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21. Supported by NASA grants NGR-10-007-008 and NAS 9-8101. Contribution No. 166 of the Institute of Molecular Evolution. Production of amino acids under the conditions described here was first reported in the symposium on the Total Synthesis of Food at the annual meeting of the American Association for the Advancement of Science, Boston, 26 December 1969.

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Vascular Lesions: Possible Pathogenetic Basis of the Generalized Shwartzman Reaction

Abstract. *Evidence of vascular injury was found in rabbits after a single small dose of endotoxin from* Escherichia coli. *Eighty percent of the treated animals developed circulating endothelial cells, leukopenia, and thrombocytopenia, and 50 percent had aortic endothelial lesions as determined by electron microscopy. Prior anticoagulation with heparin did not prevent this response. No control animals showed these abnormalities.*

The generalized Shwartzman reaction has long been thought to result from the activation of blood coagulation factors, with consequent fibrin formation (*1*). The lesions have been considered essentially embolic in nature, with the trapping of fibrin and platelets in small vessels. As an alternate hypothesis it is proposed that the generalized Shwartzman reaction is primarily a vascular reaction, with in situ thrombi forming at areas of endothelial cell detachment and exposed basement membrane (*2*). Such multiple localized areas of endothelial desquamation could account for the widespread consumption of platelets and labile clotting factors, which would lead to their depletion in the generalized Shwartzman reaction. The studies to be described provide support for the above hypothesis by demonstrating the presence of circulating endothelial cells in the blood of rabbits injected with endotoxin (*3*), and the presence of corresponding endothelial lesions in major vessels of these animals. These abnormalities were not prevented by concurrent anticoagulation with heparin.

Adult male New Zealand rabbits (2.5 to 4.0 kg) were used. Each rabbit in group A was given a single intravenous dose of *Escherichia coli* 0127:B8 endotoxin (Difco; 40 to 120 μg/kg) which was suspended in pyrogen-free isotonic saline, at a concentration of 40 μg/ml. Group B was similarly treated, but each rabbit was given 1.0 to 1.5 ml of intravenous sodium heparin (Liquaemin Sodium "10," Organon, Inc.; 1000 U.S.P. units per milliliter) immediately before the endotoxin. Rabbits in group C received sterile saline solution in volumes equivalent to those used in group A, and group D rabbits were given both heparin and saline.

The rabbits were anesthetized with intravenous sodium pentobarbital supplemented with ether. Blood was collected by free flow from the aorta. This maneuver was designed to preclude aspiration of endothelium at the tip of the needle. The first 3 to 5 ml of blood was discarded to remove any endothelial cells obtained from the vessel wall at the site of puncture. The optimum time for collection of specimens for circulating endothelium was 30 to 60 minutes after injection of endotoxin.

Blood treated with sodium ethylenediaminetetraacetic acid to inhibit coagulation was examined for total leukocyte count with a Coulter-S counter. Mean normal values were 8300 cell/mm³ in 33 rabbits. Platelets counted by the method of Brecher-Cronkite (*4*) had a mean value of 321,000/mm³ in 57 rabbits. Coagulation times for whole blood in glass were determined as described by Lee and White (*5*).

The presence of circulating endothelium was determined by light and electron microscopy (*6*, *7*). Briefly, 5 ml of blood was collected into 20 ml of a buffered, citrated solution containing approximately 1 percent formaldehyde (*6*). Red blood cells were hemolyzed with a 1 percent saponin solution, and the nucleated cells were collected by centrifugation for 10 minutes at 340*g*. For light microscopy, small portions of the "leukoconcentrate" (that is, the elements remaining after saponization) were smeared on glass slides and stained with Wright's solution and then by Giemsa stain. Endothelial cells were selected for electron microscopic examination as described (*7*). Leukoconcentrates were suspended in a few drops of buffer and were immediately fixed in a large volume of phosphate-buffered glutaraldehyde. During osmium fixation, dehydration, and

Table 1. Changes in mean maximum count of white blood cells (WBC) and platelets, changes in aortic endothelium as determined by electron microscopy, and presence of endothelial cells in the circulating blood of rabbits. Group A was treated with *E. coli* endotoxin; group B with endotoxin and heparin; group C with saline solution; and group D with saline and heparin. Data were analyzed by the Wilcoxon Rank Sum Test (*19*) for differences between the means. Grading the condition of aortic endothelium was as follows: −, negative; ±, equivocal; and +, positive.

Group	WBC		Platelets		Animals with aortic endothelial lesions			Animals with circulating endothelium	
	Animals (No.)	Change (%)	Animals (No.)	Change (%)	− (No.)	± (No.)	+ (No.)	− (No.)	+ (No.)
A	16	−57*	24	−44*	3	0	6	8	30
B	15	−40*	26	−34	0	3	2	4	19
C	12	− 4	12	+ 1	5	4	0	13	0
D	3	0	4	+ 2	1	0	0	4	0

* Difference between mean count prior to and after treatment is significant ($P < .005$).

embedding the cells were kept in suspension and were finally polymerized in a thin sheet of Epon in the shape of a slide. Endothelial cells identified by light microscopy in these slides were then excised and mounted on Epon blocks.

Grading of animals for "endothelemia" was performed on the Wright-Giemsa smears which were evaluated "blind," but the smears from animals treated with endotoxin were easily recognized by the paucity of neutrophils. The results were considered negative if two or fewer endothelial cells were present in two slides, and positive if there were more than five endothelial cells from a given animal. Blood pressures were determined in a cannulated femoral artery with a Statham P23D strain gauge, and were recorded on an "Electronics for Medicine" 8-channel recorder.

Rabbit aortas were fixed for electron microscopy by perfusion with 5 percent glutaraldehyde in 0.067*M* phosphate buffer in situ at 20°C for 20 minutes. The unmanipulated segment of aorta was removed to fresh fixative, cut into segments approximately 1 mm^3, and further fixed for 1 to 2 hours. After fixation in 1 percent osmium tetroxide in 0.067*M* phosphate buffer for 1 to 2 hours at 4°C, the unsectioned tissue was incubated in saturated uranyl acetate in 50 percent ethanol (*8*), serially dehydrated, and embedded in Epon (*9*). Ultrathin sections were cut on a Sorvall MT-2 ultramicrotome, stained with uranyl acetate and then with lead citrate (*10*), and examined in a Siemen's 1A electron microscope.

After injection with endotoxin alone (group A), or heparin and endotoxin (group B), there were significant decreases in the mean leukocyte and platelet counts (Table 1). Platelet counts fell almost immediately on injection of endotoxin, reached a nadir after 5 to 10 minutes, then rose to 50 to 60 percent of values prior to treatment between 30 and 60 minutes, where they remained for several hours. Leukopenia, primarily of granulocytes, also began immediately, but the counts decreased more gradually for approximately 30 to 60 minutes, and persisted at about 50 percent of values prior to treatment for more than 3 hours. No significant changes were noted in these tests for groups C and D rabbits. Clotting times for whole blood in all of the heparinized rabbits were greater than 1 hour, whereas for group A rabbits the clotting times were 2.5 to 9 minutes.

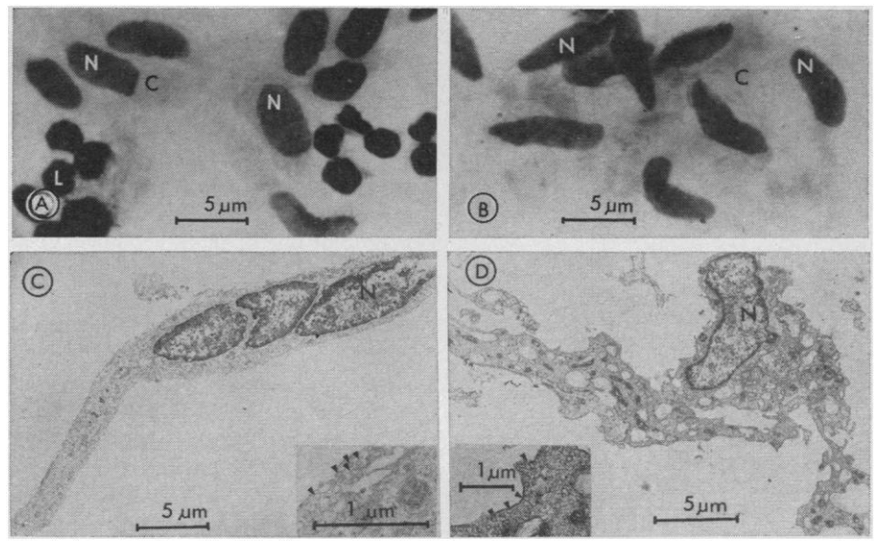

Fig. 1. (A) and (B) Light micrographs. (C) and (D) Electron micrographs. (A) and (C) Circulating endothelial cells in the blood of an endotoxin-treated rabbit. (B) and (D) Endothelial cells scraped from a normal rabbit aorta. Nuclei (*N*), cytoplasm (*C*), and lymphocytes (*L*). Pinocytotic vesicles (arrowheads) are apparent in both specimens (insets).

Of the animals treated with endotoxin (group A), 80 percent were positive for cells resembling those identified by Herbeuval (*6*) as circulating endothelium. Prior treatment with heparin did not significantly alter the ability of endotoxin to induce this phenomenon, since 82 percent of group B rabbits were positive as well (Table 1). In most animals treated with endotoxin, individual endothelial cells, from dozens to hundreds in number, were found. In addition, numerous clumps of endothelial cells were frequently seen (Fig. 1A). In some rabbits, these cells appeared in the blood at least as early as 5 minutes after injection of endotoxin, but in the majority of animals they were most readily found at 30 to 60 minutes after injection. It was not possible to evaluate quantitative or temporal changes in this test because of wide variations in the degree of response of individual rabbits and because of the large volume of blood necessary for repeated tests. Thus, it was not possible to correlate the presence of these cells with changes in leukocyte or platelet counts. No animals in groups C and D were positive for circulating endothelium.

Leukoconcentrates from four rabbits with circulating endothelium, as indicated by light microscopy, were also prepared for electron microscopic study. The morphology of such cells in circulation was compared with that of endothelium which was gently scraped from the aorta of a normal rabbit and added to its blood, and which was then processed identically. Figure 1B is a light micrograph of such endothelial scrapings. Comparison with Fig. 1A demonstrates that the elongated configuration, wrinkled nucleus and fine chromatin pattern, and nucleolar morphology are identical in both the circulating cells and the aortic scrapings. These similarities are confirmed in electron micrographs (Fig. 1, C and D). Both the circulating cells (Fig. 1C) and the scrapings (Fig. 1D) also show the numerous pinocytotic vesicles that characterize endothelial cells. Although the cells are poorly preserved, they clearly are not normal circulating blood elements. The cytolysis and vacuolization seen is presumed to be due to the trauma of mechanical injury or to the effect of endotoxin, as well as to artifacts induced by preparation (*7*).

To rule out the possibility that endotoxic "shock," with hypotension and attendant anoxia had not induced the endothelial lesions necessary to produce "endothelemia," intra-arterial blood pressures were continuously measured in two group A rabbits. Blood samples obtained 30 minutes after injection of endotoxin revealed the typical leukopenia, thrombocytopenia, and circulating endothelium of the group. During the experiment, systolic blood pressure varied from 15 mm-Hg below to 10 mm-Hg above baseline pressure of 120 mm-Hg. Fluctuations seemed to be more related to depth of anesthesia than to endotoxin administration. Two addi-

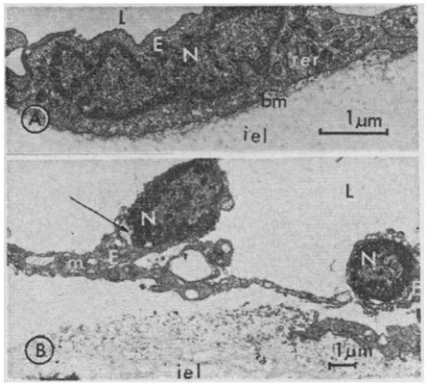

Fig. 2. Electron micrographs of aortas of rabbits treated with endotoxin. (A) Normal endothelial cells (*E*) with prominent rough endoplasmic reticulum (*rer*). Plasma membrane is in close contact with basement membrane (*bm*). Internal elastic lamina (*iel*) underlies basement membrane. (B) Endothelial cells completely detached from underlying structures. Nuclear chromatin (*N*) is condensed, and cytoplasm shows vacuoles, lysis (arrow), and mitochondrial swelling (*m*); *L*, lumen of aorta.

tional rabbits were rendered hypotensive by phlebotomy, their systolic blood pressures being lowered to 50 mm-Hg. As pressures returned toward baseline, repeated smaller phlebotomies were performed to maintain the pressure below 80 mm-Hg. Blood samples obtained after 30 minutes of hypotension were negative for circulating endothelium and showed a slight increase in count of white blood cells and platelets over values prior to treatment.

Three randomly selected areas from each of 24 rabbit aortas were examined for endothelial lesions by electron microscopy 30 to 60 minutes after injection with the experimental or control reagents. Specimens were reviewed by a double-blind method, and endothelial abnormalities were graded as follows: Intact, normally appearing endothelium was graded 0 (Fig. 2A). Specimens with cells showing prominent and dilated rough endoplasmic reticulum and a slight amount of subendothelial edema were graded ±. Since damage of this degree was seen in control vessels occasionally, and since it might be an artifact of perfusion, it was considered insignificant. Specimens with separation of the cells from their basement membranes and occasional small lytic areas in the cytoplasm were graded +. More severe abnormalities consisted of larger lytic areas in the cytoplasm or the presence of large vesicles bounded by a double unit membrane and filled with amorphous material. Such vesicles have been described in ischemic and heat-injured vessels (*11*) and are thought to be due to invagination of a swollen injured endothelial cell into an adjacent, more intact cell. The most severely injured vessels showed desquamation of one or more endothelial cells (Fig. 2B). Two-thirds of group A, and 40 percent of group B rabbits had severe endothelial abnormalities, whereas no animals in groups C and D showed significant damage (Table 1). Three rabbits in group A had aortic endothelium that was apparently normal. Since they all had demonstrated circulating endothelial cells, the lack of intimal abnormality in these aortas may reflect a sampling error related either to random selection of areas for electron microscopic study or to the small numbers of blocks examined. Significantly, the lesions were seen in major vessels that were not likely to be sites of embolic deposits.

The above data are derived from studies with single doses of endotoxin, but it is reasonable to suppose that the lesions would be extended in the generalized Shwartzman reaction seen after the second dose—which produces similar although more severe changes in blood and tissue (*12*). Accordingly, the following hypothesis may be constructed: In the generalized Shwartzman reaction endotoxin is directly toxic to vascular endothelium, or exerts its effect through leukocytes (*13*), perhaps by eliciting liberation of lysosomal enzymes. The consequent damage to endothelial cells leads to detachment of these cells, which then circulate in blood. Basement membrane is exposed and platelet accretions form at such thrombogenic sites (*14*). In the otherwise untreated animal there is additional initiation of clotting and deposition of fibrin; in animals in which coagulation was inhibited with heparin the reaction is limited to platelet accumulation. A possible explanation for the 24-hour interval required for the generalized Shwartzman reaction is that endotoxin requires plasma cofactors to produce the vascular lesion, and these are transiently depleted after the first dose. On the other hand, after injury, replacement of endothelial cells begins only after a 24-hour delay (*15*). Thus, maximum damage might occur only in the presence of adequate cofactors and prior to significant relining of vessels.

Although studies have been reported in conformity with the present hypothesis, a vascular basis for the generalized Shwartzman reaction had evidently not been emphasized. Rubenstein *et al.* (*16*) noted vascular localization of endotoxin injected into dogs, and McGrath and Stewart (*17*) reported the development of vascular lesions after massive intracardiac administration of endotoxin in rabbits. Evans and Mustard (*18*) showed that the generalized Shwartzman reaction could be suppressed by salicylates in doses which inhibited platelet aggregation without affecting clotting. On the other hand, no convincing data are available to account for the degree of coagulant activity which would be required to produce the generalized Shwartzman reaction on an embolic basis secondary to fibrin formation in flowing blood.

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