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EFFECT OF ENDOTOXEMIA AND GRAM-NEGATIVE SEPSIS ON HOST PERIPHER--ETC(U)  
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| Previous studies demonstrated that luminol-assisted endogenous polymorphonuclear leukocyte (PMN) chemiluminescence (CL) was elevated in rats and guinea pigs infected with live vaccine strain <u>Francisella tularensis</u> , but not in guinea pigs infected with Pichinde virus. To evaluate further the diagnostic potential of the CL phenomenon for bacterial infections, PMN CL was measured following challenge of rats with a gram-negative endotoxin-bearing microorganism, <u>Salmonella typhimurium</u> or <u>Escherichia coli</u> endotoxin. Rats inoculated with $10^7$ or $10^8$ live |                       |                                                             |

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Effect of Endotoxemia and Gram-Negative Sepsis on Host  
Peripheral Granulocyte Endogenous Chemiluminescence<sup>1</sup>

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<sup>1</sup>In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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Abstract. Previous studies demonstrated that luminol-assisted endogenous polymorphonuclear leukocyte (PMN) chemiluminescence (CL) was elevated in rats and guinea pigs infected with live vaccine strain Francisella tularensis, but not in guinea pigs infected with Pichinde virus. To evaluate further the diagnostic potential of the CL phenomenon for bacterial infections, PMN CL was measured following challenge of rats with a gram-negative endotoxin-bearing microorganism, Salmonella typhimurium or Escherichia coli endotoxin. Rats inoculated with  $10^7$  or  $10^8$  live or heat-killed S. typhimurium had a 6-7-fold increase in endogenous PMN CL 24 hr postinjection compared to saline controls. By 72 hr endogenous PMN CL measured from rats injected with heat-killed S. typhimurium returned to saline control values. Endogenous PMN CL measured from rats injected with live bacteria remained significantly elevated for 7 days. A progressive 9-29-fold increase in endogenous PMN CL was also measured during the 48 hr after injection of 250  $\mu$ g E. coli endotoxin. PMN CL response to endotoxin was linearly dose-dependent between 1.0 and 100  $\mu$ g. Results suggest that elevated endogenous PMN CL measured following injection of the gram-negative bacteria S. typhimurium may be partially attributed to its endotoxin. Further, these data, combined with results from other studies, suggest that the elevated endogenous PMN CL response may be stimulated in part by hormonal factors, such as leukocyte endogenous mediator, released following injection of bacteria or the administration of endotoxin.

The primary function of phagocytic polymorphonuclear leukocytes (PMN) is to protect the host against infection by ingesting and destroying invading microorganisms. When exposed in vitro to opsonized zymosan (1), bacteria (2), virus (3), or soluble stimuli (4, 5), PMN emit light, a phenomenon termed chemiluminescence (CL). Stimulation of the PMN activates membrane-associated nicotinamide adenine dinucleotide phosphate (NADP) oxidase causing a shift in the  $\text{NADPH}/\text{NADP}^+$  ratio (6, 7). Elevated concentrations of  $\text{NADP}^+$  stimulate the hexose monophosphate shunt pathway (2) resulting in increased nonmitochondrial oxygen consumption and leading to activation of a series of reactions which produce the microbicidal agents singlet oxygen ( $^1\text{O}_2$ ), superoxide anion ( $\text{O}_2^{\cdot -}$ ), hydroxyl radical ( $\cdot\text{OH}$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) (8-11). This series of events has been collectively termed the respiratory burst.

The PMN CL phenomenon has been attributed to the release of energy following the relaxation of  $^1\text{O}_2$  to triplet state (2); however, the exact origin of the CL is still controversial. The cyclic hydrazide, luminol, can be used effectively to amplify the CL response (12-14). Luminol reacts with  $^1\text{O}_2$ ,  $\text{O}_2^{\cdot -}$ ,  $\cdot\text{OH}$  and  $\text{H}_2\text{O}_2$  resulting in the production of the excited aminothalate ion which relaxes to ground-state anion while releasing energy as photons.

Previous studies from this laboratory (15) demonstrated for the first time that PMN isolated from rats and guinea pigs infected with various doses of live vaccine strain Francisella tularensis had significantly elevated endogenous luminol-assisted CL compared to noninfected controls. However, guinea pigs infected with Pichinde virus did not have an elevated PMN CL response. These results prompted us to perform additional studies measuring endogenous rat PMN CL following infection with a gram-negative endotoxin-containing microorganism,

Salmonella typhimurium, or injection of Escherichia coli lipopolysaccharide (LPS) to evaluate further the CL phenomenon. Consistent with our earlier studies, results suggest that endogenous PMN CL may be a valuable aid for differentiating bacterial and viral infection in the host.

Materials and Methods. Animals. Male, Fisher Dunning rats weighing 200-300 g were purchased from Harlan Industries, Indianapolis, Indiana, or M.A. Bioproducts, Walkersville, Maryland, and housed five per cage in a room lighted from 6 a.m.-6 p.m. and maintained at 22-24°. Rats were fed and watered ad libitum before and during all experimental procedures.

Infection and endotoxin administration. Rats were injected ip with varying doses of live or heat-killed S. typhimurium ( $10^7$ - $10^8$ /100 g body weight, BW). S. typhimurium cultures were prepared as previously described (16) and bacteria were heat-killed at 68° for 1 hr using a water bath (17). Control animals received equal volumes of tryptose-saline culture medium ip. Other rats were injected ip with varying doses (0.01-250 µg per 100 g BW of E. coli (LPS). Lipopolysaccharide (Difco Laboratories, Detroit, Mich.) was resuspended in sterile water prior to injection. Control rats were injected ip with equal volumes of sterile water. Rectal temperature was used as an indicator of illness during these studies.

Cell isolation procedure. Whole blood collection and PMN isolation were performed as previously described (15). Briefly, peripheral PMN were isolated from the combined heparinized whole blood (10-45 ml) of control and experimental groups containing 3-8 rats by dextran sedimentation and ficol-hypaque centrifugation. Excess red blood cells were removed

by NaCl lysis; total and differential cell counts were obtained by hemocytometer. Final cell suspensions contained at least 80% PMN with greater than 99% viability as determined by trypan blue dye exclusion (4).

Measurement of luminol-assisted CL. Endogenous PMN CL measurements were performed as previously described (15) using a tricarb liquid scintillation spectrometer, model 3375 (Packard Instrument Co., Downers Grove, Illinois). Chemiluminescence was measured for a minimum of 9 min; results are expressed as cpm. Data in the figures represent the mean  $\pm$  SEM of at least three assays using a single pool of PMN. Standard error bars were omitted when the values were too small to be clearly shown in the figures.

Statistical analysis. Significance of differences between experimental and control group means was determined by analysis of variance.

Results. Bacterial infection. The temporal endogenous PMN CL response 24 hr following the injection of  $10^7$  living or killed S. typhimurium is presented in Fig. 1. Previous studies (data not reported) have shown that injection of  $10^7$  S. typhimurium in rats causes 10% mortality within 6 days. Endogenous PMN CL measured from rats injected with live or heat-killed bacteria was significantly elevated ( $P < 0.01$ ) compared to tryptose-saline control values. At the time of cell isolation, infected rats had significant fever ( $38.2 \pm 1.1^\circ$ ,  $P < 0.01$ ) compared to rats injected with heat-killed bacteria ( $37.3 \pm 0.1^\circ$ ) or tryptose-saline culture medium ( $37.0 \pm 0.1^\circ$ ).

Fluctuations in the endogenous PMN CL response during the course of the S. typhimurium infection were measured in a longitudinal study. Rats were injected with  $10^8$  live or heat-killed bacteria; endogenous PMN CL was measured at various time intervals for 13 days. The dose of microorganisms was increased 10-fold in this study to augment the severity of the infection. Compared to saline control values ( $37.4 \pm 0.1^\circ$ ) a significant fever ( $P < 0.01$ ) was measured in rats 24 hr postinjection of live ( $38.7 \pm 0.2^\circ$ ) or heat-killed ( $38.5 \pm 0.1^\circ$ ) bacteria (Fig. 2A). Infected rats remained febrile through day 7, becoming hypothermic on day 9. Rats injected with heat-killed bacteria were afebrile by day 3 ( $37.6 \pm 0.2^\circ$ ). Maximum or peak endogenous PMN CL measured from infected rats was significantly elevated ( $P < 0.01$ ) compared to saline control values throughout the study (Fig. 2B). Rats injected with heat-killed bacteria had significantly elevated endogenous PMN CL ( $P < 0.01$ ) 24 hr postinjection; however, the CL response returned to control values by day 3. Rats injected with live S. typhimurium sustained a 65% mortality by termination of the study (Fig. 2C).

Endotoxin administration. Significant fever ( $P < 0.01$ ,  $39.1 \pm 0.2^\circ$ ) was measured from rats 24 hr postinjection of E. coli LPS (250  $\mu$ g) compared with sterile water controls ( $38.3 \pm 0.1^\circ$ ). Endogenous PMN CL was significantly elevated ( $P < 0.01$ ) 5 hr postinjection of LPS reaching a maximum response at 48 hr (Fig. 3). By 72 hr, endogenous PMN CL was approaching control values.

The elevated endogenous rat PMN CL response to LPS 24 hr postinjection was dose-dependent between 1.0 and 100  $\mu$ g (Fig. 4). PMN CL was maximally stimulated with 100-250  $\mu$ g LPS ( $P < 0.01$ ) compared to control values. Significant fever ( $P < 0.01$ ;  $39.1 \pm 0.1^\circ$ ) was measured 24 hr postinjection from rats injected with 100  $\mu$ g LPS. All other groups were afebrile at

the time of cell isolation. The 250- $\mu$ g dose of LPS caused 20% mortality in the experimental group within 24 hr.

Discussion. Recent technological advances and increasing interest in the potential diagnostic capabilities of CL have stimulated investigations examining the possible applications of CL for differentiating infections in the host. Mills et al. (1) described a luminol-assisted PMN CL assay for identifying heterozygote chronic granulomatous disease patients. Konishi et al. (18) used a CL-linked immunoassay to detect mumps virus antibodies in human serum. Studies by McCarthy et al. (15) demonstrated that host endogenous PMN CL was significantly elevated during live vaccine strain F. tularensis infection in rats and guinea pigs, but remained within control values during Pichinde virus infection in guinea pigs. Consistent with our earlier bacterial studies (15), rats infected with S. typhimurium had a significantly elevated endogenous PMN CL response compared to saline controls. Further, the time course of enhanced endogenous PMN CL during prolonged infection suggests a possible relationship between the CL response and the course of the disease.

The elevated endogenous PMN CL response measured from rats following injection of heat-killed S. typhimurium was relatively brief and may be attributed, at least in part, to the endotoxin associated with the bacterial inoculum. Endotoxins have a high affinity for biological membranes associating with them by edge attachment (19). Incorporation of endotoxins into the membrane may cause physiological changes which could initiate a variety of biological effects. For example, the procoagulant activity of leukocytes is stimulated following endotoxin injection (20); results presented here demonstrate that rats injected with endotoxin have a prolonged elevated endogenous PMN CL response

which appears to be dose-dependent. However, the mechanism(s) involved in generating the endotoxin- and bacteria-induced increase in endogenous PMN CL in vivo remains unclear. Studies by Nolan (21) demonstrated that endotoxin (100 µg) was rapidly cleared from rat serum during the initial 24 hr following ip injection. Although we did not quantitate the serum endotoxin levels, these results suggest that rats injected with a comparable dose of endotoxin (250 µg) had maximum endogenous PMN CL stimulated when circulating endotoxin was apparently at a low level (48 hr postinjection). Several possible explanations of these results include the stimulation of PMN oxidative metabolism by humoral factors, such as leukocyte endogenous mediator (LEM), released as a physiological response to the endotoxin injection and resulting in the elevated CL response. Pekarek et al. (22) showed that LEM is released into the serum shortly after injection of endotoxin, bacteria, or other inflammatory stimuli. Recent studies (23) have also demonstrated that highly purified human leukocytic pyrogen stimulated the oxidative metabolism of human PMN in vitro and rabbit PMN in vivo. In addition, purified and partially purified LEM stimulated rat PMN CL in vitro and in vivo, respectively (24). Furthermore, recent experiments have indicated that the endogenous PMN CL response following the injection of endotoxin or heat-inactivated S. typhimurium is significantly elevated ( $P < 0.01$ ) compared with the CL response from PMN exposed in vitro to equivalent amounts of either substance (data not shown). Whether the elevated endogenous PMN CL response following injection of bacteria or endotoxin is the result of direct interaction of host phagocytic cells with these substances and/or circulating humoral factors released following their injection remains unclear at this time. However, these studies suggest that the transient resistance to certain infections observed in animals

several hours following pretreatment with endotoxin (25) or LEM (24, 26) may be partially due to the stimulation of the phagocytic parameters in PMN.

These preliminary studies also suggest that host endogenous PMN CL may be a viable alternative to the controversial nitroblue tetrazolium (NBT) test. The NBT test, introduced by Park et al. in 1968 (27), was described as a new, simple means for differentiating certain types of bacterial infection from nonbacterial diseases. However, follow-up studies (28-30) suggest that the NBT test has a number of serious deficiencies. The production of luminol-assisted CL and the reduction of NBT by PMN are dependent on the respiratory burst of the cell (30, 31). Studies indicate that both methods are at least partially dependent on the production of the microbicidal agent  $O_2^-$  (11, 30). Although more studies are required, our preliminary results suggest that the PMN CL assay appears to be a more objective method compared to the NBT test for differentiating bacterial infections from nonbacterial diseases. Furthermore, fluctuations noted in the PMN CL response during the course of the bacterial infections (15) indicate that the CL assay may have a possible application in the prognosis of infectious diseases.

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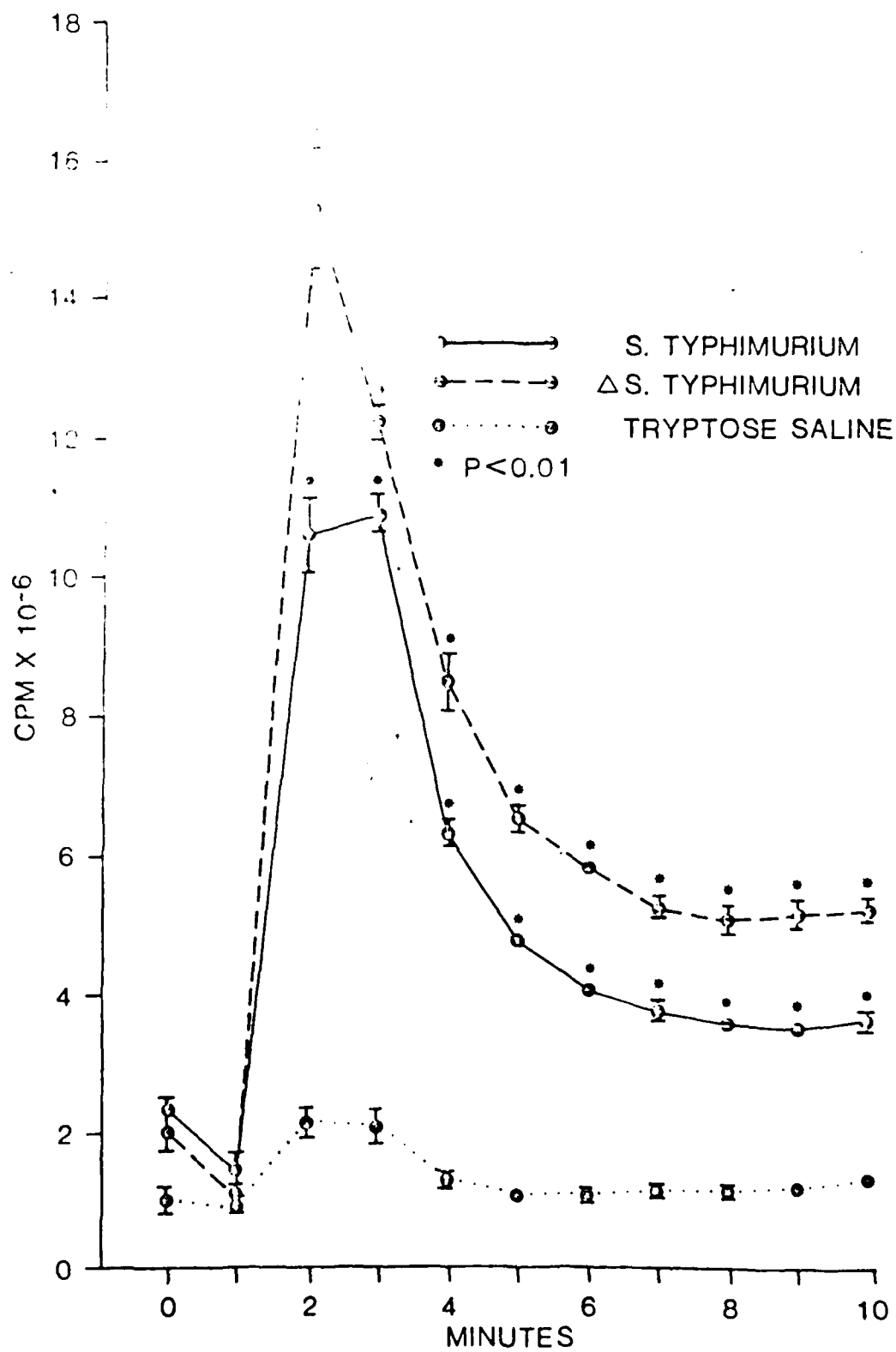
## Figure Legends

FIG. 1. Temporal luminol-assisted CL measured from  $10^6$  rat PMN 24 hr after ip injection of  $10^7$  live or heat killed (  $\Delta$  ) S. typhimurium/100 g BW. CL data represent the mean  $\pm$  SEM of 3 replicate assays using PMN isolated from experimental groups containing 8 rats. \*  $P < 0.01$ , compared to tryptose saline control.

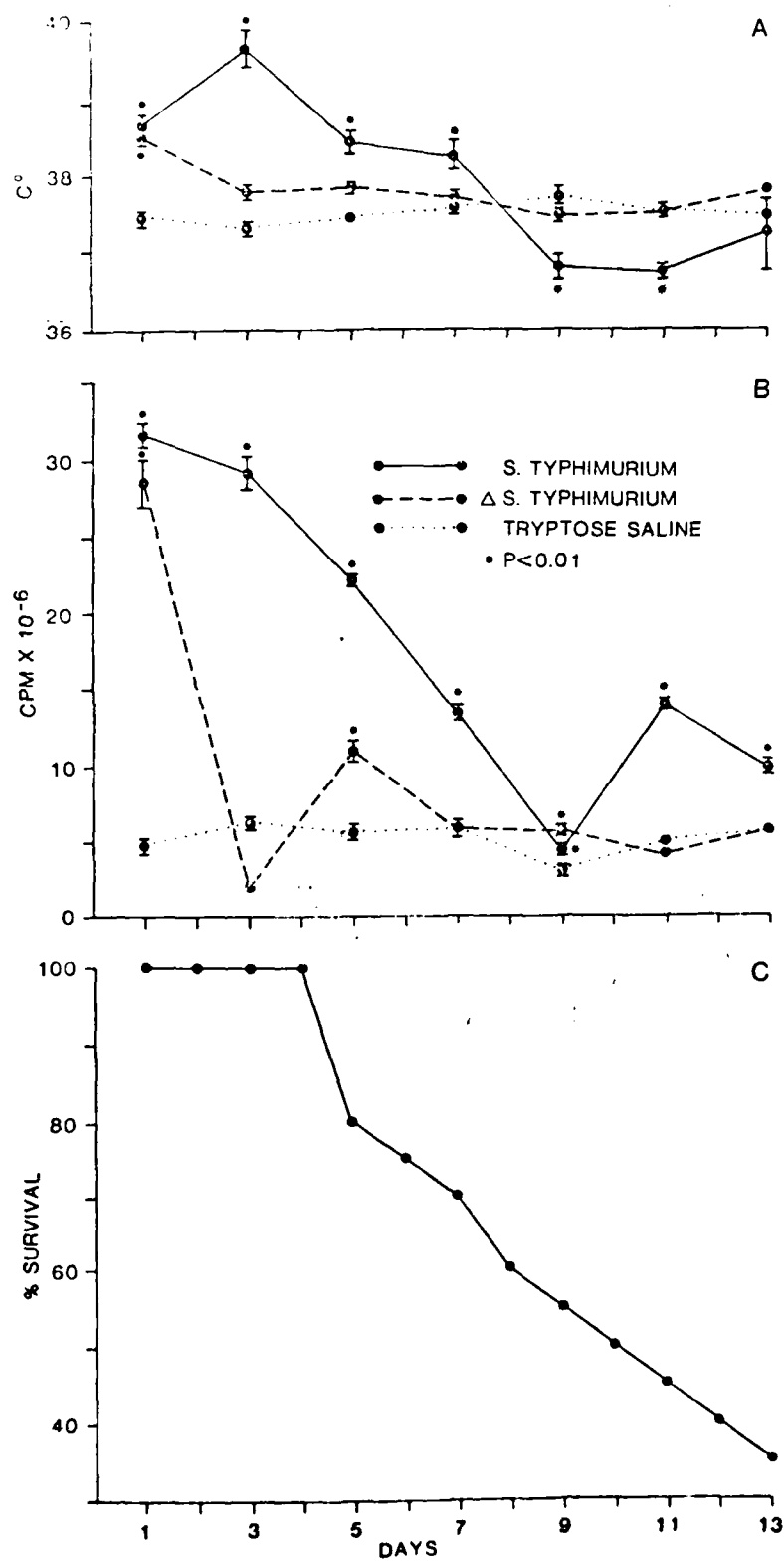
FIG. 2. Observations of rats during the 13-day period following ip injection of  $10^8$  S. typhimurium/100 g BW. (A) Rectal temperatures of 5 rats; (B) Mean peak luminol-assisted PMN CL of 3 assays of groups of 3 rats; (C) Cumulated mortality rate of 20 rats given live organisms.

FIG. 3. (A) Temporal luminol-assisted CL measured from  $10^6$  rat PMN at 5, 24, 48 and 72 hr after ip injection of 250  $\mu$ g LPS/100 g of BW. Each point represents the mean  $\pm$  SEM of 3 assays. PMN were isolated from the pooled whole blood of 5 rats. (B) Peak luminol-assisted PMN CL values from data shown in A. \*  $P < 0.01$  compared to controls.

FIG. 4. (A) Temporal luminol-assisted CL measured from  $10^6$  rat PMN 24 hr after injection of 0.01, 1.0, 10, 100 or 250  $\mu$ g LPS/100 g BW. Each point represents the mean  $\pm$  SEM of 3 assays. PMN were isolated from the pooled whole blood of at least 4 rats. (B) Peak luminol-assisted PMN CL values from data shown in A. \*  $P < 0.01$ .

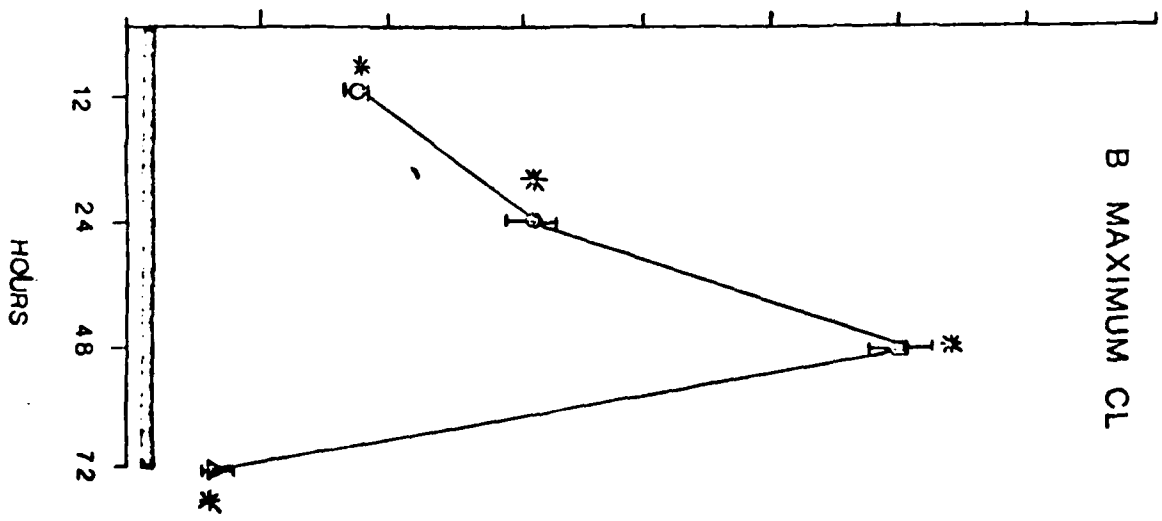
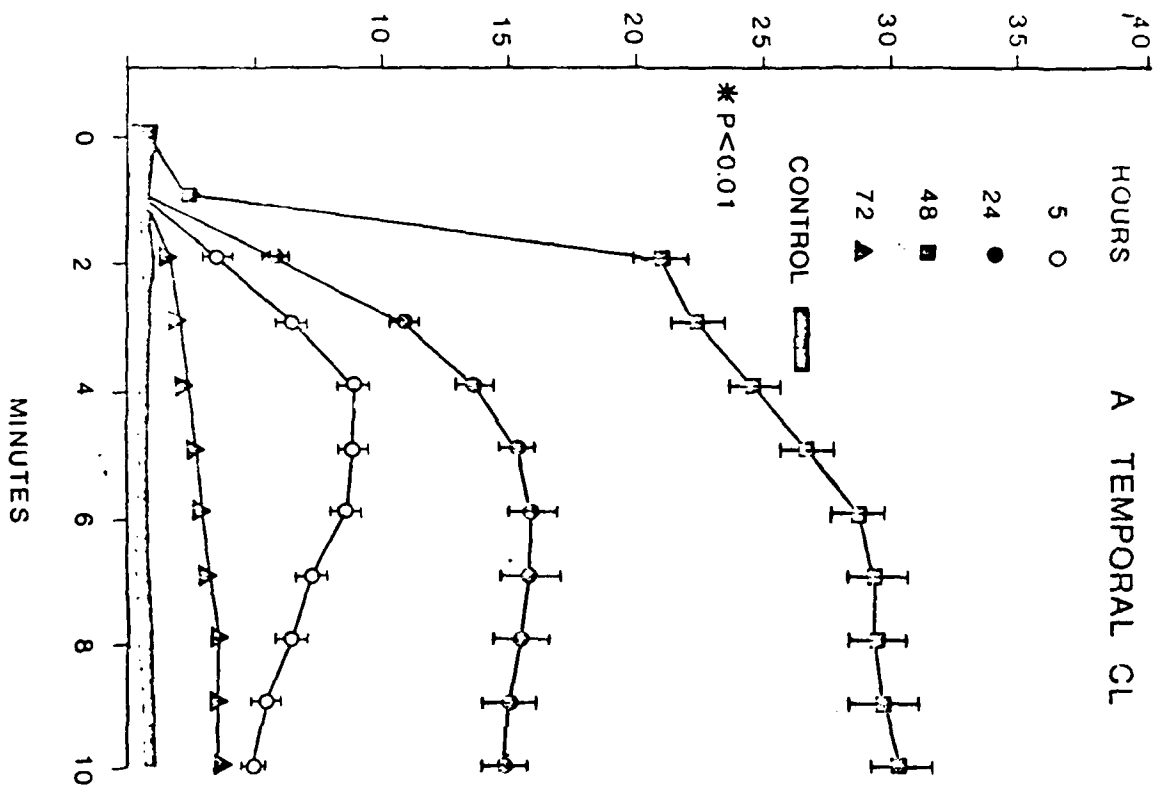


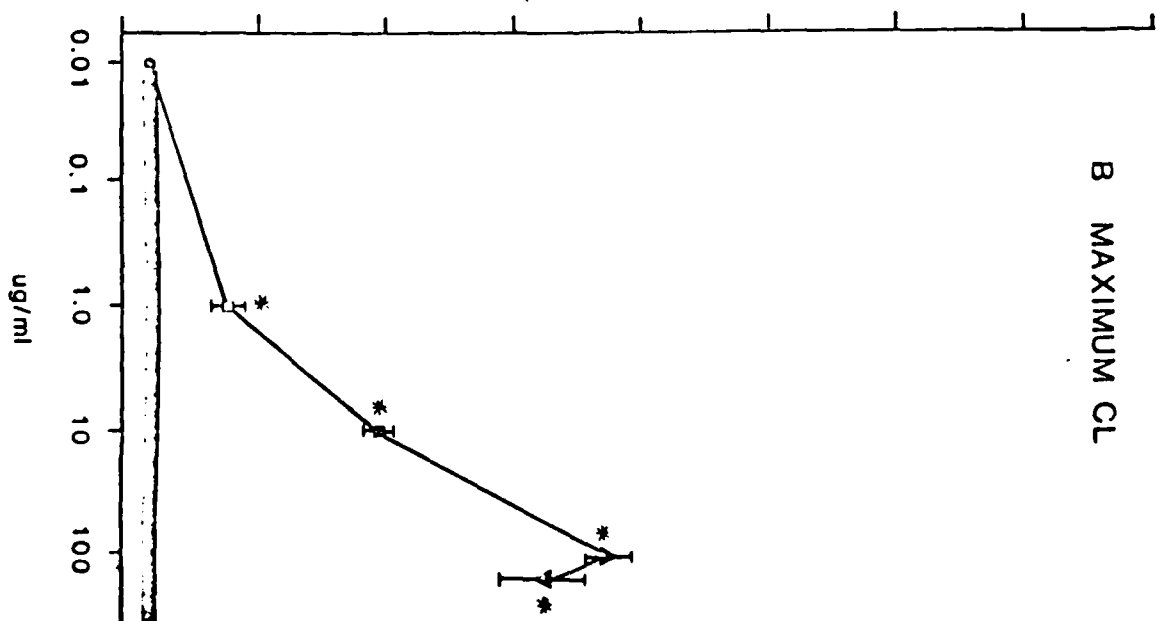
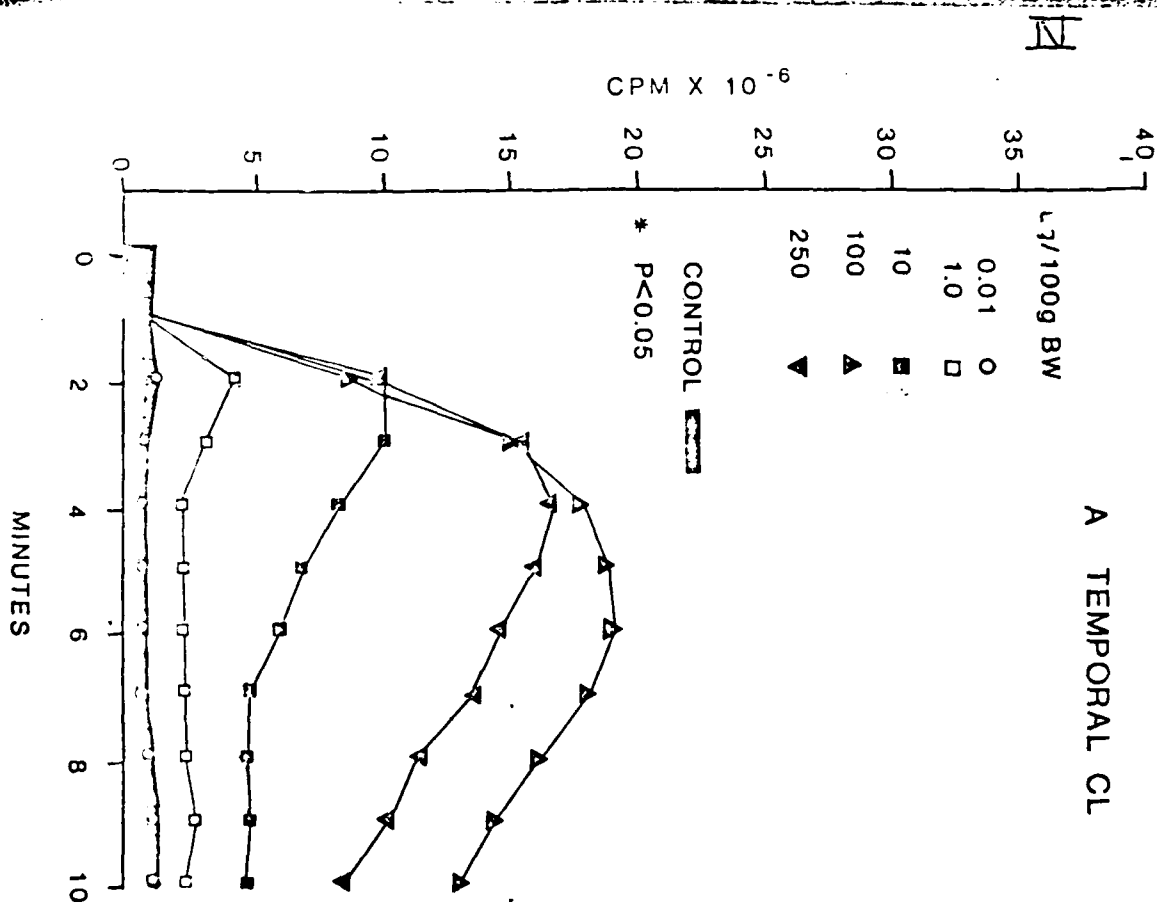
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