

Generation of osteoclast-inductive and osteoclastogenic cell lines from the *H-2K^btsA58* transgenic mouse

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ABSTRACT The development of osteoclastic cell lines would greatly facilitate analysis of the cellular and molecular biology of bone resorption. Several cell lines have previously been reported to be capable of osteoclastic differentiation. However, such cell lines form at best only occasional excavations, suggesting that osteoclastic differentiation is either incomplete or that osteoclasts represent a very small proportion of the cells present. We have used the recently developed *H-2K^btsA58* transgenic mouse, in which the interferon-inducible major mouse histocompatibility complex *H-2K^b* promoter drives the temperature-sensitive (ts) immortalizing gene of simian virus 40 (*tsA58*), to develop cell lines from bone marrow with high efficiency. Bone marrow cells were incubated with γ interferon at 33°C, then cloned, and expanded. The cell lines were characterized at 39.5°C in the absence of γ interferon. First, stromal cell lines were established that induced osteoclast formation (resorption of bone slices) when cocultured with hemopoietic spleen cells. Some of the stromal cell lines so generated were able to resorb ≈ 30 mm²/cm² of bone surface. We then established cell lines of hemopoietic origin, several of which possess osteoclastic potential. When these osteoclast-precursor cell lines were cocultured with stromal cell lines, extensive bone resorption was observed. Osteoclast formation did not occur if the precursor cell lines were incubated on bone slices without stromal cells; osteoclast formation was also dependent upon the presence of 1 α ,25-dihydroxyvitamin D₃. These cell lines represent a model for osteoclast formation and a valuable resource for identification of the mechanisms and factors that regulate osteoclast differentiation and function.

A major impediment to an analysis of the cellular and molecular biology of bone resorption has been the nature of the cell populations available for study. As primary cells, osteoclasts are available in only very small numbers in a nonproliferative state and are contaminated by other cell types (1); attempts at purification have only been partially successful. Although bone-resorbing cells can be formed in hemopoietic cultures (2–4), osteoclasts remain a minor component of a heterogeneous population. Thus, very little is known of the factors that specifically direct osteoclastic differentiation and regulate osteoclastic function, and virtually nothing is known of the factors that may be produced by osteoclasts themselves.

In other tissues, the development of cell lines has greatly assisted the investigation of the cellular and molecular interactions in development and physiology. Several cell lines have been reported to be capable of osteoclastic differentiation (3, 5–7). However, these cell lines are either incapable of bone resorption when cultured on bone slices or form only very occasional resorption lacunae. Where no resorption occurs, either osteoclastic differentiation is incomplete or

such cell lines are not osteoclastic at all; where resorption does occur, this is so sparse that the bone-resorbing cells generated represent only a minor subpopulation in the cultures. In either case, such cell lines do not represent a reliable or productive resource for investigation of the biochemical and molecular basis of bone resorption.

As osteoclasts are rare cells and little is known about the phenotype of osteoclast precursors, the use of *in vitro* strategies for the generation of cell lines by introduction of immortalizing oncogenes (8–12) is unlikely to prove an optimal approach to the generation of osteoclastogenic cell lines. For the immortalization of a cell as rare as the osteoclast, what is needed is a system whereby immortalization can be induced in the absence of extensive tissue culture manipulations, under circumstances that favor the immortalization of osteoclastic precursors over other cell types.

To generate osteoclastogenic cell lines, we have used the recently developed *H-2K^btsA58* transgenic mouse. These transgenic mice harbor a temperature-sensitive mutant of the simian virus 40 large tumor antigen (T antigen) under the control of γ interferon (IFN- γ)-inducible *H-2K^b* promoter. Under permissive conditions (33°C in the presence of IFN- γ), a high proportion of cells from such animals are conditionally immortalized (13–15). Such cells can be grown indefinitely in permissive conditions but are still able to undergo differentiation upon transfer to nonpermissive conditions (39.5°C in the absence of IFN- γ). We first established bone marrow stromal lines capable of inducing hematopoietic stem cells to differentiate into osteoclasts and then used the stromal cell lines to support the generation of further cell lines capable of undergoing extensive osteoclastic differentiation.

MATERIALS AND METHODS

Hepes-buffered medium (Flow Laboratories) was used for cell isolation. Incubations were performed in Eagle's minimal essential medium (MEM; GIBCO) supplemented with 2 mM glutamine, 100 units of benzylpenicillin per ml, 100 μ g of streptomycin per ml, and 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) (MEM/FBS) (all from GIBCO). Incubations were performed in humidified 5% CO₂/95% air. 1 α ,25-Dihydroxyvitamin D₃ (1 α ,25-(OH)₂D₃) was supplied by Duphar (Da Weesp, Holland) and was used at 10 μ M throughout. Salmon calcitonin (CT) was a gift from Sandoz Pharmaceutical. Bone slices (3 \times 3 \times 0.1 mm) were prepared as described (16) from bovine cortical bone. Bovine parathyroid hormone was provided by J. Zanelli (Hampstead, U.K.). Recombinant human macrophage colony-stimulating factor

Abbreviations: M-CSF, macrophage colony-stimulating factor; IFN- γ , γ interferon; BM, bone marrow; CT, calcitonin; CTR, calcitonin receptor; ts, temperature sensitive; 1 α ,25-(OH)₂D₃, 1 α ,25-dihydroxyvitamin D₃; large T antigen, large tumor antigen; FBS, fetal bovine serum.

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(M-CSF) was provided by Genetics Institute (Cambridge, MA).

Establishment of Bone Marrow (BM) Stromal Cell Lines. Transgenic mice (6–8 weeks old) were killed by cervical dislocation. Femora and tibiae were aseptically removed and dissected free of adherent tissue. The marrow cavity was flushed out with medium 199 (GIBCO). BM cells were washed twice and resuspended in MEM/FBS with 2 units of murine IFN- γ (GIBCO) at serial limiting dilution in 96-well plates. These cultures were incubated at 33°C in humidified 5% CO₂/95%. Cultures were fed with fresh medium every 3 days. After incubation for 3 weeks, the plates were inspected, and visible colonies were lifted with trypsin/EDTA. Each colony was then recloned by limiting dilution under the conditions described above.

Assessment of the Ability of Stromal Cell Lines to Support Osteoclast Formation. Stromal cell lines were harvested with trypsin/EDTA and resuspended in MEM/FBS at 5×10^4 cells per ml. Of this suspension, 200 μ l was added to the wells of a 96-well plate containing a bone slice and a 6-mm thermanox coverslip (Lux Scientific; Flow Laboratories) before incubation for 5 days at 39.5°C. Spleen cell suspensions were prepared from 7-day-old MF1 mice and added (1×10^6 cells per ml) to wells containing coverslips and bone slices in the presence or absence of 10 mM 1,25-(OH)₂D₃. Cultures were fed every 3 days by replacing 100 μ l of culture medium with fresh medium and hormone or vehicle. No attempt was made to replace nonadherent cells. After incubation, bone slices were prepared for scanning electron microscopy, and the coverslips were prepared for ¹²⁵I-labeled CT autoradiography, as described later.

Establishment of Osteoclast Cell Lines. BM cells from transgenic mice were harvested as described above and incubated at a density of 1×10^5 cells per 25-cm² flask (Falcon) in MEM/FBS in the presence of 5 ng of M-CSF per ml at 37°C. After incubation for 2 days, the flasks were washed free of nonadherent cells, and the cells were then removed from the flask by scraping with a plastic scraper (Costar). The cells were resuspended in MEM/FBS containing M-CSF in the presence of 2 units of IFN- γ per ml and incubated at 33°C. Cells were cloned by limiting dilution. For routine subculture cells were harvested by scraping the flasks with a plastic scraper. To assess the potential of the cell lines to form osteoclasts, the cell lines (10^3 cells per ml) were incubated on bone slices and coverslips with or without 1,25-(OH)₂D₃/M-CSF in MEM/FBS at either 33°C or 39.5°C for up to 14 days. Alternatively the cell lines (10^3 cells per ml) were cocultured with osteoclast-inductive stromal cell lines on bone slices and coverslips as described above but without M-CSF.

Measurement of Bone Resorption. After incubation, cells were removed from the bone slices by immersion in 10% sodium hypochlorite (BDH) for 10 min. Bone slices were then dehydrated in ethanol and sputter-coated in gold, and the surface of each bone slice was examined in a Cambridge S90 scanning electron microscope. Resorption by BM cells was quantified by using a 1-cm² grid at $\times 200$ screen magnification. Although relatively tedious, this is a more reliable marker for osteoclastic function than quantification of calcium release (17).

Phenotypic Assessment. Expression of CT receptors (CTR) was assessed by ¹²⁵I-labeled CT as described (18). CT was iodinated by a modification of the chloramine-T method (19). Labeled CT (0.2 nM) was incubated with cultures in medium 199 containing 0.1% bovine serum albumin for 1 hr at 22°C. Nonspecific binding was assessed by including excess (300 nM) unlabeled CT in some wells. After incubation, coverslips were fixed in formalin before extensive washing in water. The coverslips were coated with K5 nuclear emulsion (Ilford), developed after 14 days at 4°C, and counterstained with

Meyers hematoxylin. CTR⁺ cells were scored as those that demonstrated sufficient grain density to outline the cell clearly. The number of CTR⁺ cells present in 10 random fields per coverslip at $\times 100$ magnification was counted. [³H]Thymidine autoradiography was performed as described above except that [³H]thymidine was incubated with cultures for 2 hr at either 33°C or 39.5°C.

Macrophage differentiation was identified in acetone-fixed preparations by binding to F4/80 (S. Gordon, Oxford, U.K.) and MoMa-2 (Serotec), antibodies that bind to murine monocytes and macrophages (20) but do not bind to murine osteoclasts (21, 22); a standard immunoalkaline phosphatase staining technique (rat anti-murine and rabbit anti-rat IgG-alkaline phosphatase conjugate; both from Serotec) was used followed by counterstaining with hematoxylin.

RESULTS

BM Stromal Cell Lines. When BM cells from the *H-2K^b* transgenic mouse were cultured *in vitro*, stromal cell lines were readily established. Some of these cell lines ($\approx 30\%$) were found to possess the ability to induce spleen cells to resorb bone when cocultured on bone slices for 7 days in the presence of 1,25-(OH)₂D₃ (see Fig. 1). The most potent osteoclast-inductive stromal cell line (tsD4) was selected for further experiments. Switching of the tsD4 stromal cell line from permissive to nonpermissive conditions was associated with differentiation and a reduction in proliferation (Table 1 and Fig. 2 *a* and *b*). Most importantly, tsD4 cells cultured at 33°C in the presence of IFN- γ exhibited little or no ability to induce the generation of osteoclasts from hematopoietic cells isolated from spleen. In contrast, tsD4 cells grown at 39.5°C

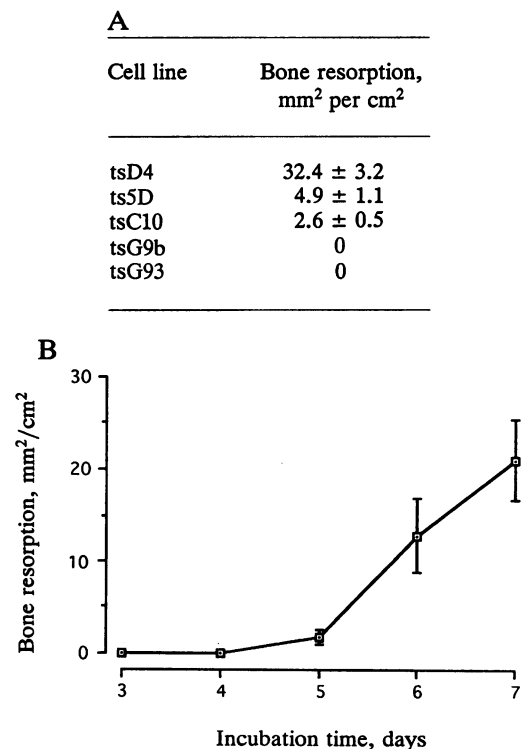


FIG. 1. Induction of bone resorption by BM stromal cell lines. (A) Cell lines from *H2K^btsA58* transgenic mice were incubated on bone slices for 5 days at 39.5°C. Spleen cells (10^6 cells per ml) were then added to the cultures and incubated at 39.5°C, together with 10 mM 1,25-(OH)₂D₃. Results expressed as mean \pm SEM of bone resorption ($n = 12$ bone slices). (B) Time course of osteoclast formation induced by tsD4 cells. Results are expressed as means \pm SEM ($n = 6$ bone slices per point). No bone resorption was observed by spleen cells alone or by any of the cell lines incubated without spleen cells.

Table 1. Conditionality of tsD4 cell behavior on temperature and IFN- γ

Culture conditions	Bone resorption, mm ² /cm ²	[³ H]Thymidine incorporation, %	Alkaline phosphatase, μ mol of pNPP per min per mg of protein
33°C/IFN- γ	0	47.0 \pm 6.6	0.1 \pm 0.1
33°C	0.9 \pm 0.5	22.6 \pm 8.3	0
39.5°C/IFN- γ	7.1 \pm 2.0	19.0 \pm 8.2	0.5 \pm 0.3
39.5°C	28.8 \pm 6.7	9.3 \pm 4.3	2.1 \pm 1.2

tsD4 cells were incubated at 33°C or 39.5°C with or without IFN- γ . Bone resorption was measured after 7 days of incubation on bone slices with 10⁶ spleen cells per ml and 10 nM 1,25(OH)₂D₃. Thymidine incorporation was measured by autoradiography after a 2-hr pulse of [³H]thymidine, 5 days after initiation of culture; alkaline phosphatase was measured 5 days after commencement of culture. Results are means \pm SEM for five cultures per variable. Control cultures of 3T3 cells showed no change in [³H]thymidine incorporation under identical conditions. pNPP, *p*-nitrophenylphosphate.

for 5 days induced the generation of sufficient numbers of osteoclasts to resorb up to 28.8 \pm 6.7 mm²/cm² of bone for every 10⁶ spleen cells added to the coculture. Growth at nonpermissive conditions (i.e., at 39.5°C with IFN- γ) was associated with 5- to 20-fold increases in activity of alkaline phosphatase, an enzyme characteristic of BM stromal cells, and with a reduction in cell division (as judged by incorporation of [³H]thymidine). To date, the tsD4 stromal cell line has been maintained in continuous culture at 33°C in the presence of IFN- γ for >12 months and has maintained throughout this time the capacity to induce bone resorption of \approx 30 mm²/cm² of bone surface per 10⁶ spleen cells.

Osteoclastogenic Cell Lines. To generate cell lines with the capacity to differentiate into osteoclasts, we first established 11 M-CSF-dependent cell lines from the BM of adult *H-2K^btsA58* transgenic mice (Fig. 2, *c* and *d*). The ability of these cell lines to generate osteoclasts was assessed by growing them in the presence of the tsD4 stromal cell line.

Of the 11 M-CSF-dependent cell lines examined, 6 were induced to generate osteoclasts by coculture with tsD4 cells at 39.5°C (Table 2). When cocultured with tsD4 stromal cells on bone slices for 14 days with 1,25-(OH)₂D₃, these 6 cell lines yielded cultures capable of resorbing bone at levels between 0.5 and 3.3 mm²/cm² per 10³ M-CSF-dependent cells added to the coculture. Moreover, in two of the resorption-competent cell lines examined further (tsA2.4 and ts4C), ¹²⁵I-CT autoradiography revealed the presence of mononuclear cells that specifically bound ¹²⁵I-CT in cultures grown with tsD4 cells at 39.5°C (Fig. 2 *e* and *f*).

Differentiation of the M-CSF-dependent cell lines into osteoclasts was dependent upon the presence of stromal cells, and no bone resorption was observed when these cell lines were incubated on bone slices alone at either 33°C or 39.5°C, even when cells were grown in the presence of 1,25-(OH)₂D₃ for 14 days. Similarly, M-CSF-dependent cell lines, grown in the absence of tsD4 stromal cells, were not labeled by ¹²⁵I-CT and thus did not apparently express CTRs. In addition, induction of differentiation required coculture of the cell lines with tsD4 stromal cells at 39.5°C; as for spleen cells, tsD4 cells grown at 33°C did not induce the appearance of bone-resorbing activity.

All M-CSF-dependent lines contained cells that were labeled by the F4/80 and MoMa-2 antibodies, two markers of macrophages. In either the presence or absence of tsD4 stromal cells at 39.5°C, the M-CSF-dependent populations contained \approx 90% F4/80⁺ cells and 90% MoMa-2⁺ cells (Fig. 2*g*). However, CTR⁺ cells were not labeled by either of these antibodies.

Further studies were conducted on the tsA2.4 cells, which exhibited the highest level of ¹²⁵I-CT labeling and were one of the most effective resorbers of bone. Under permissive conditions (33°C in the presence of IFN- γ), these cells were plastic-adherent, with a doubling time of \approx 2 days. Cells were resistant to removal from the substrate with trypsin and therefore were routinely subcultured with a plastic scraper. Growth conditionality of the tsA2.4 cells was determined by [³H]thymidine autoradiography. We found that 36.8% \pm 3.9% and 19.8% \pm 5.8% of the total population of tsA2.4 cells incorporated [³H]thymidine over a 2-hr period under permissive and nonpermissive conditions, respectively. This conditionality of growth correlated with the expression of large T antigen, detectable by immunocytochemistry (Fig. 2*h*), with strong nuclear staining of cells under permissive conditions but with little or no detectable staining after 5 days under nonpermissive conditions. At 33°C the tsA2.4 cells were also less well spread, more weakly MoMa-2⁺ positive and F4/80⁻.

DISCUSSION

Osteoclast differentiation has been shown to occur through a contact-dependent interaction between hemopoietic precursors and BM stromal cells in the presence of 1,25-(OH)₂D₃ (3, 23). Although BM stromal cell lines have been previously found (23) or developed (24) that are capable of osteoclast induction, in our experience osteoclast-inductive capacity in these cells has been limited to early passages. Therefore, as a first step in our development of cell lines able to differentiate into osteoclasts, we initially generated BM stromal cell lines with a high and sustained capacity for osteoclast induction. We found that several of the BM stromal cell lines obtained from the *H2K^btsA58* transgenic mouse provided a highly effective osteoclast-inductive environment, such that spleen cells were induced to form osteoclasts to a similar extent to that achieved by BM cell cultures (18). This stromal osteoclast-forming activity showed no evidence of decline over the duration of the present experiments (12 months). This prolonged maintenance of an original phenotype may be facilitated by the ability to suppress simian virus 40 gene expression and by the stability of integration of the immortalizing gene in the transgenic system used. Certainly, phenotype appeared to be regulated by culture conditions: under permissive conditions, the stromal cells were proliferative and unable to induce osteoclast formation from spleen cells, even though control primary cultures of BM cells were capable of osteoclast formation under the same conditions (data not shown); under nonpermissive conditions, proliferation was suppressed, and stromal osteoclast-forming activity was expressed.

Because osteoclasts form a very small proportion of the cells that develop in hemopoietic cultures or in cultures of spleen cells on tsD4 cells, it did not seem an optimal approach to the generation of osteoclasts to incubate unpurified populations of hemopoietic cells on the tsD4 cells—only a very small proportion of cell lines so induced would be likely to be osteoclastic. Instead, we exploited the known dependence of osteoclastic precursors on M-CSF (25–28): incubation of hemopoietic cells in M-CSF at low density induces proliferation of cells that express M-CSF receptors (29), which are precursors of macrophages and osteoclasts (21). We found that some of the cell lines so obtained showed various degrees of ability to form bone-resorbing cells when cocultured with tsD4 cells under nonpermissive conditions.

The proportion of cells expressing osteoclastic resorptive function in our cell line cultures was several orders of magnitude greater than has previously been achieved from cell lines. From HL-60 cells, very occasional excavations have been reported (7); we found \approx 4 mm² of excavation per

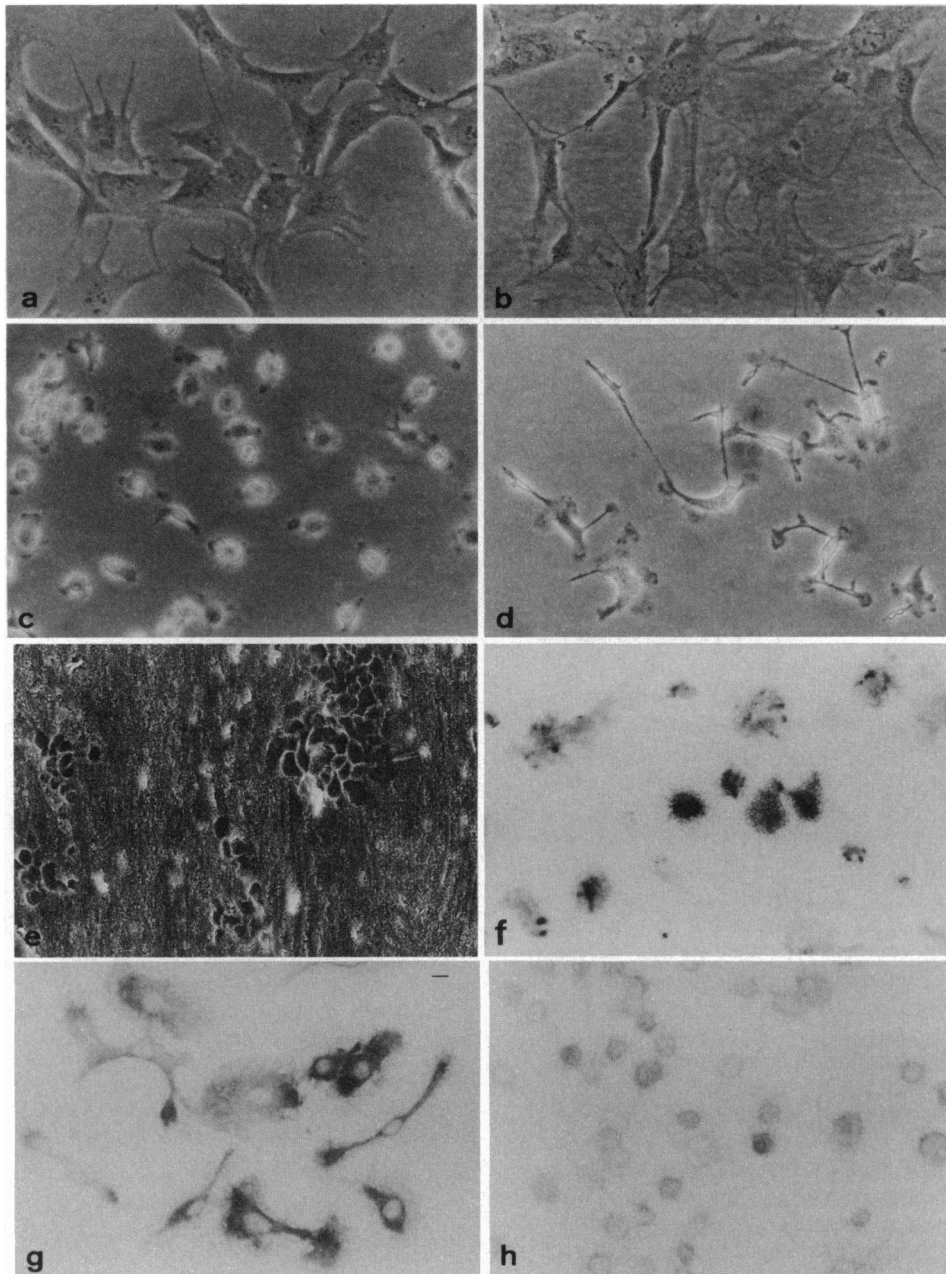


FIG. 2. (a and b) tsD4 cells after incubation at 33°C with IFN- γ (a) and at 39.5°C without IFN- γ (b) for 4 days. Incubation at the nonpermissive temperature is associated with increased spread area. (Phase contrast; $\times 625$.) (c and d) ts4C cells have been incubated in M-CSF at 33°C with IFN- γ (c) or at 39.5°C without IFN- γ (d). Nonpermissive culture conditions are associated with increased cell spreading. ($\times 625$.) (e) Bone slice after coculture of tsD4 cells with ts4C cells shows excavations in bone surface that are characteristic of osteoclastic functional activity. ($\times 155$.) (f) Incubation of cocultures with ^{125}I -labeled CT (^{125}I -CT) followed by autoradiography and immunocounterstaining reveals induction of ^{125}I -CT-binding cells by tsD4 cells three densely-grained cells at the center. (Immunoalkaline phosphatase labeled with MoMa-2; $\times 550$.) (g) ts4C cells labeled by MoMa-2 antibodies after 4 days with M-CSF at 39.5°C. (Immunoalkaline phosphatase, no counterstain; $\times 850$.) (h) ts4C cells incubated at 33°C in the presence of M-CSF and IFN- γ and exposed to the antibody to large T antigen. ($\times 850$.)

5×10^5 cells in proliferative cultures of FDCP cells (28) compared with the 50- to 300-fold higher levels of activity yielded by the transgenic cell lines. Indeed, the extent of resorption suggests that a considerable proportion of the cells in osteoclastogenic cultures resorbed bone. We generally find that when spleen cells are incubated on tsD4 cells, a plan area of up to $\approx 30 \text{ mm}^2$ is resorbed per 10^6 spleen cells added (30), 2 orders of magnitude less than that of the transgenic cell lines ($0.5\text{--}3 \text{ mm}^2$ per 10^3 cells) despite limited proliferative capacity in the latter. CTR autoradiography suggested that 5–8% of cells present after incubation are osteoclastic. Under similar experimental conditions we find $\approx 10^5 \mu\text{m}^2$ of surface

excavation per CTR $^+$ cell in BM (18), a figure very similar to that achieved by tsA2.4 cells. This suggests that, although the cell lines produce both osteoclasts and macrophagic cells, bone resorption can be accounted for by a subpopulation of CTR $^+$ cells of full resorptive capacity. The osteoclastogenic cell lines have remained proliferative through many passages for at least 9 months in IFN- γ at 33°C. They remain M-CSF-dependent unless cocultured with tsD4 cells, which may act in common with other BM stromal cells (31, 32) as a source of M-CSF.

The cells in our cultures that did not differentiate into osteoclasts differentiated instead into macrophages, as

Table 2. Bone-resorbing capacity of M-CSF-dependent cell lines

Cell line	Bone resorption, mm ² /cm ²	CTR ⁺ cells, no. per cm ²	Hemopoietic cells, no. per cm ²
ts50C	3.3 ± 1.4	ND	ND
ts4B	0.5 ± 0.3	ND	ND
ts4C	1.7 ± 0.3	15 ± 8	390 ± 250
ts4D	3.0 ± 1.0	ND	ND
tsA2.4	3.0 ± 1.2	36 ± 21	450 ± 250
ts4D5	0.9 ± 0.3	ND	ND
ts1Ad	0	0	810 ± 300
tsD IIA	0	ND	ND
tsC9II	0	0	570 ± 300
ts4C1	0	ND	ND
tsH7	0	ND	ND

To test for bone-resorbing capacity, M-CSF-dependent cell lines (10³ cells per well) were cocultured with tsD4 stromal cells at 39.5°C on bone slices for 14 days in the presence of 10 nM 1,25-(OH)₂D₃. Results are expressed as means ± SEM of at least five cultures. Some cell lines were also tested for CTR expression by similarly incubating M-CSF-dependent cell lines with tsD4 cells on Thermanox coverslips for 14 days before ¹²⁵I-CT autoradiography (five coverslips per cell line). Hemopoietic cells were recognized as relatively small, round, or fusiform cells morphologically readily distinguishable from tsD4 stromal cells. ND, not done.

judged by labeling with F4/80 and MoMa-2 antibodies. We suggest that this observation is consistent with the view that osteoclasts and at least some macrophages may be derived from a common precursor cell. We do not believe that osteoclasts in our cultures were derived from macrophages because ¹²⁵I-labeled CTR⁺ cells were consistently MoMa-2⁻ and F4/80⁻. In addition, the cell lines that generated osteoclasts were F4/80⁻ and only weakly MoMa-2⁺ when grown at 33°C in the presence of IFN-γ, suggesting that expression of a macrophage phenotype also required further differentiation. In addition, several of the cell lines we derived only gave rise to macrophages and thus may serve as a valuable tool for helping to distinguish between those precursors that can generate osteoclasts and those that cannot do so.

Our results suggest that the cell lines we have generated consist of precursors that are not yet committed between osteoclasts and macrophages. Thus, the cell lines should be useful not only for investigation of the function of mature osteoclasts but also, together with the stromal cells, for the identification of the signals that regulate the commitment of osteoclast precursors, including the nature of stromal osteoclast-forming activity itself, which cannot be replaced by any known cytokine so far tested, including c-kit ligand; interleukins 1, 3, 6, 7, 9, and 11; transforming growth factor β; or M-CSF (data not shown). These studies also demonstrate clearly the potential usefulness of *H-2K^btsA58* transgenic mice in the isolation of cell lines capable of undergoing differentiation, even when the precursor cells of interest are rare and cannot be readily distinguished by available markers. Moreover, they demonstrate the relative ease with which it is possible to generate from *H2K^btsA58* mice a complete differentiation system, including both inducing and responding cells, in the absence of retroviral (or otherwise mediated) insertion of immortalizing genes *in vitro*.

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