Leukofibrinolysis

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Glycogen-stimulated dog peritoneal leukocytes phagocytize particulate fibrin in a manner similar to that described for phagocytosis of bacteria. After phagocytosis the fibrin is solubilized, and degradation products, called here MISFI (molecules immunologically similar to fibrinogen), are released into the surrounding media. Whole, viable leukocytes are necessary; disrupted cells have little or no activity. This process of leukocytic ingestion and digestion of fibrin is called leukofibrinolysis. It is inhibited by many of the same substances that inhibit fibrinolysis. Serum, a potent fibrinolytic inhibitor, does not inhibit leukofibrinolysis.

Phagocytosis of foreign material by polymorphonuclear neutrophilic leukocytes (PMNs) was recognized over a century ago by Virchow. Recent advances in cytochemistry and electron microscopy have brought extensive descriptions of the process of phagocytosis, particularly of bacteria. PMNs can phagocytize other materials, such as tissue debris, antigen-antibody complexes, and fibrin. This report describes the phagocytosis and intracellular solubilization of particulate fibrin, a process that we have termed leukofibrinolysis.

Fibrin serves at least two major functions. First, it is the insoluble matrix of the hemostatic plug that prevents excessive loss of blood after injury. Second, and less well understood, fibrin has an extravascular function in forming a protective barrier to contain undesirable materials. Either one of these useful functions may go awry, leading to disease rather than to simple defense. Intravascular coagulation, either overt (thrombosis) or covert (diffuse) may lead to devastating results from mechanical blockage or a consumption coagulopathy. Extravascular deposition of fibrin, for example in arthritis, may also play a damaging, perhaps even perpetuating, role in disease.

The mechanisms by which unwanted fibrin is removed are far from clear. Although blood contains a potentially potent fibrinolytic enzyme system, evidence of its activation is rarely found. Cellular phagocytosis of fibrin may be of considerable importance. Lee and Gans have shown that fixed cells (RES in liver and spleen) can remove circulating fibrin. To digest fixed fibrin...
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a mobile system seems necessary, and the PMN is suitably designed. Not only does it circulate and pass freely through vascular walls, but it is also endowed with neatly packaged hydrolytic enzymes.8

Early studies by Rulot9 and Opie10 suggested that intact cells, presumably PMNs, were important to fibrin dissolution. Riddle and Barnhardt4 obtained morphologic evidence for fibrin ingestion by neutrophils in inflammatory lesions. In addition, Barnhardt et al.11 found fibrinogen/fibrin split products in arthritic synovial fluid. That leukofibrinolysis may be important in the dissolution of thrombi is suggested by the studies of Henry,12 who found leukocytes concentrated in artificial thrombi at 15 times their numbers in peripheral blood.

Many studies have dealt with proteolytic enzymes isolated from leukocytes. The phenomena described in this report require intact cells and involve ingestion of particulate fibrin and digestion to soluble products, MISFI, (molecules immunologically similar to fibrinogen) that are released into the media.

MATERIALS AND METHODS

Dog Fibrin

Citrated plasma was recalcified by adding 1/50th vol of 1 M CaCl2.13 As clotting started, the fibrin was wound out on a large stirring rod and was then transferred to a blender. After mincing and dispersing in a large volume of 0.154 M sodium chloride, the small fibrin strands were sedimented at 1500 g for 10 min and then redispersed in fresh saline. This washing procedure was repeated at least 20 times. The fibrin was dried by washing with alcohol and ether and was then ground to a fine white powder. Each lot was tested for contamination with thrombin by incubating a few milligrams in 1 ml of 0.5% human fibrinogen (Merck Sharp and Dohme). Lots that caused clotting were discarded. Fibrin was used in the preparation of antidog fibrin antiserum or labeled with 125I for use as a substrate.14 A sample of the labeled fibrin was incubated for 1 hr with streptokinase (500 U/ml). If more than 5% of the 125I was solubilized, it was assumed that this lot of fibrin was contaminated with profibrinolysin and it was discarded.

Dog Fibrinolysin

The recalcified serum from which the fibrin had been removed was diluted 19 times with distilled water, and the pH was adjusted to 5.2 with 1% acetic acid. The mixture was allowed to stand at 4°C overnight, during which time a precipitate settled to the bottom. Most of the supernatant was decanted, the remainder was centrifuged, and the precipitate (euglobulin) was dissolved in one-fourth the original volume of saline buffered at pH 7.4. This solution was stored at 4°C for about 4 wk, during which time fibrinolytic activity developed. The solution was tested at intervals for spontaneous and streptokinase-activated fibrinolytic activity.14 When added streptokinase caused no increase in activity, the enzyme solution was frozen.

Antidog Fibrin Antiserum

Dog fibrin suspended in Al(OH)3 (5 mg fibrin/ml Al(OH)3) was injected into the foot pads of rabbits. This was repeated three times at weekly intervals. The rabbits were bled into one-tenth part of 0.1 M sodium oxalate, and the plasma was separated. It was heated to 56°C for 10 min to remove fibrinogen and was treated with 50 mg/ml BaSO4 (Baker) to adsorb coagulation factor II. Lyophilized dog serum was added to each lot of antiserum until the antiserum gave precipitin lines only to fibrinogen, its breakdown products, or fibrin breakdown products. Molecules that react with the antiserum are called MISFI15,16 —molecules immunologically similar to fibrinogen. Part of the antiserum was conjugated
with fluorescein isothiocyanate, was passed through a Sephadex G-25 column that separated unbound fluorescein, and was incubated with buffy coat obtained from an equal volume of normal dog blood to lessen nonspecific staining. Unconjugated antiserum was used in immunoelectrophoresis, using % agar in barbital buffer at pH 8.6, ionic strength 0.075. Four microliters of material to be tested were electrophoresed for 45 min at 6 V/cm. Approximately 100X1 antiserum were placed in the slot, and the slide was incubated 48 hr.

**Leukocytes**

Dogs were lightly anesthesized, and 1–2 liters of 0.1% oyster glycogen (Eastman Organic Chemicals, Rochester, N.Y.) in sterile saline was infused intraperitoneally. Peritoneal fluid was removed 18–20 hr later. The cell count was about 20,000/cu mm, and 80%–91% were polymorphonuclear leucocytes (PMNs). Trypan blue staining showed that 70%–90% of the cells excluded the stain and, thus, were considered viable. The cells were packed by gentle centrifugation at 950 g for 15 min in plastic bags. If the cell concentrate was red and appeared to contain many RBCs it was discarded. The supernatants were decanted, and, in all experiments except that first shown in Fig. 2, they were boiled for 6 min. This destroyed any fibrinolytic activity that might have been present in the untreated fluid. After the fluid had cooled, the cells were resuspended to give a count of 50–80,000/cu mm. Supernatant fluids had an average pH of 7.4 and a protein content of 7 mg/ml. They lacked fibrinogen.

**Experimental Technique**

One milliliter of 125I-fibrin, containing approximately 10 mg fibrin, was pipetted into each 16 X 150 mm plastic screw top tube (Falcon 2045). The tubes were counted on a Picker Automatic Scintillation Counter to obtain total radioactivity. Either 4 ml of cell suspension or 3 ml of cells plus 1 ml of an additional reagent were added to the iodinated fibrin. Sufficient Terramycin was added to bring concentration to 30 μg/ml. Duplicate fibrin-cell mixtures were prepared for each incubation time. The tubes were rotated in a roller-tube apparatus at 0.20 rpm at 37°C. After incubation the tubes were chilled, and the cells and residual fibrin were sedimented by centrifugation. The supernatant was filtered, radioactivity in an aliquot was counted, and the per cent fibrin solubilized was calculated. The supernatant was also used for immunoelectrophoresis with the antidog fibrin antiserum. The cells were resuspended in saline and after 5 min, during which time any undissolved fibrin settled, they were pipetted off and used for electron microscopy and/or immunofluorescent studies.

For immunofluorescent studies the cells were washed three times in citrated saline; smears were prepared, fixed with cold absolute methyl alcohol, air-dried, and stored at −20°C for periods up to 1 wk. Before staining with the fluorescent antiserum, smears were flooded with normal rabbit serum for 1 hr. They were washed, stained for 2 hr, rewashed, and examined in a Zeiss ultraviolet microscope.

For electron microscopy (EM) the cells were fixed with an equal volume of 5% glutaraldehyde for 30 min. The sediment was postfixed in 1% osmic acid for another hour, then dehydration, embedding, and sectioning were done in the usual fashion. A Phillips-200 electron microscope was used.

**RESULTS**

**Rate and Degree of Fibrin Solubilization**

Figure 1 illustrates the lysis of fibrin achieved with peritoneal cells in ten consecutive experiments. The final white blood cell (WBC) count in each experiment is indicated. The rate and degree of fibrin solubilization appear to be independent of the WBC count, if this is above 35,000/cu mm. In nine of ten experiments the cells were resuspended in boiled peritoneal fluid, and in the control test (boiled fluid without cells) less than 1% of the radioactive label was solubilized.
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Figure 2 shows the first experiment in more detail. The final WBC count was 132,800/cu mm, and the cells were resuspended in and diluted with untreated top fluid. In the control (without cells), 4% of the $^{125}\text{I}$-fibrin were lysed in 6 hr. (The small degree of lysis observed in this control was the reason for boiling the supernatant fluids used in other experiments.) Testing of serial dilutions of the cells showed that there was no increase in the rate or amount of fibrin lysed at counts above 66,000/cu mm. At lower counts, leukofibrinolysis appears to be proportional to the number of PMNs present.

Fig. 1. Leukofibrinolysis. Percentage of fibrin lysed in ten consecutive experiments. WBC count per cu mm is indicated.

Fig. 2. Effects of dilutions of PMNs. Suspending fluid was not boiled.
Dependence of Leukofibrinolysis on Whole Viable Cells

Figure 2 shows that viable cells are essential to leukofibrinolysis. In this experiment, sonicated cells had no activity. In other experiments, peritoneal PMNs were mechanically disrupted, freeze-thawed, heated to 56°C for 30 min, or sonicated, always with complete or almost complete loss of activity.

Comparison of Leukofibrinolysis with Fibrinolysis

In the experiment illustrated in Fig. 3, the ability of dog fibrinolysin and cells to solubilize ^125I-fibrin was compared. The rate of lysis was faster with the fibrinolysin, but the degree of solubilization was only slightly greater than that achieved with the cells. There appears to be a lag period in leukofibrinolysis apparently related to the actual process of phagocytosis.

Effects of pH

The pH optimum for leukofibrinolysis falls between 7.2 and 7.4. Below pH 6.7 and above 8.3 very little fibrin is lysed.

Appearance of Cells by Immunofluorescence

Without incubation no yellow-green fluorescence after staining with conjugated, antidog fibrin antiserum was seen, although an occasional bright strand of fibrin was found. At 30 min–1 hr (Fig. 4A), a few very bright cells were seen. By 2–4 hr (Fig. 4B), most of the cells were fluorescing, and the intensity varied from bright to dull. By 6 hr, the fluorescence was diffuse and quite dull, and the preparations appeared “mushy,” apparently due to cellular disintegration.

Appearance of Cells by Electron Microscopy

Figure 5A shows a typical peritoneal PMN. In Fig. 5B, strands of fibrin
Fig. 4. Cells stained with fluorescent antidog antiserum. (A) After 30-min incubation. (B) After 2-hr incubation.
Fig. 5. Electron photomicrographs. (A) PMN without exposure to fibrin. $\times 13,800$. (B) PMNs + fibrin, fixed immediately. $\times 51,250$. (C) PMNs + fibrin,
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fixed after 2-min incubation. × 13,800. (D) PMNs + fibrin, fixed after 6 min. × 10,750. (E) PMNs + fibrin, 3-hr incubation. × 22,750.
are present between PMNs. In Fig. 5C, the mixture was fixed after only 2-min incubation. A few intracellular vacuoles are seen, suggesting that some fibrin has already been ingested. At 6 min (5D), this PMN has multiple vacuoles and pseudopodia. Distinct granules can still be seen but appear to be far fewer in number than in the unexposed cell (5A). The final picture (5E) shows the lacy appearance after 3 hr of incubation. There are few granules and multiple vacuoles in this disintegrating cell.

Release of MISFI

In most experiments, the supernatant fluids obtained after centrifugation and filtration of PMN—$^{125}$I-fibrin mixtures were subjected to immunoelectrophoresis using antidog fibrin antiserum. One example is shown in Table 1 and Fig. 6. MISFI (molecules immunologically similar to fibrinogen) were absent before incubation. After 1 and 3 hr, product D was seen in the supernatant and, after 6 hr both D and E (Fig. 6) were found. Both of these were stable after heating to 56°C for 6 min.

Effects of Inhibitors

The effects of certain materials that inhibit dog serum fibrinolysin were tested for their inhibitory action on leukofibrinolysis. One milliliter of the agents listed in Table 2 was added to 3 ml of concentrated cells (61,000/cu mm) or fibrinolysin. Dog serum did not inhibit leukofibrinolysis, in contrast to its marked inhibitory effects on fibrinolysis. In the illustrated experiment,
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Fig. 6. Diagramatic representation of MISFI. Product D remains close to the application well. Product E moves toward anode.

serum appeared to have an enhancing effect on leukofibrinolysis, but in other experiments this was not always observed. Epsilon aminocaproic acid (EACA) was used in concentrations that cause partial inhibition, and in this experiment, the effects were greater on the leukocytes than on the fibrinolysin. In higher concentrations, both lytic systems are almost completely inhibited. The possibility that EACA might damage the PMNs was investigated by preincubating the cells with EACA for 1 hr and then washing and resuspending them in boiled fluid. These washed, resuspended cells dissolved as much \( ^{125}I \)-fibrin as did the control cells, while in another sample that was left in contact with EACA lytic activity was greatly diminished. Lima bean inhibitor (LBI) and soybean inhibitor (SBI) were effective as inhibitors of both systems.

Leukofibrinolysis could also be inhibited by such substances as sodium fluoride, sodium cyanide, N-ethylmaleimide, nicotinic acid, Novocaine, and Nembutal.

DISCUSSION

The studies reported here demonstrate that dog leukocytes can ingest or phagocytize insoluble fibrin and release soluble, recognizable MISFI (molecules immunologically similar to fibrinogen). We had named this process leukofibrinolysis, which may be abbreviated LFL. In vitro LFL, as described here, requires intact viable cells and an environmental pH of 7.2–7.4, which is near the pH range for the vascular system (7.35–7.45). The immunofluorescent studies show a few brightly fluorescent cells early in the fibrin–PMN incubation. It would appear that these are cells which have phagocytized fibrin and are beginning to digest it. Later on, almost all of the cells are fluorescent, suggesting that they either contain or are coated by MISFI. The EM studies may be interpreted along the same lines. Fig. 5C, taken after only 2 min incubation, shows cell vacuoles containing material in which the characteristic fibrin banding is no longer evident. The cells at 6 min (Fig. 5D) appear to be actively phagocytosing, with many large vacuoles whose contents

<table>
<thead>
<tr>
<th>Incubation Time (hr)</th>
<th>(^{125}I) in Supernatant (%)</th>
<th>Immunoelectrophoresis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>9.7</td>
<td>Faint D</td>
</tr>
<tr>
<td>3</td>
<td>35.2</td>
<td>Sharp D</td>
</tr>
<tr>
<td>6</td>
<td>61.5</td>
<td>D + E</td>
</tr>
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* WBC = 59,400/cu mm; 10 mg fibrin/tube.
Table 2. Effects of Inhibitors on Leukofibrinolysis and Fibrinolysis

<table>
<thead>
<tr>
<th>Agent</th>
<th>Fibrin Lysed (1 ml)</th>
<th>Leukofibrinolysis (%)</th>
<th>Fibrinolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boiled fluid</td>
<td>52.0</td>
<td>73.6</td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>62.0</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>EACA* 25 mg/ml</td>
<td>12.6</td>
<td>25.3</td>
<td></td>
</tr>
<tr>
<td>2.5 mg/ml</td>
<td>17.2</td>
<td>63.9</td>
<td></td>
</tr>
<tr>
<td>LBII 3 mg/ml</td>
<td>9.6</td>
<td>21.8</td>
<td></td>
</tr>
<tr>
<td>SBI§ 11 mg/ml</td>
<td>4.9</td>
<td>4.4</td>
<td></td>
</tr>
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</table>

* EACA, epsilon amino caproic acid; Amicar, Lederle Laboratories.
† Amount dissolved in 1 ml boiled fluid.
‡ Lima Bean Trypsin Inhibitor, Worthington Biochemical, Freehold, N. J.
§ Soybean Trypsin Inhibitor, Worthington Biochemical, Freehold, N. J.

cannot be identified. Control cells, incubated without fibrin, showed no changes at this time.

The released MISFI are called products D and E because their electrophoretic mobilities are similar to products derived from dog fibrinogen or fibrin by fibrinolysis. In this system (pH 8.6, ionic strength .075) for immuno-electrophoresis, dog fibrinogen forms an arc extending from the application well toward the cathode. This is shown in a previous study from this laboratory. Product D forms an arch that extends slightly toward the anode and E is found well toward the anode (a1 globulin position). D derived from dog fibrinogen by enzymatic degradation is 56°C labile and clot inhibitory. D derived from fibrin is stable and not inhibitory. Thus, the leukofibrinolytic product D appears similar to its fibrinolytic counterpart. Neither are labile nor inhibitory.

LFL is in many ways similar to fibrinolysis. If sufficient cells are present, fibrin can be rapidly lysed. Both processes are inhibited by common antiproteases, but only fibrinolysis is inhibited by serum.

Estimation of the importance of LFL as an in vivo mechanism for removal of unwanted fibrin will need additional studies. Using a skin window technique, Riddle and Barnhart demonstrated fibrin phagocytosis by neutrophils and eosinophils in inflammatory exudates. In a study from this laboratory, fibrin labeled with 131I was injected intravenously or intraarterially, and soluble labeled products were observed in the circulation. In unpublished extensions of these studies, immunofluorescent stains of peripheral blood Buffy coats and immuno-electrophoretic tests for fibrin derivatives in serum were both positive by 1–2 hr after injection of the fibrin.

REFERENCES

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