PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 36-41 (1976)

Variations in Elastaselike Esterase Activities in Human Leucocytes during Cell Maturation[1] (39322)

GAD FEINSTEIN[2] AND AARON JANOFF[3]

Department of Pathology, State University of New York at Stony Brook, Stony Brook, New York 11794

The involvement of leucocyte enzymes in the pathogenesis of inflammatory diseases has been the subject of several recent reviews and symposia (1-4). Human leucocytes contain several different neutral esterases and proteases (5, 6), most of which are located in the azurophil granules of these cells (6). One of these enzymes, an elastaselike esterase which migrates slowly in acrylamide gel during acid-pH electrophoresis, requires the presence of Triton to hydrolyze synthetic substrates (5). In this regard, the slow-migrating enzyme is different from the well-characterized, rapidly migrating elastase isozymes present in the same leucocyte granules (7-10), since the latter enzymes are capable of hydrolyzing substrates in the absence of the detergent. In the course of recent work on the purification of leucocyte elastases (10), we observed that granular extracts from preleukemic and leukemic individuals contained the slow-moving, Triton-activatable elastaselike esterase (SE), but no elastase isozymes. One possibility that was suggested by this observation was that SE might be an inactive zymogen or precursor form of leucocyte elastase found mainly in immature cells, but partly retained as the cells matured.

The purpose of the present studies was to explore the relationship between cellular maturation and the SE content of human leucocyte granules. We also studied some of the biochemical and immunological properties of leucocyte SE.

Materials. Human blood from healthy individuals was purchased from the N.Y. Blood Center. Bone marrow aspirates from nonleukemic patients were kindly provided by Dr. R. Prasad of the V.A. Hospital, Northport, N.Y. Leucocytes from both sources were processed within a few hours. Enzyme substrates and inhibitors were the same as reported earlier (10). Rabbit antiserum to human leucocyte elastase was prepared in this laboratory as described before (10), and GARG[4] was purchased from Meloy Laboratories, Inc., Springfield, Virginia. The latter antiserum contained 9.0 mg/ml of antibody protein. Elastatinal was a gift from Dr. T. Sugimura.

Methods. Human leucocytes and leucocyte granules were isolated from venous blood and bone marrow aspirates by the procedure of Janoff and Scherer (11). Lysis of the granules in neutral-buffered 0.10 *M* NaCl to provide a crude extract of granule proteins was carried out as described elsewhere (10). Acrylamide gel electrophoresis, at pH 4.3, was performed according to Reisfeld *et al.* (12). Protein concentrations were determined by the method of Lowry *et al.* (13) using bovine serum albumin as a standard. Enzymatic staining reactions in acrylamide gels using Ac-DL-Ala-1-ONap was done according to Sweetman and Ornstein (5) with slight modifications (14). Enzymatic assays for elastase using Boc-Ala-ONp as a substrate were carried out as described before (10).

A double immunodiffusion experiment to test for antigenic cross-reactivity between SE and the rapidly-migrating leucocyte elas-

[1] Supported by NIH Grant No. HL-14262, and by a Phebe Cornell Maresi Center Grant of the New York State Arthritis Foundation.

[2] Permanent address: Department of Biochemistry, Tel-Aviv University, Ramat-Aviv, Tel-Aviv, Israel.

[3] Address reprint requests to Dr. A. Janoff, Department of Pathology, State University of New York at Stony Brook, Stony Brook, New York 11794.

[4] Abbreviations: *N*-acetyl-L-alanyl-L-alanyl-L-prolyl-L-alanine chloromethyl ketone, Ac-$(Ala)_2$-Pro-$AlaCH_2Cl$; *N*-acetyl-L-alanyl-L-alanyl-L-alanine chloromethyl ketone, Ac-$(Ala)_3CH_2Cl$; carbobenzoxy-L-alanine chloromethyl ketone, Z-$AlaCH_2Cl$; *N*-*t*-butyloxycarbonyl-L-alanine-*p*-nitrophenol, Boc-Ala-ONp; *N*-acetyl-DL-alanine-α-naphthyl ester, Ac-DL-Ala-1-ONap; Diisopropylfluorophosphate, Dip-F; Goat anti-rabbit immunoglobulins antiserum, GARG; slow-moving esterases, SE.

tase isozymes was performed as follows. Two-hundred-sixty-seven micrograms of granule extract protein were loaded onto each of four acrylamide gels, and standard cationic disk-electrophoresis was carried out (12). After electrophoresis, the anodal segment of each gel was removed. These segments contained the SE proteins, as judged from other gels stained for protein content and for esterase activity against Ac-DL-Ala-1-ONap. Care was taken to exclude portions of the gel containing the rapidly migrating elastase isozymes. The anodal segments were then lyophilized, pooled, pulverized with a glass rod, and extracted in 5.0 *M* NaCl + 0.05 *M* sodium phosphate buffer, pH 7.5, for 6 hr in the cold. The extract was clarified by centrifugation, concentrated by ultrafiltration (Amicon XM-50 membrane), dialyzed vs H_2O, and lyophilized. The lyophilate was restored to 0.1-ml vol with H_2O. Forty microliters were subjected to cationic electrophoresis in each of two new acrylamide gels, which were subsequently stained either for protein or for esterase activity vs Ac-DL-Ala-1-ONap. This was done to check the original gel extract for recovery of active SE as well as for the presence of contaminating elastase isozymes. [Previous work (14) has shown that as little as 0.7 μg of elastase isozymes can be detected in gels stained with Ac-DL-Ala-1-ONap.] The remainder of the reconstituted lyophilate (20 μl) was used to fill the well of an immunodiffusion agarose plate. An adjacent well was filled with 3 μg of purified leucocyte elastase isozymes (10), and a third well received 20 μl of Amicon filtrate. The center well of the plate was filled with a monospecific rabbit antiserum prepared against purified human leucocyte elastase isozymes (10). Precipitin lines which subsequently developed were amplified by secondary immunodiffusion of the exhaustively washed agarose plate against goat anti-rabbit immunoglobulin antiserum (GARG).

Results and discussion. Typical electrophoretic gel patterns of human blood leucocyte granular extracts are shown in Fig. 1. Gel 1A was stained for proteins and shows multiple bands. The four leucocyte elastase isozymes are in the middle third of the gel and are marked with single arrows in Fig. 1A. Gel 1B was stained with Ac-DL-Ala-1-ONap. The elastase isozymes are revealed in the midportion of the gel as zones of reaction product, while the anodal part of the gel (top) contains an additional zone of similar esterase activity. This is the slow-moving esterase (SE).

The esterase activity of SE required the presence of Triton in the gel (0.4%) whereas that of the more rapidly moving isozymes did not, an observation previously reported by Sweetman and Ornstein (5) and confirmed by one of us (7). These results are shown in Fig. 1C, which represents a disk gel stained for esterase activity in the absence of Triton.

The slow rate of migration of SE relative to the elastase isozymes can be seen in Fig.

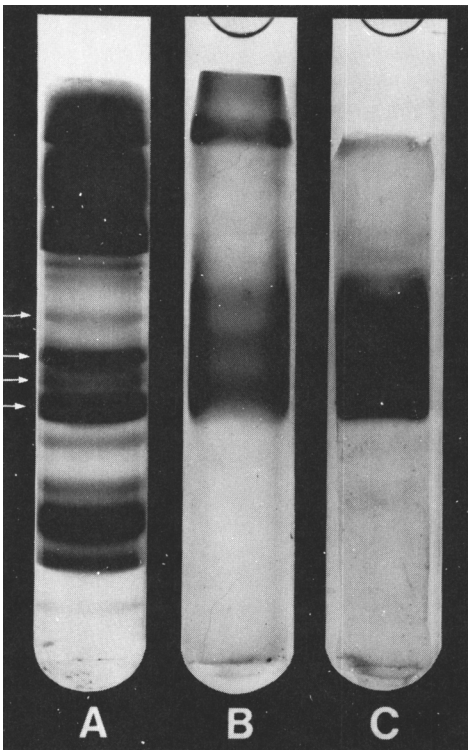

FIG. 1. Acrylamide electrophoretic gel patterns of human blood leucocyte granule extracts. Migration from top to bottom. Each gel was loaded with 535 μg of granular proteins. (A) Buffalo Black stain; (B) Ac-DL-Ala-1-ONap stain; (C) same as (B), but without Triton in the acrylamide gel. Arrows in gel (A) mark the location of four elastase isozymes. (Note that although gel (C) is shorter than gels (A) and (B), its top corresponds to the interface between spacer and separation gels as do the tops of the other two gels shown in the figure.)

1. Quantitative determination of esterase activity in gels stained with Ac-DL-Ala-1-ONap (14) showed that 10–15% of the total granule activity against this substrate was due to SE in the anodal region.

The effect of Triton on the enzymatic activity of SE against Boc-Ala-ONp was also tested as follows. After electrophoresis of leucocyte granular extract, the anodal parts (3–4 mm) of the gels were cut off and lyophilized. The lyophilized gel was extracted with neutral, 0.1 *M* phosphate buffer, and the extracts were concentrated and lyophilized again. Boc-Ala-ONp esterase activity of the final redissolved lyophilate was then determined. When 0.4% Triton was included in the enzymatic reaction mixture, the esterase activity was 61% greater than in the case of reaction mixtures not containing Triton. Detergents like Triton may affect either the conformation or state of aggregation of SE allowing it to become active against Ac-DL-Ala-1-ONap and to increase its activity against Boc-Ala-ONp, both typical leucocyte elastase substrates.

Several additional experiments were carried out to further characterize the activation and inhibition properties of SE. Treatment of leucocyte granule extract with 6.6×10^{-4} *M* Dip-F prior to electrophoresis resulted in complete inhibition of the esterase activity of elastases as well as of SE (Fig. 2B). Preincubation with 6.6×10^{-4} *M* Ac-$(Ala)_2$-Pro-AlaCH_2Cl, a powerful elastase inactivator (15), completely inactivated the elastase isozymes with no effect on SE (Fig. 2C). The inability of this elastase inactivator to affect SE was observed, even though Triton was present during the preincubation. Sweetman and Ornstein (5) reported that Ac-$(Ala)_3CH_2Cl$, in low concentration, did not affect SE while it did inactivate the elastase isozymes. When they increased the inactivator concentration to 2×10^{-3} *M*, they achieved appreciable inactivation of SE as well. In our experience, neither 6.6×10^{-4} *M* Ac-$(Ala)_2$-Pro-AlaCH_2Cl nor 6.6×10^{-4} *M* Z-AlaCH_2Cl was able to affect SE in gels. However, we did not test 10^{-3} *M* concentrations of these inactivators. Preincubation of granule extract with elastatinal, a powerful microbial peptide inhibitor of pancreatic elastase (16), did not affect the esterase activities in acrylamide gels of either the elastase isozymes or SE. This result was consonant with earlier observations of the present authors, namely that elastatinal is a rather weak inhibitor of human leucocyte

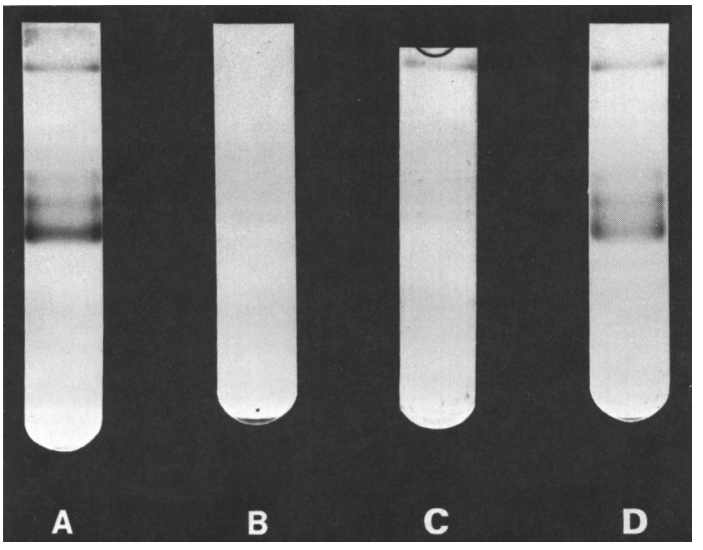

FIG. 2. Acrylamide electrophoretic gel patterns of human blood leucocyte extracts pretreated with inhibitors. Migration from top to bottom. Each gel was loaded with 107 μg of granular proteins. The granular extracts, before being loaded onto the gels, were preincubated for 1 hr at pH 7.5, at room temperature, with various inhibitors. All the gels were stained with Ac-DL-Ala-1-ONap. (A) Control (incubated without inhibitor); (B) preincubated with 6.6×10^{-4} *M* Dip-F; (C) preincubated with 6.6×10^{-4} *M* Ac-$(Ala)_2$-Pro-AlaCH_2Cl; (D) preincubated with 400 μg of elastatinal.

elastase, in comparison to porcine elastase (17).

Acrylamide gel electrophoretic patterns of 0.1 *M* NaCl granule extracts from two preparations of human bone marrow leucocytes are shown in Fig. 3. As can be seen, the gel electrophoretic patterns (protein stains) of bone marrow leucocyte extracts proved to be significantly different from those of peripheral blood leucocytes (compare Fig. 1A to Fig. 3A and B). From the protein staining, it appeared that the content of elastase isozymes (middle region) was much lower in bone marrow leucocyte granule extracts than in granule extracts from peripheral blood leucocytes. This difference was even more strikingly demonstrated when the esterase activities against Ac-DL-Ala-1-ONap were compared (see Fig. 1B vs Fig. 3a and b). No trace of elastase isozyme activities (middle region) appeared in the gels containing bone marrow leucocyte granule extracts. The only visible esterase activity against Ac-DL-Ala-1-ONap occurred in the anodal portions of the gels. Although the esterase stains shown in Fig. 1B and C (blood leucocyte granules) were obtained with large quantities of granular protein (535 μg), we previously showed that the Ac-DL-Ala-1-ONap gel-staining method is sensitive enough to detect 0.7 μg of pure leucocyte elastase isoenzymes (14) and that this method easily demonstrates these enzymes in as little as 10 μg of crude granular protein derived from blood leukocytes (7). Therefore, the total absence of isoenzyme staining in Fig. 3 (gels a and b), in which 94 and 200 μg of marrow leucocyte granular protein were tested, clearly suggests that enzymatically mature elastase isoenzymes are deficient in marrow leucocytes. The differential leucocyte count of these two bone marrow samples is given in Table I. Morphologically mature leucocytes made up about 50% of the total. Thus, despite the presence of a significant number of morphologically mature cells, enzymatic maturity was apparently still lacking, at least in the case of the elastase isozymes.

For a final experiment, SE was separated from the elastase isozymes by preparative gel electrophoresis of granule extracts prepared from peripheral blood leucocytes. Various fractions extracted from the preparative acrylamide gels (as described in *Methods*) were then tested by double immunodif-

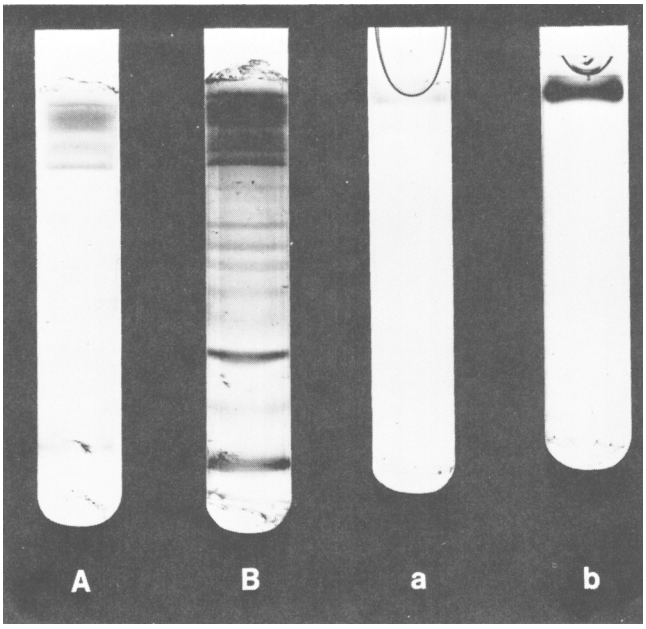

FIG. 3. Acrylamide electrophoretic gel patterns of human bone marrow leucocyte granule extracts. Migration from top to bottom. Gels (A) and (a) were each loaded with 94 μg of granular proteins from bone marrow No. 1. Gels (B) and (b) were each loaded with 200 μg of granular proteins from bone marrow No. 2. Gels (A) and (B), Buffalo Black stain; gels (a) and (b), reacted with Ac-DL-Ala-1-ONap for esterase activity.

fusion against a monospecific rabbit antiserum to purified leucocyte elastase isozymes (10). Evidence of antigenic cross-reactivity between the isozymes and SE was obtained (see Fig. 4). A precipitin line (single arrow) developed between the antiserum well and the well containing SE, and the line was continuous with the precipitin line (double arrow) given by pure leucocyte elastase isozymes reacting against this same antiserum. Electrophoretic analysis of the SE fractions used in this experiment revealed active SE, as expected, but failed to detect any contamination of the fractions with elastase isozymes.

Overall, the foregoing studies suggest several conclusions. The first is that during the maturation process of leucocytes, there are not only morphological changes in the cells but also biochemical-functional changes in their enzymatic contents. Elastase isozymes are stored in the azurophil granules of neutrophil leucocytes (6) and these azurophil granules appear, morphologically, in the promyelocyte stage of development (18). Yet, in our experience, active elastase isozymes could not be demonstrated functionally in marrow leucocytes, the preponderant majority of which were beyond the promyelocyte stage of their maturation process (see Table I). Morphologically mature leucocytes made up about 50% of the total. Thus, despite the presence of a significant number of morphologically mature cells, enzymatic maturity was apparently still lacking, at least in the case of the elastase isozymes. We had previously noticed that extracts of preleukemic and leukemic leucocyte granules were also deficient in elastase isozymes, but did contain SE activity.

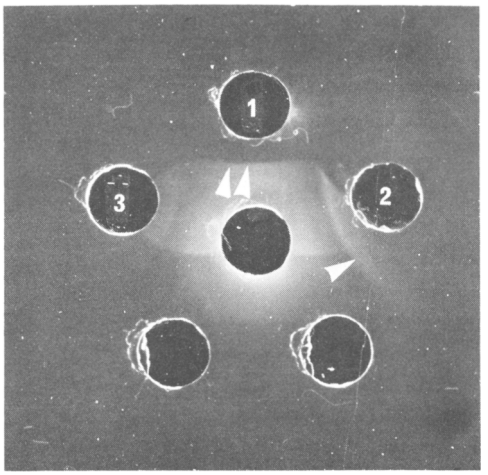

FIG. 4. Double immunodiffusion between rabbit antiserum to leucocyte elastase and SE. Center well, 8 μl of antiserum; well 1, 3.0 μg of elastase isozymes; well 2, 20 μl of acrylamide gel extract containing SE; well 3, 20 μl of Amicon filtrate (see Methods). The pattern was amplified by secondary diffusion against goat antirabbit immunoglobulin antiserum. For explanation of arrows, see text. (Note that the hexagonal-shaped zone of precipitation surrounding the center well is an artifact of the amplification procedure, which occurred despite extensive washing of the agarose prior to its treatment with the goat antiserum.)

TABLE I. DIFFERENTIAL COUNT OF BONE MARROW LEUCOCYTE PREPARATIONS.[a]

Cell type	Percentage of total cells	
	Marrow 1	Marrow 2
Blasts	3.5	0.5
Promyelocytes	0.5	0.5
Myelocytes	8.5	12.0
Metamyelocytes	34.5	24.5
Total Immature	47.0	37.5
Bands	21.0	13.0
Polymorphs	22.5	39.5
Total Mature	43.5	52.5
Eosinophils	1.0	0.5
Lymphocytes	6.5	8.5
Erythrocytes	2.5	1.5

[a] Cells were smeared, stained, and counted at the final stage in their processing. This was after removal of most erythroid precursors and mature red cells by hypotonic lysis, and of platelets by differential centrifugation.

A second point raised by our results is that the presence of SE in functionally immature leucocytes, which do not yet have active elastase isozymes, suggests that SE may be a precursor or zymogen form of these enzymes. The observation of antigenic cross-reactivity between SE and the elastase isozymes of mature cells is consistent with the above interpretation. The fact that SE is Triton-activatable suggests that it may be an aggregated precursor form of the isozymes, and its slow migration into 13% acrylamide gels also supports this view. Several trials with elastin-containing acrylamide gels, prepared as described by Taylor and Crawford (9), failed to reveal lytic activity of SE against bovine ligament elastin; whereas in this test all three elastase isozymes were active. SE has the specificity required to attack elastin, since it hydrolyses Ac-L-Ala-L-Ala-L-Ala-1-ONap, a highly specific elas-

tase synthetic substrate (unpublished observations of the present authors and (5)). However, an aggregated form of the enzymes might be sterically prevented from interacting with macromolecular substrates like elastin, while still being able to attack low MW substrates. On the other hand, the relative resistance of SE to low MW chloromethyl ketone inactivators is difficult to interpret. Many questions remain concerning the properties and function of SE in human neutrophil leucocytes, and further study will be required to resolve them.

Summary. Granules of human peripheral blood leucocytes contain four well-characterized elastase isozymes and one or two slow-moving elastaselike esterases (SE) which have not been as well characterized. SE are capable of hydrolyzing typical elastase synthetic substrates such as *N*-acetyl-DL-alanine-α-naphthyl ester (Ac-DL-Ala-1-ONap) and *N-t*-butyloxycarbonyl-L-alanine-*p*-nitrophenyl ester (Boc-Ala-ONp), but unlike the highly basic elastase isozymes, SE barely migrate into 13% acrylamide gels during cationic electrophoresis at pH 4.3. Hydrolysis of Ac-DL-Ala-1-ONap by SE requires the presence of Triton in the gel, and hydrolysis of Boc-Ala-ONp by the same enzyme(s) is also enhanced in the presence of the detergent. Triton is not required for these activities, in the case of the elastase isozymes. Diisopropylfluorophosphate (Dip-F) inactivates both SE and the elastase isozymes, whereas Ac-$(Ala)_2$-Pro-AlaCH$_2$Cl (a powerful inactivator of the leucocyte elastase isozymes at 10^{-4} *M* concentration) does not inactivate SE at the same concentration. Immunochemical studies revealed antigenic cross-reaction between the rapidly migrating leucocyte elastase isozymes and SE. Two preparations of leucocyte granules from nonleukemic bone marrow cells showed no activity of the rapidly migrating elastase isozymes, but did contain SE activity. SE may be a precursor or zymogen form of the elastase isozymes, present in immature cells and partly retained through later stages of development.

We would like to thank the New York Blood Center for their cooperation in supplying human peripheral venous blood, and Dr. R. Prasad (V.A. Hospital, Northport, L.I.) for supplying samples of bone marrow for the collection of immature human leucocytes. We also thank Drs. James C. Powers and Peter M. Tuhy for generously supplying chloromethyl ketone derivatives, and Dr. Takashi Sugimura for generously supplying elastatinal. We thank Jules M. Elias for differential counting of bone marrow leucocytes, and Rosemarie Dearing for her technical assistance throughout the experiments.

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Received November 24, 1975. P.S.E.B.M. 1976, Vol. 152.