Proteases of Human Leukocytes

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The existence of considerable proteolytic activities associated with leukocytes has previously been reported, but little characterization of the enzymes has been carried out.1–3 Experiments with leukocyte preparations using newer enzyme inhibitors are very limited and it seemed probable that improved characterization might result from studies of this type. It was also hoped that the studies might indicate whether leukocyte protease activity could be sufficient to contribute appreciably to the breakdown of damaged tissues invaded by a high concentration of white cells. The present report describes the results of preliminary studies indicating the existence of at least three intracellular proteolytic enzymes in human leukocytes.

Materials and Methods

Leukocyte preparation.—Leukocytes were collected from fresh human blood by a modification of the method of Minor and Burnett.4 Blood was collected from active Negro tuberculous patients having high sedimentation rates and leukocytosis with a preponderance of polymorphonuclear cells of 55 to 85 per cent. The following procedure was used for the isolation of leukocytes: 10 ml. of fresh blood were drawn into a syringe containing 0.6 ml of 0.1 per cent heparin solution and transferred to a test tube; a human fibrinogen solution containing 40 mg in 1.25 ml of distilled water was added to the blood and the contents of the tube mixed by inversion. The fibrinogen solution was freshly prepared and incubated at 37 C. for 5 minutes before use. The mixture of blood and fibrinogen was pipetted into a dry test tube (leaving the part above the sample clean), and incubated for 1 hour at 37 C. with the tube slanted at a 60 degree angle. The erythrocytes sedimented, leaving a suspension of leukocytes in the supernatant plasma; the latter was pipetted into a 12 ml. conical centrifuge tube and centrifuged at 3000 to 3500 rpm for 5 minutes. The supernatant fluid was discarded and the cell precipitate retained for further purification.

This method recovered 60 to 80 per cent of the total leukocytes and eliminated most of the erythrocytes. At this stage, the leukocyte precipitate contained 0.1 to 0.2 per cent of erythrocytes (i.e., 5,000 to 10,000 RBC/cu.mm.). Results from preliminary experiments showed that these remaining erythrocytes could be removed from the leukocytes by differential hemolysis as follows: Two to three ml. of 4 per cent albumin in 0.32 per cent NaCl was added to the cell precipitate, stirred well, centrifuged and the supernatant fluid discarded. The process was repeated and resulted in almost complete hemolysis of the erythrocytes (0 to 100 RBC/cu.mm.). There was an appreciable swelling of the leukocytes due to the osmotic effect. In a few cases, the removal of erythrocytes was less effective; these were in samples from patients with a dormant stage of sickle cell anemia or with sickle cell trait, which is well known to exist in the Negro race and to be associated with...
a reduced osmotic fragility. In no instance was the final RBC concentration more than 500 to 1000 RBC/cu.mm.

The white cell suspension was finally washed twice with 4 per cent albumin in 0.9 per cent NaCl. This reduced the size of the leukocytes almost to the original volume by the reverse osmotic effect. A cell count was carried out before the last centrifugation in order to determine the final volume of the suspending medium required to bring the cell count to a uniform one of 25,000 cells/cu.mm. The suspending medium was 4 per cent albumin in 0.9 per cent NaCl but a few samples were suspended in their original plasma, plasminogen solutions or inactivated plasmin solutions.

It was found that the leukocytes suspended in 4 per cent albumin per 0.9 per cent saline remained intact for 24 to 48 hours at temperatures between 2 C. and room temperature. Storage over night in a frozen state ruptured 10 to 30 per cent of the cells. The dilute saline solutions used for hemolysis of the erythrocytes resulted in alteration of the cellular structure of the leukocytes which was observed as granulation in the nucleus.

Proteolytic assays.—The hydrolytic activity of proteolytic enzymes was determined by the rate of hydrolysis of 1 per cent casein or 2 per cent denatured hemoglobin and measurement of the increase in absorbance at 280 m\(\mu\) of material not precipitated by 5 per cent w/v trichloroacetic acid at 50 C. Absorbance measurements for casein and hemoglobin hydrolysis were made with a Beckman DU spectrophotometer. Assays were carried out at 37 C. rather than at 23 C., as is common with proteolytic enzymes, since the increased rate was advantageous for the low rates of hydrolysis obtained with small samples. Enzymes such as trypsin and chymotrypsin (used for control experiments), substrates and inhibitors were obtained from commercial sources with the exception of di-n-butyl fluorophosphate. This compound, an excellent inhibitor of trypsin and chymotrypsin, was prepared by an exchange reaction between dibutyl chlorophosphate and sodium fluoride as previously described.

EXPERIMENTAL

Preparation of enzyme extracts.—Muller and Jochman found that fresh leukocytes incubated at 37 C. did not produce hydrolysis of a serum-agar plate; if, however, the suspensions were heated to 55 C. to destroy the cells and liberate the enzymes, activity resulted. Dry ether-alcohol powders obtained from leukocytes are also active. In some of our own preliminary experiments, acetone powders of leukocytes were prepared but variable results were obtained, possibly because of the difficulty of handling and drying the small samples.

Most of our leukocyte samples were stored in a deep freeze overnight; after thawing, proteolytic activity was observed. Active suspensions could also be obtained by ultrasonic rupture of the white cells or by disintegration on treating with surface active agents. Ultrasonic rupture was produced by the supersonic frequencies generated in a “Sonoblast” cleaner; the polymembrane cells showed a greater fragility than did the lymphocytes but it was not possible to use this difference for separation of the two types of cells. In unpurified cell extracts it is impossible to define a baseline for maximal enzyme activity. While ultrasonic rupture of the cell wall produces proteolytic activity, this treatment might not break down small intracellular particles with which the enzymes may be associated; on the other hand, surface-active agents, although liberating the enzymes, may be inhibitory to some components of the system.

Rossiter in studies of the esterases of leukocytes produced cellular disintegration by treatment with surface-active agents. In the present study the
After standing in contact with the active agent for 24 hours at 0 to 2 °C., the suspensions were centrifuged at high speed and the supernatant solution separated; any precipitate was redispersed in a small amount of phosphate buffer (pH 7.4). The proteolytic activity of each sample was determined. At concentrations leading to complete cellular dissolution, the synthetic detergents inhibited protease activity appreciably, but saponin gave good results, yielding an active enzyme extract. Rossiter and Wong found that saponin did not seem to inhibit the esterase activity of the white cell; however, when tested on proteolytic enzymes it was found to be an inhibitor of pepsin but not of chymotrypsin or trypsin (table 1). After a two hour treatment in the “Sonoblast,” a sample of leukocytes was compared for proteolytic activity with a saponin-treated sample. The results indicated that there was some inhibition of proteolytic activity at pH 7.6. The majority of the following experiments were performed with “Sonoblast”-treated samples; the remainder were carried out with saponin-treated material.

**Studies of proteolytic activity.**—The effect of pH on the rate of hydrolysis of casein was studied first. Typical results are shown in figure 1. The three maxima in the curve suggest that at least three proteolytic enzymes are present. In view of the possibility of contamination with serum enzymes or by plasminogen from the fibrinogen used in the cell preparation, determinations were made with and without the addition of streptokinase. On addition of streptokinase the leukocyte preparation hydrolyzed fibrin clots slowly but only a small potentiation (less than 10 per cent) of casein hydrolysis was observed in pH ranges above 7.5; in the absence of streptokinase, it would appear that the contribution of plasminogen to the observed proteolytic activity of the cell extracts is negligible. The influence of enzyme concentration and time of reaction was determined at pH 7.6 and 3.0 (figs. 2 and 3).

In order to obtain further information about the nature of the leukocyte proteases, the effects of a number of potential inhibitors and activators were determined with the white cell extracts. In the preliminary survey, each reagent was allowed to react with the enzyme for 30 minutes at pH 7 before the addition of substrate, and the effect on activity was determined at pH 3.0, 6.0 and 8.0. The results of these experiments are presented in table

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**Table 1.—Effect of Saponin on Proteolytic Enzymes**

<table>
<thead>
<tr>
<th>Enzyme Type</th>
<th>pH</th>
<th>Concentration</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pepsin (pH 3)</td>
<td>100</td>
<td>(a)</td>
<td>100</td>
</tr>
<tr>
<td>Chymotrypsin (pH 8.0)</td>
<td>&lt;10</td>
<td>(b)</td>
<td>0</td>
</tr>
<tr>
<td>Trypsin</td>
<td>10</td>
<td>(c)</td>
<td>0</td>
</tr>
<tr>
<td>Leucocyte extract</td>
<td>&lt;10</td>
<td>(d)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Control value that of sample of cells ruptured in “Sonoblast.”

*Saponin concentration: (a) = 1%; (b) = 0.1%; (c) = 0.01%; (d) = 0.001%.*
Fig. 1.—Effect of pH on hydrolysis of casein by human leukocyte extract. Open circles, with no inhibitor; full circles, after treatment with $10^{-3}$ M di-n-butyl fluorophosphate. Dotted line represents difference between the two curves and corresponds to activity of the alkaline protease of leukocytes.

2. In this survey, no potent inhibitors of either the enzyme with an optima at pH 3 or that with optimal activity in the region of pH 6 were observed. The results might be due in part to the use of crude cell extracts in which the inhibitor could react preferentially with other materials present. Several inhibitors of the pH 8.0 enzyme were noted. The best of these were di-n-butyl fluorophosphate and a related toxic organophosphorus compound, bis(2-chloroethyl)-1-ethoxy-2,2'-dichlorovinyl phosphate. A sample of the leukocyte extract was treated with the fluorophosphate ($10^{-3}$ M) and the pH activity curve of the preparation determined. The results are also included in figure 1 and show that this inhibitor abolished one peak in the enzyme activity curve. The contribution of the inhibited enzyme could then be obtained by difference as shown in the figure: It showed that the enzyme has a very similar pH-dependence curve to that of trypsin or chymotrypsin. It should be noted that ovimucoid has no inhibitory effect on the activity but that there was some inhibition by soybean inhibitor. In this respect, the similarity is closer to chymotrypsin than it is to trypsin. The alkaline protease of leukocytes was relatively much less sensitive to inhibition by di-n-butyl-1-ethoxy-2,2'-dichlorovinyl phosphate than were chymotrypsin or trypsin.

A study was made of the relative proteolytic activities of a series of leukocyte preparations from blood samples. It was found that the activity at pH 8 could be correlated with the number of polymorphonucleocytes, while pro-
Fig. 2 (at left).—Effect of time of incubation at 37 C. on hydrolysis of 1 per cent casein by human leukocyte extract at pH 3.0 and pH 8.0.

Fig. 3 (at right).—Relationship between volume of enzyme extract and extent of hydrolysis of 1 per cent casein by human leukocyte extract. Incubation time 2 hours at 37 C.

teolysis at pH 3 and 5.5 was proportional to the number of lymphocytes (fig. 4). These studies were carried out with cell preparations all containing a total of 25,000 cells/cu.mm. After a differential count, the numbers of polymorphonuclear cells and lymphocytes per unit volume in each preparation could be determined; these are the figures represented as the abscissa in the figure.

Preliminary studies of substrate specificity have been made. When denatured hemoglobin was used as the substrate, the pH activity curve differed from that with casein (fig. 5). At pH 7.6 there was some hydrolysis of acetyl tyrosine ethyl ester but the rate was low compared to that anticipated if the relative rates of hydrolysis of casein and acetyl tyrosine ethyl ester were similar to those found with chymotrypsin. Benzoyl arginine methyl ester was not hydrolyzed at this pH. Further studies of the properties of the component enzymes in this system will be carried out when purified fractions are obtained.

Conclusions

The results presented show that human leukocyte preparations contain three active proteolytic enzymes. Two of these have pH optima at about pH
The alkaline protease of leukocytes is apparently derived from the polymorphonuclear cells. Inhibition studies with organophosphorus compounds, soybean inhibitor and ovimucoid and an ability to hydrolyze acetyl tyrosine

**TABLE 2.—Effect of Chemical Reagents on the Activity of Leukocyte Proteases**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Conc.</th>
<th>pH 3.0</th>
<th>pH 6.0</th>
<th>pH 8.0</th>
</tr>
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<tbody>
<tr>
<td>Soybean trypsin inhibitor</td>
<td>.1 mg. ml.</td>
<td>0</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Ovimucoid</td>
<td>.1 mg. ml.</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Di-n-butyl fluorophosphate</td>
<td>$10^{-3}$ M</td>
<td>0</td>
<td>25</td>
<td>97</td>
</tr>
<tr>
<td>Bis(2-chloroethyl)-1-ethoxy-</td>
<td>$4 \times 10^{-4}$ M</td>
<td>-</td>
<td>-</td>
<td>74</td>
</tr>
<tr>
<td>2,2'-dichloro vinylphosphate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Di-n-butyl-1-ethoxy-2,2'-dichloro</td>
<td>$4 \times 10^{-4}$ M</td>
<td>-</td>
<td>-</td>
<td>16</td>
</tr>
<tr>
<td>vinylphosphate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iodine</td>
<td>$4 \times 10^{-5}$ M</td>
<td>0</td>
<td>12</td>
<td>70</td>
</tr>
<tr>
<td>Ethylenediamine tetraacetate</td>
<td>$10^{-3}$ M</td>
<td>10</td>
<td>-</td>
<td>15</td>
</tr>
<tr>
<td>Mercuric ion</td>
<td>$10^{-3}$ M</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>18</td>
</tr>
</tbody>
</table>

*The following compounds ($10^{-3}$ M) had negligible effect at the three pH values: iodoacetamide, sodium azide, reduced glutathione, Co$^{++}$, Mn$^{++}$.**

**FIG. 4.—Correlation between (a) cell counts of lymphocytes and proteolytic activity of extract at pH 5.5 and 3.0, (b) cell count of polymorphonuclear cells and proteolytic activity at pH 8.0.**

3 and pH 5.5 for the hydrolysis of casein, respectively, and are probably derived from lymphocytes. Selective inhibition of these two enzymes was not achieved. The enzyme active at pH 3.0 did not appear to be inhibited by saponin; this contrasts with pepsin, which is inhibited by saponin concentrations as low as 0.001 per cent. In the crude cell extracts employed in the present preliminary study, thiol reagents did not inhibit the enzyme with an optimum in the region of pH 5.5; certain cathepsins of similar pH optimum are considered to be SH enzymes, and these properties may afford a basis for differentiation.
ethyl ester indicate a class similarity to chymotrypsin, rather than to trypsin, although there is no evidence for the existence of an inactive precursor form corresponding to chymotrypsinogen. The resemblance to trypsin is shown in the inhibition of the alkaline protease of leukocytes by serum protease inhibitors. Early studies by Jobling and Strouse indicated that proteolytic digestion by white cell enzymes was not so complete as with trypsin; in this respect, it may be noted that preliminary results from the present study showed only very low rates of hydrolysis of synthetic substrates (e.g., acetyl tyrosine ethyl ester), compared to relatively high rates of proteolysis.

The concentration of potential proteolytic activity in tissue injury in which there is invasion by leukocytes is high. Jochman has suggested that digestion products produced by these enzymes may contribute to the physiologic responses resulting from infection. Further studies of the purification and properties of the proteases of leukocytes are in progress. The availability of new technics now makes it possible to isolate and characterize the degradation products of proteins; investigations of the possible formation of peptides with pharmacologic activity by enzymatic breakdown of tissue components by leukocyte proteases may contribute to knowledge of the physiologic effect of injury.

**SUMMARY**

Cell extracts possessing considerable proteolytic activity have been obtained from human leukocytes by ultrasonic breakdown or by treatment with surface active agents. Three proteolytic enzymes are present; one with an optimum at approximately pH 8 is inhibited by organophosphorus compounds and resembles chymotrypsin. This enzyme is derived from polymorphonuclear leukocytes. Lymphocytes contain two proteases with pH optima of 3.0 and 5.5, respectively.
PROTEASES OF HUMAN LEUKOCYTES

SUMMARIO IN INTERLINGUA

Extractos cellular que possede grados considerabile de activitate proteolytic eseva obtenite ab leucocytos human per decomposition ultrasonic o per tractamento con agentes a activitate superficial. Tres enzymes proteolytic eseva presente. Un de illos, con activitate optimal a pH 8 es inhibite per compositos a phosphoro organic e resimila chymotrypsina. Iste enzyma es derivate ab leucocytos polymorphonuclear. Lymphocytos contine duo proteases con valores optimal de pH a 3,0 e 5,5 respectivemente.

REFERENCES

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