LEUKOCYTE RESPONSE AND HYPOGLYCEMIA
CO-DETERMINANTS IN THE ALTERED SURVIVAL
TO SUPERLETHAL ENDOTOXIC SHOCK

Oklahoma University Health Sciences
Center, Oklahoma City

13 December 1976
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Gary L. White, Linda T. Archer, Beverly K. Beller,
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University of Oklahoma Health Sciences Center
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ABSTRACT

A prime goal of this laboratory has been to develop animal shock models more closely approximating septic shock in man. Anesthesia in dogs has been found to depress leukocyte concentration, body temperature, and central nervous system activities. This laboratory has documented a progressively developing hypoglycemia associated with systemic hypotension, hepatosplanchnic pathology and death in endotoxin-shocked dogs. Recent data documented accelerated uptake of glucose in blood following endotoxin, with certain components of the buffy coat responsible for the increased uptake. The present study explored the use of the awake dog as an improved shock model and assayed a possible protective role of leukocytes against the lethal effects of endotoxin. Experiments were conducted on unanesthetized healthy dogs with initial WBC counts between 7,000 and 20,000/mm³. Dogs were divided into paired groups: saline controls (Group I) and endotoxin experimentals (Group II). Group II animals (N=5) were injected intravenously with sublethal doses of E. coli endotoxin on Days 1 and 2, LD₁₀₀ on Day 3, and 2 X LD₁₀₀ on Day 4. The control group (N=5) received equal volumes of saline on Days 1, 2 and 3, but on Day 4 received an identical superlethal dose of endotoxin (2 X LD₁₀₀). Data document that the awake dog becomes febrile and exhibits initial leukopenia with subsequent marked leukocytosis in response to endotoxin. Lethal hypoglycemia is not seen in animals demonstrating leukocytosis on the day of superlethal endotoxin challenge, while animals with normal leukocyte counts die with low glucose concentrations (mean, 40 mg%). Results suggest that leukocytosis and sustained gluconeogenic function are important co-determinants of survivability to endotoxin shock.

KEY WORDS: endotoxin, leukocyte, febrile response, awake dog, glucose concentration, leukocytosis, phagocytosis, gluconeogenesis, shock model
The development of animal models for the study of endotoxin shock more closely approximating the state of septic shock in man has long been the goal of clinically oriented research laboratories. A persistent problem has been the adverse effects of surgical levels of anesthesia which question the validity of the majority of animal models for comparative clinical application. The use of anesthesia in dogs has been found to depress the white blood cell concentration (1), body temperature (2), and central nervous system activities (3).

Progressively developing hypoglycemia associated with systemic hypotension, hepatosplanchnic pathology and death has been observed in the dog subjected to endotoxin shock (4) and the cause has been proposed to be a depressant effect of endotoxin on liver function, specifically gluconeogenesis (5-10) combined with increased glucose need (11,12). Recent studies have demonstrated accelerated uptake of glucose in the blood following endotoxin, with certain components of the buffy coat responsible for the increased uptake (11,13). Endotoxin is efficiently phagocytized by polymorphonuclear leukocytes (14,15), and an associated increase in glucose utilization by neutrophils has been reported to occur (16,17).

The purpose of the present study was to explore the use of the awake dog as an improved shock model and to assay a possible protective role of leukocytes against the lethal effects of endotoxin. The unanesthetized dog model was utilized since it was considered to more closely parallel the pathophysiological alterations observed in human septic shock. Results reveal that the awake dog becomes febrile and exhibits initial leukopenia with subsequent leukocytosis in response to sublethal, lethal and superlethal doses of endotoxin. Lethal hypoglycemia is not seen in animals demonstrating leukocytosis at time of endotoxin challenge. Data are consistent with the view that leukocytosis and sustained liver gluconeogenic function are important co-determinants of
### Abstract

A prime goal of this laboratory has been to develop animal shock models more closely approximating septic shock in man. Anesthesia in dogs has been found to depress leukocyte concentration, body temperature, and central nervous system activities. This laboratory has documented a progressively developing hypoglycemia associated with systemic hypotension, hepatosplanchnic pathology and death in endotoxin-shocked dogs. Recent data documented accelerated uptake of glucose in blood following endotoxin, with certain components of the buffy coat responsible for the increased uptake. The present study explored the use of the awake dog as an improved shock model and assayed a possible protective role of leukocytes against the lethal effects of endotoxin. Experiments were conducted on unanesthetized healthy dogs with initial WBC counts between 7,000 and 20,000/mm³. Dogs were divided into paired groups: saline controls (Group I) and endotoxin experimentalns (Group II). Group II animals (N=5) were injected intravenously with sublethal doses of E. coli endotoxin on Days 1 and 2, LD100 on Day 3, and 2XLD100 on Day 4. The control group (N=5) received equal volumes of saline on Days 1, 2 and 3, but on Day 4, received an identical superlethal dose of endotoxin (2XLD100). Data document that the awake dog becomes febrile and exhibits initial leukopenia with subsequent marked leukocytosis in response to endotoxin. Lethal hypoglycemia is not seen in animals demonstrating leukocytosis on the day of superlethal endotoxin challenge, while animals with normal leukocyte counts die with low glucose concentrations (mean, 40 mg%). Results suggest that leukocytosis and sustained gluconeogenic function are important co-determinants of survivability to endotoxin shock.
survivability to endotoxin. The unanesthetized animal administered endotoxin was found to be a useful model for septic shock in man.

METHODS

Experiments were designed to follow the changes of the peripheral white blood cell (WBC) counts, rectal temperatures and blood glucose values during sublethal, lethal, and superlethal doses of *E. coli* endotoxin (Difco, Detroit) without the influence of anesthetics. Ten awake mongrel adult dogs of random sex, selected for freedom of clinical signs of disease, were used in the present study. Each dog was screened for microfilaria of heartworms and eliminated if positive and was treated for intestinal parasites. Animals were allowed a stabilization period of 3 to 6 weeks prior to use in the study. Only dogs with initial WBC counts between 7,000 and 20,000/mm$^3$ and hematocrits exceeding 37% were utilized.

Dogs were divided into paired saline control and endotoxin experimental groups (Groups I and II, respectively). The endotoxin group received sublethal doses of endotoxin of 0.003 mg/kg body weight on days 1 and 2 (i.e., 1/1,000 LD$_{100}$), 3 mg/kg on day 3 (i.e., LD$_{100}$) and a challenge dose of endotoxin of 6 mg/kg on day 4 (i.e., 2 X LD$_{100}$). The control group received equal volumes of saline on days 1, 2 and 3, but on day 4 received a 6 mg/kg challenge dose of endotoxin (i.e., 2 X LD$_{100}$). The LD$_{100}$ of *E. coli* endotoxin (3 mg/kg) had been previously established in a group of approximately 25 dogs. White blood cell counts, differential WBC, rectal temperatures and blood glucose were measured initially (i.e., before endotoxin or saline injection) and then each 60 to 90 minutes for 8 1/2 hours during each of the 4 days of observation. Animals surviving injection of endotoxin were sacrificed following a 30-day observation period.

The WBC counts were measured with an automatic particle counter (Coulter Z$^+_p$; Hialeah, Florida) and the differential WBC by microscopic
examination of blood smears stained with Wright's stain (100 cells counted). Blood glucose concentrations were determined using a Beckman glucose analyzer (Beckman Instruments; Fullerton, Calif.) with an accuracy of ±3 mg%, and rectal temperatures were obtained using a Tele-Thermometer probe (Yellow Springs Instruments; Yellow Springs, Ohio). The blood samples were obtained by venipunctures of either the cephalic or saphenous veins, then placed in vacutainers containing ethylenediamine-tetraacetic acid (EDTA; Becton, Dickinson, and Co.), and immediately placed on ice. The injection of saline or endotoxin was by the intravenous route utilizing either the cephalic or saphenous vein. The results were analyzed using the t test for paired or unpaired data.

RESULTS

Leukocyte, temperature and blood glucose alterations for days 1, 2 and 3 for both endotoxin experimental and saline control groups

Results from Figure 1 reveal significant increases (p<0.05) in rectal temperature at all time intervals between 60 and 510 minutes post-endotoxin injection for days 1 and 3 and from 60 through 240 minutes for day 2 when contrasted with the control group receiving saline instead of endotoxin. The initial mean WBC counts for the endotoxin group and saline control group were 12,840/mm³ and 12,540/mm³, respectively. Figure 2 demonstrates significant elevations of WBC count after endotoxin administration on days 1, 2 and 3 at 420 and 510 minutes (p<0.05) when compared with the saline control group.

Alterations in blood glucose concentration are arrayed in Figure 3. There were no significant changes in the blood glucose on days 1 and 2, but
on day 3 lower blood glucose values were observed at 240 through 420 minutes (p<0.05).

Leukocyte, body temperature and blood glucose responses to superlethal challenge of endotoxin

Figure 4 presents a comparison of changes in WBC count, rectal temperature and blood glucose that occurred on day 4, when both the experimental endotoxin and the saline control groups were administered 6 mg/kg of endotoxin (2 X LD_{100}). The endotoxin experimental group had an initial WBC count of 39,000/mm^3 at zero time on day 4, which is significantly elevated (p<0.001) when compared with its simultaneous time for the saline control group. Initial blood glucose values for the endotoxin group were not significantly different from either zero time on day 1 for the same group or zero time on day 4 for the saline group. WBC count was lowest (p<0.01) for both groups at approximately 5,000 WBC/mm^3 at 60 minutes post-endotoxin. From 60 through 510 minutes post-endotoxin, the WBC count progressively rose to near control values for the endotoxin group and to above control values for the saline group. On day 4 after injection of endotoxin, a significant difference in WBC count between the two groups occurred at 120 minutes (p<0.05). Significant febrile responses are seen with both groups from 60 through 180 minutes when compared with zero time (p<0.05). Comparing the two groups on day 4, a markedly elevated (p<0.005) rectal temperature was observed at 330 minutes in the saline group. The mean glucose concentration significantly fell (p<0.01) from 95 mg% at zero time on day 4 to 67 mg% at 120 minutes for the endotoxin experimental group. At 60 minutes after endotoxin injection, for the saline control group mean glucose concentration rose from 91 mg% to 108 mg%, and then progressively declined below the zero time control values until death. When comparing the two groups on day 4, the 60 and 120 minute measurements show that mean blood glucose for the experi-
mental group was lower (p<0.02) than the saline control group. Blood glucose concentration in the endotoxin experimental group steadily rose from 120 to 330 minutes, where it stabilized near control values.

Changes in differential leukocyte counts in endotoxin experimental and saline control groups

The responses of the total WBC, lymphocytes and mature and immature neutrophils are shown in Tables 1 and 2. The progressive leukocytosis (p<0.005) seen in the endotoxin group (Group II) on days 2 through 4 can be attributed to the increased numbers of mature neutrophils (p<0.05). In these animals, absolute leukocyte and lymphocyte counts remained constant days 1 through 4, while mature neutrophils increased (p<0.05) on day 2 and immature neutrophils decreased (p<0.025) on days 2 and 3. On day 4 in Groups I and II, the marked leukopenia (p<0.02) observed at 60 minutes post-endotoxin appeared to be a function of the depressed (p<0.02) mature neutrophil counts while depression of the immature neutrophils also occurred in the saline control group.

Mortality rates following superlethal challenge of E. coli endotoxin

Both groups received a 6 mg/kg (2 X LD_{100}) E. coli endotoxin after zero time values on day 4. In the endotoxin experimental group (Group II), 100% of the animals (N=5) survived the 2 X LD_{100} endotoxin challenge (30-day survival). In the saline control group all dogs (N=5) died within an average of 6 hours when challenged with 2 X LD_{100} endotoxin (see Table 3).

DISCUSSION

The unanesthetized animal model

The purpose of the present study was to explore the use of the unanesthetized dog as a model for septic shock and to determine the leukocyte and febrile response and changes in blood glucose concentrations. Results from the present experiments reveal a rapid leukopenia followed by a leukocytosis
in response to sublethal and lethal, as well as superlethal injections, of endotoxin. Results showed that animals receiving daily sublethal injections of endotoxin survive a superlethal challenge of endotoxin and are permanent survivors. Animals not receiving sublethal injections are very susceptible to the adverse effects of endotoxin, including the full pathophysiological impact of shock, and die in an average time of 6 hours. The unanesthetized animal model described in this study appears to be well suited for application and comparison of findings with septic shock in man.

Response of the white blood cell to endotoxin

Mulholland and Cluff reported that endotoxin causes granulocytes to adhere to the capillary endothelial cells and then later leave the circulation and move into the tissue (18). These findings corroborate the data in the present study and would explain the early leukopenia seen post-endotoxin. Leukocytosis associated with endotoxin is reported to occur via entry of new leukocytes from the bone marrow into the circulation (19). The circulating neutrophils have been demonstrated to be the key cellular components in the clearance of bacterial organisms from the blood of dogs (20). Blood from endotoxin-treated rabbits has been shown to possess a significant phagocytic capacity (18) and leukocytes were found to detoxify endotoxin in vitro in rabbit serum (21). Glucose has been demonstrated to be the substrate essential for phagocytosis by polymorphonuclear leukocytes (22).

Animals in the experimental group exhibited a marked leukocytosis (39,000/\text{mm}^3) after endotoxin by day 4, and the increased white cell count can be mainly accounted for by increased numbers of neutrophils. In the present study all of the animals in the endotoxin-pretreated group survived the massive endotoxin challenge while every animal in the control group succumbed to the identical challenge dose. Since Balis et al. (14) and Cline et al. (15) have documented that neutrophils phagocytize endotoxin, the increased numbers of neutrophils
seen in the present study may have effected an efficient and rapid clearance of endotoxin leading to the 100% survivability results. Efficient removal of endotoxin was further suggested in the present experimental group since the animals exhibited minimal clinical signs of endotoxin administration and were eating and drinking normally within 9 hours post-endotoxin.

**Blood glucose changes after endotoxin: Role of hypoglycemia**

Recent studies in dogs administered endotoxin have documented the development of hypoglycemia concomitant with the systemic hypotension, hepatosplanchnic pathology and death (4). Hypoglycemia has been attributed to depressed glucose production due to defective hepatic function (5-10), while accelerated uptake of glucose by the blood has recently been reported (13). Early hypoglycemia seen in the endotoxin-pretreated animals of the present study in response to the 2 X LD_{100} of endotoxin may suggest an early increased glucose uptake in response to leukocyte phagocytosis.

Hypoglycemia has been reported to play a significant role in the pathophysiology of endotoxin shock (8,11-13). Recent studies from this laboratory have documented a positive correlation between levels of blood glucose and survival in endotoxin shock (12,13). Further, the early hyperglycemia seen in various shock states has been attributed to sympathetic-stimulated glycogenolysis as well as alpha-adrenergic suppression of beta cell insulin release in the pancreas (8,23). Clearly, the "unprotected" saline group responded with a significant hyperglycemia during the first hour after superlethal endotoxin challenge, suggesting a greater stress to this group. At 5 1/2 hours post-endotoxin, the significantly decreased blood glucose level of the saline animals suggests an impairment of liver gluconeogenesis capacity, as has been reported by Groves et al. and Filkins et al. in endotoxin shock (7,8).

The recovery from hypoglycemia observed in the endotoxin experimental group indicates that the ability of the liver to perform gluconeogenesis in response
to superlethal endotoxin challenge has been protected. Normoglycemia was observed in the "protected" animals in contrast to hypoglycemia in the saline control dogs at 5 1/2 hours post-endotoxin. The lethal hypoglycemia previously observed in the laboratory in canine endotoxin shock was progressive but animals reached lethal levels of 12 mg% at 5 hours post-endotoxin. The sharp contrast observed in blood glucose concentration between the two groups and the 100% survival versus 100% mortality seen in the two groups support a liver-sparing role of the circulating neutrophil.

**Temperature response to endotoxin**

Data from the present experiments in unanesthetized dogs show significant increases in rectal temperature with a mean range from +1.4 to 3.2°C above control values. The rise in temperature in the endotoxin "protected" group corresponds with the time when blood glucose concentrations in these animals were decreasing, suggesting that fever may add an additional drain to the available glucose. Studies have shown that the neutrophil can release a pyrogen which will produce a febrile response in rabbits when administered endotoxin (24). Other reports found evidence that endotoxin produces fever by direct action upon the brain (25). It is known that anesthetized dogs in endotoxin shock become progressively hypothermic; while major trauma patients as well as septic animals and man spike fevers in the disease course (26,27). The awake dog thus appears to be a more valid model for application to the clinical septic state, since marked febrile responses were seen following both sub- and superlethal injections of E. coli endotoxin. Furthermore, leukopenia, followed by leukocytosis concomitant with the febrile responses, appears to more closely approximate changes seen in the septic patient.

**Role of the RES and white blood cell in endotoxin shock**

(a) **Tolerance.** Review of the literature describes the elicitation of a tolerant state following sublethal injections of endotoxins (28-30). Greisman
and Hornick have suggested possible mechanisms by which man develops tolerance to bacterial endotoxins and have separated the tolerant condition into two phases (30). Early tolerance appears to be mediated by a non-antibody mechanism entailing a transiently occurring refractory state of mainly the hepatic macrophages (Kupffer cells), while late tolerance appears to be mediated by anti-endotoxin antibodies directed against both O and common core antigens. At present there is no clear consensus of opinion on the role of the reticuloendothelial system (RES) and the peripheral cellular components on increased tolerance and resistance to endotoxin. Beeson (31) found that an increased resistance to endotoxin occurs because of increased functional capacity of the RES to remove bacterial toxins unassociated with antibody formation. Tolerance to endotoxin involves acceleration of the degrading and detoxifying systems which exist in circulating blood as well as other factors (28,30,32,33) and it is not transferred passively with serum from resistant animals (32). It was not the purpose of this laboratory to re-evaluate the mechanisms involved in tolerance but rather to try to determine the importance of leukocytes in the total host-defense mechanism. Leukocytes have been reported to exert a significant role in phagocytosis, although serum and opsonins may also perform key roles (21,34). It is known that active phagocytosis requires increased substrate in the form of glucose (16,17), but it may be the most effective defense against the hepatic dysfunction observed in shock. A study utilizing radioactively-labelled endotoxin traced its passage from the plasma to the buffy coat and finally to the liver during the early clearance period associated with leukopenia, fever and diarrhea (35). If phagocytosis in the buffy coat were sufficiently effective, it seems that the liver could be spared the added stress of endotoxin detoxification (6) and the possible depression of gluconeogenic function from the action of endotoxin (7-9). Fukuda and Akiyama (36) propose that
it is not an increased RES activity but a greater stability of carbohydrate metabolism that provides the tolerance to endotoxin. Again in the present study, the return of blood glucose concentration to normal in the "protected" group reveals a possible association of leukocytosis with preserved liver gluconeogenic function, thus melding increased RES (circulating and sessile) function and normal carbohydrate metabolism.

(b) Therapy. This study suggested the protective influences of leukocytosis stimulated by sublethal injections of endotoxin. Since survivability seemed to be associated with leukocytosis, there are possible therapeutic implications regarding the role of the white blood cell in endotoxin shock. Recent reports have described beneficial effects of transfused white blood cells as a treatment for septicemia in neutropenic patients (37) and dogs (38,39). Granulocyte transfusions markedly reduced the number of circulating Pseudomonas aeruginosa bacteria in dogs, as contrasted to non-transfused controls (39). Leukopenic dogs in E. coli septicemia when given leukocyte transfusion had prolonged periods of sterile blood cultures and extended survival times compared with leukopenic septic controls (38). Graw et al. (37) reported a significant increase in survival rate in patients with gram-negative sepsis when transfused with granulocytes compared with septic patients. Administration of hypertonic glucose has resulted in an increased clearance of E. coli from the blood of dogs (40), suggesting that supplying substantial metabolic support for accelerated leukocyte activity may enhance survival in septic shock. By using the awake dog as an improved shock model and by studying the febrile and leukocyte response to varying doses of endotoxin, we hoped to observe a combination of clinical signs that would be a clue as to the appropriate time for initiating treatment via activation of natural host-defense mechanisms or supplying the host's first natural line of defense (41) against infection—leukocytes via transfusion.
REFERENCES


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\[a\] Saline pretreated control group (Group I). On Days 1, 2 and 3 saline was administered intravenously in volumes equal to the endotoxin given to experimental animals. On Day 4, 2 x LD\(_{100}\) (6 mg/kg) \textit{E. coli} endotoxin was injected. All values represent mean±SE.

\[b\] Initial measurements in each group on four consecutive days. Day 4 mean±SE serves as the control measurement for the time designations of hours.

\[c\] P values represent paired comparisons from Day 1 control values through the time designations of days. Through the time designations of hours, p values are paired from Day 4 control value.
Table 2. Effects of *E. coli* endotoxin on leukocyte, mature neutrophil, lymphocyte and immature neutrophil counts

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**Immature Neutrophils**

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*p* values represent paired comparisons from Day 1 control values through time designations of days. Through the time designations of hours, *p* values are paired from Day 4 control value.

*a* Endotoxin pretreated experimental group (Group II); E. coli endotoxin administered intravenously Days 1 and 2, 1/1,000 LD$_{100}$ (0.003 mg/kg); Day 3, LD$_{100}$ (3 mg/kg); and Day 4, 2 x LD$_{100}$ (6 mg/kg). All values represent mean±SE.

*b* Initial measurements in each group on four consecutive days. Day 4 mean±SE serves as the control measurement for the time designations of hours.

*c* *p* values represent an unpaired comparison from saline pretreated control group.
Table 3. Lethality response to superlethal doses of *E. coli* endotoxin$^a$

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$^a$2 X LD$_{100}$ endotoxin (6 mg/kg) injected intravenously on Day 4.

$^b$On Days 1, 2 and 3 saline was administered intravenously in volumes equal to the endotoxin given to experimental animals.

$^c$*E. coli* endotoxin administered intravenously; Days 1 and 2, 171,000 LD$_{100}$ (0.003 mg/kg) and Day 3, LD$_{100}$ (3 mg/kg).
FIGURE LEGENDS

Figure 1. Effect of intravenous administration of *E. coli* endotoxin on rectal temperature (T_r - °C) in dogs. Results (mean±SE) from 10 dogs are depicted in two groups: saline controls (n=5) and endotoxin experimental (n=5). Experimental animals received sublethal doses of *E. coli* endotoxin; 1/1,000 LD_{100} on Days 1 and 2 and LD_{100} on Day 3. The control group received equal volumes of saline on Days 1, 2 and 3. P values represent an unpaired comparison between control and experimental groups.

Figure 2. Effect of intravenous administration of *E. coli* endotoxin on white blood cell concentration (mm$^3$) in dogs. Results (mean±SE) from 10 dogs are depicted in two groups: saline controls (n=5) and endotoxin experimental (n=5). (See Figure 1 for details of experiments.)

Figure 3. Effect of intravenous administration of *E. coli* endotoxin on blood glucose concentration (mg%) in dogs. Results (mean±SE) from 10 dogs are depicted in two groups: saline controls (n=5) and endotoxin experimental (n=5). (See Figure 1 for details of experiments.)

Figure 4. Effect of intravenous pretreatment with *E. coli* endotoxin on rectal temperature (°C), white blood cell concentration, and blood glucose concentration following 2 X LD_{100} *E. coli* endotoxin (6 mg/kg). Results (mean±SE) from 10 dogs are depicted; 5 from experimental group pretreated with sublethal and lethal doses of endotoxin ** and 5 control animals receiving saline injections in place of endotoxin *. Both groups received 2 X LD_{100} endotoxin on day of challenge (Day 4). P values represent an unpaired comparison between control and experimental group.
FIGURE 3
FIGURE 4
FIGURE 5