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Induction of osteoclast characteristics in cultured avian blood monocytes; modulation by osteoblasts and 1,25-(OH)₂vitamin D₃

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Summary. It has been established, that the osteoclast is derived from the haemopoietic stem cell, but its exact lineage is still controversial. It is sometimes suggested, that osteoclasts and monocytes/macrophages are related cells. It has also been suggested that osteoclast differentiation is regulated by osteoblasts and 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃). In the present paper we addressed the question whether avian monocytes can differentiate into osteoclasts *in vitro*, using an array of immunocytochemical, enzyme cytochemical and function markers. We have also determined the effects of osteoblasts, osteoblast conditioned medium and 1,25-(OH)₂D₃ on the expression of osteoclastic features on monocytes during culture. Monocytes developed tartrate resistant acid phosphatase (TRAcP) enzyme activity and antigens for all anti-osteoclast antibodies tested, during culture. However, they did not acquire the ability to resorb dentine and still showed phagocytosis of latex spheres. This indicates that the monocytes developed into cells resembling osteoclasts but lacking their function while retaining the function of macrophages. Osteoblast conditioned medium stimulated TRAcP enzyme activity and proliferation of monocytes in cultures. Addition of osteoblasts or osteoblast conditioned medium to monocyte cultures on dentine in the presence or absence of 1,25-(OH)₂D₃ did not result in the generation of genuine osteoclasts, nor in pit formation. 1,25-(OH)₂D₃ appeared to be cytotoxic to the avian monocytes in concentrations considered optimal for mouse osteoclast formation. These results suggest that avian monocytes do not readily differentiate into osteoclasts under *in vitro* conditions that stimulate osteoclast differentiation from bone marrow derived haemopoietic cells. Furthermore, labelling with anti-osteoclast antibodies and TRAcP as osteoclast-markers should be used only with great caution in the identification of osteoclasts formed *in vitro*.

Keywords: osteoclast, avian, monocyte, resorption

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Although it has recently been established that the osteoclast is derived from the haematopoietic stem cell (Scheven *et al.* 1986; Hagenars *et al.* 1989; Hattersley *et al.* 1991), the exact lineage of the osteoclast is still

unknown and its relation to the monocyte/macrophage lineage is still a controversial issue.

Osteoclasts share several features with monocytes and macrophages. Both cell types express acid phosphatase activity and are able to fuse into multinucleated giant cells. Osteoclasts exhibit a number of macrophage antigens (Athanasou *et al.* 1988). On the other hand, osteoclasts lack several other macrophage antigens (Athanasou & Quin 1990) and osteoclast specific antibodies have also been reported (Nijweide *et al.* 1985; Horton *et al.* 1985; Oursler *et al.* 1985; Hentunen *et al.* 1990; James *et al.* 1991).

Walker (1973) and Göthlin and Ericsson (1976) have shown in parabiosis experiments that a precursor cell of the osteoclast is present in rat peripheral blood. Helfrich *et al.* (1984) showed the same in cocultures of fetal mouse metatarsal bones and adult mouse blood cells. Recently, Hentunen *et al.* (1990) reported that a small number of cells of the monocyte fraction in chicken blood express osteoclast antigens. Teti *et al.* (1988) showed that a small percentage of cells in this fraction is able to fuse with isolated osteoclasts and recently Quin *et al.* (1994) reported that a significant proportion of the peripheral blood mononuclear cells could give rise to bone resorbing cells. These results and the above mentioned immunocytochemical similarities between osteoclasts and cells of the monocyte/macrophage lineage have lead several authors to suggest that cells of the monocyte/macrophage lineage are the precursors of the osteoclast (Burger *et al.* 1982; Teti *et al.* 1988; Udagawa *et al.* 1990; Suda *et al.* 1992). The exact stage at which the osteoclast lineage diverges from the monocyte/macrophage lineage is, however, still controversial.

Udagawa *et al.* (1990) have reported the generation of osteoclasts from mouse monocytes and alveolar macrophages, when cocultured with a stromal cell line in the presence of 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃). Chambers and Horton (1984), Hattersley *et al.* (1991) and Kerby *et al.* (1992), however, were unable to generate osteoclasts from mouse monocytes and macrophages under similar conditions, whereas haematopoietic cells with a multilineage potential did give rise to resorbing osteoclasts. Alvarez and co-workers generated osteoclast-like cells from chicken bone marrow macrophages (Alvarez *et al.* 1991) and blood monocytes (Alvarez *et al.* 1992). The osteoclast-like cells were characterized by tartrate resistant acid phosphatase (TRAcP) activity and by labelling with anti-osteoclast antibodies (Oursler *et al.* 1985; Horton *et al.* 1985). Furthermore, populations of these osteoclast-like cells, when seeded onto bone slices, were found to give rise to resorption pits. On the other hand, it has been shown

that under culture conditions monocytes and macrophages may acquire osteoclast characteristics (Hattersley & Chambers 1989; Modderman *et al.* 1991). In addition, Alvarez *et al.* (1991; 1992) did not give any quantitative information about how many of the cells thus generated were genuine bone resorbing cells. It is therefore possible that a small percentage of their starting population were genuine osteoclast precursors, while the majority were monocytes.

In this study, using an array of osteoclast markers, we investigated whether avian blood monocytes can differentiate *in vitro* into genuine bone resorbing osteoclasts. In addition we tested the effects of osteoblasts, osteoblast conditioned medium and 1,25-(OH)₂D₃ on the expression of these markers in monocytes in culture.

We raised six monoclonal antibodies (mAb) directed against quail osteoclasts. Two of these mAbs have been described before (Nijweide *et al.* 1985). We studied the development of the markers recognized by these mAbs, the activity of tartrate resistant acid phosphatase (TRAcP), the reaction with the 23C6 anti-osteoclast mAb (Horton *et al.* 1985) and resorption activity in cultures of monocytes. Phagocytosis was used as marker for macrophages.

Materials and methods

Materials

Culture media, chemicals and other materials were obtained from the following companies: all culture media and fetal calf serum (FCS) from Gibco; radio-labelled methionine from Amersham; collagenase and naphthol-ASBI-phosphate from Sigma; FITC conjugated anti-mouse antibodies from Nordic Immunology; protein A coupled to Sepharose beads from Pharmacia; all chemicals used for the preparation of SDS polyacrylamide electrophoresis (SDS PAGE) gels from Biorad; monodisperse fluorescent latex microspheres (diameter 0.57 µm) from Polysciences. Monoclonal 23C6 was kindly provided by Dr M.A. Horton (St Bartholomew's Hospital Medical College, London UK).

Cell isolation and culture

Monocytes were isolated from heparinized quail blood by Ficoll Isopaque density centrifugation (Alvarez *et al.* 1992). The monocyte and lymphocyte rich layer was collected, washed with phosphate buffered salt solution (PBS), spun down and resuspended in αMEM supplemented with 10% FCS. Cells were grown on glass coverslips for immunolabelling and in multiwell culture

plates for TRAcP staining and enzyme activity measurements. Generally cells were allowed to adhere for 3 days; the non-adherent cells were then removed by washing with Hanks' balanced salt solution (HBSS), fresh medium (α MEM + 10% FCS) was added and culture was continued.

Chicken osteoclasts and osteoclast progenitors were isolated from tibiae and femora of 18-day-old fetal chickens. Cells were flushed out of the dissected bones, submerged in HEPES buffered HBSS (pH 7.4), by pressing with a silicone rubber stop. The cell suspension was filtered through a 120- μ m nylon filter to remove bone fragments and was used to obtain either an osteoclast enriched population or an osteoclast progenitor containing population.

A cell suspension enriched in osteoclasts was obtained by sedimentation at unit gravity for 30 min at room temperature in siliconized 10 ml test-tubes. After this period the top 75% of the cell suspension was removed, the remaining cells were spun down and resuspended in Iscove's modification of Dulbecco's MEM (IDMEM) supplemented with 10% FCS at pH 6.9 (Arnett & Dempster 1986). The cell suspension was seeded onto glass coverslips or multiwell culture plates. The cells were allowed to adhere for one hour and non-adherent cells were removed by washing with HBSS. Osteoclast adherence is increased in IDMEM at pH 6.9, while adherence of contaminating stromal cells is decreased (unpublished observations). Osteoclasts were cultured in α MEM + 10% FCS.

An osteoclast progenitor containing population was obtained by layering the filtered cell suspension (see above) on Ficoll Isopaque and harvesting the cells at the interface after centrifugation. The cells were washed with PBS, resuspended in α MEM supplemented with 10% FCS and seeded in culture flasks. After 24 hours of culture the non-adherent cells were removed and used for osteoclast formation studies.

Quail osteoclasts were isolated as described by Nijweide *et al.* (1985). In short, the medullary bone of egg laying quails was removed from femora and tibiae five hours prior to oviposition. The medullary bone tissue was cut into small pieces in cold PBS containing 1% bovine serum albumin. The pieces were gently shaken and the resulting cell suspension was filtered through a nylon filter with a pore size of 120 μ m. The cell suspension was layered on a discontinuous Percoll gradient of 15, 30 and 60% Percoll in HBSS and centrifuged for 20 minutes at 600 g. The cells, accumulated at the interface of the 15 and 30% layers, were removed and were washed twice in HBSS. About 10% of the cells in this fraction were multinucleated osteoclasts.

Virtually all of these osteoclasts were, however, damaged and did not adhere to the culture dish when seeded. Quail osteoclasts were used for the immunization of mice and biochemical TRAcP measurements.

Chicken osteoblasts were isolated from calvariae of 18-day-old fetal chickens (van der Plas & Nijweide 1992). First, the fibrous layers of the periosteum were removed by dissection. The calvariae were then treated with collagenase (2 mg/ml, crude collagenase type 1) in HEPES buffered HBSS for 30 and 45 min. These two cell fractions were pooled, washed with HBSS, supplemented with 10% chicken serum and resuspended in culture medium (α MEM + 10% FCS). To obtain osteoblast conditioned medium (ObCM), osteoblasts were seeded in a 75-cm² culture flask at a cell density of 2×10^4 cells/cm² and cultured in α MEM supplemented with 2% FCS for 5–7 days. The ObCM was harvested and sterilized by filtration.

Monoclonal antibody production

A series of monoclonal antibodies was prepared as earlier described (Nijweide *et al.*, 1985). Isolated quail osteoclasts were used as antigen.

Immunocytochemistry

Cells grown on glass coverslips or cryosections of various tissues were air dried, preincubated with 1% BSA in PBS for 5 min to block non-specific labelling and then exposed for 45 min to various monoclonal antibodies. Specific binding was assessed by incubation for 30 min with anti-mouse antibodies conjugated to FITC. As a negative control, the first antibody was substituted with 1% BSA in PBS (Nijweide *et al.*, 1985). Immunoreactivity was graded from – to +++ depending on intensity.

TRAcP

Cytochemical staining of cultures for TRAcP was performed as described by Barka and Anderson (1962). In short, cultured cells were fixed in buffered formalin (pH 7.4), rinsed with PBS and incubated with naphthol-ASBI-phosphate, hexazotized pararosaniline and 30 mM tartrate in acetate buffer (pH 5.5) at 37°C for one hour.

TRAcP enzyme activity was determined biochemically by incubating formalin fixed cultures (chicken osteoclasts) or cell isolates (quail osteoclasts) with naphthol-ASBI-phosphate (85 μ g/ml) in an acetate buffer (pH 5.5) containing 30 mM tartrate for one hour at 37°C. The reaction was terminated and the naphthol-

ASBI precipitate dissolved by the addition of NaOH to a final concentration of 0.1 M. The amount of naphthol-ASBI produced was measured fluorometrically (excitation at 405 nm, emission at 515 nm) (Modderman *et al.* 1991). A calibration curve of known concentrations of naphthol-ASBI was used to calculate the enzyme activities. In parallel cultures, the number of cell nuclei per culture well (chicken osteoclasts) or per cytospin preparation (quail osteoclasts) was estimated by counting five randomly chosen microscope fields and extrapolating this number to the total number of nuclei present. In chicken osteoclast cultures, only the nuclei of TRAcP-positive cells were counted. In quail osteoclast isolates only the nuclei of multinucleated cells were counted. TRAcP staining of such isolates showed that the number of TRAcP-positive mononuclear cells was very small when compared to multinucleated TRAcP-positive cells.

Immunoprecipitation

Quail monocytes were isolated and cultured in 6-well plates (5×10^6 cells/well) for at least 7 days as described above. The cultures were transferred to HBSS for one hour and subsequently labelled with ^{35}S -methionine (25 μCi /well, specific activity 1000 Ci/mmol) in HBSS for 2 hours. The cells were homogenized in 300 μl 1% Triton X100 in phosphate buffer pH 8.1. Cell debris was removed by centrifugation (13 000 g for 15 minutes). Protein A coupled to Sepharose beads (75 μg /300 μl homogenate) and anti-osteoclast mAb (5 μg /300 μl homogenate) were added to the homogenate. The anti-osteocyte mAb OB7.3 (van der Plas & Nijweide 1992) was used as a control. Immunoprecipitation was allowed to proceed overnight at 4°C. The Sepharose beads were spun down and washed five times. TRAcP activity of the precipitated antigens and of the supernatants was determined fluorometrically as described above. To determine the molecular weight of the antigens, the beads were boiled for 5 minutes in 25 μl gel sample buffer (Laemmli 1970). The samples were run on a 12% SDS PAGE according to Laemmli (1970). Protein bands were visualized by autoradiography.

Monocyte cultures were used for the immunoprecipitation studies because of the relatively high purity of these populations when compared to isolated chicken osteoclast populations. Furthermore the antigens can easily be induced in monocytes in culture (see below) which facilitates the incorporation of ^{35}S -methionine into the antigens.

Resorption assay

Resorption was assessed according to the method

described by Takada *et al.* (1992). Monocytes were cultured on elephant tusk dentine slices in 96-well tissue culture plates for 1, 2 or 3 weeks in 100 μl αMEM supplemented with 10% FCS. Monocytes were plated at densities of 1×10^5 , 3×10^5 and 5×10^5 cells per well in the presence or absence of $1,25\text{-(OH)}_2\text{D}_3$ (10, 1 or 0.1 nM) and/or osteoblasts (8×10^3 cells/well). Non-adherent, osteoclast progenitor containing cell populations were plated at 3×10^5 cells per well with osteoblasts (8×10^3 cells/well) on dentine slices in 100 μl αMEM + 10% FCS and cultured for 14 days. After 2 days, one group of cultures was terminated and the dentine slices were inspected for pits. This group served as a control group, representing resorption activity of already existing osteoclasts contaminating the cell population.

At the end of the culture period, the cells were removed from the slices by rubbing with a silicone rubber stop. The slices were stained with Harris' haematoxylin for 3 minutes, rinsed with distilled water and examined. All experiments were performed at least in triplicate.

Dentine resorption by chicken osteoclasts was assayed essentially as described above for monocytes, except for the fact that the osteoclasts were cultured on the dentine slices for only 2 days. The number of osteoclasts seeded per dentine slice was equivalent to 1/8 of the number of osteoclasts retrieved from the long bones of one fetal chicken.

Phagocytosis assay

Blood monocytes were cultured in 24-well culture plates for 5 days as described above. Chicken osteoclasts were isolated and cultured for one day as described above. The cells were rinsed with HBSS and incubated with monodisperse fluorescent latex spheres suspended in HBSS for 2 hours at 37°C. Phagocytosis of the latex spheres was determined by examination of the cultures with a fluorescence microscope.

Statistics

Data are presented as means with standard deviation. The significance of effects caused by the various treatments was evaluated with Student's *t*-test. Differences were scored as being significant when *P* was less than 0.05.

Results

Osteoclast markers

When cultured on dentine slices, chicken osteoclasts

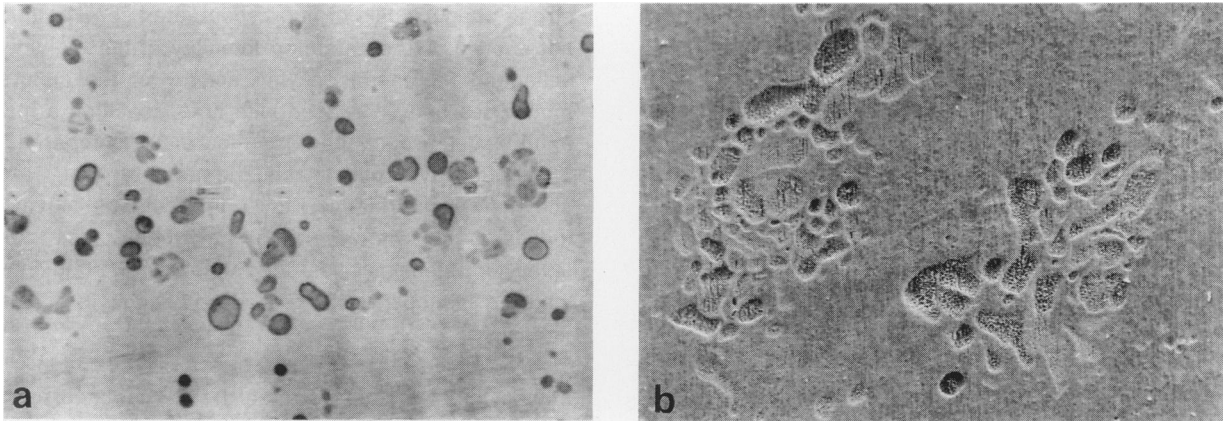


Figure 1. Resorption of dentine slices by isolated chicken osteoclasts. Chicken osteoclasts were cultured on dentine slices. After 2 days all cells were removed and the slices examined with light or scanning electron microscopy. a, Stained with acid haematoxylin, $\times 90$. b, Sputter-coated with gold and examined with a scanning electron microscope. $\times 170$.

formed numerous resorption pits after 48 hours of culture (Figure 1). They did not phagocytize fluorescent latex spheres (data not shown). Isolated chicken osteoclasts and osteoclasts in quail medullary bone cryosections were strongly positive for TRAcP and also showed a strong labelling with the 23C6 mAb (not shown).

The immunization procedure yielded six mAbs, OC6.3, OC6.9, OC6.12, OC6.13, OC6.14 and OC6.16, that reacted with osteoclasts in cryosections of quail medullary bone. All of these mAbs were of the IgG class. All mAbs but OC6.13 also labelled air dried isolated chicken osteoclasts, but not intact living osteoclasts. As OC6.13 was also found to label only air-dried quail cells, but not intact living cells, we conclude that all monoclonals labelled only cytoplasmic antigens, including mAbs OC6.3 and OC6.9 which were originally reported to stain cell surface antigens (Nijweide *et al.* 1985). Some time after the publication of the first report on the isolation of the osteoclast specific monoclonals, it

was found that virtually all osteoclasts isolated from the medullary bone of egg laying quails were permeable or became permeable for IgGs during the subsequent incubation and were not able to adhere to a culture dish. In fact this forced us to isolate osteoclasts capable of adhering and resorption from fetal chickens.

The monoclonals most specifically labelling osteoclasts were OC6.13 and OC6.16. Monoclonals OC6.3 and OC6.12 also labelled tissue macrophages, but except for Kupffer cells these were not as strongly labelled as osteoclasts. Monoclonal OC6.9 also labelled the bile capillaries of the liver and some as yet unidentified parts of the nephron (Nijweide *et al.* 1985). Although OC6.14 stained exclusively osteoclasts in medullary bone cryosections, this antibody also recognized a wide variety of antigens in other tissues.

We were able to precipitate labelled antigens from ^{35}S -methionine labelled cultured monocytes with all six mAbs. After electrophoresis, several polypeptide bands for each antigen could be detected by autoradiography

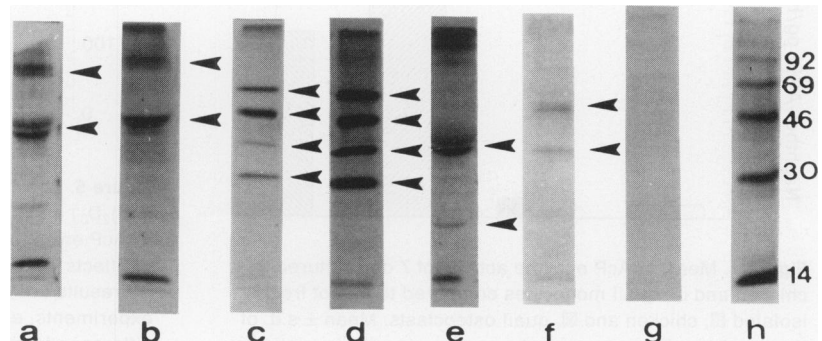


Figure 2. Autoradiogram of the radiolabelled precipitated antigens of the anti-osteoclast mAbs after electrophoresis. Lane a, OC6.14; lane b, OC6.3; lane c, OC6.13; lane d, OC6.9; lane e, OC6.12; lane f, OC6.16; lane g, control (Ob7.3); lane h, molecular weight standards.

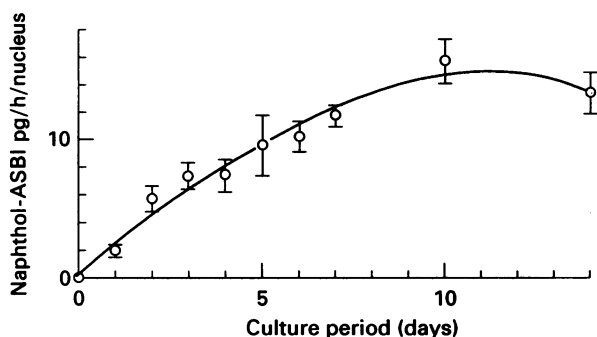


Figure 3. Development of TRAcP enzyme activity in monocyte cultures with time. Results are the mean values \pm s.d. of three independent experiments, each performed in triplicate. On day 0 the TRAcP enzyme activity of a freshly isolated leucocyte fraction was measured.

(Figure 2). Monoclonals OC6.9 and OC6.13 precipitated antigens showing an identical peptide band pattern, with bands at 65, 54, 42 and 32 kDa. The monoclonals OC6.3 and OC6.14 also precipitated antigens with identical peptide band patterns, with double bands at 96 and 50 kDa. The remaining two antibodies both precipitated different antigens. OC6.12 precipitated peptides with a molecular weight of 44 and 23 kDa and OC6.16 precipitated peptides of 50 and 40 kDa. None of the precipitated antigens exhibited TRAcP enzyme activity and none of the supernatants showed a decrease in TRAcP enzyme activity after immunoprecipitation. Therefore none of the mAbs precipitated the TRAcP enzyme.

Development of osteoclast markers on monocytes in culture

All of the cells in 7-day-old monocyte cultures exhibited

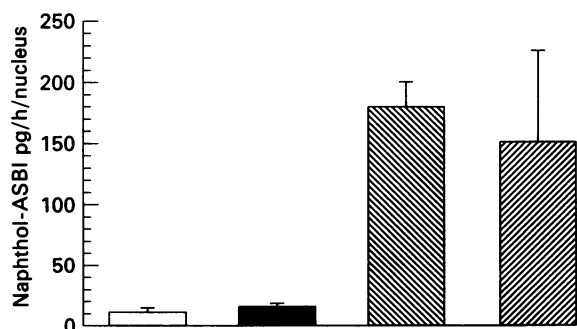


Figure 4. Mean TRAcP enzyme activity of 7 day cultured ■, chicken and □, quail monocytes compared to that of freshly isolated ▨, chicken and ▩, quail osteoclasts. Mean \pm s.d. of three independent experiments, each performed in triplicate.

strong phagocytosis of fluorescent latex spheres (data not shown). TRAcP staining intensity of the individual cells and the mean enzyme activity of the cultured monocytes both increased with time, reaching a maximum level around day 10 (Figure 3). Compared to TRAcP activity of osteoclasts, the mean level of TRAcP activity was still relatively low (Figure 4). The activity of individual cells, however, may have been close to the level of osteoclasts as the histological staining of cultured monocytes showed a wide range in staining intensity.

All selected mAbs reacted in a similar fashion with monocytes. They did not label freshly isolated monocytes, showed weak labelling of 1-day cultured cells, moderate labelling of 4-day cultured cells and strong labelling of 7-day cultured cells. The staining intensity of cells labelled with 23C6 (Horton *et al.* 1985) was only slightly less than that of cells labelled with the other six mAbs. All mAbs (except 23C6) labelled only air-dried and not living, cultured monocytes, again indicating that these mAbs react with cytoplasmic antigens.

No resorption pits could be detected on slices on which monocytes were cultured for up to 3 weeks in four independent experiments.

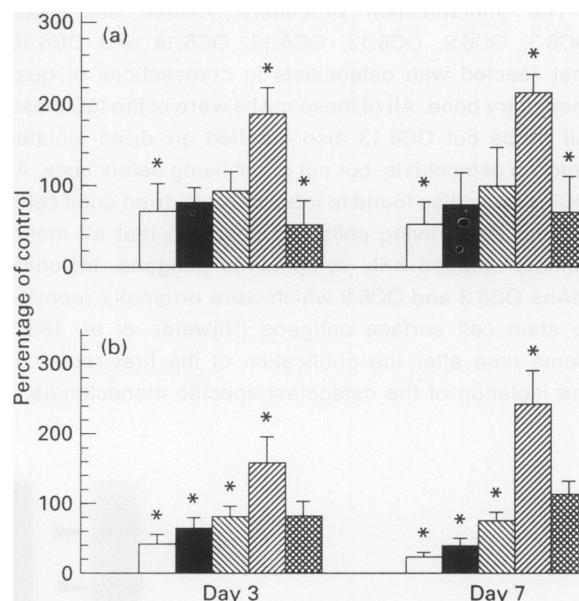


Figure 5. Effects of ▨, ObCM; □, 10; ■, 1; ▩, 0.1 nM D₃ (1,25-(OH)₂D₃) and ▨, ObCM + D₃ on quail monocytes. a, Effects on TRAcP enzyme activity per nucleus at day 3 and 7 of culture. b, Effects on cell number per well at day 3 and 7 of culture. All results are expressed as mean \pm s.d. of three independent experiments, each performed in triplicate. * Significant difference from control.

Effects of 1,25-(OH)₂D₃ and ObCM

When quail monocytes were cultured for 7 days in the presence of 10 nM 1,25-(OH)₂D₃, the immunolabelling intensity of the cultured monocytes appeared to be slightly decreased as compared to control cultures. Treatment of the cells with 1,25-(OH)₂D₃ resulted in a dose dependent decrease of mean TRAcP enzyme activity per nucleus and in a decreased number of cells per well (Figure 5). The morphology of the cells was also affected. The cytoplasmic structure of the cells became rather granular and many cells showed a stand-off morphology.

When quail monocytes were cultured in the presence of ObCM, their immunolabelling intensity was at least as high as that of control cultures. Treatment with ObCM resulted in an increased mean TRAcP enzyme activity per nucleus and cell number per well (Figure 5). The increase in cell number was not due to an increase in cell adherence. When ObCM was added after the monocytes had been allowed to adhere for 2 days in the absence of ObCM, the increase in cell number was still observed. Compared to control cultures, the fraction of mononuclear cells was increased in ObCM treated cultures (Figure 6). In contrast, 1,25-(OH)₂D₃ appeared to have no effect on the composition of the cell population in this respect.

The addition of 10 nM 1,25-(OH)₂D₃ to cells in the presence of ObCM terminated the increase in cell

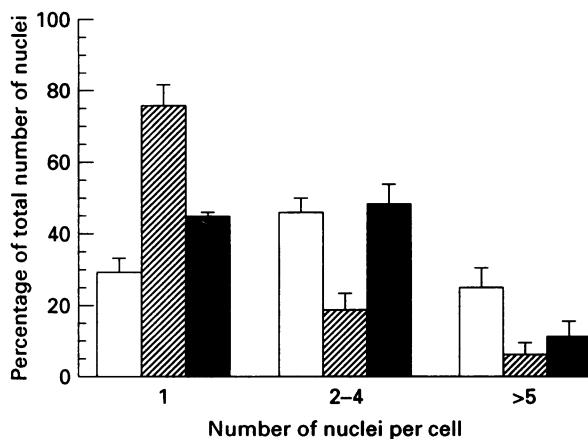


Figure 6. Effects of ObCM and 10 nM 1,25-(OH)₂D₃ on multinucleation of cultured monocytes; □, control. Quail monocytes were cultured for 7 days. The cells were fixed with buffered formalin and stained with haematoxylin. The number of nuclei per cell was counted. Of each culture well the first 300 cells were counted. The values in the graph represent the average of two independent experiments, each performed in triplicate.

number and mean TRAcP enzyme activity per nucleus (Figure 5).

In all three experiments performed, the addition of 1,25-(OH)₂D₃ and/or ObCM to the monocyte cultures did not result in the formation of resorption pits. Neither did the coculture of monocytes and chicken osteoblasts in the presence or absence of 1,25-(OH)₂D₃ result in the generation of resorbing cells (three additional experiments).

On the other hand, in the populations of non-adherent bone marrow cells cultured together with osteoblasts on dentine slices, osteoclasts did develop. Dentine slices inspected after 2 days of culture were virtually devoid of pits (in one experiment four pits per three dentine slices; in the other two experiments, none were found), demonstrating that the non-adherent cell population did not contain differentiated osteoclasts. After 14 days of culture 10–50 pits per slice (three independent experiments) were found.

Discussion

The immunization procedure yielded six different mAbs directed against quail osteoclasts. They were selected because of their specificity for osteoclasts in cryosections of medullary bone from egg laying quails or in freshly prepared cell suspensions from bone and bone marrow. All of these antibodies label cytoplasmic antigens only. Of these mAbs, OC6.13 and OC6.16 are highly specific for osteoclasts and do not react with other cells in sections of freshly isolated quail tissues. Monoclonals OC6.3, OC6.9 and OC6.12 also label a limited number of other cell types, although not as strongly as osteoclasts. The least specific mAb is OC6.14, which labels a wide variety of cell types. Monoclonal OC6.14 precipitated the same antigen as OC6.3. Because of the differences in cross-reaction between these two mAbs, we conclude that these mAbs bind to different epitopes on the same antigen. Monoclonals OC6.9 and OC6.13 also precipitated identical antigens. OC6.9, however, labels chicken as well as quail osteoclasts, whereas OC6.13 labels only quail osteoclasts; therefore, these antibodies probably also recognize different epitopes on the same antigen. None of the mAbs precipitated the TRAcP enzyme. At this stage no further attempt was made to characterize the antigens; here, it sufficed to show that the mAbs stained different antigens. The binding sites of these six mAbs, together with TRAcP, resorption of dentine and the antigen of 23C6, represent seven different markers of osteoclast differentiation.

We have shown that avian monocytes are able to differentiate into cells that express osteoclast-like

characteristics in culture. Under culture conditions these cells acquired TRAcP activity, although not at the same level as isolated osteoclasts. Furthermore, after 3 days of culture, the cells reacted with all of the anti-osteoclast mAbs, including mAb 23C6 (Horton *et al.* 1985). This could indicate that the antibodies are directed against antigens that are induced upon activation of the monocytes/macrophages and osteoclasts. However, the osteoclasts we used in the immunization procedure were inactive osteoclasts isolated from quail medullary bone at the stage of the egg laying cycle when almost all calcified bone had disappeared. The most distinguishing feature of genuine osteoclasts is the ability to resorb bone. The resorption of dentine slices by isolated chicken osteoclasts showed that dentine is a suitable substrate for osteoclastic resorption. The experiments with non-adherent bone marrow cells showed that the culture conditions used in this study allowed the differentiation of precursor/progenitor cells into mature, resorbing osteoclasts. The cultured monocytes failed to resorb dentine. As Udagawa *et al.* (1990) reported that high macrophage cell density inhibits osteoclast formation, we used several different cell densities. We found no evidence of dentine resorption at any cell density. On the other hand, the cultured monocytes showed a high capacity to phagocytize latex beads, while osteoclasts did not. We concluded therefore that the cultured monocytes have not differentiated into genuine osteoclasts.

Our results are in agreement with the results of several other investigations who reported the lack of osteoclast generation from mouse monocytes, macrophages or CFU-M (Burger *et al.* 1982; Schneider & Relfson 1988; Kerby *et al.* 1992). In contrast, others reported the generation of osteoclasts from mouse (Udagawa *et al.* 1990) and chicken (Alvarez *et al.* 1991; 1992) monocytes and macrophages. The discrepancies between these conflicting results might be explained by differences in the species of the experimental animals, in the tissues from which the putative precursors were isolated, or in cell isolation and cell culture procedures.

Alvarez *et al.* (1992), who used similar experimental conditions, did find pit formation by monocytes isolated from chickens that were fed a calcium deficient diet. These investigators, however, gave no quantitative results as to the number of pits formed or the rate of pit formation per number of cells seeded. Haemopoietic stem cells may be present in peripheral blood of adult animals (Goodman & Hodgson 1962). One may therefore expect osteoclast formation from blood leucocytes under the right conditions and it has been shown to occur by several investigators (Burger *et al.* 1982; Helfrich *et al.* 1984; Udagawa *et al.* 1990; Quinn *et al.*

1994). The difference between the results of Alvarez *et al.* and ours is perhaps due to a difference in number of stem cells present in the peripheral blood of calcium deprived laying chick hens and standard fed laying quail hens. Whether blood monocytes may differentiate into osteoclasts is again clearly another matter.

The ability of cultured monocytes to resorb dentine could not be induced by addition of $1,25\text{-(OH)}_2\text{D}_3$ or ObCM to the culture medium, by co-culture with osteoblasts or by a combination of these factors. Udagawa *et al.* (1990) and Suda *et al.* (1992) stress the importance of $1,25\text{-(OH)}_2\text{D}_3$ and the presence of osteoblast-like cells for osteoclast differentiation in mouse cell cultures. In the experiments described here, the concentration of $1,25\text{-(OH)}_2\text{D}_3$ these authors found to be optimal for osteoclast formation (10 nM), resulted in a decrease of expression of markers for osteoclast differentiation rather than an increase. Furthermore, the number of cells per culture well was decreased and the morphology of the cells was affected. This indicates that 10 nM $1,25\text{-(OH)}_2\text{D}_3$ may be cytotoxic for quail monocytes. Teti *et al.* (1988) reported that $1,25\text{-(OH)}_2\text{D}_3$ had no effect on the fusion of chicken monocytes with osteoclasts. Alvarez *et al.* (1991) generated osteoclast-like cells with avian bone marrow in the absence of $1,25\text{-(OH)}_2\text{D}_3$. This may indicate that in birds, in contrast to mammals, $1,25\text{-(OH)}_2\text{D}_3$ does not play an important role in osteoclastogenesis.

In contrast to $1,25\text{-(OH)}_2\text{D}_3$, ObCM did stimulate TRAcP activity and staining intensity. The formation of multinuclear cells, however, was inhibited. The relative decrease in the number of multinucleated cells might be due to the stimulation of proliferation of the cells. Chambers (1980) and Rodan and Martin (1981) postulated that osteoclastic bone resorption is regulated by osteoblasts. Since then, several groups have reported osteoblast derived, osteoclast stimulating activities (McSheehy & Chambers 1987; Oursler & Osdoby 1991; Teti *et al.* 1991; Greenfield *et al.* 1992; Collin *et al.* 1992; Perry & Gurbani 1992). In a recent study by Greenfield *et al.* (1992) who used chicken bone marrow cultures, the main target for the resorption stimulating activity appeared to be the osteoclast precursors. The blood monocytes in the present study behaved like osteoclast precursors in that the expression of osteoclast markers was enhanced by ObCM in these cells. However, the addition of ObCM to the culture medium did not result in the generation of dentine resorbing cells. At present the exact nature of the stimulating factor(s) in ObCM is not known, nor its (their) relation to other osteoblast derived factors reported in the literature. The identification of the factor(s) is now under investigation. In preliminary

experiments the activity was found to be present in the 10 000–30 000 Mw fraction when ObCM was fractionated by ultrafiltration.

The release of osteoclast stimulating activity by osteoblasts has been reported to be stimulated by 1,25-(OH)₂D₃ in the rat (McSheehy & Chambers 1987; Collin *et al.* 1992). In our experiments, co-cultures of osteoblasts and monocytes on dentine slices in the presence of 1,25-(OH)₂D₃ did not result in resorption of the dentine. When 10 nM 1,25-(OH)₂D₃ was added to monocytes cultured in the presence of ObCM, the TRAcP and proliferation-stimulating effects of the ObCM were abolished. This again indicates that there may be considerable differences in the role of 1,25-(OH)₂D₃ in bone resorption or induction of osteoclast markers between mammals and birds.

In sum, the results presented in this paper do not sustain the notion that quail monocytes, although closely related to osteoclasts, are the precursor cells of the osteoclast. Furthermore, as has been reported before, TRAcP staining is not of itself a reliable marker for osteoclast formation (Teti *et al.* 1988; Hattersley & Chambers 1989; Modderman *et al.* 1991). The enzyme activity level of cultured monocytes, however, was generally considerably lower than that of osteoclasts; therefore, the rate of the enzyme activity may be used as an osteoclast marker. Our results also show that the antigens recognized by the anti-osteoclast mAbs that label osteoclasts specifically *in vivo*, are induced in non-osteoclastic cells *in vitro*. At present we do not know the identity or the functions of the antigens recognized by the antibodies, except for 23C6 (Davies *et al.* 1989). It is not clear what causes the expression of osteoclast markers including TRAcP in monocytes. Neither do we know the identity of the factor or factors produced and secreted by osteoblasts that even further stimulate this induction of osteoclast markers. The results give a clear warning that the identity of putative osteoclasts formed in cell culture and the specificity of putative osteoclast inducing factors have to be established with great care. The suggested difference in the action of 1,25-(OH)₂D₃ in avian systems as compared to mammalian systems clearly warrants further investigation.

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