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Cellular effects of endotoxin in vitro: mobility of endotoxin in the plasma membrane of hepatocytes and neuroblastoma cells

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Lipopolysaccharide labeled with fluorescein isothiocyanate (FITC-LPS) was used to examine interactions between endotoxin and plasma membrane in isolated rat hepatocytes and mouse neuroblastoma NB41A3 cells. At the same endotoxin to cell ratio, hepatocytes bound more toxin than did neuroblastoma cells. At a dose of 12 μg/mg dry wt, a bound mobile fraction of between 60 and 75% of FITC-LPS was found on hepatocytes at 25°C with a lateral diffusion coefficient (D) of $4.8 \cdot 10^{-9}$ cm^2/s. In neuroblastoma cells, the mobile fraction was larger (85–90%), with D $1.0 \cdot 10^{-8}$ cm^2/s. D was temperature-dependent between 10 and 37°C and increased from $1.8 \cdot 10^{-9}$ to $1.0 \cdot 10^{-8}$ cm^2/s in hepatocytes and from $9.4 \cdot 10^{-9}$ to $1.9 \cdot 10^{-8}$ cm^2/s in neuroblastoma cells. In both types of cell, nonviable (cells which did not exclude Trypan blue) as compared to viable cells showed different recovery patterns and 100% of the probe molecules were mobile. These results suggest that: (1) endotoxin binding to mammalian cells consists of two subpopulations with different mobilities; (2) binding of the immobile fraction is dependent on cellular integrity; and (3) the differences in binding, lateral mobility, and size of the immobile fraction in hepatocytes and neuroblastoma cells may be due to variations in membrane composition and/or number of binding sites.

Introduction

Little is known about the mechanism of action of endotoxins at the cellular level, although their effects in clinical situations and experimental animals have been characterized extensively. Previous studies from this laboratory [1–3] have used isolated cells as an in vitro model system and explored early cellular responses (changes in metabolites, ion contents and enzyme activities) to endotoxin exposure. In the present work, we have used fluorescein isothiocyanate labeled lipopolysaccharide to examine directly the mechanism of toxin attachment to mammalian cell membranes. Earlier investigations have shown that endotoxins have the ability to bind to and interact with almost all types of mammalian cells [4–9]. Whether this interaction occurs through nonspecific lipid-lipid contacts, which cause membrane destabilization and/or changes in fluidity, or through binding to a specific membrane component, which initiates cellular perturbations, remains to be determined.

In vivo studies have shown that the liver is an important site for clearance of endotoxin and that parenchymal cells as well as Kupffer cells are involved in this process [9,10]. It is also known that not all cell types bind endotoxin with the

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Abbreviations: FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide.

same affinity or are equally susceptible to its toxic effects [4,5,7]. In particular, transformed cells are far more resistant to the toxic effects of endotoxin than are primary cells [5,11]. Therefore, in the present study, we examined the binding characteristics and lateral mobility of endotoxin in two cell types, primary cells (hepatocytes isolated from perfused rat livers) and transformed cells (mouse neuroblastoma NB41A3), using the method of fluorescence recovery after photobleaching. This is a relatively new technique which can be used to study lateral mobility of membrane proteins, glycoproteins, and lipids (for reviews, see Refs. 12–14). The lateral mobility of these membrane components is important in controlling cellular responses to the external environment. Because membrane proteins and lipids have different diffusion properties on the cell surface, it should be possible, using the fluorescence recovery after photobleaching technique, to determine whether endotoxin is binding to either a protein or lipid component of the plasma membrane. This knowledge should, in turn, not only allow us to gain some insight into the mechanism of endotoxin attachment but also to test the possibility that the initial interaction of the toxin with the plasma membrane influences cellular susceptibility to its action.

Materials and Methods

Cell culture. Mouse neuroblastoma cells (NB41A3) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and penicillin/streptomycin. The cultures were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C for 2–3 days. The cells were detached mechanically from their solid support, washed in Hanks' medium [15] containing 5–10 mM glucose and 0.2% bovine serum albumin, and resuspended in the same medium to a final concentration of 4–5 mg dry weight cells/ml ($(8–10)\cdot10^5$ cells/ml). The cell suspension was passed through a wire mesh (60–80 μm) to ensure homogeneity. Incubations were carried out at 25°C in a Dubnoff metabolic shaker, in open flasks (to ensure adequate oxygenation). The dry weights of cells in suspension and of the medium were determined for each preparation.

Hepatocyte preparation. Hepatocytes were isolated from perfused rat livers according to the method of Berry and Friend [16] with modifications described by Krebs et al. [17]. Male Sprague-Dawley rats (200–300 g) were starved for 48 h then anesthetized with sodium pentobarbital (Nembutal, Abbott Laboratories, Chicago, IL) by intraperitoneal injection at a dose of 5 mg/100 g body wt. Livers were perfused with Krebs-Henseleit saline (pH 7.4) equilibrated ~~with Krebs-Henseleit saline (pH 7.4) equilibrated~~ with 95% O_2/5% CO_2 and containing 0.4 mg collagenase/ml (Boehringer-Mannheim, Indianapolis, IN) and 1% bovine serum albumin. Perfusions were carried out with flow rates of 40 ml/min and at 37°C. After isolation, the cells were suspended in Krebs-Henseleit buffer containing 2% dialyzed bovine serum albumin to a cell concentration of 5 mg dry wt./ml. Cell suspensions were maintained in an atmosphere of 5% CO_2 in O_2 and continuously shaken at 25°C. Dry weights of the cells in suspension and of the medium were determined for each cell preparation.

Experimental design. Primary and transformed cells were isolated and suspended as described above and incubated with various concentrations (0.2–50 μg/mg dry wt.) of fluorescein isothiocyanate-lipopolysaccharide conjugate (FITC-LPS) (List Biological Laboratories, Campbell, CA). In this preparation, the lipopolysaccharide was isolated from *Escherichia coli* O55:B5 and the FITC probe was attached onto the lipid A portion of the molecule (24.1 mol FITC/mol LPS). At various incubation times, two aliquots of cells were removed. One aliquot was used without any further manipulation and the second was washed by diluting 10-fold in endotoxin-free medium to approximately the same cell concentration (5 mg dry wt./ml). In both samples (washed and unwashed), fluorescence intensity was measured using a Perkin-Elmer 650-10S fluorimeter with 465 nm and 520 nm as excitation and emission wavelengths, respectively. Bound and free concentrations of FITC-LPS were determined from the fluorescence intensity. The washed cell suspension destined for the fluorescence recovery after photobleaching measurements was mixed with Trypan blue (10:1) to differentiate viable from damaged cells. The Trypan blue-cell suspension (5–10 μl) was placed on a glass microscope slide and secured with a

coverslip for photobleaching recovery experiments. Protein concentrations were determined for washed and unwashed cell suspensions according to the method of Lowry et al. [18].

Fluorescence recovery after photobleaching. Diffusion coefficients (D) and mobile fractions (fractions of the fluorophores which are laterally mobile in the experimental time-scale) were determined by fluorescence recovery after photobleaching. The apparatus was designed along conventional lines [19,20]. A detailed description of the equipment and data analysis can be found elsewhere [21]. Diffusion coefficients of the fluorescent probe were determined from the time dependency of fluorescence recovery. Recovery curves were routinely measured for 5 min following a 1 s bleach.

Results

Functional test for toxicity of FITC-LPS

The toxicity of fluorescein labelled endotoxin was assessed in both cell types by measuring alterations in various metabolic parameters. In mouse neuroblastoma cells, the effect of FITC-LPS on cellular [ATP]/[ADP] ratio was determined. ATP and ADP concentrations were measured enzymatically by the methods of Lamprecht and Trautschold [22] and Jaworek et al. [23], respectively. It was found that a dose of 12–24 μg FITC-LPS/mg dry wt. produced a 24% decline in [ATP]/[adP] ratios within 1 h incubation (control = 8.72 ± 0.52 and FITC-LPS treated = 6.61 ± 0.19, values are the means ± S.E. for three separate experiments). In isolated hepatocytes, 8 μg FITC-LPS/mg dry wt. produced a 60% increase in [β-hydroxybutyrate]/[acetoacetate] ratios within the same time interval (control = 0.48 ± 0.03 and FITC-LPS treated = 0.77 ± 0.04, $n = 5$). Both metabolites were measured according to Williamson et al. [24].

Lateral mobility of endotoxin bound to hepatocytes and neuroblastoma cells

Lateral mobility of FITC-LPS was determined in hepatocytes following a 30 min incubation with 12 μg FITC-LPS/mg dry wt. at 25°C (Fig. 1). (This corresponds to an in vivo dose of endotoxin of approx. 3–5 mg/100 g. The estimate was based on the calculation of Krebs et al. [17] that 65% of rat liver is composed of parenchymal cells and on the report of Zydaszyk and Moon [9] that follow-

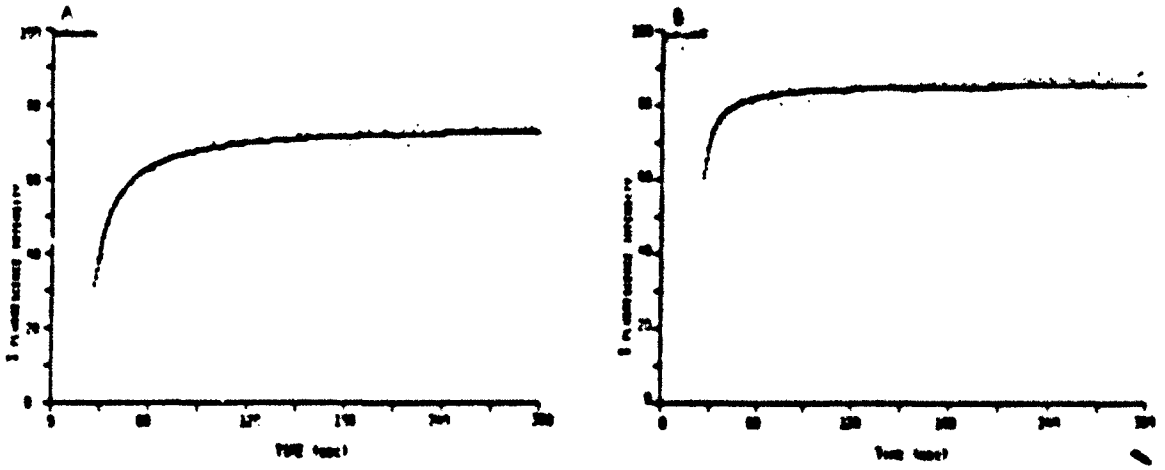

Fig. 1. Representative fluorescence recovery profiles of FITC-LPS-labelled isolated hepatocytes and neuroblastoma cells. (A) Rat hepatocytes (5 mg dry wt./ml) were incubated with 12 μg FITC-LPS/mg dry wt. for 30 min at 25°C. The individual points represent experimental data for fluorescence recovery values and the solid line shows the computer best fit to the experimental data. Diffusion coefficient is $4.8 \cdot 10^{-9}$ cm²/s with fluorescence recovery of 58%. (B) Neuroblastoma cells (5 mg dry wt./ml) were incubated with 12 μg FITC-LPS/mg dry wt. under the same experimental conditions as described in (A). Diffusion coefficient is $1.62 \cdot 10^{-8}$ cm²/s with fluorescence recovery of 76%.

ing intravenous injection of endotoxin. 80% is recovered in the liver.) The fractional recovery of fluorescence after the first bleach was used as a measure of the mobile fraction of the fluorophores. It was found that 65% of the FITC-LPS bound to hepatocytes was mobile in the plasma membrane with a diffusion coefficient of $4.5 \cdot 10^{-9}$ cm^2/s. After the second and further bleaches of the same area the curves returned to their original pre-bleach levels, which demonstrates that the recoveries were 100% complete within the limits of experimental error (see Fig. 2). Moreover, there was no significant difference between diffusion coefficients derived from the first and successive bleaches at the same membrane location. This behavior indicates that the laser beam had no adverse effect on the cellular plasma membrane, which is in agreement with numerous studies of other investigators [12–14,24,29,31].

Fig. 1b shows a representative fluorescence recovery after photobleaching profile for a neuroblastoma cell incubated with FITC-LPS under the same experimental conditions as described for hepatocytes. In neuroblastoma cells, lateral mobility of FITC-LPS was greater, with an average diffusion coefficient of $1 \cdot 10^{-8}$ cm^2/s. There was also an increase in fractional recovery of fluorescence to 80% as compared to 65% in the hepatocytes.

Effect of temperature on lateral mobility of endotoxin

Lateral mobility of FITC-LPS in both primary and transformed cells was measured at 10, 25 and 37°C to determine whether diffusion was temperature dependent. Cells were incubated for 30 min with 12 μg FITC-LPS/mg dry wt. at the three temperatures, then washed and samples prepared as described in Methods. Measurements of fluorescence recovery after photobleaching were carried out using a thermostatically controlled stage so that the incubation temperature could be maintained constant. The results of these experiments are shown in Table I. In both primary and transformed cells, lateral mobility of FITC-LPS increased as the temperature was raised from 10°C to 37°C. In hepatocytes, the diffusion coefficient increased 6-fold, from $1.78 \cdot 10^{-9}$ cm^2/s at 10°C to $1.00 \cdot 10^{-8}$ cm^2/s at 37°C, with no significant change in the size of the mobile fraction over this temperature range. In neuroblastoma cells there was a 2-fold increase in the diffusion coefficient, from $9.4 \cdot 10^{-9}$ cm^2/s at 10°C to $1.97 \cdot 10^{-8}$ cm^2/s at 37°C, while the mobile fraction appeared to be somewhat smaller (by about 10%) at 37°C.

Effect of incubation time on binding and lateral mobility of endotoxin

The effect of length of incubation time on bi-

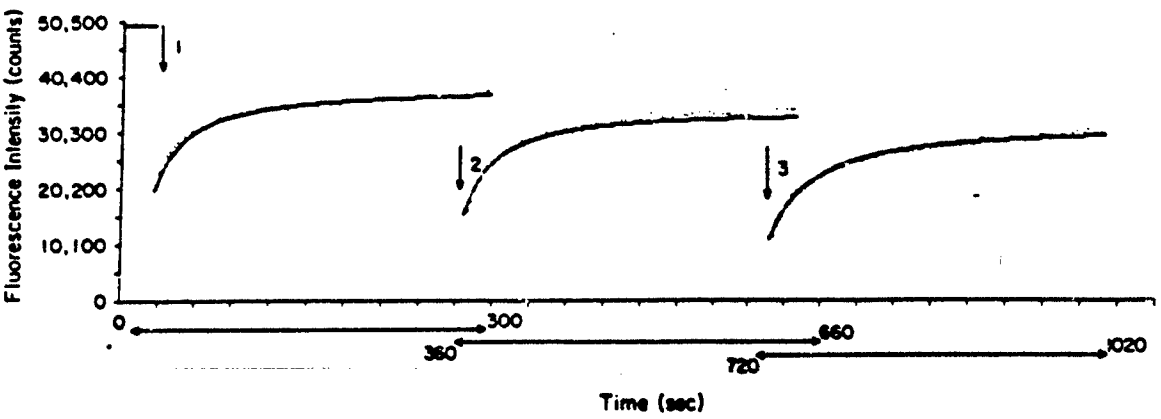

Fig. 2. Fluorescence recovery profiles for three consecutive bleaches of FITC-LPS-labelled hepatocytes. Hepatocytes were incubated with 12 μg FITC-LPS/mg dry wt. at 25°C for 30 min. Diffusion coefficient is $1.12 \cdot 10^{-9}$ cm^2/s and fluorescence recovery: (1) 56%; (2) 85% and (3) 89%.

TABLE I

EFFECT OF TEMPERATURE ON LATERAL MOBILITY OF FITC-LPS

Isolated hepatocytes and neuroblastoma cells were incubated with 12 μg FITC-LPS/mg dry wt. for 30 min. The values are means ± S.E. of the number of measurements of fluorescence recovery after photobleaching in parentheses. D = diffusion coefficient; % recovery = apparent fluorescence recovery after photobleaching. Statistical analysis. Effect of temperature. Hepatocytes: D 37°C vs. 10°C, $P < 0.005$; 25°C vs. 10°C, $P < 0.01$, for % bound all not significant. Neuroblastoma: both D and % bound at all temperatures not significant. Hepatocytes vs. neuroblastoma: 10°C for D, $P < 0.001$, for % bound, $P < 0.001$; 25°C for D, $P < 0.001$, for % bound $P < 0.01$; 37°C, D, not significant, % bound $P < 0.0025$ (*t* statistic for two means).

Temperature (°C)	Hepatocytes		Neuroblastoma	
	D (cm²/s)	% recovery	D (cm²/s)	% recovery
10	$(1.78 \pm 0.32) \cdot 10^{-9}$ (9)	63.0 ± 2.8 (9)	$(9.4 \pm 2.11) \cdot 10^{-9}$ (8)	82.3 ± 4.8 (9)
25	$(4.58 \pm 1.15) \cdot 10^{-9}$ (9)	68.7 ± 2.2 (9)	$(1.35 \pm 0.35) \cdot 10^{-8}$ (9)	82.9 ± 4.8 (9)
37	$(1.00 \pm 0.36) \cdot 10^{-8}$ (8)	64.6 ± 1.8 (9)	$(1.97 \pm 0.67) \cdot 10^{-8}$ (8)	74.4 ± 2.6 (8)

nding and lateral mobility of FITC-labelled endotoxin was examined in both primary and transformed cells and the results are shown in Table II. The cells were incubated for 10, 45, and 90 min at 25°C with 12 μg FITC-LPS/mg dry wt. It was found that neither lateral mobility nor size of the mobile fraction (data not shown) was dependent on incubation time in either type of cell (hepatocytes, $D = 3.63 \cdot 10^{-9}$ cm²/s; neuroblastoma N841A3, $D = 1.0 \cdot 10^{-8}$ cm²/s).

Conventional fluorometric technique was then used (see Methods) to determine whether the binding of FITC-LPS was time-dependent in either primary or transformed cells. It was found that when cells were incubated with 12 μg/mg dry wt. at 25°C for 10–90 min the amount of labeled toxin bound to cells did not vary significantly with time, although hepatocytes bound 3.5-times more FITC-LPS than did neuroblastoma cells.

Effect of endotoxin concentration on binding

Equilibrium binding studies at different endotoxin concentrations were performed on both hepatocytes and neuroblastoma cells as described in Materials and Methods and the results are shown in Fig. 3. Cells were incubated for 30 min at 25°C with between 1 and 250 μg FITC-LPS/ml cell suspension. Over this concentration range, there was no evidence of saturation of binding sites in either cell type. However, it was observed, at each endotoxin concentration, that hepatocytes bound between 1.5–3-times more endotoxin/mg protein than neuroblastoma cells.

Scatchard analysis of the binding of FITC-LPS

TABLE II

BINDING AND LATERAL MOBILITY OF FITC-LABELED ENDOTOXIN IN PRIMARY AND TRANSFORMED CELLS

Isolated hepatocytes and neuroblastoma cells were incubated for the times indicated with 60 μg FITC-LPS/ml cell suspension at 25°C. The results presented are from four separate cell preparations of each cell type. The values are means ± S.E. for the number of measurements in parentheses. * $P < 0.001$; ** $P < 0.005$ (neuroblastoma were evaluated with respect to the corresponding results for hepatocytes using *t*-statistics for two means).

Incubation time (min)	Rat hepatocytes		Neuroblastoma N841A3	
	D (cm²/s)	% bound	D (cm²/s)	% bound
10	$(3.51 \pm 0.43) \cdot 10^{-9}$ (10)	11.14 ± 1.41 (7)	$(9.49 \pm 2.54) \cdot 10^{-9}$ (6) *	3.80 ± 0.41 (4) *
45	$(3.84 \pm 0.67) \cdot 10^{-9}$ (11)	14.43 ± 1.25 (7)	$(1.88 \pm 0.45) \cdot 10^{-8}$ (6) **	3.38 ± 0.30 (4) *
90	$(3.53 \pm 0.88) \cdot 10^{-9}$ (6)	14.67 ± 1.20 (7)	$(9.93 \pm 3.21) \cdot 10^{-9}$ (6) **	2.75 ± 0.25 (4) *

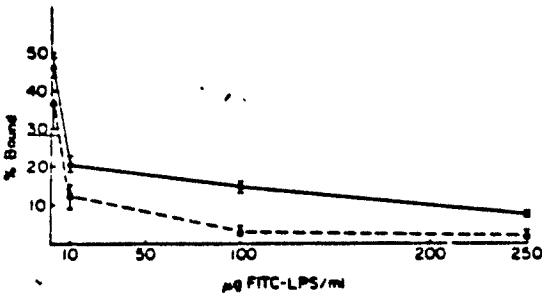

Fig. 3. Binding of FITC-LPS to hepatocytes (●) and neuroblastoma cells (▲) as a function of toxin concentration. Hepatocytes and neuroblastoma cells (5 mg dry wt./ml) were incubated with FITC-LPS at the indicated concentrations for 30 min at 25°C. Binding was determined fluorometrically. Values are the mean ± S.E. for four different cell preparations.

to hepatocytes is shown in Fig. 4. The binding curve was found to be non-linear, which suggests the existence of two types of FITC-LPS binding site: one with a high affinity which constitutes a small fraction of total sites and one with much lower affinity composed of a relatively large population of sites. The number of FITC-LPS high-affinity binding sites on hepatocyte cells was calculated to be approx. $1 \cdot 10^5$ per/cell, a value similar to that reported by Larsen and Sullivan [25] for human monocytes.

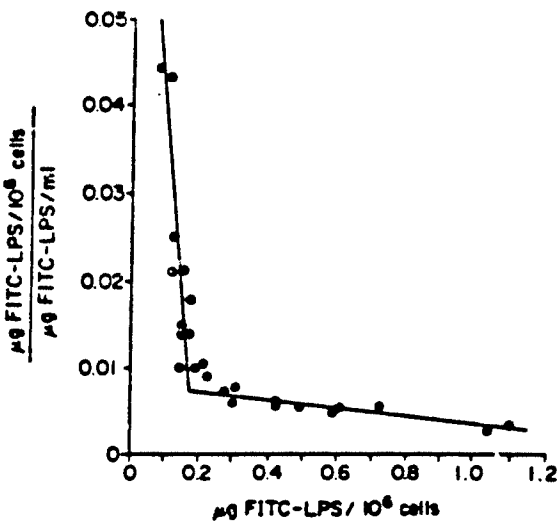

Fig. 4. Scatchard analysis of binding of FITC-LPS to hepatocytes. Concentrations of free and bound FITC-LPS were determined assuming molecular weight of lipopolysaccharide to be $1 \cdot 10^6$. r was 0.84. Values are from five different hepatocyte preparations.

It is worth mentioning that in the range of endotoxin concentration of 0.1–10 μg/ml the immobile fraction on the hepatocyte became greater. Although it is tempting to speculate that the immobile fraction represents specific binding of lipopolysaccharides, interference by background fluorescence in these measurements does not allow us, at the present time, to draw a definite conclusion.

Lateral mobility of endotoxin bound to viable and nonviable cells

Further information on the nature of endotoxin interaction with the plasma membrane was obtained by comparing the fluorescence recovery profiles of viable and nonviable cells. The diffusion coefficient for FITC-LPS bound to viable hepatocytes was ($4.58 \pm 1.15 \cdot 10^{-9}$ cm²/s and the immobile fraction was about 35% (see Table I). In a nonviable hepatocyte (i.e., that which did not exclude Trypan blue) the rate of lateral mobility of FITC-LPS was higher (D ($2.63 \pm 1.04 \cdot 10^{-8}$ cm²/s) and the fluorescence intensity curve showed 100% recovery. (Values are means ± S.E. for nine separate experiments.) Fluorescence recovery of 100% was also obtained when nonviable neuroblastoma cells were bleached (data not shown).

Discussion

An area of particular interest in understanding the mechanism of action of endotoxin at the cellular level is the mode of attachment of endotoxin to the surface of mammalian cells. It is reasonable to assume that before endotoxin can elicit cellular perturbations, it must first come in contact with and/or interact with the plasma membrane. Studies with radioactive labeled endotoxin showed that the binding of endotoxin to a variety of mammalian cells was rapid (within minutes) and independent of temperature [4,5,8,25]. It has also been shown that pretreatment of cells with unlabeled endotoxin decreased the binding of labeled toxin [8,26]. However, if cells were first labeled with ^{14}C-labeled than exposed to unlabeled LPS, very little displacement was observed, which may

suggest that endotoxin binds to cell membranes with high affinity [8]. This finding seems, however, to contradict the reports that endotoxin binding to a number of different cell types does not follow saturation kinetics [8,27,28]. Hence, it is difficult to ascertain whether interaction of endotoxin with mammalian cells is accomplished through direct attachment to a specific membrane component which generates a transmembrane signal or through nonspecific lipid-lipid contacts which produce membrane destabilization and/or alteration in membrane fluidity. Either type of endotoxin-plasma membrane association could conceivably produce the cellular perturbations that have been observed.

The results of this study have shown that endotoxin bound to mammalian cells consists of two fractions with different mobilities. A larger, mobile fraction has a diffusion coefficient of between $1 \cdot 10^{-8}$ (neuroblastoma) to $1 \cdot 10^{-9}$ cm^2/s (hepatocytes) at 25°C, which is dependent on temperature in the range 10–37°C but independent of length of incubation time between 10 and 90 min. These values for diffusion coefficients are similar to those reported for the lateral mobility of membrane lipids [29–31] and, most likely, represent nonspecific binding of endotoxin to these membrane constituents.

The remaining fraction of endotoxin molecules bound to cells is relatively immobile with a diffusion coefficient of less than 10^{-12} cm^2/s. The size of this immobile fraction is independent of temperature between 10 and 37°C and independent of length of incubation between 10 and 90 min. The immobile fraction is absent from nonviable cells in which all of the endotoxin bound to cells was mobile with a diffusion coefficient of approx. 10^{-8} cm^2/s. Hence, the immobile fraction must represent a type of binding, the existence of which depends on ability of the cell to maintain its vital function, i.e., to produce energy.

The nature of the immobile fraction of endotoxin molecules is of great interest. At least three possibilities can be entertained: (1) aggregation of endotoxin molecules on the cell surface; (2) internalization of a subpopulation of endotoxin molecules; or (3) binding of endotoxin to a specific membrane component which is anchored on the cell surface and not freely mobile.

It is unlikely that the immobile fraction represents the binding of a mixed population of endotoxin molecules (i.e., aggregates of different sizes), or aggregation of endotoxin molecules on the cell surface either spontaneously or due to chemical crosslinking produced by the photobleaching. It has been shown that successive bleaches of the same area of the cell surface result in recovery profiles which approach 100%. If the immobile fraction were a result of crosslinking of the endotoxin molecules, then successive bleaches of the same spot would continue to produce an 'immobile' fraction of fluorophores. In addition, either binding of various sizes of aggregates or formation of aggregates of endotoxin molecules should be observed in both viable and nonviable cells. We did not observe an immobile fraction in nonviable cells, indicating that cellular integrity rather than aggregation of endotoxin molecules is important for the presence of an immobile fraction on the cell surface.

It is more difficult to ascertain whether the immobile fraction represents specific binding to a membrane component or internalization of a fraction of the endotoxin molecules. However, internalization, which most likely involves changes in both the protein and lipid components of the plasma membrane, is known to be inhibited at low temperatures [33,34] and increases with longer incubation times [33]. By contrast, our studies show that the immobile fraction was temperature- and incubation-time-independent. Hence, it can be suggested that this smaller fraction of bound endotoxin molecules, which is relatively immobile on the cell surface, represents a population of sites which interact with a specific membrane component, most likely a protein, since the latter is known to diffuse in the plane of the membrane at a slower rate [12,13,20,29,31].

In comparing binding of endotoxin to hepatocytes and neuroblastoma cells, we found the following differences: (1) hepatocytes bound more endotoxin/mg protein than neuroblastoma cells; (2) the lateral mobility of endotoxin on the cell surface was lower in hepatocytes; and (3) the size of the immobile fraction was larger in hepatocytes. The reasons for these differences are not clear at present. One possibility is that the presence of nanogram quantities of endotoxin in fetal calf

serum, a component of the culture medium, causes transformed cells to become selectively resistant to the toxin. An alternative is that various cells exhibit intrinsic differences in both the lipid and protein composition of their plasma membrane and hence bind different amounts of endotoxin. This means that the number of binding 'sites' for lipopolysaccharide on plasma membranes of various cells may be different. Since in our hands hepatocytes appeared to be more sensitive to endotoxin than neuroblastoma cells it is tempting to speculate that the amount of lipopolysaccharide bound per cell and/or the size of the immobile fraction is correlated positively with cellular susceptibility to the toxin.

Acknowledgments

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Navy Contract N00014-84-K-0514

FINAL REPORT

Two types of studies were carried out under the above Navy research contract.

1. Interactions between endotoxin and plasma membrane were examined in isolated hepatocytes and neuroblastoma cells. The results suggested that: i) endotoxin binding to mammalian cells consists of two subpopulations with different mobilities; ii) binding of the immobile fraction is dependent on cellular integrity; iii) differences in binding, lateral mobility and size of the immobile fraction in hepatocytes and neuroblastoma cells may be due to variation in membrane composition and/or number of binding sites.

   Summarized in: "Cellular effects of endotoxin in vitro: mobility of endotoxin in the plasma membrane of hepatocytes and neuroblastoma cells" L. Kilpatrick-Smith, G. Maniara, J.M. Vanderkooi and M. Erecinska. Biochim. Biophys. Acta 847, 177-184, 1985.

2. Effects of intraperitoneal administration of a sublethal dose of E. coli endotoxin were evaluated in fasted rats. The results demonstrated that there was an early impairement of nitrogen metabolism in endotoxemia which may be of significant clinical importance.

   Summarized in: "Endotoxin-induced changes in nitrogen metabolism" L. Kilpatrick-Smith, M.C. Yoder, R.A. Polin, S.D. Douglas and M. Erecinska. Res. Commun. Chem. Pathol. Pharmacol. 53, 381-398, 1986.

ENCLOSURE (1)

ENDOTOXIN INDUCED CHANGES IN NITROGEN METABOLISM

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Running Title: Endotoxin Induced Changes in Nitrogen Metabolism

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ABSTRACT

Intraperitoneal administration of a sublethal dose of E. coli endotoxin to fasted rats produced within 12 hr a rise in plasma ammonia, urea, glutamine, glutamate and aspartate as compared to controls. At this time, hepatic urea levels also increased as did glutamine, glutamate, aspartate, alanine and asparagine. Hepatic [ATP]/[ADP] ratios declined in endotoxin-treated animals and there was an oxidation of mitochondrial pyridine nucleotides and a reduction of cytosolic pyridine nucleotides. The rise in blood ammonia and amino nitrogen concomitant with increased ureagenesis suggests that endotoxin administration markedly enhances protein catabolism.

KEY WORDS

hyperammonemia

ureagenesis

proteolysis

amino acids

Sprague-Dawley rats

INTRODUCTION

Studies utilizing radiolabelled endotoxin have shown that following an IV injection, the toxin accumulates in the liver (17,19,29) where it binds both to parenchymal and Kupffer cells (20,24,29). These observations indicate that the liver plays an important role in the clearance and detoxification of endotoxin.

The liver is also the primary site for gluconeogenesis, ketogenesis and ureagenesis and serves a vital role in supplying the body's energy needs. While the effect of endotoxin on hepatic gluconeogenesis and ketogenesis has been studied in some detail (For reviews, see 1,2,9,18,23), its action on nitrogen metabolism is less well understood (1,28). Preliminary studies from our laboratory have shown that a sublethal dose of E. coli endotoxin increases plasma ammonia levels (27) which suggests that hepatic clearance of this compound may have been impaired. Therefore, the object of this investigation was to examine, in more detail, the effect of endotoxin on hepatic function and in particular on nitrogen metabolism.

METHODS

Sample Preparation

Male Sprague-Dawley rats (250-300 gr) were fasted for 12 hours then injected intraperitoneally (IP) with either a sublethal dose (0.2 mg/kg) of E coli 0111:B4 endotoxin (Difco Laboratories, Detroit MI) or placebo (5% dextrose in water). With the particular batch of endotoxin used, a dose of 0.2 mg/kg produced no parenchymal inflammation or cellular necrosis within 12 hr (27). There were no changes in serum SGOT or SGPT levels and the livers of the animals were essentially normal 48 hrs post endotoxin. Other histological studies (27) showed no evidence of

inflammation or cellular necrosis in the brain, nor was there any indications of vascular damage. It does not appear that this level of endotoxin produced intestinal damage since the animals did not have diarrhea or show any other evidence of gastrointestinal disfunction. In addition, at autopsy there was no evidence of gross intestinal damage such as apparent hemorrhages or necrotic areas.

Plasma and liver samples were taken at 1, 4 or 12 hrs post-treatment. At sampling times, animals were anesthetized with sodium pentobarbital (35 mg/kg) by IP injection. In one group of control and endotoxin-treated animals, a 3 cm incision was made in the left axilla of the recumbant (supine) animals to lacerate the axillary artery. Two ml of blood were aspirated from the axillary fossa, placed in a heparinized tube and centrifuged. The plasma was removed and quickly mixed with cold perchloric acid (final concentration of 4%), centrifuged and the clear supernatant was neutralized (pH 6.8-7.2) with 2N K_2CO_3-0.58 M triethanolamine. Aliquots of the extracts were used for the assay of metabolites.

The second group of animals (control and endotoxin treated) was anesthetized as described above. A 3 cm incision was made in the right upper quadrant of the abdomen to expose the liver. The animals were quickly turned 180° to allow the liver to protrude vertically from the incision and a section of the liver was freeze-clamped using aluminum tongs which had been precooled in liquid N_2. (Care was taken during this procedure not to obstruct blood flow and introduce anoxic conditions to the lobe of the liver to be removed.) The liver samples were stored in liquid N_2 until metabolites were extracted according to the method of Williamson and Corkey (26) and neutralized as described above. Wet

weights of the liver samples were determined by weighing the freezed clamped tissue prior to extraction with perchloric acid.

A 12 hr urine collection was obtained from 5 control and 5 endotoxin treated rats. Animals were housed in hanging rodent metabolic cages (Acme, Cincinnati, Ohio). Urine was collected for 12 hrs post treatment, measured, and a 1 ml sample was mixed with perchloric acid (final concentration 4%). Samples were neutralized as described previously and aliquots were utilized for determination of urine ammonia and urea concentrations.

Measurements of Metabolites

The concentrations of ATP and ADP were measured enzymatically using the methods of Lamprecht and Trautschold (15) and Jaworek et al (10), respectively. Ammonia and urea were determined by the method of Gutmann and Bergmeyer (7), acetoacetate and 3-OH-butyrate according to Williamson et al (25), lactate according to Gawehn and Bergmeyer (6) and pyruvate by the procedure of Czok and Lamprecht (5).

Measurement of amino acids

The concentrations of various amino acids in both plasma and liver samples were determined by high pressure liquid chromatography (HPLC) of their O-phthaldialdehyde 2-mercaptoethanol derivatives and fluorescence detection (8). Sample preparation was carried out as described previously (11).

Reagents

Enzymes were obtained from Boehringer-Mannheim or Sigma Chemical Co. All other reagents were of the highest purity commercially available.

RESULTS

Effect of endotoxin on plasma [amino acids]

The concentrations of amino acids in the plasma of control and endotoxin-treated animals were measured at 1, 4 and 12 hr post treatment. Figure 1 shows a profile of selected amino acids involved in nitrogen metabolism over this time. At 1 and 4 hours, there were no significant changes in the levels of aspartate, alanine, glutamate, arginine, glutamine or asparagine in endotoxin-treated as compared to control animals. However at 12 hr after endotoxin treatment, several changes were observed: both aspartate and glutamate levels increased by 33%, while arginine rose by 24% as compared to controls. The greatest change was in glutamine levels which increased by 65% in endotoxin-treated animals.

Effect of endotoxin on plasma [urea] and [NH_4^+].

The concentrations of urea and NH_4^+ were also determined in plasma obtained from control and endotoxin-treated animals at 1, 4 and 12 hr post treatment (Table I, Part A). Plasma NH_4^+ in control animals remained stable at approximately 60 uM. In endotoxin treated animals, the [NH_4^+] was within the same range as controls at 1 and 4 hr but after 12 hr, had risen by 50%, i.e. to 99 uM. Urea concentrations were also relatively stable in control animals over 12 hr with an average [urea] of 7 mM. In endotoxin-treated animals, there was a slight elevation in urea to 8.02 and 8.41 mM at 1 and 4 hrs, respectively but by 12 hr, a significant 41% increase to 10.05 mM was observed.

Effect of endotoxin on urine [NH_4^+] and [urea]

In order to ascertain whether the rise in plasma NH_4^+ and urea in endotoxin-treated animals was due to an alteration in hepatic nitrogen

metabolism or a change in renal clearance of ammonia, urine samples were collected from both control and treated animals as described in Methods. It was found that there was no statistically significant difference in the volume of urine excreted by the two groups. The urine [NH_4^+] was 368±2 uM in control and 356±20 uM in endotoxin-treated animals (values are the mean ± SD for 5 animals in each group). In controls, the urine urea content was 103.6±6.2 mg/12 hr while in endotoxin-treated animals there was a significant ($P < 0.01$) 38% increase to 138.7±18.5 (as determined using a t-test for 2 means with an n of 5 for each group).

Effect of endotoxin on hepatic [amino acids]

The profile of liver amino acids was determined in control and endotoxin-treated animals at 1, 4 and 12 hr post treatment (Figure 2). One hr after treatment there were no statistically significant differences between endotoxin-treated animals and controls. At 4 hr, glutamate levels increased by 53% and those of glutamine by 41% as compared to controls. At 12 hr, there were statistically significant increases in the following amino acid concentrations; aspartate 42%, glutamate 72%, glutamine 76%, asparagine 16% and alanine 96%.

Effect of endotoxin on hepatic [urea] and [NH_4^+]

Hepatic ammonia and urea concentrations are shown in Table I, Part B. There was no change in urea concentration in endotoxin-treated animals at 1 hr as compared to controls. At 4 hr there was a slight increase (24%) and at 12 hr the urea concentration had risen by 67%. In control animals, the hepatic ammonia concentration remained stable at 0.7 umol/gr wet wt over 12 hr. Endotoxin-treated animals had similar NH_4^+ levels at 1 and 4 hr, but at 12 hr post treatment there was a 24% increase to 0.88 umol/gr wet wt.

Effect of endotoxin on hepatic energy production

Several parameters of hepatic energy production are shown in Table II. [ATP]/[ADP] ratios were similar in the two groups of animals at 1 and 4 hrs. However at 12 hrs, endotoxin-treated animals showed a 25% decrease in [ATP]/[ADP] as compared to controls (3.42 and 2.47, respectively). The redox state of the mitochondrial pyridine nucleotides was determined from the [3-OH-butyrate]/[acetoacetate] ratios. It was found that there was no difference at 1 and 4 hr post treatment but at 12 hr, the [3-OH-butyrate]/[acetoacetate] in endotoxin-treated animals had declined by 53%. This indicates that there was oxidation of the mitochondrial pyridine nucleotides at that time. It should be pointed out that even though the [3-OH-butyrate]/[acetoacetate] ratio declined in these animals, there was no change in the concentration of total ketones. [Lactate]/[pyruvate] ratios were also determined at 1, 4 and 12 hr post treatment. At 12 hr there was a 54% increase in the [lactate]/[pyruvate] in endotoxin-treated animals as compared to controls. This rise was due to an increase in hepatic lactate concentration, while the pyruvate concentration remained unaltered. The [lactate]/[pyruate] is a reflection of the redox state of the cytosolic pyridine nucleotides and the increase indicates their reduction.

DISCUSSION

In this study, we have found that a single sublethal dose of endotoxin given to fasted rats produced alterations in plasma and hepatic concentrations of several metabolites within 4 hr. Plasma levels of urea began to increase at 4 hr and by 12 hr both plasma urea and ammonia levels were significantly elevated as compared to controls. There were also increases in plasma levels of aspartate, glutamine, glutamate and arginine

12 hr after endotoxin. In the liver, glutamate, glutamine and urea were increased by 4 hr after treatment and by 12 hrs, glutamate, glutamine, aspartate, alanine, asparagine, urea and lactate levels had risen significantly. In addition, there was oxidation of mitochondrial pyridine nucleotides, a reduction of cytosolic pyridine nucleotides and a decline in the [ATP]/[ADP] ratios.

One of the most striking alterations produced by a sublethal dose of endotoxin was the large increase in plasma ammonia levels, 12 hr post treatment. A similar rise in blood ammonia has been reported by Yoshino (28) in rabbits that were injected intravenously with Shigella flexneri endotoxin (1 mg/kg). Ammonia is extremely toxic to animals for reasons that are not yet clear (4) and its blood level is normally maintained within narrow limits at very low concentrations ($\sim$ 60-70 uM in fasted rats (16)) through conversion to urea. Hyperammonemia can occur in an animal for several reasons; 1) increased absorption from production by intestinal flora; 2) decreased excretion due to renal failure; or 3) increased protein catabolism and/or decreased ability of the liver to metabolize ammonia. Because we used a preparation of purified E. coli endotoxin and the animal showed no signs of intestinal damage (see Methods), it does not seem likely that the increased blood NH_4^+ is due to increased bacterial activity in or enhanced absorption from the gut. We can also rule out altered renal function because endotoxin-treated animals did not have decreased urine output or ammonia excretion as compared to controls. In addition, we found that the urea concentration in urine from endotoxin- treated animals was elevated as compared to controls.

The two remaining possible explanations are that endotoxin either inhibits hepatic ureagenesis or increases protein catabolism for which hepatic nitrogen metabolism can not compensate. The former seems unlikely because we found increased urea levels in both the plasma and urine of endotoxin-treated animals. Furthermore, the hepatic concentration of this compound was increased by 24% at 4 hr and by 67% at 12 hr post endotoxin. These observations indicate that endotoxin enhances rather than inhibits urea synthesis.

It has been observed that in both sepsis and after endotoxin administration amino acids are mobilized in peripheral tissues for synthesis of proteins and preservation of liver and other central organs (3,21,22). Our results are consistent with these findings in that they show increases in the levels of several amino acids in both the plasma and liver of endotoxin-treated animals. Hence increased protein catabolism seems to be the most likely explanation for increased blood ammonia levels, although the protein source and the site of the lesion remain to be established.

It is worth pointing out that synthesis of urea in the liver is not the only pathway for detoxification of ammonia. There are two other biosynthetic reactions which are important in the regulation of ammonia levels (16); formation of glutamate (from NH_4^+ + α-ketoglutarate) and glutamine (from glutamate + NH_4^+). Our results show that there are large increases in the hepatic concentrations of both glutamate and glutamine which suggest that these amino acids may also be products of hepatic ammonia detoxification and not solely of protein catabolism. This suggestion is consistent with studies using ^{15}N labeled ammonium compounds <u>in vivo</u> which have shown that the label appeared not only in

urea but also in several amino acids, specifically glutamate, aspartate, glutamine and asparagine (16).

The question that remains to be answered is why is there still a rise in plasma ammonia level when the liver appears to be responding to an increased load appropriately by stimulation of detoxification mechanisms? A possible explanation is that glutamine synthesis and urea production are not rapid enough to deal with the overload. It is well established that both of these processes require ATP. Our measurements of hepatic energy parameters showed that there is an alteration in the energy level in endotoxin-treated animals. The oxidation of intramitochondrial pyridine nucleotides and concomitant decline in the [ATP]/[ADP] suggest that due to the decrease in the reducing power of the intramitochondrial metabolites, the liver cannot control the increased amino acid load. Consequently, blood ammonia rises in spite of increased urea synthesis and stimulation of glutamine and glutamate formation. These findings support our in vitro studies (12,13) which showed that one of the earliest perturbations produced by endotoxin was a suppresion of the aerobic metabolic capacity and thereby of the cellular energy levels.

A frequent observation following the administration of endotoxin is depressed hepatic gluconeogenesis (2,9,23). Assuming that our observation of increased ureagenesis is a general feature of endotoxemia, then this decrease in gluconeogenesis must occur simultaneously with increased nitrogen metabolism. Ureagenesis and gluconeogenesis are interrelated on 2 levels, by sharing intermediate steps and through competition for ATP (14). It is tempting to speculate that endotoxin produces hepatic conditions which favor urea synthesis over gluconeogenesis and that the

decreased gluconeogensis often observed following endotoxin administration is a secondary effect resulting from increased nitrogen metabolism.

ACKNOWLEDGMENTS

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Table 1

Effect of Endotoxin on Plasma and Liver [Urea] and [Ammonia]

Time	Plasma		Liver	
	Urea (mM)	NH_4^+ (μM)	Urea (μmol/gr wet wt)	NH_4^+ (μmol/gr wet wt)
1 hr				
Control	6.99 ± 0.62 ($\bar{5}$)	60 ± 10 ($\bar{5}$)	6.43 ± 0.31 ($\bar{6}$)	0.69 ± 0.19 ($\bar{6}$)
Endotoxin-Treated	8.02 ± 0.75 ($\bar{5}$)	68 ± 14 ($\bar{5}$)	6.51 ± 0.69 ($\bar{6}$)	0.75 ± 0.17 ($\bar{6}$)
4 hr				
Control	6.94 ± 1.37 ($\bar{5}$)	60 ± 3 ($\bar{5}$)	5.88 ± 0.92 ($\bar{5}$)	0.71 ± 0.09 ($\bar{5}$)
Endotoxin-Treated	8.41 ± 0.54 ($\bar{5}$)	64 ± 3 ($\bar{5}$)	7.29 ± 0.92 ($\bar{5}$)	0.70 ± 0.03 ($\bar{5}$)
12 hr				
Control	7.12 ± 0.74 ($\bar{5}$)	60 ± 2 ($\bar{5}$)	6.55 ± 1.49 ($\bar{10}$)	0.71 ± 0.14 ($\bar{10}$)
Endotoxin-Treated	10.05 ± 0.98* ($\bar{5}$)	99 ± 12** ($\bar{5}$)	10.96 ± 2.64** ($\bar{10}$)	0.88 ± 0.16 ($\bar{10}$)

Urea and NH_4^+ concentrations were determined as described in Methods. Values are means ± SD for the number of experiments in parenthesis. *P<0.0005 as determined by t-test for two means. **P<0.005.

Table II

Effect of Endotoxin on Hepatic [ATP]/[ADP],

Cytosolic and Mitochondrial Redox States

	1 Hr	4 Hr	12 Hr
[ATP]/[ADP]			
Control	4.16 ± 1.19 (4)	4.12 ± 1.13 (4)	3.42 ± 0.73 (7)
Endotoxin-Treated	4.17 ± 1.48 (4)	3.80 ± 0.91 (4)	2.47 ± 0.14* (7)
[3-OH-Butyrate] / [Acetoacetate]			
Control	2.08 ± 0.73 (4)	2.36 ± 0.52 (4)	2.60 ± 0.84 (7)
Endotoxin-Treated	2.26 ± 1.51 (4)	2.61 ± 0.35 (4)	1.38 ± 0.43** (7)
[Lactate]/[Pyruvate]			
Control	26.12 ± 5.49 (4)	35.37 ± 6.01 (4)	28.96 ± 4.63 (5)
Endotoxin-Treated	25.35 ± 9.89 (4)	34.39 ± 10.32 (4)	44.79 ± 9.85* (5)

Metabolite concentrations were determined as described in Methods.

[ATP + ADP] = 2.36 - 2.74 μmol/gr wet wt and [3-OH-butyrate + acetoacetate] = 0.83 - 1.14 μmol/gr wet wt in both control and endotoxin-treated animals. In controls, [lactate] = 1.28 - 1.39 μmol/gr wet wt and in endotoxin-treated animals 1.25 at 1 hr and 2.15 at 12 hr.

Values are means ± SD for the number of experiments in parenthesis.

*P<0.01, **P<0.001 as determined using t-test for 2 means.

Figure Legend

Figure 1

Effect of Endotoxin on Plasma Amino Acids

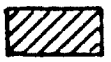
The levels of plasma amino acids were determined by HPLC in control and endotoxin-treated animals at 1, 4 & 12 hrs post treatment. Control =  Endotoxin-treated =  (1 hr),  (4 hr) and  (12 hr) Values are means ± SEM (N=5).

Figure 2

Effect of Endotoxin on Hepatic Amino Acids

The concentrations of hepatic amino acids were determined by HPLC in control and endotoxin-treated animals at 1,4 and 12 hr post treatment. Control =  Endotoxin-treated =  (1 hr),  (4 hr) and  (12 hr). Values are means ± SEM for the number of experiments in parenthesis.

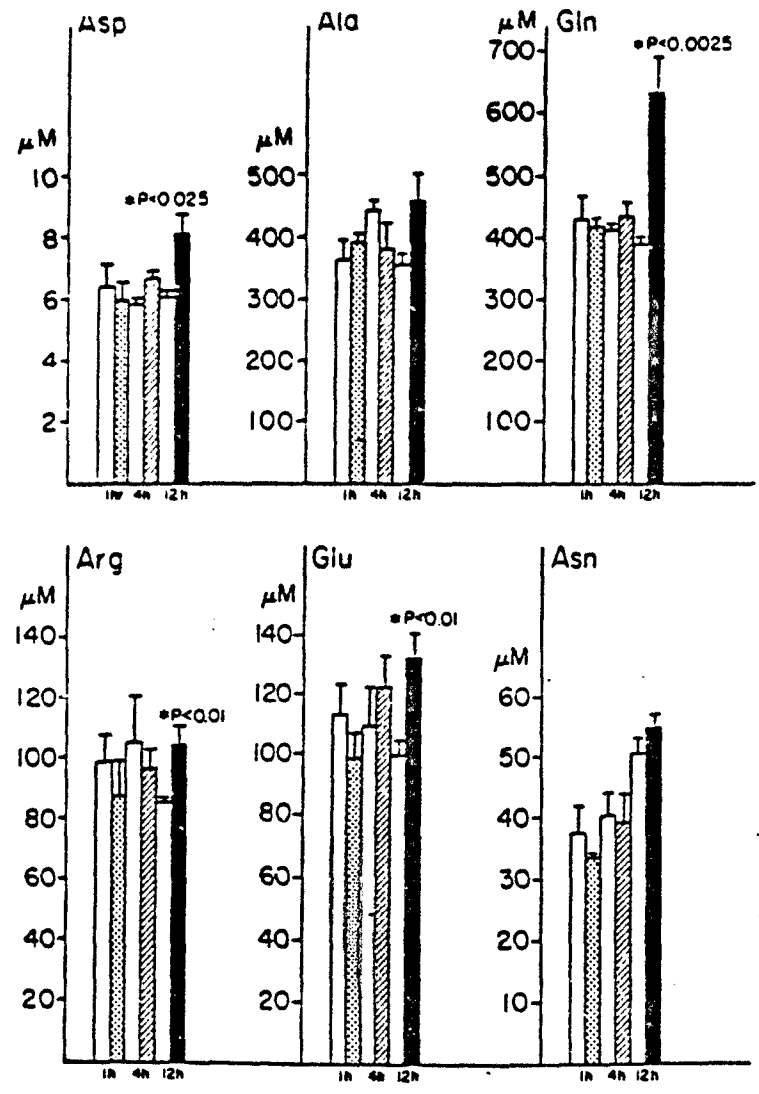


Figura 1

MAE-836