The Eosinophil as a Source for Profibrinolysin in Acute Inflammation

By Jeanne M. Riddle and Marion I. Barnhart

The possibility that eosinophils have a function in fibrinolysis grew out of our finding that profibrinolysin (plasminogen) was localized in the eosinophilic granules of developing and adult eosinophils in human bone marrow. Immature eosinophils appeared to be the sites for synthesis of profibrinolysin. It seemed reasonable to speculate on a role for eosinophils and their packets of profibrinolysin in conjunction with accumulations of either fibrin or fibrinogen. In normal physiology, some think there is a continual slow formation of fibrin along blood vessel walls. Fibrin is present in some thrombi, and extravascular fibrinogen is considerable and may approach 50 per cent of the total body fibrinogen. Following trauma with inflammation and during wound healing, fibrin deposition and its subsequent removal is imperative. Of these, we elected acute inflammation as the condition most easily controlled for our study of eosinophil function.

The skin window technic was adopted as our tool since it provides a method for observing the emigration sequence and cytology of acute inflammation in man and animals. A predictable pattern of cellular migration follows when a nonpyrogenic substance, such as diphtheria toxoid, is added as a stimulant. The nonimmune response is characterized by an early influx of large numbers of polymorphonuclear neutrophils. These subsequently decrease and are followed by mononuclears (lymphocytes, hypertrophied lymphocytes and macrophages). In contrast, the presence of eosinophils in the area of inflammation occurs in a less predictable pattern. Previous investigations have established that the magnitude of the eosinophil response does not parallel the circulating eosinophil level. A variety of materials have induced random migration of eosinophils, but no unifying concept for the "cosinotactic trigger mechanism" has been elucidated.

In the present study using the skin window technic in dogs, fibrin and fibrinogen were found to selectively attract eosinophils into an area of acute inflammation. These extravascular eosinophils contained profibrinolysin in their granules and fluoresced vividly when reacted with rhodamine antiprofibrinolysin. Distinct granular changes occurred which involved modification of granule size and variations in staining intensity with fluorescent antiprofibrinolysin. Frequently, a single granule or a coalesced mass of granules were extruded from the eosinophils. These data provide strong support for the view that eosinophilic leukocytes can act as carriers for a lytic mechanism.
of importance in inflammation and wound healing. A specific chemical stimulus for mobilization of eosinophils into an area of inflammation appears to be fibrinogen or fibrin.

**Materials and Methods**

Eleven dogs were studied at intervals over a 2-year period. Although outwardly healthy when first studied, the dogs were more carefully examined and if necessary, wormed before they were subsequently restudied. Peripheral blood eosinophil levels comprised from 4–8 per cent of the differential count thus falling within the previously reported normal range. Some 600 skin windows were evaluated during the 2 years. From 4–8 skin window lesions were placed at any one time on an individual dog so that control and experimental lesions could be assessed under closely similar conditions.

**Skin Window Technic**

Although the procedure of Re buck and Crowley was the basis of our technic, several modifications were adopted for this study on dogs. Some of the more pertinent details of the modified procedure follow.

1. Preparation of coverglass kits and tape rectangles for securing the cover glasses. Number two, 15 mm. square coverglasses were alcohol cleaned and a wax pencil R was placed on one side to later identify the surface on which there were no exudative cells. Slightly larger cardboard squares were cut from unlined file cards, and a single coverglass and cardboard square were wrapped in aluminum foil. Fifty to 60 of these coverglass kits were sterilized in a petri dish. Tape rectangles, approximately 3 inches long, were cut from an ordinary roll of 2-inch adhesive tape. Three sides of a 1 inch square were cut to form a central flap in the tape rectangle.

2. Preparation of the broad skin area. Hair was removed from the dog’s back with an electric animal clipper (size 40 head). Nair, a commercial cream depilatory, was applied to the clipped surface for 10 to 20 minutes. The cream and stubble were scraped away and the skin surface was thoroughly washed with warm tap water. To eliminate nonspecific irritation induced by the chemical or mechanical trauma of this procedure, the skin surface was prepared 12–18 hours preceding the experimental lesion.

3. Production of the skin lesion. Lesions were produced on the dorsal surface of the back, lateral to the spinal column (fig. 1). In this position, the coverglasses were not broken when the dog moved. A small area approximately 3 by 5 mm. was denuded by scraping with a sterile No. 22 Bard-Parker blade. The depth of the lesion was adequate when the papillary layer of the corium was reached and fine bleeding points were observed. The depth was visually controlled by noting when blood accumulated on the edge of the blade with each successive stroke.

4. Application of the chemical stimulant to the skin window lesion. The following stimulants were investigated in this study: diphtheria toxoid (aluminum phosphate absorbed, Parke, Davis and Company), a cathepsin concentrate (from Dr. W. Thomas, Armour Pharmaceutical Co.; prepared from pig spleen and rich in cathepsin C), Tryptar (crystallized trypsin, Armour Pharmaceutical Co.), bovine resin thrombin (courtesy Dr. W. H. Seegers; IRC-50 resin thrombin had a specific activity of 45,000 U/mg. tyrosine), fibrinogen, fibrin and plasma clots. When the stimulants were dissolved in either buffer, saline or a glycerol-saline mixture, several drops were delivered from a syringe onto the sterile coverglass surface opposite the wax R. The fibrin and plasma clots were prepared in small beakers by adding bovine resin thrombin to purified fibrinogen or by recalcifying sterile plasma. The resultant fibrin networks were transferred to sterile coverglasses. Coverglass and stimulant were placed onto the lesion with the surface bearing the wax R opposite the denuded area and surmounted by the cardboard square. The adhesive rectangle was oriented so that the central flap was directly over the lesion. A smaller piece of tape was placed over the central flap to firmly secure the coverglass (fig. 1) and to prevent it from being moved or lost.
Collection and processing of exudative cells. Cells of the inflammatory exudate migrated onto the coverglass surface where they become attached and flattened. The lesion was sampled hourly for a period of 10 hours. Each change was rapidly air-dried and immediately replaced with another coverglass so that the lesion did not dry. After each sample was removed, the wax R and debris were removed and the coverglass surface without exudative cells was mounted on a microscope slide. A very small amount of mounting media (Permout) was used since a large quantity added depth and made viewing with the ultraviolet illumination impossible.

Cellular Evaluation

The exudative cells were studied after staining with either fluorescent antiprofibrinolysin or Leishman’s stain. Following fixation for 10 minutes in Wolman-Behar solution at –20 C., rhodamine antiprofibrinolysin was applied. The dye concentration in all the fluorescent antibody preparations was kept uniform by adjusting the optical density of the solutions at 560 nm to 0.200. Such procedure provided excellent specific staining while minimizing nonspecific staining. Fluorescent antibody treatment was performed at room temperature with an exposure of 40 minutes. Tap water was used to rinse the slides. A Zeiss photomicroscope with BG 12 and OG 4 filters was employed with the fluorescent slides.

To assess the eosinophilic response to the various chemical stimulants, a 500 cell differential count was made of a representative area of each cellular sample. The percent eosinophils in this population was compared with the migration pattern obtained in control lesions. Since the cellular responses are related to time, sequential evaluations were made at hourly intervals up to 10–14 hours for each lesion. We recognized that this procedure was only an approach to exact quantitation.
Preparation and Characterization of the Antibody Used as the Cell Marker

Canine profibrinolysin was concentrated according to several different procedures. Immunologic methods were used to characterize the profibrinolysin. The best products exhibited 2 or 3 precipitin bands when tested by double diffusion in agar or by immunoelectrophoresis. The major component was profibrinolysin, fibrinogen was always present and a minor constituent sometimes present could not be identified. By using small amounts of the best profibrinolysin products, we hoped to discourage antibody formation to the minor contaminants. These partially purified profibrinolysin products (1 mg./Kg. body weight) were injected with Al (OH)₃ subcutaneously into rabbits as a single injection according to our previously described procedure. Rabbits readily produced antibodies with precipitin titers of 1/1024-1/2048 when reacted with partially purified dog profibrinolysin. The resulting antisera were collected and studied individually for their reactivity with such test reagents as dog plasma, serum and purified proteins such as dog profibrinolysin, fibrinogen, prothrombin and albumin. Even the best antisera generally showed a constituent that formed a precipitin with highly purified fibrinogen. In some cases, therefore, the antisera were treated with purified fibrinogen to remove this antibody which was not of interest to this study. However, antifibrinogen and antiprofibrinolysin are attracted to totally different cell types so their association need not be confusing.

Only the most selective antisera were converted into fluorescent antiprofibrinolysin. According to previously described procedure gamma globulin concentrates were complexed with either rhodamine sulfonyl chloride or fluorescein isothiocyanate. Such fluorescent antiprofibrinolysin products were used to identify cells that contained profibrinolysin. Only the eosinophilic series in dog bone marrow and eosinophils mobilized into areas of acute inflammation reacted specifically with fluorescent antiprofibrinolysin.

RESULTS

Previous work with human material led us to the conclusion that eosinophilic leukocytes in bone marrow synthesize profibrinolysin. This unique function for eosinophils might have been investigated further by a study of inflammation in humans. However, undesirable side reactions seemed possible if partially purified proteins were applied to a denuded area on a human. We could not minimize the risks to our satisfaction and so elected dogs as experimental animals. A necessary prerequisite was to determine whether or not bone marrow eosinophils in the dog produced profibrinolysin as in the human. With identical eosinophil-profibrinolysin relationships established, it became feasible to study the precise function of canine inflammatory cells.

Localization of Profibrinolysin in Eosinophilic Leukocytes

Fluorescent antiprofibrinolysin was applied to bone marrow and peripheral blood smears as well as to imprints from liver, spleen, lung and lymph node (table 1). Specific fluorescence was limited to eosinophilic leukocytes. Developing eosinophils in the bone marrow were particularly reactive (fig. 2). The brilliance of fluorescence paralleled the stage of eosinophil differentiation. As the eosinophilic granules matured and increased in number, they attracted more of the fluorescent antiprofibrinolysin. Such observations encourage the view that developing bone marrow eosinophils are the source of profibrinolysin.

Peripheral blood eosinophils from dogs in a normal physiologic state gen-
Table 1.—Cellular Fluorescence after Exposure to Rhodamine Antiprofibrinolysin

<table>
<thead>
<tr>
<th>Organ and Cell Type</th>
<th>Relative Fluorescence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>0</td>
</tr>
<tr>
<td>Kupffer cells</td>
<td>0</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>2+</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
</tr>
<tr>
<td>Plasma cells</td>
<td>0</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>2+</td>
</tr>
<tr>
<td>Lymph Node</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
</tr>
<tr>
<td>Eosinophils</td>
<td>2+</td>
</tr>
<tr>
<td>Bone Marrow Series</td>
<td></td>
</tr>
<tr>
<td>Eosinophilic</td>
<td>1 to 4+</td>
</tr>
<tr>
<td>Neutrophilic</td>
<td>0</td>
</tr>
<tr>
<td>Basophilic</td>
<td>0</td>
</tr>
<tr>
<td>Lymphocytic</td>
<td>0</td>
</tr>
<tr>
<td>Megakaryocytic</td>
<td>0</td>
</tr>
<tr>
<td>Plasmacytic</td>
<td>0</td>
</tr>
<tr>
<td>Erythrocytic</td>
<td>0</td>
</tr>
</tbody>
</table>

*Intensity of fluorescence was estimated as 1+ indicative of the weakest reaction while 4+ designated the most intense granular fluorescence observed.

Generally reacted poorly or not at all with the fluorescent antiprofibrinolysin. Even so, such eosinophils were stained with the acid dye present in Leishman’s stain. Localization of profibrinolysin in eosinophils as indicated by reaction with fluorescent antiprofibrinolysin reflects at least one property distinct from the general staining of the eosinophil granule by eosin or any acid dye. Why don’t these blood eosinophils of normal dogs react with fluorescent antiprofibrinolysin? It may be that the eosinophil has released most of the profibrinolysin which was synthesized and stored during maturation in the bone marrow. It is conceivable too that the profibrinolysin is masked in some way so that a complex is not formed with the fluorescent marker. In any event, eosinophils in normal blood appear to contain only a small amount or no profibrinolysin.

Extravascular eosinophils, occasionally present in tissue sections, generally fluoresced specifically in response to the application of rhodamine antiprofibrinolysin. The intensity of fluorescence was seldom as great as that noted in the adult bone marrow eosinophil.

The distribution of canine eosinophils which reacted with fluorescent antiprofibrinolysin was similar to that observed previously with human material. Attempts were next made to evaluate the contribution of eosinophils to a local fibrinolytic state which may occur in acute inflammation.

Cellular Responses during Acute Inflammation with a Nonpyrogenic Reagent

The pattern of cellular migration in sterile canine inflammatory lesions resembled that reported in human skin windows. Either saline or diphtheria
Fig. 2.—Photomicrograph of a normal dog bone marrow smear stained with rhodamine antiprofibrinolysin. The central eosinophilic metamyelocyte (EM) is filled with distinct, brilliantly fluorescent granules, several of which overlay the indented, dark nucleus. The mature bone marrow eosinophil (E) shows identical granular staining. In addition, eosinophil granules lying free in the smear (arrows) are specifically stained. By contrast, a neutrophilic band (NB) at lower left did not stain. Tri X film, exp. 5 min.

toxoid, the latter a common control substance in human skin window studies, was used as control reagents. The nonimmune response was characterized by an early influx of polymorphonuclear neutrophils. These leukocytes were present in large numbers as early as 2 hours and for several additional hours represented nearly 100 per cent of the cell population. From 6 through 14 hours, mononuclear cells (lymphocytes, hypertrophied lymphocytes and macrophages) appeared in increasing numbers. Eosinophils were observed in small numbers between 3–7 hours and seldom comprised more than 5 per cent of the cell population in the skin window (fig. 3). As did the eosinophils in the bone marrow, these extravascular eosinophils reacted with fluorescent antiprofibrinolysin indicating the presence of cellular profibrinolysin in the area of inflammation.

Selective Attraction of Eosinophils into Skin Windows Containing Proteolytic Enzymes

Cathepsin and thrombin were selected for study as constituents reasonably present in a lesion produced by abrading the surface epithelium. Trypsin was chosen because it has similar synthetic substrate preferences to those of cathepsin B and thrombin. In each lesion after the addition of one of these proteolytic enzymes, eosinophils appeared in numbers exceeding that seen in the
Fig. 3.—A comparison of the sequential eosinophil responses to purified dog fibrinogen (7.6 mg./ml.) and diphtheria toxoid. The arrow labeled Blood indicates the peripheral blood eosinophil level of this animal.

control lesion by as much as 24 per cent. Generally, a multiphasic pattern was obtained with peak eosinophil migration between 4–6 hours. The first wave of eosinophil migration occurred at 4 hours; the second wave at 6 hours. This was the typical pattern seen with either cathepsin or Tryptar (fig. 4). Thrombin, however, elicited a monophasic eosinophil response of 16 and 24 per cent (fig. 5). Each of these proteolytic enzymes can promote fibrin formation in an environment such as provided in skin window lesions. The possibility that fibrinogen or fibrin might be effective mobilizers for eosinophils was next investigated.

Extravascular Migration and Accumulation of Eosinophils in Skin Windows Containing Fibrinogen and Fibrin

Purified dog fibrinogen at different concentrations was placed in skin windows. A monophasic response occurred and was clearly concentration de-
Fig. 4.—Multiphasic eosinophil responses elicited when Tryptar and cathepsin were applied as stimulants in the skin window procedure. Per cent eosinophils reflects to what extent the eosinophilia elicited by the enzymes exceeded a control lesion run concurrently with diphtheria toxoid.

pendent. With the highest concentration of fibrinogen (7.6 mg./ml.) the peak eosinophil reaction occurred at 4 hours when eosinophils comprised 50 per cent of the cellular exudate or 47 per cent if the diphtheria toxoid control value of 3 per cent was subtracted (fig. 3). In response to a smaller concentration of fibrinogen (1.5 mg./ml.), eosinophils comprised 21 per cent of the cell population. In another animal the peak eosinophil response was 38 per cent with 7.6 mg. fibrinogen and 9 per cent with 1.5 mg. fibrinogen (fig. 6).

Fibrinogen from 2 other species, also, was effective in attracting eosinophils. When human fibrinogen (9.97 mg./ml.) was present in the skin window, the eosinophil migration pattern was 13.2 per cent at 5 hours. Bovine fibrinogen elicited the peak response at 4 hours when 36 per cent of the cell population was eosinophils. The reaction subsided with 19 per cent eosinophils at 5 hours, and 11 per cent at 7 hours.

Fibrin networks were especially effective in promoting and prolonging the eosinophilic phase of inflammation. Similar results were obtained with either dog or human fibrin. The pattern of eosinophil migration varied from monophasic to multiphasic. The continued presence of fibrin in the lesion modified the characteristic timing of cells found in control and single exposure windows.
Fig. 5.—A comparison of the monophasic eosinophil response elicited by thrombin in skin window lesions from a female and a male animal. Correction has been made for the small eosinophilic response to diphtheria toxoid in the control lesion. Thus in one male dog at 4 hours the eosinophilic response exceeded that in the control lesion so that an additional 24 per cent of the cell population was eosinophils.

at comparable hours. With repeated addition of fibrin, both eosinophilic and neutrophilic granulocytes could be maintained in the inflammatory site during the hours ordinarily dominated by the mononuclear response. In an experiment with dog fibrin for 4 hours, 32 per cent of the cell population was eosinophils. Hourly changes of the fibrin network maintained the eosinophilic response at values exceeding 20 per cent for 8 hours (fig. 6). During this entire time the polymorphonuclear neutrophil was the predominant cell type.

An Antihistamine Reaction of Eosinophils Could Not Account for the Eosinophil Mobilization

All of the evidence thus far focuses attention on fibrinogen or fibrin as unique agents capable of attracting and sustaining eosinophils at inflammatory sites. Before this view could be accepted completely, it seemed necessary to consider the possibility of some other common relationship as responsible for the described eosinophil migration. Eosinophils have been previously reported to have a capacity for neutralizing histamine. Certainly histamine is an agent which might be expected in areas of injury. Our results seem unrelated to the small variations of histamine that could have occurred in preparing the 6–8 skin windows in a single experiment. Neither control lesions with saline nor
THE EOSINOPHIL AS A SOURCE FOR PROFIBRINOLYSIN

Fig. 6.—Eosinophil migration to several concentrations of fibrinogen and fibrin. Control lesion values have been subtracted from the eosinophil response to fibrin and fibrinogen to arrive at the actual per cent eosinophils attracted by the specific stimulus. The single eosinophil peak response to purified dog fibrinogen was clearly concentration dependent. Fibrin promoted eosinophil migration and sustained increased numbers of eosinophils in the lesion as long as fibrin was added.

Diphtheria toxoid as stimulants attracted eosinophils in numbers exceeding 6 per cent of the cell population. A more general experience for control lesions in the 3 to 6-hour period was to find 1–2 per cent. Even so experiments were run with several concentrations of histamine (0.1 mg./ml. – 4.0 mg./ml.). Histamine windows were placed on 6 dogs (4 females, 2 males) and studied during the period when peak eosinophil responses were to be expected—namely 3–7 hours. In no case did the eosinophil mobilization to histamine approach that elicited by fibrinogen, fibrin or proteolytic enzyme. Results are given for 1 dog who exhibited a marked eosinophilic response to a fibrinogen window—namely 47 per cent of the cell population (fig. 3). This dog was followed for 10 hours with one window containing 2 mg. histamine/ml. solution and
Fig. 7.—Mobilization of eosinophils by fibrin formed by recalcification of plasma. Note that the same degree of eosinophil migration was achieved with a dog clot made from plasma containing 1.5 mg./ml. as from purified fibrinogen of 1.5 mg./ml. (see fig. 6). Human fibrin also was effective in selectively attracting eosinophils into the inflammatory site. Correction has been made for the small number of eosinophils that appeared in concurrent control lesions so that per cent eosinophils represents actual response to the added fibrin and clots.

Another window with 4 mg. histamine/ml. At 3 and 10 hours these exudative cell populations did not differ from control Diphtheria Toxoid values. From 4–9 hours, slightly more eosinophils appeared in the histamine windows than in controls but a significant difference is questionable since the peak eosinophil response showed only 3 per cent of the population as eosinophils. The histamine responses, also, were insignificant in comparison to the eosinophilic reactions elicited by weak fibrinogen (fig. 6) and a dog clot (fig. 7).

_Eosinophilic Migration to Fibrin Was Not a Nonspecific Protein Response_

One function assigned to eosinophils is defensive and involves the phagocytosis of foreign protein. Many of the proteins employed in this study were
either of bovine or human origin. Further, the possibility existed that the purification of dog fibrinogen could be drastic enough to alter the molecule sufficiently to make it, in a sense, a foreign protein. To test this idea two obviously foreign proteins, bovine crystalline albumin and purified human serum albumin were placed in skin windows. Even though concentrations ranged from 1–10 mg./ml., eosinophils were not attracted into the skin windows in numbers exceeding 2 per cent during the 9–10 hours of observation. These results do not reflect a nonreactive state of the dogs under test because in adjacent skin windows with fibrinogen present eosinophils were mobilized and constituted from 10–40 per cent of the cell population.

**Plasma Clots Elicited an Eosinophilic Phase in Inflammation**

That the eosinophilic responses achieved with purified fibrinogen were not a reaction to denatured protein was further amplified by our finding that fibrin nets prepared by recalcification of the animal’s plasma were competent reagents. These plasma clots provoked an eosinophil migration of 9 per cent by 4 hours (fig. 7). A similar pattern and magnitude for eosinophils was achieved in an adjacent skin window containing purified dog fibrinogen of exactly the same concentration as the original dog plasma; namely, 150 mg./per cent (fig. 6). Washed human clots also attracted eosinophils, as did fibrin nets prepared from purified human fibrinogen (fig. 7). By 4 hours 18 per cent of the exudative cell population was eosinophils. Such data as described leave little alternative except the view that fibrin is an agent which has special competence in attracting eosinophils with their packaged pro fibrinolysin into the inflammatory area.

**Functional and Morphologic Features of Eosinophils Appearing in Extravascular Areas of High Fibrin Content**

It was clear that eosinophils underwent functional changes at their extravascular location in response to the reagents present in the inflammatory area. The most striking alteration was a variation in size and number of granules within the eosinophils (fig. 8). Individual granules fused in many cases to form large coalesced masses which displaced the nucleus peripherially (fig. 8B). Such concentrated packets of eosinophil granules ultimately were released from the intact leukocyte (fig. 8C). The shedding of cytoplasmic buds was the mechanism of granule extrusion by other eosinophils. In some cases, granules were individually released leaving clear vacuoles in the eosinophil’s cytoplasm. The presence of fibrin augmented granule coalescence and release. Eosinophilic and neutrophilic leukocytes were frequently seen aligning the outer surface of fibrin strands or embedded within the fibrin mass.

Many variations in granule fluorescence were observed when rhodamine antiprofibrinolysin was applied to the cellular exudates. This response was limited to the eosinophilic leukocytes (fig. 9). These exudative eosinophils lost their responsiveness to rhodamine antiprofibrinolysin if they were treated first with unlabeled antiprofibrinolysin serum. However, the eosinophils retained their specific staining response when the pretreatment reagent was
Fig. 8.—Photomicrograph of Leishman stained exudative eosinophils in fibrin (A, C) and fibrinogen (B) skin window preparations. Many emigrated eosinophils (1) contain an abundance of eosinophil granules which show little size variation. Other eosinophils exhibit diminution in the number of granules (2) with subsequent increase and variability in granule size (3). A few eosinophils contain only a single coalesced granule mass which displaces the nucleus peripherally (4). Free eosinophil granules and coalesced masses (arrows) were released from the eosinophils.

either antifibrinogen, antiprothrombin or antialbumin. Neither rhodamine antigamma globulin nor free rhodamine in concentrations similar to rhodamine antiprofibrinolysin reacted with these eosinophils. Such experiments as these leave the firm impression that the staining of eosinophil granules by fluorescent antiprofibrinolysin reflects specifically their concentration of profibrinolysin.

Variations in the intensity of granule staining from brilliant orange to pale yellow were also observed within a single eosinophil. In addition, released granules and coalesced masses exhibited a similar range of fluorescent intensity. These results of diminished fluorescent intensity with the passage of time can reasonably be interpreted as morphologic evidence for the release of profibrinolysin at the inflammatory site.

**Discussion**

Numerous investigations have been initiated and pursued with the purpose of elucidating the precise function(s) of the eosinophilic leukocyte. Most of
Fig. 9.—Exudative eosinophils stained with rhodamine antiprofibrinolysin. These are eosinophils at 5 hours in a skin window which contained dog fibrin. Numerous eosinophils (E) show an abundance of brilliantly marked discrete granules. Intracellular and extracellular coalesced masses (CM) also are specifically stained, as are eosinophil granules (arrows) which lie free in the exudate. Tri X film, exp. 6 min.

These studies were indirect and recorded numerical fluctuations of the circulating eosinophil level in response to disease processes or chemical substances. Several published observations suggest the participation of eosinophils in the process of inflammation. Opie suggested, on the basis of his animal experimentation, that the eosinophil performed a function in the process of inflammation. Rebuck et al. observed increased numbers of eosinophils in a 7-hour skin window from a polyarteritis nodosa patient. Eitzmann and Smith working with 1½- to 21-day-old infants reported eosinophilias ranging from 10 to 93 per cent of the exudative cells in the early hour lesions. Riis found half of the exudative cells were eosinophils in cases of Felty's syndrome. Torre and Leiken investigated patients with blood eosinophilias and found that eosinophils did not consistently appear in the area of inflammation; however, some patients mobilized 50 per cent in the early hours of the inflammatory process. Eidinger et al. studied tissue eosinophilia in individuals showing the immediate type of positive skin reactions. Eosinophils appeared in these test lesions at 4 hours and gradually increased until at 24 hours they were the predominant cell. Hu et al. reported eosinophils as the most striking feature in 33- to 48-hour lesions of subjects who had contact sensitization to Rheus- oleoresin. These studies clearly indicate that the magnitude of the inflam-
matory eosinophil response does not parallel the available circulating eosinophils. Further, a single unifying concept for a common eosinotactic substance was not provided by these reports.

An attractive hypothesis in view of the experimental data given in the present report is that fibrin is an eosinotactic substance. The presence of fibrinogen, fibrin or degradation products of these proteins could account for some of the eosinophilias previously reported. Differences in concentration of fibrin could explain the inconsistencies encountered by others in their studies of acute inflammation with the skin window technic. In our experience with adult dogs, the eosinophilic phase of inflammation was consistently evoked when fibrin or reagents capable of producing fibrin were present. The eosinophilia produced in the inflammatory area exceeded the circulating eosinophils generally 2 to 10 times.

Several proteolytic enzymes have been implicated in eosinophilic responses. Chapman reported that enzymes such as pepsin and ptyalin provoked an eosinophilia in the peritoneal exudate of mice. Stoughton reported that there was a substance, probably a polypeptide, in the human epidermis which was capable of attracting eosinophils out of the blood vessels of the corium into the surrounding connective tissue. He further noted that trypsin liberated this eosinotaxin from the epidermis. In our studies cathepsin and Tryptar provoked multiphasic eosinophil responses. Based on the in vitro evidence that both of these enzymes convert prothrombin to thrombin, we propose that these enzymes promote eosinophil migration by activating the coagulation mechanism in the area of inflammation with the end result being fibrin formation. The shape of the curve indicates that the initial fibrin deposited is removed, or altered by the first wave of eosinophils. However, by continuous enzyme action more fibrin is deposited and countered by a second wave of eosinophil migration in the later hours of the inflammatory process. The formation of fibrin during the process of wound repair might also explain the inflammatory eosinophilias seen during the very late hours of inflammation (33 or 48 hours) when the coverglass has not been disturbed for a period of 12 or more hours. The presence of eosinophils in the early hours of some inflammatory exudates may reflect fibrin deposition as a consequence of the mechanical trauma of the skin window technic which undoubtedly releases variable amounts of tissue thromboplastin and cathepsins into the lesion. Thus, proteolytic enzymes as eosinotactic substances may not operate with separate and distinct mechanisms but rather through their capacity to form fibrin. This sort of reasoning provides a unifying concept (common denominator) for many seemingly unrelated tissue eosinophilias.

Fibrin formation might also be offered as an alternate explanation for the eosinophilia which accompanies the formation of immune complexes. It has been suggested that the union of antigen-antibody complexes liberate histamine, and Murray et al. have reported that in vitro purified prothrombin is transformed to thrombin in the presence of histamine, calcium, platelet extracts, and small amounts of thrombin. Thrombin, a proteolytic enzyme with
a propensity for catalyzing the conversion of fibrinogen to fibrin, evoked a monophasic pattern of eosinophil migration identical with the response to fibrinogen and fibrin.

An indicator of the dynamic quality of eosinophils in areas of acute inflammation was provided by the specific fluorescence with rhodamine antiprofibrinolysin. Clearly eosinophils transport packets of profibrinolysin into the inflammatory site. Great variability in staining was encountered which may mean that profibrinolysin was released slowly from the cells. Also, the granules of the extravascular eosinophils exhibited progressive structural alterations characterized by granule fusion into coalesced masses which stained brilliantly. Some of these masses, as well as individual granules, were ultimately released from the cells. In many cases, fluorescent antiprofibrinolysin stained extracellular granules. Sometimes this was not the case even though the staining response with eosin persisted for those granules which had presumably lost all of their profibrinolysin.

The heterogeneity of fluorescent staining of the granules might be interpreted as reflecting various states of granule maturity. A “ripening” process is observed in bone marrow eosinophils and is characterized by changes in the size, shape and staining qualities of the eosinophil granule. However, it seems unlikely to us that there was a local production of eosinophils since there was no morphologic evidence of nuclear immaturity in the extravascular eosinophils. In the dog, band and bilobed nuclei were typical of eosinophils in the circulating blood as well as in the extravascular location of the inflammatory site.

The question of origin of extravascular eosinophils has concerned many investigators. For the reasons given before, it appears unlikely that exudative eosinophils arise locally. More probably exudative eosinophils originate in the bone marrow at an earlier time. Following their release from the bone marrow, they leave the blood shortly by penetration of the vascular endothelium. Marchesi and Florey\cite{47,38} have published excellent micrographs showing the pseudopodial formation by which eosinophils pass through intercellular endothelial junctions. It may be that in such an extravascular environment eosinophils are not encouraged to release their packages of profibrinolysin unless some specific stimulus triggers the granule alterations. It is also possible that it is the circulating eosinophil which becomes the exudative eosinophil when an eosinotactic substance is present in an area of inflammation. Although our experience in normal animals has shown only a poor specific fluorescence of circulating eosinophils suggestive of a reduced profibrinolysin content, it may be that the packaged profibrinolysin is simply masked in this environment. Information is not available at present to permit a selection among the possibilities.

Whatever the origin of these exudative eosinophils, their transport function for profibrinolysin seems established. Once the profibrinolysin is released into the inflammatory exudate, mechanisms exist for activating the proenzyme and promoting fibrinolysis. It was recently shown that thrombin and cathepsin...
can activate profibrinolysin. All of these enzymes are likely constituents of an injured area. Thus, an active fibrinolytic reaction may occur in the area of inflammation to promote rapid wound healing.

**Summary**

The emigration sequence and cytology of acute inflammation produced by a variety of stimulants was studied in dogs using a modified skin window procedure. Control lesions revealed the typical acute inflammatory cycle with an early influx of polymorphonuclear neutrophils and a later increase in lymphocytes, hypertrophied lymphocytes and macrophages.

Either fibrinogen, fibrin or proteolytic enzymes that promote fibrin formation selectively attracted eosinophils into the inflammatory site. Neither histamine nor albumin produced significant eosinophil migration in the 10 hours under study.

The fluorescent antibody technic was employed to assess the level of cellular profibrinolysin. Only inflammatory eosinophils and bone marrow eosinophils were marked with rhodamine antiprofibrinolysin. Staining was confined to intra- and extracellular eosinophil granules and coalesced masses. The intensity of fluorescence varied somewhat and perhaps reflected profibrinolysin release into the inflammatory exudate.

Exudative eosinophils like all members of the bone marrow eosinophilic series contain profibrinolysin localized in the specific granules. Eosinophils which migrate and collect at an inflammatory site clearly transport profibrinolysin to an area of fibrin deposition. Granule release of profibrinolysin provides one mechanism for fibrinolysis that likely facilitates wound repair.

**Summario in Interlingua**

Le sequentia migratori e le cytologia del acute inflammation causate per un varietate de stimulantes esseva studiate in canes per medio de un modificite technica a fenestra cutanee. Lesiones de controlo revelava le typic acute cyclo inflammatori con un influxo precoce de neutrophilos polymorphonucleari e un subsequente augmento del lymphocytos, de hypertrophiate lymphocytos, e de macrophagos.

Tanto fibrinogeno como etiam fibrina e enzymes proteolytic que promote selectivemente le formation de fibrina traheva eosinophilos ad in le sito inflammatori. Ni histamina ni albumina provocava un significative migration de eosinophilos in le curso del 10 horas de observation.

Le technica a anticorpore fluorescente esseva usate pro evaluare le nivello de profibrinolysina cellular. Solmente eosinophilos inflammatori e eosinophilos de medulla ossee esseva marcate con antiprofibrinolysina rhodamina. Le tincturation esseva restringite al granulos eosinophilic intra- e extracellular e a massas coalescite. Le intensitate del fluorescentia variava in un certe mesura lo que reflecte possiblemente un liberation de profibrinolysina ad in le exsudato inflammatori.

Eosinophilos exsudative—como omne le membros del serie eosinophilic in le medulla ossee—contine profibrinolysina localisate in le granulos specific.
Eosinophilos que migra e que se collige a un sito inflammatori transporta clarmente profibrinolysina ad un area de deposition de fibrina. Le liberation granular de profibrinolysina provide un mechanismo pro le fibrinolyse que resulta probablemente in un simplification del processo de restauration in le vulner.

REFERENCES


Jeanne M. Riddle, Ph.D., Department of Laboratories, Henry Ford Hospital, Detroit, Mich.

Marion I. Barnhart, Ph.D., Department of Physiology and Pharmacology, Wayne State University School of Medicine, Detroit, Mich.
The Eosinophil as a Source for Profibrinolysin in Acute Inflammation

JEANNE M. RIDDLE and MARION I. BARNHART