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SIGNIFICANCE OF LEUKOCYTES IN ENDOTOXIN SHOCK

OKLAHOMA UNIVERSITY

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<p>Peripheral blood leukopenia and the sequestration of large numbers of polymorphonuclear leukocytes in pulmonary capillaries have been observed in experimental shock. It has been suggested that release of leukocytic lysosomal enzymes contributes to systemic hypotension and pulmonary capillary damage. This study was undertaken to evaluate the role of leukocytes in endotoxin shock. Six rhesus monkeys rendered leukopenic by total body irradiation (mean white blood cells, 358/cu mm) were compared with 6 normal monkeys (mean white blood cells, 10,550/cu mm) for 2 hrs after injection of E. coli endotoxin. The effects of irradiation were evaluated in 3 additional animals which did not receive endotoxin. Following the injection of endotoxin, the mean cardiac output and systemic pressure decreased more than 50% in both the leukopenic and normal groups. Metabolic acidosis developed in both groups. The mean arterial pO<sub>2</sub> was unchanged, however, the alveolar-arterial O<sub>2</sub> gradients increased. Differences between the groups were not significant in these parameters. Light and electron microscopy demonstrated sequestered polymorphonuclear leukocytes and platelets in pulmonary capillaries in the non-radiated group, but leukocytes were virtually absent in sections from lungs of the leukopenic animals. In spite of this difference, significant endothelial swelling and perivascular edema were demonstrable in both groups. No significant histologic abnormalities were noted in the three radiated leukopenic control animals who did not receive endotoxin. Leukopenia provided no protection from the hemodynamic effects or the histological damage in pulmonary capillaries observed after administration of endotoxin.</p>			

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## ABSTRACT

Peripheral blood leukopenia and the sequestration of large numbers of polymorphonuclear leukocytes in pulmonary capillaries have been observed in experimental shock. It has been suggested that release of leukocytic lysosomal enzymes contributes to systemic hypotension and pulmonary capillary damage. This study was undertaken to evaluate the role of leukocytes in endotoxin shock.

Six rhesus monkeys rendered leukopenic by total body irradiation (mean white blood cells, 358/cu mm) were compared with six normal monkeys (mean white blood cells, 10,550/cu mm) for 2 hours after injection of *E. coli* endotoxin. The effects of irradiation were evaluated in 3 additional animals which did not receive endotoxin.

Following the injection of endotoxin, the mean cardiac output and systemic pressure decreased more than 50% in both the leukopenic and normal groups. Metabolic acidosis developed in both groups. The mean arterial pO<sub>2</sub> was unchanged, however, the alveolar-arterial O<sub>2</sub> gradients increased. Differences between the groups were not significant in these parameters.

Light and electron microscopy demonstrated sequestered polymorphonuclear leukocytes and platelets in pulmonary capillaries in the non-radiated group, but leukocytes were virtually absent in sections from lungs of the leukopenic animals. In spite of this difference, significant endothelial swelling and perivascular edema were demonstrable in both groups. No significant histologic abnormalities were noted in the three radiated leukopenic control animals who did not receive endotoxin.

Leukopenia provided no protection from the hemodynamic effects or the histological damage in pulmonary capillaries observed after administration of endotoxin.

## INTRODUCTION

Previous reports have documented the alterations in pulmonary physiology that occur with endotoxic shock. The development of pulmonary hypertension, increased ventilation, altered blood gas exchange, decreased lung compliance, increased airways resistance and increased surface tension of lung extracts are well documented in several species (10,12,14,24). Very little is known about the pathogenesis of these physiologic derangements. This paper explores one possible pathogenetic mechanism.

Endotoxin shock is characterized by an early fall in peripheral blood leukocyte count and sequestration of large numbers of polymorphonuclear leukocytes in the pulmonary capillaries (6,15). Ultrastructurally, these leukocytes are seen degranulated and fragmented with release of their lysosomal granules into the pulmonary capillaries (6). This phenomenon of pulmonary sequestration of leukocytes in response to certain stimuli was probably first described in 1894 (5,8); however, recently there has been renewed interest in the possible adverse effects of leukocytes in endotoxic shock (18). Furthermore, Wilson, Ratliff, Mikat, Hackel, Young and Graham (28) have speculated that white blood cells (WBC), sequestered in the pulmonary circulation, might be in some fashion related to the physiologic and morphologic abnormalities seen in other hypoperfusion states (including hemorrhagic shock and following cardiopulmonary bypass for surgery). Such interest and speculation seem warranted since granulocytes are known to contain proteolytic enzymes (7,25) as well as activators of the kinins (9,17).

This study evaluates the role of sequestered leukocytes in the pathogenesis of the hemodynamic and pulmonary changes in endotoxic shock. Rhesus monkeys with normal WBC counts were studied before and after injection with endotoxin and compared to animals rendered leukopenic by total body irradiation.

## METHODS

The 14 adult rhesus monkeys used in this study weighed from 4 to 9 kg. They were captured from the wild, inspected for transmissible disease and observed for a period in captivity prior to study. The study groups are summarized below:

I. Leukopenic Endotoxin Group -- This group of 6 animals was subjected to endotoxic shock after being rendered leukopenic by total body irradiation. They received 1100 to 1300 Roentgens from a Van de Graaff 2 MEV X-ray generator at 61.73 R/min, and a distance of 117 to 142 cm. Field size varied from 25 x 33 cm to a maximum of 30.5 x 41.5 cm. The first 3 animals received their irradiation in split doses 4 days apart to establish the dose range required to produce the desired leukopenia. The subsequent 3 animals in this group received 1200 R total dose by single treatment. Hematocrit and WBC counts were performed prior to irradiation, and at frequent intervals following treatment. The shock study was performed 5 days after the last dose of radiation. Mean WBC count/cu mm prior to radiation was 7,116 as compared to a mean count of 358/cu mm just prior to the start of the 2-hour shock period.

II. Endotoxin Control Group -- The 5 non-leukopenic animals in this group received the same dose of endotoxin and were monitored in the same fashion as Group I. Their mean WBC count prior to endotoxin injection was 10,550/cu mm.

III. Leukopenic Control Group -- This group of 3 monkeys received their 1200 Roentgen total body irradiation by single treatment dose. Field size, distance and rate were the same as in Group I. They were studied (by the same parameters as Groups I and II) for 2 hours under anesthesia. They received no endotoxin. Their mean WBC count at the onset of the 2-hour study was 11,000/cu mm.

After the animals had received 15 to 30 mg/kg of pentobarbital intravenously (I.v.), a cuffed endotracheal tube was placed in the upper trachea and connected

to a water-sealed spirometer for collection of expired gases. Cutdown was performed upon the femoral vessels and the femoral artery was cannulated with a Teflon catheter. A soft polyethylene catheter was introduced into the femoral vein and advanced into the right ventricle (or pulmonary artery when possible). Position of the catheter was confirmed by pulse contour. Parameters monitored during the study period included indicator-dilution cardiac outputs (CO), systemic arterial pressure (SAP), minute ventilation (VE), arterial blood gases, oxygen consumption (VO<sub>2</sub>), alveolo-arterial (A-a) oxygen gradients, and peripheral leukocyte counts. The hemodynamic and ventilatory determinations were performed as previously described (10). Peripheral leukocyte counts were performed in a hemocytometer upon specimens obtained from the femoral artery. After baseline measurements, the animals were given 6 mg/kg of E. coli endotoxin (Difco) i.v. over a 1-3 minute period. Repeat collections and measurements were made at 30-minute intervals until the time of sacrifice. Animals were sacrificed at 120 minutes by injections of massive doses of pentobarbital. Thoracotomy was performed immediately after sacrifice and multiple samples from both lungs were obtained for histologic examination. Statistical analysis was performed using the "t" test for unpaired variates with comparison of leukopenic endotoxin animals to non-leukopenic endotoxin controls.

For light microscopic studies, Carnoy's and Bouin's fixatives were utilized. The specimens were embedded in paraplast, and the sections were stained by hematoxylin and eosin, periodic-acid Schiff with diastase control, Van Gieson elastica and stains for fibrin including phosphotungstic acid hematoxylin, Weigert's hematoxylin and Carstairs techniques. Specimens obtained for ultrastructural study were fixed in Zetterqvist's fixative, dehydrated in ascending grades of ethanol and embedded in Epon 812 and Araldite. Thin sections were

stained with Reynold's lead citrate and uranyl acetate and examined with an RCA-EMU-3F electron microscope.

## RESULTS

The leukopenic endotoxic animals had a CO of 167 ml/min/kg prior to the administration of endotoxin and a value of 60 ml/min/kg 120 minutes after endotoxin injection, at the time of sacrifice (Figure 1); although measurements were made at 30 minute intervals throughout the study, only the baseline and 120 min data are reported since the 2-hour values are representative of the findings obtained at 30, 60, and 90 minutes. The endotoxin control (non-leukopenic) animals had a fall in CO from a baseline of 174 ml/min/kg to a value of 67 ml/min/kg at 2 hours (Figure 1). There was no significant difference in the changes in CO of the leukopenic and non-leukopenic groups. The CO was essentially unchanged throughout the study in the leukopenic control animals (Fig. 1).

The observed changes in SAP are illustrated in Figure 2. Again, comparable degrees of hypotension developed in leukopenic and non-leukopenic animals after endotoxin with no significant change in the irradiated leukopenic controls.

Metabolic acidosis was characteristic of all the animals subjected to shock. The degree of acidosis as manifested by fall in pH and  $\text{HCO}_3^-$  is actually slightly greater in the leukopenic than in the non-leukopenic monkeys during the shock period (Table I). This reflects less respiratory compensation by the leukopenic group. ... Areas minute ventilation ( $V_E$ ) increased from 210 ml/min/kg to 290 ml/min/kg and  $p\text{CO}_2$  fell from 40 mm Hg to 30 mm Hg in the non-leukopenic endotoxic controls. The baseline  $V_E$  of 170 ml/min/kg was unchanged at 2 hours and the  $p\text{CO}_2$  fell only from 38 mm Hg to 35 mm Hg in the leukopenic shock animals.

Arterial  $p\text{O}_2$  was well maintained throughout the study in all 3 groups of animals (Table I) however, the alveolo-arterial (A-a) oxygen gradients increased significantly from control values in both leukopenic and non-leukopenic animals

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given endotoxin (Table I). Again, there was no significant difference in the degree of change in the 2 groups. The A-a gradient actually decreased slightly in the leukopenic controls.

Changes in mean peripheral leukocyte count are also summarized in Table I. The characteristic fall in leukocyte count was demonstrated in the endotoxic control animals. The WBC count was low and remained low throughout the study period in both irradiated groups. At no time during the study did the WBC count rise above baseline levels in the radiated animals. Two of the leukopenic monkeys underwent bilateral iliac crest bone marrow aspirations which confirmed the sparsity of available WBC reserves. Only rare granulocytic precursors were observed on stained marrow smears.

The light microscopic findings in the Group I leukopenic endotoxin-treated animals included dilated venules and arterioles devoid of leukocytes, focal to diffuse pulmonary edema with rounding of alveolar spaces and thickening of the alveolar walls (Figure 3). In 1 of the 6 animals, a pulmonary embolus was present in a muscular pulmonary artery. Ultrastructurally, only rare leukocytes and platelets were noted. However, frequent sites of perivascular space edema were present. Endothelial cytoplasmic membrane disruption was noted (Figure 4).

Dilated vessels with abundant leukocytes, foci of pulmonary edema and many leukocytes in the alveolar capillaries comprised the light microscopic findings in the Group I, endotoxin control animals (Figure 6). Ultrastructurally, endotoxin control animals showed abundant polymorphonuclear leukocytes and platelets in the pulmonary capillaries. Leukocytic-endothelial membrane sticking, endothelial cytoplasmic membrane disruption, leukocytic degranulation and disruption, and frequent sites of perivascular space edema were significant ultrastructural findings in Group II (Figure 5).

In the Group III irradiated control animals, light microscopic findings were minimal. Red blood cell congestion of the alveolar capillaries and infre-

quant sites of pulmonary edema were the only consistent changes (Figure 7). One of the 3 animals had an embolus in a large muscular artery. Ultrastructural findings included infrequent sites of perivascular space edema, occasional Type I epithelial edema and scarce leukocytes and platelets (Figure 8).

#### DISCUSSION

The phenomenon of pulmonary leukocyte sequestration was first demonstrated over 75 years ago in the studies of Goldscheider and Jacob on anaphylactic leukopenia (8). Several subsequent investigators have observed this curious occurrence in response to certain stimuli (5,20,21,26). Previous papers from this laboratory (6,15) demonstrated that leukocytes become trapped in the lungs following endotoxin injection, and numerous other papers (12,13,16,22,28) have stressed the prominence of granulocytes in pulmonary capillaries in endotoxic and hemorrhagic shock. Recently, there has been speculation that these sequestered WBC's might be etiologically related to the altered pulmonary physiology and morphology seen in various hypoperfusion states (28), including endotoxic and hemorrhagic shock as well as following cardiopulmonary bypass for surgery. The two major pathogenetic hypotheses which incriminate the sequestered leukocytes are: (1) the sequestered granulocytes may directly damage pulmonary vasculature and alveoli by releasing proteolytic enzymes during their entrapment in the pulmonary circulation; (2) the leukocytes may be related to the hemodynamic changes in shock by activation of the kinins. In support of these hypotheses, it is well known that granulocytes contain proteolytic enzymes within their cytoplasmic lysosomes (7,25) and our prior investigation (6) has demonstrated that the sequestered granulocytes degranulate and fragment within the pulmonary capillaries. In addition, Kies, Forsyth, Williams and Melmon (18) have demonstrated release of lysosomal enzymes from the granulocytes of monkeys in endotoxic shock. In support of the second hypothesis, Melmon and Cline (17) and Greenbaum and Kim (9) have demonstrated that granulocytes contain kallikrein or a kallikrein activator.

In this study, we have evaluated the role of granulocytes in the shock lung syndrome by developing a leukopenic primate model. In a pilot study, we attempted to produce leukopenia in 4 monkeys by the administration of intravenous nitrogen mustard. Doses of 1 to 10 mg/kg were given by single injection, however, no predictable relationship between dose and level of leukopenia was found. All of the animals ultimately died and none was ever sufficiently leukopenic for study purposes. We subsequently resorted to total body irradiation for the production of leukopenia. The irradiated leukopenic animals utilized in this study are considered to be an acceptable experimental model since the pulmonary abnormalities due to radiation per se can be distinguished from those that occur in endotoxic shock. The most striking pulmonary lesions following irradiation are observed several weeks following treatment (2), but ultrastructurally capillary endothelial changes have been reported from as early as 24 hours to 2-5 days after radiation (1,19). The endothelial changes described were focal but widespread. However, the endothelial blebs and disruptions reported by Phillips (19) and the endothelial intracellular vacuolization described by Adamson, Bowden and Wyatt (1) are easily differentiated from the endotoxic effects that we have described (6). The pulmonary functional derangements reported within the first 4 weeks after pulmonary radiation are minimal (3) and finally, our group of irradiated leukopenic controls was functionally and histologically (except for the absence of WBC's) no different than a prior group of normal control animals (13).

It has been attractive to speculate that pulmonary capillary plugging by leukocytes might cause the characteristic early development of pulmonary hypertension in endotoxic shock. However, two of the leukopenic animals had continuous monitoring following the injection of endotoxin and both demonstrated the characteristic early rise in pulmonary artery pressure. This finding is in

support of our previous observation in the isolated perfused lobe of lung (15) which demonstrated increased pulmonary vascular resistance after endotoxin, even when the lung was perfused with blood from which the leukocytes had been removed by filters. We, therefore, conclude that granulocytes are not directly responsible for the pulmonary hypertension by causing mechanical occlusion.

In terms of ventilatory parameters, the leukopenic and non-leukopenic monkeys response to endotoxin were similar except for the unexplained failure of the leukopenic animals to hyperventilate in response to the metabolic acidosis during shock. The observed increase in A-a gradients seen in both groups during the shock period are very difficult to interpret. Such a change might be expected to occur coincident to the fall in cardiac output, if perfusion to well-ventilated areas is proportionately more decreased than the perfusion to poorly ventilated areas. In addition, the fall in mixed venous  $pO_2$  (and thus the low  $pO_2$  of the blood involved in physiological shunts) might be sufficient to account for the increased gradient. Hemodynamically, the changes in cardiac output and systemic blood pressure were comparable in leukopenic and non-leukopenic animals given endotoxin.

Histologically, leukocytes are sparse on sections of pulmonary parenchyma from the leukopenic shock group in contrast to the endotoxin control animals. However, in spite of the small number of granulocytes seen in pulmonary capillaries, definite morphological abnormalities are present as evidenced by the endothelial membrane damage and perivascular space edema seen on multiple sections from the irradiated shock animals.

These findings are consistent with our previous observation that removal of leukocytes from the circulation by filtration (15) has little effect on the pulmonary hemodynamic and morphologic response to endotoxin. Those studies did not, however, exclude the possibility that leukocyte lysosomes were released

into the perfusate during the process of filtration. We subsequently examined the lysosomal hypothesis with a different experimental design. Since anti-inflammatory agents are inhibitors of lysosomal enzyme release in in vitro preparations (4,22), we attempted to prevent the pulmonary damage in endotoxic shock by pretreating animals with varying doses of corticosteroids or salicylates.

It is possible that only small numbers (i.e., in the range of our leukopenic animals) of granulocytes are required to produce the observed physiologic and morphologic abnormalities; however, in light of our previous investigations we believe that a more likely conclusion is that the sequestered granulocytes are not pathogenetically significant in the development of shock lung. A more attractive hypothesis is that endotoxin produces its damage in shock lung via activation of the complement system. Conceivably, the activated complement system may play the major role as biological effector with pulmonary granulocyte sequestration occurring subsequent to complements' generation of a polymorphonuclear chemotactic factor but being unnecessary for the production of tissue damage. The latter hypothesis would be supported by the studies of Spink and Vick (23) who were able to protect dogs against endotoxin shock by transfusion of blood in which an essential serum factor, presumably complement, was depleted by heating at 56°C for 30 minutes.

From our results in the present study we can conclude: (1) while total body irradiation prevented pulmonary leukocyte sequestration, it did not prevent the pulmonary capillary endothelial injury in endotoxic shock; (2) leukopenia was of no value in preventing the hemodynamic changes in animals given endotoxin.

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#### FIGURE LEGENDS

- Figure 1. Mean cardiac output ( $\pm$  SE) in Group I (leukopenic endotoxin group), Group II (endotoxin control group) and Group III (leukopenic control group). Values are plotted for the initial pre-shock study and 120 minutes after the induction of shock.
- Figure 2. Mean systemic arterial pressure ( $\pm$  SE) for Group I (leukopenic endotoxin group), Group II (endotoxin control group) and Group III (leukopenic control group). Values are plotted for the initial pre-shock study and 120 minutes after the induction of shock.
- Figure 3. Group I, Leukopenic Endotoxin Specimen. Low power micrograph in which the pulmonary vessels are seen to be devoid of cells. Sites of pulmonary edema with resultant rounding of alveolar spaces are seen. Hematoxylin and eosin, X 126.
- Figure 4. Group I, Leukopenic Endotoxin Specimen. Several red blood cells are seen within the capillary lumen (C). The endothelial cytoplasmic membrane is disrupted at several sites (arrows). A single platelet which is undergoing fragmentation is seen. The perivascular space (P) is widened and edematous. Within the alveolar space (A) there is evidence of mild pulmonary edema. The adjoining alveolar Type I epithelium is disrupted. Uranyl acetate and lead citrate, X 8,700.
- Figure 5. Group II, Endotoxin Control Specimen. Within the capillary lumen (C) several leukocytes, one degranulated, and red blood cells are seen. There are foci of leukocytic-endothelial sticking (arrow). The endothelial cytoplasmic membrane is disrupted at several points (double arrows). Within the alveolar spaces (A), evidence of pulmonary edema is present. Uranyl acetate and lead citrate, X 4,370.

Figure 6. Group II, Endotoxin Control Specimen. Low power micrograph in which a pulmonary vessel with abundant leukocytes is seen. Focal areas of pulmonary edema is present. The alveolar walls show increased cellularity. Hematoxylin and eosin, X 126.

Figure 7. Group III, Irradiated Control Specimen. Low power micrograph showing the paucity of leukocytes within the small pulmonary vessels. The alveolar walls are thin and attenuated. Hematoxylin and eosin, X 126.

Figure 8. Group III, Irradiated Control Specimen. The capillary lumina (C) contain red blood cells and one platelet. At one site mild peri-vascular space edema (P) is seen. The alveolar spaces (A) are free from transudate. Uranyl acetate and lead citrate, X 4,600.

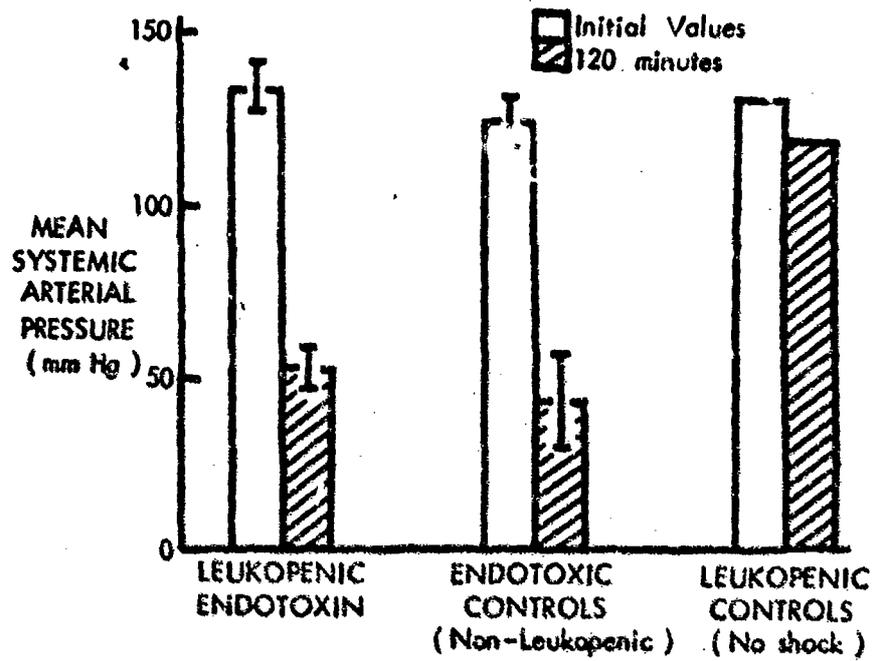


FIGURE 1

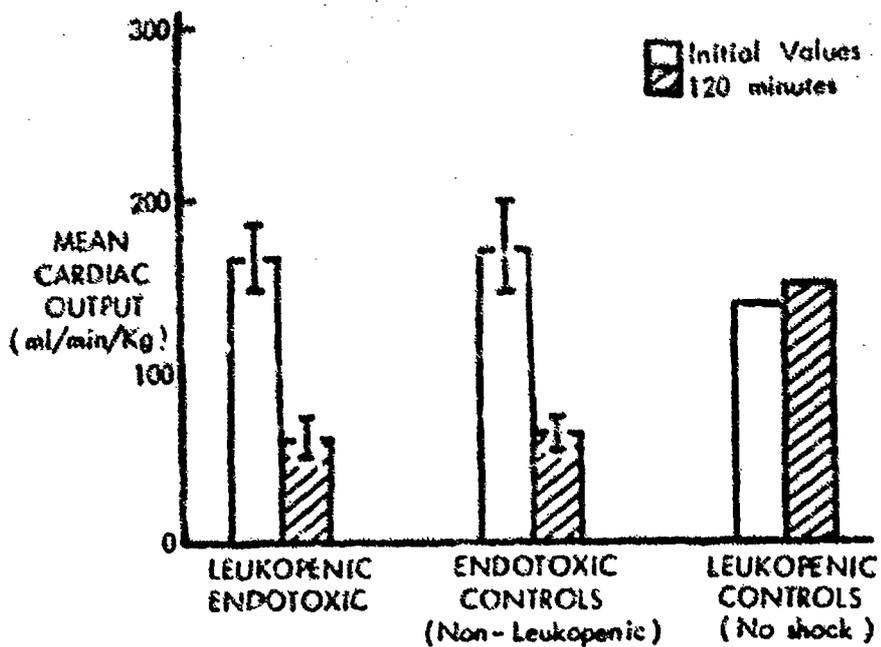


FIGURE 2 17







TABLE 1

	<u>Leukopenic</u>		<u>Endotoxic</u>		<u>Endotoxic</u>		<u>Control</u>	
	0'	120'	0'	120'	0'	120'	0'	120'
pH	mean SE	7.38 0.01	7.27 0.03	7.37 0.02	7.35 0.04	7.36 0.04	7.34	7.34
HCO <sub>3</sub> (mEq/L)	22 1.0	15.7 0.8	24 1.2	17.4 2.1	20	20	20	20
PCO <sub>2</sub> (mm Hg)	37.8 1.7	35.0 1.7	39.8	29.6	41.6	36	36	36
PO <sub>2</sub> (mm Hg)	66 4.2	68 4.5	82.4 2.6	80.8 2.5	69	83	83	83
VE (ml/min/kg)	170 20	170 20	210 20	290 26	180	240	240	240
VO <sub>2</sub> (ml/min)	6.2 0.8	3.9 0.5	5.7 0.6	5.1 0.4	5.9	6.8	6.8	6.8
A-a gradient (mm Hg)	27.2 3.2	36.1 5.0	16.6 3.9	32 2.9	18	15	15	15
WBC count/cu mm	358 113	100 34	10,550 425	3,475 1,275	145	30	30	30