Purification of a Cellular (Granulocyte) and an Extracellular (Serum) Phospholipase A₂ That Participate in the Destruction of *Escherichia coli* in a Rabbit Inflammatory Exudate*

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A granule-associated phospholipase A₂ from rabbit polymorphonuclear leukocytes and a closely similar phospholipase A₂ from rabbit serum have been purified to near homogeneity by ion-exchange and reverse-phase chromatography. The cellular (polymorphonuclear leukocyte) phospholipase A₂ has been purified >100,000-fold and the extracellular (serum) phospholipase A₂ approximately 60,000-fold. The NH₂-terminal amino acid sequence of the ascitic fluid phospholipase A₂ that we have recently purified from inflammatory exudates produced in rabbits is nearly identical (15 of 16 residues) to that of the polymorphonuclear leukocyte phospholipase A₂ and completely identical (19 of 19 residues) to that of the purified serum phospholipase A₂. The functional properties of these three phospholipases A₂ are indistinguishable. Each enzyme is active against *Escherichia coli* killed by the bactericidal/permeability-increasing protein of polymorphonuclear leukocyte, a property shared only by a subset of phospholipases A₂. The presence of structurally and functionally very closely similar phospholipases A₂ in the cellular and extracellular compartments of an inflammatory exudate is consistent with the apparent role of these enzymes in the destruction of certain microbial invaders during the acute inflammatory response.

The elimination of bacterial invaders requires sequestration by phagocytes and digestion of the killed bacteria. The determinants of the digestive process have not been clearly defined. It is evident, however, that degradation of the macromolecular constituents of bacteria killed by polymorphonuclear leukocytes (PMN) tends to be incomplete (1, 2). As part of an ongoing evaluation of bacterial digestion we have examined the degradation of the phospholipids of *Escherichia coli* ingested and killed by PMN, using sterile peritoneal inflammatory exudates produced in rabbits that provide a source of near homogeneous populations of PMN as well as extracellular host-defense factors (3–5).

In biological events involving hydrolysis of phospholipids of whole cells it has been difficult to identify the responsible enzymes, particularly because the cellular phospholipases are usually trace proteins hampering their isolation in sufficient amounts for detailed analysis. Further, even if a cellular phospholipase has been characterized and purified, its role in a given function remains uncertain if the cell of origin contains more than one phospholipase of the same type (6, 7). Although bacterial (*E. coli*) mutants are available which lack specific phospholipases, permitting demonstration of their role in specific events (8–12), such genetic manipulation of animal cells has not yet been possible.

It has become apparent, moreover, that even the highly conserved phospholipases A₂ may differ profoundly in their ability to act in a particular biological setting (13, 14). Recent progress has been reported in relating such functional differences to discrete variations in structure. For example, of many phospholipases A₂ tested, only a few hydrolyze the phospholipids of *E. coli* killed by the bactericidal/permeability-increasing protein (BPI) of PMN. This protein is a major element in the microbicidal arsenal of the PMN directed against Gram-negative bacteria (13, 15) and is also a phospholipase A₂-activating agent (10, 15, 16). A distinguishing feature of “BPI-responsive” phospholipases A₂ is the presence of a cluster of basic amino acid residues within a region of the protein’s surface including residues 6, 7, 10, 11, and 15 (7, 13–19).

Among the BPI-responsive phospholipases A₂ are two that are natural constituents of the host’s inflammatory environment, one in the PMN and one in the cell-free (ascitic) fluid (16, 20). We have established that these two phospholipases A₂ participate in the attack on the phospholipids of *E. coli* during phagocytosis (21). The ascitic fluid phospholipase A₂ has been purified, and NH₂-terminal amino acid sequence analysis revealed structural hallmarks that conform to the consensus features of BPI-responsive phospholipases A₂ (20). Until now, the scarcity of the PMN phospholipase A₂ has precluded similar structural analysis of this cellular enzyme.

We now report the isolation in pure form of a phospholipase A₂ from rabbit PMN and, in addition, a phospholipase A₂ from serum. Comparison of the structural and the functional properties of the phospholipase A₂ of the PMN, the serum, and the ascitic fluid shows that all three enzymes are very closely related. However, the PMN and ascitic fluid phospholipases A₂ differ in their NH₂-terminal residues, implying that these cellular and extracellular enzymes are structurally distinct. In contrast, the serum and ascitic fluid phospholipases A₂ appear to be identical, suggesting that the major portion of the extracellular phospholipase A₂ in the inflammatory exudate may migrate from the serum (plasma).

**EXPERIMENTAL PROCEDURES**

*Materials—* Fresh, sterile, pooled rabbit serum devoid of detectable hemoglobin was purchased from Pel Freez Biologicals (Rogers, AR),...
maintained at 0–4 °C, and used within 24 h of collection. Goat anti-human C7 and anti-human C8 sera (cross-reactive with rabbit C7 and C8, respectively) were obtained from Calbiochem; peroxidase-conjugated rabbit (anti-goat IgG) immunoglobulins were from Bio-Rad Laboratories.

Collection of Polymorphonuclear Leukocytes and Ascitic Fluid—Stable E. coli pellets were harvested and resuspended from New Zealand White rabbits 12–15 h after intraperitoneal injection with glycogen-saturated saline (3). Cells were sedimented by centrifugation at 100 × g for 10 min and washed three times with Hanks’ buffered salt solution (Microbiological Associates). Cells suspensions contained >90% PMN as judged by differential counting. Cells were either used fresh or stored at 4 °C. The cell-free supernatant was centrifuged further at 20,000 × g for 30 min to yield viscous cell-free fluid.

Growth and Labeling of E. coli—E. coli 1303 (F-pldA, bio-, endA, recA56, sup-), a pldA mutant, was kindly provided by Dr. H. Verheij (Center for Biomembranes and Lipid Enzymology, State University Utrecht, The Netherlands). The bacteria, lacking the principal envelope phospholipase (25), were grown overnight to stationary phase and then subcultured in yeast broth (26) supplemented with [3H]oleic acid or [3H]palmitic acid (Du Pont-New England Nuclear) 0.5 μCi/ml) as described previously (10). More than 90% of the incorporated [3H]oleic acid was in the 2-ester position of bacterial phospholipids, and >90% of the incorporated [3H]palmitic acid was in the 1-ester position. The labeled bacteria were resuspended in physiological saline and used as live organisms to determine the ability of various purified phospholipases to degrade the phospholipids of E. coli killed by the bactericidal/permeability-increasing protein of PMN (10, 13) or after autoclaving for 15 min at 120 °C and 2.7 kg/cm² to determine phospholipase activity (10, 24).

Analysis of Phospholipase Activity—Phospholipase A activity was measured by incubation of protein fractions with 2.5 × 10⁻⁶ M [3H]oleate or [3H]palmitate-labeled autoclaved E. coli (5 × 10⁶ cells/ml) at 37 °C. Incubation mixtures contained 40 mM Tris-HCl (pH 7.5) and 10 mM CaCl₂ (unless otherwise indicated) in a total volume of 250 μl. To determine the pH dependence of activity, the following buffers were used: sodium acetate/acetate acid (pH 4.0–6.0), Tris maleate (pH 6.0–7.5), Tris-HCl (pH 7.5–8.0), and glycine/NaOH (pH 9.0–10.5). Reactions were terminated by the addition of an equal volume of ice-cold 0.5% bovine serum albumin, and samples were spun in an Eppendorf microcentrifuge for 2 min to sediment the undegraded phospholipids that remain fully associated with the E. coli. The products of hydrolysis (free fatty acid and/or lysocompounds) released into the supernatant and trapped by association with bovine serum albumin (5%) were quantified by liquid scintillation counting. One arbitrary unit of activity is defined as 1% hydrolysis/h (1 unit = 8.3 × 10⁻⁷ IU). Calculations were made from samples producing up to 20% hydrolysis, a range in which phospholipid degradation is linearly dependent on the amount of enzyme added and time of incubation (10). To determine positional specificity of phospholipase A activity, [³H]oleic acid- and [³H]palmitic acid-labeled autoclaved E. coli were used as substrates. Extraction of the supernatant and separation (by thin layer chromatography) of labeled lysocompounds and free fatty acids were carried out as described previously (26).

Generation and Characterization of Anti-ascitic Fluid Phospholipase A₂ Serum—Antiserum was generated in rabbits immunized with the purified ascitic fluid phospholipase A₂ (immune serum) or with 1.0 ml of Tris-HCl buffer (pH 7.5) (control serum). Immunization was achieved by intraperitoneal injection of 25 mg of purified ascitic fluid phospholipase A₂ (apparent molecular mass of about 14 kDa) (Fig. 5). The extract was subsequently neutralized by prolonged dialysis against 1 mM Tris-HCl, pH 7.5. Dense precipitates were removed by centrifugation at 23,000 × g for 20 min, and the remaining clear supernatant was stored at 4 °C.

membrane Protein liquid chromatography (FPLC) was performed on a Mono S 1 HR 5/5 (Pharmacia) column equilibrated in 50 mM sodium acetate/acetate acid (pH 4.6) at room temperature. Elution of protein was carried out as described in the text and was monitored by UV absorbance at 280 or 214 nm as indicated.

Reverse-phase HPLC was performed on a C4 (Vydac; 5-μm particles, 300 Å pores) column equilibrated at room temperature in 0.1% trifluoroacetic acid (29). Further details are provided in the text.

Purification of Rabbit Ascitic Fluid Phospholipase A₂—Phospholipase A₂ was purified from rabbit ascitic fluid as described previously (20).

Electrophoretic Procedures—SDS-PAGE was carried out on a Phast Gel System (Pharmacia) using Phast Gel 8–25 gradient polyacrylamide gels, and protein bands were visualized by silver staining as described by the manufacturer.

Amino Acid Analysis—Amino acid analyses were performed using a Waters Pico-Tag amino acid analysis system. Samples were pre-treated in vacuo for 24 h at 110 °C with 5.7 N HCl containing 0.05% phenol. The resulting phenylthiohydantoin derivatives were analyzed by reverse-phase HPLC.

Amino Acid Sequence Analysis—Amino acid sequence analysis was performed on a Applied Biosystems model 477A protein/peptide Sequencer. Phenylthiohydantoin derivatives were analyzed by the Applied Biosystems model 120A PTH Analyzer.

Miscellaneous Determinations—Protein concentrations were determined by the methods of Lowry et al. (27) and Bradford (28) using bovine serum albumin as standard and by amino acid analysis. Concentrations of C7 and C8 in rabbit serum and ascitic fluid were measured immunochromatically by conventional enzyme-linked immunosorbent assays.

RESULTS

Purification of Rabbit PMN Phospholipase A₂—Previous studies in this laboratory have shown that approximately 80% of the phospholipase A₂ activity of homogenates of washed (rabbit and human) PMN that is detected using autoclaved E. coli as substrate is associated with the cytoplasmic granules (30–31). Solubilization of this intracellular phospholipase A₂ activity is readily achieved by extraction of PMN homogenates with strong acid (10). Such extracts contain 90–100% of the activity detected in whole homogenates (Table I) (4). Initial fractionation of the extract by RP-HPLC on a C4 column yielded a single discrete peak of phospholipase activity containing 95% of the total applied activity (Fig. 1). Rechromatography of the phospholipase A₂-rich fractions under the same conditions provided a further (7–8-fold) enrichment of phospholipase A₂ without loss of activity. Further purification of the phospholipase A₂ was achieved by three successive cycles of ion-exchange chromatography on a FPLC Mono S HR 5/5 column (Table I; Figs. 2 and 3). In each cycle, recovery of phospholipase A₂ activity was 60–80%. All recovered phospholipase A₂ activity was eluted as a single symmetrical peak with 1.0 M NaCl. Final purification was achieved by RP-HPLC, which yielded a single protein peak coincident with all recovered phospholipase A₂ activity (Fig. 4). The trailing of the descending limb of the peak is a feature exhibited by all purified phospholipase A₂ that we have examined when small amounts of protein are subjected to chromatography on a C4 column. Analysis of the pooled fractions within this protein peak by SDS-PAGE revealed a single band upon silver staining, co-migrating with purified ascitic fluid phospholipase A₂ (apparent molecular mass of about 14 kDa) (Fig. 5).

N²-terminal amino acid sequence analysis further confirmed...
TABLE I
Purification of rabbit PMN phospholipase A2 activity was measured as described under "Experimental Procedures." 

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein</th>
<th>Phospholipase A2 activity</th>
<th>Recovery</th>
<th>Phospholipase A2 specific activity</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg</td>
<td>units</td>
<td>%</td>
<td>units/mg</td>
<td>-fold</td>
</tr>
<tr>
<td>Homogenate</td>
<td>8,600</td>
<td>1.4 x 10^7</td>
<td>100</td>
<td>1.6 x 10^6</td>
<td>1</td>
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<tr>
<td>Acid extract</td>
<td>1,720</td>
<td>1.4 x 10^7</td>
<td>100</td>
<td>8.1 x 10^3</td>
<td>5</td>
</tr>
<tr>
<td>HPLC-1</td>
<td>160</td>
<td>1.3 x 10^7</td>
<td>95</td>
<td>8.3 x 10^4</td>
<td>50</td>
</tr>
<tr>
<td>HPLC-2</td>
<td>22</td>
<td>1.3 x 10^7</td>
<td>95</td>
<td>5.9 x 10^4</td>
<td>365</td>
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<tr>
<td>FPLC-1</td>
<td>0.830</td>
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<td>60</td>
<td>1.0 x 10^7</td>
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<td>FPLC-2</td>
<td>0.050</td>
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<td>74,000</td>
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<td>FPLC-3</td>
<td>0.025</td>
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<td>26</td>
<td>1.5 x 10^5</td>
<td>92,500</td>
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<tr>
<td>HPLC-3</td>
<td>0.006</td>
<td>1.2 x 10^6</td>
<td>8.6</td>
<td>2.0 x 10^6</td>
<td>123,500</td>
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</table>

*Protein was measured in these fractions by integration of absorbance at 214 nm elution profiles (using a known amount of purified ascitic fluid phospholipase A2 as standard) and by amino acid analysis.

Fig. 1. Reverse-phase HPLC of rabbit PMN acid extract. PMN acid extract (12 ml; 75 mg of protein) was applied to a Vydac C4 column equilibrated in 0.1% trifluoroacetic acid and eluted (right to left) with a gradient of increasing acetonitrile (0-95%) in 0.1% trifluoroacetic acid and developed in 30 min at a flow rate of 1 ml/min. Elution of protein was monitored continuously by measuring absorbance at 214 nm (solid line) and phospholipase A (PLA2) activity (dashed line) as described under "Experimental Procedures," in fractions collected at 1-min intervals.

Fig. 2. Mono S FPLC of rabbit PMN phospholipase A2 (PLA2)-rich fractions obtained from RP-HPLC. Phospholipase A2-rich fractions (see Fig. 1) were pooled, applied to a Mono S column equilibrated in 50 mM sodium acetate/acetic acid buffer (pH 4.6), and eluted at a flow rate of 1 ml/min. Elution of protein was monitored continuously at 280 nm and phospholipase A activity (dashed line) was followed as indicated in the legend of Fig. 1.

The purity of the isolated protein and its identity as a phospholipase A2 (see below). The final yield of purified phospholipase A2 was 6 µg (about 400 pmol) from 5 x 10^10 PMN, representing a recovery of approximately 9% of the total cellular phospholipase A2 activity (versus autoclaved E. coli) and a purification of >100,000-fold (Table I).

Comparison of the NH2-terminal amino acid sequences of the purified rabbit PMN phospholipase A2 and of the ascitic fluid phospholipase A2 (20) revealed that 15 of the 16 residues identified are identical (Fig. 6). Only the NH2-terminal amino acid is different, with alanine in the PMN phospholipase A2 and histidine in the ascitic fluid enzyme. A small amount of histidine (<20% of alanine) was detected in the first cycle of sequencing of the PMN phospholipase A2, which may reflect co-purification of a trace amount of ascitic fluid phospholipase A2 still associated with the washed PMN. The different end groups of the two enzymes (determined in multiple amino acid sequences) indicated that the two proteins are not the same and suggested therefore that the source of the ascitic fluid phospholipase A2 might not be the PMN as has been proposed (32, 33).

Purification of a Rabbit Serum Phospholipase A2—Following intraperitoneal infusion of glycogen, the phospholipase A2 activity in the ascitic fluid rises gradually and linearly to levels found in rabbit plasma or serum. This is accompanied by the time-dependent accumulation of several other plasma proteins including albumin, C7, and O3 (Table II), suggesting that transudation of plasma phospholipase A2 during formation of the inflammatory exudate might account for the phospholipase A2 in the ascitic fluid. Purification of rabbit serum phospholipase A2 was carried out to define further the possible relationship between plasma and ascitic fluid phospholipase A2. In contrast to the observations of others, showing increased levels of phospholipase A2 in serum versus plasma, presumably released from platelets during clot formation, we have found no difference in phospholipase A2 content of rabbit plasma and serum. One liter of fresh pooled rabbit serum...
**Fig. 3.** Mono S FPLC of highly purified PMN phospholipase A₂ (PLA₂)-rich pool. Phospholipase A₂-rich fractions recovered after two successive chromatographies on Mono S (FPLC-2; see Table I) were pooled and diluted with 50 mM sodium acetate/acetic acid buffer (pH 4.6) to reduce the NaCl concentration of the protein pool to <0.5 M. The pooled protein was reapplied to a Mono S column equilibrated in buffered 0.5 M NaCl and eluted (left to right) in buffer containing a linear gradient of increasing NaCl (0.5-1.5 M) developed in 20 min at a flow rate of 1 ml/min. Fractions were collected at 1-min intervals, and protein elution (monitored continuously at 214 nm (solid line)) and phospholipase A₂ activity (dashed line) were measured as indicated under “Experimental Procedures.”

**Fig. 4.** Reverse-phase HPLC of purified rabbit PMN phospholipase A₂. Phospholipase A₂-rich fractions recovered after three cycles of chromatography on a Mono S column (FPLC-3; Table I) were applied to a C4 column (see legend to Fig. 1) and eluted (right to left) with a linear gradient of acetonitrile (0-70%) in 0.1% trifluoroacetic acid developed in 60 min at a flow rate of 1 ml/min. Protein elution was monitored continuously by measuring absorbance at 214 nm (solid line).

**Fig. 5.** SDS-PAGE of purified rabbit PMN phospholipase A₂. Electrophoresis was carried out as described under “Experimental Procedures.” Lane 1, 100 ng of ascitic fluid phospholipase A₂ (approximate molecular mass, 14 kDa) as reference; lane 2, 200 ng of rabbit PMN phospholipase A₂.

**Fig. 6.** NH₂-terminal amino acid sequence of rabbit PMN, ascitic fluid (AF), and serum phospholipase A₂. Sequence determinations were carried out three times for the ascitic fluid phospholipase A₂ and twice for the PMN phospholipase A₂.

**Table I**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Phospholipase A₂</th>
<th>C7</th>
<th>C8</th>
<th>Total protein</th>
</tr>
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<tr>
<td>3</td>
<td>16</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
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<tr>
<td>13</td>
<td>100</td>
<td>16</td>
<td>16</td>
<td>18</td>
</tr>
</tbody>
</table>

**Fig. 7.** CM-Sepharose chromatography of rabbit serum. One liter of serum was applied to a CM-Sepharose column (6 × 20 cm), pre-equilibrated with 2.5 mM Tris-buffered 0.15 M NaCl (pH 7.4). After elution of unbound and weakly bound protein (not shown; see “Results” for details), bound protein was eluted in a stepwise manner with buffered 0.5 and 1.5 M NaCl as indicated by the arrows. Protein elution was monitored by measuring absorbance at 280 nm (open squares), and phospholipase A₂ activity (closed triangles) was measured as described under “Experimental Procedures.” Pools I, II, and III comprised fractions 20-24, 27-31, and 34-40, respectively. Each fraction contained 10 ml.

The results shown are of one of two closely similar experiments.

**Table II**

<table>
<thead>
<tr>
<th>Phospholipase A₂</th>
<th>100 ng</th>
<th>200 ng</th>
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<tbody>
<tr>
<td></td>
<td>ascitic fluid</td>
<td>PMN</td>
</tr>
<tr>
<td><strong>A₂</strong></td>
<td>ALDLFREKMIYRTTCHEAT (10)</td>
<td>ALDLFREKMIYRTTCHEAT (10)</td>
</tr>
<tr>
<td><strong>AF</strong></td>
<td>HLDLFRKMIYRTTCHEATT (10)</td>
<td>HLDLFRKMIYRTTCHEATT (10)</td>
</tr>
<tr>
<td><strong>SERUM</strong></td>
<td>YLDLFRKMIYRTTCHEATT (10)</td>
<td>YLDLFRKMIYRTTCHEATT (10)</td>
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</table>

*Fig. 7.* CM-Sepharose chromatography of rabbit serum. One liter of serum was applied to a CM-Sepharose C-50 column (6 × 20 cm), pre-equilibrated with 2.5 mM Tris-buffered 0.15 M NaCl (pH 7.4). After elution of unbound and weakly bound protein (not shown; see “Results” for details), bound protein was eluted in a stepwise manner with buffered 0.5 and 1.5 M NaCl as indicated by the arrows. Protein elution was monitored by measuring absorbance at 280 nm (open squares), and phospholipase A₂ activity (closed triangles) was measured as described under “Experimental Procedures.” Pools I, II, and III comprised fractions 20-24, 27-31, and 34-40, respectively. Each fraction contained 10 ml.

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**Table II**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Phospholipase A₂</th>
<th>C7</th>
<th>C8</th>
<th>Total protein</th>
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Purification of Rabbit Granulocyte and Serum Phospholipases A2

TABLE III
Purification of rabbit serum phospholipase A2

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein</th>
<th>Phospholipase A2 activity</th>
<th>Recovery</th>
<th>Phospholipase A2 specific activity</th>
<th>Purification</th>
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<tr>
<td>Serum</td>
<td>52,000</td>
<td>1.8 x 10^6 units</td>
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<td>3.5 x 10^5 units/mg</td>
<td>-fold</td>
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<td>CM-Sephadex</td>
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<tr>
<td>Pool I</td>
<td>3.4</td>
<td>8.0 x 10^7 units</td>
<td>44 (100)</td>
<td>2.3 x 10^7 units/mg</td>
<td>6,600</td>
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<tr>
<td>Pool II</td>
<td>2.5</td>
<td>5.6 x 10^7 units</td>
<td>31</td>
<td>2.2 x 10^7 units/mg</td>
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<tr>
<td>Pool III</td>
<td>5.0</td>
<td>5.0 x 10^7 units</td>
<td>27</td>
<td>1.0 x 10^7 units/mg</td>
<td>2,880</td>
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<td>Pool I</td>
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<tr>
<td>RP-HPLC-1</td>
<td>0.44</td>
<td>5.7 x 10^7 units</td>
<td>32 (71)</td>
<td>1.3 x 10^6 units/mg</td>
<td>37,100</td>
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<tr>
<td>RP-HPLC-2</td>
<td>0.15</td>
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<td>17 (39)</td>
<td>2.0 x 10^6 units/mg</td>
<td>57,000</td>
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Values in parentheses are percent recovery relative to activity in pool I.

Fig. 8. Reverse-phase HPLC of serum phospholipase A2 (PLA2)-rich fractions. Phospholipase A2-rich fractions (RP-HPLC-1 in Table III) were applied to a Vydac C4 column and eluted (right to left) with a linear gradient of acetonitrile (0-70%) in 0.1% trifluoroacetic acid developed in 60 min at a flow rate of 1 ml/min. Elution of protein and phospholipase A activity was measured as described in the legend to Fig. 3; solid line, absorbance at 214 nm; dashed line, phospholipase A activity.

Fig. 9. Rechromatography by RP-HPLC of highly purified serum phospholipase A2 (PLA2)-rich fractions. Phospholipase A2-rich fractions (RP-HPLC-1 in Table III) were pooled, diluted with 0.1% trifluoroacetic acid, and reapplied to a C4 column. Chromatographic conditions were identical to those described in the legend of Fig. 8. Elution (left to right) of protein (solid line) and phospholipase A activity (dashed line) was monitored as described in the legend to Fig. 3.

Acidic phospholipase was focused on these fractions. RP-HPLC of the phospholipase A2-rich fractions comprising pool I yielded a discrete protein peak containing all the recovered phospholipase A2 activity enriched an additional 5-6-fold (Fig. 8 and Table III). Rechromatography of this phospholipase A2-rich fraction under the same conditions produced a partial separation of phospholipase A2 from remaining extraneous proteins (Figs. 9 and 10). By applying the same number of phospholipase A2 activity units in fractions 27-32 to each lane of an SDS-PAGE gel we could establish the correlation between the phospholipase A2 activity and the presence of a single protein band (Fig. 10). Fractions 30 and 31 contained this protein material in nearly homogeneous form. NH2-terminal amino acid sequence analysis of these fractions confirmed their purity and revealed a protein sequence (19 residues identified) identical to that of the aspic fluid phospholipase A2, including the presence of histidine as the NH2-terminal residue (Fig. 6). Thus, the major rabbit serum phospholipase A2, by these criteria, is indistinguishable from the aspic fluid phospholipase A2.

Comparison of Structural and Functional Properties of Purified Rabbit PMN, Serum, and Aspic Fluid Phospholipases A2—In addition to the nearly identical NH2-terminal region of the PMN, serum, and aspic fluid phospholipase A2 (Fig. 6), all three proteins share a high content of basic amino acids (about 20 mol %; not shown), consistent with their highly cationic properties displayed during ion-exchange chromatography (Figs. 2, 3, and 6) (15, 34). The three enzymes also co-elute during RP-HPLC on a C4 column, suggesting similar surface hydrophobicity.

The functional properties of the three phospholipases A2 are also indistinguishable (Table IV). Hydrolysis by each enzyme of [14C]oleate- or of [14C]palmitate-labeled E. coli produced as the radiolabeled products almost exclusively free fatty acids and lysocompounds, respectively, consistent with the positional specificity of a phospholipase A2. Catalytic activity of the three phospholipases A2 was absolutely dependent on Ca2+; activity was abolished by 10 mM EDTA or 2 mM PO4. The three phospholipases A2 showed a similar pH dependence, with a maximum between pH 7.5 and 9.0. The specific activity of all three purified phospholipases A2 measured in the autoclaved E. coli assay, was approximately 2 x 105 units/mg (166 μmol/min/mg). The three enzymes were also equally active toward the phospholipids of pldA+ E. coli, killed by the BPI of rabbit PMN, a function shared only by a
subsets of phospholipases A₂ (13, 34). Antiserum generated in chicken against the purified acutic fluid phospholipase A₂ produced similar dose-dependent inhibition of the catalytic activity of each of the three rabbit phospholipases A₂. This antiserum only partially inhibited the activity of a pig pancreatic phospholipase A₂ and did not inhibit the activity of the basic Agkistrodon h. blomhoffii and pig intestinal phospholipases A₂ (also BPI-responsive enzymes).

**DISCUSSION**

The study of cellular phospholipases A₂ that are not secretory phospholipases A₂ has been seriously limited by the difficulty of obtaining sufficient quantities of these proteins, which are present as minor components in most cells. We have now succeeded in purifying a phospholipase A₂ from rabbit PMN to apparent homogeneity as judged by HPLC, SDS-PAGE, and partial NH₂-terminal amino acid sequence determination. This primary structure analysis shows that the PMN phospholipase A₂ and a recently purified extracellular phospholipase A₂, present in the same inflammatory exudate from which the PMN were obtained, share 15 of 16 residues in the NH₂-terminal region, differing only in their NH₂-terminal residues. In contrast, serum phospholipase A₂ that was also isolated in this study is identical in all first 19 NH₂-terminal residues with the ascitic fluid phospholipase A₂.

All three rabbit phospholipases are equally effective in the hydrolysis of the phospholipids of BPI-treated E. coli and share with other BPI-responsive phospholipases A₂ a cluster of basic amino acid residues in the NH₂-terminal region (residues 6, 7, 10, and 15). This feature is common to all BPI-responsive group II phospholipases A₂, in support of our previous hypothesis that this cluster of basic residues is an important determinant of phospholipase action against BPI-killed E. coli.

Until very recently, knowledge of the amino acid sequences of mammalian phospholipases A₂ was limited to the pancreatic phospholipases A₂, enzymes that all belong to the so-called group I category of phospholipases A₂ (35). It has now become evident that in many mammalian species including man, non-pancreatic phospholipases A₂ are of the group II variety. These phospholipases A₂ have been isolated from platelets and inflammatory fluids (ascitic fluid and synovial fluids) of rabbit (20), rat (36–39), and man (40, 41) and from pig intestine (42). (Partial) amino acid sequence analysis of all these phospholipases A₂ has revealed the absence of Lys³⁵, a characteristic of group I phospholipases A₂, and in the case of the rabbit acutic fluid and the human synovial fluid phospholipases A₂, a COOH-terminal extension, which, along with the different disulfide positions, defines the group II phospholipases A₂ (35).

These non-pancreatic phospholipases A₂ show species-related differences in amino acid composition and sequence but within a given species are very closely similar, as we show here for the cellular and extracellular phospholipases A₂ isolated from the rabbit. Others have reported that in both rat and rabbit the partial NH₂-terminal amino acid sequences of platelet and ascitic fluid phospholipases A₂ are nearly identical (43, 44). In the rat, a third phospholipase A₂ isolated from liver mitochondria (45) differs from the platelet enzyme only at the NH₂ terminus, the same site that distinguishes the phospholipases A₂ that we have purified from rabbit PMN, serum, and ascitic fluid. These observations may point to a limited gene family encoding group II phospholipases A₂ in several cell types. Whether expression of pancreatic-type (group I) phospholipases A₂ in some tissues (spleen, gastric mucosa, and lung) and of group II enzymes in other cells and tissues such as intestine, spleen, platelets, and PMN (38, 46, 53) reflects a structural basis for different functions remains to be explored further.

The apparent identity between the serum and ascitic fluid phospholipases A₂ that we have isolated raises the possibility that this constituent of the inflammatory environment enters from the circulatory fluid. This appears plausible because during the evolution of the inflammatory exudate after sterile peritoneal irritation, the level of phospholipase A₂ activity in the acutic fluid increases with time, reaching levels that are comparable to those in the serum or plasma (Table II). The more rapid accumulation in the acutic fluid of phospholipase A₂ than of C7, C8, and albumin is consistent with transudation of this enzyme from plasma because the smaller phospholipase A₂ (about 14 kDa) versus 65–150 kDa for the other three proteins) should diffuse faster into the inflammatory exudate. Whether the presence in serum (Fig. 7) but not in ascitic fluid of other chromatographically distinct phospholipase A₂ activities implies heterogeneity of serum phospholipases A₂ or reflects an association with other proteins remains to be explored.

The cellular source(s) of extracellular phospholipase A₂ during inflammation remain(s) uncertain. Recent evidence suggests that the predominant phospholipase A₂ in synovial fluid of patients with rheumatoid arthritis is identical to a granule-associated (secretory) enzyme present in human platelets (40, 41). In rabbit, however, comparison of partial amino acid sequences of inflammatory (ascitic) fluid and platelet phospholipases A₂ (20, 43) has revealed discrete differences, indicating that the two enzymes are closely similar but distinct (43).

Stimulated (rabbit peritoneal exudate) PMN also secrete phospholipase A₂ during degradation in vitro (32, 33), suggesting that these cells may be a source of inflammatory fluid enzyme. However, the 1–2 x 10⁶ units that are released/10⁶ cells, although representing approximately 30–50% of the total measurable cellular activity (using autoclaved E. coli as substrate), can account for only 1–2% of the activity present in an amount of acutic fluid harboring 10⁶ PMN. Because under certain inflammatory conditions PMN can synthesize and secrete various proteins (47, 48), perhaps including phospholipase A₂ (49), the PMN might be an additional source of inflammatory fluid phospholipase A₂ that is distinct from the cellular enzyme that we have purified. Other cells, including chondrocytes, fibroblasts, and macrophages, also secrete phospholipase A₂ activity when treated with mediators released during inflammation (50–59), but the molecular properties of these enzymes are unknown.
We do know, however, regardless of the still undetermined cellular source(s) of the phospholipase A₂ in rabbit serum and ascitic fluid, that both the cellular (PMN) and the extracellular (ascitic fluid) phospholipases A₂ participate in the degradation of the phospholipids of E. coli ingested and killed by the PMN (21) and also that their action is triggered by a well-defined bacterial polypeptide that only activates phospholipases A₂ with shared structural properties (5, 10, 15, 34). We have also shown that the abundant extracellular phospholipase A₂ in the inflammatory exudate, when added as the purified enzyme, acts only after the enzyme is translocated along with the bacteria into the phagocytic vacuole of the PMN where the granule-associated BPI exerts its antibacterial action (21), indicating therefore that this extracellular enzyme depends for its action on intracellular activation. These observations are consistent with the concept that the structurally and functionally closely similar phospholipases A₂ in the cellular and extracellular compartments of an inflammatory exudate fulfill a specific role in the destruction of certain microbial invaders during the host defense by the PMN.

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