Differences in Nonspecific Esterase from Normal and Leukemic Monocytes
By Perry D. Cohn, Peter D. Emanuel, and Marek J. Bozdech

Nonspecific esterase (NSE) is used to identify normal and leukemic mononuclear phagocytes cytochemically. It can be demonstrated by the hydrolysis of α-naphthyl acetate or butyrate. NSE from leukapheresed leukemic monocytes were extracted with several types of detergent and grouped into two isoelectric point (pl) categories based on the ease of solubility: pl 5.7-6.3 (6 bands) > pl 6.6-7.6 (6 bands). Normal monocytes yielded only the pl 5.7-6.3 isozymes. Isozymes from both leukemic and normal monocytes were inhibited similarly by sodium fluoride, pH < 4.7, and a serine active site inhibitor. All isozymes were bound by Sepharose-concanavalin A (Con-A) and displayed similar substrate preferences and pH v activity slopes. Under the conditions of detergent solubilization, the smallest mol wt species retaining enzymatic activity was 50 ± 5 kd. Despite these similarities, the isozymes of pl 5.7 through 6.3 and pl > 6.3 exhibited different degrees or types of inhibition by phenylmethylsulfonyl fluoride (PMSF), resistance to heat and antigenic character. Thus, the esterase isozymes represent two families of glycoproteins, both of them probable cell surface enzymes, resembling classical liver carbonyl or B-esterases (EC 3.1.1.1).

Supported by the Helen Zieske Fund and the University of Wisconsin Department of Medicine. During the course of this research, Marek J. Bozdech was a Junior Clinical Faculty Fellow of the American Cancer Society.

Address reprint requests to Wistar Institute, 36th and Spruce Sts, Philadelphia, PA 19104.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1987 by Grune & Stratton, Inc.

MATERIALS AND METHODS

Cells. Two patients with acute monocytic leukemia (French-American-British [FAB] classification M5) were therapeutically leukapheresed to reduce their dangerously high leukocyte counts. Pheresed leukocytes from both patients were >85% α-naphthyl acetate or α-naphthyl butyrate nonpunctate esterase positive (ie, mononuclear phagocytes, not T cells), using the method of Li and colleagues5 or Bozdech and Bainton2 (described below). As judged by Wright's staining, the leukapheresed mononuclear phagocytes from both patients were predominantly monoblasts. They were negative for peroxidase and weakly positive for acid phosphatase. Equal numbers of leukapheresed cells from both patients were positively stained by antimonocyte 1 monoclonal antibody (Bethesda Research Laboratories, Gaithersberg, MD) but not by OKT3 (Ortho Pharmaceutical, Westwood, MA). Based on Wright's and chloroacetate esterase staining, no platelets and only 5% each of neutrophils and lymphocytes were present. Electron cytochemistry of the esterase was performed using the methods of Bozdech and Bainton.2 NSE was localized both on the cell surface and intracellularly, as judged by thick and thin section (Figs 1 and 2). Normal monocytes were obtained from normal volunteers and purified by Ficoll-Hypaque centrifugation. Further purification (up to 90% to 95% monocytes) was effected by reversible adherence to fibronectin-coated plastic Petri dishes.11 Purified fibronectin was supplied by Deane F. Mosher. Iron carbonyl separation of monocytes was performed in some experiments.

Enzyme extraction. Leukapheresed leukemic monocytes were cleared of RBCs by 0.83% NH4Cl lysis. The cells were briefly vortexed with equal volumes of detergent solution [0.1% cetyltrimethyl ammonium bromide (CTAB) or 0.1% to 1.0% Triton X-100 or 0.1% to 3.0% chomalidopimethylammonio-1-propanesulfonate (CHAPS)] in phosphate-buffered saline (PBS) followed by centrifugation at 2,000 g. Following a 60-second extraction, the sedimented cells appeared intact by electron microscopy except for loss of the plasmalemmal membrane. No enzyme inhibitors were included because the inhibitor specificities were not yet established. The supernatant was centrifuged a second time and frozen. Thawing was accompanied by an additional centrifugation, followed in some experiments by ultrafiltration through a 0.2-μm filter.

Isoelectric focusing. Analytical polyacrylamide IEF was conducted in 5% gels incorporating 1% Triton X-100 and pH 3-10 ampholines enriched with pH 5-7 or 5-8 ampholines. Bands containing NSE activity were stained with the same techniques as used for the cells.
LEUKEMIC MONOCYTE NONSPECIFIC ESTERASE

Measurement of NSE. NSE was measured by the techniques of Bozdech and Bainton, which were adapted from Li and colleagues. This method was dependent on the hydrolysis of α-naphthyl acetate or butyrate (Sigma) in Tris or phosphate buffer (pH 7.2) followed by the reaction of the α-naphthyl leaving group with the hexazotized dye pararosaniline, producing a red color that can be monitored at 500 nm. The esterase activity was either estimated microscopically (cells), judged grossly (polyacrylamide gels), or quantitated by spectrophotometry (enzyme purification). The reaction was stopped at 1 minute with pH 4.5 buffer for spectrophotometry.

Affinity chromatography purification. Sepharose-4B beads conjugated with concanavalin A (Con A, Sigma) were washed and added to the detergent extract. Titration determined that a small amount of beads could bind all NSE activity from the detergent suspension. Based on that titration, Sepharose-Con A was used to purify the extract partially. After extensive washing in buffer, 50 mM of mannose in pH 7 PBS was used to elute the NSE from the beads. The NSE activity and Coomassie R-250 (Bradford assay) estimated protein concentration were measured at each step. The amount of protein in the eluate was 5% of that in the initial extract, whereas 70% to 80% of the enzyme activity was recovered. Analytical IEF demonstrated that all original NSE bands were present in the eluate. The eluate was dialyzed in PBS and either concentrated by lyophilization or by concentrative ultrafiltration techniques using Nuclepore CX-10 filters (Nuclepore Corp, Pleasanton, CA).

Preparative IEF. The preparative IEF purification of NSE used granulated, low electroendosmotic polyacrylamide (Biogel; Biorad, Richmond, CA) or 0.5% agarose/4% polyacrylamide gels incorporating a mixture of pH 3-10 and pH 5-8 ampholines. Crude detergent extract or extract partially purified on Sepharose-Con A, dialyzed in 2% tricine-glycine or 1% ampholytes was placed on the prefocussed gels. After the run, the NSE activity was localized by blotting with nitrocellulose paper. That area of the bed was removed, and the NSE was eluted with PBS. This method succeeded in purifying the NSE 50-fold, as judged by the change in the ratio of enzyme activity to protein concentration. The eluate was concentrated by ultrafiltration. Up to several hundred-fold purification, as judged by Coomassie staining of analytical IEF gels, was accomplished by combining Sepharose-Con A purification with preparative IEF. Individual isozymes are purified by a factor of ~14 (there are 14 bands) higher.

New Zealand white rabbits were purchased from Sprague-Dawley. Following inoculation and boosting, the animal was bled every other week. After 4 hours was allowed for clot formation, the serum was removed, heated at 56°C for 30 minutes, and frozen at −70°C.

Immunodiffusion assays of the sera were performed overnight at room temperature in 2% agarose buffered with 0.05 mol/L of Tris pH 7.2. Unprecipitated protein was dissolved out, and the gels were placed in NSE substrate solution to reveal immunoprecipitation bands containing NSE.

Immunofluorescence of cells was accomplished by testing dilutions of sera. Cells on slides were fixed at 4°C with acetone-methanol (1:1) for 60 seconds and washed in PBS. They exhibited only very weak autofluorescence. Diluted rabbit antisera was added for 1 hour, washed off, and followed by a dilution (1:40) of goat anti-rabbit immunoglobulin (heavy and light chains) (Cappel, Malvern, PA).

Mol wt estimation. Sodium dodecyl sulfate (SDS) polyacrylamide “disc” gels were prepared according to the Laemmli method. The stacking gel was made to 4% acrylamide, and the spacer gel was 7.5%. Samples were preincubated overnight with 0.1% SDS in pH 6.8 Tris-glycine. The gels were run until the bromophenol blue marker left the gel (~20 hours at 20 mA constant current). Protein standards included bovine serum albumin (BSA) (66,000), β-galactosidase (115,000), immunoglobulin G (IgG) (150,000 to 180,000), and thyroglobulin (690,000).

Gel filtration determination of mol wt was performed with G-200 Sephadex and Sephacryl S-300. PBS was the eluant. The ratio of the elution volume and the void volume were linearly correlated with the logarithm of the protein standards mol wt. Protein standards included BSA (66,000), yeast alcohol dehydrogenase (150,000), and β-amylase from potato (200,000).

RESULTS

IEF. IEF separated the isozymes from the other more acidic cellular protein. Analytical IEF revealed that the region exhibiting the NSE staining contained ~1% to 2% of
the total Coomassie staining (Fig 3). Different sets of NSE bands (Fig 4) were obtained from live cells briefly extracted with different detergents or from cells first subjected to freezing.

Brief extraction with CTAB of live cells from patient 1 (2 minutes) yielded two IEF “doublet” bands with esterase activity at pI 6.0 through 6.1 and 6.2 through 6.3 (Fig 4). These cells were mostly intact as judged by electron microscopy and the low lactate dehydrogenase (LDH) content of the extract. These apparent “doublets” were accompanied at pH 5.7 through 5.9 by four bands of weak to strong activity. These isozymes, but they did so with greater activity (Fig 4E). Leukapheresed cells of patient 2 contained more 0.1% CTAB-soluble pI > 6.3 isozymes than did the cells from patient 1 (Fig 4F). One short extraction (2 to 5 minutes) was sufficient to solubilize nearly all the NSE activity. In IEF gels stained with Coomassie R-250, the pI > 6.3 isozyme bands exhibited a degree of protein concentration similar to that exhibited by the more acidic bands. In contrast to these results with the leukemic cells, extraction of normal monocytes (Fig 4G) yields only the pI 5.7 through 6.3 bands after either 0.1% CTAB or 1% Triton X-100.

Preparative IEF enabled separation of the leukemic isozymes from patient 2 into 2 acid pH regions, 5.7 through 6.0 and 6.0 through 6.3, each containing 4 bands, and 6 neutral regions corresponding to the 6 neutral isozyme doublets. After elution, these preparations were used to test substrate and antisera specificities (see below).

That all the isozymes were glycosylated to some degree is evident from the ability of Sepharose-Con A to bind completely all esterase isozymes from CTAB or Triton X-100 extracts. No difference in binding was exhibited by the different isozymes. The binding of all isozymes was completely reversed by mannose or glucose. The bound enzyme retains considerable activity when bound to Sepharose-Con A even after storage for 1 year at 4°C. Other lectin-affinity chromatography preparations, such as agarose-bound peanut agglutinin or Lotus tetragonolobus agglutinin, did not bind or inactivate the enzyme. In contrast, serum cholinesterase, which also hydrolyzes α-naphthyl acetate, was not bound by Sepharose-Con A.

Plasmalemmal 5’ nucleotidase, cytoplasmic lactic dehydrogenase, and acid phosphatase (AcP) of lysosomal origin were present in all preparations. The latter two enzymes were up to fivefold more active or abundant in the most extensive extractions as compared with the shortest CTAB extractions. Most additional solubilized protein in extensive extractions was present in a range between pI 4 and 5.5.

**Inhibitors.** PMSF, a serine active site inhibitor, is an effective inhibitor of solubilized NSE. Activity in the crude extracts was totally inhibited by 50 mmol/L of NaF, 0.1 mmol/L of PMSF, and pH < 4.5. The I₅₀ dose of PMSF varied between 10 and 25 μmol/L depending on the batch.

A degree of differentiation emerged between the pI 5.7 through 6.3 and the pI > 6.3 isozymes in the type of inhibition by PMSF. The inhibition of the neutral isozymes by PMSF was reversed by IEF. These results could be explained by low-affinity noncovalent inhibition of the neutral isozymes, reversed as inhibitors were separated from enzyme by the IEF. The inhibition of all bands by NaF was reversed by IEF.

Neostigmine (10 to 15 mmol/L) inhibited isozymes pI 5.7 through 6.3 30% to 50% when tested with α-naphthyl butyrate in the pH range of 5 through 6. No inhibition of the isozymes was observed in the pH range of 7 through 8. By comparison, serum cholinesterase was inhibited by micromolar amounts, as expected.
Various other compounds were tested as inhibitors of α-naphthyl acetate/butyrate hydrolysis. These have included a wide range of natural and artificial protease and phospholipase inhibitors, none of which had significant effect. Sulphydryl reducing agents such as dithiothreitol, β-mercaptoethanol and p-chloromercuribenzoate; Ca²⁺/Mg²⁺ chelators like EDTA and EGTA; urea up to 8 mol/L; metal ions like Fe²⁺, Fe³⁺, Ni²⁺, and Mn²⁺; and the oxidase inhibitor sodium azide all had no effect on soluble or cell associated esterase activity. Isozymes of p1 5.7 through 6.3 were sensitive to 50°C, whereas most p1 > 6.3 forms were denatured only after heating to 62°C. These results with solubilized enzyme were cytochemically duplicated by similar treatment of leukemic and normal monocytes. Prefixation of the cells with 0.25% glutaraldehyde decreased the sensitivity of the cellular enzymes to heat. Freezing at -20°C completely preserved the extracted esterase. Dialysis to remove the detergent did not affect activity or the pattern of bands in IEF.

Substrate specificities. We measured the reaction kinetics of the isozymes with α-naphthyl acetate and butyrate at pH 6, pH 7, pH 7.5 and pH 8.5. The leukemic isozymes were separated by preparative IEF as described above. The results are presented in Fig 5 and represent the median of three experiments in which relative absorbance was plotted against pH.

In general, all isozymes, like the crude extract, displayed greatest reactivity between pH 7.5 and 9.0, with an optimum at pH 8.5. All isozymes were inactive at pH 9.5 with half-maximum reactivity at pH 9.0 (data not shown). Extracts from normal monocytes behaved very much like the isozymes from leukemic cells. Cytochemically demonstrated NSE exhibited a broader pH optimum from pH 6 to pH 9.

The substrate α-naphthyl butyrate was the most preferred by all isozymes from both normal and leukemic cells, followed in order by α-naphthyl acetate, propionate, valerate, and caproate. The caproate ester had ~25% activity of the butyrate as assayed with the crude extract. In IEF gels, the caproate ester was preferentially cleaved by the p1 6.0 isozyme doublet. Crude extract and eluted isozymes of all p1 5.7 through 6.0 and 6.0 through 6.3 can also hydrolyze α-naphthyl caprylate, laurate, and myristate, but at ~1% to 2% of the rate of α-naphthyl butyrate, even in a detergent solution.

Mol wt. Gel filtration with Sephadex G-200 or Sepharose S-300 demonstrated that esterase isozymes exist as units of 50,000 ± 10,000 daltons, which can form aggregates of ~550 kd, presuming that the aggregate is roughly spherical. Aggregates were observed in concentrated solutions of enzyme (after Sepharose-Con A purification) in PBS and when crude detergent extracts were eluted through the columns with PBS. No aggregates were observed when extracts were eluted with detergent solution. IEF showed that the low mol wt fractions from PBS-eluted filtrations were composed of predominantly acid isozyme activities. High mol wt aggregates contained predominantly neutral isozymes. The gel staining was measured only qualitatively.

Discontinuous gel electrophoresis with 0.1% SDS revealed esterase activity only at 150 ± 10 kd, regardless of whether crude detergent extract, Sepharose-Con A purified enzyme, or the 500- to 600-kd G-200 fractions were applied. Because only the activities of p1 > 6.3 isozymes survive prolonged exposure to 0.1% SDS, as judged by IEF of crude extract and gel filtration fractions, the 50-kd isozyme monomers (predominantly acid isozymes) could not be visualized with the enzymatic reaction. Because of the remaining contamination with other proteins, even with partially purified preparations, Coomassie staining of protein was not used to localize the NSE.

Antisera. Partially purified extract (composed entirely of p1 5.7 through 6.3 isozymes) from patient 1 was used for immunizing rabbits. The antisera produced two immunoprecipitin bands containing NSE activity in immunodiffusion assays against the CTAB extract from patient 1. In a similar assay, the extract of the leukemic monocytes from patient 2 exhibited one NSE-positive immunoprecipitin band that possessed an arc of identity with the major band of patient 1 (Fig 6). Immunodiffusion assays using enzyme extracts produced with different detergents (0.1% CTAB, 0.1% CHAPS, 1% Triton X-100) yielded equivalent arcs of identity (Fig 6).

Detergent extracts of normal human Ficoll-Hypaque-purified mononuclear cells or fibronectin-purified adherent cells also possess an arc of identity with the esterase from the leukemic cells (Fig 6). Isozymes separated from preparative polyacrylamide-agarose IEF gels, however, did not react
Cellular specificity of the antisera was also tested. Dilutions of 1:40 were most effective without too much background. Leukapheresed cells from patient 2 (Fig 8A) were ~90% fluorescent, which matched the proportion of NSE stained cells. Much of the reactivity was probably intracellular. Adherent normal monocytes (Fig 8B) were 50% to 90% positive, depending upon the degree of lymphocyte contamination. Cytocentrifuged nonadherent lymphocytes (Fig 8C) were <10% positive, correlating with the number of contaminating NaF-sensitive NSE-positive cells, possibly nonadherent monocytes.

**DISCUSSION**

Leukapheresed mononuclear phagocytes from two patients with acute monocytic leukemia were extracted with detergent to study the solubilized NSE. On the basis of our data, the detergent extractable NSE of the leukemic monoblasts from two patients are separable into two families: the six acid isozymes comprising the pl 5.7 through 6.3 group and the six neutral isozymes with pl 6.60 through 7.45. The common features of both families of enzymes include inhibition by fluoride ion and PMSF, sensitivity to pH < 5, a similar degree of binding to Sepharose-Con A, unit mol wt of 50 ± 10 kd, and similar substrate specificities and pH optima. The distinguishing features of the two groups of isozymes include resistance to elevated temperatures and to 0.1% SDS denaturation, lack of covalent bonding by PMSF to the pl > 6.3 isozymes, and different antigenicity. One further distinguishing feature is our observation that normal monocytes and macrophages (corroborating results of Parawesch and colleagues) possess only the acid isozymes that we find to be antigenically identical to the acid isozymes from leukemic monocytes.

The characteristics of these isozymes are remarkably similar to the liver carboxyl esterases (EC 3.1.1.1). Indeed, a portion of the liver isozymes in most studies was probably contributed by Kupffer cells, which are mononuclear phago-

Cellular specificity of the antisera was also tested. Dilutions of 1:40 were most effective without too much background. Leukapheresed cells from patient 2 (Fig 8A) were ~90% fluorescent, which matched the proportion of NSE stained cells. Much of the reactivity was probably intracellular. Adherent normal monocytes (Fig 8B) were 50% to 90% positive, depending upon the degree of lymphocyte contamination. Cytocentrifuged nonadherent lymphocytes (Fig 8C) were <10% positive, correlating with the number of contaminating NaF-sensitive NSE-positive cells, possibly nonadherent monocytes.

**DISCUSSION**

Leukapheresed mononuclear phagocytes from two patients with acute monocytic leukemia were extracted with detergent to study the solubilized NSE. On the basis of our data, the detergent extractable NSE of the leukemic monoblasts from two patients are separable into two families: the six acid isozymes comprising the pl 5.7 through 6.3 group and the six neutral isozymes with pl 6.60 through 7.45. The common features of both families of enzymes include inhibition by fluoride ion and PMSF, sensitivity to pH < 5, a similar degree of binding to Sepharose-Con A, unit mol wt of 50 ± 10 kd, and similar substrate specificities and pH optima. The distinguishing features of the two groups of isozymes include resistance to elevated temperatures and to 0.1% SDS denaturation, lack of covalent bonding by PMSF to the pl > 6.3 isozymes, and different antigenicity. One further distinguishing feature is our observation that normal monocytes and macrophages (corroborating results of Parawesch and colleagues) possess only the acid isozymes that we find to be antigenically identical to the acid isozymes from leukemic monocytes.

The characteristics of these isozymes are remarkably similar to the liver carboxyl esterases (EC 3.1.1.1). Indeed, a portion of the liver isozymes in most studies was probably contributed by Kupffer cells, which are mononuclear phago-
cytes. Like the esterase in our extracts, carboxyl esterases are, by working definition, inhibited by organophosphates but not by the acetyl-cholinesterase inhibitor, eserine, or by sulfhydryl reagents. Relatively concentrated eserine was inhibitory, although that concentration also inhibits neutrophil chloroacetate esterase. Temperature and fluoride sensitivity and pH optimum of beef liver esterases studied by Wynne and co-workers were also similar to our extracts.

Ecobichon demonstrated the presence in human liver of 180- and 65-kd forms of carboxylesterase which existed in the monomeric form below pH 4.5. Horgan and colleagues and Krisch and Krisch, using a variety of biochemical and electron microscopy techniques, showed that pig liver esterase is a 55- to 60-kd unit that can associate as a trimer of 160 kd. Our data indicates a similar unit mol wt in detergent. Without detergent, the pl > 6.3 isozymes polymerized to form a large aggregate of 550 kd, presuming a spherical shape on gel filtration. In 0.1% SDS gels, this ran as an apparent trimer of 150 kd with weak esterase activity. The pl 5.7 through 6.3, 50-kd forms of the enzymes were apparently inactivated by 0.1% SDS.

That all NSE is bound by Con A and is, therefore, glycosylated is evidence of cell surface or Golgi-endoplasmic reticulum lysosome (GERL) localization. In mature monocytes, it has been found only on the cell surface. The neutral to alkaline pH optima of both acid and neutral isozymes is consistent with esterase placement on the cell surface similar to macrophage plasminogen activator and elastase. Because the pH of macrophage phagosomes rises toward pH 8 immediately prior to lysosome fusion, some of the cell surface proteases and NSE isozymes may have a special function during this early step of internalization. Esterase isozymes may also have a function in pinocytosis since we have observed, with electron microscopy, the presence of cytochemical reaction product in pinosomes (unpublished observations, August 1982).

Curiously, the pattern of esterase isoelectric points from leukemic monocytes closely resembles the pattern of platelet (and, presumably, megakaryocyte) esterase described by Radzun and colleagues and Parawesch and co-workers. Because no platelets were present in the leukemic monocyte preparations, there may be some developmental relationship between monocytes and megakaryocyte/platelet bone marrow precursors. One subset of myeloid stem cells, colony-forming units, develops into colonies containing granulocytes, monocytes, and megakaryocytes. Platelets and monocytes do have other cell surface products in common, including complement and fibrin cascade components and fibronectin. Distribution and sharing of esterase isozymes among the various types of leukemias and lymphomas could prove to be a valuable tool in the pursuit of normal and disturbed bone marrow lineage and function.

REFERENCES

9. Scott CS, Linch DC, Bynoe AG, Allen C, Hogg N, Ainley MS, Hough D, Roberts BE: Electrophoretic and cytochemical character-
Differences in nonspecific esterase from normal and leukemic monocytes

PD Cohn, PD Emanuel and MJ Bozdech