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Enzymes and Protein Markers

Clinical Significance of Immunoassays for Type-5 Tartrate-resistant Acid Phosphatase

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Background: **Tartrate-resistant acid phosphatase (TRAP; EC 3.1.3.2) is a product of osteoclasts and a biochemical marker of bone resorption rate. However, erythrocytes and platelets contribute to total TRAP activity in serum, reducing the specificity of direct biochemical assays in serum. Osteoclast TRAP is also known as type-5 TRAP and is antigenically unique. Immunoassays are sought to improve the specificity and sensitivity of TRAP as a bone marker.**

Methods: **We developed two colorimetric microplate assays for type-5 TRAP: an enzyme capture immunoassay to measure antibody-bound enzymatic activity, and a two-site immunoassay to measure bound enzyme protein. Both use the same monoclonal antibody (14G6) to capture type-5 TRAP, which permits determination of specific activity of serum TRAP in health and disease.**

Results: **Both TRAP assays were linear from one-tenth to fivefold the mean value in 18 healthy subjects. In these subjects, the mean (SD) TRAP activity was 3.2 (0.54) U/L for the enzyme capture assay and 37 (13) μg/L for the two-site assay. Mean TRAP activity was not significantly increased in 64 patients with endstage renal disease requiring hemodialysis (HD) or 99 unselected patients with rheumatic diseases. By contrast, TRAP protein was increased in both the HD and rheumatic disease groups. The specific activity of TRAP in the 17 of 64 HD sera that had increased TRAP activity (0.088 U/μg) was similar to that in healthy subjects (0.091 U/μg). By contrast, the specific activity of TRAP in the 31 of 99 rheumatic sera with increased TRAP protein (0.035 U/μg) was significantly decreased.**

Conclusions: **Wide sample distributions for TRAP activity in HD patients and TRAP protein in rheumatic disease patients suggest the presence of subpopulations of HD patients with increased TRAP activity and of rheumatic patients with increased TRAP protein. Each assay for TRAP activity and protein may have its own biological significance and clinical applications in specific groups of patients.**

Healthy bone is remodeled continuously, requiring a balance of bone formation and bone resorption. Excessive bone growth without balanced resorption leads to conditions of osteopetrosis. Conversely, when bone resorption outpaces bone formation, osteoporosis and risk for bone fracture result. It is advantageous to use biomarkers of bone metabolism to study the pathogenesis and mechanisms of various bone diseases and to monitor treatment *(1, 2)*. For bone matrix formation and osteoblast activity, N- and C-terminal peptides of procollagen, osteocalcin, and alkaline phosphatase are markers *(3)*. For bone matrix resorption, tests for pyridinoline cross-links and N- and C-terminal cross-linked collagen peptides in urine and serum are available *(4)*. The only known marker for osteoclast activity is tartrate-resistant acid phosphatase (TRAP[5]; EC 3.1.3.2) *(5, 6)*.

TRAP is also known as "type-5" acid phosphatase because of its rapid migration in native, acidic polyacrylamide gel electrophoresis. Serum biochemical assays for TRAP have been used for many years but lack absolute specificity *(7)*. Erythrocytes and platelets release TRAP unrelated to that of bone into serum. Serum TRAP activity assays may also lack sensitivity. Type-5 TRAP is considered unstable; thus, some of the circulating enzyme protein has no activity. To increase the sensitivity and spec-

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[5] Nonstandard abbreviations: TRAP, tartrate-resistant acid phosphatase; ESRD, endstage renal disease; HD, hemodialysis; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; PBST-E, PBS containing 0.5 mL/L Tween 20, 10 mmol/L EDTA, and 20 mL/L glycerol; RBC, red blood cell; and pNPP, *p*-nitrophenyl phosphate.

ificity of TRAP assays, immunoassays that use specific antibodies to type-5 TRAP are replacing whole serum biochemical assays *(8)*. Some assays measure the type-5 TRAP activity *(9, 10)*, whereas others measure the quantity of TRAP protein *(10–14)*. It is not yet certain which of these approaches provides the greatest clinical sensitivity and specificity for all metabolic bone diseases. To this end, we developed immunoassays for type-5 TRAP activity and protein that use the same antibody to capture TRAP from serum. After standardization, these assays were compared directly for their clinical sensitivity in two groups of patients at risk for increased bone turnover; those with endstage renal disease (ESRD) requiring chronic hemodialysis (HD) and those with rheumatic diseases.

Materials and Methods

ANTIBODIES

Monoclonal anti-TRAP antibody 14G6. Antibody 14G6 was raised against purified TRAP from the spleens of patients with hairy cell leukemia. It reacts with a native epitope present on osteoclasts and macrophage TRAP *(15)*. The 14G6 IgG was conjugated to biotin by a kit method (Pierce Chemical). The protein concentration of the conjugate was estimated by the Bradford method *(16)*, using a commercial reagent (Bio-Rad Laboratories). The 14G6 IgG was conjugated to activated horseradish peroxidase (HRP; Zymed Laboratories) according to manufacturer's protocols. The concentration of IgG-HRP used in experiments was based on the assumption of full recovery of IgG after conjugation and dialysis. The 14G6 IgG was conjugated to AminoLink Plus agarose (Pierce) at 2.5 g IgG/L agarose according to the manufacturer's directions to prepare a solid-phase immunoprecipitating gel.

J1B and O1A anti-TRAP antibodies. Antibodies J1B and O1A were raised against pure human bone TRAP. Each reacts with a distinct epitope specific to human osteoclasts and macrophage TRAP *(17)*. J1B and O1A IgGs were also conjugated to biotin and HRP.

CALIBRATORS

The total serum TRAP assay and the enzyme capture immunoassays were calibrated against dilutions of *p*-nitrophenolate equivalent to 0.16–10 U (1 U = μmol of substrate hydrolyzed per min per liter of sample at 37 °C) in sample dilution buffer consisting of phosphate-buffered saline (PBS; 6.4 mmol/L Na_2HPO_4-0.9 mmol/L KH_2PO_4-137 mmol/L NaCl-2.7 mmol/L KCl, pH 7.2), containing 0.5 mL/L Tween 20, 10 mmol/L EDTA, and 20 mL/L glycerol (PBST-E). Calibrators for the two-site immunoassay were dilutions of affinity-purified TRAP protein containing 1.6–100 μg/L TRAP in sample buffer containing 100 g/L bovine albumin. TRAP was partially purified from the spleens of patients with hairy cell leukemia through the phenyl-Sepharose stage *(18)*. This preparation was then passed over a column of 14G6 antibody immobilized to AminoLink Plus (Pierce). TRAP protein was eluted with 0.1 mol/L glycine-HCl, pH 2.4, and immediately neutralized with 0.1 volume of 1.0 mol/L Tris, pH 8.0. The affinity-purified TRAP was dialyzed exhaustively against 25 mmol/L Tris-75 mmol/L NaCl, pH 7.5 (0.5× Tris-buffered saline) containing 50 mL/L glycerol and concentrated by centrifugal evaporation to 100 mg/L.

TEST ANTIGENS AND STUDY SUBJECTS

Lysates of hairy cells, platelets, and red blood cells (RBCs) were prepared to test the specificity of 14G6 for type-5 TRAP by immunoprecipitation. Hairy cells (10^8/mL), platelets (10^9/mL), and RBCs (2×10^8/mL) were lysed in a buffer of 50 mmol/L Tris, pH 7.5 containing 300 mmol/L NaCl, 10 mL/L Nonidet P-40 detergent, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mg/L each of aprotinin and leupeptin. To establish values for healthy individuals, sera were collected from 18 apparently healthy, informed and consenting hospital workers: 6 males and 12 premenopausal females. To test clinical relevance of TRAP assays, fresh sera were obtained from 64 unselected patients with ESRD on chronic HD after informed consent to participate in this study, and from 99 unselected patients with rheumatic diseases after the specimens had been used for clinical purposes (waste specimens). Sera from ESRD patients were collected just before dialysis. All sera were stored at −50 °C until analysis. This study was approved by the University of Louisville Human Studies Committee.

ASSAY METHODS

Biochemical assay of total serum TRAP. Total TRAP activity in sera, cell lysates, and purified TRAP preparations was determined by a rapid microplate colorimetric assay *(19)* with modifications. *p*-Nitrophenyl phosphate (pNPP; 7.6 mmol/L) was used as substrate in a buffer containing 100 mmol/L sodium acetate and 50 mmol/L sodium tartrate (pH 5.5). Samples (50 μL) were added to 150 μL of substrate and incubated at 37 °C for 60 min. The reaction was stopped by the addition of 50 μL of 3 mol/L NaOH; the absorbance was read at 405 nm with a Bio-Rad model 550 microplate reader, and data were analyzed with Microplate Manager 4.0 software (Bio-Rad). The activity was estimated in U, using solutions of *p*-nitrophenolate as calibrators.

Enzyme capture immunoassay. Avidin-coated wells (generously provided by Zymed Laboratories, Inc., San Francisco, CA) were first coated with 0.5 μg of biotinylated 14G6 in 100 μL of PBS for 30 min at 37 °C. The wells were rinsed once with PBS and blocked with 30 g/L gelatin in PBS, pH 7.2, for 30 min at 37 °C. The wells were washed with three changes of PBST-E. Calibrators (100 μL) or serum samples (50 μL diluted with 50 μL of PBST-E) were then added to the wells and allowed to incubate overnight at 4 °C. The wells were then washed with three changes of

PBST, after which 200 μL of pNPP substrate was added to each well. Plates were incubated at 37 °C for 1 h, and the reaction was stopped by the addition of 50 μL of 3 mol/L NaOH. Absorbance was read at 405 nm, and bound activity was expressed in U as for the biochemical assay of total serum TRAP.

Two-site immunoassay. The two-site immunoassay was modified from a published method *(17)* by substituting HRP for Eu^{3+} as an indicator. Avidin-coated wells were coated with 14G6-biotin and blocked with gelatin; samples were then added exactly as for the enzyme capture assay. After incubation overnight at 4 °C, the wells were washed with three changes of PBST. To detect bound TRAP protein, 25 ng of J1B-HRP in 100 μL of PBST was added to each well and allowed to bind for 60 min at room temperature. Wells were then washed with three changes of PBST, and the HRP activity was measured using 2.2 mmol/L *o*-phenylenediamine and 3.6 mmol/L H_2O_2 in citrate phosphate buffer (25 mmol/L citrate-50 mmol/L phosphate, pH 5.0). The substrate was incubated for 15 min at room temperature, and the reaction was stopped by the addition of 50 μL of 2 mol/L H_2SO_4. The absorbance was read at 490 nm, and bound TRAP protein was expressed in μg/L.

STATISTICS

Regression analyses, the Student *t*-test with or without Welch's correction for unequal variance for comparisons of group means, and Mann–Whitney tests for comparisons of group medians (nongaussian sample distributions) were performed with Prism 2.01 software (GraphPad Software). The value for α was set a priori at 0.05.

Results

ANTIBODY CHARACTERISTICS

Antibody 14G6 specificity. The specificity of 14G6 for type-5 TRAP is illustrated by the immunoprecipitation data shown in Fig. 1. Eight percent of type-5 TRAP from hairy cells was left in the 14G6 unbound fraction, whereas >90% of the RBC and platelet TRAP was unbound. Only 63% of type-5 TRAP was recovered in the 14G6 precipitate because there is some degree of enzyme inactivation when TRAP is bound by solid phase 14G6, a finding we reported previously *(15)*.

Relative antibody affinities. Biotinylated antibodies 14G6, O1A, and J1B were coupled to avidin-coated wells, TRAP ligand in sample dilution buffer was added, and the amount of bound and unbound TRAP activity was determined. All three antibodies bound >90% of the TRAP activity added (Fig. 2). Antibody 14G6 again showed a tendency to inactivate bound TRAP activity, although it effectively removed it from solution (Fig. 2, inset).

Epitope independence among anti-TRAP antibodies. To ensure that 14G6 could be used as a capture antibody in conjunction with J1B or O1A, an experiment was conducted to test whether the antibodies compete for epitopes. Biotinylated monoclonal anti-TRAP antibodies (1 μg in 100 μL of PBS) were added to avidin-coated wells and allowed to bind for 30 min at 37 °C. After washing, a constant dose of TRAP and increasing amounts of competing monoclonal antibody (0–10 μg/well) were added and allowed to react for 2 h at 4 °C. The amount of bound TRAP activity was then determined with pNPP substrate. Fig. 3 shows that only homologous antibodies competed significantly for antigen binding and did so in a similar dose-dependent fashion.

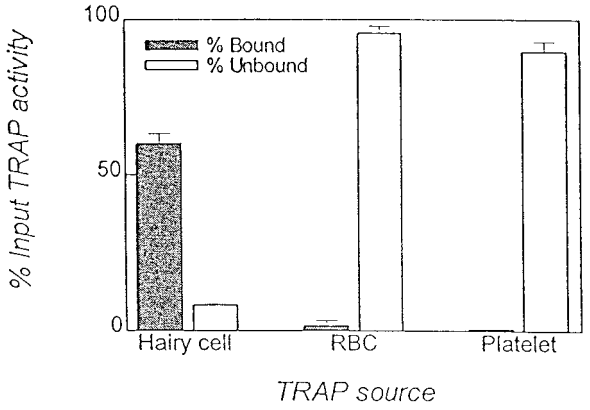

Fig. 1. Monoclonal antibody 14G6 reacts specifically with type-5 TRAP.

Cell lysates from hairy cells, RBCs, and platelets were prepared and mixed with solid-phase 14G6 antibody. Only type-5 TRAP from hairy cells was precipitated; however, the precipitated enzyme was ~30% inactivated. Results are the means ± SE of three independent experiments.

Effect of HRP conjugation. Portions of all three antibodies were conjugated to HRP and assessed for their sensitivity as detector antibodies in two-site immunoassays of all possible pairings. The assay was of low sensitivity when

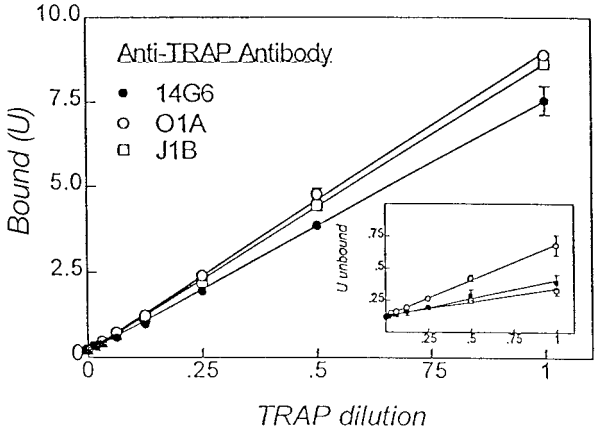

Fig. 2. Similar relative affinities of three monoclonal antibodies for TRAP.

Biotinylated antibody (0.5 μg) was bound to streptavidin-coated microtiter wells. Dilutions of pure hairy cell TRAP were added, and the amounts of bound activity were determined. At all dilutions, 14G6 appeared to bind less activity. Measurement of unbound activity (*inset*) shows that the lost activity with 14G6 was actually attributable to enzyme inactivation rather than lower affinity.

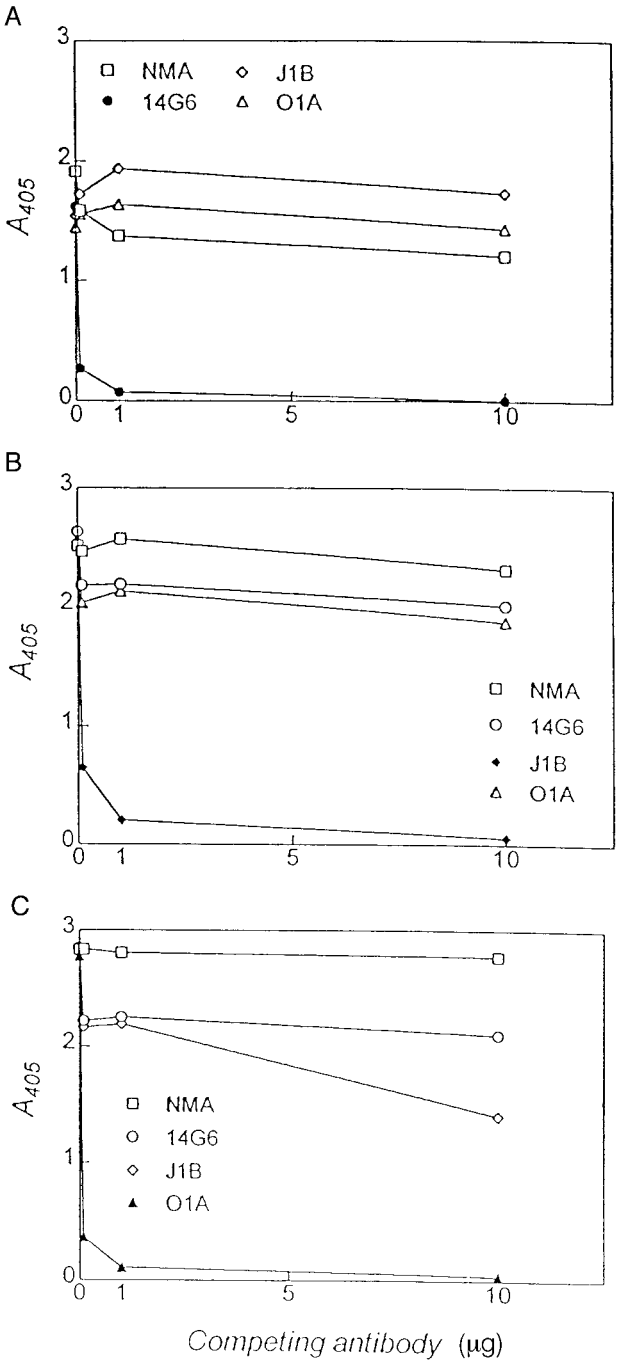

Fig. 3. Epitope competition analysis shows dose-dependent inhibition of antigen binding only by homologous antibody.

Wells were coated with 1 μg of 14G6 (*A*), J1B (*B*), or O1A (*C*). Increasing amounts of competing antibodies were added with pure TRAP. Bound activity was detected with pNPP substrate. At high concentrations, J1B competed partially with O1A (*C*).

14G6-HRP was used as detector and high sensitivity when either J1B-HRP or O1A-HRP were used. To determine the reason for this, conjugated and unconjugated antibodies (~0.25 μg of each) were applied to wells previously coated with rabbit anti-mouse IgG. Subsequently, TRAP enzyme was added, and the amount of bound activity was determined with pNPP substrate. HRP conjugation severely attenuated the ability of 14G6 antibody to bind antigen (14% bound), whereas it had only a minimal effect on J1B and O1A (78% and 82% bound). Thus, 14G6 is not suitable as a HRP-conjugated detection antibody in a two-site immunoassay but can be used as a capture antibody.

ASSAY CHARACTERISTICS

Calibration curves and specific activity of TRAP. Biotinylated 14G6 was used as a capture antibody for both TRAP activity and TRAP protein immunoassays. The enzyme capture immunoassay calibration curve was linear to 10 U with a mean absorbance at 405 nm of 2.7 at 10 U. The two-site immunoassay calibration curve was linear to 150 μg/L, with a mean absorbance at 490 nm of 2.8 at 150 μg/L. The minimum detectable TRAP was defined as 0.2 U and 2.8 μg/L. Calibrators below these concentrations had mean absorbance values (3 replicates) that could not be discriminated from that of the zero calibrators + 3 SD (16 replicates). The correlation of the two immunoassays was almost perfect ($r > 0.99$). Because the same antibody was used to capture and immobilize type-5 TRAP ligand under the same conditions in both assays, quantification of bound activity and bound protein permitted us to calculate the specific activity of TRAP from the ratio of activity (μmol substrate hydrolyzed · min^{-1} · L^{-1}) to protein (μg/L) assays. The mean specific activity of purified TRAP calibrators over the linear range of the assays, determined from three independent experiments, was 0.108 ± 0.028 U/μg.

Analytical recovery and linearity. To estimate analytical recovery, pure TRAP was added to three sera from healthy subjects, heated at 56 °C for 30 min to inactivate any confounding endogenous TRAP. TRAP assays were performed on twofold serial dilutions of each serum with and without added enzyme. For the enzyme capture immunoassay, recovery was calculated as the percentage of activity bound at each dilution. Because heat inactivation does not eliminate endogenous TRAP mass, recovery in the two-site immunoassay was calculated as the percentage of expected TRAP mass actually observed for each dilution. The expected recovery was the sum of the amount of TRAP added and the endogenous TRAP, measured at each dilution. Table 1 summarizes the results as the mean ± SD for three sera tested by both immunoassays. There was a direct relationship between recovery and dilution for both TRAP activity and TRAP protein. Undiluted serum produced the greatest interference, causing recovery to be lower than expected. At a 1:2 dilution, the recovery was >90% and gradually increased thereafter. The degree of lost activity from undiluted sera was accounted for in the unbound fraction (Table 1A), indicating a reduced binding efficiency when sera are undiluted. We sought to overcome this serum interference by increasing up to fourfold the amount of capturing

Table 1. Analytical recovery of type-5 TRAP activity by enzyme capture immunoassay and TRAP protein by two-site immunoassay.

A. Enzyme capture immunoassay

	TRAP activity,[a] U			
Dilution	**Added**	**Unbound**	**Bound**	**Recovery, %**
Undiluted	13.26 ± 0.04	3.86 ± 0.63	9.15 ± 0.72	66.5 ± 1.91
1:2	6.32 ± 0.41	0.55 ± 0.18	5.60 ± 0.05	88.9 ± 6.12
1:4	3.10 ± 0.11	0.12 ± 0.09	2.97 ± 0.09	95.8 ± 4.06
1:8	1.41 ± 0.15	0	1.60 ± 0.08	113.5 ± 16.8

B. Two-site immunoassay

	TRAP protein,[a] μg/L				
Dilution	**Added**	**Endogenous**	**Expected**	**Observed**	**Recovery, %**
Undiluted	74.4	26.6 ± 9.6	101 ± 9.6	81.68 ± 11.0	80.3 ± 5.2
1:2	36.5	26.5 ± 7.2	62.9 ± 7.2	59.0 ± 8.8	93.5 ± 3.6
1:4	19.1	14.1 ± 4.1	29.9 ± 3.4	28.8 ± 4.4	97.4 ± 20
1:8	9.8	7.7 ± 2.0	17.4 ± 2.0	15.2 ± 3.0	86.8 ± 7.6

[a] Expressed as mean ± SD for three sera, from healthy subjects, to which pure type-5 TRAP had been added.

antibody; however, the loss of linearity with undiluted serum persisted. As a result, all subsequent serum assays were conducted using a 1:2 dilution of analyte.

Assay precision. The within-run imprecision for each assay was estimated by calculating the CVs, expressed as the percentage of error [CV (%) = (mean/SD) × 100], for measurements of eight replicates of three sera. Between-run imprecision was determined for duplicate measurements of three aliquoted and frozen sera over 5 consecutive days. For the enzyme capture immunoassay, the mean within-run CV was 2.1% over a range of 2.3–8.3 U. The mean between-run CV was 13% over a range of 2.4–13.3 U. For the two-site immunoassay, the mean within-run CV was 7.9% over a range of 4.9–82.6 μg/L, and the mean between-run CV was 4.9% over a range of 41.9–213.6 μg/L. Thus, both assays were highly reproducible within runs; however, the enzyme capture assay was subject to more day-to-day variation than the two-site assay. This is probably because color development of the *p*-nitrophenolate calibrators for activity was not as influenced by minor variations in incubation time and temperature as the unknowns.

LIGAND STABILITY TESTS

Serum TRAP activity is considered unstable *(19)* because it steadily loses activity during storage at −20 °C over several months. We tested the short-term stability of serum TRAP using a total of five freshly drawn samples from healthy subjects. Aliquots of sera were incubated at 4 °C or room temperature for up to 48 h. After storage, samples were frozen at −50 °C and subsequently subjected to immunoassay for activity and protein. Immunoreactive TRAP activity was diminished by only 4% at 4 °C and 11% at room temperature over 48 h. TRAP protein was similarly reduced by only 4% at 4 °C and 7% at room temperature. This decrease was not statistically significant and was close to the interassay precision estimates.

CONCENTRATION RANGE AND SPECIFIC ACTIVITY OF TRAP IN SERA FROM HEALTHY SUBJECTS

Table 2 summarizes the results of total TRAP biochemical assay and type-5 TRAP immunoassays in healthy subjects and in hemodialysis and rheumatic disease patients. In the sera from healthy subjects, type-5 TRAP accounted for 71% of total activity. The biochemical assay for total TRAP activity and the immunoassay for type-5 TRAP activity

Table 2. TRAP activity and protein in healthy subjects and in patients with ESRD on chronic hemodialysis (HD) and patients with rheumatic arthropathies (Rheum).

			Immunoassays			
	Biochemical assay for total serum TRAP		Type-5 TRAP activity, U		Type-5 TRAP protein, μg/L	
	Mean ± SD, U	**Range, U**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
Healthy subjects (n = 18)	4.45 ± 0.71	3.46–5.93	3.2 ± 0.54	2.13–4.02	37.5 ± 12.7	22.9–68.6
Patients						
HD (n = 64)	4.83 ± 2.30	1.39–10.8	3.80 ± 1.92	0.98–9.46	69.8 ± 27.7[a]	13.4–165
Rheum (n = 99)	3.88 ± 1.55	1.58–11.5	3.10 ± 0.80	1.47–4.95	64.9 ± 44.9[b]	14.1–266

[a] $P < 0.001$ compared with healthy subjects (Welch's correction for comparison of group means of unequal variance).
[b] $P < 0.001$ compared with healthy subjects (Mann–Whitney test for comparison of group medians of nongaussian sample distributions).

correlated significantly ($r = 0.51$). The immunoassays for TRAP activity and protein in sera from healthy subjects also correlated significantly ($r = 0.62$). The mean specific activity of type-5 TRAP in sera from healthy subjects as determined by the ratio of the immunoassays for activity and protein was 0.091 ± 0.023 U/μg, which was not significantly different from that of pure TRAP calibrator (0.108 U/μg).

CLINICAL RELEVANCE OF TRAP IMMUNOASSAYS

The mean total TRAP activity, as determined by biochemical assay, and type-5 TRAP activity, as determined by immunoassay, were not significantly increased in the HD or the rheumatic disease group, and correlated closely in both groups ($r = 0.84$ for HD and $r = 0.74$ for rheumatic disease). Type-5 TRAP accounted for 79% of total activity in HD patients and 80% of total activity in rheumatic disease patients (Table 2). The close correlation between biochemical and immunochemical assays for TRAP activity and the similar proportions of immunoreactive type-5 activity in all study groups indicates (*a*) that antibody binding efficiency for type-5 TRAP is similar in all groups; (*b*) that the proportion of total activity attributable to type-5 TRAP is similar in all groups; and (*c*) that antibody inactivation of type-5 TRAP during immunoassay does not occur to a significant degree. Unlike TRAP activity, mean or median (as in the case of rheumatic disease) TRAP protein was increased in both disease groups. The sample distributions were wide for TRAP activity in the HD group and for TRAP protein in the rheumatic disease group, as shown in Fig. 4. There was a weak but significant correlation between type-5 TRAP activity and protein among HD sera ($r = 0.39$) but not among rheumatic disease sera ($r = 0.024$). The characteristics of these sample distributions as determined by immunoassay suggest that subpopulations of patients may exist in both groups with respect to type-5 TRAP activity and protein. Twenty-six percent (17 of 64) of the HD sera had TRAP activity that was at least 2 SD higher than that of the healthy subjects, whereas only 7% (7 of 99) of rheumatic sera had similarly high TRAP activity. Fifty-one percent (33 of 64) of HD sera and 31% (31 of 99) rheumatic disease sera had increased TRAP protein. The mean specific activity of TRAP in the 33 HD sera with increased activity was 0.088 ± 0.052 U/μg, which was not significantly different from that of the healthy subjects. On the other hand, the mean specific activity of TRAP in the 31 rheumatic disease patients with increased TRAP protein was significantly decreased, being only 0.035 ± 0.014 U/μg.

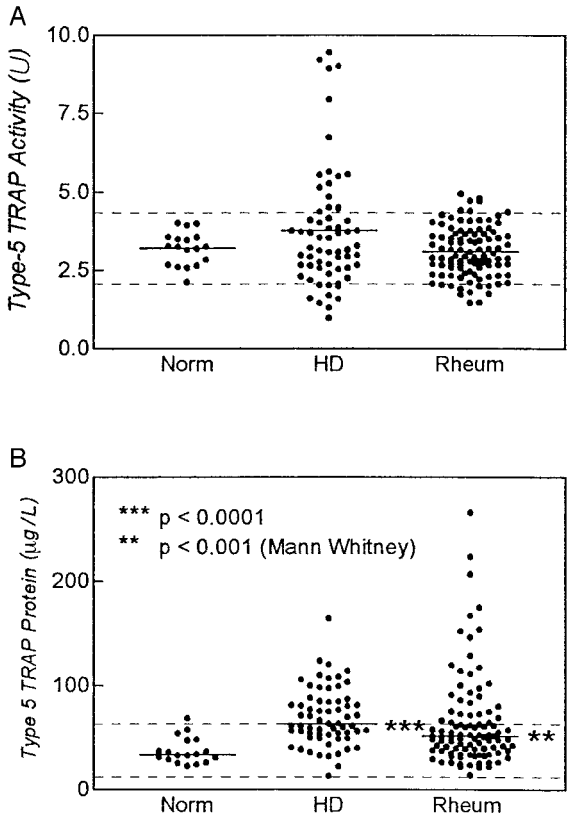

Fig. 4. Mean TRAP activity (*A*) and median TRAP protein (*B*).

(*A*), mean TRAP activity was not significantly different from healthy subjects (*Norm*) in unselected hemodialysis (*HD*) or rheumatic (*Rheum*) patients, although the sample distributions were wide, especially in the HD group. (*B*), mean and median TRAP protein was increased in *HD* patients (unpaired *t*-test, $P < 0.0001$). Median TRAP mass was increased in rheumatic (*Rheum*) patients (Mann–Whitney test, $P < 0.001$) The sample distributions again were wide, with the rheumatic disease group being nongaussian. Sample distributions suggest the presence of subpopulations with high TRAP in both patient groups. *Solid lines* depict the mean TRAP activity or median TRAP protein concentration for each group. *Dashed lines* depict the means ± 2 SD for the healthy subjects.

Discussion

We have developed two TRAP immunoassays to independently measure type-5 TRAP activity and protein. Both assays used the same capturing monoclonal antibody, ensuring that the same antigens were measured. Both immunoassays were reproducible and linear over the same range from one-tenth to fivefold higher than the values for healthy subjects. Both assays had comparable analytical recovery. When monoclonal antibodies are used as solid-phase capturing agents in microplate assays, the test is essentially of an affinity-purified analyte. Because the same anti-TRAP antibody was used in both TRAP immunoassays in this study, the specific activity of TRAP in sera could be reliably calculated as the ratio of the two immunoassay results. We wondered whether these assays would have their own clinical applications in specific disease groups or whether both types of assay together would yield more information than either assay alone by revealing differences in the specific activity of circulating TRAP.

We quantified immunoreactive type-5 TRAP activity and protein in sera from patients with ESRD requiring HD and in patients with assorted rheumatic diseases, two groups at risk for osteoporosis and increased bone resorption. The mean (or median for the rheumatic disease group) TRAP protein was increased in both the HD and the rheumatic disease groups; however, the mean TRAP

activity was not significantly different from that in the healthy subjects. Wide sample distributions suggested that both groups contained subpopulations with significantly increased TRAP. However, the abnormal subgroups differed with respect to the specific activity of the increased TRAP. In the HD patients with high TRAP activity, the mean specific activity was not significantly different from that in the healthy subjects. On the other hand, in the rheumatic disease patients with high TRAP protein, the mean specific activity was significantly decreased. Therefore, disease-related differences in the specific activity of serum TRAP may exist. Each assay may then have its own advantages for detecting increased TRAP. The finding of increased TRAP concentrations in our HD patients is consistent with those of others. Increased TRAP activity and protein have been shown repeatedly in hyperparathyroidism and chronic renal failure *(11, 12, 19–21)*. There are reports of increased TRAP-containing macrophages in synovial tissues *(22–24)*, but concordant serum TRAP concentrations were not determined. In a single study of juvenile rheumatoid arthritis *(25)*, serum TRAP activity was not significantly different from that in healthy subjects. Our finding of increased TRAP protein in sera from adult patients with rheumatic diseases is the first of its kind, and the significantly decreased specific activities in some rheumatic disease sera require explanation.

Several factors could contribute to the low specific activity of TRAP. These include extracellular inactivation and catabolism of TRAP in vivo after it is released from osteoclasts into the blood *(17)*, inactivation of TRAP activity in vitro by antibody binding *(9, 10)*, and instability of TRAP activity in vitro during storage *(19)*. However, these influences are not likely to cause disease-related differences as were seen in our data. The use of two monoclonal antibodies with independent epitopes for the assay of TRAP protein increases the probability that intact TRAP is being measured. Although it is possible that some catabolic fragments containing both 14G6 and J1B epitopes exist, it is doubtful they would predominate.

We and others have previously reported the tendency of TRAP to be partially inactivated in immune complexes *(9, 10, 15)*. This phenomenon was apparent in this study as well. Nevertheless, the recovery with monoclonal antibody 14G6 was ~65% by immunoprecipitation and 79% by immunoassay, which was much higher than the 42% *(9)* and 33% *(10)* recoveries reported by others using polyclonal antisera. This degree of inactivation would not be sufficient to account for the low activity in the 26% of our rheumatic disease cases with high TRAP protein. Furthermore, the mechanism of antibody-mediated inactivation in vitro is unlikely to be disease related.

There are conflicting reports of the degree and mechanism of TRAP instability in serum. Total serum TRAP activity declines significantly over 1 h at increased temperatures *(17)*. However, this is caused by rapid inactivation of erythrocytic TRAP. The bone-related TRAP activity declines significantly only after storage for >1 month at −20 °C. Halleen et al. *(17)* showed that endogenous TRAP forms Ca^{2+}-dependent complexes with high-molecular weight proteins that can interfere with antibody binding, and thus immunodetection, but that do not inactivate the enzyme. The complex can be dissociated easily by chelators EDTA or EGTA. For this reason, we included 10 mmol/L EDTA in the sample dilution buffer for this work. In another study, Halleen et al. *(26)* showed that TRAP mass is stable at 4 °C for up to 1 week, although significant decreases were noted at room temperature after 1 day. At apparent odds with this is the report by Brehme et al. *(27)*, who found that added exogenous recombinant TRAP forms Ca^{2+}-independent complexes with α_2-macroglobulin that interfere with antibody binding and inactivate the enzyme. This discrepancy may be attributable to differences in capturing antibodies, experimental protocol, and the use of recombinantly synthesized TRAP to supplement sera in the latter study. We were concerned only with the intrinsic stability of endogenous TRAP as it relates to the practical performance of the assays. We found that both the activity and detectable protein of type-5 TRAP are only slightly diminished over a period of 24–48 h at 4 °C or room temperature. The reduction was not significantly different and was within the interassay precision estimate.

In summary, we have developed two immunoassays to measure separately the active fraction and total protein of type-5 TRAP in serum. Our results from direct comparison of these assays lead us to advance the hypothesis that the specific activity of circulating TRAP varies greatly and may be disease related. Each assay may have its own biological significance and clinical applications in specific diseases. Continuing studies aimed to correlate TRAP activity and protein to other markers of bone metabolism and clinical evidence of bone disease are underway to test this hypothesis. Measurement of TRAP activity may give a better indication of metabolic bone disease and convey the current bone resorption rate as suggested by studies of osteoporosis in postmenopausal women on antiresorptive therapy *(28)*. The significance of increased TRAP protein with low specific activity remains to be determined.

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References

1. Risteli L, Risteli J. Biochemical markers of bone metabolism. Ann Med 1993;25:385–93.
2. Bikle DD. Biochemical markers in the assessment of bone disease. Am J Med 1997;103:427–36.
3. Delmas PD. Biochemical markers of bone turnover. J Bone Miner Res 1993;8:S549–55.
4. Bonde M, Qvist P, Fledelius C, Riis BJ, Christiansen C. Applications of an enzyme immunoassay for a new marker of bone resorption (CrossLaps): follow-up on hormone replacement therapy and osteoporosis risk assessment. J Clin Endocrinol Metab 1995;80:864–8.
5. Chen J, Yam LT, Janckila AJ, Li CY, Lam KW. Significance of "high" serum acid phosphatase in normal children. Clin Chem 1979;25: 719–22.
6. Khansur T, Yam LT, Tavassoli M. Serum monitors of bone metastasis. In: Stoll BA, Panbhoo SP, eds. Bone metastasis and its treatment. New York: Raven Press, 1983:165–80.
7. Yam LT. Clinical significance of the human acid phosphatases. A review. Am J Med 1974;56:604–16.
8. Price CP, Kirwan A, Vader C. Tartrate-resistant acid phosphatase as a marker of bone resorption. Clin Chem 1995;41:641–3.
9. Lam K-W, Siemens M, Sun T, Li C-Y, Yam LT. Enzyme immunoassay of tartrate-resistant acid phosphatase. Clin Chem 1982;28: 467–70.
10. Echetebu ZO, Cox TM, Moss DW. Antibodies to porcine uteroferrin used in measurement of human tartrate-resistant acid phosphatase. Clin Chem 1987;33:1832–6.
11. Kraenzlin ME, Lau K-HW, Liang L, Freeman TK, Singer FR, Stepan J, Baylink DJ. Development of an immunoassay for human serum osteoclastic tartrate-resistant acid phosphatase. J Clin Endocrinol Metab 1990;71:442–51.
12. Cheung CK, Panesar NS, Haines C, Masarei J, Swaminathan R. Immunoassay of a tartrate-resistant acid phosphatase in serum. Clin Chem 1995;41:679–86.
13. Chamberlain P, Compton J, Cox TM, Hayman AR, Imrie RC, Reynolds K, Holmes SD. Generation and characterization of monoclonal antibodies to human type-5 tartrate-resistant acid phosphatase: development of a specific immunoassay of the isoenzyme in serum. Clin Chem 1995;41:1495–9.
14. Halleen J, Hentunen TA, Hellman J, Vaananen HK. Tartrate-resistant acid phosphatase from human bone: purification and development of an immunoassay. J Bone Miner Res 1996;11: 1444–52.
15. Janckila AJ, Cardwell, EM, Yam LT. Characterization of monoclonal antibodies specific to human tartrate-resistant acid phosphatase. Hybridoma 1997;16:175–81.
16. Bradford MM. A rapid and sensitive method for the quantitation of micrograms quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254.
17. Halleen JM, Hentunen TA, Karp M, Kakonen S-M, Pettersson K, Vaananen HK. Characterization of serum tartrate-resistant acid phosphatase and development of a direct two-site immunoassay. J Bone Miner Res 1998;13:683–7.
18. Janckila AJ, Latham MD, Lam KW, Chow KC, Li C-Y, Yam LT. Heterogeneity of hairy cell tartrate-resistant acid phosphatase. Clin Biochem 1992;25:437–43.
19. Lau K-HW, Onishi T, Wergedal JE, Singer FR, Baylink DJ. Characterization and assay of tartrate-resistant acid phosphatase activity in serum: potential use to assess bone resorption. Clin Chem 1987;33:458–62.
20. Stepan JJ, Silinkova-Malkova E, Havranek T, Formankova J, Zichova M, Lachmanova J, et al. Relationship of plasma tartrate-resistant acid phosphatase to the bone isoenzyme of serum alkaline phosphatase in hyperparathyroidism. Clin Chim Acta 1983;133:189–200.
21. Scarnecchia L, Minisola S, Pacitti MT, Carnevale V, Romagnoli E, Rosso R, Mazzuoli GF. Clinical usefulness of serum tartrate-resistant acid phosphatase activity determination to evaluate bone turnover. Scand J Clin Lab Investig 1991;51:517–24.
22. Wilkinson LS, Pitsillides AA, Edwards JCW. Giant cells in arthritic synovium. Ann Rheum Dis 1993;52:182–4.
23. Fujikawa Y, Sabokbar A, Neale S, Athanasou NA. Human osteoclast formation and bone resorption by monocytes and synovial macrophages in rheumatoid arthritis. Ann Rheum Dis 1996;55: 816–22.
24. Gravallese EM, Harada Y, Wang J-T, Corn AH, Thornhill TS, Goldring SR. Identification of cell types responsible for bone resorption in rheumatoid arthritis and juvenile rheumatoid arthritis. Am J Pathol 1998;152:943–51.
25. Pepmueller PH, Cassidy JT, Allen SH, Hillman LS. Bone mineralization and bone mineral metabolism in children with juvenile rheumatoid arthritis. Arthritis Rheum 1995;39:746–57.
26. Halleen JM, Karp M, Viloma S, Laaksonen P, Hellman J, Kakonen S-M, et al. Two-site immunoassays for osteoclastic tartrate-resistant acid phosphatase based on characterization of six monoclonal antibodies. J Bone Miner Res 1999;14:464–9.
27. Brehme CS, Roman S, Shaffer J, Wolfert R. Tartrate-resistant acid phosphatase forms complexes with α_2-macroglobulin in serum. J Bone Miner Res 1999;14:311–8.
28. Halleen JM, Suominen H, Heikkinen JE, Vaananen HK. Tartrate-resistant acid phosphatase 5b is a promising new serum marker of bone resorption. Calcif Tissue Int 1999;64(Suppl 1):S106.