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Interleukin 4 Increases Type 5 Acid Phosphatase mRNA Expression in Murine Bone Marrow Macrophages

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Abstract Type 5 acid phosphatase is a lysosomal enzyme expressed in cells of monocyte/macrophage lineage frequently used as a marker of osteoclastic differentiation. Oligonucleotide primers for DNA amplification were designed following sequence alignment of rat bone and human macrophage type 5 acid phosphatases. DNA (330 bp in length) obtained using these primers and reverse transcribed total cell RNA from in vitro generated murine osteoclastic cells was cloned and sequenced. DNA sequence analysis of two clones demonstrates that the amplified material was 91% and 96% identical to rat bone type 5 acid phosphatase at the nucleotide and amino acid level, respectively. Northern blots of murine tissue RNA show the presence of 1.5-kb transcripts that are most highly expressed in the long bones. Total cell RNA from the osteoclastic cells contain a marked level of type 5 acid phosphatase mRNA when compared to the levels seen in the tissue samples. Additionally, osteoclastic cell RNA contains two additional transcripts of 2.5 and 5 kb. Bone marrow macrophages grown in the presence of M-CSF express low levels of the 1.5-kb transcript with no signal observed for either of the two larger transcripts that were seen in the osteoclastic RNA samples. Importantly, bone marrow macrophage 1.5-kb type 5 acid phosphatase transcript levels are increased by interleukin 4 treatment in both a time and concentration-dependent manner. These findings indicate that type 5 acid phosphatase, while a cytochemical marker for osteoclasts, can be induced in macrophages by agents that block in vitro osteoclastic differentiation. Increased type 5 acid phosphatase may play a role in interleukin 4-stimulated monocyte activities.

Key words: vitamin D, glucocorticoids, polymerase chain reaction, osteoclasts, M-CSF Abbreviations: TRAP, tartrate resistant acid phosphatase; RT-PCR, reverse transcription-polymerase chain reaction; IL-4, interleukin 4

INTRODUCTION

The expression of type 5 (tartrate-resistant) acid phosphatase (TRAP) is frequently utilized as a marker of osteoclast differentiation and function [Minkin, 1982]. This enzyme, also expressed by monocyte-macrophages, is found at high levels in patients with Gaucher's disease [Robinson et al., 1980] and hairy cell leukemia [Yam et al., 1972]. Amino acid sequences obtained from TRAPs purified from a variety of tissues and cells suggest that TRAP activity may be expressed by a number of distinct, yet highly related, proteins [Ek-Rylander et al., 1991]. Recently, cDNAs have been identified for a number of different TRAP enzymes including both human macrophage TRAP [Lord et al., 1990] and rat bone TRAP [Ek-Rylander et al., 1991], enzymes thereby classified as type 5 acid phosphatases. When used as probes, these sequences may prove useful as markers of cellular differentiation. In fact, an early study of human peripheral blood monocyte type 5 TRAP gene expression indicates that TRAP mRNA increases during in vitro differentiation and is sensitive to macrophage-activating treatments [Bevilacqua et al., 1991]. During in vivo osteoclast formation, it appears that TRAP expression (detected histochemically) first occurs in mononuclear cells near the bone surface prior to the appearance of osteoclasts (multinucleated TRAP+ cells) [Van de Wijngaert et al., 1986; Baron et al., 1986]. These mononuclear TRAP+ cells are considered to be osteoclast precursors and such cells increase in parallel with osteoclast-like cells in in vitro osteoclast-generating culture systems [Udagawa et al., 1989].

Our laboratory has been examining the impact of IL-4 on in vitro murine osteoclast differentiation and we have found that IL-4 profoundly suppresses osteoclast generation [Shioi

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et al., 1991]. Because TRAP expression likely appears in mononuclear cells prior to their fusion to become osteoclasts, we wished to determine the impact of IL-4 on TRAP gene expression by osteoclast progenitor cells. This report describes the reverse transcription-polymerase chain reaction (RT-PCR) cloning of a portion of murine osteoclast TRAP that is highly homologous to rat bone TRAP. Furthermore, we show that mRNA for this enzyme is highly expressed in murine bone when compared to other tissues. In contrast to its suppressive effects on osteoclast generation, IL-4 promotes the expression of TRAP mRNA levels dose- and time-dependently in bone marrow macrophages. Histochemically, TRAP staining is increased in IL-4-treated bone marrow cell cultures. These findings indicate that bone marrow macrophage TRAP mRNA induction may be induced by factors that do not necessarily promote osteoclast formation and suggest that increased TRAP levels may be involved in IL-4-stimulated monocyte activities.

METHODS

Cytokines

Purified murine M-CSF (stage 1) was prepared from serum-free, L929 cell supernatants as previously described [Stanley et al., 1983] and was provided by Dr. Steven Teitelbaum (Washington University, St. Louis, MO). Recombinant interleukin 4 was expressed and purified from baculovirus transfected SF-9 (spodoptera frugiperda) cell culture supernatants as described (Ohara et al., 1987) (Ohara, 1989) and was provided by Dr. Jun-ichi Ohara. Vitamin D_3 metabolites were the kind gift of Milan Uskokovic (Hoffmann-LaRoche) and were dissolved as concentrated stock solutions in absolute ethanol and stored in the dark at −20°C.

Bone Marrow Cell Preparation

Murine bone marrow cells were prepared by a modification of a method described previously [Tushinski et al., 1982]. Briefly, the bone marrow of C3H/HeN mice (4-10 weeks of age) (Harlan-Sprague Dawley, Indianapolis, IN) was flushed from femurs and tibiae with ice-cold α-minimal essential media (α-MEM). The cells were collected, pelleted, resuspended in α-MEM supplemented with 10% heat-inactivated fetal calf serum (HIFCS) containing 500 U/ml of stage 1 CSF-1 (50 ml media/8 bones/150 mm tissue culture dish) and subsequently incubated for 24 h (37°C, 5% CO_2). The nonadherent cells were then collected, pelleted (1,000 rpm, 7 min, 4°C) and resuspended (1×10^7 cells/ml) in α-MEM and layered onto 15 ml of cold Ficoll-Hypaque (d = 1.077) and centrifuged at 200*g* for 15 min at room temperature. The cell suspension from the top of the gradient was carefully removed, washed with α-MEM, centrifuged (200*g*, 10 min, 4°C), and the cell pellet suspended in α-MEM containing 10% HIFCS (day 1 cells). To obtain adherent M-CSF responsive macrophages, these cells we cultured at $0.5–1 \times 10^6$ cells/ml/2 cm^2 for various periods with α-MEM containing 10% HIFCS and 2000 U/ml M-CSF. To obtain osteoclast-like cells, the day 1 bone marrow cells were cocultured with ST2 cells (stromal cell line, RIKEN Cell Bank, Tsukuba, Japan) for 10 days as described [Shioi et al., 1991]. TRAP activity was identified histochemically using a kit (Sigma, St. Louis, MO). To remove stromal cells, collagenase P (Boehringer-Mannheim, Indianapolis, IN) was diluted (1 mg/ml) in α-MEM and then added to the osteoclast-containing cocultures (2 h, 37°C). The cells were then gently rinsed with media or Dulbecco's phosphate-buffered saline.

Partial Murine TRAP Cloning

To identify potential sites in the previously cloned rat [Ek-Rylander et al., 1991] and human [Lord et al., 1990] sequences suitable for PCR primers, the sequences were aligned with the use of a computer program (GeneJockey Sequence Processor, BIOSOFT, Cambridge, England). While a number of areas demonstrated stretches where the cDNAs approached 100% identity at the nucleotide level, we selected two sites that predicted a PCR product of 330 bp. The 5′ primer (359-372; 5′-CAATGACAAGAG-GTTCCAGGAGAC-3′) contains the lysosomal targeting sequence DKRFQ. The 3′ primer (638-609; 5′-TGTTTTTTGAGCCAGGACAGCTTG-3′) is in a region of high homology between the human macrophage and rat bone TRAP (1 mismatch at 633). At the amino acid level, the 3′ region is also highly homologous with human placental TRAP, but less so with porcine uteroferrin, and bovine spleen TRAP [Ek-Rylander et al., 1991]. Both primers were obtained from Genosys (Woodlands, TX).

Total cell RNA from murine osteoclastlike cells generated in vitro was purified (for method see below) from cultures following their collage-

nase treatment (to remove stromal cells). First strand synthesis utilizing random hexamer primers and total cell RNA (2 μg, total volume, 50 μl) was performed as described [Lacey et al., 1993]. An aliquot of this cDNA (5 μl) was then used for PCR under the following conditions: Buffer: 20 pmol 5′ and 3′ primers, 1× Taq buffer, 400 μM dNTP's, 2.5 mM MgC12, 1 U Taq polymerase, (total volume, 50 μl); Thermocycler (Perkin-Elmer, Norwalk, CT) settings: 95°C (3 min)-[95°C (45 sec), 55°C (1 min) −72°C (2 min) × 30 cycles −72°C (7 min)]. An aliquot of the amplified DNA was ligated into Invitrogen's (San Diego, CA) TA PCR cloning vector according to their instructions. Two of the isolated clones were PCR sequenced using ^{35}S-dCTP and a direct incorporation method using a kit (fmol Sequencing Kit, Promega, Madison, WI). The resulting sequence was aligned with the human and rat cDNA sequences and the deduced amino acid sequence derived using the software noted above.

RNA Isolation and Northern Analysis

Total cell RNA from tissues and cells was isolated utilizing guanidinium isothiocyanate extraction (Brinkmann Polytron, Westbury, CT, 10-s generator, setting 7, 30 sec) and cesium chloride (5.7 M) centrifugation (Beckman, Arlington Heights, IL, TL100 ultracentrifuge, TLS55 rotor, 55,000 RPM, 2.5 h, 22°C) [Chirgwin et al., 1979]. Prior to the cesium chloride gradient step, the tissue extracts were centrifuged at 500*g* for 10 min, to remove debris. Total cell RNA was quantified by absorbance spectroscopy (1 A_{260} = 40 μg/ml), samples electrophoresed in 1% agarose gels under denaturing conditions [Chaplin et al., 1983], and the gels electroblotted onto Zeta probe membranes (BioRad, Richmond, CA) in a Transblot apparatus (BioRad) according to the manufacturer's instructions. DNA to be labeled for the TRAP probe was generated via PCR utilizing the TRAP sequence-containing vector as a template and TRAP primers using the same conditions noted above. Following electrophoresis in low melting temperature agarose (FMC, Rockland, ME), the DNA in the excised band was isolated using a kit (Magic PCR Preps, Promega, Madison, WI). Aliquots (25 ng) of this material were labeled to high specific activity (10^8–10^9 cpm/μg) using ^{32}P-dCTP (3,000 Ci/mmol, ICN, Irvine, CA) and a random priming method (Prime-a-Gene Kit, Promega). Prehybridization, hybridization, and rinsing (all at 56°C) were performed as recommended by the membrane's manufacturer (Bio-Rad). For autoradiographs, the blots were exposed to Kodak XAR film at −80°C prior to developing.

RESULTS

Using the TRAP primers and murine osteoclast-like cell RNA, we identified a single amplified band following RT-PCR on agarose gels that migrated close to the distance predicted by the primers' positions in the rat bone TRAP sequence (~330 bp, data not shown). Subsequently, DNA from the PCR reaction was ligated into a PCR cloning vector and two independent clones were sequenced yielding identical results. As shown in Figure 1A, the murine osteoclast, rat bone and human macrophage TRAP nucleotide sequences are similar within the amplified area with the murine sequence having an overall 91% and 84% homology with the rat bone and human macrophage TRAPs, respectively. When assessed at the amino acid level (Fig. 1B), the murine osteoclast TRAP sequences are 96% and 82% homologous with the rat and human TRAP proteins, respectively. The few differences between the murine and rat sequences reflect conservative substitutions except for the positively charged lysine at position 154 that in the rat is a negatively charged amino acid, glutamic acid.

Next, the murine sequence was used as a probe for Northern blots of total RNA obtained from a number of tissues as well as from the in vitro generated osteoclastic cells. As shown in Figure 2, the strongest signal from the tissue samples was observed in the long bones with detectable signals observed in all other sites excepting the brain and heart. In these tissues, the hybridizing band migrates just ahead of the 18s RNA consistent in size (1.5 kb) with the transcripts observed in both human [Lord et al., 1990] and rat material [Ek-Rylander et al., 1991]. The RNA from the murine osteoclast-like cells gave a hybridization signal greatly exceeding that observed in any of the tissue samples indicating that TRAP mRNA is highly expressed by these cells. In this sample, two additional, less intense, bands were observed that migrated just above and below the 28s RNA consistent with the presence of larger transcripts of ~5 and 2.5 kb. These larger species may represent distinct, yet highly related mRNAs or they may result from alternative splicing of the same gene prod-

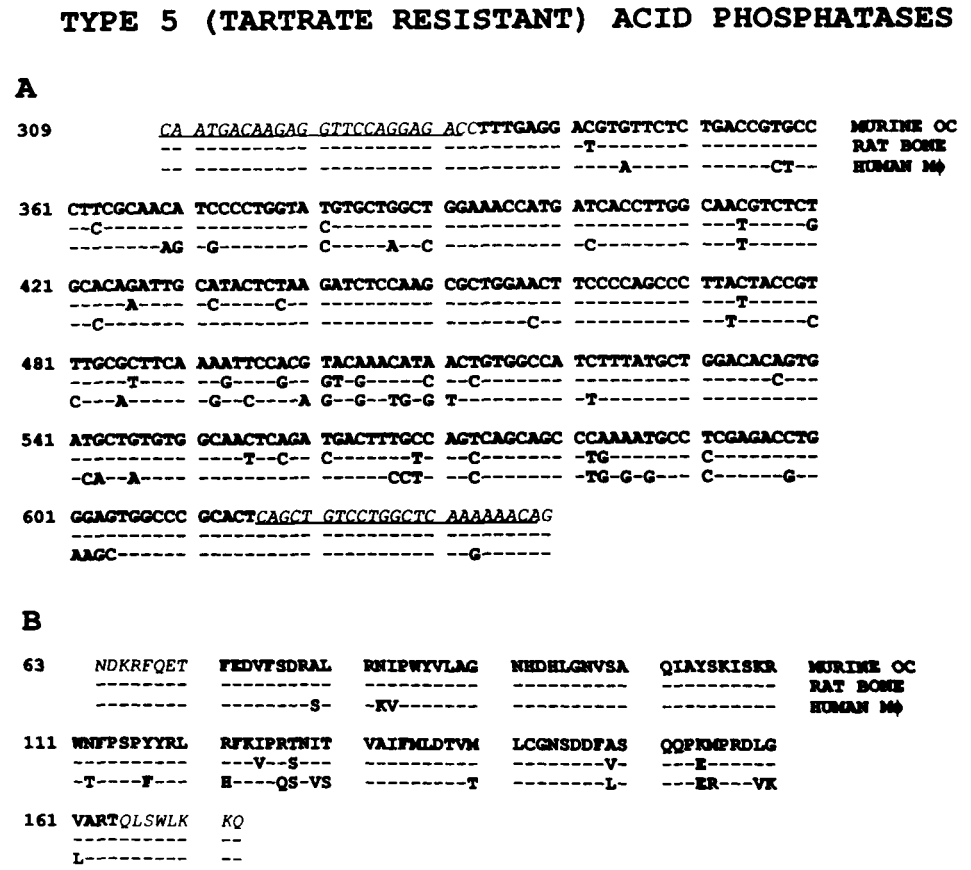

Fig. 1. Nucleotide and predicted amino acid sequences of murine osteoclastic TRAP. The numbers displayed are based on the previously published rat bone cDNA sequence (6). In A, the murine TRAP nucleotide sequence is compared to rat bone TRAP and human macrophage TRAP. The regions used to design the PCR primer sequences are underlined and italicized. In B, the deduced amino acid sequences for the three different species' TRAPs are presented. The amino acids from the region used to design the primers are italicized.

uct. Whether these mRNAs are unique to the osteoclastic cells or are observed because of the marked level of TRAP mRNA expression is unknown.

The major impetus for this project was to determine whether TRAP expression was inhibited in osteoclast progenitors by interleukin 4, as we have observed that this cytokine inhibits osteoclast generation in an in vitro model of osteoclast formation [Shioi et al., 1991]. To address this question, bone marrow cells were cultured in the presence of M-CSF, a growth and differentiation factor for monocyte/macrophages, for 4 days. The resultant macrophage population can develop into osteoclast-like cells when cocultured with ST2 cells and 1,25 dihydroxyvitamin D3 and dexamethasone (data not shown) indicating that they function as osteoclast progenitors. Figure 3 shows the results of an experiment where day 4 bone marrow macrophages were treated for various periods up to 48 h with 200 U/ml IL-4. Total cell RNA was collected and analyzed by Northern blotting using the ^{32}P-TRAP probe. Murine bone marrow macrophages express transcripts similar in size to that observed in the tissue RNA samples ($\sim$1.5 kb). The larger TRAP transcripts were not observed, possibly due to the much lower level of TRAP mRNA expression generally exhibited by the macrophages that we grew in culture. Importantly, IL-4 augmented the level of TRAP mRNA in a time-dependent fashion with increases in TRAP mRNA routinely detectable by 24 h and in some experiments as early as 8 h of cytokine exposure. The maximal IL-4 effect was observed at 48 h.

In the next series of experiments, the concentration-dependence of IL-4's effect on TRAP

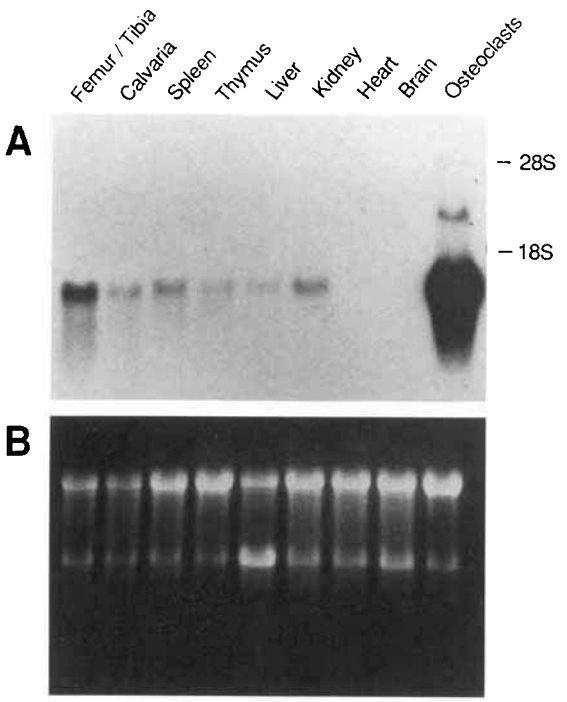

Fig. 2. Autoradiograph of tissue and osteoclast Northern blot hybridized with the murine TRAP probe. Total RNA from tissues of an 8-week-old C3H/HeN mouse and murine osteoclastic cells were isolated as described and aliquots (15 μg) used to prepare Northern blots. The blot was hybridized with the ^{32}P-labeled TRAP probe described in Figure 1 and rinsed. **A,B:** The autoradiograph and the ethidium bromide stained gel are exhibited, respectively.

mRNA levels was assessed. Total cell RNA was harvested from day 4 bone marrow macrophages treated for 48 h with various amounts of IL-4. Northern blots were prepared and probed with the ^{32}P-TRAP probe. As shown in Figure 4, the IL-4 effect on TRAP expression was dose dependent with detectable effects observed at 10 U/ml IL-4 and the maximal effect attained with 200 U/ml IL-4. To determine whether the IL-4 treated bone marrow cells expressed more TRAP activity, day 4 bone marrow macrophages were treated for 48 h with 200 U/ml IL-4 and then assessed for TRAP activity. Using enzyme histochemistry with napthol-AS-B1 phosphoric acid as the substrate and fast garnet as the coupler, IL-4 treated cells stained more darkly than untreated controls (Fig. 5) indicating that they expressed increased TRAP activity.

DISCUSSION

In this paper, we report a partial sequence that likely represents the murine equivalent of type 5 acid phosphatase (TRAP), a monocytic lysosomal enzyme previously cloned from rat and human tissues and cells. First, the nucleotide and predicted amino acid sequences of the amplified material are highly homologous to both the rat and human enzymes (Fig. 1). Second, the TRAP transcripts are most highly expressed in bone and are markedly expressed by murine

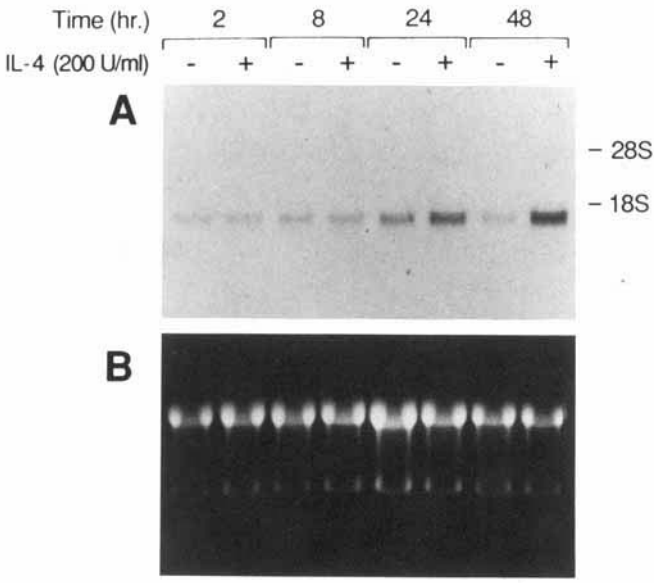

Fig. 3. IL-4 increases murine bone marrow macrophage TRAP mRNA expression in a time-dependent manner. Murine bone marrow macrophages were grown in the presence of CSF-1 for 4 days and then treated with IL-4 (200 U/ml) for various additional periods up to 48 h. Total cell RNA (10 μg) was used to make Northern blots that were hybridized with the TRAP probe as described. **A, B:** the autoradiograph and the ethidium bromide stained gel are exhibited, respectively.

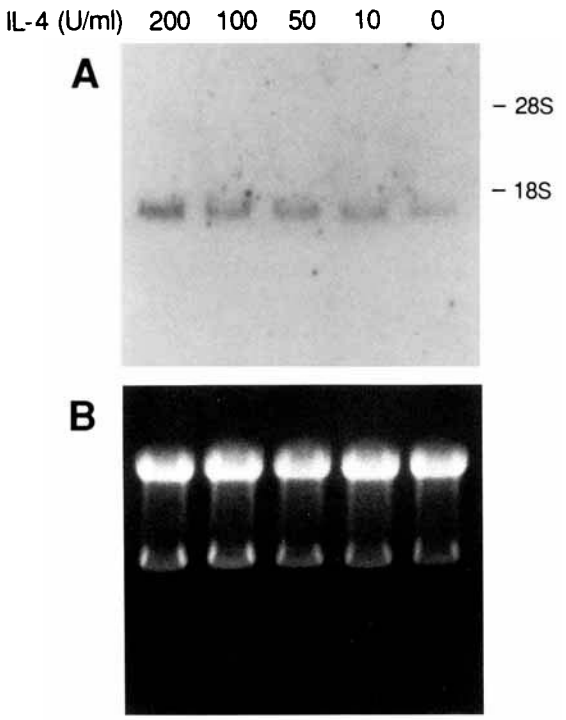

Fig. 4. IL-4 increases murine bone marrow macrophage TRAP mRNA expression in a dose-dependent manner. Murine bone marrow macrophages were grown in the presence of CSF-1 for four days and then treated with various concentrations of IL-4 for 48 additional hours. Total cell RNA (10 μg) was used to make Northern blots that were hybridized with the TRAP probe as described. **A, B:** the autoradiograph and the ethidium bromide stained gel are exhibited, respectively.

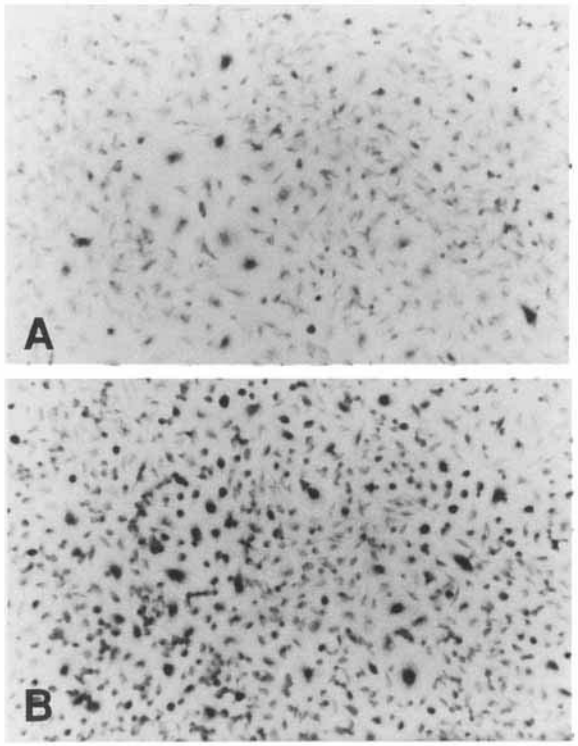

Fig. 5. IL-4 increases histochemically detectable TRAP activity in bone marrow macrophages. Murine bone marrow macrophages were cultured on glass coverslips as in Figure 4, except that the cells were treated with and without 200 U/ml IL-4 for the entire 48 h. The coverslips were then stained for TRAP activity as described and photomicrographs (×100) taken. **A,B:** The control and IL-4-treated cells are shown.

osteoclastic cells, similar to rat bone TRAP [Ek-Rylander et al., 1991]. Third, the major murine transcript that hybridized with our TRAP probe was 1.5 kb in length, which is the size of the TRAP transcript observed in human macrophage RNA [Lord et al., 1990] and the size of the largest transcript observed in rat bone RNA [Ek-Rylander et al., 1991]. The smaller transcripts noted in rat bone RNA were not observed in either the murine tissue or cell RNA blots. Finally, the murine TRAP probe recognized a 1.5-kb mRNA in human monocyte RNA (data not shown).

The expression of type 5 acid phosphatase by cells of the monocytic lineage depends on the macrophage source and, in in vitro studies, type of treatment. In addition to osteoclasts, specialized-bone resorbing cells of monocytic lineage, alveolar macrophages also express appreciable amounts of this enzyme [Lord et al., 1990]. While the function(s) of this enzyme in the cellular activities performed by these cells in these two quite dissimilar tissues is unclear, both cells are highly phagocytic suggesting that increased TRAP levels may simply reflect a general increase of lysosomal enzyme content. In in vitro studies of human monocytes [Bevilacqua et al., 1991], TRAP mRNA levels are quite low in freshly isolated cells but the level of expression increases with time. Additionally, exposure of the cells to either endotoxin or interferon gamma appears to inhibit the expression of TRAP mRNA.

In conditions leading to the formation of osteoclasts, TRAP appears to be highly expressed in mononuclear, putative osteoclast precursor cells prior to their fusion [Baron et al., 1986; Van de Wijngaert and Burger, 1986]. These observations indicate that TRAP induction is part of the osteoclast differentiation pathway. Based on this line of reasoning, we were surprised to see that a treatment (IL-4) that, in other circumstances, inhibits in vitro osteoclast formation [Shioi et al., 1991] actually increased TRAP mRNA in bone marrow macrophages. This observation may well be due to differences in the culture

systems employed. In the osteoclast generating experiments, bone marrow cells are combined with a stromal cell line in the presence of 1,25 dihydroxy-vitamin D3 and dexamethasone. Clearly this system generates osteoclastic cells [Shioi et al., 1991] that express high levels of TRAP (Fig. 2). In contrast, the experiments reported here were done with homogeneous monocyte/macrophage cells in the absence of stromal cells and the steroids. Importantly, we never observed the same level of TRAP mRNA expression by IL-4 treated monocyte/macrophages as we observe in the osteoclastic cells. Furthermore, the two larger transcripts seen in the osteoclastic cell RNA were never seen in the RNA from the IL-4 treated cells. While the potential role of increased TRAP expression in interleukin 4-directed monocyte activities is unknown, IL-4 is expressed by a subset of T-helper cells (type 2) [Mosmann et al., 1989] that appear to be involved in host defenses against parasitic infections suggesting that TRAP may play a role in mediating this host response. In any event, the capacity for IL-4, an osteoclast inhibitory factor, to augment the expression of TRAP in putative osteoclast progenitors further underscores the potential unreliability of TRAP expression [Chambers, 1989] as a selective marker of osteoclast differentiation. Therefore, TRAP induction, while being maturation-dependent, occurs in monocytes developing along multiple as opposed to singular differentiation pathways.

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