1965

Septic shock : the effect of hydrocortisone on the localization of endotoxin in the peripheral vascular system as determined by fluorescent antibody technics

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SEPTIC SHOCK: THE EFFECT OF HYDROCORTISONE ON THE LOCALIZATION OF ENDOTOXIN IN THE PERIPHERAL VASCULAR SYSTEM AS DETERMINED BY FLUORESCENT ANTIBODY TECHNICS

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Submitted in Partial Fulfillment for the Degree of
Doctor of Medicine

College of Medicine, University of Nebraska

February 1, 1965

Omaha, Nebraska

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INTRODUCTION

Although beneficial results of pharmacologic doses of hydrocortisone have been described in the treatment of endotoxin shock, in experimental animals and in patients, no one has demonstrated the mechanism or site of action of this drug. (1, 6-10, 14-16, 18, 20, 22-26)

Lillehei (6, 9) proposes that the basic mechanism of endotoxin shock is the sympathomimetic action of endotoxin and its ability to call forth increased levels of circulating catechol amines. He further believes that the site of action of hydrocortisone is at the alpha adrenergic nerve endings; thus functioning as an alpha adrenergic blocking agent.

Rubenstine, Fine and Coons (13) have demonstrated endotoxin localization in the endothelium of the peripheral vascular system, in circulating leukocytes and other sites and postulate that the presence of endotoxin in blood vessel walls may be acting directly on those structures to produce the peripheral vascular collapse of lethal endotoxemia.

In this study, I have attempted to determine if either pre-treatment or post-treatment of endotoxin shock with hydrocortisone alters the localization of endotoxin.

MATERIAL AND METHODS

Ten adult, mongrel dogs averaging twelve kg. in weight were selected at random. All were treated in the same manner except as dictated by the experimental procedure.

Control Studies

Group A. One dog was killed with intravenous sodium pentobarbital and specimens of lung, spleen, heart, adrenal, aorta, muscle, liver, small bowel, bladder and kidney were secured and quick-frozen. Duplicate samples of tissue from each dog were cut into blocks of the approximate dimensions of 2 x 6 x 12 mm. These blocks were placed near the bottom of a Wasserman tube, with the thickest surface flush against the wall of the tube. The tube was corked and quick frozen in a dry-ice and 95 percent ethanol mash and kept in the mas for one hour. The blocks were then stored at -20° C until time of sectioning.

Sections, 3 to 5 mcg. thick, were cut in a cryostat at -20° C. Two sections were placed on each slide and were quickly thawed by placing a finger on the undersurface of the slide, immediately below each section. The slides were then placed for one hour in front of a fan to promote rapid evaporation of tissue moisture and were then placed in a dessicator containing $CaCl_2$ for one to ten days.

The sections were stained by the method of Rubenstein et al. (13)

The slides were first placed in absolute acetone for ten minutes, air-dried for thirty minutes, and then immersed in a staining buffer 1. in Coplin jars. After removal from the staining buffer, the excess solution around the sections was carefully absorbed with bibulous paper. Next a drop of rabbit anti-E. coli, $O111B_4$ serum[2] was placed on each section. The slides were incubated in a moist chamber for thirty minutes; and the excess serum washed off with cold staining buffer and the slides returned to Coplin jars, rinsed with five changes of staining buffer over a fifteen minute period, removed from the Coplin jar and excess moisture again being absorbed.

Next, a drop of horse anti-rabbit gamma globulin conjugated with fluorescein isothiocyanate[3]. was placed on each section, and incubated in a moist chamber for 30 minutes. The slides were again gently washed with staining buffer, returned to the Coplin jars and rinsed with five changes of staining buffer over a fifteen minute period, removed from the Coplin jar with the excess moisture again being absorbed. One drop of buffered glycerol (nine parts glycerol, one part staining buffer) was placed on each section. The slides were then mounted with cover slips.

I examined the slides immediately or stored them at 4° C and studied them within four days.

The controls for the sandwich technic of immuno-fluorescent staining were:

a. Sections from normal dogs were stained with rabbit anti-endotoxin antisera as previously described.

b. Sections from dogs with and without pre or post-treatment with hydrocortisone were stained as described except that the rabbit serum was absorbed with E. coli $0111B_4$ prior to staining.

Group B. - Three dogs were injected intravenously with endotoxin[4]. 4 mg./kgm. body weight, and were killed four hours later by the intravenous administration of sodium pentobarbital. Selected tissues were quick-frozen.

Experimental Studies

Group A. - Three dogs were pretreated with intravenous hydrocortisone, 15 mg./kgm. body weight, each day for four days. On the fourth day, endotoxin, 4 mg./kg. body weight was administered intravenously one hour after the final injection of hydrocortisone. Four hours after the injection of the endotoxin, these dogs were killed by the intravenous injection of sodium pentobarbital and selected tissues were quick frozen.

Group B. - Three dogs were injected intravenously with endotoxin, 4 mg./kg. body weight, followed in fifteen minutes by the intravenous injection of hydrocortisone, 50 mg./kg. body weight. These dogs were killed four hours after the injection of endotoxin by the intravenous injection of sodium pentobarbital and the selected tissues were quick frozen.

DISCUSSION

Jacob (5) first described the phenomenon of septic shock fifty-six years ago. Since that time many have reported proposed mechanisms and therapy but only in the last ten years have significant advances appeared in the literature.

Ribi (12) characterized endotoxin biochemically and demonstrated that the biologically active portion is a lipopolysaccharide which thus far has defied structural characterization but contains fatty acids, nitrogenous material and phosphorous. The molecular weight of purified endotoxin extracted by chloroform or phenol extraction methods from the antigenic material of the cell wall seems to be approximately 100,000. (17)

In small doses, endotoxin elicits a variety of responses; production of fever, stimulation of resistance to infection and protection against radiation injury. In larger doses, the endotoxins cause acute shock or death. (27)

Gilbert (4) described the species differences in various laboratory animals but found that even though hemodynamic effects vary from species to species, some features of septic shock are common to all; early vasoconstriction followed at some later stage by vascular collapse, decreased cardiac output, hypotension, decreased venous return and the pooling of blood.

The sympathomimetic effect of endotoxin shock causes an intense vasospasm in the arterioles and venules, especially in the splanchnic areas of experimental animals and man. (69)

Cremer and Watson (3) utilized fluorescent antibody studies to demonstrate endotoxin in the reticuloendothelial system of rabbits. They found that pre-treatment with hydrocortisone did not inhibit phagocytosis but seemed to inhibit the degradation and elimination of endotoxin.

Braude et al. (2) attempted to localize endotoxin *in vivo* by tagging the endotoxin with radioactive sodium chromate. They found that after intravenous injection the endotoxin was carried in plasma but not in erythrocytes. At one hour the concentration of endotoxin in the plasma decreased to 3/5 and at two hours 1/2 of the original titer. This period of removal from the plasma was accompanied by fever, leukopenia, hypotension and diarrhea. The liver and the buffy coat take up the largest portion of endotoxin. The leukopenia occured during the time the endotoxin was accumulating in the buffy coat. The lung and spleen were also sites of high concentrations of endotoxin.

Rubenstein, Fine and Coons (13) reported on the localization of endotoxin as determined by the fluorescent antibody technic. They treated three dogs with *E. coli* endotoxin and took samples of various organs for study when the dogs died or were sacrified. The

sandwich technic of immunofluorescence was used. They found endotoxin in the walls of the peripheral vessels in endothelium of capillaries and venules, intra and extra cellular, in vein walls, in the media of arteries and arterioles, in reticuloendothelial cells in polymorphonuclear leukocytes free in the circulation, in renal glomerular vessels, in hepatic sinusoids, and in adrenal medullary sinuses.

In 1954, Smith, Muller and Hinshaw (18) reported that pharmacologic doses of hydrocortisone improved blood pressure and cardiac output as well as survival.

In 1954, Thomas and Smith (22) reported that hydrocortisone protected against early prostration, shock and death. Hydrocortisone also protected against the Schwartzman phenomenon.

In 1958, Melbey (11) and Spink (19) found in circulating plasma cortisol during shock demonstrating that the shock was not due to adrenal insufficiency.

In 1958, Lillehei and MacLean (7) demonstrated that hydrocortisone treatment of endotoxin shock in dogs prevented the original hypotension, plasma loss and hemoconcentration.

In 1959, Lillehei and MacLean (8) reported that hydrocortisone increased the survival of experimental animals subjected to endotoxin shock.

In 1961, Weil and Miller (25) demonstrated that hydrocortisone was therapeutically effective in endotoxin shock in dogs when used early and in large doses.

Anderson and Kadner (1) used pharmacologic doses of hydrocortisone in septic abortions with vascular collapse and felt that hydrocortisone contributed to the reduced mortality and morbidity.

Other investigators (14-16, 23) reported that corticosteroids caused a significant increase in the cardiac index, no significant change in arterial pressure, a 25 percent decrease in peripheral resistance and no change in venous pressure.

Weil, Shulin, and Biddle (26) reported that in endotoxin shock in human subjects who had been treated with pharmacologic doses of hydrocortisone, the survival was significantly greater (p=less than 0.01) than in patients not treated with hydrocortisone.

Lillehei (6, 9) showed that pre-treatment with hydrocortisone produced a 90 percent survival in dogs in endotoxin shock and a 90 percent mortality in dogs without this treatment. If 50 mg./kgm. of hydrocortisone was administered intravenously thirty minutes after the induction of endotoxemia, the following effects were noted: absence of the marked increase in individual organ or total peripheral resistance, increased perfusion of bowel and viscera and a significant decrease in the plasma volume loss.

RESULTS

Table I shows the results of fluorescent microscopy.

The staining of the tissues examined was specific for endotoxin. The tissues of the dog which did not receive endotoxin or hydrocortisone, did not fluoresce.

The negative control tissues on each slide occasionally fluoresced but, with one exception, the fluorescence was less than or equal to the fluorescence of the tissue to which the anti-endotoxin serum was added. This exception may be explained by the postulation that the serum was applied to the wrong tissue. The fluorescence of the negative controls may have resulted from the mixing of anti-sera after application to the slide.

The fluorescence was most marked in the tissues of the dogs which received only endotoxin. In this group, the lung, spleen, kidney and small bowel were positive for fluorescence. In these tissues, fluorescence was present within the lumen of blood vessels and the tissue. Further localization of the endotoxin could not be made with the fluorescent microscope.

The tissues of the animals pre-treated with hydrocortisone were less fluorescent than the tissues of the animals which had received endotoxin alone.

The major differences in the degree of fluorescence were observed in lung, liver, gastrointestinal tract and kidney.

The tissues of the animals given endotoxin then treated with hydrocortisone fluoresced more than the tissues of the hydrocortisone-pre-treated-animals but less than the tissues of the animals which received endotoxin alone.

SUMMARY

The comparison of the localization of endotoxin in dogs pre-treated with hydrocortisone and animals treated with hydrocortisone after endotoxin was made with the fluorescent microscope.

The tissues of the animals pre-treated with hydrocortisone were less fluorescent than the tissues of the animals given endotoxin alone and the tissues of the animals given endotoxin followed by hydrocortisone.

TABLE I.

	CONTROL				EXPERIMENTAL					
	GROUP A	GROUP B			GROUP A			GROUP B		
Lung	X*	4+	2+	4+	0	0	2+	X	X	0
Neg. Cont.	X	0	2+	2+	0	0	0	0	0	0
Spleen	0	4+	0	2+	2+	0	2+	4+	X	4+
Neg. Cont.	X	2+	0	0	2+	1+	1+	2+	0	3+
Heart	0	0	2+	0	0	0	0	0	2+	2+
Neg. Cont.	X	0	0	0	0	0	0	0	2+	2+
Adrenal	0	1+	0	2+	0	0	1+	X	1+	0
Neg. Cont.	X	0	0	0	0	0	0	0	0	0
Aorta	0	0	0	0	0	0	X	0	0	0
Neg. Cont.	X	0	0	0	0	0	0	0	0	0
Muscle	0	X	0	0	0	0	0	0	1+	0
Neg. Cont.	X	0	0	0	0	0	0	0	0	0
Liver	0	4+	2+	4+	0	0	0	2+	3+	2+
Neg. Cont.	X	1+	0	0	0	0	0	1+	2+	0
Gastrointestinal	0	4+	2+	0	0	0	0	0	0	0
Neg. Cont.	X	0	0	0	0	0	0	0	0	0
Bladder	0	4+	0	0	0	0	0	0	0	0
Neg. Cont.	X	0	0	0	0	0	0	0	0	0
Kidney	0	4+	1+	4+	0	1+	0	0	1+	2+
Neg. Cont.	X	X	0	2+	0	1+	0	0	X	0

*X - either not examined, e.g. negative controls in Group A, or sections were washed from the slide during the staining procedure.

FOOTNOTES

1. The staining buffer was composed of: 6 grams $NaH_2PO_4 \cdot H_2O$, 28.5 grams of $Na_2HPO_4 \cdot 7H_2O$, 120 grams NaCl and distilled water q.s. for 15 L.

2. Rabbit Anti-endotoxin Serum. - A 50 ml. aliquot of tryptic digest broth was innoculated with a loup full of organisms from a stock culture of E. coli $01111B_4$[5]. and this preparation was incubated for eight hours with constant swirling on a magnetic stirring device. The organisms were then centrifuged at 2,800 RPM for one half hour and the supernatent discarded. The organisms were re-suspended in staining buffer in a 25 ml. test tube and boiled in a water bath for one hour. Additional buffer was added and the suspension re-centrifuged at 2,800 RPM for 30 minutes. The organisms were re-suspended, washed and centrifuged three more times and then suspended in staining buffer so that the density was equivalent to the density of a McFarland standard #5 (21) as measured in a photoelectric cell. This preparation was administered within one hour after preparation to four adult albino rabbits weighing an average of 3 kg. via the ear veins according to the schedule:

Day 1, 0. 25 ml.; Day 3, 0.15 ml.; Day 5, 0.75 ml.; Day 7, 1.0 ml.; Day 9, 1.25 ml.; and Day 11, 1.5 ml. On the fourteenth day the blood was harvested by intracardiac puncture, allowed

to stand at room temperature for two hours, the clot rimmed, and stored at 4° C for eighteen hours. The serum was decanted, centrifuged at 2,000 RPM for 30 minutes at 4° C and stored at 4° C.

The effectiveness of the antiserum was opposed by a sample of endotoxin used to produce endotoxin shock. Two to five bands of precipitation developed in four days. The antisera that does not demonstrate at least two bands are ineffective for immuno-fluorescent staining.

Control Serum. - Five ml. of the anti-endotoxin antisera was absorbed with boiled E. coli, $0111B_4$. Approximately 500 ml. of tryptic digest broth incubated for ten hours at 37° C provided sufficient organisms for absorption of 5 ml. of serum.

Both test and control sera were preserved with equal volumes of glycerol and stored at 4° C.

For staining purposes, one drop of test or control sera was diluted with 20 drops of staining buffer. One drop of the test serum was placed on one of two sections on the slide, and one drop of control serum on the other.

3. The horse anti-rabbit Gamma Globulin labelled with fluorescein isothiocyanate was purchased from Difco Laboratories, Detroit, Michigan.

4. The endotoxin was purchased from Difco Laboratories, Detroit, Michigan, and was lipopoly saccharide extracted by the Boivin method from Escherichia coli, $0111B_4$.

5. The stock culture of Escherichia coli, $0111B_4$, was kindly supplied by Dr. Howard S. Rubenstein, Department of Microbiology, Harvard Medical School, Boston, Mass.

ACKNOWLEDGEMENTS

I wish to express my gratitude to Dr. Merle M. Musselman, Professor and Chairman of the Department of Surgery for his advice, constructive criticism and support; to Dr. Earl E. Walker, Senior Resident in the Department of Surgery for his invaluable advice and assistance; to Drs. H. W. McFadden and M. Tremaine, Department of Microbiology, Drs. C. A. McWhorter, A. L. Larsen, and J. Scott, Department of Pathology and Dr. V. Wilder, Department of Biochemistry for their advice and assistance.

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