Prevention of Degradation of Human Polymorphonuclear Leukocyte Proteins by Diisopropylfluorophosphate

By Philip C. Amrein and Thomas P. Stossel

Proteases can complicate the characterization of proteins from cells, especially human polymorphonuclear leukocytes (PMN), which contain abundant neutral proteases. We tested the ability of agents to inhibit proteolysis, with special reference to the subunit polypeptides of the contractile proteins actin, myosin, and actin-binding protein (ABP). Phenylmethylsulfonyl fluoride (PMSF), O-phenanthroline, EGTA, EDTA, N-ethylmaleimide, alone or in combinations, failed to prevent extensive proteolysis of the PMN proteins during solubilization of cells with dodecyl sulfate. These inhibitors and also a-1-antitrypsin and soybean trypsin inhibitor similarly could not prevent proteolysis during homogenization of cells in cold isomolar sucrose. Treatment of PMN with DFP under conditions inhibiting proteolysis did not extract. suggesting that the efficacy of DFP may result from its permeation of intact cells and granules before barriers are disrupted by detergents or homogenization. Treatment of PMN with DFP under conditions inhibiting proteolysis did not affect their rate of phagocytosis. We recommend the use of DFP in future studies correlating functions and protein structure of PMN.

THE ACTION OF PROTEASES can complicate the identification, quantification, and characterization of proteins in cells. This complication is potentially most serious in cells such as polymorphonuclear leukocytes (PMN) and macrophages, which are endowed with large quantities of proteolytic enzymes. PMN contain numerous neutral proteases including elastase-like enzymes, collagenase, and cathepsins-

To minimize proteolysis, we and other investigators have homogenized cells in media containing isoosmolar sucrose to prevent rupture of lysosomes prior to their removal by centrifugation and have included antiproteases, such as PMSF, and divalent cation chelators in the media. In this article, we document that these maneuvers are sufficient in studies of rabbit lung macrophage proteins. However, proteolysis in human PMN is preventable only with DFP, possibly because this agent can penetrate intact cells.

MATERIALS AND METHODS

Isolation of Cells

Venous blood was drawn from healthy human volunteers into syringes containing 0.25 volumes of ACD anticoagulant. Neutrophils were purified from the blood by a modification of Boyum's method.14 One volume of 2% dextran (170,000 molecular weight) was added to the blood, and the mixture was left standing for 30 min, during which time erythrocytes sedimented. The neutrophil-rich supernatant fluid was transferred to 50-ml conical plastic centrifuge tubes. Two volumes of ice-cold 0.87% NaCl were added to lyse the erythrocytes.16 The leukocytes were immediately centrifuged at 4°C at 200 g for 8 min, and the leukocyte pellet was suspended in 4 ml of 0.15 M NaCl. The suspension was layered over a 3-ml aqueous solution of 5.7 g Ficoll 400 and 9.0 g diizzleter sodium per 100 ml (Ficoll-Paque, Pharmacia Fine Chemicals), and centrifuged at 500 g at 20°C for 30 min. The pelleted neutrophils were resuspended and washed in ice-cold 0.15 M NaCl, then suspended in an equal volume of 0.15 M NaCl, and kept on ice until used further. Wright’s stained smears showed 90%–100% PMN, 0%–3% lymphocytes, 0%–6% monocytes, and 0%–1% eosinophils. Macrophages were obtained from the lungs of rabbits by intratracheal lavage as previously described.17 The macrophages were washed with 0.15 M NaCl at 4°C, suspended in an equal volume of 0.15 M NaCl, and kept on ice.

Treatment of Cells With Protease Inhibitors

The neutrophils and macrophages were reacted with diisopropylfluorophosphate (DFP) (Sigma Chemical Co., obtained as a stock solution of 0.1 M in propylene glycol) in one of two ways in a fume hood. (1) To a test tube in an ice bath containing the cell suspension was added 0.02 volumes of 0.1 M DFP stock solution or dilutions of the stock solution in 0.1 M Tris HCl, pH 8. After 5 min, the cells were washed once in 100 volumes of ice-cold 0.15 M NaCl, the end of the test tube being sealed with parafilm, and the supernatant discarded in the fume hood. The cells were kept on ice until aliquoted for individual experiments. All pipette tips were flushed with 5 M NaOH to inactive the DFP. (2) The cell suspensions were first diluted in 3 volumes of 0.1 M Tris HCl containing 5 mM EDTA and 5 mM EGTA, pH 7.4, and aliquoted into test tubes for individual experiments. In a fume hood, the 0.1 M DFP stock

From the Hematology-Oncology Unit, Massachusetts General Hospital, Department of Medicine, Harvard Medical School, Boston, Mass.

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Address reprint requests to Philip C. Amrein, M.D., Hematology-Oncology Unit, Massachusetts General Hospital, Boston, Mass. 02114.

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solution was diluted with an appropriate amount of 0.1 M Tris HCl, pH 6.8, and 0.08 ml was added for each ml of cell suspension. As above, the cells were washed once in 100 volumes of 0.15 M NaCl, test tubes were sealed with parafilm, and the pipette tips were alkalinized before disposal.

Neutrophils were incubated in ice-cold 0.15 M NaCl containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma) for intervals up to 60 min.

**Preparation of Cell Extracts**

To the cell suspension was added an equal volume of a homogenizing medium containing 0.34 M sucrose, 5 mM EGTA, 20 mM imidazole chloride, 0.5 mM ATP, 5 mM dithiothreitol (DTT), 0.25 mg/ml soybean trypsin inhibitor, 0.25 mg/ml α-1-antitrypsin, and 1 mM PMSF, pH 7.0, as previously described. In some experiments, 5 mM EDTA was added and the soybean trypsin inhibitor and the α-1-antitrypsin were omitted. PMSF was added to the homogenizing medium as a concentrated solution in 95% ethanol, prepared within 1 hr of use. The cells were broken in a Dounce homogenizer with a tight-fitting pestle, and the homogenates were centrifuged at 15,000 g at 4°C for 30 min. The supernatants (cell extracts) were decanted and kept in an ice bath. A portion of each supernatant was removed for determination of protein concentration by the method of Lowry. For electrophoresis, the extracts were diluted with 1 volume of electrophoresis buffer (2% SDS, 8 M urea, and 2% β-mercaptoethanol or 1% SDS, 8 M urea, 4.7% β-mercaptoethanol, 10% sucrose, 4 mM EDTA, 40 mM Tris HCl, pH 7.4) and 1 volume of a bromophenol blue solution (bromophenol blue dye in 10% sucrose and 40 mM Tris HCl, pH 7.4) and boiled for 2 min. Some extracts were incubated at 37°C for 10 min before, and for 10 min after, addition of electrophoresis buffer.

**Solubilization of Cells**

To the cell suspension were added 3 volumes of ice-cold 0.1 M Tris HCl, pH 7.4, containing 5 mM EDTA and 5 mM EGTA. In some experiments (see Results), the Tris buffer also contained different concentrations of PMSF, 20 mM EDTA, 20 mM EGTA, 10 mM N-ethylmaleimide (NEM), 10 mM orthophenanthroline, and 5 mM DTT. The cells were then solubilized by addition of 16 volumes of a solution at room temperature containing 8% SDS, 4 M urea, and 1% β-mercaptoethanol. If an inhibitor was present in the Tris buffer, it was also in this solubilizing medium. Some samples were then incubated at 37°C for various times. Immediately after addition of solubilizing medium or else after incubation at 37°C, 4 crystals of sucrose (about 11 mg) and 1 volume of bromophenol blue dye solution per 25 volumes of sample were added to all samples, which were then boiled for 2 min. Solubilizing medium caused PMN, but not macrophages, to form a gel, probably because of the relatively high concentration of DNA in these cells. Addition of 0.15 volumes of 1 N HCl to solubilized PMN made the highly viscous samples fluid enough to pipette.

**Incubation of Cell Extracts With Solubilized Proteases**

Most of the neutral protease content of human PMN resides in cytoplasmic granules. A solution containing PMN proteases was prepared by detergent extraction of a granule-enriched PMN particle preparation. Human PMN were isolated as described above, diluted with an equal volume of homogenizing medium (0.34 M sucrose, 20 mM imidazole chloride, 2 mM ATP, pH 7.5), homogenized, and centrifuged at 12,000 g at 4°C for 30 min. The pellets were suspended in 2 volumes of Triton X-100 (0.1% in homogenizing medium), incubated for 4°C for 20 min, and then centrifuged at 12,000 g at 4°C for 10 min. The supernatants, containing 1 mg/ml protein, were stored at -20°C.

Some of the Triton extracts were incubated in 1 mM DFP or 1 mM PMSF by adding 1 volume of a 10 mM solution of the inhibitor to 9 volumes of the Triton solution and leaving it on ice for 10 min before use. Cytoplasmic extracts were incubated at 37°C for 20 min with 0.2 volumes of the control or inhibitor-treated Triton extracts. The solutions were then prepared as above for electrophoresis in dodecyl sulfate.

**Electrophoresis**

Samples were subjected to electrophoresis for 6–15 hr in 5%-15% polyacrylamide gradient slab gels in the presence of dodecyl sulfate according to the technique of Laemmli. Gels were then stained with Coomassie brilliant blue and scanned for quantitative analysis of protein bands by means of a gel scanner (Densitometer, E-C Apparatus Corp.). ABP purified from rabbit lung macrophages, and myosin and actin purified from rabbit skeletal muscle were used as standards to identify the corresponding polypeptide subunits in gels after electrophoresis of reduced and denatured human PMN and rabbit lung macrophages or their extracts. The respective polypeptides from these diverse cell types have been shown to have equal electrophoretic mobilities in dodecyl sulfate. The density of peaks corresponding to these standards was taken as the concentration of the respective polypeptides in the cells or extracts. The proteins are designed ABP subunit, myosin heavy chain, and actin monomer bands, although it is recognized that other proteins might contribute to these peaks, especially of "actin" after lysis of higher molecular weight proteins. The area under the respective peaks was shown to be directly proportional to the quantity of protein applied to the gels for electrophoresis. All samples were adjusted to protein concentrations such that about 500 μg of protein was applied to gel columns.

**Phagocytosis**

Human PMN were isolated and suspended 1:1 and 0.15 M NaCl as described above. One-half of the cells was reacted with 2 mM DFP and one-half kept on ice. The PMN were then diluted with 99 volumes of a modified Krebs-Ringer phosphate medium containing 1 mM Mg and 1 mM Ca, pH 7.4, and aliquoted for tests of phagocytosis as described elsewhere. To 0.8 ml of each PMN suspension was added 0.2 ml of dodecyl phthalate-Oil Red O particles coated with Escherichia coli lipopolysaccharide B (Difco Laboratories, Detroit, Mich.) and opsonized with human serum. Phagocytosis was initiated by incubation at 37°C with agitation and stopped after 4 min with the addition of 6 ml of ice-cold 1 mM N-ethylmaleimide in 0.15 M NaCl. After washing the PMN, the amount of dodecyl phthalate ingested was determined by extracting the Oil Red O with 1 ml dioxane and calculating the concentration of the dye in the dioxane by spectrophotometry at 525 nm. Knowing the original concentration of dodecyl phthalate per optical density at 525 nm, a standardized rate of ingestion can be calculated for 107 PMN/ml/min.

**RESULTS**

**Solubilization of Polymorphonuclear Leukocytes**

Polypeptides comigrating with the ABP subunit, myosin heavy chain, and G-actin were resolved on Coomassie-blue stained polyacrylamide slab gels after electrophoresis of whole PMN solubilized with sodium.
Fig. 1. Coomassie-blue-stained polyacrylamide slab gels of solubilized human PMN and rabbit alveolar macrophages and of PMN and macrophage cytoplasmic extracts. Details concerning solubilization of cells and preparation of extracts are described in Materials and Methods. Lane 1, human PMN reacted with 0.5 mM DFP, solubilized as described in the text, incubated at 37°C for 20 min, and heated at 100°C for 1 min. Lane 2, human PMN treated as described for lane 1, except that the cells were first reacted with 1 mM PMSF instead of DFP, and the solubilizing medium contained 1 mM PMSF. Lane 3, human PMN solubilized and incubated as described for lanes 1 and 2, but without use of either DFP or PMSF. Lane 4, cytoplasmic extract from human PMN reacted with 2 mM DFP before homogenization. After addition of the electrophoresis buffer, the extract was incubated for 10 min at 37°C. Lane 5, cytoplasmic extract from human PMN homogenized in the presence of 0.5 mM PMSF and prepared for electrophoresis as in Lane 4. Lane 6, rabbit macrophages reacted with 2 mM DFP, solubilized, and incubated at 37°C for 20 min. Lane 7, rabbit macrophages solubilized in the presence of 0.3 mM PMSF and incubated as in lane 6. Lane 8, rabbit macrophages solubilized without either DFP or PMSF and incubated as in lanes 6 and 7. Lane 9, cytoplasmic extract from rabbit macrophages treated with 2 mM DFP before homogenization. After addition of electrophoresis buffer, the solution was incubated for 10 min at 37°C. Lane 10, extract from rabbit macrophages homogenized in the presence of 1.0 mM PMSF and prepared for electrophoresis as in lane 9. Arrows and horizontal bars indicate the position of bands comigrating with ABP subunit, myosin heavy chain, and G-actin.

dodecyl sulfate, provided that the cells had been previously reacted with ≥ 0.5 mM DFP. As shown in Fig. 1, these polypeptides were visible on the gels after the solubilized, DFP-treated cells were incubated at 37°C for 20 min and then heated briefly at 100°C. However, if the cells were not treated with DFP, extensive polypeptide breakdown occurred. The ABP and myosin heavy chain bands disappeared, and the actin band was markedly reduced in intensity. One millimolar PMSF retarded the loss of the actin band but was only minimally effective in preventing the degradation of the ABP and myosin bands, even if incubated with the cells up to 60 min prior to solubilization.

The concentrations of polypeptides of solubilized PMN were quantified by densitometry. After solubilization, the concentrations of bands comigrating with ABP subunit, with myosin heavy chain, and with actin monomers, respectively, of DFP-treated cells were 0.8% ± 0.2%, 2.2% ± 0.5%, and 16.9% ± 1.4% (means ± SD for 6 preparations). For PMSF-treated cells the corresponding concentrations were 0.1% ± 0.1%, 0.5% ± 0.4%, and 9.9% ± 2.3% (means ± SD for 9 preparations). Figure 2 depicts a representative experiment that shows that the decrease in intensity of actin and myosin bands in PMSF-treated cells compared to DFP-treated cells was evident immediately after solubilization and did not change with incubation at 37°C. On the other hand, the ABP band of PMSF-treated cells completely disappeared during the incubation. Figure 3 shows the effect of different PMSF and DFP concentrations on the concentrations of ABP, myosin, and actin bands of cells solubilized and then incubated 20 min at 37°C. DFP concentrations of 0.5 mM maximally inhibited the disappearance of these bands. Concentrations of DFP higher than 1 mM did not increase the quantity of these polypeptides (data not shown). PMSF (0.5–1.0 mM) was slightly effective in retarding the degradation of the myosin and actin bands but was ineffective in preventing the loss of the ABP band. Ten millimolar N-ethylmaleimide, 10 mM orthophenanthroline, 20 mM EDTA, and 20 mM EGTA were not helpful, either with or without 1 mM PMSF, in inhibiting the lysis of these proteins. Addition of cells to boiling dodecyl sulfate or to ice-cold trichloroacetic acid before solubilization were also ineffective maneuvers in preventing proteolysis in this setting.
Polymorphonuclear Leukocyte Extracts

Cytoplasmic extracts of human PMN prepared in the presence of 1.0 mM PMSF, 5 mM EGTA, 0.25 mg/ml soybean trypsin inhibitor, 0.25 mg/ml α-1-antitrypsin, and 5 mM DTT lost half of the myosin heavy chain and two-thirds of the ABP subunit bands compared with PMN treated with DFP. The actin band concentrations were similar (Table 1). Extracts of DFP-treated PMN were incubated at 37°C for 20 min in the presence of a Triton X-100 extract of PMN particles as a source of proteases. Under these conditions, the ABP subunit band was degraded by 50% ± 25% (mean ± SD of 3 preparations) and the myosin heavy chain band was degraded by 20% ± 11% (mean ± SD of 3 preparations). However, when the Triton extracts containing the proteases were treated with either 1 mM PMSF or 1 mM DFP prior to incubation with the extracts, there was no degradation of the ABP or myosin bands.

Effect of DFP on PMN Function

As reported by others,25 there was no significant inhibition of phagocytosis if PMN were washed after DFP treatment. Treatment of PMN with DFP at concentrations up to 5 mM by the procedures that were effective in inhibiting proteolysis had little influence on the initial rate of phagocytosis, provided that either the cells were washed after DFP treatment or the DFP was diluted 100-fold by the phagocytosis medium (Table 2).

Table 1. Effect of DFP on Content of Actin, Myosin, and ABP Polypeptides in Extracts of Polymorphonuclear Leukocytes

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>Treatment of Cells before Homogenization</th>
<th>Content Percent of Total Stainable Protein</th>
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<tr>
<td></td>
<td>2 mM DFP</td>
<td>Zero Time 37°C, 20 min</td>
</tr>
<tr>
<td>Actin</td>
<td>-</td>
<td>27 ± 1† 23 ± 1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>29 ± 2 29 ± 1</td>
</tr>
<tr>
<td>Myosin heavy chain</td>
<td>-</td>
<td>1.8 ± 0.1 0.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>3.4 ± 0.2 2.2 ± 0.1</td>
</tr>
<tr>
<td>ABP subunit</td>
<td>-</td>
<td>0.5 ± 0.1 0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.5 ± 0.1 1.1 ± 0.1</td>
</tr>
</tbody>
</table>

*The homogenizing medium contained 1 mM PMSF, 5 mM EGTA, 0.25 mg/ml soybean trypsin inhibitor, 0.25 mg/ml α-1-antitrypsin, 5 mM DTT.
†Mean and range of two separate preparations.
Solubilization of Macrophages

In contrast to PMN, macrophages dissolved in dodecyl sulfate, 5 mM EDTA, and 5 mM EGTA had the same concentrations of ABP subunit, myosin heavy chain, and actin bands whether or not PMSF or DFP was present (Fig. 1). Incubation of the solubilized cells at 37°C did not alter the percentages of cell protein corresponding to the bands (data not shown).

Macrophage Extracts

No degradation of polypeptides occurred when macrophage extracts were prepared from DFP-treated macrophages or from macrophages homogenized in medium containing PMSF (Fig. 1). However, extracts with neither DFP nor PMSF were found to contain 50% less of the ABP subunit band, although the proportion of the myosin and actin bands were unchanged (Table 3). Incubation of inhibitor-treated extracts at 37°C did not change the content of these polypeptides. Extracts not treated with PMSF or DFP contained one-third the original ABP band after incubation at 37°C for 60 min (data not shown).

DISCUSSION

The principal finding of this work is that of various methods tested to inhibit proteolysis, only pretreatment of cells with DFP was effective in preventing the total or partial destruction of proteins of human PMN. The efficacy of DFP was particularly evident with high molecular weight polypeptides and in cells solubilized for electrophoresis in dodecyl sulfate. The high molecular weight polypeptides are particularly suitable for detecting proteolysis by the technique of electrophoresis in dodecyl sulfate, because their disappearance is easily noted and because degradation of yet higher molecular weight polypeptides does not complicate the analysis by generating overlapping bands.

The major effect of DFP implicates the neutral serine proteases of human PMN as the principal factors responsible for the observed degradation of contractile proteins. EDTA, dithiothreitol, and β-mercaptoethanol, all collagenase inhibitors, were also included in solubilizing or homogenizing media, but these agents were clearly ineffective without pretreatment of cells with DFP.

PMSF, also a serine protease inhibitor, alone or in combination with orthophenanthroline, N-ethylmaleimide, EDTA, and EGTA, was inadequate to prevent proteolysis of PMN during solubilization or homogenization. Time-course experiments indicated that proteolysis in the absence of DFP occurred rapidly after addition of dodecyl sulfate, and then slowed or stopped. Heating briefly at 100°C inactivates proteolytic enzymes, but this procedure cannot be done until solubilization of cells has occurred, a process that takes a finite amount of time. The findings indicate that dodecyl sulfate released granule-associated proteolytic enzymes during solubilization faster than it inactivated them. However, the continued destruction of ABP during the incubations at 37°C is further evidence that proteolytic enzymes can be active in sodium dodecyl sulfate solutions. The results also suggest that considerable destruction of the high molecular weight polypeptides occurred during homogenization, before inhibitors in the homogenizing medium could retard the action of proteases present. Once the extract was prepared and incubated at 37°C, PMSF in the medium was as effective in inhibiting proteolysis as pretreatment of the cells with DFP. PMSF and DFP are essentially equally reactive with serine proteases in solution. We infer, therefore, that DFP can penetrate intact cells and granules and neutralize an important fraction of the proteolytic activity. Since some degradation of polypeptides took place with both inhibitors, proteases other than serine proteases and metalloproteases were probably present.

In contrast to PMN, proteolysis in solubilized rabbit lung macrophages or in macrophage extracts was preventable with a combination of inhibitors in the solubilizing or homogenizing medium without utilizing DFP. Possibly the divalent cation chelators present were the most important inhibitory agents, because the proteases of rabbit lung macrophages characterized so far appear to be metalloproteases. However, our results do not rule out that the reaction of macrophages with DFP might be necessary to prevent degradation of proteins other than those studied or during prolonged storage.

Table 2. Effect of DFP Treatment of PMN on Phagocytosis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial Rate of Phagocytosis</th>
<th>Percent Control</th>
</tr>
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<tbody>
<tr>
<td>No DFP</td>
<td>404 ± 31 (4)*</td>
<td>100</td>
</tr>
<tr>
<td>2.0 mM DFP diluted</td>
<td>385 ± 40 (4)</td>
<td>95</td>
</tr>
</tbody>
</table>

*Mean ± SD (number to tests).

Table 3. Effect of PMSF and DFP on Content of Actin, Myosin, and ABP Polypeptides in Extracts of Rabbit Macrophages

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>No Inhibitor</th>
<th>2 mM DFP</th>
<th>1 mM PMSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABP</td>
<td>0.6 ± 0.0 (3)*</td>
<td>0.9 ± 0.3 (3)</td>
<td>1.2 ± 0.4 (3)</td>
</tr>
<tr>
<td>Myosin</td>
<td>1.4 ± 0.2 (3)</td>
<td>1.3 ± 0.4 (3)</td>
<td>1.8 ± 0.6 (3)</td>
</tr>
<tr>
<td>Actin</td>
<td>26 ± 3 (3)</td>
<td>32 ± 3 (3)</td>
<td>28 ± 1 (3)</td>
</tr>
</tbody>
</table>

*Mean ± standard deviation and number of experiments.
This study documents that techniques for avoiding proteolysis cannot be assumed to be effective for a given cell type until proven and that DFP must be used for work with PMN proteins if proteolysis is to be avoided. Since DFP-treated PMN appear unimpaired with respect to a motile function, phagocytosis, the use of DFP in future studies correlating structure and function in PMN can be highly recommended. Although the applicability of our findings to other ameboid cells requires experimental verification, possibly the use of DFP will simplify and resolve some complexities and controversies concerning polypeptide structure between different cell types.

REFERENCES


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PC Amrein and TP Stossel