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Comparative *in vivo* and *in vitro* models to approach the cellular basis of endotoxic shock. The role of sinusoidal liver cells

R. Pagani[1], M.T. Portolés[1], R. Arahuetes[2], M.J. Ainaga[1], C. Machín[3] and C. Rua[3]

[1]Department of Biochemistry and Molecular biology, Faculty of Chemistry, [2]Department of Animal Biology II, Faculty of Biology and [3]Department of Cellular Biology, Faculty of Biology, Complutense University, Madrid, Spain

Summary. During endotoxic shock, the liver exerts a lipopolysaccharide (LPS) clearance function with the participation of both parenchymal and sinusoidal cells. Liver damage could be caused by LPS direct action, hypoxia and/or inflammatory mediators released by Kupffer cells.

The aim of this study is to establish an experimental model that could allow us to understand the direct *E. coli* 0111:B4 LPS action on sinusoidal cells. A comparative study was carried out, *in vivo* and *in vitro*, using either a rat reversible endotoxic shock model or sinusoidal cell cultures.

The LPS was found to induce important and similar morphological alterations both *in vivo* and *in vitro*, specially in Kupffer cells. These cells present mitochondrial damage, nuclear membrane swelling, and increased number of phagosomes, including lamellar bodies. An immunocolloidal gold technique shows, *in vitro*, the LPS mainly located on Kupffer cell membrane and in phagosomes. The LPS binding to membrane, as a primary step of Kupffer cell activation, increases the phagocytosis. This effect could be related to a decrease of fluidity on the external membrane portion.

Key words: Kupffer cells, Endotoxins, Lipopolysaccharide, Immunocolloidal gold technique, Membrane fluidity

Introduction

Severe endotoxemia, induced either by the release of Gram-negative bacterial endotoxins (lipopolysaccharides, LPS) or following the experimental exogenous administration of endotoxins, is a pathophysiological phenomenon accompanied by extended serum parameter alterations and structural damages in various target organs, such as adrenal glands, kidneys, lung and liver (Bosch et al., 1988; García et al., 1990).

Endotoxin administered intravenously is cleared from the systemic circulation principally by the liver (Prytz et al., 1976; Jacob et al., 1977; Nolan et al., 1977; Triger et al., 1978; Mathison and Ulevitch, 1979; Freudenberg et al., 1982).

It is generally accepted that the elimination of endotoxin from the circulation is effected mainly by the cells of the reticuloendothelial system (RES) and to a lesser degree by the granulocytes. In agreement with earlier studies, macrophages are the most important cells of primary LPS uptake due both to their ubiquitous presence and their high capacity for storing endotoxin (Braude, 1964; Mathison and Ulevitch, 1979; Freudenberg et al., 1984, 1985; Kang et al., 1992).

The liver, the largest RES organ, contains Kupffer cells, tissue-fixed macrophages derived from monocytes (Rogoff and Lipsky, 1981). Their placement within the liver sinusoids, suggests that they represent a step beyond gut-associated lymphoid tissues and mesenteric lymph nodes in the physiological mechanisms responsible for dealing with the clearance and detoxification of gut-derived substances.

Sinusoidal cells (Kupffer and endothelial cells) and parenchymal cells (hepatocytes) seem to be involved in the process of endotoxin uptake, although the relative contribution of sinusoidal cells versus hepatocytes is a controversial topic.

It has been shown that rat Kupffer cells, rather than endothelial and parenchymal cells, are responsible for the clearance of intravenously administered ^{51}Cr-endotoxin (Praaning-van Dalen et al., 1981; Ruiter et al., 1981). *In situ* immunoperoxidase staining of sections of rat organs, or autoradiographs after ^{3}H-LPS injection, support this pathway, revealing that endotoxin is first associated with Kupffer cells and, after 2/7 days, appears in parenchymal cells (Freudenberg et al., 1985; Van Bossuyt and Wisse, 1986).

In vitro data have shown that Kupffer cells can express classical macrophage functions after stimualtion with endotoxin (Rogoff and Lipsky, 1981; Keller et al., 1984, 1985; Shiratori et al., 1984).

Offprint request to: Dr. R. Pagani, Departamento de Bioquímica y Biología Molecular, Facultad de Química, Universidad Complutense, 28040 Madrid, Spain

The activity of LPS-carrying macrophages and LPS-activated macrophages may play an important role in the induction of the pathophysiological endotoxin effects. Using *in vitro* experiments, it was found that the LPS is directly cytotoxic to explanted hepatic macrophages, and acts by increasing several catabolic enzymes levels (Maier and Ulevitch, 1981).

LPS-activated macrophages, such as Kupffer cells, may increase tissue injury by releasing different mediators into the blood (prostaglandins and other products of the arachidonic acid cascade, C3, interleukin-1 and tumor necrosis factor (TNF)) (Chensue et al., 1991; Ogle et al., 1991). Additional studies showed that Kupffer cells exposed to endotoxin caused a decrease in hepatocyte protein release via heat labile soluble mediators (West et al., 1986).

Since Kupffer cells are the primary site for the clearance of endotoxin and are exposed to this substance, it is necessary to understand how these cells are affected by the toxin and how they can modify their microenvironment, which could induce some changes in parenchymal cell metabolism.

In summary, endotoxic damage on liver could be caused by direct LPS action on different cells, hypoxia and/or the action of a variety of inflammatory mediators mainly released by macrophagic cells.

The complexities imposed on the *in vivo* studies by factors such as cellular heterogeneity and homeostatic mechanisms, may alter the determination of specific cellular disfunctions induced by the LPS itself. In order to clarify the LPS action, we have used *in vitro* cell cultures that allow us to observe the behaviour of different cell types. In previous studies, using parenchymal cell cultures, we have shown LPS binding and localization and its direct or mediated action on different parameters (cytochrome P_{450}, cytochrome b_5, $[Ca^{2+}]_i$, pH, and lipid peroxidation) (Pagani et al., 1987, 1988, Díaz-Laviada et al., 1991; Portolés et al., 1989, 1991, 1993). Although the parenchymal cell response is the clue to the liver function, sinusoidal cells must play a central role in the first steps of the liver LPS clearance and the endotoxic shock induction.

The use of a rat reversible endotoxic shock model and parenchymal and/or sinusoidal cell cultures, allows us to compare the *in vivo* and *in vitro* response to the LPS in an attempt to understand the LPS action at a cellular level.

In this study sinusoidal cell cultures (with major reference to Kupffer cells) were used to reveal the localization of endotoxin by an immunocytochemical technique, and the LPS effect on membrane fluidity as a first step of endotoxin uptake and cell activation.

Materials and methods

Endotoxin

LPS from *Escherichia coli* 0111:B4, obtained according to the method of Westphal et al. (1952), was supplied by Difco (Detroit, Michigan, USA). The A_{260}/A_{240} ratio, was determined for purity verification (Romanowska, 1970). This LPS was used either for the *in vivo* experiments or the cultured cell treatment.

In vivo endotoxin administration

Male Wistar rats, weighing 200-250 g, were used. Standard diet and water were given *ad libitum*. *E. coli* 0111:B4 LPS (1.6 mg/100 g body weight) was administered intravenously (i.v.) in saline (1.6 mg/0.2 ml). Rats were killed after different times according to the experiment. Control animals received equivolume injections of saline. Blood samples and biopsies were taken at different times (1, 2, 4, 6, 24, 72 h). The experiments described were performed in adherence to the EU (86/609) and Ministerio de Agricultura (Spain, BOE 223/1988) guidelines for care and use of laboratory animals.

Isolation and culture of parenchymal cells

Parenchymal cells were isolated by the perfusion technique, using collagenase in Krebs-Ringer-bicarbonate solution (KRB medium) according to the general method of Berry and Friend (1969). Cell suspensions were purified by repeated centrifugations at 165g for 20 s in William's E sterile medium, with 500 U penicillin and 0.1 mg/ml streptomycin. Cell viability was routinely monitored by the Trypan blue exclusion test obtaining 85-90% viability. Yield of viable hepatocytes was $100\pm20 \times 10^6$ per rat liver. The isolated hepatocytes were cultured in the presence of 10% foetal calf serum, at 37 ºC in 25 cm^2 bottles ($6x10^6$ cells/bottle) under a CO_2/O_2 (5%/95%) atmosphere for 24 h, up to the formation of a continuous monolayer. Cell culture was performed under sterile conditions in a Telstar CAM 7001 laminar flow chamber.

Isolation and culture of liver sinusoidal cells

Adult rat liver sinusoidal cells were isolated from male Wistar rats (150-200 g), fasted for 18 h, by a collagenase/pronase recirculating perfusion method according to Van Bossuyt et al. (1988) with minor modifications.

Cell suspensions were purified by repeated centrifugation (200g for 10 min) in Gey's Balanced Salt Solution (GBSS), with the addition of antibiotics (400 UI/ml Penicillin; 100 µg/ml Streptomycin, Antibioticos S.A. (León, E) and 50 µg/ml Gentamycin) in a refrigerated RC-5 Sorvall. Purification was completed with a gradient Nycodenz (Nycomed, AS, Oslo, N) centrifugation (1400g/15 min). Floating cells were washed twice in Dulbecco's Minimal Essential Medium (DMEM) with 548 mg/l glutamine, 20 mM Hepes and 10 mM $NaHCO_3$.

Cell viability, tested by Trypan Blue exclusion assay, was 90-95%. Maintenance of cell structure and

identification of endothelial and Kupffer cell percentage was verified by phase-contrast (Leitz Laborlux K), electron microscopy (Zeiss 902) and flow cytometry techniques. Flow cytometry allows the distinction between Kupffer and endothelial cells due to their different shape and intracellular complexity. The percentage of Kc was determined by incubating the cell suspension, 30 min, with the Kupffer cell specific monoclonal antibody ED1 linked to R. Phycoerythrin (RPE) (MCA34LP, Serotec, UK) (270 µl cell suspension in PBS, 1% foetal calf serum and 30 µl ED1). The RPE fluorescence was excited by 2W Laser tunning to 488 nm and the emitted fluorescence was measured at 575 nm in a FACStar Plus Becton Dickinson flow cytometer. Percentages were calculated with the LYSIS II Program of Becton Dickinson. Yield of viable sinusoidal cells was $100x10^6 \pm 50 \times 10^6$ per rat liver. Relative percentage of endothelial and Kupffer cells was 75/25% respectively.

The isolated and purified sinusoidal cells were cultured in DMEM/RPMI (2/3:1/3, v:v) medium (Flow Lab., Irvine, Ayrshire, UK) supplemented with 20% foetal calf serum in 25 ml plastic bottles (Costar, Cambridge, MA, USA). Cells were cultured under CO_2/O_2 (5%:95%) (Heraeus B5061) atmosphere during 48 h.

In order to obtain enriched (80%) Kupffer cell cultures, after 20 h, the media with unattached cells were discarded and replaced by fresh complete media (differential adhesion procedure), then 48 h cultures were used for assays. Cell culture was performed under sterile conditions in a laminar flow chamber, Telstar CAM 7001.

In vitro LPS treatment

After 48 h cultures, DMEM/RPMI medium was removed and replaced by an LPS suspension (10 to 200 µg/ml) in the same medium, serum free. LPS was previously sonicated for 5 min in Ultrason and sterilized by filtration through Millex-GO 0.22 µm (Millipore Corp., Bedford, MA, USA). Then monolayers were incubated for 15 and 60 min at 37 ºC in a CO_2/O_2 (5%:95%) atmosphere. Controls with DMEM/RPMI medium, LPS free, were always carried out.

Light and electron microscopy

Organ histology

Organ histology was performed in standard conditions after 3% glutaraldehyde fixation of liver biopsies. Samples were post-fixed in buffered osmic acid, dehydrated and embedded in Araldite. For routine light microscopy (LM), thick sections (1 µm), lightly stained with 1% toluidine blue-borax or Masson staining, were examined in a Leitz Laborlux microscope. For transmission electron microscopy (TEM), ultrathin sections (70-80 nm), stained with uranyl acetate and lead citrate, were examined in a Zeiss 902 microscope.

Fixation and embedding of cultured cells

Sinusoidal cultured cells, control and LPS-treated, were washed in Phosphate Buffered Saline (PBS) at 4 ºC and fixed in 0.5% glutaraldehyde and 0.2% tannic acid in PBS for 10 min. Then, fixative was removed, monolayers were scraped off, resuspended in 2 ml PBS and centrifuged for 5 s x 900 g. The resulting pellet was incubated for 10 min at 4 ºC in 0.4 M NH_4Cl.

Dehydration of the pellet was performed in 30% and 50% ethanol/H_2O (v/v), 2 h at 4 ºC, 70% ethanol/H_2O (v/v) and 100% ethanol, 2 h at -20 ºC overnight. Then, the pellet was embedded in 50%, 70% Lowicryl K4M/ethanol (v/v) and 100% Lowicryl K4M at -20 ºC for 1 h. Afterwards 100% Lowicryl K4M was renewed and samples were polymerized in capsules with ultraviolet radiation (360 nm) for 24 h at -20 ºC and finally, for 48 h at room temperature.

Immunocytochemical labelling

Ultrathin sections (70-80 nm) of cultured sinusoidal cells, mounted on gold grids, were processed according to a modified Beesley method (1985). After several washes in PBS/albumin (0.5%), grids were transferred to a drop of diluted and purified anti-LPS serum (1/100) and incubated overnight at 4 ºC. Polyclonal anti-LPS serum was obtained by Dr. F. Díaz de Espada (Clínica Puerta de Hierro, Madrid, Spain). Afterwards, grids were washed with PBS and incubated in the protein A-gold complex (Amersham, UK) (10 nm), diluted 1/20, for 1 h at room temperature. The grids, washed three times in PBS, were rinsed in double distilled and sterile water and dried at room temperature. Negative staining with 2% uranyl acetate was performed before examination in a Zeiss 902 electron microscope.

Control preparations were always performed incubating grids with same procedure substituting the anti-LPS serum for a non-immune serum from normal rabbits and PBS.

Membrane microviscosity measurements

The probes DPH (1,6-diphenylhexa-1,3,5-triene) and TMA-DPH (1-[4-(trimetilamino)-phenyl]-6-phenyl-hexa-1,3,5-triene) (9 µM) were incorporated into the membrane bilayer by incubating isolated cells in suspension (30 min, 37 ºC) (Portolés et al., 1987). Parenchymal and sinusoidal (Control and LPS treated) cells were gently washed with culture medium before fluorescence polarization determination. Fluorescence polarization was measured in a Perkin-Elmer MPF-44E spectro-fluorimeter equipped with a polarization attachment. Microviscosity (η) was calculated from P values using the relationship (η=2P/(0.46-P) (Shinitzky and Barenholz, 1978).

Statistical analysis

Data given in tables represents the mean values±SD of 5 samples. Comparison between groups has been carried out by Student's t-test. Results were considered statistically significantly different when $p<0.05$.

Results

Histological observations

Reversible endotoxic shock was induced in adult Wistar rats by intravenous injection of *E. coli* 0111:B4 LPS. Serum samples and biopsies from liver were studied at different times after the LPS treatment to evaluate the progression of the shock state. Major histological changes in liver were observed during the acute-phase of shock (2-6 h post LPS injection). Liver histology showed disseminated intravascular coagulation and cellular degeneration around central veins, whereas the periportal areas remained normal. Dilatation and congestion of sinusoids and hepatic veins were more marked in the area of central zone with necrotic cells (Fig. 1A). Light microscopy revealed a consistent increase in intracellular lipid and a short number of inflammatory cells (Fig. 1B).

A detailed electron microscopy study revealed damage in both parenchymal and sinusoidal cells, mainly in Kupffer cells.

Kupffer (Fig. 1C), like parenchymal cells (García et al., 1990), presented mitochondrial damage and nuclear membrane dilatation. Major histological changes could be observed in the perisinusoidal space of Disse that contained conspicuous lamellar bodies (Fig. 1D) similar to those observed in Kupffer and parenchymal cells. Both of them represented an increase in the phagocytic vacuoles that could include residual mitochondrial membranes (pseudolamellar bodies) (Fig. 1E,F).

Sinusoidal cell culture

Using a recirculating perfusion with collagenase/pronase and further gradient centrifugation, we obtained a purified suspension of endothelial and Kupffer cells with good rates of viability (90-95%) and good adhesion capacity on a simple plastic substrate.

Previous light microscopy and flow cytometry techniques have shown a mixed culture of endothelial (70-75%) and Kupffer (25-30%) cells (Portolés et al., 1994), reaching 80% of these cells in the enriched cultures obtained by differential adhesion procedure.

After 48 to 72 h culture, cells were used for morphological, immunochemical and binding studies, presenting a monolayer (Fig. 2A,B) tending to confluence, that could be reached at longer time culture (1 week). The ultrastructural morphology, observed by electron microscopy, was normal (Fig. 2C,D).

These cultures, treated with LPS (100-200 µg/ml, 20 min) and observed by phase-contrast and electron microscopy, evidenced the *in vitro* response to the endotoxin.

The LPS induced vacuolation, even at low LPS doses (10 µg/ml), nuclear membrane swelling, mitochondrial damage and a phagosome increase (including lamellar bodies), coinciding with the *in vivo* alterations (Fig. 2E,F).

Immunocytochemical studies

Binding, localization and distribution of *E. coli* 0111:B4 LPS, into Kupffer cells was studied by the protein A-gold imunolabelling technique, using purified Kupffer cell monolayers (Fig. 2A,B) incubated with LPS (100 µg/ml) for 15 or 60 min at 37 ºC.

From the immunocytochemical labelling a time-dependent endotoxin uptake could be observed. After 15 min of endotoxin treatment, LPS was bound to plasma membrane of Kupffer cells, mainly associated to microvilli, or internalized in the peripheric area (Fig. 3A,B).

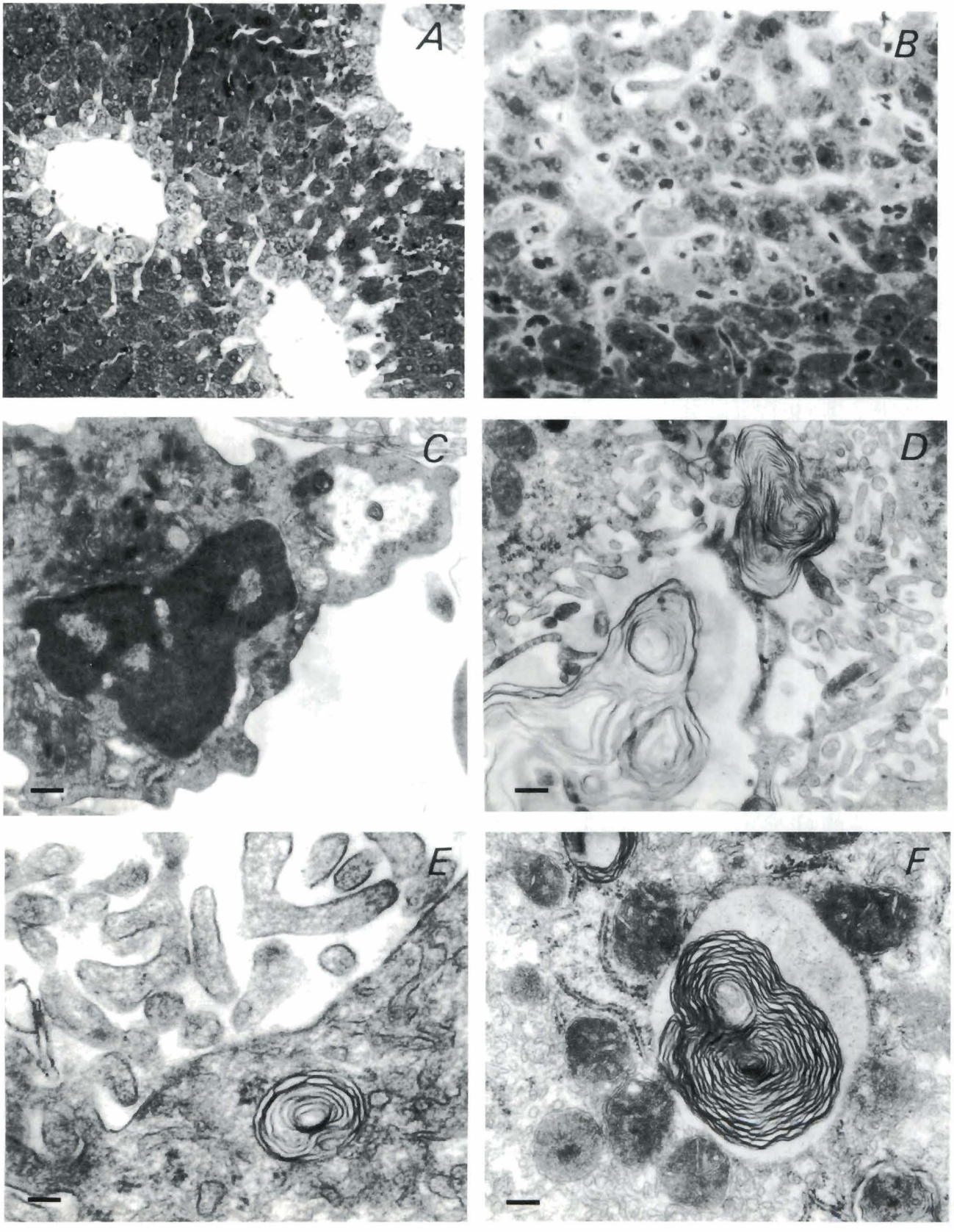
At longer times (60 min) the endotoxin was mainly distributed into phagocytic vesicles (Fig. 3C, D-E) associated to phagosomes with residual mitochondrial membranes (Fig. 3F). The label was observed in mitochondria and, rarely, inside the nucleus.

Membrane microviscosity

Direct LPS interaction with cells could modify physical parameters of the membrane. The use of the probes DPH and TMA-DPH allowed us to observe the microviscosity modification in the hydrophobic portion of the membrane (DPH) or its external portion (TMA-DPH).

Parenchymal cells exhibited an increase (25.5%) of microviscosity on the membrane external portion when treated with LPS (100 µg/ml) although there was a slight diminution into the hydrophobic portion of the membrane (Table 1). On sinusoidal cells we could observe an inverse behaviour: a diminution of microviscosity on the membrane external portion and an increase of this parameter in the hydrophobic portion of the membrane (Table 2).

Fig. 1. Micrographs of liver after *in vivo* reversible endotoxic shock induction (acute phase). Mayor hepatotoxic signs are: **A.** Swollen and palely stained parenchymal cells around the terminal hepatic venules with dilatation of sinusoids. Masson staining, light microscopy. x 170. **B.** Parenchymal cells with cytoplasmic lipid droplet inclusion. Toluidine staining, light microscopy. x 510. **C.** Mitochondrial damage, nuclear membrane dilatation and increased number of phagosomes in Kupffer cells. x 12,000; Bar: 500nm. **D.** Space of Disse including lamellar bodies. x 12,000; Bar: 500nm. **E.** Magnification of parenchymal cell containing lamellar phospholipidic material (pseudolamellar bodies). x 48,000. Bar: 125nm. **F.** Magnification of parenchymal cell showing mitochondrial damage and pseudolamellar body formation. x 18,000; Bar= 330 nm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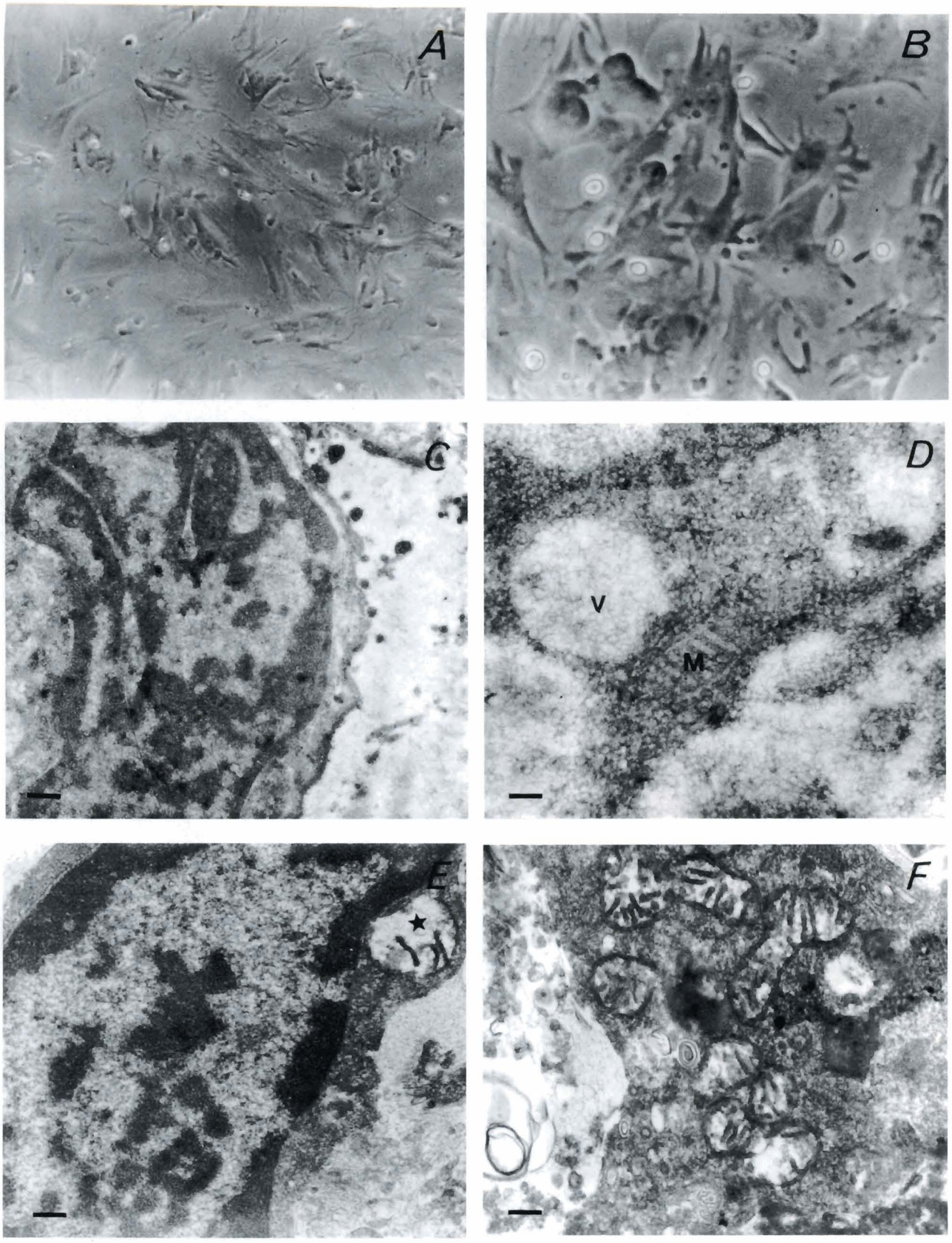
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Discussion

While it is generally accepted that the LPS has mainly a hepatic clearance, the mechanisms involved in the hepatic damage have not been fully elucidated. Liver damage could be caused by LPS direct action, hypoxia and/or inflammatory mediators. The organ complexity, with different cell populations (parenchymal and sinusoidal cells), leads to a difficult approach when the process is considered.

A reversible endotoxic shock model was previously established (Bosch et al., 1988) to study liver damage during the different *in vivo* phases of shock.

Morphological study, by light and electron microscopy, of livers from LPS-treated rats, has evidenced a major damage around centrolobular veins during the acute-phase process (2-6 h), altering both parenchymal and sinusoidal cells and perisinusoidal space of Disse. Mayor cell histological changes can be observed in parenchymal and Kupffer cells with mitochondrial alterations and nuclear membrane dilatation. An increased rough reticulum was observed in parenchymal cells that can be correlated with the high rate of acute-phase protein synthesis (García et al., 1990). The perisinusoidal space of Disse includes a large number of lamellar bodies that can be a result of the increased phagocytic activity of Kupffer cells. These cells exhibit a large number of phagosomes filled with pseudolamellar bodies, probably derived from the mitochodrial degradation.

Although *in vivo* studies indicate that parenchymal and Kupffer cells are structurally damaged, in order to elucidate their contribution to the endotoxic process, it is necessary to study the extent of the direct LPS action on their functionality.

A useful approach could be the establishment of *in vitro* models using liver cell cultures that allow the study of the LPS action at a cellular level.

In previous studies, cultures of parenchymal cells (Pagani et al., 1987) were used to observe the direct action of the LPS from *E. coli* 0111:B4.

Experiments with *E. coli* 0111:B4 [^{14}C]LPS had previously shown the absence, under our conditions, of specific binding sites on isolated and cultured parenchymal cells (Pagani et al., 1981, 1988; Díaz-Laviada et al., 1991). The LPS binds to the cell membrane and microvilli, after short incubation times, and is then internalized into cells showing time and temperature-dependent kinetics (Díaz-Laviada et al., 1991) supporting the two-step model proposed by Price and Jacob (1986). But parenchymal cells need high doses of LPS to show morphological damage *in vitro*, this fact suggests that the *in vivo* response could be enhanced through other factors like hypoxia or soluble mediators released by surrounding cells (i.e., transient macrophages or sinusoidal cells).

Since sinusoidal cells, mainly Kupffer cells, are involved in the primary liver detoxification mechanisms, and may play a key role in the process, the study of their LPS-uptake and localization can be a contribution to understand the molecular basis of the liver LPS action.

Isolation, characterization and culture of liver sinusoidal cells are the first steps to establish the experimental model that will complete the study on parenchymal cells and will allow a comparative study on both fractions.

Within sinusoidal cells (Kupffer, endothelial, fat storing and pit cells), the macrophagic population of Kupffer cells seem to play a key role in the process. The isolation of a pure (95-98%) Kupffer cell fraction is difficult, time wasting and needs a complex elutriation technique. In this study a more simple mixed culture of enriched Kupffer (80%) and endothelial cells is proposed.

To obtain these cells we have used a recirculating perfusion with collagenase/pronase method and to

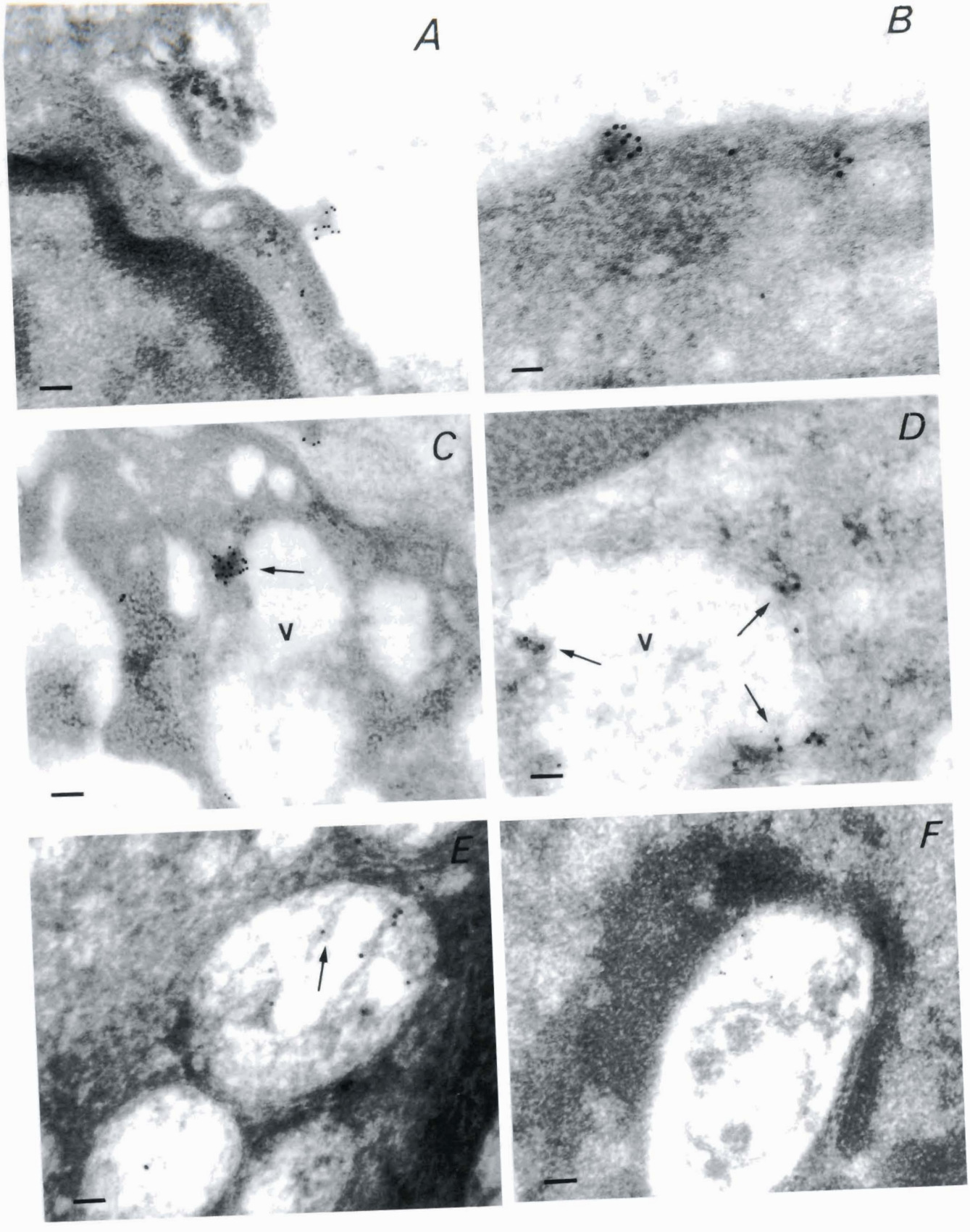
Table 1. Effect of LPS on the plasma membrane microviscosity ($\bar{\eta}$) of liver parenchimal cells. $p<0.001$

MEMBRANE PORTION	TREATMENT (30 min, 37 ºC)	$\bar{\eta}$ (Pa.s)
External	Control	0.364±0.007
	50 µg LPS/ml	0.400±0.006
	100 µg LPS/ml	0.457±0.007
	200 µg LPS/ml	0.414±0.005
Hydrophobic	Control	0.171±0.005
	50µg LPS/ml	0.142±0.006
	100µg LPS/ml	0.150±0.005
	200 µg LPS/ml	0.164±0.007

Table 2. Effect of LPS on the plasma membrane microviscosity ($\bar{\eta}$) of liver sinusoidal cells. $p<0.001$

MEMBRANE PORTION	TREATMENT (30 min, 37 ºC)	$\bar{\eta}$ (Pa.s)
External	Control	0.471±0.007
	50 µg LPS/ml	0.414±0.006
	100 µg LPS/ml	0.375±0.014
	200 µg LPS/ml	0.350±0.010
Hydrophobic	Control	0.310±0.005
	50µg LPS/ml	0.328±0.006
	100µg LPS/ml	0.371±0.005
	200 µg LPS/ml	0.400±0.007

Fig. 2. Micrographs showing enriched Kupffer cell culture. Control cultures (A, B, C, D) and *in vitro* LPS treated culture (100 µg/ml *E. coli* 0111:B4, 20 min at 37 ºC) (E,F). **A,B.** Optic phase contrast microscopy of 72 h cultured Kupffer cells from adult Wistar rats. A, x 275; B, x 1,100. **C.D.** Electron microscopy showing a normal ultrastructural morphology of Kupffer cells (TEM). V: Phagocytic vacuole; M: mitochondria. C, x 11,500, Bar: 520 nm; D, x 46,000; Bar: 130 nm. **E.** Cytoplasmic lesions with mitochondrial damage (star) induced by LPS in cultured Kupffer cells (TEM). x 17,000, Bar: 350 nm. **F.** Magnification of mitochondrial swelling and autophagosome formation induced by LPS in cultured Kupffer cells (TEM). x 17,000; Bar: 350 nm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	200 µg LPS/ml	0.400±0.007

Fig. 2. Micrographs showing enriched Kupffer cell culture. Control cultures (A, B, C, D) and *in vitro* LPS treated culture (100 µg/ml *E. coli* 0111:B4, 20 min at 37 °C) (E,F). **A,B.** Optic phase contrast microscopy of 72 h cultured Kupffer cells from adult Wistar rats. A, x 275; B, x 1,100. **C.D.** Electron microscopy showing a normal ultrastructural morphology of Kupffer cells (TEM). V: Phagocytic vacuole; M: mitochondria. C, x 11,500, Bar: 520 nm; D, x 46,000; Bar: 130 nm. **E.** Cytoplasmic lesions with mitochondrial damage (star) induced by LPS in cultured Kupffer cells (TEM). x 17,000, Bar: 350 nm. **F.** Magnification of mitochondrial swelling and autophagosome formation induced by LPS in cultured Kupffer cells (TEM). x 17,000; Bar: 350 nm

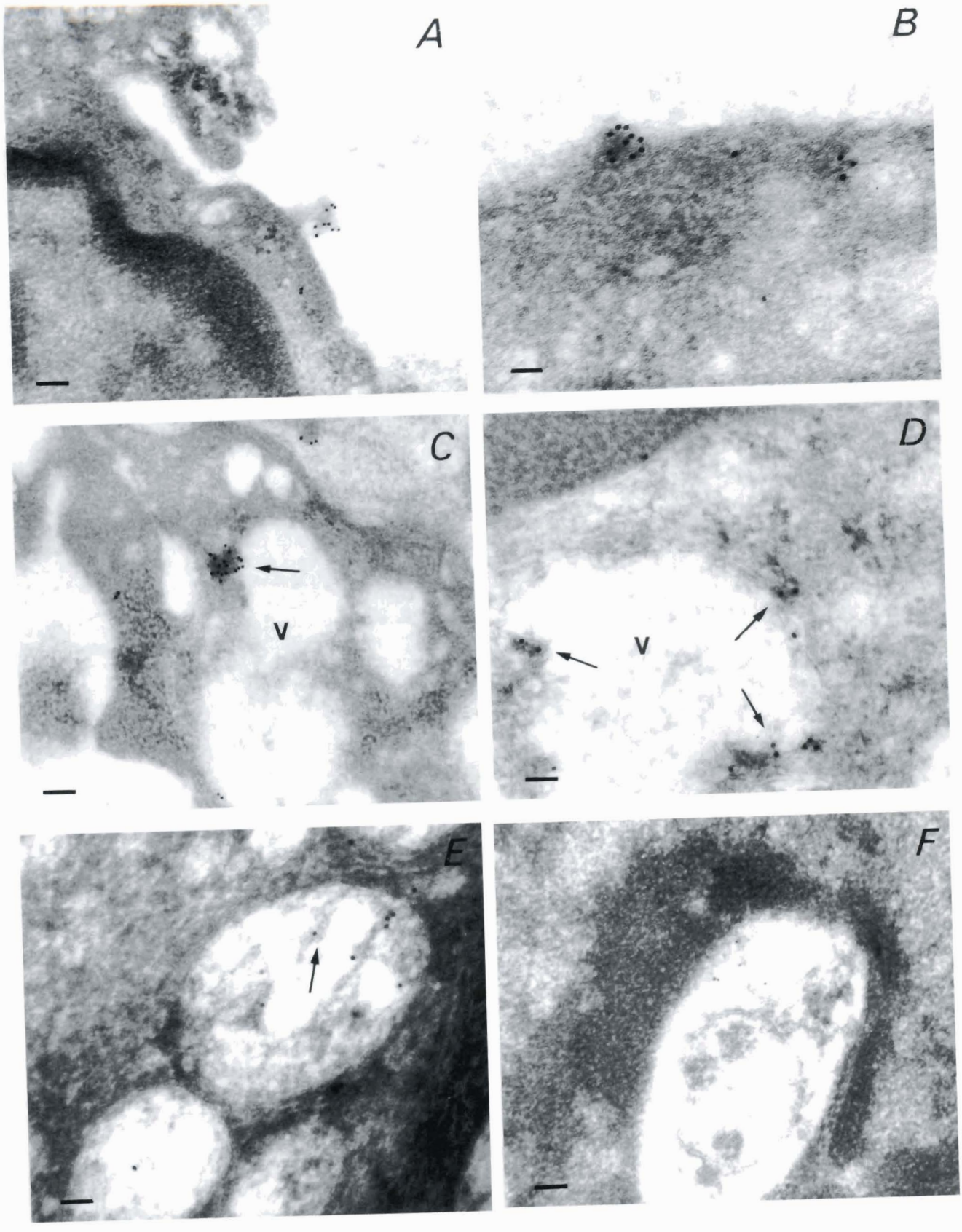
A
B
C
V
D
V
E
F

Fig. 3. Immunocytochemical studies. **A,B.** Electron micrographs of 72 h cultured Kupffer cells treated with *E. coli* 0111:B4 LPS (100 µg) (15 min, 37 ºC). The immunogold labelling shows the endotoxin associated to cell surface (A) being internalized (B). A, x 48,000, Ba: 125 nm; B, x 80,000, Bar: 75 nm. **C-F.** Electron micrographs of 72 h cultured Kupffer cells treated with *E. coli* 0111:B4 LPS (100 µg) (60 min, 37 ºC). The immunogold labelling (arrows) shows the endotoxin associated to phagocytic vacuoles (V), internal membranes (C, D, E) and residual mitochondrial membranes (F). C,F, x 48,000, Bar: 125 nm; D,E, x 80,000, Bar: 75 nm).

characterize these cultures we have used microscopical and flow cytometry techniques.

Cultures can be useful for morphological and immunocytochemical studies and were used to study the *E. coli* 0111:B4 LPS uptake and location. Kupffer cells showed the same morphological damage observed *in vivo*, suggesting an increased phagocytic response and a mitochondrial damage induced by the direct interaction of LPS. These results do not totally agree with *Salmonella abortus* action (Van Bossuyt et al., 1988); there the enhanced phagocytosis is evidenced but is not a direct action on cells. This can be related to a different mechanism of action depending on the LPS type.

The immunocytochemical studies have shown a rapid uptake of the LPS that can be evidenced, after 15 min, associated to plasma membrane and to phagocytic vacuoles. Longer times of exposure (60 min) allowed us to observe the LPS associated to residual mitochondrial membranes in phagosomes, supporting a direct damage of LPS on mitochondria.

When treated with LPS, Kupffer cell behaviour is different from parenchymal cells, first of all Kupffer cells are more sensitive to low doses (10-200 µg) of LPS although parenchymal cells need doses up 500 µg in order to observe morphological alterations, such as cytoplasmic retraction and diminished adhesion capacity (Pagani et al., 1988). As macrophagic cells, Kupffer cells are activated by low doses of LPS and their phagocytic capacity increases; for these reasons we can observe the LPS mainly associated to phagosomes instead of to plasma membrane or in cytoplasm as in parenchymal cells.

Activation of Kupffer cells has been correlated with a rapid formation of free radicals (Portolés et al., 1994) and a release of different mediators. This activation process is not shown in endothelial cells (Portolés et al., 1994).

The difference on microviscosity modification observed after the LPS treatment on parenchymal and sinusoidal cells supports the increased phagocytic activity on Kupffer cells, the opposite to the more static binding on the surface of parenchymal cells (Pagani et al., 1981, 1988; Portolés et al., 1987).

The different cellular uptake and response can justify the biphasic behaviour that can be observed in the endotoxin-induced shock: a direct action of the endotoxin with the cell membrane of parenchymal and sinusoidal cells (mainly Kupffer cells) that can lead to synthesize different mediators and to a complex influence between cells and general homeostatic mechanisms.

The activation of inflammatory cells has been described as the clue to the increase of a wide spectra of serum mediators (arachidonic acid metabolites, hydroperoxiderivatives, leukotrienes and tromboxanes, and acute-phase proteins). Previous studies using primary cultures of parenchymal cells, showed the importance of serum mediators in the alteration of some cellular parameters, such as aspartate aminotransferase (GOT) and albumin. Both parameters are not altered by direct LPS action on parenchymal cells, but they are modified by the addition of serum obtained from LPS-treated animals, although LPS seems to act directly on cytochrome P450 (Pagani et al., 1987), cytochrome b5 (Portolés et al., 1989), superoxide dismutase (SOD) activities (Portolés et al., 1993) and Ca^{++} levels (Portolés et al., 1991).

In conclusion, using sinusoidal cell cultures as an *in vitro* model, we have shown a good correlation with the *in vivo* alterations due to a direct action of the LPS on Kupffer cells. The LPS, through a rapid uptake, can account for the Kupffer cell activation, triggering the mediators synthesis involved in the propagation of shock.

Although further studies are needed, cell cultures can be used as a good model to approach the cellular LPS action avoiding the use of a high number of experimental animals and allowing the distinction between direct and indirect actions of LPS.

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References

Beesley J.E. (1985). Colloidal gold: a new revolution in marking cytochemistry. Proc. RMS 20, 187-197.

Berry M.N. and Friend D.S. (1969). High-yield preparation of isolated Parenchymal cells. A biochemical and fine structural study. J. Cell Biol. 43, 506-520.

Bosch M.A., García R., Pagani R., Portolés M.T., Díaz-Laviada I., Abarca S., Ainaga M.J., Risco C. and Municio A.M. (1988). Induction of reversible shock by *Escherichia coli* lipopolysaccharide in rats. Changes in serum and cell membrane parameters. Br. J. Exp. Pathol. 69, 805-812.

Braude A.I. (1964). Absorption, distribution and elimination of endotoxin and their derivatives. In: Bacterial endotoxins. Landy M. and Braun M. (eds). Rutgers University Press. New Brunswick. New York.

Chensue S.W., Terebuh P.D., Remick D.G., Scales W.E. and Kunkel S.L. (1991). *In vivo* biologic and immunohistochemical analysis of interleukin-1 alpha, beta and tumor necrosis factor during experimental endotoxemia. Kinetics, Kupffer cell expression, and

glucocorticoid effects. Am. J. Pathol. 138, 395-402.

Díaz-Laviada I., Ainaga M.J., Portolés M.T., Carrascosa J.L., Municio A.M. and Pagani R. (1991). Binding studies and localization of *Escherichia coli* lipopolysaccharide in cultured hepatocytes by an immunocolloidal-gold technique. Histochem. J. 23, 221-228.

Freundenberg M.A., Freudenberg N. and Galanos C. (1982). Time course of cellular distribution of endotoxin in liver, lung and kidney of rats. Br. J. Exp. Pathol. 63, 46-56.

Freudenberg M.A., Kleine B. and Galanos C. (1984). The fate of lipopolysaccharide in rats: evidence for alteration in the molecule. Rev. Infect. Dis. 6, 483-487.

Freudenberg N., Freudenberg M.A., Bandara K. and Galanos C. (1985). Distribution and localization of endotoxin in the reticuloendothelial system (Res) and in the main vessels of the rat during Shock. Pathol. Res. Pract. 179, 517-527.

García R., Díaz-Laviada I., Bosh M.A., Abarca S., Portolés M.T., Risco C., Ainaga M.J., Pagani R., Municio A.M. and Escalona J. (1990). Morphological studies of cytotoxic lesions in reversible endotoxic shock. Adrenal glands, liver, lung. Histol. Histopathol. 4, 43-48.

Jacob A.I., Goldberg P.K., Bloom N., Degenshein G.A. and Kozinn P.J. (1977). Endotoxin and bacteria in portal blood. Gastroenterology 72, 1268-1270.

Kang Y., Lee C. and Monroy R.L. (1992). Uptake, distribution and fate of bacterial lipopolysaccharides in monocytes and macrophages: an ultrastructural and functional correlation. Electron. Microsc. Rev. 5, 381-419.

Keller F., Wild M.T. and Kirn A. (1984). *In vitro* cytostatic properties of unactivated rat Kupffer cells. J. Leukocyte Biol. 35, 467-474.

Keller F., Wild M.T. and Kirn A. (1985). *In vitro* antiviral properties of endotoxin activated rat Kupffer cells. J. Leukocyte Biol. 38, 293-303.

Maier R.V.R. and Ulevitch J. (1981). The response of isolated rabbit hepatic macrophages (H-Mo) to lipopolysaccharide (LPS). Circ. Schok 8, 165-181.

Mathison J.C. and Ulevitch R.J. (1979). Clearance, tissue distribution, and cellular localization of intravenously injected LPS in rabbits. J. Immunol. 23, 2133-2143.

Nolan J.P., Hare D.K., McDevitt J.J. and Ali A.V. (1977). *In vitro* studies of endotoxin absorpiton. Gastroenterology 72, 434-439.

Ogle C.K., Wu J.C., Alexander J.W., Fischer J.E. and Ogle J.D. (1991). The effects of *in vivo* administration of endotoxin on the functions and interaction of hepatocytes and Kupffer cells. Prostaglandins 41, 169-183.

Pagani R., Portolés M.T. and Municio A.M. (1981). The binding of *Escherichia coli* endotoxin to isolated rat hepatocytes. FEBS Lett. 131, 103-107.

Pagani R., Portolés M.T., Bosch M.A., Díaz-Laviada I. and Municio A.M. (1987). Direct and mediated *Escherichia coli* lipopolysaccharide action in primary hepatocyte cultures. Eur. J. Cell Biol. 43, 243-246.

Pagani R., Portolés M.T., Díaz-Laviada I. and Municio A.M. (1988). Morphological damage induced by *E. coli* lipopolysaccharide in cultured hepatocytes. Localization and binding properties. Br. J. Exp. Pathol. 69, 537-549.

Portolés M.T., Pagani R., Díaz-Laviada I. and Municio A.M. (1987). Effect of *Escherichia coli* lipopolysaccharide on the microviscosity of liver plasma membranes and hepatocyte suspensions and monolayers. Cell Biochem. Funct. 5, 55-61.

Portolés M.T., Díaz-Laviada I., Ainaga M.J., Pagani R. and Municio A.M. (1989). Involvement of cytochrome b5 in the cytotoxic response of *Escherichia coli* lipopolysaccharide. Mol. Cell. Biochem. 87, 79-84.

Portolés M.T., Ainaga M.J., Municio A.M. and Pagani R. (1991). Intracellular calcium and pH alterations induced by *Escherichia coli* endotoxin in rat hepatocytes. Biochim. Biophys. Acta 1092, 1-6.

Portolés M.T., Ainaga M.J. and Pagani R. (1993). The induction of lipid peroxidation by *E. coli* lipopolysaccharide on rat hepatocytes as an important factor in the etiology of endotoxic liver damage. Biochim. Biophys. Acta 1158, 287-292.

Portolés M.T., Arahuetes R.M. and Pagani R. (1994). Intracellular calcium alterations and free radical formation evaluated by flow cytometry in endotoxin-treated rat liver Kupffer and endothelial cells. Eur. J. Cell Biol. 65, 200-205.

Praaning-van Dalen D.P., Brouwer A. and Knook D.L. (1981). Clearance capacity of rat liver Kupffer, endothelial, and parenchymal cells. Gastroenterology 81, 1036-1044.

Price R.M. and Jacobs D.M. (1986). Fluorescent detection of lipopolysaccharide interactions with model membranes. Biochim. Biophys. Acta 859, 26-32.

Prytz A., Hots-Christensen J., Korner B. and Liehr H. (1976). Portal venous and systemic endotoxemia in patients without liver disease and systemic endotoxemia in patients with cirrhosis. Scand. J. Gastroenterology 11, 857-863.

Rogoff T.M. and Lipsky P.E. (1981). Role of the Kupffer cell in local and systemic immune responses. Gastroenterology 80, 854-860.

Romanowska E. (1970). Sepharose gel filtration method of purification of lipopolysaccharides. Ann. Biochem. 33, 383-389.

Ruiter D.J., Van der Meulen J., Brouwer A., Hummel M.J.R., Mauw B.J., Van der Ploeg J.C.M. and Wisse E. (1981). Uptake by liver cells of endotoxin following its intravenous injection. Lab. Invest. 45, 38-45.

Shinitzky M. and Barenholz Y. (1978). Fluidity parameters of lipid regions determined by fluorescence polarization. Biochim. Biophys. Acta 515, 367-394.

Shiratori Y., Okano K., Matsumoto K. and Murao S. (1984). Antigen presentation by Kupffer cells in the rat. Scand. J. Gastroenterology 19, 733-739.

Triger D.R., Boyer T.D. and Levin J. (1978). Portal and systemic bacteremia and endotoxemia in liver disease. Gut 19, 935-939.

Van Bossuyt H. and Wisse E. (1986). Time course of cellular and subcellular distribution of endotoxin (with emphasis on lipid A) in rat liver. In: Proc XIth Int. Cong. Electron Microscopy Jap. Soc. Electron Microscopy. Imura T., Maruse S. and Suzuki Y. (eds). Tokyo, Japan. pp 2911-2912.

Van Bossuyt H., Desmaretz C., Rombaut B. and Wisse E. (1988). Response of cultured rat Kupffer cells to lipopolysaccharide. Arch. Toxicol. 62, 316-324.

West M.A., Keller G.A., Hyland B.J., Cerra F.B. and Simons R.L. (1986). Further characterization of Kupffer cell/macrophage-mediated alterations in hepatocyte protein synthesis. Surgery 100, 416-421.

Westphal O., Lüderitz O. and Bister F. (1952). Über die Extraktion von Bakterien mit Phenol/Waser. Z. Naturforsch. 7b, 148-156.

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