THE EFFECTS OF ENDOTOXIN ON VASCULAR ENDOTHELIUM

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Collapse of the peripheral circulation induced by endotoxins (lipopolysaccharides) from Gram-negative bacteria results ultimately from the response of the vascular smooth muscle to some stimulus. The changes in circulatory derangements during endotoxin shock have been extensively studied (Bennett and Cluff, 1957; Gilbert, 1960; Hardaway and Elovitz, 1966; Martin et al., 1965; Thomas, 1954). However, the basic mechanisms responsible for these derangements remain largely unknown.

Since the original observation by Gerber of hyaline thrombi in the lungs and liver of rabbits subjected to endotoxin (Gerber, 1936), similar thrombi have been observed in the pulmonary arteries, coronary arteries, and the sinusoids of the spleen, as well as in the lungs and liver (Brunson et al., 1955a, 1955b, Brunson, 1964; Cavanagh and Albores, 1966; Gilbert, 1960; Thomas, 1954). Hyaline thrombi are also characteristic findings in autopsy material from human and canine victims of endotoxemia (Weil, 1964).

Since thrombi are now considered to form only in areas where connective tissue is exposed (Bounameaux, 1959, 1961; French and Poole, 1963; French et al., 1964; Seegers, 1962; Zucker and Borelli, 1962), these observations indicate that the vascular endothelium suffers extensive damage resulting in denuded areas during endotoxin shock. However, the extent and type of such damage has not been systematically studied. This is partly due to the difficulty of observing an adequate number of cells by classical histological techniques.

In this study the above limitation was circumvented by a technique which made it possible to observe large areas of endothelium as monolayered sheets of cells stripped from the other vascular components. The extent of damage or desquamation of endothelial cells and the formation of blood cell aggregates on the vascular surface caused by bacterial endotoxin were investigated.

Materials and Methods

Animals.—Young, apparently healthy white rabbits weighing between 4 and 6 lb. were divided into three groups: group I, control animals; group II, rabbits injected intracardially with endotoxin 1 hr before perfusion; and group III, rabbits injected intracardially with endotoxin 24 hr before perfusion.

Treatments.—An LD_{50} dose of endotoxin (7.5 mg of endotoxin [Bacto Lipopolysaccharide, Difco Laboratories, Detroit, Mich.] in 15 ml of 0.9% saline) was injected intracardially while

the animals were lightly anesthetized with ether. The animals were observed during recovery from the injection, and then returned to their cages for the specified length of time.

Perfusion.—The rabbits were kept under deep ether anesthesia during preparation for perfusion. The left ventrolateral surface of the abdominal region and the ventral surface of the neck were shaved. An incision in the abdominal region exposed the vena cava which was then separated from the surrounding tissue. A hemostat was applied to the vessel anterior to the point at which it was to be severed to allow drainage of blood and perfusion fluid. The carotid arteries were clamped off to divert the perfusion fluid to the abdominal region.

After the three hemostats were in place, the chest cavity was quickly opened, a 15 gauge needle inserted directly into the left tip of the heart, and the flow of perfusion fluid begun. As soon as the fluid started to flow, the vena cava was cut.

1000 ml each of 0.5, 1.0, and 1.5% glutaraldehyde in Millonig's phosphate buffer (Pease, 1964) at pH 7.0 were employed in the perfusion. Saturated alcoholic methylene blue was added so that the course of perfusion could be observed. Containers for the perfusion fluids were mounted about 6 ft above the level of the operating area, and the perfusion fluid passed into the heart through a 15 gauge needle connected to a suitable arrangement of plastic tubing with clamps.

After the 0.5, 1.0, and 1.5% buffered glutaraldehyde had perfused the vessels, parts of the mesentery were immediately removed, transferred to 5% glutaraldehyde in buffer, and refrigerated at 4°C overnight. After fixation, small pieces of mesentery containing blood vessels were dehydrated by passage through 25, 50, 75, 95, and 100% ethanol and then transferred to acetone for 5 min to dissolve fat deposits. The tissue was returned to absolute alcohol during removal of the connective tissue surrounding the blood vessels. This was stripped away with fine forceps under a Bausch and Lomb stereoscopic dissecting microscope.

When the extraneous tissue had been removed, a section of an artery was cut from the network (arteries were differentiated from veins by their smaller size and their resiliency). This section was slit longitudinally with a scalpel and each half transferred to a sheet of cork where it was kept moist with ethanol, flattened out, and firmly affixed to the cork with fine mounting pins.

After the alcohol had evaporated, a solution of 3% Formvar in ethylene dichloride (Ladd Research Industries, Burlington, Vt.) was pipetted onto the flat surface of the vessel. Any excess flowed onto the surface of the cork. The solvent evaporated within a few minutes and the Formvar-impregnated tissue was transferred to 10 N NaOH overnight, then rinsed with distilled water. A laminated film consisting of Formvar and a monolayer of endothelial cells was stripped from the luminal surface of the vessel by the use of fine forceps and a dissecting microscope.

The cell sheets were mounted temporarily on glass slides in an aqueous methylene blue medium. The edges of the cover slips were sealed with Permount (Fisher Scientific Co., Pittsburgh, Pa.).

Observation of Cell Sheets.—The sheets of endothelium containing approximately 500–1000 cells were observed by phase and bright-field microscopy (Leitz Ortholux microscope). Sheets of cells from three animals in groups I and II and from four animals in group III were photomicrographed with a Leica camera, model DPB, on 35 mm Kodachrome II-A, ASA 40. Photomicrographs were taken with phase-contrast optics at × 100, 400, and 700 and at × 400 and 900 with bright-field optics.

The characteristics of mechanical damage were studied by pricking control specimens with a fine needle, the needle marks being made in a diamond-shaped pattern to allow for positive identification.

Properties of Red Blood Cells.—Blood from control and endotoxin-treated rabbits was obtained by cardiac puncture. The samples were placed in an excess of buffered glutaralde-

hyde with methylene blue at 4°C overnight. The percentage of stained and unstained cells was determined.

Freshly drawn, unstained blood from both control and endotoxin-treated animals was examined by phase-contrast microscopy.

RESULTS

Group I.—Sheets of endothelial cells from control animals exhibited smooth, elliptical nuclei oriented parallel to the longitudinal axis of the cells. The nuclei were distributed somewhat irregularly over a smooth background with faint longitudinal striations. Aqueous methylene blue stained the nuclei a deep blue, but the cytoplasm stained so faintly that cell outlines were indistinct (Figs. 1 and 2).

Group II.—Sheets of endothelium from animals injected with endotoxin 1 hr prior to sacrifice exhibited several degrees of damage. In the least severely damaged specimens, the nuclei were spindle shaped and stained irregularly (Fig. 3). The longitudinal striations were more pronounced and many small, extremely faint round bodies were present.

In more severely damaged specimens, nuclear vacuolization and adherence of blood cells was extensive (Fig. 4).

Aggregates composed of stained and unstained red blood cells, a few white blood cells, and platelets were common. The consistent apposition of stained and unstained red blood cells in the same aggregate was dramatic (Fig. 5).

Specimens from two of the rabbits in group II exhibited about equal and extensive damage while that from the third rabbit was similar but not so extensive.

Group III.—Specimens from 24 hr posttreatment animals exhibited more extensive damage than that observed in 1 hr posttreatment animals. In some specimens from 24 hr posttreatment rabbits, barely recognizable remnants of nuclei remained (Fig. 6), while in others no evidence of the original cellular structure could be identified (Fig. 9).

In some instances, nuclei had disappeared from the endothelial sheets, or had changed staining characteristics (Fig. 7). In other areas of the same specimen all cellular structure was lost.

In specimens from one 24 hr posttreatment rabbit, it appeared that strips of underlying tissue oriented parallel to the longitudinal axis of the endothelial cells came away with the film causing dense cords which were separated by light zones (Fig. 8).

Nuclear vacuoles were prominent in only one 24 hr endotoxin-treated animal.

The number of red blood cells and platelets stuck on the vascular surface was much greater than in 1 hr samples. Red blood cells, singly, in pairs, and in clumps were a consistent characteristic of these specimens. Most were stained; however, some in close proximity to stained cells remained unstained.

In a few instances the membranes of stained red cells were broken and the contents appeared to be spilling out.

Platelets, which could be differentiated from the other formed elements of the blood by their small size, were also present in greater numbers in 24 hr than in 1 hr specimens. All were unstained. In specimens from two of these rabbits, there were unstained plaques covering some areas (Fig. 9).

Properties of Blood Cells.—Upon exposure to methylene blue in buffered glutaraldehyde, blood from a control animal contained no stained red cells, while that from an animal 1 hr after endotoxin injection contained from 21–28% of stained red cells. White blood cells were almost entirely absent in the blood from the endotoxin-treated rabbit. No platelets were stained in either sample.

In freshly drawn, unstained blood from an endotoxin-treated animal, 20–25% of the red blood cells were knobby, while the control showed no irregularly shaped cells. Aggregates of two to seven red blood cells were observed in blood from an endotoxin-treated animal but no aggregates were observed in blood from the control.

DISCUSSION

Examination of thousands of endothelial cells and their relation to each other has shown that intravascularly introduced bacterial endotoxin causes detectable changes in almost every cell in the vascular lining of the mesenteric arteries of rabbits. These alterations in the endothelium may be a direct result of action of endotoxin or they may be secondary effects mediated by a chemical substance or substances, such as the vasoactive amines. This study does not distinguish between the two possibilities.

Extensive damage to endothelial cells has also been observed during inflammation caused by thermal (Cotran, 1965) and chemical (Ham and Hurley, 1965) agents. In turpentine-induced pleurisy, endothelial cells of rats exhibited a marked degree of cytoplasmic swelling and vesiculation (Ham and Hurley, 1965), while after severe thermal injury, large vacuoles, condensation of the nucleus, and fragmentation of the cell membrane and organelles were observed in these cells. Cellular fragmentation and sloughing occurred occasionally after thermal injury (Cotran, 1965). In this study, distorted nuclei, apparent nuclear vacuolization, and missing nuclei were observed. In the studies cited above (Ham and Hurley, 1965; Cotran, 1965), the damage was not universal, but limited to certain cells, while endotoxin-induced damage was universal. Cytoplasmic swelling and vesiculation have been observed in endothelial cells after anoxia (Moore, 1959), but the vessels used in this study were large enough to make ischemia unlikely.

Mechanical injury produced with a fine needle was easily distinguished from endotoxin-induced changes by the severity and limited site of damage. Endothelial cells at the point of injury were severely disrupted, but the remainder

of the cells in the sheet appeared normal. Mechanical damage around the edges of cell sheets from endotoxin-treated animals was easily identifiable even when superimposed on endotoxin-induced damage.

Several investigators have observed platelets and leukocytes stuck to the surface of blood vessels after endotoxin injection (Rigdon, 1953; Zweifach et al., 1965), however, the sticking of red blood cells to such vessels has not been reported before to our knowledge. In this study red blood cells, singly and in clumps, were consistently and conspicuously observed on vascular surfaces from endotoxin-treated animals. Only rarely were a few single red cells seen on surfaces of sheets from control animals.

The close proximity of stained and unstained red blood cells recalls a similar phenomenon that occurs in yeast. In the case of yeast, viable cells remain unstained while dead cells readily take up methylene blue. Studies on fresh and aged human red cells showed that methylene blue stains a significant percentage of 24 day old cells, but practically no fresh red cells.[1] These results suggest that the stained cells may be damaged or undergoing degenerative changes. This possibility is supported by the observation that the membranes of a few stained red cells were broken and the contents appeared to be spilling out.

Unstained platelets and plaques resembling platelet thrombi were observed on the surfaces of vessels from endotoxin-treated animals only. Specimens from rabbits sacrificed 24 hr after endotoxin injection exhibited a greater number of platelets than those from animals sacrificed 1 hr postinjection. The adherence of platelets to damaged blood vessel walls with subsequent thrombus formation has been observed by many investigators (French et al., 1964; Poole et al., 1958, 1959; Rigdon, 1953; Warren, 1965).

The scarcity of leukocytes in blood from the endotoxin-treated animal is not surprising since several investigators have reported a pronounced decrease in the number of circulating leukocytes in animals exposed to endotoxin (Florey, 1962; Westphal, 1960).

SUMMARY

This study was undertaken to develop a technique for the preparation of sheets of endothelial cells and to investigate the effects of bacterial endotoxin on large numbers of cells from continuous sheets of vascular endothelium.

Rabbits were divided into one control and two experimental groups. The experimental animals received intracardially an LD_{50} dose of *Escherichia coli* endotoxin. 1 and 24 hr postinjection, the vessels of the animals were perfused with glutaraldehyde in Millonig's buffer with methylene blue as a marker.

Pieces of mesentery containing arteries were postfixed in buffered glutaraldehyde, dehydrated, and placed in acetone (to remove fat deposits). The sur-

[1] McGrath, J. M., and G. J. Stewart. 1967. Unpublished observations.

rounding connective tissue was stripped from the mesenteric arteries, and segments of the vessels were slit longitudinally, flattened out, and firmly affixed to a sheet of cork with fine mounting pins. A 3% solution of Formvar in ethylene dichloride was pipetted onto the luminal surfaces of the vessels. The endothelial cells were impregnated with and adhered to the Formvar and, after soaking overnight in 10 N NaOH, could be stripped from the vessel walls as monolayers. Sheets of Formvar-impregnated cells were temporarily mounted on glass slides in aqueous methylene blue and examined by phase and bright-field microscopy. Methylene blue stained the nuclei a deep blue and the cytoplasm faintly, but cell outlines were indistinct.

Endothelial sheets from control rabbits had smooth, elliptical nuclei oriented parallel to the longitudinal axis of the cells and irregularly distributed over a smooth background with faint longitudinal striations.

Essentially every cell in endothelial sheets from endotoxin-injected animals appeared to be severely damaged. Cell sheets from 24 hr posttreatment animals exhibited the same type of, but more extensive, damage than that observed in 1 hr posttreatment animals. The most prominent features of the damaged endothelium were distorted nuclei, apparent nuclear vacuolization, and missing nuclei.

Unstained platelets and plaques were present on the surfaces of the specimens from the experimental animals only. Stained and unstained red blood cells were also dispersed over the luminal surfaces of the endotoxin-treated vessels.

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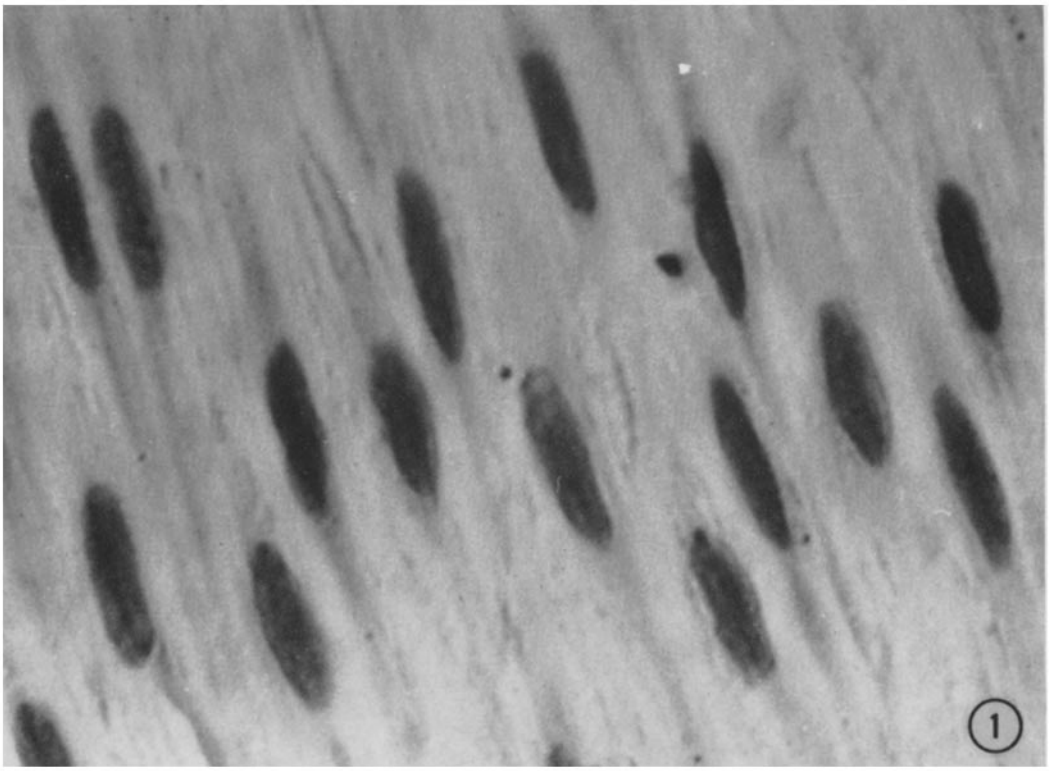
FIG. 1. Sheet of endothelial cells from a control animal. The nuclei are dark, smooth ovals irregularly spaced over a lightly textured background. Cell outlines are indistinct. × 3250.

FIG. 2. Sheet of endothelial cells from a control animal at higher magnification. The dark nuclear material is surrounded by a membrane like structure which stains less intensely. The light, longitudinal striations of the background are more evident. × 5500.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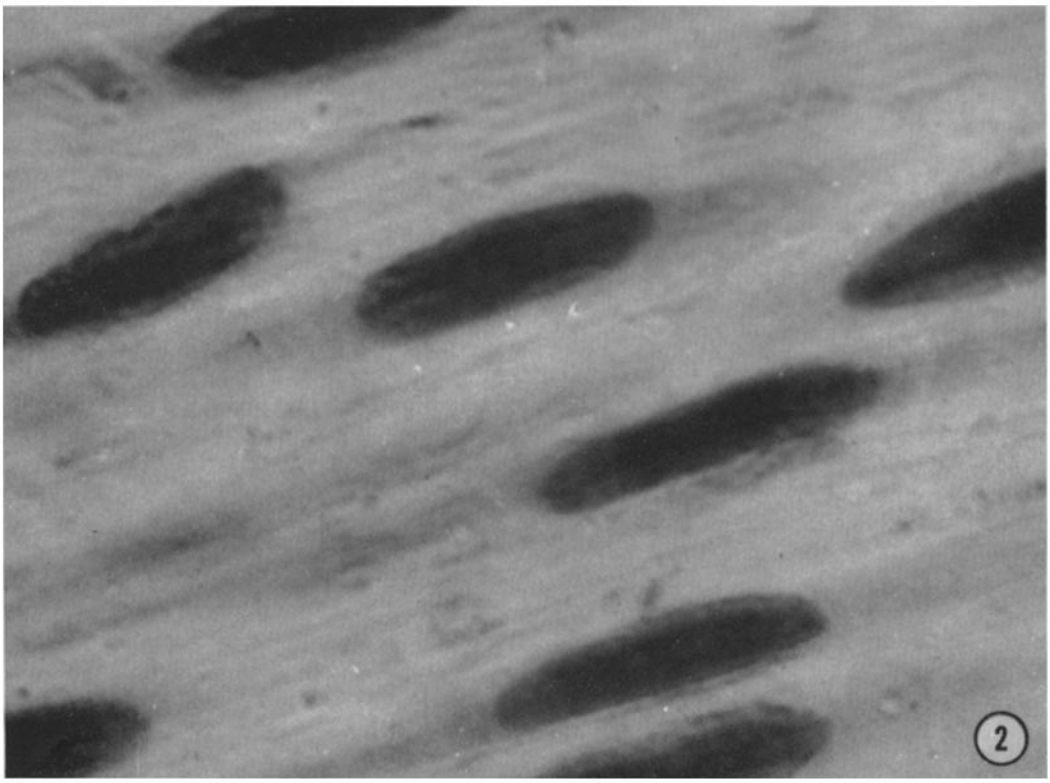
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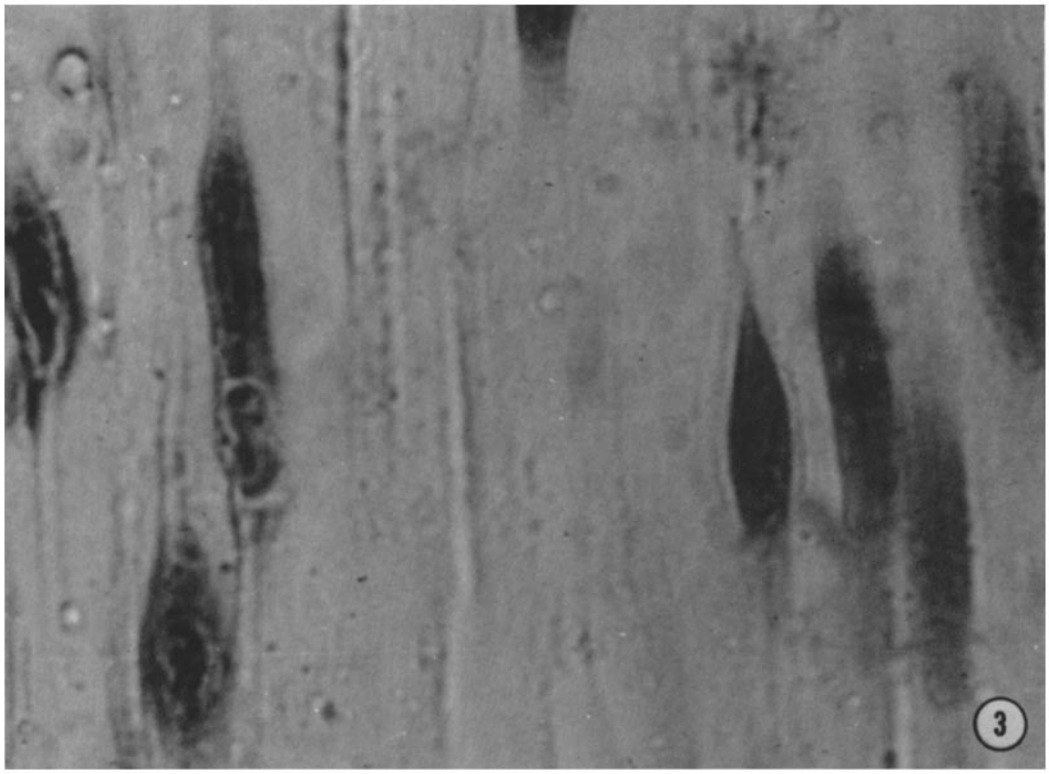
FIG. 3. Sheet of endothelial cells from a rabbit 1 hr after endotoxin injection. The nuclei are withered spindles which stain irregularly. The background has assumed a mottled appearance, and the longitudinal striations have become more prominent and irregular in staining than those of controls. This animal suffered relatively mild endothelial damage. × 5750.

FIG. 4. Sheet of endothelial cells from a rabbit 1 hr after endotoxin injection. This specimen shows extensive damage in the form of extreme nuclear distortion and vacuolization. A large number of small, dense structures surrounded by a light zone adhere to the background. These may be white cells with stained nuclei surrounded by unstained cytoplasm. × 7500.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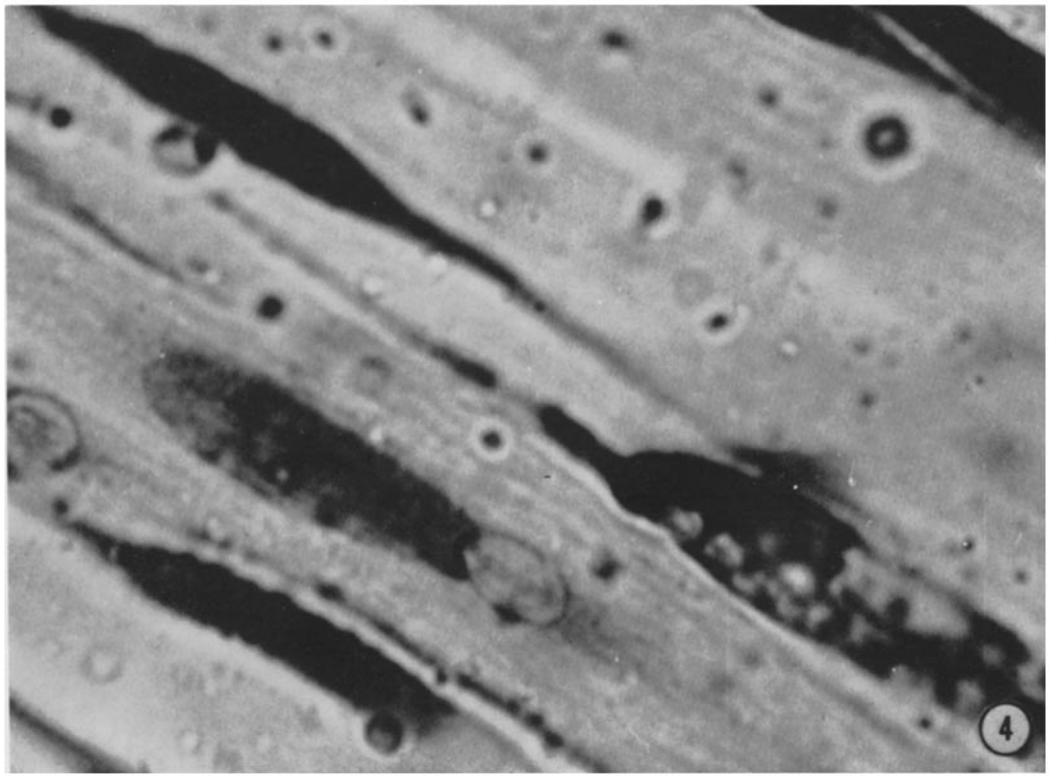
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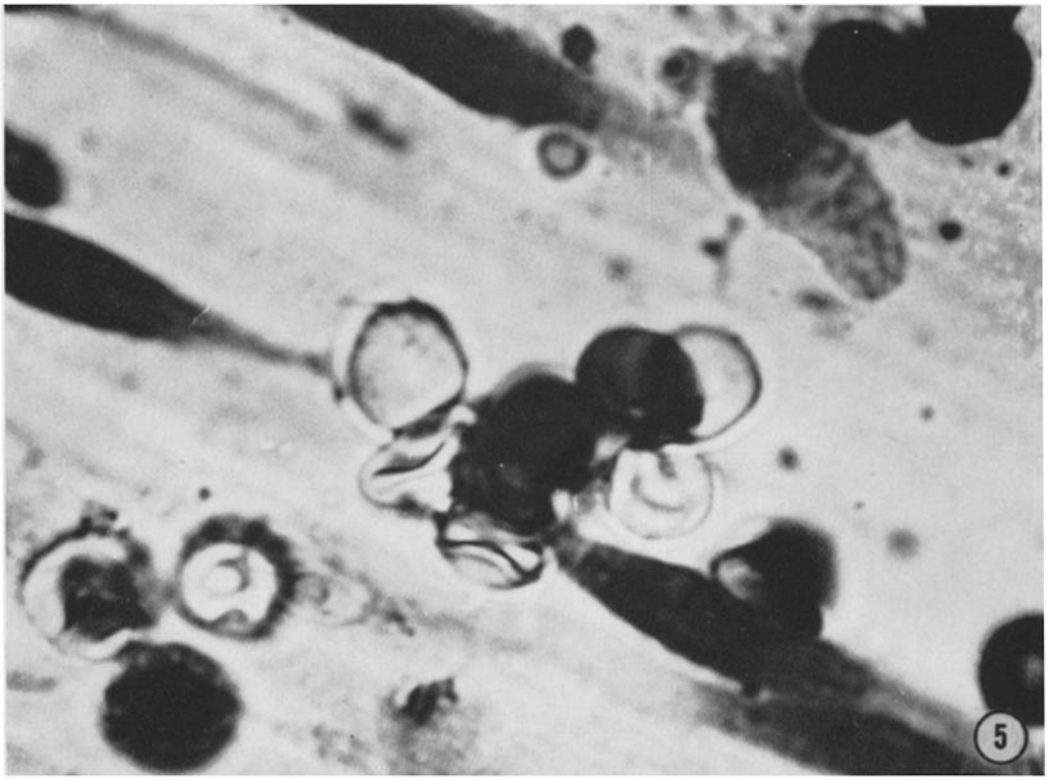
FIG. 5. Sheet of endothelial cells from a rabbit 1 hr after endotoxin injection. In addition to the features described in the two other specimens, this specimen exhibits aggregates of red blood cells in which stained and unstained cells lie in close apposition. × 6000.

FIG. 6. Sheet of endothelial cells from a rabbit 24 hr after endotoxin injection. Few recognizable remnants of nuclei remain while longitudinal striation has become pronounced. A few red cells adhere to the surface. × 5500.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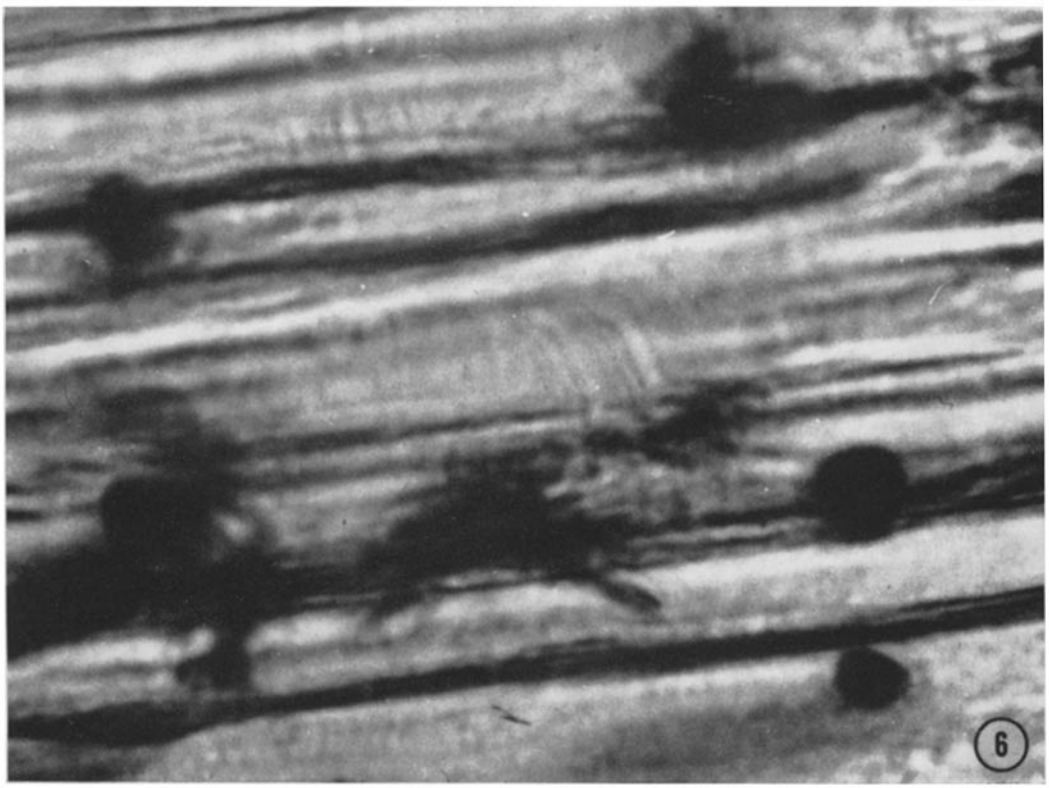
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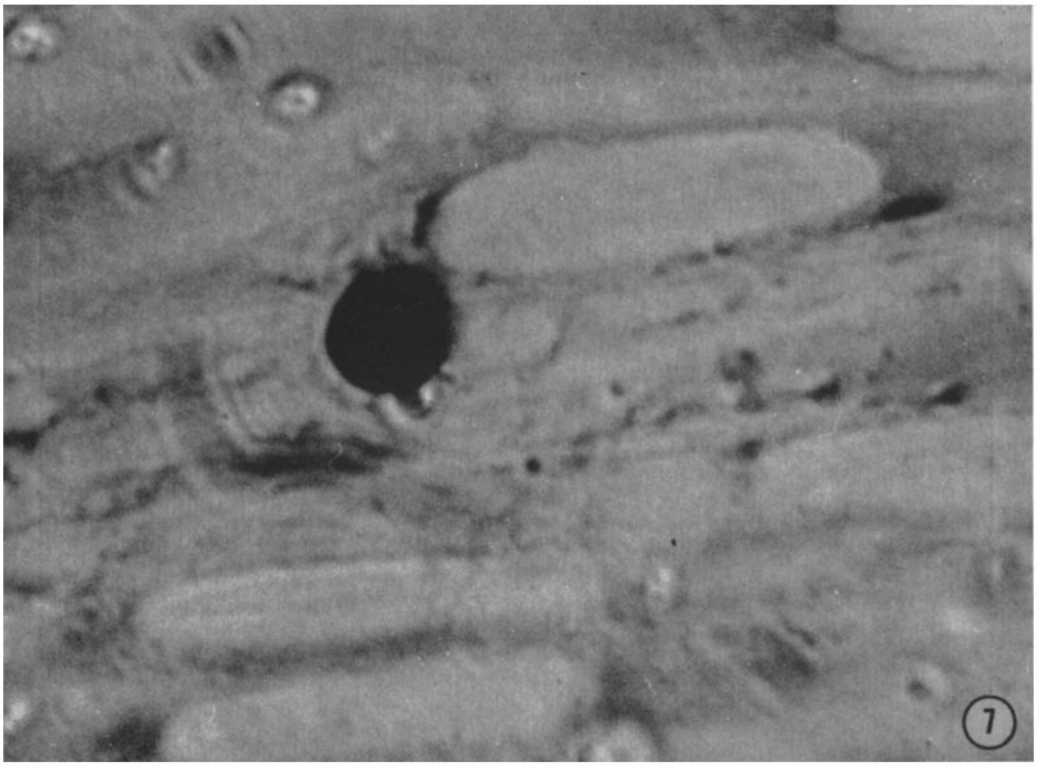
FIG. 7. Specimen from another rabbit 24 hr after injection with endotoxin. In this specimen the nuclei in the central area of the print have either been lost from the cell sheet or have undergone a change in staining characteristics. The regions at the upper left and lower right have lost all evidence of cellular structure. × 7500.

FIG. 8. Cell sheet from a third rabbit 24 hr after injection with endotoxin. Strips of underlying tissue adhering to parts of the endothelial cell sheet impart a dense appearance to these areas. The most prominent feature of this specimen is the longitudinal cords of intermediate density separated by light strips. The density of the cords is probably due to the presence of underlying tissue which adhered to the sheet. Densely staining, ovoid nuclei are all of the structure which remains recognizable. × 5750.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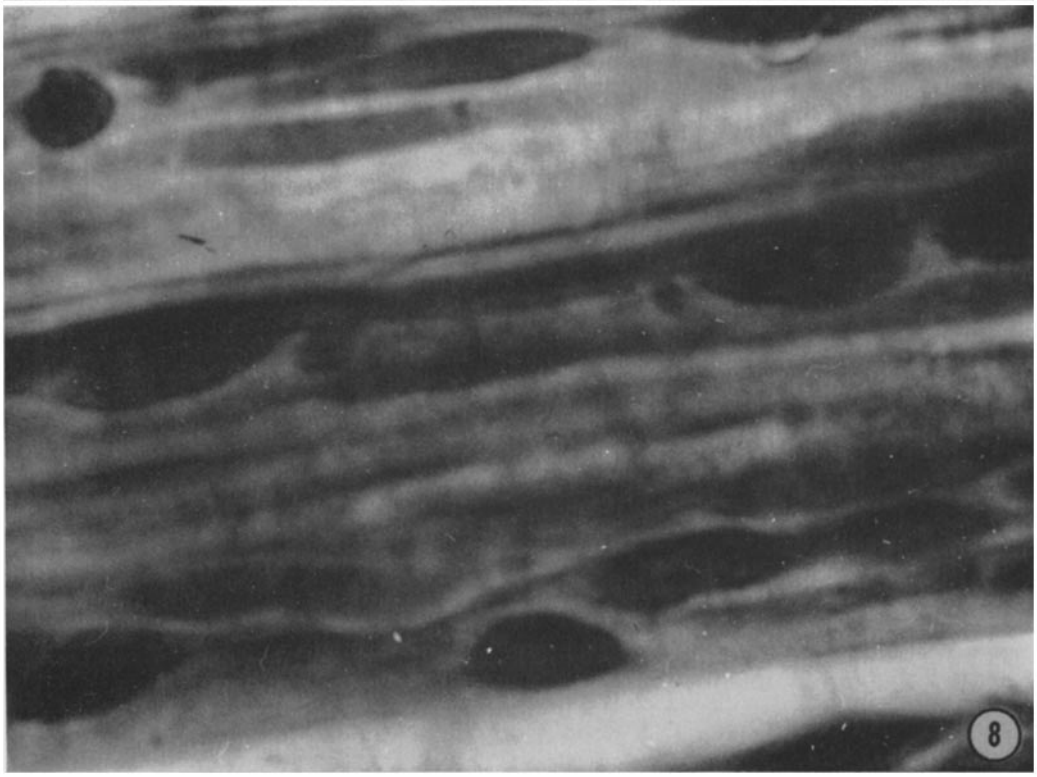
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FIG. 9. Cell sheet from a fourth rabbit 24 hr after endotoxin injection. Plaques resembling platelet thrombi are dispersed over the endothelial surface, as are a number of stained red blood cells. No recognizable endothelial cell structure remains. × 6500.