MEM, penicillin/streptomycin, L-glutamine, and sodium pyruvate were obtained from the Tissue Culture Support Center, Washington University School of Medicine, St. Louis, MO.

Antibodies. Anti-murine M-CSF mAb 5AI and D24 (27) were a generous gift of H. S. Lin, Washington University. A rabbit polyclonal antibody (purified IgG) against IFN-α/β was purchased from Lee Biomolecular Research Laboratories, San Diego, Ca M1/70 (anti-murine CR3) (28) IgG was purified from tissue culture supernatant by using an ammonium sulfate precipitation. Monoclonal anti-murine IFN-α and anti-murine IFN-β were from Seikagaku America (Rockville, MD). The anti IFN-α neutralized 10 U of IFN-α at a dilution of 1/500; the anti-IFN-β had an equivalent neutralizing titer at 1/400,000. Anti-murine IL-6 was the kind gift of Dr. Robert Schreiber. At 1 μg/ml this antibody completely neutralized 5 ng of IL-6.

Cytokines. Purified murine M-CSF with a sp. act. of 5×10^7 U/ml (27) was a gift of H. S. Lin, Washington University. Human rM-CSF was a gift from the Genetics Institute, Cambridge, MA. rIL-4 was a gift from A. Levine, Monsanto Company, St. Louis, MO. rIFN-γ, rTNF-α, and rIL-2 were generous gifts from R. D. Schreiber, Washington University. rIL-1 was kindly provided by D. P. Chaplin, Washington University. rIFN-α and rIFN-β were from Lee Biomolecular Research, San Diego, CA.

LCM. L929 (mouse fibroblast) cells were grown in α-MEM supplemented with nonessential amino acids, L-glutamine, sodium pyruvate, and penicillin/streptomycin. Cells were plated at 2.5×10^5 cells/50 ml and allowed to grow to confluence, at which time the supernatant was collected, filtered, and stored at −20°C. Repeated freeze-thawing was avoided.

Macrophages. Peritoneal macrophages were obtained by lavage with cold PBS (Whittaker Bioproducts, Walkersville, MD) from 16- to 18-g female CD-1 mice (virus antibody free; Charles River Laboratories, Wilmington, MA) as described (29). After removing the peritoneal fluid, the cells were washed once in cold PBS, counted, and plated at a density of 4×10^6 cells/ml (or, for some experiments, $r \times 10^5$ cells/ml) onto 12-mm round glass coverslips (Fisher Scientific, Orangeburg, NY) and allowed to adhere for 2 h at 37°C. After four to five washes in 37°C PBS, the coverslips were placed in individual wells of a 24-well tissue culture cluster (Costar, Cambridge, MA) with the media preparation to be tested and incubated for 2 days unless otherwise indicated. The medium used for culturing peritoneal macrophages consisted of DME with 20% FCS supplemented with penicillin, streptomycin, L-glutamine, and sodium pyruvate ("control" medium). In some cases this was modified by the addition of LCM (30% v/v) the indicated concentrations of purified or recombinant cytokines, and/or monoclonal or polyclonal antibodies.

Phagocytic targets. E were opsonized with IgG or guinea pig C components as previously described (30). Briefly, E were washed three times in VBS (Veronal buffered saline, Whittaker Bioproducts) and the concentration was adjusted to 10^9 cells/ml by using a spectrophotometer. For C opsonization, the E were then mixed with an equal volume of 1:200 anti-sheep E IgM (Diamedix Corporation, Miami, FL) in VBS and incubated for 10 to 15 min at 37°C. After two washes in gelatin-dextrose-VBS, the volume was adjusted to yield a cell concentration of 1.5×108 cells/ml. Guinea pig C1, human C4, and guinea pig C2 and C3 (Diamedix) were added sequentially to make C3b-opsonized E (EC3b). For preparation of EC3bi, EC3b were further incubated for 1 h at 37°C with a 1/200 dilution of human serum in EDTA-VBS, pH 6.0 (29). EIgG were made by incubating 10^9 E with an equal volume of 1/1000 anti-sheep E IgG (Diamedix) in VBS for 15 min at 37°C. Both preparations were used at a final concentration of 5×10^8 E/ml.

Phagocytosis assay. Peritoneal macrophages cultured for 2 days on glass coverslips were removed from assay medium and placed in 1 ml of 37° DME with penicillin/streptomycin. To this was added 25 μl of opsonized sheep E. After a 45-min incubation at 37°C, coverslips were washed gently two to three times with room temperature PBS. To lyse externally bound red cells, 1/5 × PBS was added for 30 s. (For adherence assays, this step was omitted.) After another rinse with PBS, the coverslips were overlaid with 1% glutaraldehyde in PBS for 5 min, rinsed in distilled water, and then strained with Giemsa stain (Fisher Scientific). The coverslips were then air dried and mounted upside-down on glass slides with Permount (Fisher Scientific, Fair Lawn, NJ). Ingestion of E by the macrophages was determined by using light microscopy, and in most cases the person scoring the assay had no knowledge of the experimental groupings. The results are expressed as the P.I., which is equal to the number of E ingested per 100 macrophages. Each experimental point was performed in duplicate or triplicate.

Quick-freeze, freeze-dry electron microscopy. Resident peritoneal macrophages were adhered to glass coverslips and cultured for 2 days in DME with or without 30% LCM. After a brief wash with "simplified" PBS (70 mM NaCl, 30 mM HEPES, 2 mM $CaCl_2$, pH 7.2) the monolayers were fixed with 1% formaldehyde and then quenched with simplified PBS containing 50 mM lysine and 50 mM NH_4Cl. Primary antibody (M1/70 or an irrelevant rat IgG control) was added in simplified PBS with 1% BSA (Sigma Chemical Co., St. Louis, MO) and allowed to adhere at room temperature for 1 h. After several washes with BSA-PBS, secondary antibody (goat-anti-rat IgG) conjugated to 15 nm gold (E-Y Laboratories, IN, San Mateo, CA) was added in BSA-PBS and allowed to adhere at room temperature for 1 h. After several rinses with PBS, the macrophage monolayers were postfixed with 1% glutaraldehyde. Preparation of samples for electron microscopy was performed as previously described (31). Briefly, the cells were frozen in liquid nitrogen, freeze dried for 15 min, at −80°C, then platinum replicated with 2 nm of platinum applied at an angle of 24°C above the horizontal. The replicas were examined in transmission electron microscopy uncleaned to preserve the gold under the replica.

RESULTS

LCM stimulates phagocytosis by resident peritoneal macrophages. Murine resident peritoneal macrophages were adhered to glass coverslips and cultured for 48 h in the presence or absence of 30% (v/v) LCM in DME with 20% FCS. Those macrophages incubated in the presence of LCM phagocytized an average of 4.5 (± 1.9, $n = 43$) times more EC3b than those incubated without LCM (Fig. 1*a*). Phagocytosis of EC3bi also was higher in LCM-treated macrophages (Fig. 1*b*). Phagocytosis of EC3bi and EC3b proceeded via distinct receptors, because binding of EC3bi was inhibited by M1/70, but binding of EC3b was not, as previously reported (31). The murine macrophage equivalent of CR1, the C3b receptor, is not yet well described. However, its function was enhanced equivalently to CR3 function by culture in L cell supernatant. Potential contamination with LPS is unlikely to have any role in this activation of phagocytosis, because resident peritoneal macrophages from C3H/HeJ mice also increased ingestion of EC3b (2.5 ± 0.4-fold increase, $n = 3$) and EC3bi (2.5 ± 0.24-fold increase, $n = 3$) in response to LCM (data not shown).

LCM-induced stimulation is dependent upon M-CSF. Because L cell supernatant contains large amounts of M-CSF (9), antibodies were added to the cultures to determine if the stimulation of phagocytosis was dependent upon M-CSF. mAb to murine M-CSF (27) were added to cultures of adherent peritoneal macrophages at the be-

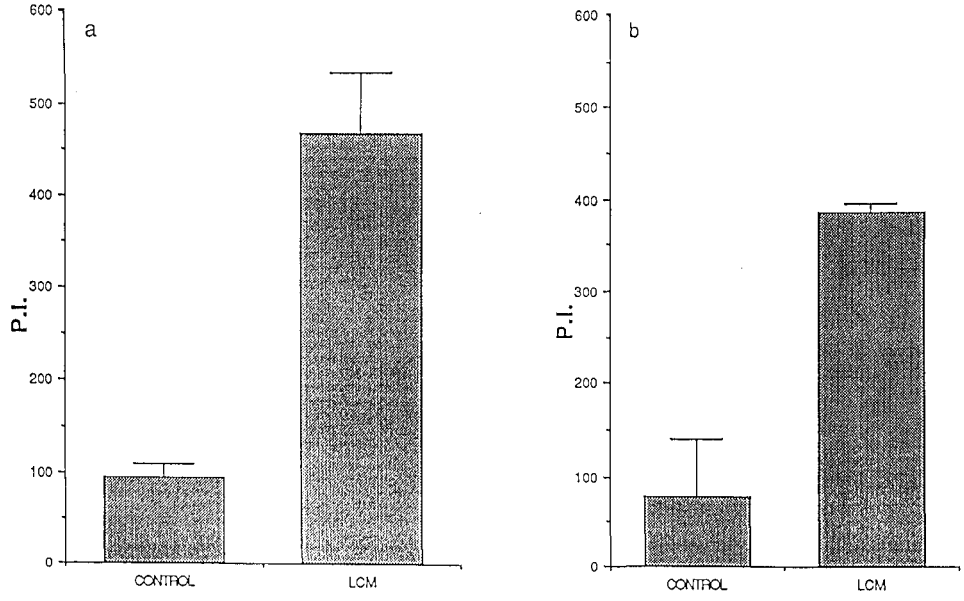

Figure 1. LCM stimulation of C phagocytosis. Fresh cultures of resident peritoneal macrophages were incubated in the presence (*LCM*) or absence (*Control*) of L cell-conditioned medium (30% v/v in DME with 20% FCS). After 2 days, a phagocytosis assay was performed with either EC3b (*a*) or EC3bi (*b*) targets. Shown are representatives of 43 and 4 experiments, respectively.

ginning of the 48-h incubation. 5AI, a mAb that neutralizes the colony-stimulating activity of M-CSF, completely inhibited the LCM stimulation of C3b-mediated phagocytosis. D24, an anti-M-CSF monoclonal that binds, but does not block its activity, had no effect (Fig. 2). Therefore, M-CSF is necessary for the stimulation of C3b-mediated phagocytosis.

To show that M-CSF alone is sufficient for this increase, purified murine M-CSF was added to the beginning of 48-h cultures of resting peritoneal macrophages. M-CSF stimulated phagocytosis of EC3b with a maximum at a dose of 100 U/ml (Fig. 3). Similar results were obtained with human rM-CSF (not shown).

Purified IL-4 also stimulates C3b- and C3bi-mediated

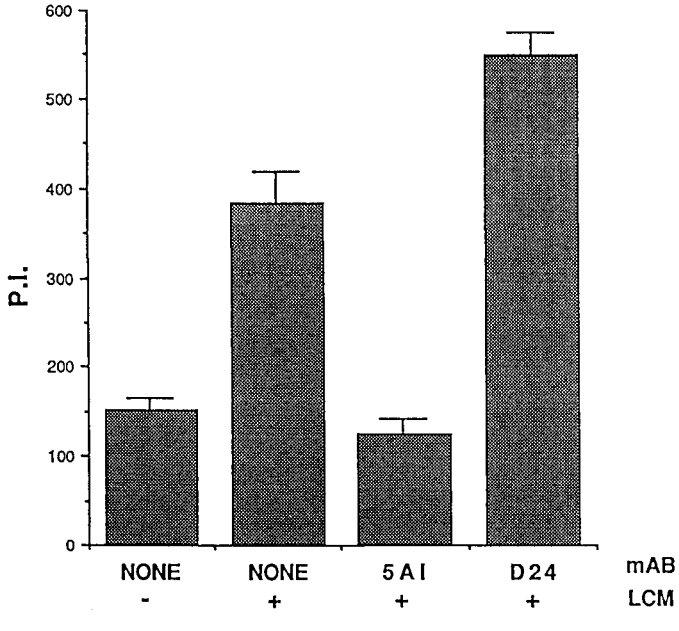

Figure 2. LCM stimulation of phagocytosis is blocked by an anti-M-CSF neutralizing antibody. 5AI (a neutralizing mAb to M-CSF) or D24 (an anti-M-CSF antibody that is non-neutralizing) ascites (1/2500 final dilution) was added to fresh cultures of resident peritoneal macrophages with 30% LCM. After 2 days of incubation, a phagocytosis assay was performed with EC3b targets. A representative experiment from nine performed is shown.

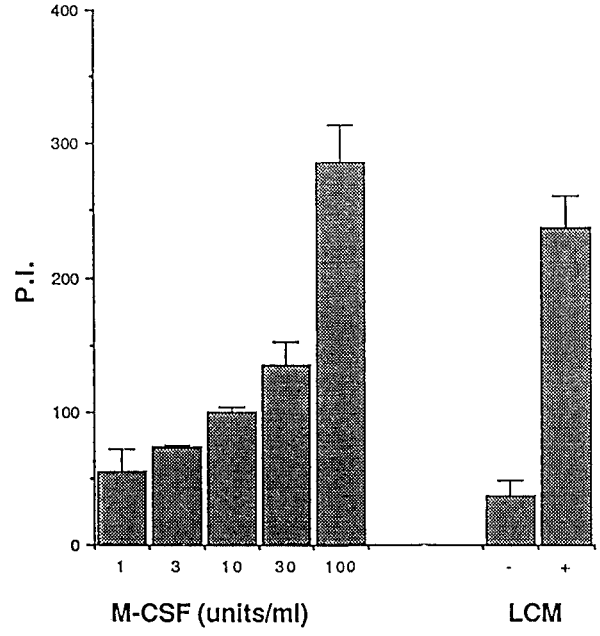

Figure 3. Dose response of purified murine M-CSF. Indicated concentrations of purified murine M-CSF were added to fresh cultures of resident peritoneal macrophages. After a 2-day incubation, the cultures were tested for phagocytosis with EC3b targets. Thirty percent LCM, shown as a positive control, is estimated to contain approximately 900 U/ml of M-CSF activity.

phagocytosis. Several other cytokines were tested for their ability to affect C receptor-mediated ingestion. rIL-1, rTNF-α, rIL-2, and rIFN-γ, each at 1000 U/ml, had no effect of EC3b ingestion when added individually to macrophage cultures (data not shown). However, rIL-4 enhanced C3b-mediated ingestion equivalently to CSF-1 (Fig. 4). Maximum ingestion was achieved at a dose of about 100 U/ml (Fig. 4). A total of 100 U/ml of IL-4 also induced EC3bi ingestion (data not shown).

Kinetics of M-CSF and IL-4 stimulation. Kinetic studies were performed to further define the mechanism of M-CSF and IL-4 enhancement of phagocytosis. Resident peritoneal macrophages adherent to glass coverslips were transferred from control medium (DME with 20% FCS) to medium containing 30% LCM at various time points dur-

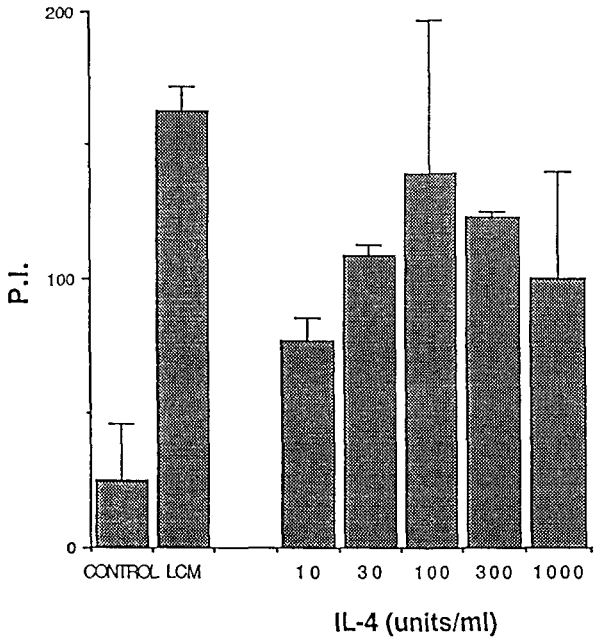

Figure 4. IL-4 stimulation of C3b phagocytosis. Indicated concentrations of murine rIL-4 were added to fresh cultures of peritoneal macrophages. After a 2-day incubation, phagocytosis of EC3b was assayed. Twenty percent LCM is shown as a positive control. Shown is a representative experiment of five performed.

ing the 48-h incubation. Those exposed to LCM at time 0 or before the 16th h of culture had full phagocytic capacity (compared with macrophages incubated for 2 days in control medium) when tested at 48 h. A half-maximal point was achieved at 24 h (Fig. 5*a*). In addition, coverslips transferred from medium containing LCM to control medium had maximal phagocytic capacity only if the transfer occurred after 32 h with half-maximal effect after transfer to LCM-free media at 24 h (Fig. 5*b*). Together these data suggest that although at least 32 h of LCM treatment is necessary to obtain the maximal phagocytic increase, this treatment can occur either during the first or the last 32 h of culture. These data also show that once full phagocytic capacity is achieved, this capacity is retained for at least 16 h in the absence of LCM.

Similarly, IL-4 required prolonged incubation to achieve maximum stimulation. Resident peritoneal macrophages were cultured for 48 h in 20% DME, and 300 U/ml of murine rIL-4 were added at various times during the culture. When IL-4 was added 1 or 7 h before assessing ingestion, there was no increase in phagocytosis. Only macrophages receiving IL-4 for the entire 48 h showed markedly enhanced ingestion (Fig. 5c).

These experiments show that the mechanism of M-CSF and IL-4 stimulation differs from that of the cytokine described by Griffin and Griffin, which can stimulate C3b-mediated phagocytosis in resident peritoneal macrophages when added immediately before the phagocytic assay (3). Moreover, it distinguishes enhanced ingestion from macropinocytosis, which also begins after a few minutes of incubation with CSF-1 (15).

Stimulation of phagocytosis is dependent upon cell density. We also sought to address whether the effects of M-CSF and IL-4 on ingestion were related to the morphologic alterations of increased size and vacuolization that occur prominently in macrophages incubated with M-CSF (32). Against this hypothesis were the facts that the morphologic changes were quite apparent with as little as 3 U/ml M-CSF, whereas maximal ingestion required 30 times more; that IL-4 effects on morphology were much less prominent than M-CSF effects, even though the two cytokines were both potent for stimulating ingestion; and that the morphologic changes occurred

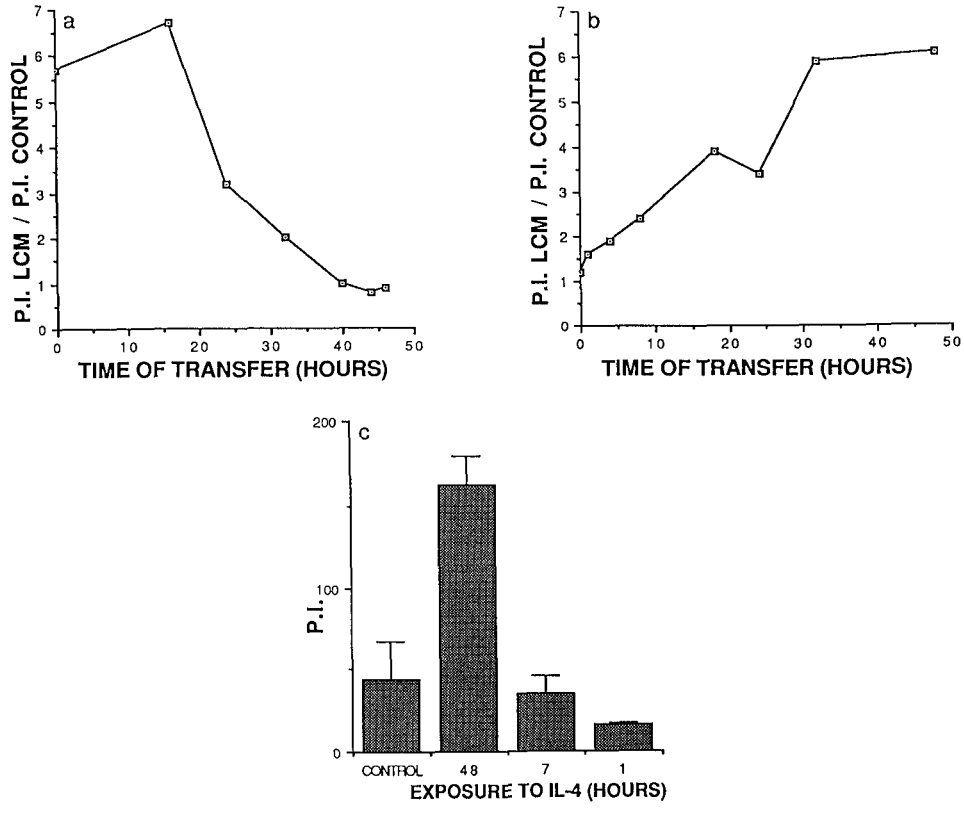

Figure 5. Kinetic studies of the LCM and IL-4 enhancement of C phagocytosis. *a*, Fresh cultures of resident peritoneal macrophages were incubated in control medium (without LCM) and at the indicated time points duplicate coverslips were transferred to warmed medium containing 30% (v/v) LCM. Phagocytosis assays were performed with EC3b after a total of 48 h in culture. Shown is a compilation of three experiments, with the data expressed as the ratio of the average P.I. of LCM-stimulated cultures to the average P.I. of unstimulated cultures at each time point. *b*, Same as *a*, except coverslips were transferred from 30% LCM to control medium. *c*, rIL-4 (300 U/ml) was added to cultures of resident peritoneal macrophages at 48, 7, or 1 h before the assay of C3b phagocytosis on day 2. Shown is a representative experiment of three performed.

much earlier than the effects on ingestion (data not shown). To confirm that the phagocytic effects were unrelated to morphologic changes, we examined the effect of cell density on morphology and ingestion. Macrophages plated at 4×10^5/ml showed morphologic changes similar to cells plated at 4×10^6/ml in medium containing either 100 U/ml rM-CSF or 300 U/ml rIL-4. However, there was a marked effect of cell density on phagocytic function (Fig. 6). Neither rM-CSF nor rIL-4 supported EC3b ingestion in cells plated at low density, whereas those plated at 4×10^6 cells/ml did show a significant increase (Fig. 6).

IL-4 and M-CSF increase in C receptor phagocytosis requires endogenously produced IFN-β. Because the effects of both M-CSF and IL-4 on ingestion required a prolonged period of incubation and were dependent on cell density, we hypothesized that they might be indirect.

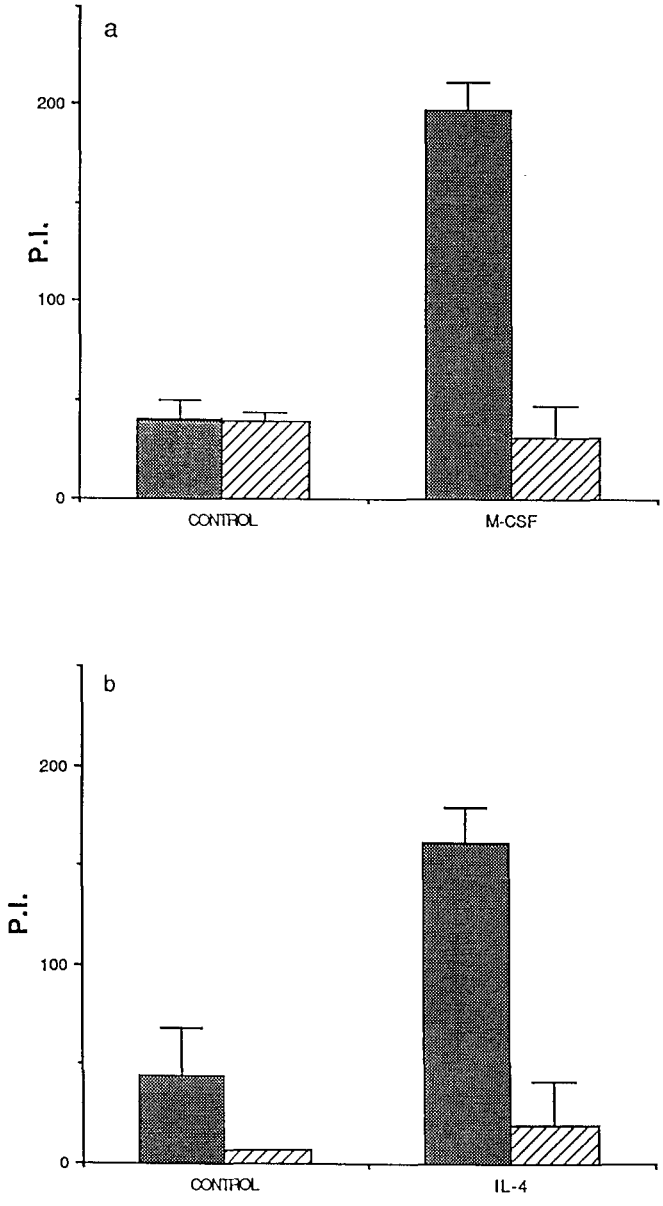

Figure 6. Stimulation of phagocytosis is dependent upon high cell density. Resident peritoneal macrophages were plated at a density of 4×10^6 cells/ml (*solid bars*) or 4×10^5 cells/ml (*hatched bars*) and incubated for 2 days in either 30% LCM (*a*) or 300 U/ml IL-4 (*b*) before being assayed for C3b phagocytosis. One experiment representative of seven experiments is shown.

For example, these two phagocytosis-stimulating cytokines might depend on macrophage synthesis and secretion of another mediator. IFN-α/β has been shown to enhance phagocytosis by peritoneal macrophages in vitro, and IFN-α/β is produced by bone marrow macrophages in response to M-CSF (11, 12). Therefore, we tested the role of IFN α/β in the stimulation of C3b-mediated phagocytosis by M-CSF and IL-4.

Addition of a polyclonal anti-IFN-α/β antibody (200 inhibitory units) inhibited the increase in C3b-mediated phagocytosis in both IL-4- and rM-CSF-stimulated cultures (Fig. 7). Because this antibody might have multiple specificities, we used mAb to IFN-α and IFN-β in similar assays. Anti-IFN-α at a final dilution of 1/50 (sufficient to neutralize 100 U IFN-α) had no effect on C3b-mediated ingestion stimulated by IL-4 or M-CSF. On the other hand, anti-IFN-β, at a dilution of 1/40,000 (sufficient to neutralize 100 U IFN-β) decreased M-CSF-enhanced ingestion by 80%, and decreased IL-4-enhanced ingestion by 59% (Table I). Because polyclonal anti-IFN-α/β might contain anti-IL6, we tested a neutralizing antibody to murine IL-6 under the same conditions. In two experiments monoclonal anti-IL-6 at 3.9 μg/ml (sufficient to neutralize 20 ng of IL-6) inhibited M-CSF-enhanced C3b phagocytosis by only 18%, and had no effect on IL-4-stimulated phagocytosis. Thus, we concluded that IFN-β, at least, was necessary for both M-CSF- and IL-4-stimulated C3b-mediated ingestion. IL-6 did not appear to play a role. Although anti-INF-α had no effect, its inhibitory titer was significantly lower than the other two antibodies, leaving open the possibility of an additional role for IFN-α. Interestingly, neither rIFN-α nor rIFN-β added to peritoneal macrophages at concentrations of 25 to 650 U/ml could substitute for IL-4 or M-CSF in stimulation of C3b-mediated ingestion. At no concentration of either IFN was C-mediated ingestion above base line. These data show that IFN-β is necessary but not sufficient to stimulate efficient C receptor phagocytosis.

These results predicted that M-CSF and IL-4 should also enhance ingestion of EIgG, because this is a known effect of IFN-α/β (33, 34). This was indeed the case (Fig. 8).

M-CSF induction of C3bi-mediated phagocytosis is not caused by receptor clustering. Detmers et al. have hypothesized that phorbol ester-stimulated C3bi-mediated phagocytosis in human neutrophils correlates with ligand-independent clustering of C3bi receptors on the surface of the cells (35). We examined the effect of LCM on clustering of surface CR3 in peritoneal macrophages by using quick-freeze, freeze-dry electron microscopy. M1/70, a mAb specific for CR3, was allowed to bind to the surface of either control of LCM-treated macrophages. A second antibody (goat anti-rat IgG) conjugated to 15 nm gold particles was then added to allow viewing of receptor distribution. Figure 9 shows that culturing in LCM did little to change the distribution of gold particles bound to the cell surface in this assay. This suggests that the stimulation of C3bi-mediated phagocytosis by LCM is not caused by ligand-independent clustering of surface receptors.

DISCUSSION

The discovery that phagocyte C receptors, not normally capable of ingestion, could be activated to mediate phago-

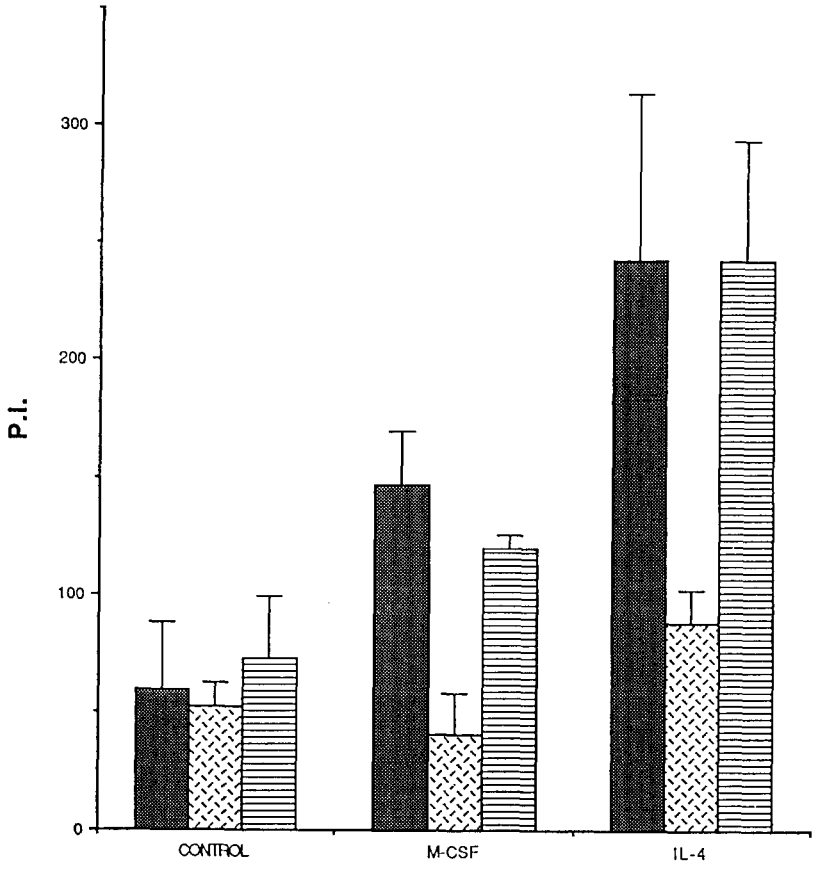

Figure 7. Inhibition of M-CSF and IL-4 stimulation of phagocytosis with anti-IFN-α/β polyclonal antibodies. A total of 200 neutralizing units of an anti-IFN-α/β polyclonal antibody was added to fresh cultures of resident peritoneal macrophages along with 100 U/ml of either human rM-CSF or rIL-4. These cultures were then assayed for C3b phagocytosis after 2 days. *Solid bars*, no antibody; *stippled bars*, anti-IFN-α/β polyclonal (200 neutralizing units/ml (206 μg/ml)); *striped bars*, control rabbit IgG (200 μg/ml). A representative experiment of four experiments is shown.

TABLE I

Inhibition of C3b-mediated phagocytosis by anti-IFN mAb

Stimulus	Phagocytic Index[a]		
	Antibody		
	Control[b]	IFN-α	IFN-β
0	22	27	13
M-CSF	148	180	46
IL-4	172	157	8

[a] One experiment, performed in triplicate, representative of two separate experiments. Phagocytic index determined as described in *Materials and Methods*.

[b] Macrophages incubated with irrelevant mouse mAb.

cytosis by inflammatory signals in vivo and in vitro (1–3) brought into focus an important problem in the cell biology of inflammation. What were the signals that could regulate C receptor phagocytosis in vivo, and what were the molecular interactions required for the dramatic alteration in function of these receptors on phagocytic cells? Griffin and Griffin described a lymphokine activity that could effect this transformation in vitro (2, 3), but it has never been characterized sufficiently to determine its in vivo relevance. Well characterized cytokines have not been systematically tested for their effect on C receptor-mediated ingestion. For human phagocytes, several pharmacologic and potentially physiologic activators of C receptor phagocytosis have been described. From these studies, the hypothesis has been advanced that ligand-independent receptor aggregation may be necessary for C mediated ingestion (35). Moreover, the process of macropinocytosis has recently been described in macrophages (15). Macropinocytosis is similar in several ways to C-mediated phagocytosis. First, both processes are induced by exposure of macrophages to phorbol esters or M-CSF; second, both require an intact cytoskeleton. Thus, it is possible to hypothesize that C-mediated ingestion is simply a special form of macropinocytosis.

In this work we undertook a study to characterize the effects of well studied purified and recombinant cytokines on C receptor-mediated ingestion. With the use of these activators we have studied the mechanism of C receptor activation, and have examined the necessity for receptor aggregation and the relationship of C-mediated phagocytosis to macropinocytosis. We have shown that two different cytokines, M-CSF and IL-4, are able to activate resident peritoneal macrophages to ingest C-opsonized targets. These agents also induce enhanced phagocytosis of IgG opsonized targets, so their effect seems to be on the ingestive mechanism, rather than on a specific receptor. In this they are different from the lymphokine described by Griffin and Griffin (2, 3), which activates C-mediated phagocytosis, but has no effect on IgG-mediated ingestion. M-CSF and IL-4 can also be distinguished from Griffin's activity by the kinetics of activation of ingestion: both M-CSF and IL-4 required at least 8 h in culture to have any effect on ingestion, with maximal stimulation requiring more than 30 h, whereas the activity of Griffin's lymphokine was discernible within minutes of exposure to resident peritoneal macrophages (3).

The effects of M-CSF and IL-4 on ingestion were not reproduced by IL-2, IFN-γ, IL-1, or TNF-α. It is not clear whether the two activating ligands act through similar receptor signaling pathways. The M-CSF receptor, *c-fms*, is a protein tyrosine kinase, and it is therefore intriguing to speculate that tyrosine phosphorylation is a necessary element in the induction of C-mediated ingestion. Phag-

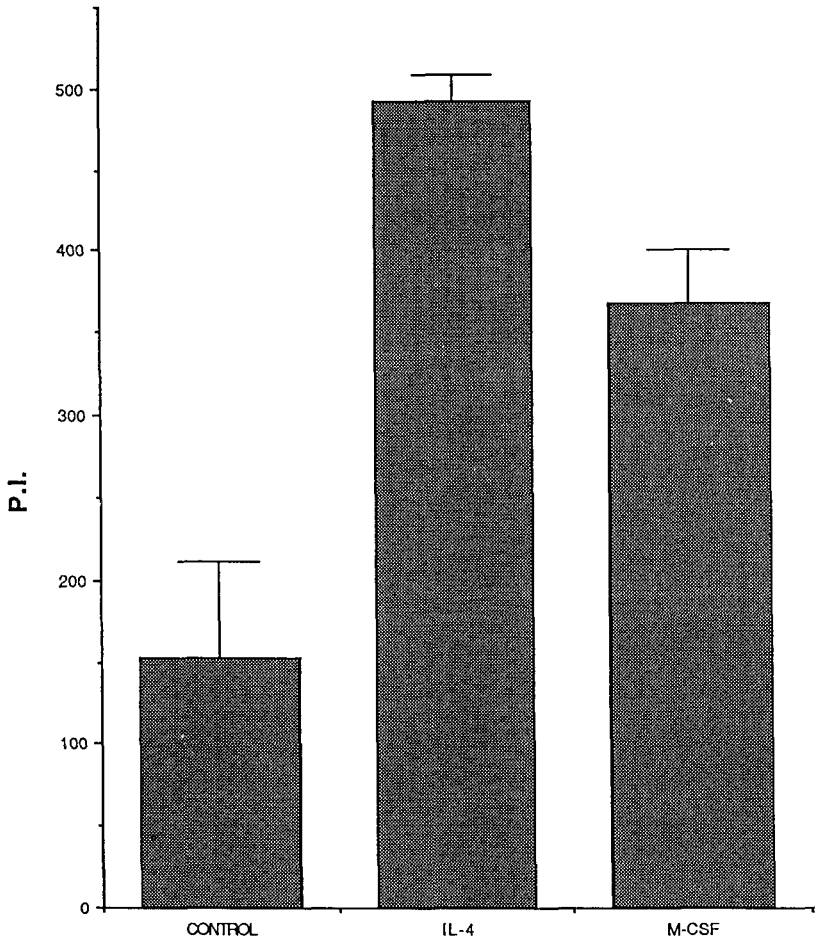

Figure 8. Enhancement of IgG phagocytosis by M-CSF and rIL-4. A total of 100 U/ml of rIL-4 or human rM-CSF were added to fresh cultures of resident peritoneal macrophages. After 2 days, these cultures were assayed for phagocytosis with EIgG. Shown is a representative experiment of six experiments.

ocytic cells are relatively abundant sources of tyrosine kinases, even though they do not proliferate much, if at all. This would suggest that tyrosine kinase activity in these cells has a function other than induction of mitogenesis, the activity with which tyrosine kinases have been most closely associated. It is a very appealing hypothesis that phagocyte tyrosine kinases regulate cell activation for phagocytosis or other inflammatory functions. However, there is no known connection between the IL-4R and tyrosine kinase activity. Nonetheless, IL-4 can act as a growth factor for some cells, and it is possible that there is some as yet undiscovered pathway for activation of tyrosine kinase activity by IL-4.

Both M-CSF and IL-4 would only enhance ingestion if macrophages were cultured at high density. Moreover, both required long incubations to enhance ingestion, were not required during the ingestion step, and could be removed as much as 16 h earlier without affecting the enhancement of ingestion. These data led us to hypothesize that neither cytokine acted directly to increase C-mediated ingestion, but required synthesis and secretion of a more proximate mediator. This turned out to be IFN-α/β from macrophages. IFN-α/β has been shown to stimulate IgG-dependent ingestion by macrophages (33, 34). We are not certain that M-CSF and IL-4 caused the synthesis of IFN-β in this system, although such an effect for M-CSF has been demonstrated in the past (11, 12). In our studies, IFN-β was required for activation of both CR1- and CR3-dependent phagocytosis, as assessed by mAb inhibition. However, IFN-β itself could not stimulate C receptor phagocytosis, suggesting that other, as yet unidentified, signals may contribute to the process. These may come from IL-4 or M-CSF directly, or may depend on other autocrine factors stimulated by the binding of these cytokines.

With the use of quick-freeze, freeze-dry electron microscopy we studied C receptor distribution on unstimulated and M-CSF-stimulated cells. We examined distribution of CR3, the C3bi receptor, because CR1, the C3b receptor of murine macrophages, is not yet well described. By using this technique, we could find no difference in density or distribution of the gold particles as a function of LCM treatment. Thus, for cytokine activation of C receptor ingestion, there is no apparent alteration in receptor distribution, which suggests that ligand-independent receptor redistribution is not a prerequisite for C-mediated internalization. However, it is possible that the more acute alterations in receptor function induced by phorbol esters may relate to ligand-independent changes in the location of CR3.

Finally, we have tested the relationship of macropinocytosis and of receptor aggregation to phagocytic activation of the C receptors. Macropinocytosis could be distinguished from activation of ingestion by kinetics: the former occurs within minutes of exposure to M-CSF (15), whereas the latter requires more than 24 h of incubation. Thus, the phenomena of macropinocytosis and C receptor-mediated phagocytosis are apparently unrelated. There is no information available as to whether IL-4 might induce macropinocytosis. However, it does not induce vacuolization of cells nearly to the extent of M-CSF, so we believe that it probably does not activate macropi-

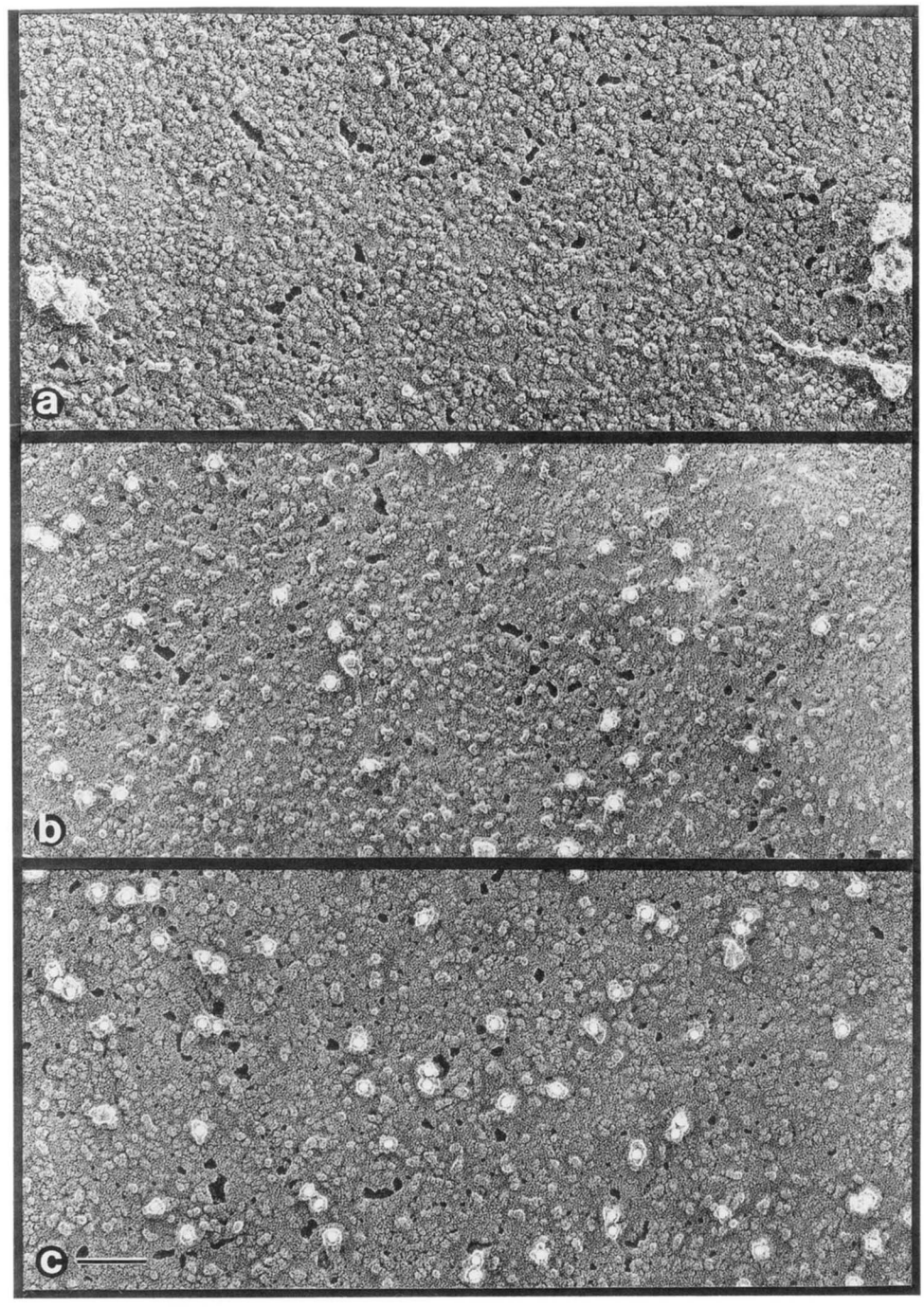

Figure 9. LCM treatment does not cause ligand-independent receptor clustering. Cultures of resident peritoneal macrophages incubated for 2 days in DME with or without 30% LCM were fixed with formaldehyde and treated with M1/70 (anti-CR3) or a control rat IgG. The cultures were then treated with 15-nm gold particles conjugated to goat-anti-rat IgG, washed, and prepared for quick-freeze, freeze-dry microscopy. *a*, Control antibody, LCM; *b*, M1/70, no LCM; *c*, M1/70, LCM. *Bar* equals 0.1 μm. Shown are pictures representative of the results of three separate experiments.

nocytosis.

In summary, these studies have determined the ability of several recombinant cytokines to activate complement-mediated phagocytosis. Of the cytokines tested, only M-CSF and IL-4 had this effect. Interestingly, both required synthesis of IFN-β. It is possible that this is the mechanism via which in vivo inflammatory agents such as thioglycollate activate peritoneal macrophage C receptors. Systemic administration of IFN inducers is known to activate resident macrophages (36).

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