Studies on Distribution of *Escherichia coli* Endotoxin in Mice.* (24525)

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Although physiological changes resulting from their injection have been described, the lethal mechanism of lipopolysaccharide materials derived from gram-negative bacteria is not known. Indeed, until recently, there has been no good qualitative technic for *in vivo* detection of minute qualities of endotoxins. Such a technic has been developed by Braude, *et al.*(1) who have shown that Cr^{51} can be used as a reliable tag. This study was undertaken in the hope that changes in distribution could be correlated to toxicity and that this, in turn, would provide some insight as to mode of action of endotoxins.

Materials and methods. The endotoxin preparation used was prepared from a strain of *E. coli* isolated from a fatal human septicemia. The bacteria were grown in synthetic medium with aeration, harvested by centrifugation and killed and dried with acetone. Endotoxin was removed by extraction with trichloroacetic acid and was further purified by ethyl alcohol-salt (NaCl) fractionation (2). Nitrogen content of endotoxin was 2.3% by weight. All dilutions were made with 0.9% NaCl solution. Tagging of the endotoxin was carried out as follows: Approximately 1 mc of sterile $Cr^{51}Cl_3$ (Abbott Labs., Oak Ridge, Tenn.—specific activity approximately 1 mc/mg) was added to saline solution of 100 mg endotoxin and kept at room temperature. After 24 hours the mixture was dialyzed at room temperature against 5000 ml volumes of 0.067 M phosphate buffer, pH 7.2, which was changed at 24-hour intervals until appearance of Cr^{51} in the dialysate was negligible, usually 72 to 96 hours. Tagging by this procedure did not alter the original toxicity of the endotoxin. All mice were Bagg strain albino, obtained from Walter Reed Army Medical Center colony. The mice were housed in glass jars where food (containing no antibiotics) and water were available. Some mice were injected with trypan blue to alter their susceptibility to endotoxin by intraperitoneal administration of 2.5 mg trypan blue in distilled water daily for 3 successive days. On the fourth day mice were injected with endotoxin. To measure radioactivity, entire organs were removed and each was digested by boiling in 2 ml of concentrated HNO_3. Foaming was prevented by adding small amount of antifoam (Dow Corning Silicone Defoamer—Antifoam A) to each digestion tube. Blood was obtained by cardiac puncture and digested as above. All measurements of radioactivity in blood were based on total blood volume, considered to be 8% of body weight. Radioactivity of each sample was measured in well-type scintillation counter which had an efficiency of approximately 3%. The counting error at the 0.95 level was 23 to 28 cpm for the 2-minute counting intervals.

Results. Comparison of distribution patterns in mice of Cr^{51} tagged endotoxin and free $Cr^{51}Cl_3$ one hour after intravenous administration showed that most Cr^{51} bound to endotoxin was in the liver and remained there for long periods. Free $Cr^{51}Cl_3$ was more generally distributed and appeared early in urine in considerable amounts. Toxicity studies showed that routes of injection greatly influenced lethality of this preparation. The LD_{50} dose was 145 μg intravenously and intracranially, 290 μg intraperitoneally and 580 μg intramuscularly. Distribution studies were carried out to explain these differences.

Distribution of endotoxin following intravenous administration. There was an appreciable rise in amount of endotoxin in the blood when the amount of toxin administered exceeded the lethal level, *i.e.*, 150 μg (Table I). However, it should be noted that this does not necessarily represent overflow endotoxin because it is apparent that amounts in the organ continued to increase with increases in dosage. Using larger groups of mice, showed

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TABLE I. Distribution of Cr^{51} Tagged Endotoxin Administered Intravenously in 20-22 g Mice (Sacrificed 3 Hr after Injection).

# of mice	Toxin dose (μg)	Endotoxin in blood (μg)	Endotoxin in liver (μg)
9	1	.1	.69
4	10	.20	7.7
17	100	2.2	62.2
17	150	6.4	79.8
10	175	17.5	86.5
15	200	27.2	103.8
10	225	41.6	131.1

that most injected endotoxin was found in the liver within 5 minutes following injection and the amount detectable remained constant for long periods. An experiment was carried out to determine length of time (as measured by radioactivity) the endotoxin distribution pattern remained unchanged. Three groups of mice were injected intravenously with 1, 5, or 50 μg of endotoxin, sacrificed at intervals, when various organs were checked for radioactivity. Measurable radioactivity in livers and spleens remained constant for 30 days while most of the radioactivity disappeared from blood and lung specimens much earlier. Approximately 60% of injected radioactivity was still present in the liver 60 days after injection of endotoxin.

Distribution of endotoxin following intracranial administration. Using a 27 guage needle, mice weighing 18 to 20 g were injected intracranially with 0.2 ml saline containing varying dosages of endotoxin and sacrificed as described earlier. The LD_{50} dosage of 150 μg was used in most instances. The results showed that distribution pattern of LD_{50} dose by this route was similar to the same LD_{50} dose by intravenous route. Egress from the cranium was rapid. It was not determined whether the 20 to 40% of injected dose remaining in the head was bound by brain tissue. When endotoxin is administered by other routes it is not found in appreciable amounts in the head and in rabbits, at least none is found in the brain. It is of interest to note that dosage and time of death by intracranial route was very similar to dosage and time of death by intravenous route. When the overwhelming dosage of 870 μg/mouse was used, percentages of injected dosages found in blood were again strikingly similar to that found using intravenous administration. It is tempting to postulate a threshold liver or blood concentration above which death from endotoxin occurs and below which survival is the rule. Such a hypothesis, however, did not hold when intramuscular route of administration was used.

Distribution of tagged endotoxin following intramuscular administration. The LD_{50} of this endotoxin prepartaion by intramuscular route was about 580 μg/mouse, which was the dosage used to study distribution by this route of administration. The distribution pattern was quite different by this route than by intravenous or intracranial routes (Table II). From one-third to one-half of the injected toxin remained at site of injection and not more than 4% of the toxin was found in all other areas assayed. Blood levels of endotoxin increased rather than decreased with time but total amount detected in the liver was far less than that following intravenous or intracranial injection. Thus lethality by this route could not be correlated to liver saturation of endotoxin.

Effect of premedication with trypan blue on endotoxin distribution. Administration of trypan blue reportedly blocks the reticuloendothelial system and results in greater susceptibility to endotoxin. The increased susceptibility was confirmed, ranging from increase of 4-fold to 10-fold depending on route of administration of endotoxin. Table III gives a comparison of the distribution pattern in normal mice and mice premedicated with trypan blue when the intravenous route

TABLE II. Distribution of Cr^{51} Tagged *E. coli* Endotoxin* in Normal Mice after Intramuscular† Injection.

# of mice	Time of sacrifice	Endotoxin (μg)			
		Blood	Lungs	Liver	Site of inj.
6	5 min.	.6	.6	3.6	393.2
6	30	1.8	.6	4.8	377.0
4	1 hr	1.8	.6	7.8	372.4
4	2	1.8	.6	5.4	366.0
4	3	1.8	.6	8.4	345.1
6	4	1.8	.6	9.0	335.2
4	5	3.0	.6	13.8	349.2
2	6	3.6	6.6	6.0	315.5
6	24	3.0	.6	14.4	301.0

* 580 μg.
† Inj. vol was 0.2 ml into gluteal area.

TABLE III. Comparison of Endotoxin Distribution in Normal Mice and Mice Premedicated with Trypan Blue.

# of mice	Premedication	Time of sacrifice (hr)	Toxin admin. (μg)	Route of admin.	μg of endotoxin in		
					Blood	Liver	Site of inj.
10	Trypan blue	3	145	I.V.	14.1	66.9	
17	Saline	3	145	"	6.4	79.8	
6	Trypan blue	3	145	I.M.	.1	1.9	
6	*Idem*	3	290	"	.9	2.0	
4	Saline	3	580	"	1.8	8.4	345
5	Trypan blue	24	145	"	3.8	3.9	50
4	*Idem*	24	290	"	5.5	6.1	97
6	Saline	24	580	"	3.0	14.4	301

of administration was used. The amount found in blood was increased in the trypan blue mice, but the amount in other organs assayed was not appreciably altered. When trypan blue mice were injected intramuscularly, distribution 3 hours after injection was similar to that of normal mice. At 24 hours after injection, however, the blood level of trypan blue mice was as great or greater than that of normal mice even when dosage administered to the trypan blue mice was 25% that of normal mice (Table III).

Discussion. A number of technics have been used to detect *in vivo* endotoxins. Braude(3) and his co-workers working with rabbits found that following intravenous administration, endotoxin was present in plasma but not the erythrocytes. It then passed to the buffy coat and liver. Accumulation in the buffy coat was accompanied by a pronounced leucopenia, primarily a fall in lymphocytes and neutrophils. Very little of the endotoxin could be found in spleen or lung and none in the brain. In a continuation of this study Carey *et al.*(4) studied the effect of tolerance on distribution. They found that although normal and tolerant animals both cleared sublethal doses of endotoxin rapidly and at approximately equal rates, a great difference was noted between tolerant and non tolerant animals. Hepatic localization and plasma clearance of massive quantities of endotoxin were much more rapid in tolerant animals. The authors felt that tolerance to lethal doses of endotoxin depends on permanent removal of circulating endotoxin into hepatic and other cells.

Rowley *et al.*(5) found that when 5-60 μg of P^{32} phosphorus labelled endotoxin was injected intravenously into mice, 60 to 75% was eliminated from the circulation in 8 minutes and that at least half of the removed radioactivity appeared in liver and spleen and smaller amounts appeared in lungs, adrenals and kidneys. They noted that endotoxin removed by reticuloendothelial cells appeared to be firmly held by them. Using the fluorescent antibody technic Cremer and Watson(6) studied distribution of endotoxin in rabbits following intravenous injection. In normal animals the toxin was removed from the circulating blood quickly by the reticuloendothelial system. There was a reduction of toxin with time in spleen and lungs, but the liver maintained a rather constant pattern. In cortisone-treated or irradiated animals the amount of endotoxin remained high in spleen and lungs as well as in liver. When premedicated with thorotrast, little or no endotoxin was removed by the reticuloendothelial system and there was an increased death rate.

The above studies suggest that lethality of endotoxin is associated with failure of the reticuloendothelial system to remove circulating endotoxin. Some results in this study tend to support this theory. These are: 1) Partial blockade of the reticuloendothelial system by trypan blue increased susceptibility of mice to endotoxin and resulted in high blood levels of endotoxin. 2) Amounts of circulating endotoxin increased rapidly when lethal amounts of endotoxin were injected via intravenous or intracranial routes. The latter finding, however, does not necessarily represent reticuloendothelial system saturation because the liver continued to take up endotoxin when greater amounts were injected. There is, of course, the possibility that this in-

creased endotoxin is in hepatic cells rather than Kupffer cells.

The relatively constant percentages of radioactivity remaining in liver and spleen suggest a number of possibilities. First, radioactivity in liver represents cleaved Cr^{51}. This seems unlikely because it has been shown that free Cr^{51} does not tend to accumulate in liver or spleen. A second possibility is that the Cr^{51} is attached to some degraded or detoxified endotoxin in these organs. The possibility of a liver "endotoxinase" must be considered. A third possibility is that the cells of the reticuloendothelial system protect the animal by holding the endotoxin and releasing it very slowly over long periods. One finding in this study that is difficult to relate to reticuloendothelial system saturation as a factor in death from endotoxin is the pattern of distribution following intramuscular administration of endotoxin. Amount and distribution of radioactivity, and presumably endotoxin, found in the reticuloendothelial system following intramuscular injection is compatible with life as demonstrated by intravenous injection of sublethal amounts of endotoxin, yet the animals injected intramuscularly, died. One possible explanation is that the tag is cleaved in muscle tissue with the Cr^{51} being excreted or more generally distributed and the Cr^{51}-free endotoxin is present but undetectable in liver. Another possible explanation is that a lethal mechanism of endotoxin is its persistence in the blood. Initially blood levels of endotoxin following intramuscular injection are low, but, in contrast to the pattern seen when other routes of injection were used, levels continued to rise and persist for at least 24 hours. The lethal mechanism of endotoxin may be vascular in this instance. Some evidence for such a time-dose type of theory is that mice rarely die from a barely lethal dose of endotoxin within the first 8 hours after injection and most are dead within 24 hours after injection.

Summary. 1. Intravenous injection of Cr^{51} tagged endotoxin into mice resulted in marked increase of circulating endotoxin as dosages reached the lethal range. 2. Distribution pattern of endotoxin in mice injected intracranially was similar to that of mice injected intravenously. 3. Distribution pattern of endotoxin in mice injected intramuscularly differed markedly from those found in mice injected intravenously or intracranially. 4. Premedication of mice with trypan blue resulted in greater susceptibility to endotoxin and increased amounts of circulating endotoxin in mice injected intramuscularly or intravenously. 5. Using the technique described, lethality of endotoxin for mice could not be related to any one distribution pattern.

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Chromatography of McCaslan Pole Bean Hemagglutinin on DEAE-Cellulose. (24526)

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Phytohemagglutinin of McCaslan Pole Bean (*Phaseolus vulgaris*) has been a material of interest since discovery of its ability to agglutinate human erythrocytes (rbc). Boyd (1) observed that it agglutinates cells of blood groups A, B, and O (all Rh positive). Part of the interest in this material stems from its utility in preparing high purity leukocyte sus-