THE EFFECTS OF ENDOTOXIN AND HUMORAL MEDIATOR(S) DERIVED FROM ENDOTOXIN-TREATED RATS ON THE HORMONAL INDUCTION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE IN REUBER H-35 HEPATOMA CELLS

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ABSTRACT

THE EFFECTS OF ENDOTOXIN AND HUMORAL MEDIATOR(S) DERIVED FROM ENDOTOXIN-TREATED RATS ON THE HORMONAL INDUCTION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE IN REUBER H-35 HEPATOMA CELLS

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An in vitro assay system utilizing cultured Reuber H-35 hepatoma cells was employed to study the effects of endotoxin, modified forms of endotoxin, the by-products of endotoxin-induced complement activation, and serum-borne mediators on the inducibility of phosphoenolpyruvate carboxykinase (PEPCK) by the synthetic glucocorticoid, dexamethasone.

The addition of 150 ug of LPS directly to hepatoma cell cultures had no effect upon induction of PEPCK. Addition of endotoxemic rat serum significantly inhibited this induction. The inhibitor was found to be present immediately after endotoxin challenge (30 seconds) and to persist 8 hours post-injection, at which time its activity began to diminish. Inhibitory activity could not be associated with either a low density form of endotoxin or with the by-products of endotoxin-induced complement activation. These data support an indirect mechanism of action for endotoxin

and suggest that the inhibitor is a pre-formed molecule released upon endotoxin challenge.

To my parents, Claire and Harold Keitelman
To my brother, Edward Neil
To my sister, Marian Sue

for their love, encouragement, and support.

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INTRODUCTION

For over a century the chemical and biological properties of endotoxin have been under investigation. Endotoxin has been found to elicit an impressive array of different biological activities including shock, lethality, interaction with complement, abortion, cytotoxicity, Shwartzman reaction, vascular effects, release and sensitization to histamines, endocrinological changes, metabolic changes, pyrogenicity, immunogenicity, leukopenia and leukocytosis, protection against irradiation, effect on the reticuloendothelial system, development of tolerance, enhancement of nonspecific resistance, mobilization of interferon, and changes in blood clotting (12, 79). Until recently it had been difficult to imagine how the lipopolysaccharide molecule or the biologically active lipid-A moiety could produce such a wide array of host responses. Current evidence suggests that endotoxin acts to stimulate the production and/or release of mediators which are thought to be responsible for the wide range of biological activities seen in the susceptible host.

The intent of this study is to examine, <u>in vitro</u>, the effects of endotoxin, modified forms of endotoxin, the by-products of endotoxin-induced complement activation, and serum-borne mediators on the inducibility of PEPCK by the

synthetic glucocorticoid, dexamethasone in Reuber H-35 hepatoma cell cultures. It is hoped that these studies will help to elucidate the mechanisms underlying the mediated actions of endotoxin and the protective role of glucocorticoids in endotoxin lethality.

LITERATURE SURVEY

Chemical Structure of Endotoxin

Endotoxins (lipopolysaccharides) are constituents of the outer cell membrane of gram-negative bacteria. The lipopolysaccharide (LPS) molecule consists of three regions: an O-specific polysaccharide, a core polysaccharide, and a lipid moiety termed lipid A. Aggregation of monomers and complex formation with other constituents of the cell walls has led to varying molecular weights of 1 to 20 million, depending upon the method of isolation and the degree of purification (79). The core polysaccharide, often identical for large groups of bacteria, links the lipid moiety to the O-specific polysaccharide. Chemical analysis has shown this region to consist of 5 basal sugars, phosphate, and O-phosphorylethanol amine (39). The O-polysaccharide structure, chemically unique for each type of organism, is composed of repeating oligosaccharide units and determines the O-antigenic specificity of the bacteria (40). Lipid A, unique to bacterial endotoxins, consists of a β1-6 linked diglucosamine backbone containing both ester- and amide-linked long-chain fatty acids as well as pyrophosphate groups (31). LPS isolated from the Re595 mutant of Salmonella minnesota, consisting only of a lipid A and core trisaccharide 2-keto-3-deoxyoctulosonate (KDO), was shown to be endotoxically as

active as the parent LPS from the wild-type (S) strain. Chemical modification of the glycolipid by succinylation which results in the esterification of free hydrolysis, did not diminish the toxicity of the glycolipid suggesting that KDO residues did not contribute to endotoxicity (27)(85). These findings suggest that the polysaccharide moiety of LPS does not play any major role in endotoxicity and that the biologically active component of LPS is lipid A (54)(55).

Serum Detoxification of Endotoxin

There is much controversy concerning the role of humoral and cellular defenses in detoxification of endotoxin. The reticuloendothelial system has been widely implicated as the system of major importance in the uptake and detoxification of circulating endotoxins. Skarnes (103), on the other hand, suggests that components in the circulating plasma play a major role in detoxification and are largely responsible for survival of the endotoxemic host. He defines detoxification as a two step mechanism, initial degradation followed by detoxification of exposed toxic groups. Chromatographic studies led to the separation of a potent detoxifying fraction containing two serum alpha globulins having esterase activity of the non-specific carboxylic type and shown to interact with endotoxin. Initial interactions of endotoxin with a heat-stable alpha lipoprotein (esterase) led to degradation of the large molecules. Detoxification of exposed toxic groups was effected by interaction with the other, heat-labile alpha globulin (esterase). Physiologic

concentrations of serum Ca^{2+} were shown to inhibit the formation of the endotoxin-alpha lipoprotein complex, and thus, detoxification. Data from other studies (102) showed that the concentration of Ca^{2+} gradually decreased in post-endotoxin serum accompanied by a concomitant increase in the heat-stable carboxylic esterase level and a marked increase in the rate of detoxification of endotoxin.

Ulevitch and Johnston (114) showed that normal rabbit serum (or plasma) reduced the buoyant density (d) of LPS from Escherichia coli 0111:B4 (d=1.44 g/cm^3) and Salmonella minnesota (d=1.38 g/cm^3) to less than 1.2 g/cm^3. This density shift was shown to be associated with the inhibition of a number of endotoxic activities including pyrogenic activity, the ability to produce an immediate neutropenia in rabbits, and anti-complementary activity. The toxicity of the LPS was only partially diminished as a result of the density reduction. Preliminary results did not support Skarnes' hypothesis that enzymatic degradation of LPS accompanied serum (or plasma) induced detoxification of LPS.

More recent work by Ulevitch et al. (115) has shown that the reduction of the buoyant density of LPS requires plasma (or serum) lipid. Delipidation of the plasma (or serum) by extraction with n-butanol/diisopropyl ether prevented a density shift. Reversal of the effect of delipidation was achieved upon addition of physiologic concentrations of high density lipoprotein (HDL). Addition of low density lipoprotein or very low density lipoprotein had no

effect. Their experimental findings also suggested that LPS undergoes modification before interaction with HDL. Addition of HDL to the parent LPS in the absence of delipidated serum did not result in a significant reduction of density, indicating that delipidated serum might be involved in modifications, possibly disaggregation, of the LPS prior to interaction with HDL. Freudenberg et al. (26), using the method of crossed immunoelectrophoresis, have also shown that LPS interacts with plasma HDL of rats, forming a complex with characteristic electrophoretic mobility. The interaction of LPS with HDL occurred rapidly, and the complex formed persisted while the LPS remained in the circulating plasma. Plasma clearance studies, carried out with ^{14}C-labeled LPS, resembled that of the disappearance of the LPS-HDL complex. These findings are in agreement with Skarnes' demonstration that LPS forms complexes with plasma lipoproteins. In contrast, the authors do not support the mechanism of humoral detoxification of LPS, but suggest that HDL represents a transport protein for LPS in plasma to organs of clearance or to other cellular targets.

Biological Activity of Endotoxin

The biological effects of endotoxin on the susceptible host are diverse and numerous. Some of the major biological activities associated with endotoxemia include pyrogenicity, immunogenicity, leukopenia and leukocytosis, development of tolerance, enhancement of nonspecific resistance, activation

of complement, metabolic changes, Shwartzman effect, effects on the reticuloendothelial system, shock, and lethality (12) (79). Because of the diverse nature of these activities it has been difficult to ascertain the mechanism of action of endotoxin. Recent research lends support to the hypothesis that many of the biological effects of endotoxin are elicited by mediators released into the blood of the host upon endotoxin challenge. Mediators recognized to date include endogenous pyrogen or leukocytic endogenous mediator (EP or LEM), interferon, colony stimulating factor (CSF), tumor necrosis factor (TNF), glucocorticoid antagonizing factor (GAF), prostaglandins, kinins, interleukin-1, and interleukin-2 (12).

Effects of Endotoxin on Carbohydrate Metabolism

Bacterial endotoxins have long been known to deplete an animal of its carbohydrate reserves (68). Numerous studies suggest that the cause for carbohydrate loss in endotoxin poisoning is an impaired ability to carry out gluconeogenesis. Berry et al. (17) demonstrated that an injection of a lethal dose of killed Salmonella typhimurium into mice resulted in a reduction of liver glycogen and an almost complete loss in total body carbohydrate. Shands et al. (98) clearly showed impaired gluconeogenesis to be responsible for the decreased conversion of pyruvate-2-^{14}C into blood glucose in Mycobacterium bovis BCG-primed mice challenged with endotoxin. Studies by La Noue et al. (58), in

which rat liver slices were employed, showed the overall rate of gluconeogenesis to decrease in gram-negative infection.

Evidence indicates that alteration of certain inducible enzymes in endotoxin poisoned mice may be responsible for this impaired regulation of body carbohydrate. Inhibition of hormonal induction of phosphoenolpyruvate carboxykinase (PEPCK) has been observed in mice treated with endotoxin (16). PEPCK, a key enzyme involved in glucoenogenesis, catalyzes the GTP- or ITP-dependent carboxylation of oxaloacetate and effectively bypasses the thermodynamically unfavorable conversion of pyruvate to phosphoenolpyruvate (38). Endotoxin has been shown to antagonize the hormonal induction of tryptophan oxygenase (TO) (16) and glycogen synthase (67) as well. Induction of tyrosine aminotransferase (TAT) was not inhibited (15) whereas pyruvate kinase (PK) activity was actually enhanced (107) by endotoxin administration. Rippe and Berry (87)(88), utilizing a radial immunodiffusion technique, established that endotoxin blocked the hormonal induction of PEPCK and TO by preventing synthesis of the enzyme. In an earlier report Shtasel and Berry (100) demonstrated an overall increase in protein and messenger ribonucleic acid (mRNA) synthesis in livers of endotoxin poisoned mice as determined by the uptake of ^{14}C-leucine into acid-insoluble peptides and by the incorporation of ^{14}C-labeled orotic acid into liver mRNA. Thus, the impairment of synthesis of only certain inducible liver enzymes implies a highly

selective mode of action for endotoxin. Endotoxin, when added to isolated rat hepatocytes (24) or to liver homogenates (16) was shown to have no direct effect on either gluconeogenesis or PEPCK activity. Such evidence suggests that the action of endotoxin on regulatory mechanisms involved in the control of hepatic synthesis is mediated.

The Mediation of Endotoxin: Glucocorticoid Antagonizing Factor

Evidence for a mediator responsible for inhibiting the hormonal induction of PEPCK has been found in the serum of zymosan-primed mice 2 hours after an injection of endotoxin. When endotoxin-tolerant mice were injected with serum derived from endotoxin-treated normal mice, the hormonal induction of PEPCK was inhibited. PEPCK induction was not inhibited when these animals were injected with endotoxin (74). Thus, enzyme inhibition cannot be attributed to any contaminating endotoxin present in the serum but must be due to the presence of a mediator. This mediator has been called glucocorticoid antagonizing factor (GAF). Moore et al.'s work (74) suggested that macrophages are involved in the production of GAF. The ability of endotoxin to block PEPCK induction was eliminated when macrophages were selectively incapacitated by an injection of anti-macrophage serum 1 hour prior to endotoxin and hydrocortisone injection. Secondly, when supernatant fluid from a culture of mouse peritoneal exudate cells (PEC) incubated overnight in

the presence of endotoxin (100 ug/ml) was injected into endotoxin-tolerant mice, PEPCK induction was inhibited. These results do not rule out lymphocyte involvement in mediator production since the PEC cultures may be contaminated with lymphocytes.

Congenitally athymic nude mice (nu/nu) were used to determine thymus or T-lymphocyte involvement in mediator production (75). PEPCK was found to be fully inducible in athymic nude mice receiving an injection of endotoxin. Euthymic heterozygous littermates (nu/+) exhibited inhibition of hormonal induction upon endotoxin administration. These results indicate that the nude mutation may be responsible for this abnormal behavior. Nude mice, treated with serum containing GAF obtained from donor CD-1 mice, exhibited no inhibition of hormonal induction of PEPCK. When the concentration of GAF in the serum of the donor mice was increased by pretreatment with zymosan, hormonal induction of PEPCK was significantly inhibited in nude mice. These studies suggest that nude mice are sensitive to GAF but require larger amounts in order to be inhibitory. Thus, GAF is either not produced in congenitally athymic nude mice upon endotoxin administration or, if it is produced, in concentrations too low to be inhibitory. Further evidence for the importance of reticuloendothelial cells in the formation of GAF was demonstrated by the injection of a suspension of PEC from zymosan pretreated conventional mice and endotoxin into hydrocortisone treated nude mice. Following

this treatment hormonal induction of PEPCK was inhibited. These observations suggest that T-lymphocytes are needed for GAF production or that macrophage in athymic nude mice are deficient in GAF production and confirms Moore et al.'s earlier work (74) suggesting that GAF is cellular in origin.

Goodrum et al. (34) undertook studies with indomethacin, a non-steroidal, anti-inflammatory agent, to determine whether it could suppress endotoxemic responses. They found indomethacin able to diminish the formation of GAF in vivo as well as in PEC cultures while exerting no effect upon an animal's response to an exogenous source of GAF. It was not determined whether these effects were due to indomethacin's ability to inhibit prostaglandin synthesis.

C_3H/HeJ mice, known to be extremely resistant to endotoxin lethality (110), were shown not to respond normally to phenol-water extracted LPS (76). A phenol-water extracted LPS failed to inhibit the hormonal induction of PEPCK whereas a Boivin (trichloroacetic acid) extracted LPS did. When serum from phenol-water extracted LPS poisoned conventional mice was injected into C_3H/HeJ mice hormonal induction of PEPCK was inhibited. Moore et al.'s findings implicate macrophages and/or B-cells in the release or formation of GAF since both cell types have been found by Rosenstreich et al. (89) to be abnormal. Therefore, C_3H/HeJ mice appear to be deficient in their ability to form GAF while possessing normal susceptibility to GAF.

To date GAF has been only partially characterized. GAF was estimated, by elution from Sephadex G-200, to have a molecular weight of about 150,000 daltons. One hour at 56°C had no effect on its inhibitory activity, whereas 1 hour at 75°C virtually eliminated any GAF effect upon PEPCK induction. In addition, GAF activity was shown to be sensitive to trypsin (500 ug/ml) (76).

The Mediation of Endotoxin: Interferon

Interferon, in addition to its classical antiviral activity, has been shown to be immunostimulatory as well as immunosuppressive (23), to enhance macrophage activities (42), and to stimulate natural killer cells (41). Similar effects observed due to endotoxin suggest the importance of interferon in endotoxemia. Interferon has also been found to sensitize adult mice (72) and rats (59) to LPS. Thought to be a glycoprotein, interferon is believed to have a polymeric structure with an estimated molecular weight ranging from 25,000 to 85,000 daltons (37).

In early reports only the macrophage obtained from the peritoneal cavity of mice and rabbits was consistently shown to produce interferon in response to endotoxin (18)(106). Jullien and De Maeyer (43) reported that following x-irradiation, interferon production in endotoxin treated mice was normal. This study suggested that the radioresistant macrophage might be involved in interferon production. In contrast, Postic et al. (84) observed that corticosteroids

significantly decreased the production of endotoxin-induced interferon in rabbits. Sensitivity to lysis by corticosteroids implicated lymphocytic involvement. Together, these conflicting reports suggest that perhaps more than one cell type is involved in the production of interferon in response to endotoxin.

Maehara and Ho (62) utilized purified cells to study endotoxin-induced interferon. They found interferon induced by endotoxin in the mouse macrophage to be significantly different than that induced by B-lymphocytes. Macrophage derived interferon was labile at 56°C, stable at pH 2, neutralized by anti-mouse fibroblast interferon at high serum dilutions, and present in the serum 1 hour after endotoxin challenge. B-lymphocyte interferon, obtained from the serum at later times, was heat stable and neutralized at low dilutions of antiserum. No production of interferon was detected from purified T-cell populations. Study of interferons induced by Newcastle disease virus (NDV) and the synthetic double-stranded polyribonucleotide polyinosinic-polycytidylic acid (poly I:C) as well as endotoxin indicated that the heterogeneity of interferon was not due to the different modes of induction but was dependent solely upon cellular origin (63).

Interferon has been implicated as a mediator responsible for the antagonism of glucocorticoid induction of certain hepatic enzymes. Moore et al. (73) have shown that mice injected with either living or ultraviolet-irradiated

Sindbis virus were unable to induce TO, PEPCK, and TAT in response to hydrocortisone treatment. Induction of TAT was not inhibited by the administration of endotoxin (15). Poly I:C treatment was also found to inhibit the induction of PEPCK and TO (72). In addition, Beck et al. (10) have shown that crude preparations of interferon in relatively high concentrations inhibited steroid induction of TAT in cultured hepatoma cells. However, observations with commercially available interferon raise doubt as to whether interferon is in fact the mediator responsible for blocking hormonal induction of these enzymes (73). Only studies utilizing highly purified preparations of interferon will ascertain its role as a mediator in endotoxemia.

The Mediation of Endotoxin: Tumor Necrosis Factor

For almost 40 years bacterial endotoxin has been known to cause hemorrhagic necrosis in certain mammalian tumors (99). The fact that endotoxin does not kill tumor cells *in vitro* suggests that its mode of action is mediated by a soluble host factor. Carswell et al. (20) have shown that serum obtained from *Mycobacterium bovis* BCG-infected mice treated with endotoxin contained a substance, termed tumor necrosis factor (TNF), which was as effective as endotoxin in causing tumor necrosis. Sera from normal mice, endotoxin-treated mice, and BCG-infected mice did not contain TNF. The requirement for pretreatment of donor mice with macrophage-activating agents such as BCG implicates this cell as the

source of TNF. Studies by Mannel et al. (64)(65), using LPS-responsive mice (C_3H/HeN) and LPS-unresponsive mice (C_3H/HeJ), as well as athymic nude mice, supports the idea that activated macrophage release a soluble tumor cytotoxin after LPS challenge. Only C_3H/HeN mice were able to produce the cytotoxic serum factor. When C_3H/HeJ mice were lethally x-irradiated and reconstituted with C_3H/HeN bone marrow cells they gained the ability to release the tumor cytotoxin into the serum. These data suggest that LPS acts on some bone marrow derived cell type to induce the release of the cytotoxin. Serum from nude mice infected with BCG and treated with LPS contained as much cytotoxic activity as did that from heterozygous littermates indicating that mature T-cells are not required for the generation of serum cytotoxic activity. Recent work directly implicate the macrophage as the cellular source of TNF. Macrophage-enriched cell populations from BCG-infected mice, macrophage-like tumor cells (PU5-1.8), and peritoneal macrophages propagated with macrophage growth factor released cytotoxic activity when stimulated in vitro with minute (nanogram) quantities of LPS. Rabbit antiserum directed against serum-derived TNF from BCG-infected, LPS-challenged mice inhibited all of the cytotoxic activities generated in vitro suggesting that macrophage-derived cytotoxins are identical to the serum-derived TNF (66).

The partial purification and preliminary characterization of TNF from mouse sera has been accomplished by Green

et al. (36). TNF, purified 20- to 30-fold, migrated with α-globulins, is a polymer composed of at least 4 subunits, and has an estimated molecular weight of 150,000 daltons. Biochemical analysis indicates that the active factor is a glycoprotein containing sialic acid and galactosamine. The physiocochemical characteristics of the cytotoxins from LPS-stimulated macrophage cultures were compared to those of serum-derived cytotoxic factor. In addition to the similarity in kinetics for appearance of the cytotoxins (maximal levels present 2 to 3 hours after LPS challenge) the biochemical data obtained were similar. The activity eluted in a broad peak in the molecular weight range of 50,000 to 60,000 daltons as determined by gel filtration. The cytotoxin was stable for 30 minutes at 56°C, destroyed completely after 10 minutes at 100°C, and stable at pH 6 to 10 (66).

The Mediation of Endotoxin: Colony Stimulating Factor

Mouse and human granulocyte/macrophage progenitor cells proliferate and differentiate *in vitro* to form colonies of mature granulocytes and macrophages. The clonal growth of these progenitor cells in soft agar cultures is dependent on the presence of colony stimulating factor (CSF) (19)(83). LPS, particularly the lipid A portion of the molecule, has been shown to be a potent inducer of CSF (2)(5). After administration of endotoxin, a rise in tissue and serum CSF, as well as a rise in the number of granulocyte/macrophage

progenitor cells in the marrow and spleen has been observed in conventional mice (6). C_3H/HeJ mice do not respond with proliferation of granulocyte/macrophage progenitor cells or generation of CSF after endotoxin challenge. However, C_3H/HeJ mice were shown to respond normally in the presence of an external source of CSF obtained from post-endotoxin serum from C_3H/eB mice. Lethal irradiation of low-responder C_3H/HeJ mice, followed by reconstitution with C_3H/eB bone marrow cells, restored serum CSF response to endotoxin. These data indicate that C_3H/HeJ mice may be defective in the production of CSF and suggest that the cells generating CSF in response to endotoxin are of bone marrow origin (82). Studies carried out by Apte et al. (3)(4) emphasize the role of macrophages and lymphocytes in the generation of CSF after endotoxin administration. Resident peritoneal macrophage were shown to release high levels of CSF upon direct interaction with endotoxin. However, the interaction of spleen cells with endotoxin to generate CSF required a collaboration between macrophage and lymphocytes. Nonadherent lymphocytes produced insignificant amounts of CSF. Only when supplemented with a small, critical number of macrophages was the ability to secrete CSF restored. Supernatant fluid from macrophage cultured in the presence of LPS contained factors that subsequently stimulated B-lymphocytes to secrete CSF in the absence of LPS. Thus, macrophage appear to play some regulatory role in the process of elaboration of CSF by B-lymphocytes.

The Mediation of Endotoxin:
Leukocytic Endogenous Mediator (Endogenous Pyrogen)

Fever resulting from LPS administration is believed to be due to an endogenous pyrogen (EP). This factor is thought to be identical to or closely related to leukocytic endogenous mediator (LEM) (49)(70) which has been shown to cause a wide array of biological effects in addition to pyrogenicity. Merriman et al. (70) have shown highly purified preparations of EP from rabbit peritoneal granulocytes or human blood monocytes, when subjected to sequential purification and injected back into rabbits and rats, produced all of the activities associated with LEM. A variety of phagocytic cells including peritoneal granulocytes, peritoneal macrophages, Kupffer cells, blood leukocytes, and blood monocytes have been used to prepare LEM. All cell types excluding peritoneal granulocytes were shown to require activation by endotoxin or phagocytosis of foreign material in order to produce LEM (45). Lymphocytes seemed not to be directly involved in LEM production. However, evidence suggests that activated lymphocytes may release a soluble agent which, in turn, activates the phagocytic cells to produce LEM (7).

Injection of LEM into rats and rabbits caused a pronounced decrease in plasma iron (50) and zinc (51) with minimum amounts reached, respectively, 6 to 10 hours and 5 to 6 hours post-injection. Significant increases in serum copper levels were also observed in the rat (81). LEM has

also been shown to cause release of lysosomal enzymes and to elevate serum levels of acute phase alphaglobulin, ceruloplasmin, and mucoprotein (53)(81). A rapid release of neutrophils from the bone marrow into the blood stream has been associated with the injection of LEM into rats (46). Kampschmidt and Upchurch (52) reported that repeated injections resulted in increased amounts of CSF in the serum and suggested that LEM may act on mature macrophage to cause the release of CSF. LEM, when given 24 hours prior to an intraperitoneal (i.p.) challenge of a lethal dose of Salmonella typhimurium, increased nonspecific resistance to infection with an effectiveness comparable to an injection of endotoxin (47). LEM did not produce a tolerance upon repeated injection, was active in endotoxin-tolerant rats, and was destroyed by heating, thus differentiating this factor from bacterial endotoxin (50). LEM was shown to be heat labile, inactivated by trypsin, and to have a limited solubility in organic solvents (50). The molecular weight varied depending on cell type and animal species and ranged from 10,000 to 30,000 daltons (48)(80).

The Mediation of Endotoxin: Prostaglandins

Agents such as acetylsalicylic acid (aspirin) and the non-steroidal, anti-inflammatory compound indomethacin, capable of interfering with the biosynthesis of prostaglandins (116), have been shown to inhibit certain endotoxin effects including diarrhea, lethality, generalized

Schwartzman reaction, hypothermia, and release of GAF (86). Such evidence implicates prostaglandins as a possible mediator of LPS action. Prostaglandins appear to be primarily involved in the detrimental effects of endotoxin including fever (22)(101), the early phase of shock (25), and abortion (104), although investigators have not ruled out the possibility that certain prostaglandins released during endotoxin shock may exert beneficial effects. This is indicated by the finding that arachidonic acid, an immediate precursor in prostaglandin biosynthesis, when infused into LPS-treated rabbits, provided significant protection from irreversible shock (25). With LPS, a dose dependent release of prostaglandins has been demonstrated in thioglycollate-stimulated mouse peritoneal macrophages (119), human blood monocytes (57), and macrophage tumor cell lines (57). In contrast, peritoneal macrophages from C_3H/HeJ mice did not release prostaglandins on incubation with phenol-extracted LPS (LPS-Ph) (119). C_3H/HeJ macrophages did, however, release increased amount of prostaglandins in response to butanol-extracted LPS (LPS-Bu) and lipid-A associated protein (LAP) demonstrating that they are capable of being stimulated <u>in vitro</u>. This is consistent with the finding that LPS-Bu is significantly more toxic to this mouse strain than LPS-Ph (119). Rietschel et al. (86) have shown that on exposure to endotoxin macrophages from LPS-hyperreactive mice released significantly larger amounts of prostaglandins than did controls. In contrast, macrophages from animals made tolerant to LPS were completely

refractory in their ability to release prostaglandins on incubation with LPS. On incubation with zymosan, however, macrophages obtained from tolerant animals were shown to release significant amounts of prsotaglandins indicating that the capacity to produce prostaglandins was intact. These findings suggest that increased prostaglandin release may be related to the state of LPS hyperreactivity, that impaired prostaglandin synthesis may be linked to the state of LPS refractoriness (tolerance), and that prostaglandins may play a role in endotoxin activity.

Lymphoreticular Involvement in the Mediation of Endotoxin

Recent progress in the field of endotoxin research can be attributed to the identification and characterization of the C_3H/HeJ mouse strain (109). The C_3H/HeJ LPS defect has been found to be due to a mutation in a single autosomal gene locus (termed Lps) involved in the regulation of expression of a number of endotoxic reactions (120). This strain has been shown to be hyporesponsive to almost all of the biological effects of endotoxin studied thus far, including mitogenicity (111), immunogenicity (120), adjuvanticity (105), lethality (109), the extravascular accumulation of leucocytes (109), and the enhancement of nonspecific resistance to bacterial infection (21). That a single mutation could affect so many different biological processes suggests that some common mechanism, defective in C_3H/HeJ mice, is responsible for the initiation of all biological activities associated with endotoxin. Thus, the endotoxin-unresponsive

C_3H/HeJ strain has become a valuable tool for analyzing the mechanism of action of endotoxin.

Studies of the interaction between endotoxin and various cell types derived from C_3H/HeJ mice are suggestive of lymphoreticular cell involvement in the expression of endotoxicity in the host. Early evidence implicated cells from the spleen as contributing to lethality. Agarwal et al. (1) demonstrated a greater resistance to a lethal dose of endotoxin in splenectomized animals. It was suggested that the change in sensitivity may have been due to a decrease in the number of endotoxin-sensitive lymphoid cells following splenectomy. Adoptive transfer studies (29) demonstrated that spleen cells did contribute significantly to the lethality associated with endotoxemia. After irradiation (450 R) of recipients, spleen cells were adoptively transfused between C_3H/HeJ mice and the histocompatible C_3H/HeN LPS-responsive strain. C_3H/HeJ mice were found to be sensitized to LPS after adoptive transfer of C_3H/HeN spleen cells while C_3H/HeN mice reconstituted with C_3H/HeJ spleen cells were shown to become more resistant to LPS. Michalek et al. (71) utilized an improved adoptive transfer technique (adoptive transfer of bone marrow cells; 850 R irradiation of recipients) to evaluate the contribution of lymphoreticular cells to immunogenicity, adjuvanticity, lethality, induction of interferon, and induction of CSF. C_3H/HeJ chimeras were shown to be as sensitive to LPS as the responsive C_3H/HeN strain for all parameters examined except

lethality, for which a 3-fold greater resistance was observed. C_3H/HeN mice, reconstituted with C_3H/HeJ bone marrow cells, were resistant to all the effects of LPS studied except induction of CSF. This observation is consistent with reports indicating that a wide variety of radio-resistant tissues and cells of nonlymphoid origin are capable of CSF production (77)(97). These observations demonstrated that endotoxin responsiveness required the presence of some radio-sensitive, bone marrow derived cell(s) and clearly suggests that LPS-sensitive lymphoreticular cells are involved as primary mediators of endotoxemia.

The defect in LPS sensitivity of the C_3H/HeJ mouse strain has been demonstrated at the cellular level and has been found to be expressed in vitro in macrophages (28)(30), B-lymphocytes (56)(105)(111), T-lymphocytes (56), and embryonic fibroblasts (94). Sultzer and Nilsson (111) demonstrated a refractoriness of C_3H/HeJ spleen cells to the induction of mitogenesis by LPS in vitro. This failure to observe B-cell mitogenic stimulation appeared to be specific to LPS since another B-cell mitogen, the purified protein derivative of tuberculin (PPD), elicited a mitogenic response in C_3H/HeJ in spleen cells. T-lymphocytes have also been found to be defective in C_3H/HeJ mice. Although LPS has been shown to induce phenotypic differentiation for both B- and T-cells (95), highly purified preparations of LPS did not induce phenotypic differentiation of either B-cell precursors or T-cell precursors from C_3H/HeJ mice (56).

The induction mechanism of phenotypic differentiation was shown not to be impaired for this strain since precursor cells were readily induced by dibutyryl cAMP. Glode et al. (28) investigated the sensitivity of macrophages derived from C_3H/HeJ and C_3H/HeN mice to the *in vitro* cytotoxic effect of LPS. They found LPS to be cytotoxic for C_3H/HeN macrophages at concentrations nontoxic for lymphocytes. C_3H/HeJ macrophages were found not to be killed by LPS. Use of a CBA/N strain, containing an X-linked recessive gene which produces defective B-cell maturation (96), suggested that mature B-lymphocyte subpopulations were not required for LPS to exert its effects (90). CBA/N spleen cells responded poorly to the mitogenic effect of LPS. However, unlike C_3H/HeJ macrophages, CBA/N macrophages were shown to be sensitive to LPS. CBA/N macrophages were stimulated by LPS to produce the monokine, lymphocyte activating factor (LAF) and prostaglandins. LPS was also shown to be cytotoxic for CBA/N macrophages.

Most recently attention has been focused on the macrophage as the primary cellular target and effector of endotoxicity. C_3H/HeJ mice have been found to possess major defects in macrophage function independent of exogenous administration of endotoxin (91)(112). The defective macrophage has been shown to lack the capacity to achieve a stimulated or an activated state. Vogel and Rosenstreich (118) found that Fc-mediated functions of cultured, thioglycollate-induced C_3H/HeJ macrophages was abnormal. Macrophage

from the nonresponder strain lost the ability to phagocytose opsonized sheep red blood cells (SRBC) after 24 hours *in vitro* while C_3H/HeN macrophage exhibited a marked increase in phagocytosis. Phagocytosis of latex particles by C_3H/HeJ macrophages was found to be normal. This Fc receptor defect was found to be secondary to a failure to maintain a differentiated state *in vitro*. Treatment of C_3H/HeJ macrophages with supernatant derived from conconavalin A (con A) stimulated spleen cells corrected this defect. This observation is consistent with Ruco and Meltzer's report (92) demonstrating that C_3H/HeJ macrophages responded to LPS to kill tumor cells *in vitro* only after activation with lymphokine. Thus, it seems that only macrophages having attained a certain activation state are capable of being triggered by LPS. Vogel et al.'s recent findings (117) further emphasize the close relationship between LPS sensitivity and macrophage activation. These studies demonstrated that C_3H/HeJ mice, preinfected with BCG, displayed enhanced sensitivity to the *in vivo* effects of LPS including enhanced lethality, elevation of serum interferon, production of acute phase reactant serum amyloid (SAA), and hypoglycemia. This artificial activation of C_3H/HeJ macrophage *in vivo* with BCG infection (61) serves to confirm the hypothesis relating the differentiated state of macrophages to their ability to respond to endotoxin.

MATERIALS AND METHODS

Animals

Sprague-Dawley male rats (Harlan Industries, Indianapolis, Indiana), weighing between 300-350 grams, were used as a source for mediator production. Rats were anesthesized by an intraperitoneal (i.p.) injection of 0.5 mls sodium pentobarbital (65 mg/ml; W. A. Butler Co., Columbus, Ohio). Animals were given Purina Laboratory Chow and water *ad libitum*.

Endotoxin

Lyophilized *Salmonella typhimurium* lipopolysaccharide (LPS), extracted by the Westphal procedure and purchased from Difco Laboratories (Detroit, Michigan), was dissolved in sterile, pyrogen-free saline (Cutter Laboratories, Berkeley, Calif.) at a concentration of 1 mg/ml and stored at -20°C until used. Stock endotoxin solutions were diluted in saline to desired concentrations prior to being injected.

Induction of Tolerance

Rats were made tolerant to endotoxin by i.p. injections of increasing doses of endotoxin in 0.25 mls saline. The schedule for induction of endotoxin tolerance is that described by Ruggiero et al. (93): day 1, 0.01 mg; day 2,

0.025 mg; day 3, 0.1 mg; day 4, 0.2 mg; day 5, 0.6 mg; days 6 and 7, no treatment; day 8, 1 mg; day 9, 1 mg. Rats were challenged with 500 ug endotoxin and bled at 4 hours on day 11. Control rats were bled without receiving endotoxin.

Rat Serum

Blood, obtained from anesthesized rats by cardiac puncture, was allowed to clot for 1 hour at room temperature (25°C) and then centrifuged at 10,000 x g for 30 minutes at 4°C to obtain serum. Serum was pooled and stored at -70°C until used. Serum containing mediator was obtained from rats given an intravenous (i.v.) injection of 500 ug of endotoxin 30 seconds, 1 hour, 2 hours, 4 hours, and 8 hours prior to bleeding.

Cell Culture Conditions

Reuber H-35 cells, derived from the H-35 rat hepatoma, were obtained from Dr. John Koontz, Department of Pharmacology, University of Colorado Medical Center in Denver. Stock cultures were grown as monolayers in Eagle's Joklik-Modified Minimum Essential Medium (Grand Island Biological Co., New York) containing 5% fetal bovine serum and 10% newborn calf serum. Cultures were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO_2-95% air.

Experimental Conditions

Cells used for experimentation were plated in 60 x 15 mm tissue culture dishes (Corning Glass Works, N.Y.). When

dishes were near confluence, growth medium was replaced with fresh serum-free medium of the same formulation containing either LPS (60 ug/ml culture medium) or 10% experimental rat serum. The final volume was 2.5 mls/dish. After 1 hour incubation dexamethasone (9α-fluoro-16αMethylprednisolone; Sigma Chemical Co., St. Louis, Missouri) or $N^6,O^{2'}$-dibutyryl-adenosine 3'-5'-cyclic monophosphoric acid and theophylline (Sigma Chemical Co., St. Louis, Mo.) were added at final concentrations of 1 uM, 0.5 mM, and 1 mM, respectively. Enzyme activity was assayed after incubation overnight (12 hours).

Phosphoenolpyruvate Carboxykinase (PEPCK) Assay

Enzyme activity was measured in the cytosol fraction of Reuber H-35 cells. Cell monolayers were washed 3 times with and then suspended in 0.6 mls of 0.15 M KCl-0.001 M EDTA solution, pH 7.6. Cells were fractured by freeze-thawing 3 times in a dry ice-95% EtOH bath. The cytosol fraction was isolated by centrifugation for 20 minutes at 20,000 x g, 4°C. PEPCK activity was measured by the $NaH^{14}CO_3$ fixation assay as described by Ballard and Hanson (8) with the following minor modifications: 1) the bicarbonate fixation assay mixture contained all reagents listed except dithiothreitol, 2) assays were carried out for 12 minutes at 37°C, and 3) samples were gassed for 45 minutes after reaction occurred to remove any free $^{14}CO_2$ not incorporated into the end-product, malate. Data is presented as relative specific activity where experimental values were normalized against

controls (untreated hepatoma cell cultures) representing basal levels of PEPCK activity. Specific activity is expressed as the number of enzyme units per milligram of protein, where enzyme units were nanomoles (nmoles) bicarbonate fixed per minute. Protein concentration was measured by the method of Lowry et al. (60).

Tyrosine Aminotransferase (TAT) Assay

Tyrosine aminotransferase (TAT) activity was assayed in the cytosol fraction of Reuber H-35 cells following a modification of the procedure described by Granner and Tomkins (35). Cytosol fractions were prepared as described above. The reaction was run for 15 minutes at 37°C and then stopped by the addition of 0.3 mls 100% trichloroacetic acid (TCA) with immediate mixing. The solution was incubated 30 minutes at room temperature (25°C) prior to reading the optical density at 850 mu. Data is presented as relative specific activity where experimental values were normalized against controls representing basal levels of TAT activity. Enzyme activity is expressed as umoles of p-hydroxyphenylpyruvate formed per minute per milligram protein. Protein concentration was measured by the method of Lowry et al. (60).

In Vitro Preparation of Serum-Modified Endotoxin

200 ug of endotoxin was added to 1 ml of pooled control rat serum and maintained at 37°C in a shaker water bath

for 30 minutes. The serum-modified endotoxin preparation was used immediately after incubation.

Chromium-51 Labeling of Endotoxin

Radiolabeled endotoxin was prepared as described by Zladeszyk and Moon (121). Chromium-51 ($Na^{51}CrO_4$; New England Nuclear Corp., Boston, Mass.) was purchased in 2 mCi amounts with a specific activity of about 300-500 Ci/g. 1.5 mCi of chromiun-51 was added to 100 mg of lyophilized lipopolysaccharide. Pyrogen-free saline and 10% sodium azide were added to give a final volume of 10.0 mls. The mixture was incubated with constant stirring at 37°C for 48 hours and then dialyzed at 4°C for 3-5 days against frequent changes of deionized water. The preparation was centrifuged at 8,000 x g for 1 hour. The supernatant was then recentrifuged at 100,000 x g for 8 hours. The pellet from the second centrifugation was resuspended in 4.0 mls of deionized water and stored at -20°C. The ^{51}Cr-labeled endotoxin was diluted in saline to desired concentration prior to injection.

Isopycnic Density Gradient Ultracentrifugation

Cesium chloride solutions having an average density (d) of 1.45 g/cm^3 were prepared containing serum samples diluted 1:7 in deionized water. Serum samples were prepared by injecting 500 ug of ^{51}Cr-labeled endotoxin into rats. Rats were bled immediately (30 seconds) or 30 minutes after injection and serum was prepared as described above. 200 ug

of ^{51}Cr-labeled endotoxin was added to 1 ml of control rat serum and prepared as described for an *in vitro* preparation. 5 ul of ^{51}Cr-labeled endotoxin was added to water to determine the density of toxic endotoxin. Samples were centrifuged to equilibrium in the Spinco model L3-50 Preparative Ultracentrifuge (Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.) with a SW50.1 rotor at 40,000 rpm, 20°C for 60 hours. 0.1 ml fractions were collected. The presence of endotoxin in each fraction was determined by the measurement of radioactivity in a Packard gamma counter. Densities were calculated from the index of refraction as determined from a refractometer (Bausch and Lomb, Rochester, N.Y.).

Assay for Hemolytic Complement

Hemolytic complement titers were measured in 50% hemolytic complement (CH_{50}) units for experimental rat seras following a modification of Kabat and Mayer's procedure (44). Sheep erythrocytes, kindly donated by Dr. Meyer (Animal Care Department, State Laboratory, Lansing, Mi.), were sensitized with rabbit anti-erythrocyte hemolysin (Microbiological Associates, Walkersville, Md.). 0.5 mls of sensitized cells in Veronal buffered saline containing 1.5×10^{-4} M Ca^{2+} and 5×10^{-4} M Mg^{2+}, pH 7.4 was added to 0.5 mls of serially diluted rat serum and incubated at 37°C for 1 hour. After incubation 2 mls of buffer was added to each tube, cells were then centrifuged at 1000 x g for 10 minutes and absorbance was read at 541 mu.

Statistics

Statistical significance between means was determined by an analysis of variance (ANOVA) and Duncan's multiple range test (108).

RESULTS

Effect of Lipopolysaccharide (LPS) on the Induction of Phosphoenolpyruvate Carboxykinase (PEPCK) in Cultured Hepatoma Cells Exposed to Dexamethasone

PEPCK activity was assayed as described in Materials and Methods in Reuber H-35 hepatoma cell cultures exposed to dexamethasone after overnight incubation (12 hours) with (experimental) and without (induction controls) Salmonella typhimurium LPS (60 ug/ml culture medium) to determine the direct effect of the LPS molecule on induction of PEPCK by the synthetic glucocorticoid, dexamethasone. Addition of LPS to hepatoma cell cultures in the absence of dexamethasone had no effect upon basal levels of PEPCK activity (Table 1). After overnight incubation of Reuber H-35 cell cultures in the presence of 1 uM dexamethasone a two-fold increase over basal PEPCK activity was observed. Hepatoma cell cultures exposed to 1 uM dexamethasone and incubated overnight in the presence of LPS displayed no significant inhibitory effect on induced PEPCK synthesis.

Table 1: Induction of PEPCK in Reuber H-35 Hepatoma Cells Treated with Endotoxin

TREATMENT	RELATIVE PEPCK ACTIVITY[a]
None	1.00 ± .16
Dexamethasone[b]	2.00 ± .34[d]
Endotoxin[c]	.98 ± .29
Endotoxin and dexamethasone	1.84 ± .89[d]

[a]Mean relative activity expressed as nmoles $NaH^{14}CO_3$ fixed per minute per milligram protein ± standard deviation (s.d.).

[b]1×10^{-6} M

[c]150 ug

[d]Not significant (NS)

Effect of Serum from Endotoxemic Rats on the Induction of Phosphoenolpyruvate Carboxykinase (PEPCK) in Cultured Hepatoma Cells Exposed to Dexamethasone

Sera obtained from untreated rats (controls) and rats made endotoxemic by an intravenous (i.v.) injection of 500 ug of LPS (in 0.5 mls saline) and bled at 4 hours (experimental) was studied for effects upon PEPCK inducibility in Reuber H-35 cells. Sera to be tested for inhibitory activity on PEPCK induction by dexamethasone was added to hepatoma cell cultures to a final concentration of 10% (v/v) in the culture medium and incubated overnight prior to assay. Addition of control serum to hepatoma cell cultures resulted in lowered induced levels of PEPCK activity (Table 2) when compared to the level of induction seen in the absence of

Table 2: Induction of PEPCK in Reuber H-35 Hepatoma Cells Treated with Rat Serum[a]

TREATMENT	RELATIVE PEPCK ACTIVITY[b]
Control serum[c]	1.00 ± .16[e]
Control serum and dexamethasone[d]	1.62 ± .42[f]
Endotoxemic serum	.84 ± .24[e]
Endotoxemic serum and dexamethasone	.83 ± .18[f]

[a]Serum was obtained as described in Materials and Methods.

[b]Mean relative activity expressed as nmoles $NaH^{14}CO_3$ fixed per minute per mg protein ± s.d.

[c]Serum added to 10% (v/v) final concentration.

[d]1×10^{-6} M.

[e]NS.

[f]$p < .01$.

serum (Table 1). Experimental serum added to hepatoma cell cultures exposed to dexamethasone significantly ($p < .01$) inhibited the induction of PEPCK. Addition of experimental serum in the absence of dexamethasone had no significant effect upon the base level of PEPCK activity (Table 2).

Time Course for the Production of an Endotoxin-Induced Humoral Mediator Able to Inhibit the Induction of Phosphoenolpyruvate Carboxykinase (PEPCK)

Serum was collected from rats at timed intervals (30 seconds, 1 hour, 2 hours, 4 hours, and 8 hours) following an intravenous (i.v.) injection of 500 ug of LPS and tested for inhibitory activity on PEPCK induction in hepatoma cell

cultures exposed to dexamethasone. As shown in Table 3, serum collected as early as 30 seconds after LPS administration was found to significantly ($p < .01$) inhibit the induction of PEPCK. Significantly more inhibitory activity was found in serum collected at 1 hour, 2 hours, and 4 hours post-LPS administration than that collected at 30 seconds as demonstrated by the increased inhibition of induction of PEPCK ($p < .01$). By 8 hours post-injection the inhibitory activity began to diminish as shown by a significant decrease ($p < .01$) in the inhibition of PEPCK induction. Inhibitory activity in serum obtained from endotoxemic rats appeared to be maximal at 1 to 2 hours post-LPS administration (Figure 1a).

Effect of Serum Obtained from Endotoxin-Tolerant Rats on the Induction of Phosphoenolpyruvate Carboxykinase (PEPCK) in Cultured Hepatoma Cells Exposed to Dexamethasone

Serum obtained from endotoxin-tolerant rats was tested for the presence of a mediator able to inhibit the glucocorticoid induction of PEPCK in Reuber H-35 hepatoma cell cultures. Rats were made tolerant to endotoxin as described in Materials and Methods. On day 11 of the tolerance schedule serum was collected from untreated endotoxin-tolerant rats receiving 500 ug of LPS and bled at 2 hours. Sera was added to hepatoma cell cultures to a final concentration of 10% (v/v) in the culture medium and incubated overnight prior to assay. As shown in Table 4, addition of control and experimental sera allowed for significant ($p < .01$) induction of

Table 3: Time Course for the Production of an Endotoxin-Induced Humoral Mediator Involved in the Inhibition of Induction of PEPCK by Dexamethasone

TREATMENT	RELATIVE PEPCK ACTIVITY[a]
Control serum[b]	1.00 ± .15
Control serum and dexamethasone[c]	1.35 ± .15[d]
Endotoxemic serum (30 sec) and dexamethasone	1.06 ± .17[d,e]
Endotoxemic serum (1 h) and dexamethasone	.75 ± .08[e,f]
Endotoxemic serum (2 h) and dexamethasone	.71 ± .11[f]
Endotoxemic serum (4 h) and dexamethasone	.83 ± .18[f,g]
Endotoxemic serum (8 h) and dexamethasone	1.09 ± .15[g]

[a]Mean relative activity expressed as nmoles $NaH^{14}CO_3$ fixed per minute per mg protein ± s.d.

[b]Serum added to 10% (v/v) final concentration.

[c]1×10^{-6} M.

[d]$p < .01$.

[e]$p < .01$.

[f]NS.

[g]$p < .01$.

PEPCK in Reuber H-35 hepatoma cell cultures exposed to dexamethasone. However, the induction of PEPCK seen in the presence of the experimental serum was significantly lower ($p < .01$) than that seen in hepatoma cultures treated with the control serum.

Table 4: Induction of PEPCK in Reuber H-35 Hepatoma Cells Treated with Serum Obtained from Endotoxin-Tolerant Rats[a]

TREATMENT	RELATIVE PEPCK ACTIVITY[b]
Tolerant control serum[c]	1.00 ± .12[d]
Tolerant control serum and dexamethasone[e]	1.54 ± .18[f]
Tolerant endotoxemic serum and dexamethasone	1.33 ± .14[d,f]

[a]Rats were made tolerant to endotoxin as described in Materials and Methods.

[b]Mean relative activity expressed as nmoles $NaH^{14}CO_3$ fixed per minute per mg protein ± s.d.

[c]Serum added to 10% (v/v) final concentration.

[d]$p < .01$.

[e]1×10^{-6} M.

[f]$p < .01$.

Effect of an In Vitro Preparation of Serum-Modified Endotoxin on the Induction of Phosphoenolpyruvate Carboxykinase (PEPCK) in Cultured Hepatoma Cells Exposed to Dexamethasone

Table 5 shows the effect of *in vitro* serum-modified LPS on the induction of PEPCK by dexamethasone in Reuber H-35 hepatoma cell cultures. 200 ug of LPS was added per 1 ml of pooled control rat serum and maintained at 37°C for 30 minutes prior to assay (experimental serum). Control serum was prepared by maintenance of pooled control rat serum at 37°C for 30 minutes in the absence of LPS. Sera to be tested for inhibitory activity on PEPCK induction by

Table 5: Induction of PEPCK in Reuber H-35 Hepatoma Cells Treated with an In Vitro Preparation of Serum-Modified Endotoxin[a]

TREATMENT	RELATIVE PEPCK ACTIVITY[b]
Control serum[c]	1.00 ± .09
Control serum and dexamethasone[d]	1.42 ± .22[e]
Serum containing modified endotoxin and dexamethasone	1.40 ± .15[e]

[a]Serum-modified endotoxin was prepared *in vitro* as described in Materials and Methods.

[b]Mean relative activity expressed as nmoles $NaH^{14}CO_3$ fixed per minute per mg protein ± s.d.

[c]Control rat serum was maintained at 37°C for 30 minutes. Serum added to 10% final concentration.

[d]1×10^{-6} M.

[e]NS.

dexamethasone was added to hepatoma cell cultures to a final concentration of 10% (v/v) in the culture medium and incubated about 12 hours prior to assay. As seen in Table 5, addition of serum containing the modified form of LPS had no significant inhibitory effect upon the induction of PEPCK.

The Modification of Salmonella Typhimurium Lipopolysaccharide (LPS) by Rat Serum (or Plasma)

Isopycnic density gradient ultracentrifugation in cesium chloride, as described in Materials and Methods, was employed to study the interaction of *Salmonella typhimurium* LPS with serum (*in vitro*) and with plasma (*in vivo*). As shown in Table 6, native *Salmonella typhimurium* LPS was found

Table 6: The Effect of Rat Serum (or Plasma) on the Buoyant Density of Salmonella Typhimurium Lipopolysaccharide (LPS)

TREATMENT	DENSITY (g/cm^3)[a]
Native LPS	1.46
LPS in endotoxemic serum (30 sec)[b]	1.38
LPS in endotoxemic serum (30 min)[b]	1.36
LPS in tolerant endotoxemic serum (30 sec)[b]	1.36
Serum-modified LPS (*in vitro*)[c]	1.46
2 peaks:	1.35

[a]Reduction in buoyant density was determined as described in Materials and Methods.

[b]Rats were injected with 500 ug ^{51}Cr-LPS and bled 30 seconds or 30 minutes post-injection.

[c]200 ug ^{51}-Cr-LPS was added to 1 ml of control rat serum and maintained at 37°C for 30 minutes.

to have a buoyant density (d) of 1.46 g/cm^3. After addition of 200 ug of ^{51}Cr-LPS to 1 ml of control rat serum and maintenance at 37°C for 30 minutes a new peak (about 25% of the added radioactivity) appeared at d=1.35 g/cm^3. The predominance of the radioactivity remained at d=1.46 g/cm^3. This density shift of LPS induced by serum was also found to occur *in vivo*. 500 ug of ^{51}Cr-LPS was injected i.v. into rats (normal and tolerant) and bled 30 seconds or 30 minutes post-injection. Results indicate that a reduction in the buoyant density of LPS occurred almost immediately (30 seconds) and to completion in normal (d=1.38 g/cm^3) and tolerant (d=1.36 g/cm^3) animals. Normal animals bled 30

minutes post-LPS administration showed no further significant reduction in LPS buoyant density.

Activation of Complement by Lipopolysaccharide (LPS) in Serum Obtained from Rats

The degree of complement activation as a result of LPS administration was examined in serum obtained at timed intervals (30 seconds, 1 hour, 2 hours, 4 hours, and 8 hours) from rats receiving 500 ug of LPS i.v., in serum obtained from tolerant rats receiving 500 ug of LPS and bled at 2 hours, and in an *in vitro* preparation of serum containing modified LPS. Hemolytic complement titers, measured in 50% hemolytic complement (CH_{50}) units, were determined as described in Materials and Methods. Data is presented as percent of original CH_{50} titer where original CH_{50} titer was determined in control rat serum. As shown in Table 7 and Figure 1b, CH_{50} levels in endotoxemic rat serum decreased rapidly (30 seconds) and remained depressed for about 2 hours after LPS injection. By 4 to 8 hours post-LPS injection complement levels were nearly restored to control levels.

Addition of LPS to serum *in vitro* and incubation at 37°C for 30 minutes, resulted in the activation of complement to the same degree as that seen *in vivo* 1 hour post-LPS administration. Complement levels were also found to be markedly reduced (41% of original CH_{50} titer) in endotoxemic serum obtained from tolerant rats 2 hours after LPS challenge.

Table 7: Complement Activation by Lipopolysaccharide (LPS)

TREATMENT	% OF ORIGINAL CH_{50} TITER[a]
Control serum (Normal)	100
endotoxemic serum (30 seconds)	80 ± 7
endotoxemic serum (1 hour)	64 ± 13
endotoxemic serum (2 hours)	86 ± 11
endotoxemic serum (4 hours)	94 ± 27
endotoxemic serum (8 hours)	91 ± 38
control serum (tolerant)	100
tolerant endotoxemic serum	41 ± 2
control serum (*in vitro*)	100
serum containing modified LPS[b]	66 ± 1

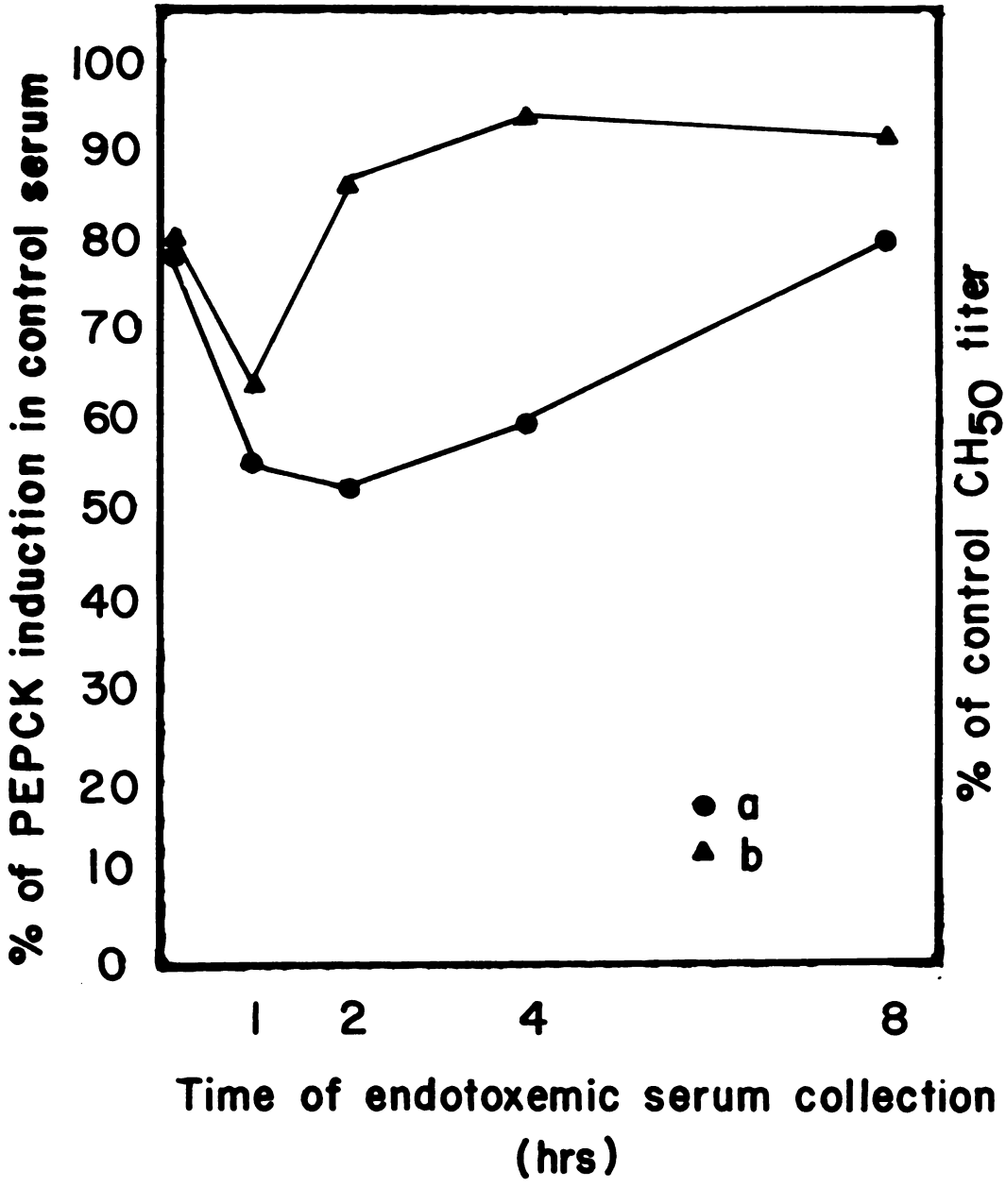
[a] CH_{50} titers were determined as described in Materials and Methods.

[b] 1 ml control rat serum containing 200 ug of LPS maintained at 37°C for 30 minutes.

Figure 1: Comparison of the Time Courses for the Production of an Endotoxin-Induced Humoral Mediator Able to Inhibit the Glucocorticoid Induction of PEPCK and for LPS-Induced Complement Activation in Rats.

(a) Serum was added to a final 10% (v/v) concentration in Reuber H-35 hepatoma cell cultures. PEPCK activity was determined after 12 hours of exposure to 1 uM dexamethasone. Values represent the percentage of control PEPCK induction expressed as the mean.

(b) Hemolytic complement titers were measured in 50% hemolytic complement (CH_{50}) units for rat sera collected at timed intervals following endotoxin administration. Values represent the percentage of CH_{50} for control rat serum expressed as the mean.


% of PEPCK induction in control serum
100
90
80
70
60
50
40
30
20
10
0
% of control CH50 titer
a
b
1
2
4
8
Time of endotoxemic serum collection
(hrs)

DISCUSSION

In recent years it has become widely accepted that many of the biological effects of endotoxin are elicited by mediators released into the serum of the host upon endotoxin challenge (12)(78). Although a mechanism for protection remains to be elucidated, glucocorticoid hormones have been found to suppress or diminish many of the biological effects associated with endotoxin in the host (11). Glucocorticoids stimulate gluconeogenesis through induced synthesis of critical enzymes including the rate-limiting enzyme, PEPCK. It is believed that endotoxin interferes indirectly with the regulatory effect of glucocorticoids through a mediator termed glucocorticoid antagonizing factor or GAF (74). Although not firmly established, GAF is thought to interfere with transcription of enzyme mRNA, which glucocorticoids are believed to increase during the induction process (9)(13).

Data presented in Tables 1 and 2 support an indirect mechanism of action for endotoxin. The addition of 60 ug of LPS/ml culture medium (or 150 ug of LPS/hepatoma cell culture) directly to hepatoma cell cultures had no inhibitory effect upon PEPCK inducibility by dexamethasone (Table 1). Therefore, the inhibition of PEPCK induction observed upon the addition of endotoxemic rat serum to hepatoma cell

cultures cannot be attributed to any residual toxic endotoxin remaining in the serum. Inhibition of hormonal induction may be explained by the presence of a humoral factor(s) released by the host as a result of endotoxin administration. The suggestion that background levels of inhibitory activity may be present in normal serum and functioning as a normal metabolic and immunologic regulator (32) may explain the slight, but statistically insignificant, decrease in induction of PEPCK by dexamethasone seen in the presence of control rat serum. The observation that as little as 1 ug of LPS injected i.v. into mice was sufficient to inhibit hydrocortisone-induced PEPCK synthesis (unpublished results), presumably through the release of endotoxin-induced mediators, provides further evidence for a mediated rather than direct effect of endotoxin.

Time course studies revealed the presence of inhibitory activity as early as 30 seconds post-LPS administration. Previous reports (32)(33) found significant inhibitory activity no earlier than 1 hour post-LPS injection in zymosan-primed mice. These same studies found the inhibitor to disappear by 4 hours post-injection. Data presented in Table 3 show inhibitory activity to remain significant but begin to diminish by 8 hours post-injection. Since inhibition of hormonal induction of PEPCK has been shown to be a dose-dependent phenomenon (32) these discrepancies may be the result of differences in the amounts of endotoxemic-serum (2% versus 10%) and, thus, the amount of inhibitor

added to hepatoma cell cultures and/or to differences in the amount of inhibitory activity generated by the two species (zymosan-primed mice versus untreated rats). The significance of the presence of inhibitory activity immediately after LPS challenge suggests that the inhibition of PEPCK induction may be due to the release of a pre-formed molecule by the host or to some immediate alteration in the LPS molecule following LPS-plasma interactions.

Isopycnic density gradient ultracentrifugation in cesium chloride of endotoxemic rat serum revealed an immediate (30 second post-injection) and complete reduction in buoyant density of LPS from 1.46 g/cm^3 to 1.38 g/cm^3. It is thought that the direct interaction of LPS with humoral as well as cellular elements may lead to the production of injurious mediators (78). Thus, it was postulated that this plasma-modified low density form (LDF) of LPS may be the inhibitory factor effecting PEPCK induction by glucocorticoids. To test this hypothesis a LDF of LPS was prepared *in vitro* and added to hepatoma cell cultures exposed to dexamethasone. No inhibitory activity was found with this serum-modified LPS. These results do not conclusively disprove the hypothesis since an *in vitro* preparation results only in an incomplete reduction of buoyant density of LPS (Table 6).

Studies involving serum obtained from endotoxin-tolerant rats present a stronger argument against the low density form (LDF) of LPS playing any role in the mediation of inhibition of glucocorticoid induction of PEPCK. Serum

obtained from endotoxin-tolerant rats 2 hours after receiving a dose of LPS contained either an absence of or decreased amounts of the inhibitory factor since it allowed for the significant induction of PEPCK by dexamethasone in Reuber H-35 cells. Although inhibitory activity was minimal to nonexistent, plasma from endotoxin-tolerant rats was able to cause a reduction in the buoyant density of LPS to completion within 30 seconds post-injection. It has been suggested that this interaction of LPS with plasma (or serum components leading to a reduction in buoyant density may, in fact, be a major pathway of LPS detoxification (114).

Another report (113) suggests that LPS, prior to inactivation and/or clearance, may interact with plasma protein systems, such as complement, and result in the production of biologically active, short-lived mediators. Biological activities significant in the pathophysiology of endotoxin and produced in vitro as a consequence of interactions between endotoxin and complement include release of histamine and heparin from mast cells, adherence and degranulation of platelets, and production of anaphylatoxin and chemotactic factors (69). Present studies examined the idea that by-products of LPS-induced complement activation may be active biologically and may be responsible for the inhibitory effect upon PEPCK induction by glucocorticoids. Although complement activation and production of inhibitory activity were both found to be immediate events, CH_{50} levels were restored to normal levels at a time when significant

inhibitory activity remained (Figure 1). Thus, the time course for LPS-induced complement activation does not correspond to that of mediator production. LPS was also found to activate complement to a greater degree in tolerant rats than in control rats and to activate complement *in vitro* to the same degree as that seen *in vivo* 1 hour post-injection even though no inhibitory activity was found to be associated with either preparation.

These results, in combination with other studies (74)(75)(76), provide strong evidence that the inhibition of hepatic PEPCK synthesis upon endotoxin challenge occurs via a mediator(s) released by the host. The involvement of the host reticuloendothelial system, particularly the macrophage, has been implicated in the production of this mediator(s) (74). Further *in vitro* studies are needed to conclusively determine the cellular source of this mediator(s). Once this source is identified, researchers can proceed with the purification and characterization of this inhibitor. Unfortunately the current *in vitro* assay system employing Reuber H-35 hepatoma cell cultures appears to be somewhat less than ideal for these studies (see appendix). Currently, there is a need for the identification and characterization of a new cell-line suited for the continuation of these studies.

APPENDIX

APPENDIX

THE LOSS OF ENZYME INDUCIBILITY IN REUBER H-35 HEPATOMA CELL CULTURES

The development of an *in vitro* assay system utilizing cultured Reuber H-35 hepatoma cells by Goodrum and Berry (32) has been invaluable in the study of an endotoxin-induced humoral mediator responsible for the inhibition of induction of PEPCK by dexamethasone. Advantages of this system over *in vivo* assays include an insensitivity to endotoxin and the ability to detect about 10 times less mediator than that detectable *in vivo*. However, recent evidence suggests that the disadvantages of an assay employing Reuber H-35 hepatoma cell cultures may outweigh any advantages. Stock cultures of Reuber H-35 hepatoma cells were found to exhibit a gradual reduction in enzyme inducibility by dexamethasone over short periods of time. After maintenance of stock cultures for about 2 months, with passage by trypsinization twice a week, PEPCK was found to no longer be inducible by dexamethasone. In these cultures $N^6,O^{2'}$-dibutyryladenosine 3'-5'-cyclic monophosphoric acid (DBcAMP) was found to induce PEPCK, although to levels significantly lower than that reported in the literature (Table A-1). Barnett and Wicks' (9) studies of the effects of

glucocorticoids and DBcAMP in Reuber H-35 cells found PEPCK to be induced 2- to 3-fold by dexamethasone and DBcAMP. To determine if this loss of inducibility was specific for PEPCK, another hepatic enzyme, tyrosine aminotransferase (TAT), was studied. Although Barnett and Wicks (9) reported a 5- to 8-fold increase of TAT by dexamethasone in Reuber H-35 cells, as shown in Table A-2, only a 1.93-fold increase in activity occurred upon exposure to dexamethasone. A second assay at a later date (about 1-1/2 months after initial assay date) showed a further reduction in TAT inducibility to levels comparable to control levels. These studies suggest that the aging process of Reuber H-35 hepatoma cell cultures may interfere with enzyme inducibility and, thus, questions the usefulness of this particular cell line as an in vitro assay system for endotoxin research.

Table A-1: Loss of Phosphoenolpyruvate Carboxykinase (PEPCK) Inducibility in Reuber H-35 Hepatoma Cell Cultures

TREATMENT	RELATIVE PEPCK ACTIVITY[a]
None	1.00 ± .02
Dexamethasone[b]	1.05 ± .10
DBcAMP[c]	1.65 ± .42

[a]Mean relative activity expressed as nmoles $NaH^{14}CO_3$ fixed per minute per mg protein ± s.d.

[b]1×10^{-6} M.

[c]5×10^{-4} M $N^6,O^{2'}$-dibutyryladenosine 3'-5'-cyclicmonophosphoric acid and 1×10^{-3} M theophylline.

Table A-2: Loss of Tyrosine Aminotransferase (TAT) Inducibility in Reuber H-35 Hepatoma Cell Cultures

TREATMENT	RELATIVE TAT ACTIVITY[a]
None	1.00 ± .35
Dexamethasone[b] (assay 1)	1.93 ± .35
Dexamethasone[b] (assay 2)[c]	1.20 ± .16

[a]Mean relative activity expressed as umoles of p-hydroxyphenylpyruvate formed per minute per mg protein at 37°C ± s.d.

[b]1×10^{-6} M.

[c]About 1-1/2 months after assay 1.

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