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Osteoclast formation and activity in the pathogenesis of osteoporosis in rheumatoid arthritis

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Abstract

Objective. Rheumatoid arthritis (RA) is often complicated by generalized osteopenia due to increased bone resorption by osteoclasts. We analysed a number of cellular and humoral factors that influence osteoclast formation from circulating precursors in RA patients.

Methods. Monocytes isolated from RA patients and normal controls were cultured with macrophage colony-stimulating factor (M-CSF) and nuclear factor-κB ligand (RANKL), or with RANKL-expressing UMR106 cells and 1,25 dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]. Osteoclast differentiation was assessed by expression of tartrate-resistant acid phosphatase (TRAP) and vitronectin receptors (VNR) and lacunar resorption.

Results. Osteoclasts formed from RA patients exhibited increased resorptive activity but there was no difference in the relative proportion of circulating osteoclast precursors between RA patients and normal controls. Osteoclast precursors in RA patients were not more sensitive to the osteoclastogenic effects of 1,25$(OH)_2D_3$, M-CSF or RANKL. Dexamethasone, but not interleukin (IL) 1β, tumour necrosis factor α and IL-6, increased osteoclast formation and lacunar resorption.

Conclusion. There is an increase in the extent of lacunar resorption carried out by osteoclasts formed from circulating precursors in RA patients. This is not due to an increase in the number of circulating precursors or increased sensitivity to the osteoclastogenic effects of 1,25$(OH)_2D_3$, M-CSF, RANKL or inflammatory cytokines. Our findings suggest that increased osteoclast functional activity rather than osteoclast formation is more likely to play a role in the generalized bone loss that occurs in RA, and that corticosteroids stimulate osteoclast formation and resorption.

KEY WORDS: Rheumatoid arthritis, Osteoarthritis, Osteoclast, Bone resorption.

Rheumatoid arthritis (RA) is a systemic inflammatory disorder in which there is destruction of articular cartilage and juxta-articular bone. Patients with RA are also known to have lower bone mineral density and are at risk of pathological fracture [1–5]. Although the cause of generalized bone loss in RA is not known, changes in hormones (e.g. insulin, corticosteroids), vitamin D and mineral metabolism and in circulating levels of cytokines [e.g. interleukin (IL) 1 and tumour necrosis factor α (TNF-α)] have been implicated in the pathogenesis of systemic bone loss in RA. Other important factors include the immobilization induced by the joint disease of RA itself and the fact that both RA and primary osteoporosis occur much more frequently in women than men [6, 7]. Corticosteroid treatment, which is a recognized cause of secondary osteoporosis, is also likely to contribute to systemic bone loss in RA [8, 9]. The above pathogenic factors are thought to be mediated at the cellular level by increased osteoclastic bone resorption [10].

Osteoclasts are specialized multinucleated cells (MNCs) which carry out bone resorption; they are formed from mononuclear precursors that circulate in the monocyte fraction of peripheral blood [11]. These mononuclear precursors express the monocyte/macrophage antigens CD11b and CD14 and are entirely negative for phenotypic markers of osteoclasts, including tartrate-resistant acid phosphatase (TRAP), vitronectin receptors (VNR) and calcitonin receptors, and lack the ability to carry out bone resorption. It has been shown that CD14-positive cells in the monocyte fraction, which express the receptor activator of nuclear factor κB (RANK), can differentiate *in vitro* into functional osteoclasts in the presence of cells that express macrophage colony-stimulating factor (M-CSF) and

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RANK ligand (RANKL), including osteoblasts, fibroblasts and T lymphocytes [12, 13]. Inflammatory macrophages isolated from the RA synovium are also capable of differentiating into osteoclasts in the presence of M-CSF and RANKL [14].

A role for increased osteoclastic bone resorption in the generalized osteoporosis that occurs in RA is suggested by studies which have shown that there is an increase in biochemical markers of bone resorption in RA patients with low skeletal bone mineral density measurements [10]. Whether this increase in bone resorption is due to an increase in osteoclast formation or to an increase in osteoclast bone-resorbing activity is not known. In this investigation, our aim was to analyse osteoclast formation and the functional activity of RA. We examined whether there is an increase in the relative proportion of mononuclear precursors primed for osteoclast formation in the circulating monocyte population of RA patients. We also assessed whether these precursors are more sensitive to RANKL, M-CSF and 1,25 dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], humoral factors which are known to be essential for osteoclast formation *in vitro*. As corticosteroids and cytokine factors, such as IL-1, TNF-α and IL-6, have been implicated in the pathogenesis of generalized bone loss in RA, we also assessed the effects of these factors on osteoclast formation and activity.

Methods

Reagents

All cell incubations were performed in alpha minimal essential medium (αMEM) (Gibco, Paisley, UK) [supplemented with glutamine (2 mM) and benzyl penicillin (100 IU/ml], streptomycin (10 μg/ml) and 10% heat-inactivated fetal calf serum (FCS) (Gibco) (αMEM/FCS) in a humidified atmosphere of 5% CO_2 at 37°C. Cloned, hormone-responsive, calcitonin receptor-negative, osteoblast-like UMR106 cells (from a rat osteosarcoma-derived cell line) were obtained from Professor T. J. Martin (Melbourne, Australia). $1,25(OH)_2D_3$ (Solvay Duphar, Weesp, The Netherlands) and dexamethasone (Sigma, Poole, UK) were dissolved in absolute alcohol and stored at −20°C. Amgen (Thousand Oaks, CA, USA) kindly provided the soluble RANKL. Human M-CSF, IL-1β, TNF-α, IL-6, soluble IL-6 receptor (sIL-6R), all purchased from R&D Systems (Abingdon, UK), were dissolved in αMEM/FCS and stored at −20°C.

Isolation and culture of monocytes and the effect of the osteoclastogenic factors M-CSF, RANKL and $1,25(OH)_2D_3$ on osteoclast formation in RA patients and normal controls

Twenty patients were studied: 10 seropositive RA patients and 10 age- and sex-matched normal control patients. The RA population ranged in age from 35 to 74 yr and included five males and five females. The control population ranged in age from 35 to 72 yr and also included five males and five females. Peripheral blood of RA patients and controls was collected into heparinized tubes, diluted 1:1 in αMEM, layered over Ficoll–Hypaque (Pharmacia, St Albans, UK) and centrifuged at 693 *g* for 18 min at 4°C. The peripheral blood mononuclear cell (PBMC) layer was removed and washed in αMEM and the cell pellet was resuspended in αMEM/FCS. The number of PBMCs in the cell suspension was counted in a haemocytometer after lysis of red cells with 5% v/v acetic acid solution.

PBMCs (2×10^5 cells/well) were added to 7 mm wells of a 96-well tissue culture plate. These wells contained either dentine slices (6 mm diameter), prepared as described previously [11], or glass coverslips (6 mm diameter) on which 2×10^4 UMR106 rat osteoblast-like cells had been cultured in αMEM/FCS for 24 h. PBMCs were settled onto the coverslips and dentine slices for 2 h. The coverslips and dentine slices were then removed from the wells, washed vigorously in αMEM/FCS to remove non-adherent cells and cultured in 24-well tissue culture plates containing 1 ml αMEM/FCS in the presence or absence of $1,25(OH)_2D_3$ (10^{-10} to 10^{-7} M), M-CSF (5–25 ng/ml) and dexamethasone (10^{-8} M). All cultures were incubated for 24 h and 14 and 21 days and the culture medium containing these factors was replenished every 3–4 days.

PBMCs, isolated as detailed above, were also cultured alone (i.e. in the absence of UMR106 cells) in the presence of RANKL (5–30 ng/ml), M-CSF (5–25 ng/ml) and dexamethasone. PBMCs were seeded at 2×10^5 cells/well onto dentine slices and coverslips in 96-well tissue culture plates. After washing, dentine slices and coverslips were placed in 24-well tissue culture plates containing 1 ml αMEM/FCS, in the presence of RANKL (5–30 ng/ml), M-CSF (5–25 ng/ml) and dexamethasone (10^{-8} M).

Cytochemical assessment of osteoclast formation

All cells cultured on coverslips for 24 h and 14 days were assessed histochemically for the expression of TRAP. Histochemical staining for TRAP was carried out using a commercially available kit (Sigma). Cell preparations were fixed in citrate/acetone solution and stained for acid phosphatase, using naphthol AS-BI phosphate as the substrate, in the presence of 1.0 M tartrate; the product was reacted with fast garnet GBC salt [15].

Cell preparations on coverslips were also stained immunohistochemically by an indirect immunoperoxidase technique with the monoclonal antibody 23C6 (Serotec, Oxford, UK); this is directed against CD51, the vitronectin receptor (VNR), a highly osteoclast-associated antigen [16]. Cell preparations were similarly stained with the monoclonal antibody JML-H14, directed against CD14, a monocyte/macrophage-associated antigen which is not expressed by osteoclasts [17]. Coverslips were examined by light microscopy. On each coverslip, the number of $TRAP^+$ MNCs was counted in four (10×objective) high-power fields (HPF) and the mean number of $TRAP^+$ MNCs per HPF was calculated. As some variation was noted between

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individuals (which is partly associated with age and gender [18]), our results were scored as: + = 1–5 MNCs/HPF; + + = 5–10 MNCs/HPF; + + + = 10–15 MNCs/HPF; + + + + = > 15 MNCs/HPF.

Functional evidence of osteoclast differentiation

Functional evidence of osteoclast differentiation was determined with a lacunar resorption assay system using cell culture on dentine slices [11, 14]. These slices provide a smooth-surface mineralized substrate for the assessment of lacunar resorption. After the cells had been cultured on dentine slices for 24 h and 21 days, the slices were removed from the wells, rinsed in phosphate-buffered saline and placed in 0.25% trypsin for 15 min. They were then washed vigorously in distilled water and left overnight in 1 M ammonium hydroxide. The dentine slices were then sonicated to remove cell debris and stained with 0.5% toluidine blue. Resorption pits were examined by light microscopy and the percentage surface area of lacunar resorption on each dentine slice was measured using an image analysis system.

Comparison of osteoclast formation from mononuclear precursors in RA patients and normal controls

To determine the relative proportions of circulating osteoclast precursors in the monocyte fraction of whole blood in RA patients and controls, serial dilutions of PBMCs (1×10^1 to 1×10^5 cells) were added to each well, which had been seeded earlier with UMR106 cells. The co-cultures were maintained in the presence of $1,25(OH)_2D_3$ (10^{-7} M), dexamethasone (10^{-8} M) and M-CSF (25 ng/ml) for up to 21 days. Osteoclast formation in these co-cultures was assessed by TRAP/VNR expression and lacunar resorption.

Co-cultures of UMR106 cells and PBMCs isolated from RA patients and normal controls were also incubated in the presence of $1,25(OH)_2D_3$ (10^{-7} M), dexamethasone (10^{-8} M) and M-CSF (25 ng/ml) for 1, 4, 7, 14 and 21 days. Differences in the expression of the osteoclast markers TRAP and VNR and in the extent of lacunar resorption were assessed after each period of incubation in co-cultures prepared with cells from RA patients and normal controls.

Assessment of the effects of corticosteroids and inflammatory cytokines/prostaglandins on osteoclast formation

To determine the effect of corticosteroids on monocyte-osteoclast formation, PBMCs (2×10^5 cells/well) were cultured for 24 h and 14 and 21 days in the presence and absence of dexamethasone (10^{-8} M) either with RANKL (30 ng/ml) and M-CSF (25 ng/ml) or with UMR106 cells, $1,25(OH)_2D_3$ (10^{-7} M) and M-CSF (25 ng/ml). To determine the effect of cytokines on osteoclast formation, PBMCs (2×10^5 cells/well) were cultured for 24 h and 14 and 21 days in the presence or absence of IL-1β (1 ng/ml), TNF-α (10 ng/ml) or IL-6 (100 ng/ml) with sIL-6R (100 ng/ml) with either RANKL (30 ng/ml), M-CSF (25 ng/ml) and dexamethasone (10^{-8} M) or with UMR106 cells, $1,25(OH)_2D_3$ (10^{-7} M) and M-CSF (25 ng/ml) and dexamethasone (10^{-8} M). Osteoclast formation in these co-cultures was assessed by TRAP/VNR expression and the extent of lacunar resorption.

Statistical analysis

Each experiment was carried out in triplicate (i.e. for each control and each treatment three dentine slices were set up). Data are presented as the mean percentage lacunar resorption $\pm$ S.E.M. Statistical analysis of the measurements of mean percentage area resorbed was performed using the Mann–Whitney *U*-test. *P* values < 0.05 were considered significant.

Results

Osteoclast differentiation from circulating precursors in RA patients and normal controls

In 24-h cultures, PBMCs from both RA patients and controls expressed the monocyte/macrophage marker CD14 and were negative for the osteoclast markers TRAP and VNR. No evidence of lacunar resorption was seen in 24-h PBMC cultures on dentine slices. These isolated cells thus expressed the cytochemical and functional phenotype of monocytes and not osteoclasts.

Osteoclast differentiation in co-cultures of UMR106 cells and PBMCs isolated from all RA patients and controls was evidenced by the generation of $TRAP^+$ and VNR^+ MNCs and the formation of lacunar resorption pits (Fig. 1A–C). Generation of $TRAP^+$ MNCs in co-cultures of UMR106 cells and PBMCs from RA patients and controls occurred after the same period of incubation. In both RA patients and controls, $TRAP^+$ cells were first noted after 7 days of incubation.

The number of $TRAP^+$ MNCs formed in co-cultures of UMR106 cells and PBMCs derived from RA patients and controls varied from case to case but was generally rated as either + (1–5 MNCs/HPF) or + + (5–10 MNCs/HPF). No significant difference in the mean number of $TRAP^+$ MNCs formed in UMR106–PBMC co-cultures from RA patients and controls was noted, indicating that the number of osteoclasts formed from circulating precursors in RA and control patients was similar.

Numerous $TRAP^+$ and VNR^+ MNCs were found in cultures at day 14, when lacunar resorption pit formation on dentine slices was also evident. After 21 days of incubation, extensive lacunar resorption pit formation was seen in co-cultures of UMR106 cells and PBMCs from RA patients and controls. PBMCs incubated in the presence of RANKL and M-CSF also differentiated into osteoclasts, as evidenced by the formation of numerous $TRAP^+$ and VNR^+ MNCs in 14-day cultures on coverslips and the production of numerous lacunar resorption pits in 21-day cultures on dentine slices. The relative proportion of osteoclast precursors in the monocyte fraction appeared to be similar in RA and control patients. When serial dilutions of PBMCs (1×10^2 to 1×10^5 cells/well) from both RA patients

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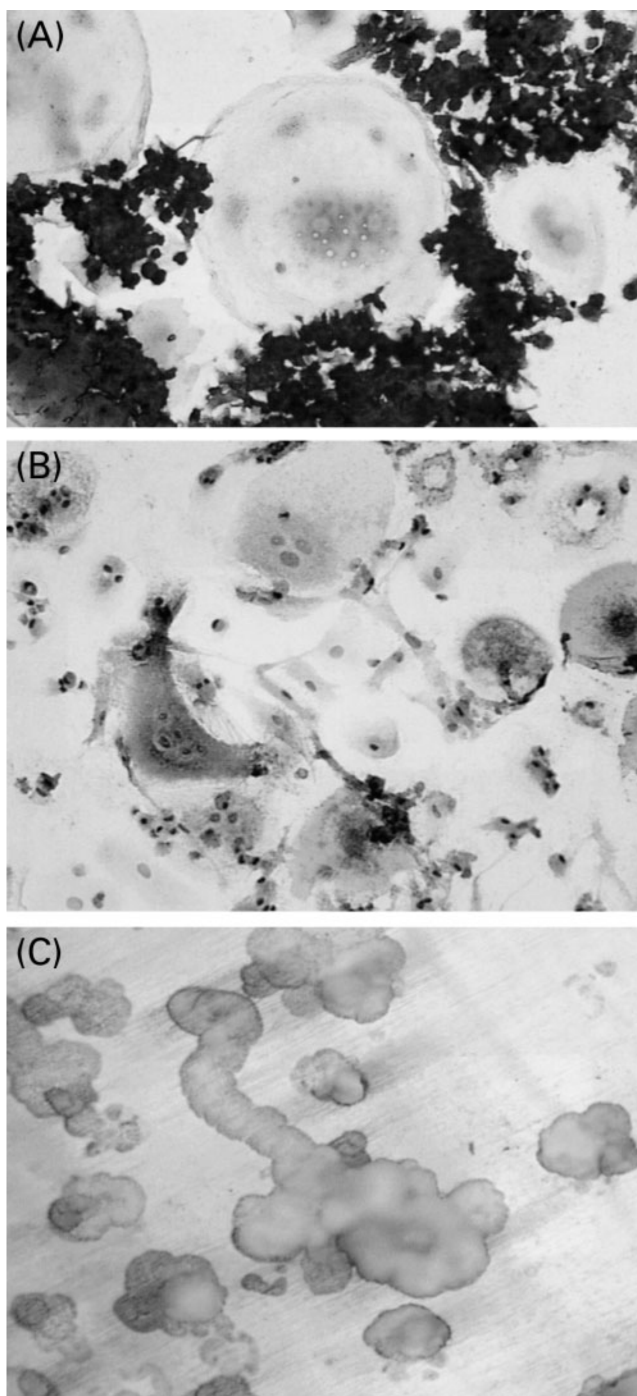

FIG. 1. Formation of (A) $TRAP^+$ and (B) VNR^+ MNCs on glass coverslips and (C) lacunar resorption pits on dentine slices in co-cultures of RA PBMCs and UMR106 cells incubated with M-CSF and $1,25(OH)_2D_3$. Original magnification: (A) ×400; (B) ×200; (C) ×20.

and controls were cultured with RANKL, M-CSF and dexamethasone or with UMR106 cells, M-CSF, $1,25(OH)_2D_3$ and dexamethasone, osteoclast generation, as shown by the formation of $TRAP^+$ MNCs and lacunar resorption pits, was first observed in both RA

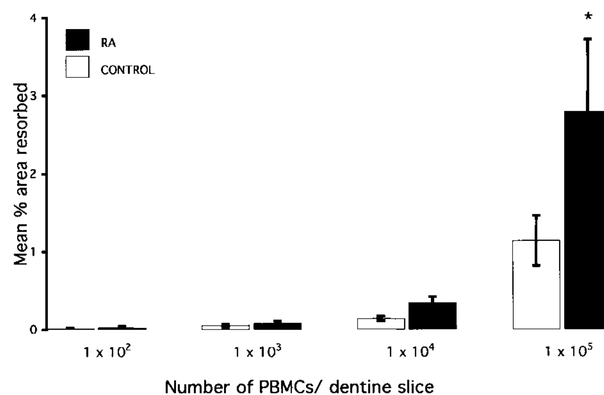

FIG. 2. The effect on lacunar resorption of different dilutions of RA and control PBMCs in the presence of RANKL, M-CSF and dexamethasone. The results are expressed as the mean percentage area resorbed per dentine slice ± S.E.M. $*P<0.05$ relative to 1×10^5 PBMC control cultures. RA patients, $n=10$; control patients, $n=10$. All experiments for each patient were carried out in triplicate.

and control patients when as few as 100 cells were seeded onto dentine slices; no resorption was seen when fewer than this number of cells were cultured on dentine slices.

Although there was no difference in the relative proportion of osteoclast precursors in peripheral blood or the number of $TRAP^+$ MNCs formed in monocyte cultures from RA patients and controls incubated with M-CSF and UMR106 cells or RANKL, it was found that lacunar resorption was three times greater in cultures of 1×10^5 PBMCs derived from RA patients compared with normal controls ($P=0.02$) (Fig. 2). This finding suggests that there is an increase in the resorbing activity of osteoclasts formed from RA monocyte precursors.

Sensitivity of circulating osteoclast precursors to osteoclastogenic factors in RA patients and controls

$1,25(OH)_2D_3$. When serial dilutions of $1,25(OH)_2D_3$ were added to co-cultures of UMR106 cells and PBMCs from either RA or control patients, the formation of $TRAP^+$ MNCs and lacunar resorption in RA and controls was first noted at a concentration of 10^{-9} M $1,25(OH)_2D_3$. No osteoclast formation was noted in co-cultures to which 10^{-10} M $1,25(OH)_2D_3$ was added. No difference in the number of $TRAP^+$ MNCs was noted in UMR106-PBMC cultures from RA and control patients. However, more lacunar resorption was noted in RA compared with control cultures (Fig. 3).

RANKL. The addition of RANKL (5–30 ng/ml) to PBMC cultures showed no difference in $TRAP^+$ MNC formation between RA patients and controls. Lacunar resorption, however, was significantly increased in RA compared with controls when RANKL 5 ng/ml ($P=0.0004$), 10 ng/ml ($P=0.05$) and 30 ng/ml ($P=0.009$) was added to PBMC cultures (Fig. 4).

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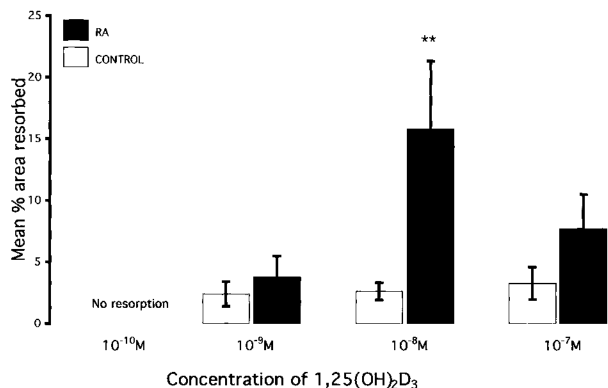

FIG. 3. The effect of $1,25(OH)_2D_3$ on lacunar resorption of RA and control PBMCs co-cultured with UMR106 cells in the presence of M-CSF and dexamethasone. The results are expressed as the mean percentage area resorbed per dentine slice ± S.E.M. $^{**}P<0.005$ relative to control culture. RA patients, $n=3$; control patients, $n=3$. All experiments for each patient were carried out in triplicate.

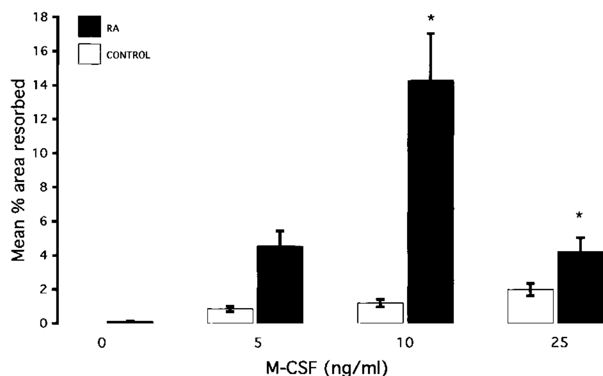

FIG. 5. The effect of M-CSF on lacunar resorption of RA and control PBMCs cultured in the presence of RANKL and dexamethasone. The results are expressed as the mean percentage area resorbed per dentine slice ± S.E.M. $^{*}P<0.05$ relative to control culture. RA patients, $n=3$; control patients, $n=3$. All experiments for each patient were carried out in triplicate.

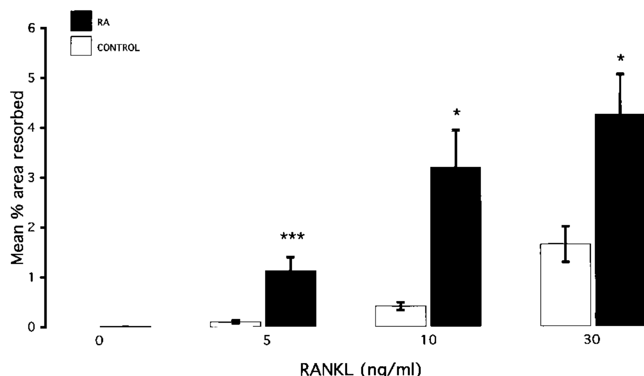

FIG. 4. The effect of RANKL on lacunar resorption of RA and control PBMCs in the presence of M-CSF and dexamethasone. The results are expressed as the mean percentage area resorbed per dentine slice ± S.E.M. $^{***}P<0.0005$ relative to control culture. RA patients, $n=9$; control patients, $n=10$. All experiments for each patient were carried out in triplicate.

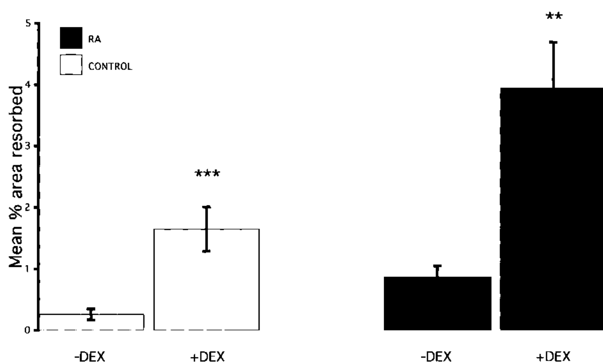

FIG. 6. The effect of dexamethasone on lacunar resorption of RA and control PBMCs cultured in the presence of RANKL and M-CSF. The results are expressed as the mean percentage area resorbed per dentine slice ± S.E.M. $^{***}P<0.0005$ and $^{**}P<0.005$ relative to cultures to which no dexamethasone had been added. RA patients, $n=10$; control patients, $n=10$. All experiments for each patient were carried out in triplicate.

M-CSF. The addition of M-CSF to cultures of RA and control PBMCs incubated for 14 days in the presence of RANKL showed no difference in $TRAP^{+}$ MNC formation. However, in 21-day PBMC cultures, a significant increase in lacunar resorption was noted in RA relative to controls when M-CSF was added at 10 ng/ml ($P=0.02$) or 25 ng/ml ($P=0.05$). Osteoclast formation did not occur in any of the cultures to which M-CSF had not been added (Fig. 5).

Corticosteroids. Corticosteroids are known to influence bone remodelling, long-term use being associated with an increased risk of osteoporosis in RA patients [8, 9]. The addition of dexamethasone (10^{-8} M) to both RA and control cultures of PBMCs incubated with RANKL (and M-CSF) resulted in a marked increase [+++ (10–15 MNCs/HPF) and ++++ (>15 MNCs/HPF)] in $TRAP^{+}$ MNCs formed on coverslips. An increase in the extent of lacunar resorption in cultures of PBMCs derived from both RA ($P<0.001$) and control ($P=0.0001$) patients was also noted (Fig. 6).

IL-1β, IL-6, TNF-α. The addition of the proinflammatory cytokines IL-1β, IL-6 and TNF-α did not stimulate osteoclast formation, as assessed by the formation of $TRAP^{+}$ MNCs in 14-day cultures or the extent of lacunar resorption in 21-day cultures. The addition of TNF-α significantly inhibited lacunar resorption in RA PBMC cultures (Fig. 7).

Discussion

Patients with early RA and those with inactive disease are known to have low bone mineral density measurements which correlate with increased levels of

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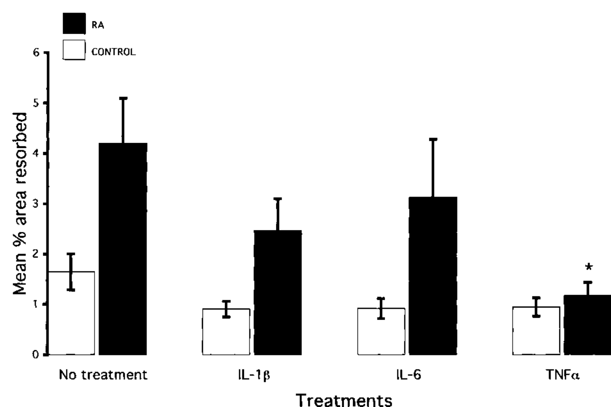

FIG. 7. The effects of IL-1β, TNF-α and IL-6 (with sIL-6R) on lacunar resorption of RA and control PBMCs cultured in the presence of RANKL, M-CSF and dexamethasone. The results are expressed as the mean percentage area resorbed per dentine slice ± S.E.M. *$P<0.05$ relative to RA culture to which no cytokine had been added. RA patients, $n = 8$; control patients, $n=10$. All experiments for each patient were carried out in triplicate.

biochemical markers of bone resorption [10]. This increase in bone resorption results in generalized osteopenia and an increased risk of fracture in RA patients compared with sex- and age-matched normal controls [1, 2]. Osteoclasts could contribute to the increased bone loss that occurs in RA as a result of increased formation from circulating precursors and/or increased lacunar bone-resorbing activity. In this study, we show that there is an increase in the extent of bone resorption carried out by osteoclasts that are formed from circulating precursors in RA, and that this increased osteolysis is not due to enhanced osteoclast formation. As assessed by $TRAP^+$ MNC formation in PBMC cultures, RA patients did not have more circulating osteoclast precursors in the monocyte fraction. The addition of M-CSF, RANKL and $1,25(OH)_2D_3$ did not promote osteoclastogenesis in RA patients, although corticosteroids enhanced osteoclast formation and resorption in RA patients and controls.

Osteoclasts form part of the mononuclear phagocyte system. Osteoclast precursors are marrow-derived cells which circulate in the $CD14^+$ fraction of PBMCs [11, 19]. Although enhanced generation of $CD14^+$ monocyte-lineage cells in cultures of marrow cells from RA patients has been noted [20–22] we did not observe either increased formation of osteoclasts or an increase in the proportion of circulating osteoclast precursors in RA patients relative to normal controls. As osteoclast precursors form a small subset of the circulating monocyte fraction of PBMCs [11, 19], these results suggest that most of the $CD14^+$ cells produced in RA do not represent osteoclast precursors. This finding is in keeping with previous *in vitro* studies, which have shown that most monocyte-lineage cells formed in cultures of bone marrow cells derived from RA patients exhibit accelerated maturation towards cells that express HLA-DR [21], an antigen which is expressed by macrophages but not by osteoclasts [17].

M-CSF is essential for the proliferation, differentiation and survival of cells of the monocyte–macrophage lineage, including osteoclast precursors [23, 24]. In combination with the recently discovered osteoclast differentiation factor RANKL, M-CSF induces human osteoclast formation *in vitro* [12]. In the presence of bone stromal cells, which express RANKL, osteoclast differentiation from circulating mononuclear precursors requires the presence of $1,25(OH)_2D_3$ as well as M-CSF [25]. Our finding that suboptimal doses of RANKL, M-CSF and $1,25(OH)_2D_3$ did not stimulate $TRAP^+$ MNC formation in cultures of PBMCs from RA patients relative to controls suggests that osteoclast precursors in RA are not primed or more sensitive to these osteoclastogenic factors. We did, however, note an increase in lacunar resorption by osteoclasts formed in cultures of monocytes derived from RA patients. These cultures were incubated in the presence of RANKL, M-CSF and $1,25(OH)_2D_3$, factors which are known not only to be required for osteoclast formation but also to promote osteoclast-resorptive activity. M-CSF inhibits osteoclast apoptosis and may thus be acting in our cultures to increase the extent of lacunar resorption by prolonging the survival of functional osteoclasts which have been generated *in vitro* [23]. RANKL is also known to decrease osteoclast apoptosis and has been shown to be a potent stimulator of osteoclastic bone resorption [26]. $1,25(OH)_2D_3$ is known to promote osteoclastic bone-resorbing activity mediated via stimulation of osteoblastic cells [27]. UMR106 cells are an osteoblast-like cell line [28] and were present in the monocyte cultures to which $1,25(OH)_2D_3$ was added. Thus, it would appear that the consistent increase in lacunar resorption noted in all PBMC cultures from RA patients is most likely accounted for by stimulation of osteoclast bone-resorbing activity and enhanced osteoclast survival rather than an increase in osteoclast formation.

Monocytes are activated in patients with RA both at the onset of the disease and during its chronic phase [29]. These cells produce increased amounts of inflammatory cytokines and prostaglandins [30–32]. These proinflammatory factors are known to promote osteoblast stimulation of osteoclast bone-resorbing activity [33]. They are also known to modulate osteoclast formation in bone marrow cell cultures by up-regulating bone stromal cell expression of RANKL and down-regulating osteoprotegerin, the decoy receptor for RANKL [13]. Osteoclast formation from mouse marrow precursors is stimulated by TNF-α, which may either substitute for RANKL or promote osteoclast formation in the presence of permissive levels of RANKL [34, 35]. IL-6 is also known to promote the development of osteoclast progenitor cells and, in combination with sIL-6R, stimulates osteoclast formation in mouse and human bone marrow cultures [36]. IL-1, IL-6 and TNF-α are present in increased amounts in the inflamed RA synovium and are thought to play a role in the formation of marginal erosions in

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RA [37, 38]. We did not find that the cytokines IL-1, IL-6 and TNF-α stimulated RANKL-induced osteoclast formation or lacunar resorption. On the basis of finding increased levels of the cytokines IL-1, IL-6 and TNF-α in RA patients who had low bone mineral density and showed increased urinary excretion of markers of bone resorption, Gough *et al*. [10] postulated that the generalized bone loss that occurs in RA is due to these humoral factors. If this is the case, our findings suggest that these factors do not act directly on circulating osteoclast precursors to stimulate osteoclast formation. Our results do not exclude the possibility that these factors may be acting primarily on bone stromal cells to stimulate osteoclast formation and activity.

Corticosteroid therapy has been implicated as a major cause of secondary osteoporosis in RA [8, 9]. Bone changes in patients receiving steroid treatment characteristically show increased bone turnover with an increase in the number of osteoclasts as well as an increase in osteoclast bone-resorbing activity [39]. Our findings indicate that the synthetic corticosteroid dexamethasone increased osteoclast formation from PBMCs derived from both RA patients and controls. Dexamethasone has been shown previously to stimulate the formation of osteoclasts from marrow and circulating precursors *in vitro* [25, 40]. Corticosteroids could thus induce generalized osteopenia by stimulating osteoclast formation and bone resorption, and in this way are likely to play a role in pathogenesis of secondary osteoporosis in RA patients receiving steroid therapy.

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