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Study of chemical sympathectomy in endotoxin-induced lethality and fibrin deposition

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Study of chemical sympathectomy in endotoxin-induced lethality and fibrin deposition. Shock and the generalized Shwartzman reaction are well known features of endotoxin which have been shown to involve the sympathetic nervous system. The mechanism of sympathetic nervous system involvement with endotoxin injection was studied in rabbits chemically sympathectomized with 6-hydroxydopamine. Endotoxin, in doses producing a spectrum of morbidity and mortality in normal rabbits, was administered i.v. to chemically sympathectomized, normal, and unilateral renal surgically sympathectomized animals. Chemical sympathectomy produced a significant depletion of tissue norepinephrine which, in endotoxin recipient animals, was associated with a significantly lower mortality rate and greatly decreased fibrin deposition in the lungs and kidneys, despite intravascular coagulation. Unilateral renal sympathectomy afforded protection to the ipsilateral kidney, but data on mortality and systemic fibrin deposition were similar to those reported for normal rabbits given endotoxin. Six-hydroxydopamine prevents significant tissue injury secondary to endotoxin in this experimental model. In addition, the data provide direct evidence that an intact reactive sympathetic nervous system is essential for development of lethal toxicity and generalized Shwartzman reaction due to endotoxin.

Etude de la sympathectomie chimique de la lethalité et des dépôts de fibrine chez animaux qui ont reçu l'endotoxine. Le choc et le phénomène de Schwartzman généralisé sont des conséquences bien connues de l'endotoxine et il a été montré que ces réactions impliquent le systéme nerveux sympathique. Le mécanisme de la mise en jeu du système nerveux sympathique par l'injection d'endotoxine a été étudié chez des lapins ayant subi une sympathectomie chimique par la 6-hydroxydopamine. L'endotoxine, aux doses qui déterminent une morbidité et une mortalité chez les lapins normaux, a été administrée par voie intraveineuse à des animaux normaux, ayant subi une sympathectomie chimique ou ayant subi une sympathectomie rénale, chirurgicale et unilatérale. La sympathectomie chimique a produit une déplétion significative de norépinéphrine tissulaire qui, chez les animaux qui ont reçu l'endotoxine, a été associée à une diminution significative de la mortalité et une diminution importante des dépôts de fibrine dans les poumons et les reins malgré la coagulation intravasculaire. La sympathectomie unilatérale a protégé le rein ipsilatéral mais la mortalité et les dépôts systémiques de fibrine ont été semblables à ceux observés chez les lapins normaux qui ont reçu l'endotoxine. La 6-hydroxydopamine diminue de façon significative les altérations tissulaires secondaires à l'endotoxine dans ce modèle expérimental. De surcroît, ces observations apportent la preuve directe de ce qu'un système nerveux sympathique intact et réactif est essentiel pour l'apparition de la léthalité et du phénomène de Schwartzman généralisé consécutifs à l'endotoxine.

The coagulation system appears to be intimately involved in the pathogenesis of many clinical and experimental renal lesions [1, 2]. The kidney may be involved primarily, with secondary insult from the coagulation system, or it may be affected as a result of generalized intravascular coagulation. The most reproducible and well-studied example of diffuse intravascular coagulation with consequent renal damage is that induced by the i.v. injection of endotoxin in an animal model [3–6]. Endotoxin injection results in the release of catecholamines and other vasoactive substances and may produce intravascular coagulation with widespread deposits of fibrin in various organs [7–14]. Large quantities of endotoxin produce shock and death, while sublethal amounts prepare the animal so that a second injection of endotoxin results in the generalized Shwartzman reaction, characterized by renal cortical necrosis [3, 4]. Despite extensive studies of this model, the sequence of events leading to the fibrin deposition is not well-understood. Catecholamines and the sympathetic nervous system have been strongly implicated in this regard, as evidenced by protection from the generalized Shwartzman reaction afforded locally to one kidney by unilateral renal sympathectomy and generally by alpha-adrenergic blockade [15–17]. Generalized sympathectomy, performed immunologically or surgically, however, has not been feasible in the adult experimental animal [18–20], and adrenergic blockers possess numerous pharmacological properties other than adrenergic blocking ability that could contribute to their protective

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effects [21]. A simple and effective technique producing specific adrenergic nerve terminal destruction by the administration of 6-hydroxydopamine hydrobromide (6-OHDA) ($C_8H_{11}NO_3 \cdot HBr$, Regis Chemical Co., Morton Grove, Ill.) has recently been introduced [18, 22]. We have employed 6-OHDA in studying the sympathetic nervous system's role in the pathogenesis of endotoxin shock and the generalized Shwartzman reaction in rabbits. This paper describes the significant protection provided by chemical sympathectomy, not only from the lethal effects of endotoxin, but from fibrin deposition in the lungs and kidneys as well.

Methods

Induction of endotoxin lethal toxicity and the generalized Shwartzman reaction. Male New Zealand white rabbits weighing 1.5 to 2.0 kg were used in these studies. The animals were not treated with antibiotics and were housed under standard conditions in the University of Virginia Vivarium. Lipopolysaccharide from *Escherichia coli* (055-B5, Difco Laboratories, Detroit, Mich.) was used for all studies. The endotoxin was freshly prepared in sterile saline for each injection. An initial i.v. injection of 0.4 mg was given slowly and was followed 18 hr later by a 0.2-mg i.v. injection. This regimen resulted in the death of approximately 30% of the animals within 18 hr of the first injection; an additional 30% died by 24 hr after the second injection. Rabbits surviving 24 hr after the second dose were killed at that time.

Sympathectomy: a) Chemical sympathectomy. 6-OHDA was stored under nitrogen in the dark at −50°C and was prepared for injection by using a modification of the technique of Thoenen, Tranzer, and Hausler [22]. The 6-OHDA was dissolved in 0.001 N hydrochloric acid, 1% ascorbic acid, kept on ice at all times, and used immediately. The first i.v. injection consisted of 50 mg/kg of body wt of 6-OHDA and was followed in 20 hr by another i.v. injection of 50 mg/kg. One week later, the rabbits received a second double course of 6-OHDA injections at doses of 100 mg/kg. The animals were entered into the study either as controls or as endotoxin recipients.

b) Unilateral renal surgical sympathectomy. Rabbits were anesthetized by i.v. injection of acetylpromazine maleate and pentobarbital until light narcosis ensued. The left kidney was approached by a flank incision, and the renal pedicle was dissected free. The renal vessels were isolated and carefully stripped of the nerves. A minority of animals (2/8) had 5% phenol [23] applied to the vessel walls after stripping, but this procedure produced no significant further depletion of tissue norepinephrine levels. The rabbits were allowed to recover for 10 days and were then entered into the study as endotoxin recipients ($N = 4$) or as controls ($N = 4$).

Tissue catecholamine determinations. Heart, lung, and kidney were sealed in plastic bags at the time of sacrifice or within two hours of spontaneous death and were frozen at −50°C until analyzed. This latter period of time did not significantly alter tissue catecholamine levels. For analysis of norepinephrine and epinephrine, the organs were thawed, washed in saline, weighed, minced, and then homogenized in cold 5% trichloroacetic acid. The homogenate was filtered; and the filtrate, pH 8.3, was adsorbed on aluminum oxide columns, washed twice with deionized water, and subsequently eluted with 0.25 N acetic acid [24]. Following elution, the differential analysis of norepinephrine and epinephrine was performed fluorometrically by the automated trihydroxyindole method of Robinson and Watts [25], using the Technicon system (Technicon Instruments Corp., Tarrytown, N.Y.).

Tissue levels are expressed as μg of norepinephrine or epinephrine per g of tissue weight. Recovery rates in our laboratory are 75 to 80%. Values reported here are not corrected for recovery rate.

Coagulation studies. Heparin-precipitable fibrin products have been shown to result from the i.v. injection of endotoxin in rabbits. They are considered to be fibrin products released during intravascular coagulation and precipitated in the cold by heparin; they are found only in small quantities in 15% of normal animals [26]. Five-milliliter specimens of whole blood were drawn into heparinized glass syringes to a final concentration of 0.1 mg of heparin/ml of blood. Samples were taken three hours after the first injection of endotoxin. Heparin-precipitable fibrin products were determined by placing one aliquot of plasma at 4°C and leaving another aliquot at 37°C. The appearance of flocculation after chilling was considered to constitute a positive test. Other groups of normal and 6-OHDA sympathectomized rabbits were tested serially for fibrin degradation products by using the staphylococcal clumping assay [27]. The quantity in μg/ml of plasma was determined using a standard curve of fibrinogen.

Immunohistology. Tissue from the lungs and kidneys, obtained at spontaneous death or sacrifice, was snap frozen in dry-ice isopentane, cut at four microns, and examined with monospecific fluorescein-labeled anti-rabbit IgG, C-3, fibrinogen, and alpha-2-macroglobulin (Cappel Laboratories, Inc., Dowington, Pa.) [28]. Sections were graded 0 to 4+ on the basis of the quantity and staining intensity of deposits and without knowledge of the animals' experimental group.

Analysis of data. Immunofluorescence scores were accumulated for each study group and presented as the mean ± SEM. Tissue concentrations of norepinephrine and epinephrine were similarly prepared. The results of five separate experiments in native and manipulated animals were combined for analysis by Student's t and χ^2 tests.

Results

Tissue catecholamine levels. Injection of 6-OHDA significantly reduced tissue concentrations of norepinephrine (Table 1). This reduction was most marked in heart and kidney, although a significant reduction occurred as well in lung. Injection of endotoxin alone also produced a significant reduction in tissue norepinephrine, but not to the same degree as that induced by 6-OHDA alone. When 6-OHDA-prepared rabbits received endotoxin, however, further significant depletion of tissue norepinephrine occurred, with the greatest change taking place in lung. Tissue levels of norepinephrine in the unilaterally sympathectomized kidney were reduced 93% ($P < 0.001$), from 0.12 ± 0.01 μg/g for the contralateral kidney to 0.008 ± 0.001 μg/g for the ipsilateral kidney in the four animals that received endotoxin. Tissue norepinephrine content in heart and lung in those surgically sympathectomized animals given endotoxin did not differ from that of normal animals given endotoxin. Analysis of the tissue norepinephrine levels in non-sympathectomized animals dying after one or two doses of endotoxin or sacrificed 24 hr after the challenge dose of endotoxin revealed no significant differences in lung or kidney at any of the three time intervals. On the other hand, there were significant differences in cardiac tissue concentrations of norepinephrine at each of these three time intervals (Fig. 1). Severe

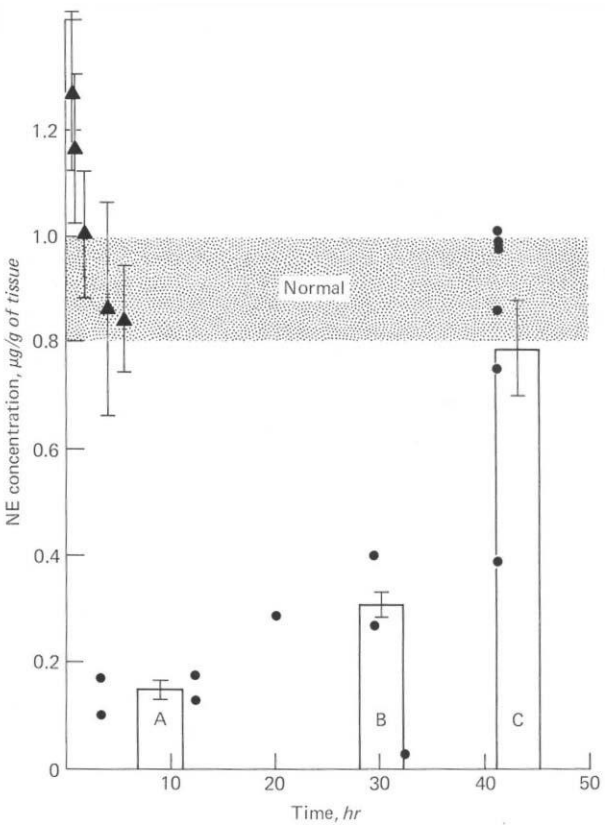


Fig. 1. *Relationship between heart norepinephrine (NE) content and spontaneous death or sacrifice in normal animals (●) and rabbits sacrificed electively (▲) after endotoxin.* Four animals were sacrificed for each time period represented by the solid triangle (▲).) *Bar A* represents animals that died after one dose of endotoxin (0.145 ± 0.018 μg/g, compared to controls, $P < 0.01$, and compared to the other two groups in the figure, $P < 0.01$), *Bar B* represents animals that died after two doses of endotoxin (0.300 ± 0.036 μg/g, compared to controls, $P < 0.01$, and compared to the other two groups in the figure $P < 0.01$). *Bar C* represents animals sacrificed 24 hr after second dose of endotoxin (0.787 ± 0.109 μg/g, compared to the other two groups in the figure, $P < 0.01$). Endotoxin was administered at 0 and 18 hours.

Table 1. Tissue catecholamine concentrations (*μg/g of tissue weight*)

Groups	No. of animals	Heart	Kidney	Lung
		Norepinephrine		
Normal control	11	0.914 ± 0.055	0.142 ± 0.010	0.027 ± 0.004
6-hydroxydopamine control	7	0.108 ± 0.012[a,b]	0.022 ± 0.004[a,b]	0.013 ± 0.005[c]
Endotoxin	21	0.528 ± 0.078[a]	0.093 ± 0.012[c]	0.016 ± 0.002[a]
6-hydroxydopamine-endotoxin	20	0.079 ± 0.010[a,b]	0.014 ± 0.001[a,b,d]	0.001 ± 0.001[a,b,d]
		Epinephrine		
Normal control	11	0.0248 ± 0.0086	0.0051 ± 0.0018	0.0016 ± 0.0005
6-hydroxydopamine	7	0.0021 ± 0.0012	0.0012 ± 0.0006	0.0069 ± 0.0033[b]
Endotoxin	21	0.0190 ± 0.0061	0.0106 ± 0.0023[e]	0.001 ± 0.001
6-hydroxydopamine-endotoxin	20	0.0034 ± 0.0006[a,f]	0.0046 ± 0.0021	0.0028 ± 0.0006[f]

[a] $P < 0.005$ vs. normal.
[b] $P < 0.005$ vs. endotoxin.
[c] $P < 0.05$ vs. normal.
[d] $P < 0.025$ vs. 6-hydroxydopamine.
[e] $P < 0.05$ vs. 6-hydroxydopamine.
[f] $P < 0.025$ vs. endotoxin.

depletion was found in animals dying as early as three hours after the first endotoxin injection. Tissue levels in a group of animals randomly sacrificed during the first six hours after endotoxin injection, however, did not differ from control levels (Fig. 1, triangle). Cardiac tissue norepinephrine content after endotoxin injection was progressively greater over time, and it was nearly normal in animals sacrificed at the end of the investigation period. Tissue concentrations of norepinephrine in 6-OHDA-endotoxin animals dying spontaneously were not different from concentrations in animals sacrificed at the study's termination.

While tissue norepinephrine concentrations decreased markedly after 6-OHDA, endotoxin, or 6-OHDA-endotoxin administration, only minor, insignificant changes in tissue epinephrine content were generally noted (Table 1), and the range of epinephrine concentrations within each type of organ was quite variable. Only the combination 6-OHDA-endotoxin produced a significant decrease in heart and lung epinephrine concentrations.

Fibrin deposition. The injection of endotoxin was followed by intravascular coagulation of a similar degree in both normal animals and those pretreated with 6-OHDA, as indicated by a positive heparin-precipitable fibrin products test and circulating fibrin degradation products (Table 2). Fibrin deposits were present in the interstitium and within vessels of the lungs of non-sympathectomized rabbits (Fig. 2). These deposits occurred in animals dying after either the first or second dose of endotoxin and were noted as well in animals sacrificed 24 hr after the second injection. Fibrin deposits in the kidneys after the first endotoxin dose were localized to glomerular capillary lumens with small amounts in peritubular capillaries (Fig. 3a). Despite the presence of glomerular fibrin and significant amounts of fibrin in the lungs after the first injection, cortical necrosis did not occur. After the second endotoxin dose was given, renal lesions were much more severe (Fig. 3b), with massive amounts of fibrin detected in glomerular capillaries. Involvement of larger vessels within the kidney after the second injection was noted, and cortical necrosis was frequently observed on gross inspection. No deposits of IgG or alpha-2-macroglobulin were observed; C-3 was present as occasional granular mesangial deposits of trace intensity in all animals, including normal and 6-OHDA-pretreated rabbits. In contrast to non-sympathectomized rabbits, chemically sympathectomized animals had either minor fibrin deposits in lung (Fig. 4) and kidney or were negative. In those animals with unilateral renal surgical sympathectomies, pulmonary deposits of fibrin were similar to those found in normal endotoxin-injected rabbits, while a decreased amount of fibrin was present in the sympathectomized kidney relative to the contralateral unmanipulated kidney. Table 3 presents the results of the fibrin-scoring. The results are given for those animals dying or sacrificed after two injections of endotoxin. Again, the propensity for lung to be affected at each stage is illustrated, and renal involvement is shown to increase significantly after the second endotoxin injection.

Endotoxin lethality. Sixty-four percent of normal rabbits died after receiving endotoxin, compared to 25% of chemically sympathectomized rabbits ($P < 0.05$). The first endotoxin injection was fatal to 41% of normal rabbits and 10% of chemically sympathectomized animals; the second injection of endotoxin resulted in death of 38% of the remaining control animals and 11% of the remaining 6-OHDA-pretreated rabbits. The mortality in unilaterally sympathectomized animals—50% at 24 hr after the second injection—was similar to that in controls.

Discussion

Chemical sympathectomy with 6-OHDA did not prevent development of heparin-precipitable fibrinogen products or fibrin degradation products in rabbits given endotoxin; it was associated, however, with significantly reduced fibrin deposition in kidney and lung. Isolated surgical denervation of one kidney also afforded protection from fibrin deposition, but to a lesser degree. In addition, our studies provided further information concerning the mechanism by which sympathetic nervous system-endotoxin inter-

Table 2. Fibrin degradation products (μg/ml of plasma) occurring after i.v. injection of endotoxin (ET)[a]

Groups	Baseline	3 hr after 1st ET	3 hr after 2nd ET	At sacrifice 24 hr after 2nd ET
6-hydroxydopamine-ET	0.7 ± 0.1 (6)	6.5 ± 3.5 (4)	37.2 ± 14.0 (3)	28.5 ± 13.2 (4)
ET[b]	0.4 ± 0.1 (8)	6.8 ± 2.8 (4)	32.0 ± 0 (2) [32.0, 32.0][b]	144 (2) [32.0, 245.8][b]

[a] Parentheses denote the number of animals.
[b] Brackets denote the actual values for individual animals.

action causes tissue damage. Despite the fact that sympathectomy with 6-OHDA has little effect on adrenal stores of catecholamines or their release [18, 22], 6-OHDA largely abrogated tissue injury due to endotoxin. This suggests that intact tissue, rather than increased circulating norepinephrine, plays a primary role in the production of endotoxin-induced organ damage. This hypothesis is supported by observations that, following depletion of tissue norepinephrine by surgical sympathectomy or in the acutely denervated organ in which norepinephrine release from the intact storage granules is prevented [15, 29], endotoxin injections cause only minimal tissue damage. Such observations suggest that norepinephrine release from sympathetic terminals within the organ at the time of endotoxin administration is a necessary prerequisite for development of pathological changes. Further, both tissue norepinephrine concentrations and intact central nervous

Table 3. Immunofluorescence score (0 to 4+)

Group	No. of animals	Kidney	Lung
Endotoxin[a]	22	1.48 ± 0.29	2.00 ± 0.25
Endotoxin[b]	14	2.00 ± 0.20	2.20 ± 0.18
6-hydroxydopamine	7	0	0
6-hydroxydopamine endotoxin[a]	20	0.11 ± 0.06[c]	0.60 ± 0.19[c]
6-hydroxydopamine endotoxin[b]	19	0.12 ± 0.06[d]	0.58 ± 0.20[d]
Unilateral renal sympathectomy	4		
left side (sympathectomy)		0.80 ± 0.07	2.10 ± 0.90
right side (native)		1.20 ± 0.44	

[a]Total rabbits receiving endotoxin.
[b]Rabbits receiving two doses of endotoxin.
[c]$P < 0.001$.
[d]$P < 0.005$.

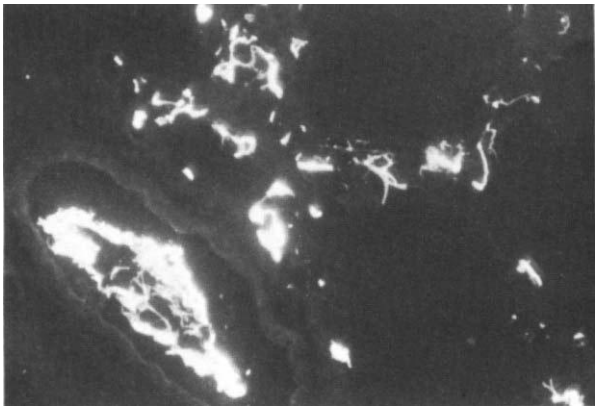
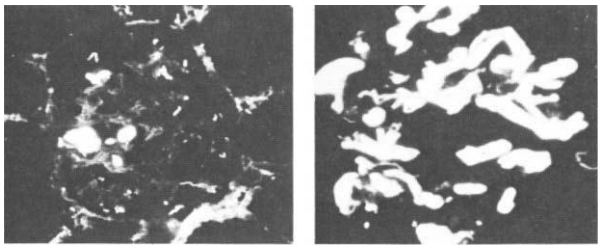
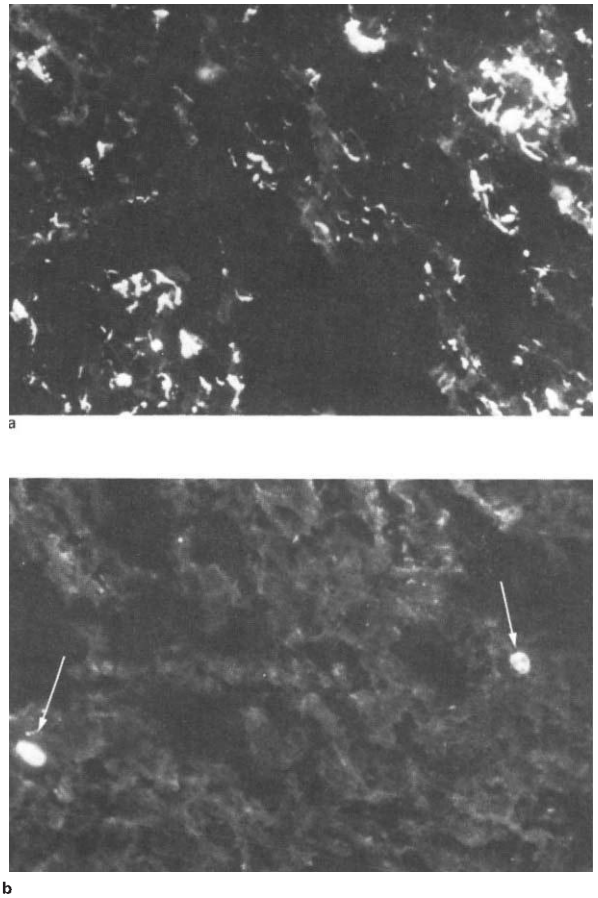


Fig. 2. *Photograph showing pulmonary fibrin deposits within swollen septa with alveolar sparing.* A large vessel is also occluded by fibrin. This is from an animal that died after one dose of endotoxin. (Magnification, × 325.)



a b

Fig. 3. *Left panel (a) is glomerulus stained for rabbit fibrin, illustrating deposits graded as 1+ intensity.* Isolated deposits of fibrin in the glomerular capillary loops and peritubular capillaries are present. This is from an animal that died after one injection of endotoxin. (Magnification, × 585.) *Right panel (b) is rabbit glomerulus stained for fibrin, showing intracapillary deposits of 4+ intensity.* This is from an animal sacrificed 24 hr after the challenge injection of endotoxin. (Magnification, × 585.)



a

b

Fig. 4. *Top panel (a) is section of lung stained for fibrin.* Numerous deposits within vessels and alveolar septa are illustrated. This is from a normal rabbit sacrificed 24 hr after challenge injection of endotoxin. (Magnification, × 325.) *Lower panel (b) is section of lung from a 6-hydroxydopamine chemically sympathectomized rabbit, stained for fibrin and photographed identically to the section in Fig. 4a.* Most fields from this animal were negative, while scant fibrin deposits are shown in the present field *(arrows).* This is from a rabbit sacrificed 24 hr after challenge injection of endotoxin. (Magnification, × 325.)

system connections appear to be important. Indirect evidence for this is suggested by the induction of a systemic-like effect with tissue depletion of norepinephrine when endotoxin is given intracerebrally [30, 31].

The mechanism of the 6-OHDA effect has not been fully clarified. Possibilities include failure of fibrin to localize with prevention of consequent hypoxemia, acidosis, and cardiopulmonary collapse [32–34] or blockade of vasospasm with resultant ischemia and endothelial damage [35]. The latter mechanism is most plausible, because the presence of heparin-precipitable fibrin products and fibrin degradation products indicates that endotoxin activates the coagulation system [11–13, 36]. Other studies indirectly support the concept that 6-OHDA affects protection through blockade of vascular spasm and ischemia. Endotoxin potentiates the action of epinephrine and norepinephrine upon peripheral vessels in rabbits and results in greatly prolonged vasoconstrictor responses in rats [37, 38]. Large doses of endotoxin lead to hyporeactivity of vessels with pooling of blood and induce alterations in organ perfusion, perhaps as a result of vascular hyporeactivity and stasis of blood [39, 40]. The intravenous infusion of epinephrine into dogs leads to severe intravascular coagulation, hemolytic anemia, and hypertension followed by cardiovascular collapse and death [41].

Normally, the physiological effects of catecholamines are terminated by at least two mechanisms: re-uptake of catecholamines into synaptic areas, and enzymatic degradation by 0-methylation and deamination. These processes require oxygen. If these mechanisms are greatly disturbed within adrenergic neurons by ischemia and acidosis [42], the effects of released catecholamines may be further exaggerated. It is also possible that norepinephrine escapes from these ischemic areas, reaches the extracellular space, and causes further vasoconstriction of local blood vessels. Concurrent intravascular serotonin and other vasoactive substances from the platelets trapped within ischemic tissue might be expected to aggravate the vasospasm and ischemic tissue injury and lead to fibrin deposition. Anticoagulation ameliorates the intravascular coagulation and hemolysis, but does not prevent death [43]. Complete protection is provided by prior alpha-adrenergic blockade.

The generalized Shwartzman reaction and endotoxin shock are associated with elevated concentrations of circulating catecholamines, and endotoxin is known to induce adrenal release of norepinephrine and epinephrine [44–46]. Endotoxemia is associated with a decrease in peripheral tissue norepinephrine concentrations [30]. The mechanism of this phenomenon is unclear. In our experiments, animals that were electively sacrificed had little reduction of norepinephrine concentrations, but those dying spontaneously had very low concentrations. The low norepinephrine concentrations after endotoxin injections, as shown here and reported by others, may reflect the degree of tissue damage in native animals. This would explain the lack of consequent protection afforded by tissue norepinephrine depletion caused by the first injection of endotoxin (Fig. 1).

Previous studies have been limited by certain technical barriers. Surgical sympathectomy is applicable only to isolated organs with anatomically simple sympathetic innervation; it is not feasible to perform generalized surgical sympathectomies. Further, it is difficult to define clearly the effects of alpha blockers referable solely to the sympathetic nervous system. Numerous vasoactive substances may be released by endotoxin [7, 9, 10] and the significant antihistamine, antiserotonin, anti-acetylecholine properties of alpha blockers might actually be involved in the protection observed with the use of these agents [21]. Thus, only indirect evidence of the importance of the sympathetic nervous system is provided by studies with alpha blockade. In addition, alpha blockers affect not only alpha receptors on effector cells, but block nerve terminal re-uptake of norepinephrine and block the negative feedback on norepinephrine release regulated by alpha receptors on the nerve terminal [47]. This produces an elevation of available local and circulating norepinephrine which could result in stimulation of non-alpha catecholamine receptors with consequent diverse physiological manifestations [48]. Given the multiple actions of alpha-adrenergic blockers, a specific technique for selective adrenergic blockade would be of value. That specificity is supplied by 6-OHDA, which is taken up selectively into the storage granules, destroying the terminal. Our studies using 6-OHDA offer data supporting the hypothesis that adrenergic blockers exert their endotoxin-protective effect via the sympathetic nervous system. We have extended this hypothesis by providing direct evidence of sympathetic nervous system involvement in endotoxin-induced tissue damage.

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