The Role of Granulocytes in Endotoxin-induced Vascular Injury

By Evelyn Gaynor

This study examines the role of neutrophils (PMN) in the pathogenesis of the endothelial lesion induced by a single sublethal dose of endotoxin. It is intended to clarify whether the margination of PMN on endothelium after endotoxin causes intimal injury or is a response to it. Neutropenic rabbits had mean PMN counts of 33/cu mm 72 hr after nitrogen mustard (HN2). They were heparinized and given either intravenous endotoxin or saline and were sacrificed 30-60 min later. Preterminal blood samples were positive for the presence of endothelium in 77% of endotoxin-treated neutropenic rabbits, in 87% of endotoxin-treated normal rabbits, and in only 12% of neutropenic rabbits given saline. Sections of aorta revealed marked abnormalities of endothelium in rabbits receiving endotoxin, whether neutropenic (90% had lesions) or normal (85% had lesions). Endothelial abnormalities included vacuolation and lysis, marked subendothelial edema, and desquamation. Similar lesions in control neutropenic rabbits were not found, and mild abnormalities were seen only rarely. These data indicate that neutropenia does not protect rabbits from endothelial injury due to endotoxin. They further suggest that HN2 may cause endothelial damage either directly or secondary to the effects of neutropenia.

RABBITS treated with a single intravenous injection of endotoxin, in either lethal doses or amounts preparatory for the generalized Shwartzman reaction (gSr), develop widespread multifocal lesions of vascular endothelium. These vascular lesions are not generally accompanied by significant hemostatic deposit, such as major platelet aggregates or fibrin formation, in contrast to the pathologic sequence that follows a similar small dose given 24 hr later. These lesions of endothelium are not prevented by prior anticoagulation, as are the microthrombi of the full-blown gSr. They are manifested by desquamation of endothelial cells, singly and in sheets, into the circulation as early as 5 min after endotoxin, and by increased mitotic activity in the intima of the aorta and in renal and pulmonary capillary endothelium. The mechanism whereby endotoxin effects endothelial damage is not known. Some investigators have proposed that the injury is caused by substances such as histamine, serotonin, epinephrine, or kinins, since similar lesions have been described after infusion with these mediators. Other workers have suggested that the profound vascular effects of endotoxin on endothelium are...
secondary to release of lysosomal or chemical mediators from platelets or polymorphonuclear leukocytes (PMN).\textsuperscript{9-12}

Granulocytes were first shown to be an essential component for the development of the gSr by Stetson and Good in 1951.\textsuperscript{13} Their studies have been amplified more recently by Forman et al.,\textsuperscript{14} who were able to prevent the characteristic coagulation defects and renal cortical necrosis of the gSr by rendering rabbits neutropenic with nitrogen mustard (HNs). The classical manifestations of the gSr were, moreover, reinduced when the neutropenic animals were infused with viable granulocytes at the time of endotoxin injection.\textsuperscript{14} McKay et al. tested the hypothesis that injured leukocytes contribute to the development of the thrombotic features of the gSr by their release of a thromboplastic material (i.e., tissue factor). Using lysates of PMN derived from peritoneal exudates, however, these workers were not able to demonstrate thromboplastic activity either in vitro or in vivo.\textsuperscript{15} On the other hand, recent work by both Lerner et al. and Niemetz and Fani has demonstrated that viable granulocytes, after prolonged incubation with endotoxin and a serum factor, are capable of generating potent tissue factor activity.\textsuperscript{16-18}

Whether PMN are essential to the development of early endothelial lesions or are involved mainly in later events is not known. Accordingly, the purpose of the present study was to examine the early effects of endotoxin on the endothelium of rabbits rendered neutropenic with HNs.

**MATERIALS AND METHODS**

**Animals**

Adult male New Zealand rabbits weighing from 2.0 to 4.0 kg were used. These animals had free access to water and Purina rabbit pellets throughout the experiments.

**Induction of Neutropenia**

Nitrogen mustard (mechlorethamine HCl, 10 mg vial, Merck, Sharp & Dohme, West Point, Pa.) was prepared by adding 10 ml of bacteriostatic, pyrogen-free sterile water; the solution was used promptly thereafter. A dose of 1.75 mg/kg was injected into the marginal ear vein 72 hr before the animals underwent further studies.

**Induction of Endotoxemia**

*Escherichia coli* endotoxin 0127:B8 Boivin type was obtained from Difco, Detroit, Mich. and was freshly prepared for each experiment by suspension in pyrogen-free, sterile isotonic saline at a concentration of 100 µg/ml. Immediately prior to receiving endotoxin, each rabbit was injected intravenously with 1.0 ml sodium heparin (Upjohn Co., Kalamazoo, Mich., 1000 USP units/ml) to prevent any thrombin formation that might ultimately (directly or indirectly) effect injury to endothelium. Endotoxin was administered via a marginal ear vein in a single dose of from 25 to 100 µg/kg. The variation in dose was occasioned by a seasonal variation in rabbit susceptibility to the agent. Larger doses were required in the winter than in the summer to produce a similar endothelial response. The dose was determined in separate studies as the minimal amount of endotoxin necessary to induce the appearance of endothelium in the circulation of 80% of the rabbits.

**Experimental Design**

Rabbits were divided into four groups and were treated as indicated in Table 2. At the time of sacrifice, either blood or tissue, or both blood and tissue were obtained for study from each rabbit. It was not possible to perform all studies on each rabbit.
Blood was obtained from the central artery of the ear prior to any treatment and at 72 hr after nitrogen mustard, through siliconized needles and plastic tubing (Butterfly-21 Infusion Set, Abbott Labs., North Chicago, Ill.) directly into glass tubes containing 0.06 ml of 15% potassium EDTA, for baseline blood counts. At sacrifice, animals were anesthetized with sodium pentobarbital (Diabutal, Diamond Labs, Des Moines, Iowa) supplemented with ether. Blood was obtained at sacrifice by free flow from the aortic bifurcation through a No. 18 siliconized needle and plastic tubing to preclude aspiration of endothelial cells at the tip of the needle. Terminal specimens were collected for circulating endothelium, platelet counts, and leukocyte counts and differentials. Whole blood coagulation times were performed to measure the effectiveness of heparinization. The collecting tubing was then clamped, and the thoracic aorta was cannulated just below the arch for perfusion fixation of the aorta in situ. The inferior vena cava was incised at the onset of the perfusion to permit drainage of the perfusate.

Blood Counts and Other Blood Studies

Total leukocyte counts were performed on EDTA-anticoagulated blood with a Coulter S Counter. Platelets were enumerated by the method of Brecher et al.19 Differential counts were performed on 200 leukocytes in Wright’s-stained blood films. Coagulation times for whole blood in glass were determined as described by Lee and White.20

Detection of Circulating Endothelial Cells

The presence of circulating endothelium was determined by light microscopy as previously described.4 Briefly, 5 ml of blood were collected into 10 ml of a buffered citrated solution containing approximately 1% formaldehyde. Erythrocytes were hemolyzed with a 1% saponin solution in 50% ethanol, and the remaining “leukoconcentrate” was collected by centrifugation at 340 g for 10 min. Portions of the sedimented cell button were then smeared on glass slides, were stained with Wright’s and Giemsa stains, and were examined for the presence of endothelium. Specimens were examined “blind” and were considered positive if more than a total of five endothelial cells were found on two random slides from a given animal and were negative if two or fewer endothelial cells were present. These limits were established in pilot studies, where one or two endothelial cells were found in the blood of less than 1% of normal rabbits and were thought to be artifacts of collection. An arbitrary count of greater than five cells was established as positive, in order to rule out false positives. In the current study, all animals reported as negative actually had no detectable endothelium in their blood. Similar pilot studies established that there were wide variations both quantitatively and temporally in the degree of response of individual rabbits to identical doses of endotoxin, and no correlation between dose levels and numbers of endothelial cells was found. Higher doses resulted in a greater percentage of rabbits responding with circulation of endothelium, rather than greater numbers of endothelial cells per individual rabbit.

Preparation of Tissues

Aortas from 18 neutropenic and 12 normocytic rabbits were fixed by perfusion in the anesthetized animals 30–60 min after injection with endotoxin or control solutions. Two per cent glutaraldehyde in 0.067 M phosphate buffer, pH 7.2, at 37°C were perfused by gravity flow at a pressure of 120 mm Hg through the cannulated thoracic aorta for 30–45 min. The aorta was then dissected gently from surrounding tissues, and segments of thoracic and abdominal aorta were sliced into cross-sectional rings approximately 1 mm in length. After further glutaraldehyde fixation at 4°C for 1 hr, tissues were postfixed in 1% osmium tetroxide in phosphate buffer for 1 hr at 4°C followed by 1% uranyl acetate fixation for 1 hr. After dehydration through graded alcohols and propylene oxide, specimens were embedded in Epon 812. One micron sections were made of the entire aortic circumference with a Sorvall MT-1 Ultramicrotome; these were stained with lead hydroxide and uranyl acetate, and were examined in a Siemens 1A electron microscope.
All tissues were examined without knowledge of the experimental group from which they were derived and were independently evaluated for the presence and extent of abnormalities of the endothelium by two individuals. The following morphologic features were considered abnormal in the semithin and ultrathin cross sections of aorta: frank sloughing of endothelium (Fig. 3C), platelet aggregates adherent or closely adjacent to the perfused vessel wall (Fig. 2B,C), lysis and vacuolation of endothelial cytoplasm (Fig. 2B), formation of stalks of elongated endothelial cytoplasm terminating luminally with a markedly contracted nucleus (Fig. 2B), and severe subendothelial edema resulting in the formation of arcades (Fig. 2D,E).

Vessels from an additional 12 rabbits (two in each group and the remainder at various intervals after injection with HN2) were prepared for en face examination using the techniques described by Poole et al.22 They were perfused (at 1–4 hr after endotoxin or saline control solution) through the cannula in the thoracic aorta, with efflux of perfusate through a cannula in the femoral artery. Briefly, the following solutions were successively perfused over 15–30 sec each: 100 ml of 5% glucose in water, 20 ml of 0.25% silver nitrate, 20 ml of 5% glucose, 80 ml of a solution containing 3% cobalt bromide and 1% ammonium bromide, 20 ml of 5% glucose, and 100–200 ml of 4% formaldehyde. The formalin solution was continued slowly over 1 hr, after which the aorta was washed out with 100 ml distilled water and was then counterstained by perfusion with 40 ml of hematoxylin delivered over 90 sec. After a second flush with 100 ml of tap water, the aorta was dissected free of adventitia in situ, was removed and opened longitudinally, and was pinned out flat on cardboard. It was then dehydrated through alcohols and was mounted in Damar, endothelium upward, on glass slides.

In these en face preparations, the entire aorta below the cannulated upper thoracic segment was examined for the presence of either PMN or lymphocytes in the plane luminal to the endothelium. Platelets and red blood cells are not well stained with this technique, and thus, their presence could not be evaluated. Moreover, since artifactual tears in the endothelial lining occurred occasionally, due to stretching the vessel out on cardboard, accurate estimation of endothelial sloughing was precluded. This technique was used primarily to evaluate the extent and geographic distribution of inflammatory cells. Confirmation of identification as inflammatory cells was obtained using routine histologic techniques in an additional three rabbits in each group.

RESULTS

Induction of Neutropenia

Total leukocyte counts and differential counts were performed on the arterial blood of rabbits at 72 hr after intravenous injection of HN2. The results of control and 72-hr blood counts are presented in Table 1. There was a fall in total leukocyte counts, primarily accounted for by a marked neutropenia, in all the rabbits. The degree of neutropenia achieved was consistent with those levels reported necessary to prevent the gSr.14 There was no significant change in platelet counts from pretreatment levels in these rabbits.

Table 1. Effect of Nitrogen Mustard Injection (1.75 mg/kg) in 71 Rabbits

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>72 Hr After HN2</th>
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<tbody>
<tr>
<td>WBC/cu mm</td>
<td>8600 (4200–16,000)</td>
<td>1400 (400–2300)</td>
</tr>
<tr>
<td>PMN/cu mm</td>
<td>4700 (2100–6400)</td>
<td>33 (0–96)</td>
</tr>
<tr>
<td>Platelets × 10^3/cu mm</td>
<td>380 (250–520)</td>
<td>253 (135–375)</td>
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Results are given as means, with range of values given in parentheses.
animals appeared to be in good health, with the exception of two rabbits with moderately severe diarrhea.

Effects of Heparin

All rabbits were anticoagulated with heparin immediately prior to injection with endotoxin or control solutions of saline. Clotting times for whole blood were greater than 1 hr in all rabbits sacrificed up to 1 hr later.

Effects of Endotoxin

Normal rabbits treated with endotoxin exhibited a mean fall in WBC counts of 52% and a mean fall in platelet counts of 47% during the hour after treatment. There were no dose-dependent differences noted within the dosage range used. These findings are consistent with those previously reported. Although there was no significant change in mean WBC in the neutropenic rabbits after endotoxin, the mean platelet count fell from 253,000/cu mm to 130,000/cu mm, or by about 50%.

Examination for Circulating Endothelial Cells

Neutropenia conferred no protection against the desquamation of intima after injection with endotoxin. Within the hour after a single dose, endothelium was found in the blood of 87% of the normocytic rabbits, and 77% of the neutropenic rabbits. These cells were identified by their characteristic elongated wrinkled and pale-staining nuclei (Fig. 1A). They occurred either singly or in clusters up to several dozen (Fig. 1B). The incidence of circulating endothelial cells at 30–60 min after treatment is presented in Table 2.

Six of 36 control rabbits rendered neutropenic, but not given endotoxin, also had circulating endothelial cells. It may be significant that two of these animals were noted to have severe diarrhea at the time of sacrifice, although the rabbit colony as a whole was free of this ailment.
Table 2. Incidence of Endothelial Damage in Normal and Neutropenic Rabbits After Intravenous Endotoxin

<table>
<thead>
<tr>
<th></th>
<th>No. of Animals With Circulating Endothelial Cells</th>
<th>No. of Animals With Aortic Endothelial Lesions</th>
<th>No. of Animals With Either or Both Abnormalities</th>
<th>% of Animals With Either or Both Abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN&lt;sub&gt;2&lt;/sub&gt; + saline</td>
<td>6 (36)†</td>
<td>1 (19)</td>
<td>6 (46)</td>
<td>12</td>
</tr>
<tr>
<td>HN&lt;sub&gt;2&lt;/sub&gt; + endotoxin‡</td>
<td>13 (17)</td>
<td>9 (10)</td>
<td>22 (25)</td>
<td>88 &lt; 0.01</td>
</tr>
<tr>
<td>Normal + endotoxin†</td>
<td>7 (8)</td>
<td>5 (7)</td>
<td>12 (14)</td>
<td>85</td>
</tr>
<tr>
<td>Normal + saline</td>
<td>0 (7)</td>
<td>0 (7)</td>
<td>0 (7)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Neutropenia induced with intravenous HN<sub>2</sub> (1.75 mg/kg) 72 hr before treatment with endotoxin or saline control.
†Numbers in parenthesis indicate total number of animals examined.
‡Endotoxin given as a single dose of 25–100 µg/kg 30–60 min before sacrifice.

Examination of Tissues

Among the rabbits treated with endotoxin, 90% of the neutropenic, and 71% of the normal animals exhibited significant abnormalities of aortic endothelium. There was no difference between these two groups in the severity, distribution, and extent of injury, nor were there any dose-related differences within the range used. On the other hand, in the neutropenic and control untreated rabbits, there was virtually no evidence of injury to aortic endothelium; at most, the sole morphologic abnormalities consisted of rare areas with minimal subendothelial edema that could be detected only by electron microscopy. There was complete concurrence by both independent observers as to the presence and severity of abnormalities. The results of these studies are listed in Table 2. Differences between the means for the effects of neutropenia with and without endotoxin are significant at \( p < 0.01 \).

The degree of injury ranged from moderate to severe in the endotoxin-treated animals and involved more than 25% of the endothelial cells in any given aortic cross section. The most frequently found abnormalities were marked contraction of the endothelial cells (Fig. 3B) with development of areas of intracellular lysis and vacuolation, and bulging of the nuclei into the lumen of the vessel (Fig. 2B). The nucleus occasionally extended far into the lumen on a thin stalk, and in some instances it had torn free from the stem and apparently entered the circulation leaving cytoplasmic strands. In addition, in many blocks there was marked subendothelial edema. In some areas, this was severe enough to impart the appearance of arcades to the endothelium, with attachment to subendothelial structures by only a few attenuated cytoplasmic strands (Figs. 2D,E, and 3A). The most severe change noted was frank desquamation of endothelium into the lumen. In Fig. 3C, a whole sheet of endothelium has almost completely sloughed, and a few platelets and platelet pseudopods are seen adhering to the subendothelium. As expected, there was no fibrin formation in these heparinized animals. Clusters of disc-shaped, intact-appearing platelets were occasionally seen adjacent, but not adherent, to relatively normal-appearing endothelial cells (Fig. 2B). Although attempts were
Fig. 2. Photomicrographs of semithin sections of aorta from (A) neutropenic rabbit; (B), (C), and (D) neutropenic rabbits at 1 hr after injection with endotoxin; and (E) normal rabbit 1 hr after injection with endotoxin. Solid lines represent 10 μ. Note normal appearance of endothelium in neutropenic rabbit aorta, compared with abnormalities of endothelium in both neutropenic and normal animals given endotoxin. Pathologic changes include vacuolation (v), subendothelial edema (ed) resulting in arcade formation, platelet aggregates (p), and attenuated cytoplasmic stalk (arrow).

made to section the vessels serially at these sites in order to define loci of platelet adhesion, it was not always possible to recover and to examine every section by electron microscopy. Consequently, it was not possible to demonstrate the structures to which the intact platelets adhered. Presumably, such clusters were part of the periphery of an aggregate whose site of attachment was relatively distant from the plane of sectioning. Figure 2C shows such a group of platelets near the aortic wall where there is moderately severe endothelial damage.

To define the geographic distribution and extent of endothelial injury after endotoxin, as well as after HNa alone, en face preparations and longitudinal sections of the whole aorta were examined. These were obtained from rabbits at 1 hr and 4–6 hr after injection with endotoxin and at 6, 24, 48, 72, and 96 hr following treatment with HNa. Desquamation of endothelium could not be distinguished from artifacts of preparation, and platelets and red blood cells
do not stain with this technique. Therefore, the effect of treatment on the endothelium of these vessels could be accurately evaluated only in terms of the inflammatory response it evoked. Such inflammatory cells were seen as randomly scattered small foci of cells only in those animals that had received endotoxin. These consisted of clusters of round cells adherent to the luminal surface of the intima in the neutropenic group (Fig. 4B) and a combination of round cells and PMN in the group with normal WBC (Fig. 4A). In these photomicrographs of the full thickness of the aorta, the focal plane is luminal to the endothelial cells, which are outlined by the silver stain. The inflammatory cells (PMN, lymphocytes, and mononuclear cells) have small densely stained nuclei that can be clearly distinguished from the large, pale nuclei of the endothelial cells, which are just below the plane of focus. The normal appearance characteristic of the aorta in rabbits 72 hr following HN2 alone is illustrated in Fig. 4C. In the nine rabbits examined at various intervals after HN2 alone, a single small focus of round cells was noted in only one aorta at 72 hr following injection. There was no discernible predilection for these
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Fig. 4. En face preparations of aorta 1–3 hr after intravenous endotoxin in (A) normal, and (B) neutropenic rabbits. Scattering of WBC is similar in both, except for absence of PMN in neutropenic rabbit. Small dense nuclei are inflammatory cells, and large paler nuclei are endothelial cells. Precipitated silver stain outlines the endothelium. In contrast, (C) represents normal appearance of aorta from neutropenic rabbit given saline 72 hr after HN₂. × 3250.

findings in any localized area of the aorta in the six rabbits that had received endotoxin.

DISCUSSION

These studies demonstrate that PMN are not necessary for the induction of endothelial lesions by endotoxin and suggest that endotoxin may have a direct toxic effect on endothelium. The presence of severe neutropenia, at levels sufficient to prevent the thrombotic manifestations of the gSr,¹⁴ had no detectable ameliorating effect on the incidence of endothelium in the circulation or on the development of early, widespread significant endothelial lesions in the aortas of heparinized rabbits given a single nonlethal injection of endotoxin.

Of interest is the demonstration of circulating endothelium in 16% of rabbits examined at 72 hr after HN₂, at the time of maximal neutropenia.
HN2 injury may conceivably result in late desquamation of endothelium, but data to support such a concept are not available. However, additional rabbits, examined at 1, 24, 48, and 96 hr after treatment, were negative for circulating endothelium, further suggesting that this finding is not related to the effects of HN2. One is tempted to propose that the combination of residual HN2-induced injury of intestinal mucosa plus decreased host defenses resulted in entry of endogenous coliform organisms into the blood stream of the affected rabbits.

Of further interest is the incidental observation that larger doses of endotoxin resulted in increased numbers of rabbits responding positively, rather than in greater numbers of endothelial cells in the circulation. This finding implies that the endotoxin effect on endothelium may require the presence of a cofactor or factors that limit responsiveness or that endothelium is damaged secondarily. Potential candidates for such interaction with endotoxin include platelets, complement, and antibody.

Although PMN are noted to marginate in blood vessels early in endotoxemia, the concept that these cells are involved in the pathogenesis of the endothelial lesions must be reexamined in the light of the results of this study. Additional evidence in support of the present concepts has been presented by Pingleton et al., who demonstrated that radiation-induced leukopenia provided no protection from either the hemodynamic or the histologic damage in pulmonary capillaries observed after administration of endotoxin.

The above studies do not minimize the importance of PMN in the development of the full-blown gSr, with its thrombotic manifestations and consumption coagulopathy. Here, a possible role of the neutrophil is to contribute its tissue factor activity in enhancing fibrin formation at sites of endothelial injury and subsequent thrombus growth. Tissue factor generated by PMN reaches levels of significant activity after 4–6 hr of incubation with endotoxin, and it is at this time that fibrin is first seen in the gSr. Whether this tissue factor activity causes fibrin formation in the circulating blood or is generated locally after attraction of PMN to sites of platelet adhesion after endothelial injury is yet to be demonstrated.

Attraction of PMN to sites of vascular damage is suggested by their margination at areas of platelet adhesion 6 hr after mechanical injury of the aorta, but evidence suggesting activity within circulating blood has been presented by Niemetz and Fani. Leukocytes were obtained from peritoneal exudates of rabbits given intraperitoneal and intravenous endotoxin. Rapid infusion of these cells into normal rabbits was followed by massive pulmonary and right ventricular clots. When given at a slower rate, multiple microscopic pulmonary clots were found. Whether this in vitro generation of tissue factor activity is, in fact, duplicated in the intact rabbit given endotoxin is not yet known.

It is postulated that the sequence of events occurring in endotoxemia is one in which an initial endothelial injury is followed by platelet deposition and subsequent fibrin formation. Such lesions are amplified in the gSr with the second dose of endotoxin, where similar although more severe changes in
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blood and tissue have been discussed by McKay. On the basis of this study, it would appear that PMN, considered essential for the development of the thrombotic renal lesion characteristic of the gSr, are not involved in the pathogenesis of the primary endothelial lesion of endotoxemia. It is proposed that PMN may affect the gSr by favoring thrombus growth on established vascular lesions.

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