Macrophage Colony-Stimulating Factor Induces Substantial Osteoclast Generation and Bone Resorption in Human Bone Marrow Cultures

By U. Sarma and A.M. Flanagan

Macrophage colony-stimulating factor (M-CSF) is essential for murine osteoclast formation and its role in human hematopoiesis in vitro is not fully defined. Therefore, we have investigated the effect of M-CSF on the formation of human osteoclasts in vitro. M-CSF was found to induce substantial bone resorption and osteoclast formation in a dose-responsive and time-dependent manner above that induced by 1,25 dihydroxyvitamin D₃ (1,25 vitamin D₃) in cultures of human bone marrow (BM) stromal cells sedimented onto devitalized bone. By day 14 there was a mean of approximately 50% of the surfaces of the bone slices resorbed compared with only 6% in cultures treated with 1,25 vitamin D₃ alone. Osteoclasts were identified as 23c6⁺ cells (an antibody that recognizes the vitronectin receptor), 87.5% of which coexisted with the calcitonin receptor. The number of 23c6⁺ cells correlated strongly with bone resorption spatially, and in a dose-responsive and time-dependent manner; the correlation coefficient in cultures treated with 1,25 vitamin D₃ alone was 0.856 and those treated with both M-CSF and 1,25 vitamin D₃ was 0.880. Granulocyte-macrophage colony-stimulating factor, IL-1β, IL-3, IL-6, tumor necrosis factor-α, transforming growth factor-β, leukemia inhibitory factor, and IL-11 did not increase bone resorption above that in 1,25 vitamin D₃–treated cultures. We also found that 1,25 vitamin D₃ increased, to a minor but significant degree, both bone resorption and the concentration of M-CSF in the culture supernatants above that in vehicle-treated cultures, indicating that M-CSF is present in our BM cultures, but that there is insufficient to induce substantial osteoclast formation. These results define a critical role for M-CSF in the formation of human osteoclasts.

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MATERIALS AND METHODS

Human BM was aspirated under 1% lignocaine local anesthesia from the posterior iliac crest of healthy volunteers between the ages of 30 and 75 years (approved by St Mary’s Hospital medical ethics committee) into preservative-free heparin (CP Pharmaceuticals Ltd, Wrexham, UK). Marrow mononuclear cells were isolated by centrifugation over Ficol–Hypaque density gradients (Sigma Chemical Co, Poole, Dorset, UK) and resuspended in phenol red-free RPMI medium (Sigma) supplemented with 10% heat-inactivated newborn calf serum (Harlan Sera Lab, Ltd., Sussex, UK), L-glutamine, 100 IU benzylpenicillin per/mL (GIBCO Life Technologies, Paisley, UK), 1% benzylpenicillin per/mL (GIBCO Life Technologies, Paisley, UK), and penicillin G 100 IU/mL (GIBCO Life Technologies, Paisley, UK).

The absence of a well-defined and specific role for M-CSF in the formation of human hematopoietic cells and the knowledge that this cytokine is essential for osteoclast formation in mice suggests that M-CSF may be the limiting factor in the generation of osteoclasts in human BM cultures. It is also interesting that M-CSF has been reported to increase osteoclast formation in cocultures of mouse embryonic metatarsals and human cord blood. Therefore, we elected to test this hypothesis by assessing the formation of osteoclasts in vitro by the presence of 23c6⁺ cells and bone resorption (unequivocal evidence that osteoclasts have been generated).

THE ROLE OF macrophage colony-stimulating factor (M-CSF) in human hematopoiesis is not fully explained. Although originally described as a monocyte/macrophage-restricted growth factor it was found to have limited effect on human macrophage colony formation in vitro. In addition, the transcription factor, Egr-1, which has been reported as being essential for macrophage differentiation and for restricting differentiation along the macrophage lineage, is not expressed in macrophages stimulated by M-CSF. On the contrary, M-CSF induces the formation of murine macrophage colonies in a dose-responsive manner, and has more recently been shown to be essential for osteoclast formation in mice. Identification of a mutation within the coding region of the M-CSF gene in the osteopetrotic op/op mouse, together with experiments which showed that op/op mice could be cured by administration of recombinant human (rh) M-CSF demonstrated that this cytokine is essential for osteoclast formation. The osteoclast is a cell that resorbs bone and derives from a hematopoietic precursor, and although its relationship with other hematopoietic cell types is not fully elucidated there is evidence that it is closely related to the macrophage/monocyte.

Our experience and that of others is that the generation of human osteoclasts in vitro, as assessed by bone resorption, is difficult to achieve. In previous studies only a small number of osteoclasts have been generated from human bone marrow (BM) in vitro, as assessed by bone resorption and this may be a reflection of the low number of osteoclasts present in normal adult humans. Nevertheless large numbers are found in particular clinical conditions and we speculated that it should be possible to increase osteoclastogenesis in vitro if the correct osteoclast-inductive factor were added to the cultures. More recently we have improved our culture system such that osteoclastic excavations are formed reproducibly, although in small numbers. Optimization of our culture conditions for bone resorption probably explains why we have recently succeeded in generating bone-resorbing cells in human BM cultures, whereas previously we were unable to do so. This is consistent with evidence that hematopoiesis in vitro is dependent on culture conditions including serum batch, cell density, and the presence of hydrocortisone.

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Fig 1. Bone resorption (B.R.) and numbers of 23c6+ cells in human BM cultures in response to increasing concentrations of M-CSF together with 1,25 vitamin D3 compared to 1,25 vitamin D3 alone, 14 days after the cells were sedimented onto bone slices. The data were derived from four experiments each containing five or six bone slices. The BM in these experiments was from two 25-year-old men, a 30-year-old man, a 20-year-old woman, a 30-year-old man, and a 56-year-old woman. The area of bone resorption and the number of 23c6+ cells in the M-CSF cultures were compared with these parameters respectively in cultures without M-CSF. *P < .05; **P < .001.

100 µg streptomycin/mL (GIBCO) and 10-8 mol/L hydrocortisone (Sigma) and incubated in tissue culture flasks (Corning Glass Works, Corning, NY) at 37°C in a humidified atmosphere of 5% CO2/95% air. The BM cells were fed weekly by replacing half the medium. After 10 to 14 days when a heterogeneous population of cells including fibroblasts, adipocytes, macrophages, and other hematopoietic cells had formed a confluent layer, the cells were recovered from the flasks by treatment with trypsin/EDTA solution (Sigma), washed in phosphate-buffered saline (PBS) (Sigma), and resuspended in phenol red-free RPMI medium containing the supplements stated above. The recovered cells were counted using a hemocytometer; if there were insufficient cells obtained from a volunteer for an experiment, the cells were mixed with those from another donor. The cells were sedimented onto devitalized bone slices (3 x 3 x 0.1 mm) in a 96-well plate (Corning Glass Works) at 10⁴ cells/well. The cells on the bone slices were cultured in the presence of 1,25 vitamin D3 (kindly provided by Dr M.R. Uskokovic, Hoffmann-La Roche Inc, Nutley, NJ), vitamin D3 (kindly provided by Dr M.R. Uskokovic, Hoffmann-La Roche Inc, Nutley, NJ), and in rhM-CSF (Genetics Institute [GI], Boston, MA) over a range of concentrations (0.05 to 500 ng/mL) in combination with 1,25 vitamin D3, for 14 days. Time-course experiments were also performed by culturing the cells on bone slices in 1,25 vitamin D3 alone and in M-CSF at 50 ng/mL together with 1,25 vitamin D3; bone slices were removed from cultures between days 3 and 21. In other experiments, cells were cultured on bone slices in the presence of 1,25 vitamin D3 alone, in M-CSF (50 ng/mL) with 1,25 vitamin D3, sometimes in M-CSF alone and in other cytokines including rh granulocyte-macrophage CSF (GM-CSF) (a gift from Immunex, Seattle, WA), interleukin-
Fig 3. The effect of M-CSF (50 ng/mL) in the presence and absence of 1,25 vitamin D$_3$ (D$_3$) and other cytokines in the presence of D$_3$ on bone resorption and 23c$^+$ cell numbers in human BM cultures. The BM was obtained from individuals of different ages and sex; GM-CSF experiment, 80-year-old woman; IL-1$\beta$, 65-year-old man; IL-3 and IL-6, 30-year-old woman; IL-11, LF, 30-year-old woman; TNF-$\alpha$, 25-year-old man; TGF-$\beta$, from two 30-year-old women and a 60-year-old man. The area of bone resorption and the number of 23c$^+$ cells in D$_3$-treated cultures were compared with all other treatments in each experiment after 14 days. *$P<.05$ versus D$_3$-treated cultures, **$P<.001$ versus D$_3$-treated cultures.

23c$^+$ cells; (i), bone resorption.

1$\beta$ (rhIL-1$\beta$, purchased from NIBSC, Herts, UK), rhIL-3 (GI), rhIL-6 (Sandoz, UK), rhIL-11 (GI), rh leukemic inhibitory factor (LIF) (R & D Systems, Abingdon, Oxon, UK), rh tumor necrosis factor-$\alpha$ (TNF-$\alpha$) (R & D Systems), and rh transforming growth factor-$\beta$ (TGF-$\beta$) (NIBSC) over a range of concentrations from 0.05 ng/mL to 500 ng/mL together with 1,25 vitamin D$_3$ for 14 days. Bone resorption and the number of 23c$^+$ cells were studied in these cultures.

M-CSF was measured in supernatants from control and 1,25 vitamin D$_3$-treated cultures at the end of the experiments using a commercially available ELISA kit (R & D Systems). The supernatants from the wells were centrifuged at 1,500 rpm and then filtered through a 0.2-$\mu$m filter (Millipore Products, Bedford, MA). The medium was stored at $-20^\circ$C and the assay was carried out according to the manufacturer’s instructions. Bone resorption was assessed in these cultures.

The cells on the bone slices were fed by replacing half the medium every 3 to 4 days. The bone slices were fixed in acetone (Analar, Hayman Ltd, Essex, UK) and air dried. Immunohistochemistry, using monoclonal antibody (MoAb) 23c$^+$ (kindly provided by Prof T.J. Chambers, London, UK), which preferentially stains osteoclasts, was performed using conventional techniques. The number of 23c$^+$ cells were counted by light microscopy on 30% of the surface of each of the bone slices and the numbers of mononucleate, binucleate, and multinucleate (>2 nuclei per cell) cells were also determined. The total surface of each bone slice was inspected and the area of resorption was quantified by reflected light microscopy.

Salmon calcitonin (CT) (kindly provided by Rhone-Poulenc Rorer, PA) was iodinated using a modification of the chloramine T method. BM cultures were incubated in radiiodinated ($^{125}$I) CT 9 days after the cells were plated on bone slices, with or without excess unlabeled CT, washed, fixed, stained with antibody 23c$^+$, and processed for autoradiography as previously described. After development, the cultures were counter-stained with toluidine blue.

Six- to eight-week-old MF1 mice were killed by cervical dislocation. The long bones were removed, cleaned of soft tissue, and the
ends of the bones were cut so that the marrow could be flushed from the bones with PBS using a 25-gauge needle. The cells were resuspended in Minimal Essential Medium (MEM) (Sigma) with the same supplements used in the human BM cultures. Cells, $8 \times 10^6$, were sedimented onto bone slices in a 96-well plate and after 7 days 1,25 vitamin $D_3$ ($10^{-4}$ mol/L) with and without M-CSF (50 ng/mL) were added to the cultures. The cells were fed every 3 to 4 days and the experiments were terminated 14 days after the 1.25 vitamin $D_3$ and M-CSF were added to the cultures. The experiments were terminated by submerging the bone slices in sodium hypochlorite to remove the cells. Bone resorption was quantitated using reflected light microscopy. The results were analyzed using the Student’s $t$-test.

**RESULTS**

M-CSF substantially increased bone resorption and the formation of 23c6$^+$ cells above that induced by 1,25 vitamin $D_3$ alone; the effect of M-CSF was dose- and time-dependent (Figs 1 and 2). M-CSF at 50 ng/mL alone was equally effective as M-CSF at 50 ng/mL in combination with 1,25 vitamin $D_3$ at increasing bone resorption in human BM cultures (Fig 3). The bone-resorptive effect of M-CSF was found in human BM cultures irrespective of the age and sex of the volunteers

Fig 4. Strong immuno-alkaline phosphatase staining, using MoAb 23c6, of two human BM cells 14 days after the addition of 1.25 vitamin $D_3$. The 23c6$^+$ cells are large with one and two nuclei, have a dendritic appearance, and partly cover osteoclastic excavations (arrows) (A). A low power magnification of a bone slice covered by human BM cells, which has been cultured in the presence of M-CSF 50 ng/mL and 1,25 vitamin $D_3$; it shows large numbers of 23c6$^+$ cells that are closely associated with areas of bone resorption (B). The areas without osteoclastic excavations are devoid of 23c6$^+$ cells.

Fig 5. Autoradiograph of human BM cells on a bone slice incubated with M-CSF (50 ng/mL) for 9 days, before addition of $^{125}$I-CT. The autoradiograph grains clearly label the strongly stained cells (immunoalkaline phosphatase labeled with 23c6). Bone resorption is not evident in this photomicrograph because the bone slice was only weakly stained with toluidine blue to emphasize the double-labeled cells (A). A high-power magnification of three cells labeled with both 23c6 and $^{125}$I-CT, two of which lie on resorption pits (arrow) (B).
from whom the BM was obtained, nor was there any appreciable difference in the behavior of the cultures if osteoclasts were generated from a single donor or from a mixture of cells derived from two or more volunteers (Figs 1 through 3).

Osteoclasts on bone slices were identified as cells which showed strong staining, largely of the membrane, with MoAb 23c6; these large cells had a dendritic appearance with an expanded central area from which protruded slender elongated cytoplasmic extensions (Fig 4A and B). The number of nuclei was low, usually 1 or 2 and rarely greater than 3. The brightly red-stained cells stood out against the clean background of toluidine blue-stained predominantly spindleshaped cells and were spatially related to resorption sites (Fig 4). Three days after the cells were plated on bone slices all of the 23c6+ cells were mononucleate whereas 11 days later, cells with two or three nuclei and occasionally more were found in the presence of M-CSF (Table 1).

The osteoclastic phenotype of these 23c6+ cells was confirmed in two further experiments, in which there was 27.1% ± 4.1% (n = 12) of the surfaces of the bone slices resorbed, by showing that 87.5% ± 4% of the 23c6+ cells were also CT receptor+ when inspected on day 9 (Fig 5). CT receptor+ cells were always 23c6+. The addition of excess cold CT prevented CT labeling of the 23c6+ cells (not shown). Occasional polykaryons which were not labeled with 23c6 or CT were also identified in these cultures.

A part from a few very small pits, bone resorption was virtually absent 3 days after the addition of 1,25 vitamin D3 in the absence or presence of M-CSF, although even at this time there was significantly greater bone resorption in the latter: 18.60 ± 3.05/cm2 versus 31.8 ± 7.48/cm2 mean ± SEM (data derived from 18 bone slices in the time course experiments Fig 2A through C). The rate of increase in the number of 23c6+ cells accelerated just before the dramatic increase in the rate of bone resorption in both culture treatments. However, the increase in the number of 23c6+ cells in the cultures treated with both M-CSF and 1,25 vitamin D3 occurred earlier and more rapidly compared to the cultures treated with 1,25 vitamin D3 alone. The numbers of 23c6+ cells declined when the rate of bone resorption was decreasing (Fig 2A through C); this occurred at the same time in both culture treatments. This suggests that M-CSF increases the life span of 23c6+ cells.

To assess the relationship between 23c6+ cells and area of bone resorption we pooled the experimental data from eight experiments on day 14 and plotted a scatter diagram; the results from the cultures treated with 1,25 vitamin D3 alone and those treated with both M-CSF and 1,25 vitamin D3 were studied separately: the correlation coefficient (r) for the former was .856; the gradient, 0.008, and the latter was .880; the gradient, 0.003 (Fig 7). This indicates that there is a strong correlation between bone resorption and the number of 23c6+ cells in both culture treatments: this is consistent with the finding that cultures in which there was very little or no bone resorption also had very few, or no, 23c6+ cells, respectively (see Fig 3).

To extend the assessment of the correlation of bone resorption and 23c6+ cells we analyzed these parameters with respect to one another over a 7-day period. Figure 2A shows that a mean of 4,800 23c6+ cells/cm2 were formed in the presence of M-CSF between days 3 and days 10; during this time, bone was resorbed at a rate of 12% of the bone surfaces per day (12 × 10^6 μm2/d). Likewise, Fig 2B shows that a mean of approximately 3,300 23c6+ cells/cm2 were present during the same period in M-CSF whereas approximately 4% of the bone surfaces were resorbed per day. Similar calculations were made with the data from Fig 2C and the respective cultures treated with 1,25 vitamin D3 alone. The results show that approximately 1 to 3 × 10^6 μm2 was resorbed per 23c6+ cell per day in both culture treatments.

Table 1. Number of 23c6+ Mononucleate, Binucleate, and Multinucleate Cells in Human BM Cultures 14 Days After the Addition of 1,25 Vitamin D3 and M-CSF

<table>
<thead>
<tr>
<th>Experiment Type</th>
<th>Percentage of 23c6+ Mononucleate Cells</th>
<th>Percentage of 23c6+ Binucleate Cells</th>
<th>Percentage of 23c6+ Cells With 3 or More Nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-3/IL-6</td>
<td>76 ± 0.9</td>
<td>23.2 ± 0.9</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>IL-3/IL-6</td>
<td>51 ± 2</td>
<td>43 ± 1</td>
<td>6.6 ± 0.8</td>
</tr>
<tr>
<td>IL-3/IL-6</td>
<td>72 ± 1.1</td>
<td>25.2 ± 1</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>IL-1</td>
<td>83.2 ± 0.9</td>
<td>23.2 ± 1.2</td>
<td>0.3 ± 0.08</td>
</tr>
<tr>
<td>IL-1</td>
<td>77.6 ± 1.8</td>
<td>22.6 ± 1.7</td>
<td>0.3 ± 0.08</td>
</tr>
<tr>
<td>IL-1</td>
<td>75 ± 1.1</td>
<td>23.2 ± 0.9</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>Time-course</td>
<td>92 ± 3</td>
<td>8 ± 2.9</td>
<td>0</td>
</tr>
<tr>
<td>Time-course</td>
<td>89.2 ± 0.6</td>
<td>9.6 ± 0.6</td>
<td>0</td>
</tr>
<tr>
<td>Time-course</td>
<td>87.6 ± 0.5</td>
<td>12 ± 0.5</td>
<td>0</td>
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</tbody>
</table>

Data are derived from 50 23c6+ cells per bone slice, mean ± SEM, n = 6. M-CSF was used at 50 ng/mL. vit D3 = 1,25 vitamin D3.
The results were similar when calculations were made with data from days 7 to 14. This suggests that M-CSF in combination with 1,25 vitamin D$_3$ does not increase the bone resorptive activity per osteoclast compared with that of 1,25 vitamin D$_3$ alone and supports the data which show that M-CSF increases the life span of 23c6$^+$ cells in culture.

To determine whether the dramatic results obtained in these cultures were specific for M-CSF we looked at several other cytokines, over a wide range of concentrations, which have been cited as osteoclast-inductive factors. All of these cytokines failed to increase bone resorption above that induced by 1,25 vitamin D$_3$ alone and IL-1, IL-6, GM-CSF, and TGF-β at high concentrations were found to inhibit bone resorption (Fig 3). However, LIF and IL-11 increased the number of 23c6$^+$ cells above that found in the 1,25 vitamin D$_3$–treated cultures.

1,25 vitamin D$_3$ caused a small but significant increase in bone resorption compared with vehicle-treated cultures after 14 days ($10.0 \pm 0.8 \mu m^2 \times 10^3$ 1,25 vitamin D$_3$ v $5.0 \pm 1.0 \mu m^2 \times 10^3$ vehicle, mean ± SEM; $P < .05$; n = 12 from two experiments). In the same experiments 1,25 vitamin D$_3$ also significantly increased the concentration of M-CSF in the culture supernatants compared with vehicle-treated cultures ($1.80 \pm 0.05$ ng/mL 1,25 vitamin D$_3$ v $1.02 \pm 0.10$ ng/mL vehicle; $P < .05$).

Unlike in human BM cultures, M-CSF was found to significantly reduce bone resorption in long-term murine BM cultures ($9.82\% \pm 2.01\%$ surface of bone slices resorbed v
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The occurrence of mononucleate 23c6+ cells with human BM in which bone resorption and the number of 23c6+ cells were measured 14 days after the addition of 1.25 vitamin D₃ alone or 1.25 vitamin D₃ in combination with M-CSF 50 ng/mL.

DISCUSSION

The present data define a clear role for M-CSF in the formation of bone-resorbing osteoclasts. Our finding is consistent with M-CSF being essential for osteoclast formation in mice. However, until the work on the op/op mutant was published all the experimental data indicated that this growth factor inhibited bone resorption and formation of murine osteoclasts in vitro, a finding that has never been explained. One view is that osteoclasts and macrophages derive from a common precursor and that M-CSF directs the formation of macrophages in favor of osteoclasts. This suggestion appears to be inconsistent and difficult to reconcile with our results. Further experiments are needed to study the relationship between these cells.

The low numbers of 23c6+ cells and virtual absence of bone resorption 3 days after the addition of 1.25 vitamin D₃ alone or M-CSF with 1.25 vitamin D₃ to the BM cultures and the subsequent dramatic increase in these parameters suggests that functionally active osteoclasts have been generated from BM cells ex vivo. However, the rapid development of 23c6+ cells and bone resorption after the addition of the osteoclast-inductive factor suggest that osteoclast precursors are already present in the cells that were sedimented onto bone slices. The occurrence of mononucleate 23c6+ cells before the formation of the multinucleate forms is consistent with the knowledge that multinucleate osteoclasts are formed by fusion of mononucleate precursors. In addition, the expression of the α₁β₁ subunit of the vitronectin receptor, identified by the MoAb 23c6 before bone resorption is consistent with data which show that this integrin is necessary for osteoclast adhesion and bone resorption.

The 23c6+ and CT receptor+ cells in these cultures have a striking appearance; their large size, dendritic morphology, and the small numbers of nuclei bear remarkable similarity to the 23c6+ cells in rabbit BM cultures in which bone resorption occurred. This finding suggests that this characteristic morphology is not dependent on these cells being formed on bone matrix as the immunohistochemistry on the rabbit BM experiments was performed on plastic.

Increased life span of the 23c6+ cells in the presence of M-CSF in combination with 1.25 vitamin D₃ compared with that in 1.25 vitamin D₃ alone probably accounts for the different gradients in the scatter diagrams, in which bone resorption and the number of 23c6+ cells were correlated. The alternative is that M-CSF in the presence of 1.25 vitamin D₃ increases the resorptive activity of osteoclasts compared with 1.25 vitamin D₃ alone. However, we could find no evidence for increased osteoclast activity to account for this; our calculations showed that the area of bone resorbed per 23c6+ cell per day (between days 3 and 10 and days 7 and 14) was similar in cultures treated with both M-CSF with 1.25 vitamin D₃ compared with 1.25 vitamin D₃ alone in three separate time-course experiments. The finding that M-CSF in combination with 1.25 vitamin D₃ increases the life span of 23c6+ cells is entirely consistent with the finding that M-CSF increases survival of murine osteoclasts. However, the ability of M-CSF to increase osteoclast activity would be better tested by adding M-CSF to isolated mature osteoclasts because it may not be possible to elucidate an effect on resorptive activity in this long-term culture system.

The resorptive activity of osteoclasts, identified as 23c6+ cells, calculated from our experiments is approximately 1 to 3 × 10⁷ μm²/cell/day and is similar to the resorptive activity of isolated fetal osteoclasts. The bone resorptive activity in analogous murine cultures is found to be 1 to 2 × 10⁶ μm²/osteoclast/day, identified as CT receptor+ cells on plastic. This 10-fold difference may be largely explained by the enhanced osteoclast-inductive effect that occurs when BM cells are cultured on bone compared with plastic; further studies are needed to confirm this.

The finding that 1.25 vitamin D₃ increased both bone resorption and M-CSF to a minor but significant degree compared with control cultures supports the osteoclast-inductive effect of M-CSF. It also shows that endogenous M-CSF is present in our 1.25 vitamin D₃−treated cultures but fails to be induced sufficiently to bring about substantial osteoclast formation and bone resorption. Furthermore, M-CSF was specifically required to induce substantial bone resorption consistently above that seen in the presence of 1.25 vitamin D₃ alone, as shown by the absence of effect by other cytokines. These cytokines were compared with M-CSF because of their reported actions on osteoclast formation and bone resorption in vitro and in vivo. Our data show that none of these cytokines can reproduce the bone-resorptive effect of M-CSF; this implies that these cytokines can neither induce sufficient M-CSF nor can they be induced sufficiently by M-CSF to bring about the substantial increase in bone resorption that we have demonstrated. However, it is worthy of note however that LIF and IL-11 increased 23c6+ cell numbers compared to the 1.25 vitamin D₃−treated cultures and further experiments are required to determine why this occurs in the absence of an increase in bone resorption.
These experiments were not designed to determine why high levels of some of these cytokines inhibited bone resorption; it may be that they induce nitric oxide which inhibits osteoclast formation and resorption; or, nor do they exclude the possibility that these cytokines modulate osteoclast formation and bone resorption, as these cytokines may be present endogenously in optimal concentrations in our cultures.

M-CSF and GM-CSF have been shown to increase the formation of large multinucleate cells with numerous nuclei in baboon BM cultures. These cells were labeled as 'osteoclast-like' cells on the basis of their morphology but they were neither stained with tartrate-resistant acid phosphatase (TRAP), an enzyme present in osteoclasts, nor with the MoAb 23c6 and, in particular, bone resorption was not studied. This result is noteworthy because although M-CSF cured the osteoclast deficiency in op/op mutant mice, GM-CSF failed to do so. and GM-CSF did not increase osteoclasts in our current experiments. Since that publication, there have been numerous reports of formation of large numbers of 'osteoclast-like' cells in human BM cultures, and although sometimes these included photographs of small osteoclastic excavations there has been little correlation between bone resorption and the number of these 'osteoclast-like' cells.

Several factors may account for the discrepancy between these reports and our current results. Bone resorption is permanent and unequivocal evidence of osteoclast formation whereas the expression of TRAP and the vitronectin receptor are obviously transient. Furthermore, these markers may be expressed by other cells in vitro; however, it is generally accepted that if the cells stain strongly for TRAP or the vitronectin receptor they are osteoclasts. Nevertheless, analysis of histochemistry and immunohistochemistry is subjective and interpretation is liable to differ between observers and this may account for some of the differences in opinion. We only accept that cells are positive for 23c6 if they have a strong and predominantly membrane stain (as shown in our study) while we discount cells that show a diffuse, weak, cytoplasmic h~e.' Further evidence that our 23c6' cells have 1 or 2 nuclei and that cells with 3 or more nuclei are uncommon. These data, together with reports of large numbers of 'osteoclast-like' cells in cultures in which there was virtually no bone resorption, imply that the presence of multinucleate cells is not a reliable marker for osteoclasts generated in vitro.

We have shown previously that 'osteoclast-like' multinucleate cells in cultures, in which there is very limited bone resorption, have a phenotype typical for macrophages and not osteoclasts. These findings as compared with our current experiments in which there are striking numbers of dendritic 23c6' cells that correlate with the area of resorbed bone and an absence of 'osteoclast-like' multinucleate cells, suggest that M-CSF directs a common osteoclast-macrophage precursor to form osteoclasts in favor of macrophages. The alternate view, as suggested by Roodman, is that the multinucleate cells are in fact osteoclasts, which are not fully differentiated, and are unable to resorb bone. The possibility of a common osteoclast-macrophage precursor that can be induced to form one cell type in favor of the other is supported by the experimental data which shows that the absence of c-fos results in a lineage shift from osteoclasts to macrophages. It is also interesting to point out that the transcription factor Egr-1, which is essential for and restricts differentiation along the macrophage lineage, has been reported not to be expressed in M-CSF–stimulated macrophages whereas it is expressed after exposure to IL-6. It is interesting to speculate that Egr-1 induces macrophage formation in favor of osteoclasts and that this can be regulated by M-CSF. This may be the reason why M-CSF increases and IL-6 reduces osteoclast formation and bone re-
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27. Hunter WM, Greenwood FC: Preparation of iodine 131-la- 1


34. Shinar DM, Rodan GA: Biphasic effects of transforming growth factor β on the production of osteoclast-like cells in mouse bone marrow cultures: The role of prostaglandins in the generation of these cells. Endocrinology 126:3133, 1990


41. Hattersley G, Chambers TJ: Calcitonin receptors as markers for osteoclastic differentiation: Correlation between generation of bone-resorptive cells and cells that express calcitonin receptors in mouse bone marrow cultures. Endocrinology 125:1606, 1989


Macrophage colony-stimulating factor induces substantial osteoclast generation and bone resorption in human bone marrow cultures

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