Hairy Cell Identification by Immunohistochemistry of Tartrate-Resistant Acid Phosphatase

By Anthony J. Janckila, Ernest M. Cardwell, Lung T. Yam, and Chin-Yang Li

Tartrate-resistant acid phosphatase (TRAcP) has been an indispensable marker for hairy cell leukemia (HCL) for over two decades. However, the traditional TRAcP cytochemical stain cannot be performed effectively on sections of paraffin-embedded tissues that are important resources for histopathologic evaluation in diagnosis and treatment of HCL. Wide variation in expression of TRAcP activity by hairy cells (HCs) within and among patients is an interesting biologic phenomenon that has not been explained and can cause some diagnostic uncertainty as well. To solve the problem of staining TRAcP in paraffin sections and to begin to address the questions of variable TRAcP expression in HCL, we developed a monoclonal antibody to TRAcP, 9C5, for immunohistochemical identification of HCs. In smears of blood and bone marrow, immunocytochemistry of TRAcP using 9C5 was as specific but slightly less sensitive than direct cytochemical staining of enzymatic activity. In paraffin sections of spleen and bone marrow from HCL patients, immunohistochemistry with 9C5 stained the HCs with high sensitivity and specificity and clearly showed the characteristic diffuse infiltration by HCs. Other cells noted to stain strongly with 9C5 were occasional macrophages in bone marrow smears and osteoclasts and occasional tissue macrophages in paraffin sections. These cells are known to express abundant TRAcP activity. Immunohistochemistry with anti-TRAcP monoclonal antibody 9C5 may have utility as an added option in the diagnosis of HCL, as a means to evaluate residual disease in HCL patients undergoing new treatments, and as a way to address questions regarding variable expression of TRAcP activity by HCs within and among patients with HCL. Also, 9C5 has potential as a reagent for the immunoeasay of bone-derived serum TRAcP in patients with certain bone diseases and cancers with bone metastasis.

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mmol/L phenylmethylsulfonyl fluoride, and 0.05 to 0.1 trypsin-inhibitory unit of aprotinin per milliliter. The insoluble pellet was then extracted with the same citrate buffer, but containing 300 mmol/L NaCl and no detergent. TRAcP was purified from this “high-salt” extract by sequential chromatographic separations over CM cellulose, Phenyl-Sepharose, Superdex 75 (all from Pierce Chemical Co, Rockford, IL), and MonoS (Pharmacia-LKB, Piscataway, NJ). Two peaks of TRAcP were resolved (peak 1 and peak 2) with specific activities of approximately 500 and 1,000 U/mg protein, respectively. Peak 2 was concentrated by vacuum centrifugation to 1 mg/mL and was used for immunization. The antigen was denatured by boiling for 2 minutes in the presence of 1.4 mg sodium dodecyl sulfate (SDS)/mg protein. One female Balb/c mouse was primed intraperitoneally (IP) with 100 µg denatured TRAcP in Freund’s complete adjuvant. Three booster doses of 50 to 75 µg denatured TRAcP in Freund’s complete adjuvant were given IP at 2-week intervals. After immunization, the mouse was rested for 4 weeks and then challenged IP on 3 consecutive days with 50 µg denatured TRAcP in saline. Four days later, the mouse was exsanguinated and spleenectomized. Hybridomas were prepared by conventional methods of polyethylene glycol-mediated spleen cell fusion with NS-1 plasmacytoma and selection in hypoxanthine, aminopterin, thymidine medium. Screening culture supernatants for anti-TRAcP antibodies was done by indirect enzyme-linked immunosorbent assay (ELISA) using partially pure antigen. Hybridomas from positive wells were subjected to two rounds of subcloning by limiting dilution using syngeneic thymocytes as feeder cells. Antibody-con-
taining ascites were induced in pristane-primed syngeneic mice by implanting the clone, 9C5, that gave the highest antibody activity in vitro. The IgG fraction of that ascites was prepared by one round of purification over a protein A/G affinity column (Pierce) according to the manufacturer's protocol.

**Antibody characterization.** Antibody titer was estimated by an indirect ELISA assay of serial dilutions of monoclonal antibody (MoAb) versus 10 ng TRAcP that was coupled to each microtiter well in 50 mmol/L carbonate buffer, pH 9.6. Calf intestinal alkaline phosphatase was the indicator enzyme and p-nitrophenyl phosphate was the substrate. Antibody class was determined to be IgG2b using a commercial kit (Sangstat, Inc, Menlo Park, CA). Specificity of the antibody was assessed by Western analysis of both peak 1 and peak 2 TRAcP from 12% reducing and nonreducing SDS-polyacrylamide gels and from 7.5% native acid gels and by dot-blot analysis of native and denatured TRAcP.

**Immunoprecipitation.** The specificity of 9C5 was further studied by direct immunoprecipitation from HC and non-HC lysates and by Western blot of precipitated proteins. Mononuclear cells were suspended at $2 \times 10^6$/ml in 50 mmol/L Tris, 150 mmol/L NaCl, 0.1% SDS, 1% NP-40, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 µg/ml leupeptin (pH 7.5) and were lysed by incubation on ice for 30 minutes. TRAcP activity in the soluble fraction was estimated by a rapid fluorometric method using naphthol ASBI-phosphate as substrate. A solid-phase 9C5 IgG was prepared by coupling purified IgG to amino-link gel (Pierce) at 2.5 mg IgG/ml packed gel. The gel was stored as a 50% suspension in phosphate-buffered saline (PBS) with 0.02% sodium azide. Before immunoprecipitation, 100- to 200-µl lysates were denatured by addition of one-tenth volume 10% SDS and were boiled for 2 minutes. After cooling the denatured samples, NP-40 was added to a final concentration of 3%. Twenty microliters of 9C5 gel suspension was added to the denatured lysates and allowed to immunoprecipitate TRAcP overnight at 4°C with mixing. After washing the precipitates 3 times in Tris-buffered saline TBS (50 mmol/L Tris, 150 mmol/L NaCl, pH 7.5), precipitated proteins were released from the gel by boiling 2 minutes in nonreducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The proteins were subjected to SDS-PAGE and transferred to nitrocellulose. TRAcP antigen was detected by an indirect immunoenzyme alkaline phosphatase method with 9C5 IgG followed by alkaline phosphatase-conjugated goat antimouse IgG. Alkaline phosphatase was stained with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

**Patients.** A total of 24 specimens from 19 cases of HCL were included for study. In 4 cases, multiple specimens were available including: blood and BM biopsy specimen, 1 case; BM biopsy specimen, spleen cell suspension, and spleen sections, 1 case; and spleen cell suspension and spleen sections, 2 cases. All 150 non-HCL control specimens were obtained from different patients.

**Smears and tissues.** Excess specimens of blood and BM from HCL patients and randomly selected non-HCL patients were used to prepare conventional and buffy coat smears. Cytocentrifuge smears were prepared from mononuclear cells obtained by ficoll-hypaque gradient centrifugation. Tissue sections of spleen, BM, and lymph nodes were cut at 4 to 6 microns from paraffin blocks of tissue fixed in neutral buffered formalin and, in the case of BM, were decalcified for 30 minutes in a commercial decalcifier of 7% w/vol ethylenediamine tetraacetate in 1 N hydrochloric acid (Baxter Scientific Products, Obetz, OH).

**Immunohistochemistry.** Air-dried smears were fixed for 30 seconds in cold buffered formalin acetone, rinsed in several changes of deionized water, air-dried, and stained immediately. Some unfixed smears had been stored for up to 4 years at room temperature before immunocytochemical staining. Antigenic activity of TRAcP in the smears was enhanced by placing the fixed smears in boiling distilled water for 1 minute, air-drying then digesting for 5 minutes at 37°C with trypsin Type IX (Sigma Chemical Co, St Louis, MO) at a concentration of 5 µg/ml in TBS-Ca²⁺ (50 mmol/L TRIS, pH 7.6, containing 150 mmol/L NaCl and 0.04% CaCl₂). After digestion, smears were rinsed with several changes of distilled water and were air-dried again. Tissue sections were deparaffinized in two changes of xylene and were hydrated through graded alcohols to water. Antigenic activity was enhanced by placing sections in boiling water for 15 minutes, allowing to cool slowly, then digesting for 30 minutes at 37°C with trypsin Type IX at 40 µg/ml in TBS-Ca²⁺. Digested sections were rinsed with several changes of PBS (10 mmol/L phosphate, pH 7.4, containing 150 mmol/L NaCl). The same alkaline phosphatase–anti-alkaline phosphatase (APAAP) immunohistochemical method was used for both smears and sections. Primary antibody (MoAb 9C5) was used at 1/500, goat antimouse IgG (Bio-source, International, Camarillo, CA) at 1/20, and APAAP (Boehringer-Mannheim Corp, Indianapolis, IN) at 1/40. Alkaline phosphatase activity was stained using naphthol AS-phosphate as substrate and fast red-violet LB salt as coupler in a buffer of 200 mmol/L Tris, pH 8.5, containing 50 mmol/L MgCl₂ and 1 mmol/L levamisole.

**RESULTS**

**Preparation and characterization of antibody.** Of 380 wells from the cell fusion, 8 contained antibody-producing hybridomas. Clone 9C5 was selected for these studies because it produced the highest titer antibodies (Fig 1). Dot-blot analysis showed that 9C5 reacts only with denatured TRAcP and that TRAcP is partially denatured at pH 9.6 used for antigen coupling to microtiter wells (Fig 2). This explains its reactivity with 9C5 antibody in ELISA assay. By Western analysis, 9C5 was specific for the denatured 16-kD fragments of both peak 1 and peak 2 isoforms (Fig 3); it did not react with TRAcP in transfers from nondenaturing gels. To document more fully the specificity of 9C5, solid-phase antibody
was used to immunoprecipitate TRAcP directly from HC and non-HC lysates. Results of a Western blot of precipitated proteins are shown in Fig 4. HCs contained at least four TRAcP isoforms. The intensity of the immunoprecipitated bands was proportional to the amount of activity present in the cell lysates. A weak TRAcP band was immunoprecipitated from the prolymphocytic leukemia cells corresponding to weak TRAcP activity in the lysate. In 2 HCL cases, a small amount of the antigenic 16-kD subunit was present in the immunoprecipitates. No other 9C5-reactive proteins were observed.

**Immunocytochemistry.** Immunocytochemical staining with 9C5 anti-TRAcP gave results comparable in specificity with direct cytochemical staining of TRAcP activity (Table 1 and Fig 5a and b). Of the non-HCL specimens tested, a BM smear from a single case of acute myeloid leukemia of the French-American-British classification type M0 (AML-M0) contained moderate TRAcP activity in approximately 20% of the blasts. A similar number of cells were also stained by immunocytochemistry with 9C5 antibody. In freshly prepared and stained smears, direct cytochemical demonstration of TRAcP activity is more sensitive than immunodetection of TRAcP antigen (Table 2). This is apparent for both the intensity of staining and the percentage of positive cells. Air-dried smears can be stored unfixed at room temperature for up to 4 months without appreciable loss in immunoreactivity of TRAcP; after 1 year, TRAcP antigen is irretrievable. Whereas a trace of TRAcP activity can be detected in some normal blood monocytes, 9C5 failed to yield a positive immunocytochemical stain in these cells. In BM smears, variable numbers of macrophages express TRAcP activity and are stained by 9C5 as well. These macrophages are primarily located in the residual BM particles that are difficult to spread. Other than HCs, these occasional TRAcP-positive macrophages and the blasts in the single case of AML-M0 noted above, cells in blood and BM were unreactive with 9C5.

**Immunohistochemistry.** Fixation of tissues with neutral buffered formalin and embedment in paraffin inactivate TRAcP activity. After heat-denaturation and trypsin digestion, antibody 9C5 enables detection of TRAcP in such tissues with a high degree of sensitivity and specificity. Figures 5c and d show that 9C5 antibody reacts strongly with HCs in formalin-fixed BM biopsy specimens and spleen. In addition to HCs, 9C5 gives strong reactions in osteoclasts and some macrophages. Among the non-HCL control tissue specimens tested, a BM biopsy specimen from a case of splenic lymphoma with villous lymphocytes contained a few 9C5-positive lymphoid cells. This patient’s blood smear also contained numerous villous lymphocytes with moderate TRAcP activity. Blood smears were not available for immunocytochemistry. The naphthol AS-phosphate/fast red-violet LB staining method gives a heavy, bright red reaction prod-
uct, which is preferred when hematoxylin counterstains are desired. However, the methods of McGadey using 5-bromo-2-chloro indolyl phosphate and nitroblue tetrazolium (NBT/BCIP) or of Rutenburg et al using naphthol AS-phosphate and fast-blue BB may be more sensitive, giving heavy dark blue-black or bright blue products, respectively. Alternatively, excellent results are attainable with peroxidase–anti-peroxidase formats when stained with either 3,3'-diaminobenzidine or 3-amin0-9-ethylcarbaole.

**DISCUSSION**

Today, increasingly effective, possibly curative, treatments for HCL are available including interferon-α, deoxycoformycin, and 2-chlorodeoxyadenosine. Still, a percentage of patients will do well for long periods with little more than supportive care. Accurate identification of HCs in blood and BM is essential for proper use of these treatments. Also, response to treatment is judged in part by estimating the number of residual HCs left afterward. TRAcP will continue to play an important role as a selective marker for HCs in the diagnosis and management of HCL. The inability to stain TRAcP activity in paraffin sections has always been a major limitation to its even wider use as a cell marker. To approach these problems, we have made several attempts to generate MoAbs to TRAcP. Immunization of mice with partially pure TRAcP was ineffective, probably because of weak immunogenicity of TRAcP, especially when contaminated by strong antigens. Similar experience has been reported by others in attempting to immunize rabbits with TRAcP. Immunization with pure TRAcP resulted in either low titers or few unstable clones. To increase the immunogenicity of human TRAcP in mice, we elected to denature the antigen. We now report the successful production of an antibody to TRAcP useful for immunohistochemical identification of HCs and other cells expressing TRAcP.

The 9C5 antibody is an IgG2b that reacts specifically with the 16-kD fragment of denatured TRAcP. We speculate that heat and subsequent trypsin digestion generate and expose the denatured epitope required for maximum immunoreactiv-

![Image](https://example.com/image.png)
Fig 5. Immunohistochemistry of TRAcP with 9C5 MoAb shows specific reactivity with HCs in smears and sections. (a) BM smear, anemia (original magnification \( \times 1,600 \)). (b) cytocentrifuge smear, splenic HC (original magnification \( \times 1,600 \)); (c) BM biopsy specimen paraffin section, HCL (original magnification \( \times 640 \)); (d) spleen paraffin section, HCL (original magnification \( \times 256 \)). APAAP method using naphthol AS-phosphate as substrate and fast-blue BB as coupler in 0.2 mol/L Tris, pH 8.5, containing 50 mmol/L MgCl\(_2\) is shown.

Immunohistochemical staining of smears with 9C5 antibody is just as specific as direct cytochemical staining of TRAcP activity, although somewhat less sensitive. All cases of HCL with cytochemically detectable HCs also had 9C5-reactive HCs, but the staining intensity was generally less intense with immunohistochemical methods, resulting in fewer cells stained. In this series, only 2 of 150 non-HCL specimens contained 9C5-positive cells. These cases should not be considered false-positives in terms of antibody specificity, because both had detectable TRAcP activity in the leukemic cells. We anticipate improvement in sensitivity through the use of more effective microwave antigen retrieval methods and more sensitive immunodetection systems. MoAb 9C5 is also effective for staining HCs in formalin-fixed and B-5--fixed paraffin sections. This may be particularly helpful as a means to detect residual HCs in BM biopsy specimens from patients undergoing treatment. In addition, 9C5 can be used to label osteoclasts and some macrophages. Specific immunodetection of TRAcP-positive cells of macrophage lineage should have added clinical applications in diseases involving these cell types. Another clinical application for 9C5 could be to monitor serum TRAcP in patients with metabolic bone disorders or to

Table 2. Comparison of TRAcP Cytochemistry to 9C5 Immunocytochemistry for HC Identification

<table>
<thead>
<tr>
<th>HCL Specimen</th>
<th>% TRAcP-Positive MNCs</th>
<th>% 9C5-Positive MNCs*</th>
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<tr>
<td>Blood smear</td>
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<td>100</td>
</tr>
<tr>
<td>Spleen MNC</td>
<td>100</td>
<td>28</td>
</tr>
</tbody>
</table>

Abbreviation: MNC, mononuclear cell.

* In all cases, immunocytochemical staining was weaker than cytochemical staining.

† Cytocentrifuge smear from MNC preparations.
evaluate bone metastasis by certain tumors. There is wide variation in the amount of TRAcP expressed by HCs. This heterogeneous expression is an interesting biologic phenomenon that has not been explained. With anti-TRAcP antibodies, we are in a position to investigate this question and to explore the structure and biologic function of TRAcP.

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REFERENCES
Hairy cell identification by immunohistochemistry of tartrate-resistant acid phosphatase

AJ Jancka, EM Cardwell, LT Yam and CY Li