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Continuous Infusion of *Escherichia coli* Endotoxin In Vivo Primes In Vitro Superoxide Anion Release in Rat Polymorphonuclear Leukocytes and Kupffer Cells in a Time-Dependent Manner

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Continuous infusion of a nonlethal dose of *Escherichia coli* lipopolysaccharide (LPS) (0.5 mg/kg) induced early (3 h) accumulation of polymorphonuclear leukocytes (PMNL) in rat liver followed by later (30 h) greater extravasation of mononuclear phagocytes (MNP) (E. B. Rodriguez de Turco and J. A. Spitzer, J. Leukocyte Biol. 48:488–494, 1990). Nonparenchymal liver cells from rats treated for 3 and 30 h with LPS were recovered by centrifugal elutriation, yielding a 23-ml/min fraction (endothelial cells) and a 45-ml/min fraction (PMNL, Kupffer cells, and MNP), and compared for their capacity for basal and agonist-stimulated superoxide (O_2^-) production. Stimulation with phorbol myristate acetate and opsonized zymosan caused a dose-dependent release of O_2^- from the 45-ml/min fraction derived from rats treated for 3 h with saline, but not from the 23-ml/min fraction. Further purification of the 45-ml/min fraction by discontinuous density gradient centrifugation into a Kupffer and a PMNL fraction revealed that most of the agonist-induced O_2^- release was generated by infiltrating PMNL at this early time point of LPS infusion. By 30 h of LPS infusion, although enhancement of the phorbol-12-myristate-13-acetate- and opsonized zymosan-stimulated release of O_2^- was observed in the 45-ml/min fraction, but not in the 23-ml/min fraction, the maximum release of O_2^- was smaller than that observed in the rats treated for 3 h. Our results support the following conclusions: (i) after a 3-h LPS infusion, PMNL found in the liver in increased numbers are also highly primed for agonist-stimulated release of O_2^-, while Kupffer cell priming is of a lesser extent; (ii) after a 30-h infusion of LPS, infiltrating MNP found in the liver in increased numbers are primed for agonist-induced O_2^- release, while priming of PMNL has diminished; (iii) at both 3 and 30 h of LPS infusion, liver endothelial cells are not significantly primed for agonist-stimulated O_2^- release; and (iv) in vivo priming by LPS infusion at both 3 and 30 h was not reversed by the experimental method used for cell recovery (ca. 3 h), thus suggesting that in vivo LPS priming of O_2^- release may ultimately lead to severe impairment of liver function and metabolism observed during endotoxemia and sepsis if not therapeutically blocked at an early time point.

Initial observations suggesting that endotoxin (ET) might be the underlying cause of liver damage and that the intestinal flora might be the source of ET were reported several decades ago (9, 32, 38). ET or the lipopolysaccharide (LPS) cell wall component of bacteria is constantly being produced in the terminal ileum and large intestine as a result of the death of enteric gram-negative organisms and is regularly absorbed. Low-grade portal vein endotoxemia has been shown to be a normal state in humans, implicating the liver as the major target organ involved (17, 46, 49).

Experimental studies with radioactively labeled *Escherichia coli* LPS have shown that the clearance of LPS from the systemic circulation is initially via the liver (41) and occurs in association with high-density lipoprotein during a slower second phase (27, 74, 75). Since resident liver macrophages or Kupffer cells have been observed to reside in the periportal area of the liver, they are the first cells to come in contact with ET or other noxious agents that enter the circulation via the portal vein. By autoradiography LPS has been shown to accumulate in Kupffer cells and later in hepatocytes (28, 30, 60, 76), thus demonstrating the ability of the Kupffer cells to clear gut-derived ET from the portal vein.

The liver has been shown to play an essential role in the detoxification of enterically absorbed ET, and it has therefore been hypothesized that any impairment in the normal physiologic mechanisms for clearance and detoxification of gut-derived ET might constitute a major contributing factor leading to the development of hepatic disease (26). Support for this hypothesis has come from studies in which the progression of liver disease was drastically reduced by experimental procedures that limited the pathological action of ET, such as the antiendotoxin polymyxin B (48), using ET-resistant C3H/HeJ mouse strains (29), inducing ET tolerance in rats (47), or simply eliminating the source of enteric ET by gut resection (11).

Furthermore, specific impairment of the Kupffer cell and its functions has been thought to be the principal cause of liver disease, since decreased Kupffer cell function has been correlated with increased hepatic injury in several models of experimental liver injury (35, 50, 66).

Because the intravenous injection of purified LPS precipitates a clinical syndrome that has many features in common with gram-negative sepsis, LPS has been considered primarily responsible for the morbidity and mortality that accompanies gram-negative sepsis (78).

Recent evidence suggests that ET may not be directly toxic to the liver, but may instead be a potent inducer of the in vivo production and release of toxic products by the host, such as free radicals (1, 5, 10). Oxygen-derived free radicals and chemotactic lipids are implicated in several forms of cellular injury and in a variety of disease states (21, 51, 77) and, in the liver, possibly contribute to the pathogenesis of

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chemically induced (44, 64, 65) and ET-induced (1, 5, 10, 72) hepatic injury.

Mononuclear phagocytes (MNP) and polymorphonuclear leukocytes (PMNL) that have been recruited into the liver by inflammatory stimuli, such as LPS, become activated (2). They display altered functional and biochemical properties including enhanced chemotaxis, phagocytosis, and release of reactive mediators such as oxygen-derived free radicals, a sequence of events collectively termed respiratory burst activity (36, 45). When released within phagolysosomes (intracellularly), oxygen-derived free radicals play an important role in the killing of bacteria and parasites by phagocytes (34, 36, 40). However, evidence is accumulating that extracellular release of oxygen-derived free radicals by stimulated phagocytes may be implicated in the pathogenesis of host tissue damage, leading to endothelial injury and increased vascular permeability (21). In vitro studies with LPS have shown that it appears to be a poor direct stimulant for the production of oxygen radicals by hepatic macrophages, but can prime MNP to an increased response to stimuli such as opsonized zymosan (OPZ), the calcium ionophore A23187, and phorbol myristate acetate (PMA) (15, 33, 64). PMA has been shown to induce the respiratory burst in phagocytes and also to activate the NAD(P)H oxidase through activation of protein kinase C (18, 37). A23187 induces a rapid influx of divalent cations, such as Ca^{2+}, that may trigger respiratory burst in leukocytes (58, 62). OPZ is a widely used phagocytic stimulus to induce respiratory burst in macrophages and PMNL (6).

It was shown recently that O_2^- is released in vivo after a bolus injection of LPS (5). It is, however, a subject of considerable debate how closely bolus models of LPS injection mimic human septic shock, which is usually the outcome of prolonged sepsis and endotoxemia (3, 43). To monitor the time course of chronic sepsis and endotoxemia, this laboratory has for the last several years been investigating the infusion of LPS in the rat, as both a short-term and a long-term continuous infusion via a surgically implanted osmotic minipump which reproduces some of the pathophysiologic features of gram-negative sepsis as observed in the clinical setting (25). Various aspects of the metabolic, hemodynamic, and immunologic characteristics of this chronic endotoxemia model have been previously described (23, 24, 67–71). At 3 h, rats do not show signs of morbidity, yet at the hepatic cellular level the vasopressin-stimulated inositol lipid-mediated signal transduction is already impaired (54). At the 30-h time point, maximal morbidity and severe impairment of hepatocyte and adipocyte responses are observed (14, 53, 55, 71).

The purpose of this study was to determine whether a 3-h and a 30-h infusion of a nonlethal dose of *E. coli* LPS would prime nonparenchymal liver cells (NPC) (endothelial and Kupffer cells) for basal and agonist-stimulated release of O_2^-. Thus, by selecting these two time points, we would be able to compare and contrast the early adaptive changes after 3 h of LPS infusion with those manifest at the fully morbid stage, after 30 h of LPS infusion.

MATERIALS AND METHODS

Chemicals. Sources of chemicals were as follows: collagenase (CLS II batch 45 A 7260; 145 U/mg) from Cooper Biomedical, Malvern, Pa.; LPS B (*E. coli* O26:B6) from Difco Laboratories, Detroit, Mich.; ketamine-HCl from Areco Co., Fort Dodge, Iowa; xylazine from Mobay Co., Shawnee, Kans.; Trypan blue from GIBCO Laboratories, Life Technologies Inc., Grand Island, N.Y.; Wright stain (modified), Histopaque 1077 and 1119, cytochrome *c* type III (from horse heart), superoxide dismutase (from bovine liver), PMA, the calcium ionophore A23187, zymosan, and dimethyl sulfoxide from Sigma Chemical Co., St. Louis, Mo. PMA and A23187 were maintained at −20°C in a stock solution of 10 mM in dimethyl sulfoxide. Opsonized zymosan (OPZ) was maintained at −20°C in a stock solution of 15 mg/ml in phosphate-buffered saline and prepared as described previously (6). LPS was prepared by being resuspended in pyrogen-free isotonic saline (Baxter, Deerfield, Ill.).

Animal preparation and administration of *E. coli* LPS. The experiments were performed on male Sprague-Dawley rats (330 to 370 g; Charles River) that were housed in a controlled environment with a 12-h light-dark cycle and provided with standard rodent chow (Purina) and water ad libitum. Two experimental groups of rats were used. In the first group, the rats were anesthesized with an intramuscular injection of ketamine-xylazine (90 and 9 mg/kg, respectively) and catheters were implanted in the aortic arch via the left carotid artery and in the right jugular vein by using aseptic surgical techniques. The rats were caged individually and fasted for 18 h but given water ad libitum. On the following morning rats were infused intravenously through the venous catheter with pyrogen-free isotonic saline (Baxter) or *E. coli* LPS (50 μg/100 g of body weight) over 3 h at a rate of 1 ml/h as previously described (55).

In the second group, 72 h prior to the experiment the rats were anesthesized with ketamine hydrochloride-xylazine and subjected to surgery for subcutaneous implantation of the Alzet 2 ML 1 osmotic pump (Alza, Palo Alto, Calif.). In the rats fitted with a saline pump, the pump was set to deliver sterile pyrogen-free isotonic saline (NaCl at 154 mmol/liter) at a rate of 10 μl/h into the jugular vein. In the rats fitted with an LPS pump, after a 42-h postsurgical recovery period, during which saline was continuously infused, *E. coli* LPS was infused at the same rate as saline and the dosage (0.1 mg of LPS/100 g of body weight/24 h) was adjusted to cause chronic, nonlethal endotoxemia. Both saline and LPS pump rats were fed ad libitum and sacrificed 30 h after the onset of saline or LPS infusion (25).

Preparation of liver cell fractions. The rats were anesthesized with ether, the portal vein was cannulated, and the liver was perfused in situ with continually gassed (95% oxygen–5% CO_2) Hanks' solution containing collagenase (9,780 U/50 ml; Worthington Biochemical Co.) and 5 mM $CaCl_2$ as described earlier (53). Parenchymal cells were sedimented by centrifuging the cell suspension at 20 g for 2 min at 21°C. The supernatant, containing NPC (Kupffer cells, PMNL, endothelial cells, and erythrocytes), was decanted and centrifuged at 600 × *g* for 10 min. The pellet of NPC, endothelial cells, and erythrocytes was resuspended in 40 ml of Hanks' solution containing 2.5 mM $CaCl_2$, 11 mM glucose, and 0.5% bovine serum albumin.

Elutriation procedure for various NPC fractions. The NPC were loaded into the separation chamber of a JE-6 elutriator rotor in a J2-21 type centrifuge (Beckman Instruments, Palo Alto, Calif.) used at 2,500 rpm and loaded at a flow rate of 11 ml/min. A first fraction of 200 ml was collected at a flow rate of 23 ml/min (at which most of the endothelial cells were harvested). The cells were then washed with 100 ml of elutriator buffer at a flow rate of 29 ml/min. Thereafter, a second fraction of 200 ml was collected at a flow rate of 45 ml/min (at which Kupffer cells, PMNL, and MNP were harvested). Parenchymal cells did not elute from the rotor at

TABLE 1. Cell yield and distribution of the 45-ml/min NPC fraction recovered by centrifugal elutriation from saline- and LPS-treated rats

Cell type	10^6 cells (mean ± SD; $n = 4$) (%) after[a]:			
	Saline (3 h)	LPS (3 h)	Saline (30 h)	LPS (30 h)
Total	17.4 ± 10.7 (100)	44.3 ± 38 (100)	12.6 ± 7.8 (100)	89.6 ± 34.6 (100)
Kupffer	15.8 ± 9.7 (91 ± 5.8)	19.8 ± 17 (44.9 ± 8.6)	11.6 ± 7.2 (92.3 ± 3.1)	80.2 ± 31 (89.5 ± 3.9)
PMNL	0.8 ± 0.5 (4.6 ± 3.9)	22.6 ± 19.3 (50.5 ± 7.6)	0.6 ± 0.3 (4.3 ± 1.5)	6.7 ± 2.6 (7.5 ± 4.8)
Endothelial	0.76 ± 0.5 (4.4 ± 3.5)	1.9 ± 1.6 (4.4 ± 4.8)	0.4 ± 0.2 (3.3 ± 2.1)	2.7 ± 1.04 (3 ± 1)

[a] Total number of cells of each type and percentage of cell type recovered in the 45-ml/min fraction by centrifugal elutriation as described in Materials and Methods.

flow rates of 45 ml/min or lower. Both the 23-ml/min and the 45-ml/min fractions were centrifuged at 600 × *g* for 10 min and subjected to hypotonic shock to remove contaminating erythrocytes. The cells were centrifuged again, and the pellet was resuspended in HEPES buffer (pH 7.3) containing the following millimolar concentrations: NaCl, 140; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 10; KCl, 10; $CaCl_2$, 0.1; $MgCl_2$, 0.2; $NaHCO_3$, 11.9; and glucose, 5.0; it also contained 14.5 μM bovine serum albumin. The cell suspensions were adjusted to a final concentration of 1×10^6 cells per ml, and their viability was assessed by trypan blue exclusion. The cells remained more than 90% viable throughout the experimental procedure. Cell types present in the 23-ml/min and the 45-ml/min fractions was determined by using the peroxidase reaction (staining Kupffer cells and PMNL but not endothelial cells). Wright's stain was used to differentiate between Kupffer cells and PMNL on the basis of their nuclear morphology.

Since PMNL were present in large numbers in the livers of rats treated for 3 h with LPS and eluted in the 45-ml/min NPC fraction together with Kupffer cells, a discontinuous Ficoll-Hypaque density gradient centrifugation procedure was used to obtain separate Kupffer and PMNL fractions (20). The viability of the purified PMNL and Kupffer cells in these fractions was greater than 95% as determined by trypan blue exclusion. Both cell fractions were resuspended in HEPES buffer at a concentration of 10^6 cells per ml before the superoxide anion assay was started.

Assay for superoxide anion generation. O_2^- generation by all the liver cell fractions prepared was determined by superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c* by a modification of previously described protocols (12, 34). In a typical experiment, the cells (2.5×10^5) were aliquoted into 50-ml sterile round-bottom polypropylene tubes (Sarsted). Tubes of treatment 1 received ferricytochrome *c* and HEPES buffer to measure spontaneous O_2^- release from unstimulated cells. Assay blank tubes of treatment 2 contained ferricytochrome *c* (50 μM), HEPES buffer, and 700 U of SOD to inhibit ferricytochrome *c* reduction by spontaneous O_2^- release. Tubes of treatment 3 contained ferricytochrome *c*, HEPES buffer, and one of three agonists to stimulate O_2^- release: PMA at a final concentration of 10^{-5} to 10^{-12} M, OPZ at a final concentration of 500 to 3,500 μg/10^6 cells, or A23187 at a final concentration of 10^{-6} M. Control tubes of treatment 4 contained all reagents and demonstrated that SOD inhibited (>95%) ferricytochrome *c* reduction caused by the agonist used to stimulate O_2^- production. Each set of treatments was run in triplicate and in a final volume of 1.6 ml. The tubes were gassed with 100% O_2 for 10 s, capped, and then incubated for 30 min in a shaking water bath at 37°C. The production of O_2^- was terminated by plunging the tubes into ice. Tube contents were transferred to borosilicate tubes (10 by 75 mm; Baxter Healthcare Corp, McGraw Park, Ill.) and centrifuged at 600 × *g* for 10 min. The changes in A_{550} in the supernatants were determined by using a Beckmann DU-8 spectrophotometer. The difference in the amount of reduced ferricytochrome *c* in the presence and the absence of SOD was used to estimate the amount of O_2^- release by employing a molecular extinction coefficient of 21.0×10^3 M^{-1} cm^{-1}. O_2^- release was expressed as nanomoles per 10^6 cells per 30 minutes, since previous experiments showed that there was a linear relationship between the amount of O_2^- released and the concentration of cells.

Statistical analysis of the data. Data are expressed as means ± standard errors of the mean (SEM) of triplicate determinations of three to five similar experiments as detailed in the legend to each figure. Appropriate multiway analysis of variance was performed on all sets of data. When significant interaction was encountered, simple effects were tested by using a one-way analysis of variance followed by a Student-Newman-Keuls test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

In-migration of monocytes and PMNL into the liver after LPS infusion. Centrifugal elutriation is a widely used method for the separation of liver NPC to provide cell fractions enriched in Kupffer and endothelial cells (53). We have recently shown that under our experimental conditions, PMNL sequestered in the liver after 3 h of intravenous LPS infusion were recovered together with Kupffer cells in the 45-ml/min NPC fraction (57). As shown in Table 1, the cell distribution in the livers of rats infused with saline for 3 h showed that in the 45-ml/min NPC fraction, 91 ± 5.8% of the total cells recovered were Kupffer cells and 4.6 ± 3.9% were PMNL. In the rats infused with LPS for 3 h, 44.9 ± 8.6% of the total cells recovered were Kupffer cells and 50.5% were PMNL. The 3-h LPS infusion caused a 2.5-fold increase in the total number of cells recovered in the 45-ml/min NPC fraction, mainly by the infiltration of PMNL. As shown in Table 1, in the rats that received a continuous infusion of saline for 30 h, 92.3 ± 3.1% of the total cells recovered in the 45-ml/min NPC liver fraction were Kupffer cells and 4.3 ± 1.5% were PMNL. In the rats receiving a continuous infusion of LPS for 30 h, 89.5 ± 3.9% of the total cells recovered were Kupffer cells and infiltrating monocytes and 7.5 ± 4.8% were PMNL. The 30-h LPS infusion caused a sevenfold increase in the number of cells recovered in the 45-ml/min NPC fraction, mainly by the infiltration of monocytes. The 23-ml/min fraction (mostly endothelial cells) resulted in the following yields (× 10^6 cells per rat; $n = 4$): 3.8 ± 0.8 (3-h

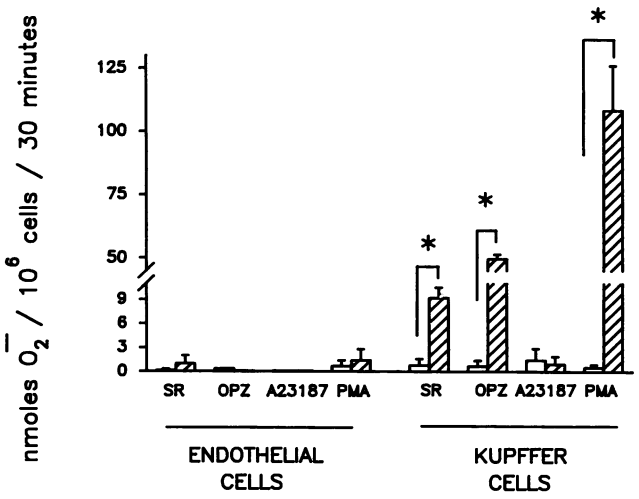

FIG. 1. Stimulation by OPZ (2,000 μg/10^6 cells), A23187 (1 μM), and PMA (1 μM) of O_2^- release (nanomoles per 10^6 cells per 30 minutes) by 23-ml/min (endothelial cells) and 45-ml/min (Kupffer cells and PMNL) hepatic NPC fractions recovered from rats infused with saline (□) and LPS (▨) for 3 h. Data are expressed as mean ± SEM of triplicate determinations of five experiments. *, $P < 0.05$; SR, spontaneous release.

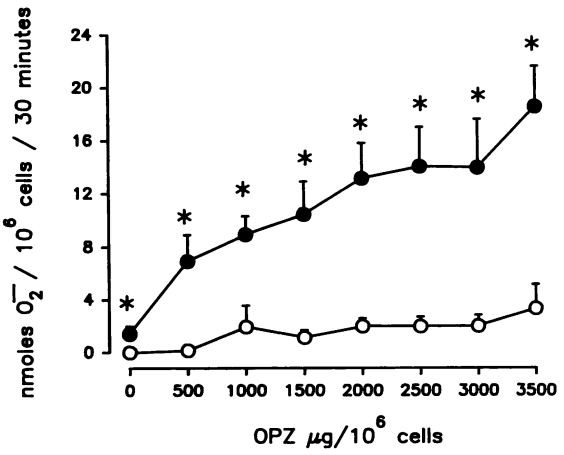

FIG. 2. Stimulation by increasing concentrations of OPZ of O_2^- release (nanomoles per 10^6 cells per 30 minutes) by 45-ml/min (Kupffer cell and PMNL) NPC fractions recovered from rats infused with saline (○) and LPS (●) for 3 h. Data are expressed as mean ± SEM of triplicate determinations of five experiments. *, $P < 0.05$.

saline), 6.3 ± 3.4 (3-h LPS), 22.9 ± 9.7 (30-h saline), and 17.3 ± 1.8 (30-h LPS).

Effect of 3-h LPS infusion on O_2^- release by liver NPC. As shown in Fig. 1, endothelial cells (23-ml/min fraction) recovered from livers of rats treated with LPS and with saline for 3 h released small amounts of O_2^- spontaneously or when stimulated by OPZ (2,000 μg/10^6 cells) and A23187 (1 μM). With PMA (1 μM), endothelial cells from LPS-treated rats showed a slight increase of O_2^- release (1.4 nmol/10^6 cells/30 min) with respect to saline-treated controls (0.7 nmol/10^6 cells/30 min). Only the 45-ml/min NPC fraction from LPS-treated rats showed an elevated spontaneous release of O_2^- (9.2 nmol/10^6 cells/30 min; $P < 0.05$). When stimulated with OPZ (2,000 μg/10^6 cells) and PMA (1 μM), the 45-ml/min NPC fraction from LPS-treated rats showed a 49-fold increase (49.7 versus 0.7 nmol/10^6 cells/30 min) and a 107-fold increase (108 versus 0.6 nmol/10^6 cells/30 min), respectively, in O_2^- release over saline-treated controls ($P < 0.05$). Although A23187 elicited a slight increase over the spontaneous release of O_2^- in the 45-ml/min fractions of saline-treated rats (1.45 versus 0.8 nmol/10^6 cells/30 min), the spontaneous O_2^- release was higher in the 45-ml/min fractions of LPS-treated rats than that elicited by A23187 (9.2 versus 0.95 nmol/10^6 cells/30 min).

Effect of 3-h LPS infusion on O_2^- release by NPC stimulated with OPZ and PMA. As shown in Fig. 2, a statistically significant dose-dependent increase in O_2^- release over basal values was observed when 45-ml/min fractions of NPC from rats treated for 3 h with saline were stimulated with OPZ. A maximum release of 3.3 nmol/10^6 cells/30 min was obtained with 3,500 μg of OPZ per 10^6 cells ($P < 0.05$ over basal O_2^- release, which was undetectable). Similarly, corresponding fractions from rats treated for 3 h with LPS showed a statistically significant dose-dependent enhancement of O_2^- release over saline-treated controls when stimulated with OPZ (500 to 3,500 μg/10^6 cells; $P < 0.05$). A sixfold increase in O_2^- production (18.5 nmol/10^6 cells/30 min) was obtained with 3,500 μg of OPZ/10^6 cells with respect to saline controls ($P < 0.05$).

As can be seen in Fig. 3, there was a statistically significant dose-dependent increase in O_2^- release when 45-ml/min fractions of NPC from rats treated for 3 h with saline were stimulated with PMA. A maximum release of 33.2 nmol/10^6 cells/30 min was obtained with 10 μM PMA, compared with a basal O_2^- release of 3.6 nmol/10^6 cells/30 min ($P < 0.05$). Similarly, the NPC from rats treated for 3 h with LPS showed a statistically significant dose-dependent enhancement of O_2^- release over saline-treated rats upon stimulation with 0.1 nM to 10 μM PMA ($P < 0.05$). A fourfold increase in O_2^- production (127.6 nmol/10^6 cells/30 min) with respect to saline controls ($P < 0.05$) was obtained with 10 nM PMA.

Effect of a 3-h LPS infusion on O_2^- release by Kupffer cells and PMNL. To determine the relative contribution of Kupffer cells and infiltrating PMNL to the total O_2^- release observed in the 45-ml/min NPC fraction (Fig. 1 and 2), a

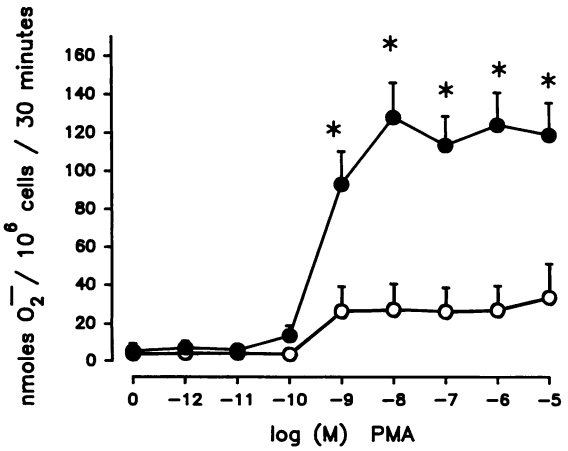

FIG. 3. Stimulation by increasing concentrations of PMA of O_2^- release (nanomoles per 10^6 cells per 30 minutes) by 45-ml/min (Kupffer cell and PMNL) NPC fractions recovered from rats infused with saline (○) or LPS (●) for 3 h. Data are expressed as mean ± SEM of triplicate determinations of four experiments. *, $P < 0.05$.

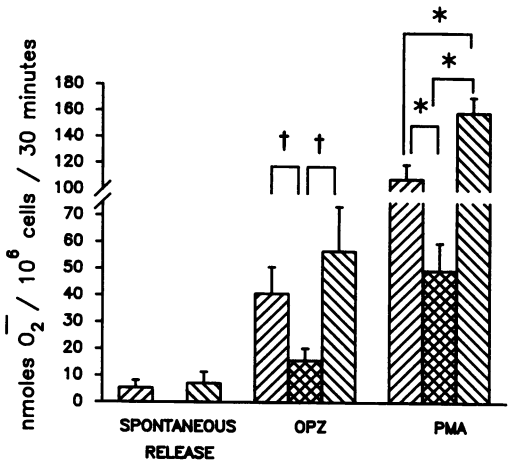

FIG. 4. Stimulation by OPZ (3,500 μg/10^6 cells) and PMA (1 nM) of O_2^- release (nanomoles per 10^6 cells per 30 minutes) by 45-ml/min liver NPC, Kupffer cell, and PMNL fractions, respectively, recovered from rats infused with LPS for 3 h. Data are expressed as mean ± SEM of triplicate determinations of four experiments. *, $P < 0.05$ (analysis of variance); †, $P < 0.05$ (paired t test). Symbols: ▨, Kupffer cells plus PMNL; ⊠, Kupffer cells; ▧, PMNL.

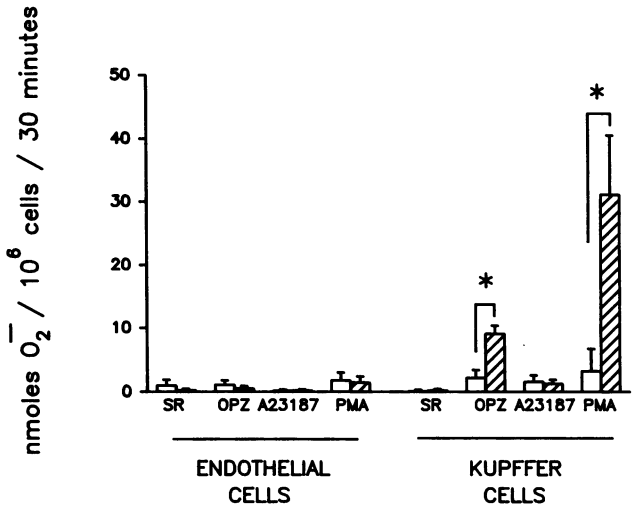

FIG. 5. Stimulation by OPZ (2,000 μg/10^6 cells), A23187 (1 μM), and PMA (1 μM) of O_2^- release (nanomoles per 10^6 cells per 30 minutes) by 23-ml/min (endothelial cells) and 45-ml/min (Kupffer cells and monocytes) NPC fractions recovered from rats infused with saline (□) and LPS (▨) for 30 h via surgically implanted osmotic pumps. Data are expressed as mean ± SEM of triplicate determinations of four experiments. *, $P < 0.05$; SR, spontaneous release.

Kupffer cell fraction and a PMNL-enriched fraction were prepared by centrifugation by using a discontinuous Ficoll-Hypaque density gradient (see Materials and Methods). The Kupffer cell fraction contained 70.8 ± 9.3% Kupffer cells and 11.8 ± 7.4% PMNL; the PMNL fraction, on the other hand, contained 21.2 ± 4.9% Kupffer cells and 84 ± 7.4% PMNL, as determined by the morphology of nuclei detected by using Wright's stain. As shown in Fig. 4, spontaneous release of O_2^- was observed in the 45-ml/min and the PMNL fractions from rats treated for 3 h with LPS. With one-way analysis of variance for OPZ (3,500 μg/10^6 cells)-stimulated O_2^- release, no statistically significant differences in the O_2^- release were observed among the three cell fractions investigated. However, a paired t test applied to the analysis of O_2^- release of the three OPZ-stimulated fractions of LPS-treated rats revealed that upon stimulation with OPZ (3,500 μg/10^6 cells), the release of O_2^- by the 45-ml/min fraction was greater than that by the Kupffer fraction (40.7 versus 15.7 nmol/10^6 cells/30 min, respectively [$P < 0.05$]) but smaller than that by the PMNL fraction (40.7 versus 56.7 nmol/10^6 cells/30 min, respectively; not statistically significant). Similarly, upon 10 nM PMA stimulation, the release of O_2^- by the 45-ml/min fraction was greater than that by the Kupffer cell fraction (108.3 versus 49.5 nmol/10^6 cells/30 min, respectively [$P < 0.05$]), but significantly smaller than that by the PMNL fraction (108.3 versus 159 nmol/10^6 cells/30 min, respectively [$P < 0.05$]). This enhanced O_2^- release by the PMNL fraction suggests that the O_2^- produced by the 45-ml/min NPC fraction is caused mainly by infiltrating PMNL.

Effect of 30-h LPS infusion on O_2^- release by hepatic NPC. As can be observed in Fig. 5, endothelial cells (23-ml/min fraction) from rats treated for 30 h with LPS or saline released small amounts of O_2^- spontaneously or when stimulated by OPZ (2,000 μg/10^6 cells) or PMA (1 μM). The 45-ml/min NPC fraction from LPS-treated rats showed a fourfold (8.8 versus 2.4 nmol/10^6 cells/30 min) and threefold (29.9 versus 10.4 nmol/10^6 cells/30 min) increase in O_2^- release over saline-treated controls, respectively, upon stimulation with OPZ (2,000 μg/10^6 cells) or PMA (1 μM) ($P < 0.05$). A23187 (1 μM) elicited a slight release of O_2^- in saline- and LPS-treated NPC (1.6 versus 1.3 nmol/10^6 cells/30 min, respectively). However, the 45-ml/min NPC fractions from LPS-treated rats and saline-treated controls showed no spontaneous release of O_2^-.

Effect of 30-h LPS infusion on O_2^- release by NPC stimulated with OPZ and PMA. As shown in Fig. 6, a statistically significant dose-dependent increase in O_2^- release over basal values was observed when 45-ml/min NPC fractions from rats treated for 30 h with saline were stimulated with OPZ. A maximum release of 4.27 nmol/10^6 cells/30 min was obtained with 3,500 μg of OPZ per 10^6 cells compared with

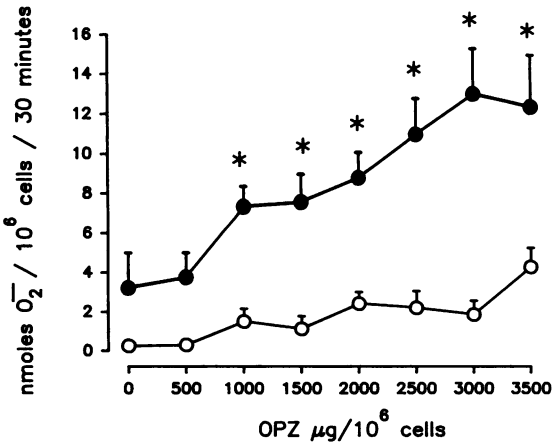

FIG. 6. Stimulation by increasing concentrations of OPZ of O_2^- release (nanomoles per 10^6 cells per 30 minutes) by 45-ml/min NPC fractions recovered from rats infused with saline (○) and LPS (●) for 30 h. Data are expressed as mean ± SEM of triplicate determinations of four experiments. *, $P < 0.05$.

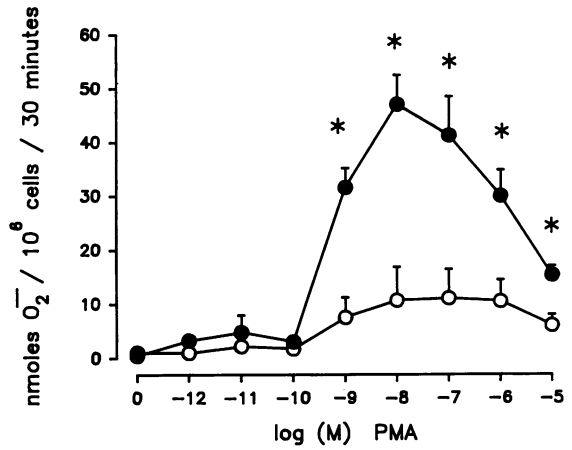

FIG. 7. Stimulation by increasing concentrations of PMA of O_2^- release (nanomoles per 10^6 cells per 30 minutes) by 45-ml/min NPC fractions recovered from rats infused with saline (○) and LPS (●) for 30 h. Data are expressed as mean ± SEM of triplicate determinations of four experiments. *, $P < 0.05$.

basal O_2^- release, which was undetectable ($P < 0.05$). Similarly, corresponding fractions from rats treated for 30 h with LPS showed a statistically significant dose-dependent enhancement of O_2^- release over that in saline-treated controls when stimulated with 1,000 to 3,500 μg of OPZ per 10^6 cells ($P < 0.05$). A threefold increase in O_2^- production (12.3 nmol/10^6 cells/30 min) was obtained with 3,500 μg of OPZ/10^6 cells with respect to saline-treated controls ($P < 0.05$). This maximal release of O_2^- was lower than the maximum production observed in rats treated for 3 h with LPS (18.5 nmol/10^6 cells/30 min).

Figure 7 documents a statistically significant dose-dependent increase in O_2^- release when 45-ml/min NPC fractions from rats treated for 30 h with saline were stimulated with PMA. A maximum release of 11 nmol/10^6 cells/30 min was obtained with 0.1 μM PMA, compared with a basal O_2^- release of 1.06 nmol/10^6 cells/30 min ($P < 0.05$). Similarly, such NPC from rats treated for 30 h with LPS showed a statistically significant dose-dependent enhancement of O_2^- release over saline-treated rats when stimulated with 0.1 nM to 10 μM PMA ($P < 0.05$). A fourfold increase in O_2^- production (47 nmol/10^6 cells/30 min) was obtained with 10 nM PMA ($P < 0.05$). This maximal production of O_2^- was considerably lower than the highest production observed with 10 nM PMA in rats treated for 3 h with LPS (127.6 nmol/10^6 cells/30 min).

DISCUSSION

In the experiments described in this study we have used a protocol of continuous infusion of LPS into rats as a clinically relevant model to study the pathophysiology of prolonged endotoxemia leading to irreversible septic shock (13). Selection of 3- and 30-h time points for analysis enabled us to compare O_2^- generation by liver NPC during the early response to LPS with that during a later phase, when morbidity is maximal and metabolic responses are at their nadir (25, 53, 55, 70).

Accumulation of PMNL was observed after a 3-h infusion of LPS, whereas after a 30-h infusion a predominance of activated mononuclear cells in the inflammatory infiltrate of the liver was clearly visible, thus confirming our previous findings with this model (57). The kinetics of the leukocyte response after a continuous infusion of LPS was similar to that reported following a bolus injection of LPS in rats (52, 65). This biphasic leukocyte response, which is typically observed in acute inflammation (61), has been observed in several experimental models of hepatic injury, in which MNP were shown to be recruited into the liver and activated by bacterial, viral, or parasitic infections (19, 22, 39).

The toxicity of free radicals has been documented in inflammatory disorders (21), circulatory shock (79), postischemic tissue injury (42), and gram-negative bacterial sepsis (5, 10, 63). Several sources of free radicals may contribute to the pathogenesis of cellular injury: (i) radicals generated in eicosanoid biosynthesis from arachidonic acid; (ii) hydrogen peroxide, O_2^-, and hydroxyl anion liberation from neutrophils and macrophages (34); and (iii) oxygen free radicals produced by the conversion of hypoxanthine to xanthine, catalyzed by xanthine oxidase (42). There is now accumulating evidence that oxygen products of the NADPH oxidase contained within phagocytic cells play an important role in the injury associated with the triggering of an inflammatory process (77).

The fact that increased generation of toxic oxygen might be associated with tissue damage in liver injury induced by LPS has been shown previously in several studies involving bolus models of LPS administration into rodents. In a severe model of hepatic injury (*Corynebacterium parvum* on day 1 followed by LPS on day 6), SOD decreased the severity of hepatic injury 24 h after treatment, suggesting that O_2^- (or other toxic oxygen species produced by O_2^-) released into the extracellular space was the most important cytotoxic factor. The ineffectiveness of allopurinol, an inhibitor of xanthine oxidase, in decreasing the severity of hepatic injury suggested that extracellular release of O_2^- from macrophages was the most important source of oxygen-derived free radicals in this model (1). These pioneering studies were subsequently confirmed for mice by Broner et al., who showed increased survival of mice receiving a bolus injection of LPS (0.1 μg per mouse) and pretreated with SOD (10). Recently, Bautista and Spitzer have gone one step further by developing an elegant in vivo technique demonstrating that in situ liver perfusion of rats receiving a bolus injection of LPS (0.1 mg/kg of body weight) shows a peak of O_2^- release 3 h after LPS injection, which can be inhibited by SOD but not by allopurinol (5). Furthermore, the release of O_2^- in vivo following an in vivo bolus injection of LPS was relatively early, of short duration, and transient.

The data reported in this paper indicate that after a 3-h continuous infusion of a nonlethal dose of LPS, PMNL are primed so that they release increased O_2^- when stimulated by several agonists. At the 3-h time point, which corresponds to the initial acute inflammatory response to LPS infusion, significant sequestration of PMNL infiltration into the liver was observed. Additionally, enhanced release of O_2^- was documented in the 45-ml/min NPC fraction (Fig. 1) upon stimulation with a soluble (PMA) and a particulate (OPZ) agonist. To determine the source of O_2^-, we separated the 45-ml/min NPC fraction by Ficoll-Hypaque density gradient centrifugation into a Kupffer and a PMNL fraction (see Results). This revealed that most of the O_2^- release originated from the infiltrating PMNL, with a minor contribution by the Kupffer cells.

Our results with continuous infusion of LPS closely resemble those observed in a bolus model of endotoxemia in which PMNL and Kupffer cells were observed to be the main contributors to agonist-induced O_2^- release 3 h after

injection of LPS (1 mg/kg of body weight), whereas no release was observed from the liver endothelial cells (4). Since the Ficoll-Hypaque density centrifugation method that we used did not permit complete separation of Kupffer and PMNL from the 45-ml/min NPC fraction, it is difficult to ascertain the significance of the contribution to O_2^- release by the Kupffer cells present, as proposed by Bautista et al. (4). However, it is reasonable to conclude that infiltrating PMNL are responsible for most of the O_2^- release after 3 h of continuous LPS infusion since in vitro studies have shown that LPS will prime PMNL to O_2^- production rapidly (less than 60 min) (31). Although Kupffer cells have been shown not to release O_2^- when stimulated directly with LPS (7) and to do so only upon prolonged incubation with LPS (>24 h) and stimulation by several agents (16, 31), the outcome of coexistence of Kupffer cells and sequestered PMNL in vivo in terms of modulation of O_2^- release by Kupffer cells is unknown. Therefore, the contribution of Kupffer cells to the observed O_2^- release in the Kupffer cell fraction is difficult to assess.

In contrast to the 3-h period, by 30 h of LPS infusion a progressive extravasation of MNP into the liver was observed. The enhanced O_2^- release measured in the 45-ml/min fraction must be interpreted as due to a heterogeneous population of cells consisting of LPS-exposed resident Kupffer cells, macrophages derived from local proliferation of Kupffer cells, and those recently recruited from the bone marrow or peripheral blood (8). No attempt was made in this study to identify or separate subgroups of MNP. Although modifications of the standard technique of SOD-inhibitable ferricytochrome *c* reduction are believed to detect O_2^- release from Kupffer cells (2), we have been able to observe O_2^- release in both OPZ- and PMA-stimulated cells in our experiments.

Although to our knowledge no report is available describing O_2^- release in long-term (more than 24 h) endotoxemia, several other investigators have reported an increase of O_2^- in Kupffer cells or infiltrating MNP in several models of bolus ET or bacterial injection. Several years ago, Hashimoto et al. observed enhanced chemiluminescence in both peritoneal and Kupffer macrophages after PMA stimulation in mice pretreated for 2 to 20 days with *Lactobacillus casei* (33). Thereafter, Arthur et al. showed that hepatic macrophages isolated 6 days after *C. parvum* elicitation in vivo showed enhanced O_2^- release when stimulated with PMA (2). More recently, Shiratori et al. reported that rat liver macrophages pretreated with LPS (100 μg per rat, 24 h earlier by bolus injection) had an increased capacity to produce large amounts of O_2^- when stimulated by PMA or latex particles in vitro (64). Similar results were observed by Mochida et al., who have shown that rat hepatic macrophages exhibited both intracellular and extracellular release of O_2^- upon PMA stimulation 6 days after pretreatment with *C. parvum* (44).

Thus, the release of O_2^- after a continuous nonlethal infusion of *E. coli* LPS into rats is produced mainly by infiltrating PMNL at an early time (after 3 h of LPS administration). Thereafter, as the presence of PMNL in the liver subsides, the production of O_2^- shifts to the resident Kupffer cells and the infiltrating MNP. Generation of O_2^- is part of the microbicidal killing mechanism of leukocytes (PMNL and MNP). When generated in excess, O_2^- may also be harmful and locally toxic to a particular organ where it may accumulate, e.g., in the lungs and liver during endotoxemia. O_2^- can, by spontaneous dismutation, change into hydrogen peroxide, and it can react with hydrogen peroxide to form more toxic radicals such as singlet oxygen and hydroxyl radicals (36) and can induce the formation of chemotactic lipid (51) that may induce the influx of more PMNL to the liver. All of these products of oxygen reduction (O_2^-, hydrogen peroxide, hydroxyl radicals) and excitation (singlet oxygen) may account for the induction of tissue injury observed in early endotoxemia (21) and may participate in the killing of hepatocytes (59, 73). By these mechanisms extracellular oxygen-derived free radicals released by infiltrating PMNL and MNP, as well as Kupffer cells, could promote hepatocyte damage.

Further studies are needed (i) to clarify the pathophysiologic role of PMNL at an early stage of endotoxemia and MNP at a later stage, (ii) to determine the precise role played by LPS and other in vivo agonists (interacting with the same receptors as PMA and OPZ) on spontaneous and agonist-stimulated O_2^- generation, and (iii) to define the molecular mechanisms involving O_2^- and other free radicals and leading to tissue damage in liver injury.

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REFERENCES

1. **Arthur, M. J. P., I. S. Bentley, A. R. Tanner, P. K. Saunders, G. H. Millward-Sadler, and R. Wright.** 1985. Oxygen-derived free radicals promote hepatic injury in the rat. Gastroenterology **89**:1114–1122.
2. **Arthur, M. J. P., P. Kowalski-Saunders, and R. Wright.** 1986. *Corynebacterium parvum*-elicited hepatic macrophages demonstrate enhanced respiratory burst activity compared with resident kupffer cells in the rat. Gastroenterology **91**:174–181.
3. **Balis, J. V., E. S. Rappaport, L. Gerber, J. Fareed, F. Buddingh, and H. L. Messmore.** 1978. A primate model for prolonged endotoxin shock. Blood vascular reactions and effects of glucocorticoid treatment. Lab. Invest. **38**:511–523.
4. **Bautista, A. P., K. Meszaros, J. Bojta, and J. J. Spitzer.** 1990. Superoxide anion generation in the liver during the early stage of endotoxemia in rats. J. Leukocyte Biol. **48**:123–128.
5. **Bautista, A. P., and J. J. Spitzer.** 1990. Superoxide anion generation by in situ perfused rat liver: effect of *in vivo* endotoxin. Am. J. Physiol. **259**:G907–G912.
6. **Berton, G., and S. Gordon.** 1983. Modulation of macrophage mannosyl-specific receptors by cultivation on immobilized zymosan. Effects on superoxide-anion release and phagocytosis. Immunology **49**:705–715.
7. **Birmelin, M., U. Karck, P. Dieter, N. Freudenberg, and K. Decker.** 1986. Distribution of endotoxin and endotoxin-stimulated prostanoid production in primary cultures of rat Kupffer cells, p. 295–300. *In* A. Kirn, D. L. Knook, and E. Wisse (ed.), Cells of the hepatic sinusoid, vol. 1. Kupffer Cell Foundation, Rijswijk, The Netherlands.
8. **Bouwens, L., and E. Wisse.** 1985. Proliferation, kinetics, and fate of monocytes in rat liver during a zymosan-induced inflammation. J. Leukocyte Biol. **37**:531–543.
9. **Broitman, S. A., L. S. Gottlieb, and N. Zamcheck.** 1963. Influence of neomycin and ingested endotoxin in the pathogenesis of choline deficiency cirrhosis in the adult rat. J. Exp. Med. **119**:633–647.
10. **Broner, C. W., J. L. Shenep, G. L. Stidhan, D. C. Stokes, and W. K. Hildner.** 1988. Effect of scavengers of oxygen-derived free radicals on mortality in endotoxin-challenged mice. Crit. Care Med. **16**:848–851.
11. **Camara, D. S., C. A. Caruana, Jr., K. A. Schwartz, M. Montes, and J. P. Nolan.** 1983. D-Galactosamine liver injury: absorption of endotoxin and protective effect of small bowel and colon resection in rabbits. Proc. Soc. Exp. Biol. Med. **172**:255–259.
12. **Cerasoli, F., Jr., P. J. McKenna, D. L. Rosolia, K. H. Albertine,**

S. P. Peters, and M. H. Gee. 1990. Superoxide anion release from blood and bone marrow neutrophils is altered by endotoxemia. Circ. Res. 67:154–165.
13. Danner, R. L., R. J. Elin, J. M. Hosseini, R. A. Wesley, J. M. Reilly, and J. E. Parillo. 1991. Endotoxemia in human septic shock. Chest 99:169–175.
14. Deaciuc, I. V., and J. A. Spitzer. 1986. Rat liver free cytosolic Ca^{2+} and glycogen phosphorylase in endotoxicosis and sepsis. Am. J. Physiol. 251:R984–R995.
15. Decker, K. 1990. Biologically active products of stimulated liver macrophages (Kupffer cells). Eur. J. Biochem. 192:245–261.
16. Decker, K., M. Birmelin, and R. Bhatnagar. 1982. The role of Ca^{2+} and prostaglandins in the response of rat Kupffer cells to external stimuli, p. 361–368. *In* D. L. Knook and E. Wisse (ed.), Sinusoidal liver cells. Elsevier Biomedical Press, Amsterdam.
17. Deitch, E. A. 1990. Translocation of the gut flora. J. Trauma 30:S184–S189.
18. Dewald, B., T. G. Payne, and M. Baggiolini. 1984. Activation of NADPH oxidase of human neutrophils. Potentiation of chemotactic peptide by a diacylglycerol. Biochem. Biophys. Res. Commun. 125:367–373.
19. Diesselhoff-Den Dulk, M. M. C., R. W. Crofton, and R. Van Furth. 1979. Origin and kinetics of Kupffer cells during an acute inflammatory response. Immunology 37:7–14.
20. English, D., and B. R. Andersen. 1974. Single-step separation of red blood cells, granulocytes and mononuclear leukocytes on discontinuous density gradients of Ficoll-Hypaque. J. Immunol. Methods 5:249–252.
21. Fantone, J. C., and P. A. Ward. 1982. Role of oxygen-derived free radicals and metabolites in leukocyte-dependent inflammatory reactions. Am. J. Pathol. 107:397–418.
22. Ferluga, J., and A. C. Allison. 1978. Roles of mononuclear infiltrating cells in pathogenesis of hepatitis. Lancet ii:610–611.
23. Fish, R. E., A. H. Burns, C. H. Lang, and J. A. Spitzer. 1985. Myocardial dysfunction in a nonlethal, nonshock model of chronic endotoxemia. Circ. Shock 16:241–252.
24. Fish, R. E., C. H. Lang, and J. A. Spitzer. 1986. Regional blood flow during continuous, low dose endotoxin infusion. Circ. Shock 18:267–275.
25. Fish, R. E., and J. A. Spitzer. 1984. Continuous infusion of endotoxin from an osmotic pump in the conscious, unrestrained rat: a unique model of chronic endotoxemia. Circ. Shock 12:135–149.
26. Fox, E. S., P. Thomas, and S. A. Broitman. 1990. Hepatic mechanisms for clearance and detoxification of bacterial endotoxins. J. Nutr. Biochem. 1:620–628.
27. Freudenberg, M. A., T. C. Bog-Hansen, U. Back, and C. Galanos. 1980. Interaction of lipopolysaccharide with plasma high-density lipoprotein in rats. Infect. Immun. 28:373–380.
28. Freudenberg, M. A., N. Freudenberg, and C. Galanos. 1982. Time course of cellular distribution of endotoxin in liver, lungs and kidneys of rats. Br. J. Exp. Pathol. 63:56–65.
29. Freudenberg, M. A., D. Keppler, and C. Galanos. 1986. Requirement for lipopolysaccharide-responsive macrophages in galactosamine-induced sensitization to endotoxin. Infect. Immun. 51:891–895.
30. Freudenberg, M. A., B. Kleine, and C. Galanos. 1984. The fate of lipopolysaccharide in rats: evidence for chemical alteration of the molecule. Rev. Infect. Dis. 6:483–487.
31. Guthrie, L. A., L. C. McPhail, P. M. Henson, and J. R. Johnston. 1984. Priming of neutrophils for enhanced release of oxygen metabolites by bacterial lipopolysaccharide. J. Exp. Med. 160:1656–1671.
32. Gyorgy, P. 1954. Antibiotics and liver injury. Ann. N.Y. Acad. Sci. 57:925–931.
33. Hashimoto, S., K. Nomoto, T. Matsuzaki, T. Yokokura, and M. Mutai. 1984. Oxygen radical production by peritoneal macrophages and Kupffer cells elicited by *Lactobacillus casei*. Infect. Immun. 44:61–67.
34. Johnston, R. B., Jr. 1984. Measurement of O_2^- secreted by monocytes and macrophages. Methods Enzymol. 105:365–369.
35. Kelly, P. M., A. R. Heryet, and J. O. McGee. 1989. Kupffer cell number is normal, but their lysozyme content is reduced in alcoholic liver disease. J. Hepatol. 8:173–180.
36. Klebanoff, S. J. 1980. Oxygen metabolism and the toxic properties of phagocytes. Ann. Intern. Med. 93:480–489.
37. Lehmeyer, J. E., R. Snyderman, and R. B. Johnston, Jr. 1979. Stimulation of neutrophil oxidative metabolism by chemotactic peptides: influence of calcium ion concentration and cytochalasin B and comparison with stimulation by phorbol myristate acetate. Blood 54:35–45.
38. Luckey, T. D., J. A. Reyniers, P. Gyorgy, and M. Forbes. 1954. Germfree animals and liver necrosis. Ann. N.Y. Acad. Sci. 57:932–935.
39. MacDonald, J. R., J. H. Beckstead, and E. A. Smuckler. 1987. An ultrastructural and histochemical study of the prominent inflammatory response in D(+)-glactosamine hepatotoxicity. Br. J. Exp. Pathol. 68:189–199.
40. Markert, M., P. C. Andrews, and B. M. Babior. 1984. Measurement of superoxide anion production by human neutrophils. The preparation and assay of NADPH oxidase-containing particles from human neutrophils. Methods Enzymol. 105:358–365.
41. Mathison, J. C., and R. J. Ulevitch. 1979. The clearance, tissue distribution, and cellular localization of intravenously injected lipopolysaccharide in rabbits. J. Immunol. 123:2133–2143.
42. McCord, J. M. 1985. Oxygen-derived free radicals in postischemic tissue injury. N. Engl. J. Med. 312:159–163.
43. McGuire, G. P., and R. G. Pearl. 1990. Sepsis and the trauma patient. Crit. Care Clin. 6:121–146.
44. Mochida, S., I. Ogata, Y. Ohta, S. Yamada, and K. Fujiwara. 1989. In situ evaluation of the stimulatory state of hepatic macrophages based on their ability to produce superoxide anions in rats. J. Pathol. 158:67–71.
45. Nathan, C. F. 1987. Secretory products of macrophages. J. Clin. Invest. 79:319–326.
46. Nolan, J. P. 1981. Endotoxin, reticuloendothelial function, and liver injury. Hepatology 1:458–465.
47. Nolan, J. P., and M. V. Ali. 1973. Endotoxin and the liver. II. Effect of tolerance on carbon tetrachloride-induced injury. J. Med. 4:28–38.
48. Nolan, J. P., and A. I. Leibowitz. 1978. Endotoxin and the liver. III. Modification of acute carbon tetrachloride injury by polymyxin B—an antiendotoxin. Gastroenterology 75:445–449.
49. Nolan, J. P., A. I. Leibowitz, and A. O. Vladutiu. 1980. Influence of carbon tetrachloride on circulating endotoxin after exogenous administration of endotoxin in rats. Proc. Soc. Exp. Biol. Med. 165:453–456.
50. Pereira, C. A., A. M. Steffan, F. Koehren, C. R. Douglas, and A. Kirn. 1987. Increased susceptibility of mice to MHV 3 infection induced by hypercholesterolemic diet: impairment of Kupffer cell function. Immunobiology 174:253–265.
51. Petrone, W. F., D. K. English, K. Wong, and J. M. McCord. 1980. Free radicals and inflammation: superoxide-dependent activation of a neutrophil chemotactic factor in plasma. Proc. Natl. Acad. Sci. USA 77:1159–1163.
52. Pilaro, A. M., and D. L. Laskin. 1986. Accumulation of activated mononuclear phagocytes in the liver following lipopolysaccharide treatment of rats. J. Leukocyte Biol. 40:29–41.
53. Rodriguez de Turco, E. B., and J. A. Spitzer. 1987. Impairments in hepatocyte phosphoinositide metabolism in endotoxemia. Metabolism 36:753–760.
54. Rodriguez de Turco, E. B., and J. A. Spitzer. 1988. Early effects of *Escherichia coli* endotoxin infusion on vasopressin-stimulated breakdown and metabolism of inositol lipids in rat hepatocytes. Biochem. Biophys. Res. Commun. 155:151–159.
55. Rodriguez de Turco, E. B., and J. A. Spitzer. 1988. Kinetics of diaclyglycerol accumulation in response to vasopressin stimulation in hepatocytes of continuously endotoxaemic rats. Biochem. J. 253:73–79.
56. Rodriguez de Turco, E. B., and J. A. Spitzer. 1988. Impairments in vasopressin-stimulated inositol lipid metabolism in hepatocytes of septic rats. Circ. Shock 25:299–307.
57. Rodriguez de Turco, E. B., and J. A. Spitzer. 1990. Eicosanoid production in nonparenchymal liver cells isolated from rats infused with *E. coli* endotoxin. J. Leukocyte Biol. 48:488–494.
58. Romeo, D., G. Zabucchi, N. Miani, and F. Rossi. 1975. Ion

movement across leukocyte plasma membrane and excitation of their metabolism. Nature (London) **253**:542–544.
59. **Rubin, R., and J. L. Farber.** 1984. Mechanism of the killing of cultured hepatocytes by hydrogen peroxide. Arch. Biochem. Biophys. **228**:450–459.
60. **Ruiter, D. J., J. Van Der Meulen, A. Brouwer, M. J. R. Hummel, B. J. Mauw, J. C. M. Van der Ploeg, and E. Wise.** 1981. Uptake by liver cells of endotoxin following its intravenous injection. Lab. Invest. **45**:38–45.
61. **Ryan, G. B., and G. Majno.** 1977. Acute inflammation. A review. Am. J. Pathol. **86**:185–276.
62. **Schell-Frederick, E.** 1974. Stimulation of the oxidative metabolism of polymorphonuclear leucocytes by the calcium ionophore A23187. FEBS Lett. **48**:37–40.
63. **Schoenberg, M. H., and H. G. Berger.** 1988. Sauerstoffradikale imseptischen Schock. Chirurg **59**:836–841.
64. **Shiratori, Y., T. Kawase, S. Shiina, K. Okano, T. Sugimoto, H. Teraoka, S. Matano, K. Matsumoto, and K. Kamii.** 1988. Modulation of hepatotoxicity by macrophages in the liver. Hepatology **8**:815–821.
65. **Shiratori, Y., M. Tanaka, K. Hai, T. Kawase, S. Shiina, and T. Sugimoto.** 1990. Role of endotoxin-responsive macrophages in hepatic injury. Hepatology **11**:183–192.
66. **Shiratori, Y., H. Teraoka, S. Matano, K. Matsumoto, K. Kamii, and M. Tanaka.** 1989. Kupffer cell function in chronic ethanol-fed rats. Liver **9**:351–359.
67. **Spitzer, J. A., and I. V. Deaciuc.** 1986. Effect of endotoxicosis and sepsis on intracellular calcium homeostasis in rat liver. Mitochondrial and microsomal calcium uptake. Circ. Shock **18**:81–93.
68. **Spitzer, J. A., and R. E. Fish.** 1986. Lipolytic patterns in isolated adipocytes of continuously endotoxemic rats. Circ. Shock **18**: 21–29.
69. **Spitzer, J. A., H. Friedman, C. Newton, R. Widen, S. Pross, and T. W. Klein.** 1987. Suppressed *in vitro* blastogenic response of rat spleen cells after continuous infusion of endotoxin by an implanted osmotic pump. Proc. Soc. Exp. Biol. Med. **186**:21–26.
70. **Spitzer, J. A., K. M. Nelson, and R. E. Fish.** 1985. Time course of changes in gluconeogenesis from various precursors in chronically endotoxemic rats. Metabolism **34**:842–849.
71. **Spitzer, J. A., E. B. Rodriguez de Turco, I. V. Deaciuc, and B. L. Roth.** 1987. Perturbation of transmembrane signaling in acute and chronic endotoxemia, p. 401–418. *In* G. Schlag and H. Redl (ed.), First Vienna Shock Forum. Alan R. Liss, Inc., New York.
72. **Spitzer, J. J., K. Meszaros, A. P. Bautista, C. H. Lang, and Z. Spolarics.** 1990. Potential contribution of phagocytic cells to liver damage after *Escherichia coli* endotoxin, p. 521–528. *In* A. Nowotny, J. J. Spitzer, and E. J. Ziegler (ed.), Cellular and molecular aspects of endotoxin reactions. Elsevier Biomedical Press, Amsterdam.
73. **Starke, P. E., and J. L. Farber.** 1985. Endogenous defense against cytotoxicity of hydrogen peroxide in cultured rat hepatocytes. J. Biol. Chem. **260**:86–92.
74. **Ulevitch, R. J., A. R. Johnston, and D. B. Weinstein.** 1979. New function for high density lipoproteins. Their participation in intravascular reactions of bacterial lipopolysaccharides. J. Clin. Invest. **64**:1516–1524.
75. **Ulevitch, R. J., A. R. Johnston, and D. B. Weinstein.** 1981. New function for high density lipoprotein. Isolation and characterization of a bacterial lipopolysaccharide-high density lipoprotein complex formed in rabbit plasma. J. Clin. Invest. **67**:827–837.
76. **Van Bossuyt, H., R. B. De Zanger, and E. Wisse.** 1988. Cellular and subcellular distribution of injected lipopolysaccharide in rat liver and its inactivation by bile salts. J. Hepatol. **7**:325–337.
77. **Ward, P. A., J. S. Warren, and K. J. Johnson.** 1988. Oxygen radicals, inflammation, and tissue injury. Free Radic. Biol. Med. **5**:403–408.
78. **Wolf, S. M.** 1973. Biological effects of bacterial endotoxins in man. J. Infect. Dis. **128**:S259–S264.
79. **Yoshikawa, T.** 1986. Endotoxin-induced disseminated intravascular coagulation and shock, and their relationship with oxygen free radicals, p. 234. *In* G. P. Novelli and F. Ursini (ed.), Oxygen free radicals in shock. S. Karger, Basel.