

Alterations induced on cytoskeleton by *Escherichia coli* endotoxin in different types of rat liver cell cultures

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Summary. Endotoxins (lipopolysaccharide, LPS) from Gram-negative bacteria are considered as the agents responsible for the induction of endotoxic shock, producing severe cellular metabolic dishomeostasis. Cytotoxic lesions, as well as functional and metabolic disturbances, occur mainly in the liver, which is one of the target organs and exerts an LPS clearance function. In an attempt to approach the molecular basis of endotoxic shock, and to propose an experimental model, we have focused this study on cytoskeleton (microtubules and microfilaments) alterations induced by different doses of endotoxin in different target liver cells. Microfilaments and microtubules were studied by immunofluorescence and different microscopy techniques (optic fluorescence microscopy and confocal laser scanning microscopy) in order to improve the cytoskeleton study resolution.

Parenchymal and sinusoidal cell morphology, severely damaged by the LPS action, is related to a disturbance on the cytoskeletal organisation, even more evident in a particular proliferating rat liver cell culture.

The most relevant changes are seen in the microtubule patterns in all liver cells tested, which could be related, depending on cell type, either to a direct LPS action or to $[Ca^{+2}]$, dishomeostasis as well as free radical and cytokine (IL-1 β and TNF- α) production.

Due to their features, proliferating rat liver cell cultures are used as a sensitive cell model to understand the effect of LPS on cytoskeleton organisation.

Key words: Endotoxin, cytoskeleton, Parenchymal cells, Sinusoidal cells, Proliferating liver cells

Introduction

Endotoxins (lipopolysaccharide, LPS), amphiphilic components of the outer membrane of Gram-negative bacteria such as *Escherichia coli*, are responsible for the multisystemic organism failure in endotoxic shock (Nowotny, 1987), which still remains as a major clinical problem despite efforts in the development of new therapies. The liver is one of the LPS target organs which presents important morphological damages *in vivo* (Pagani et al., 1996). It is accepted that parenchymal and sinusoidal liver cells play a multiple role either in detoxification processes (Mimura et al., 1995) or in inflammatory mediator production (Pagani et al., 1987).

The complexity of the liver organ suggests a different behaviour of parenchymal and non-parenchymal or sinusoidal cells (mainly Kupffer cells). Although the parenchymal cell response is the basis for the liver function, sinusoidal cells play an important role in the first steps of the liver LPS clearance (Freudenberg et al., 1985), starting a complex pathophysiological response (Maier and Ulevitch, 1981) which involves cytokines, such as tumor necrosis factor- α (TNF- α) and interleukins, important mediators of the endotoxin action (Chensue et al., 1991; Fiers, 1991; Ogle et al., 1991; Van Deuren et al., 1992; Goossens et al., 1995), oxidative stress, lipid peroxidation (Portolés et al., 1993, 1994, 1996; Catalá et al., 2002) and nitric oxide production (Harbrecht and Billiar, 1995; Steinhorn and Cerra, 1997).

Previous studies have shown that LPS from *Escherichia coli* 0111:B4 induces important morphological (Pagani et al., 1988, 1996; Vergani et al., 1999) and metabolic disturbances. An increase in intracellular Ca^{2+} and cytochrome b5, free radical formation as well as a decrease in pH and cytochrome P450 was observed in parenchymal and macrophagic Kupffer cells (Portolés et al., 1989a, 1991, 1994). It is important to emphasize that Kupffer cells, as macrophagic cells, are more sensitive to low LPS doses (Portolés et al., 1994; Pagani et al., 1996).

Nevertheless, in endotoxin-induced shock the first

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step in the cellular damage could be attributed to a direct interaction of LPS with the cell membrane. Experiments with *Escherichia coli* [C14]LPS, immunocytochemical colloidal-gold technique and flow cytometry have previously shown that, in isolated and cultured liver cells, the LPS binds to the cell membrane and then it is internalised into cells at different rates depending on the cell type (Pagani et al., 1981; Municio et al., 1990; Díaz-Laviada et al., 1991). Sinusoidal cells (mainly Kupffer cells) have shown a rapid uptake of the LPS associated to plasma membrane and to phagocytic vacuoles. At longer times, all cells show the LPS associated to residual mitochondrial membranes (autophagosomes), supporting a direct damage of LPS on mitochondria (Pagani et al., 1996; Catalá et al., 1999) and, possibly, to other structures like the cytoskeleton.

The endotoxin effect at the cellular level induces ultrastructural changes that could be related to cytoskeleton. In parenchymal non-replicative liver cells these morphological changes include cytoplasmic retraction and diminished adhesion capacity (Pagani et al., 1988). Sinusoidal cells (endothelial and Kupffer cells) show a similar reaction, but they are more sensitive to low LPS doses (Portolés et al., 1994; Pagani et al., 1996). The damage is even more evident using a proliferating liver cell type (Vergani et al., 1999). These cells have an enhanced adhesion capacity and, in contrast to non-dividing primary cultures of hepatocytes, present a high rate of mitotic divisions (Vergani et al., 1979, 1984, 1991, 1994; Colemann et al., 1993; Thorgeirsson, 1993).

The cytoskeleton involves not only the maintenance of the cell shape but also a wide variety of cell functions like co-ordination and regulation of cell metabolism and, to some extent, could be involved in the pathogenesis of various liver diseases (Mori, 1994; Liu et al., 2001; Schaffert et al., 2001).

LPS cell binding and uptake induce a change in their membrane fluidity (Portolés et al., 1987; Pagani et al., 1996) and permeability (Portolés et al., 1989b). The oxygen-derived radicals, generated mainly by Kupffer cells, can stimulate lipid peroxidation (Portolés et al., 1996), aggravating the alteration of membrane and cytoskeleton, the cytoplasmic network of unstable polymers, very sensitive to changes in $[Ca^{2+}]_i$ levels (Kraus-Friedmann, 2002) and oxyradicals (Ding et al., 2001; Lin et al., 2001).

On the other hand, the ability of LPS to interact with microtubular proteins has been demonstrated, suggesting a possible participation of microtubules in the cellular effects of endotoxins (Risco et al., 1993a; Russwurm et al., 2000) thus explaining its possible role in the endotoxin-induced inflammatory events such as cytokine production (Allen et al., 1997; Baldari and Telford, 1989).

A microtubule-associated protein has been proposed as a possible mediator of cellular responses to LPS in macrophages (Ding et al., 1990a,b, 1992). In effect, it has been reported that LPS induces different alterations

in microfilaments and microtubules in macrophages and monocytes (Isowa et al., 1999). In these cells, endotoxin causes a rapid alteration in microtubule stability and a reorganisation of microfilaments (Shinji et al., 1991; Bannerman et al., 1997, 1998).

Due to the important role of the cytoskeleton in cell function and in an attempt to approach and better define the molecular basis of endotoxic shock, we have focused this study on the cytoskeleton (tubulin and actin) alterations that can be induced in different liver cell types such as parenchymal and sinusoidal cells. Microtubules and microfilaments were studied by immunofluorescence using different microscopical techniques (optic fluorescence microscopy and confocal laser scanning microscopy (CLSM)). Our results show that a proliferating rat liver cell type could be used as a sensitive and suitable experimental model for the analysis of cytoskeletal changes induced by endotoxins. This cell model could then be used to understand the molecular mechanism involved in endotoxic shock.

Materials and methods

Isolation and culture of parenchymal and sinusoidal liver cells

Parenchymal and sinusoidal liver cells were isolated from adult male Wistar rats (150–200 g b.w.). The animals were kept and handled according to the Spanish (M^o de Agricultura - Spain, BOE 223/1988, 265/1990) and the European Committee guidelines for the care and use of laboratory animals (86/609 CEE).

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 suspension was purified by repeated centrifugation (165g for 20s) in William's E medium with 500 UI/ml penicillin and 0.1 mg/ml streptomycin. Cells were cultured in the presence of 10% foetal calf serum, at 37 °C in 25 ml plastic bottles (Costar, Cambridge, MA, USA) or on coverglasses. Cells were cultured under a CO₂ (5%) atmosphere (Heraeus B5061) for 24/72 h.

Sinusoidal liver cells were isolated by a collagenase/pronase recirculating perfusion method, according to Van Bossuyt et al. (1988), with minor modifications. Cell suspensions were purified by repeated centrifugation (200 g for 10 min) in Gey's Balanced Salt Solution (GBSS), with the addition of antibiotics (400 UI/ml penicillin, 100 mg/ml streptomycin and 50 mg/ml gentamycin; Antibioticos S.A., León, Spain) in a refrigerated RC-5 Sorvall. Purification was completed with Nycodenz's gradient (Nycomed AS, Oslo, N) and centrifugation (1400g for 15 min). Floating cells were washed twice in Dulbecco's Minimal Essential Medium (DMEM) with 37.5 mM glutamine, 20 mM Hepes and 10 mM NaHCO₃. In order to obtain enriched (80%) Kupffer cell cultures, after 20h the medium with unattached cells was discarded and

replaced by fresh complete medium (differential adhesion procedure). 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. Cells were cultured under a CO₂ (5%) atmosphere for 48/72 h.

Cell viability, tested by the Trypan Blue exclusion assay, was 90-95%. Maintenance of cell structure and identification of parenchymal and sinusoidal cells, was verified by phase-contrast (Leitz Laborlux K and Leitz Diavert microscopes, Wetzlar, Germany) and electron microscopy (Zeiss 902, Carl Zeiss, Oberkochen, Germany). Relative percentage of endothelial and Kupffer cells, after the diamine benzidine (DAB) reaction was 75%/25%, respectively (Pagani et al., 1996). The endothelial/Kupffer cell ratio was also evaluated by flow cytometry (Pagani et al., 1996).

Proliferating rat liver cells

Rat liver parenchymal cells were prepared from twenty-day-old male Wistar rats according to Vergani et al. (1991, 1994, 1999). These cells were cultured in Ham's F-10 medium (Biochrom KG, Seromed, Berlin, Germany) supplemented with 10% foetal calf serum and antibiotics (100 UI/ml penicillin, 100 mg/ml streptomycin) as well as L-glutamine (0.37 mM) in a CO₂ incubator (5% CO₂) at 37 °C. The medium was changed every three days up to the formation of a continuous monolayer. Six cultures per experimental group were used. Maintenance of cell structure was verified by phase-contrast (Leitz Laborlux K and Leitz Diavert microscopes, Wetzlar, Germany) and electron microscopy (Zeiss 902, Carl Zeiss, Oberkochen, Germany).

LPS treatment

The *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) used, obtained according to the method of Westphal et al. (1952), was supplied by Difco (Detroit, Michigan, USA). The A250/A240 ratio was determined for purity verification (Romanowska, 1970). The LPS suspensions were previously sonicated for 5 min in Bransonic12, and sterilised by filtration through 0.22 mm Millex-GS (Millipore Corp., Bedford, MA, USA) before their use for cell treatment.

Parenchymal and sinusoidal liver cells were incubated with different doses of *Escherichia coli* LPS (10, 100 or 500 µg/ml William's or DMEM/RPMI (2/3:1/3, v:v) according to the cell type), for 5, 10 or 15 min in a CO₂ incubator (5% CO₂) at 37 °C.

Proliferating rat liver cells were cultured for 48 h and, after that, Ham's F-10 medium was removed and replaced by an LPS suspension (10 µg/ml) in the same serum-free medium. Then monolayers were incubated for 5 or 15 min at 37 °C in a CO₂ incubator (5% CO₂) at 37 °C. Controls with LPS-free William's, DMEM/RPMI

or Ham's F-10 medium were always carried out.

Cytoskeleton studies

Maintenance of cell structure and identification of parenchymal, sinusoidal, and replicating parenchymal cells were verified by phase-contrast microscopy before LPS treatment and cytoskeleton studies. All cell populations were grown until monolayers were formed following previous culture conditions and were tested for ultrastructure by scanning and electron transmission microscopy (Pagani et al., 1988, 1996; Díaz-Laviada et al., 1991; Vergani et al., 1994, 1999). Non-proliferating parenchymal and Kupffer cell cultures were used before reaching the complete confluence for better observing the cytoskeletal structures.

Double immuno-labelling was performed on cell monolayers treated or not with different LPS doses at different times. Monolayers, washed with PEMP 100 (4%) polyethyleneglycol, were permeated with PEMP/ (0.5%) Triton X100, washed in PEMP and fixed in PEMP/ (3.7%) paraformaldehyde. Monolayers, washed in PBS, were treated for 1h at 37 °C with monoclonal anti-α-tubulin (Amersham Int., Little Chalfort, UK), washed in PBS and treated with anti-anti-tubulin FITC (Amersham Int., Little Chalfort, UK). Phalloidin-rhodamin (Molecular Probes Inc., Eugene, OR, USA) was used to evidence actin microfilaments in fixed monolayers. Controls were always performed incubating cultured cells with the same procedure in absence of LPS.

Fluorescence images of double-stained cells were observed with Leitz Ortholux2 (Ploemopack 2.5) and Confocal Laser Scanning Microscopy (CLSM) (Confocal Microscope Multiline Argon Laser; Carl Zeiss, Germany) using Normanski optics.

Results

The effect of the LPS from *Escherichia coli* 0111:B4 on the cytoskeleton components (microtubules and microfilaments) was observed in different cultured liver cells. Three types of cells were used: parenchymal non-replicating cells, sinusoidal cell enriched with Kupffer cells and a particular type of replicating liver parenchymal cell.

The liver cells were isolated and cultured in specific conditions according to their requirements. Cultures were grown on plastic or coverglass supports and checked by optic phase-contrast microscopy to assure their morphological integrity and viability. All cell populations were previously tested for ultrastructure by scanning and electron transmission microscopy (Díaz-Laviada et al., 1991; Pagani et al., 1988, 1996; Vergani et al., 1994, 1999). Adult non-proliferating parenchymal cells showed a normal polygonal morphology (Fig. 1a) and sinusoidal cell cultures, enriched up to 85% in Kupffer cells, also showed a normal morphology with a high adhesion to substrate (Fig. 1b). Both types of

cultures were used before reaching confluence in order to better observe the cytoskeleton. Parenchymal proliferating cells showed a very homogeneous polygonal shape, a central nucleus with one or, frequently, several nucleoli and a granular cytoplasm (Fig. 1c). As previously observed, these cells showed mitosis with normal mitotic figures (Vergani et al., 1999)

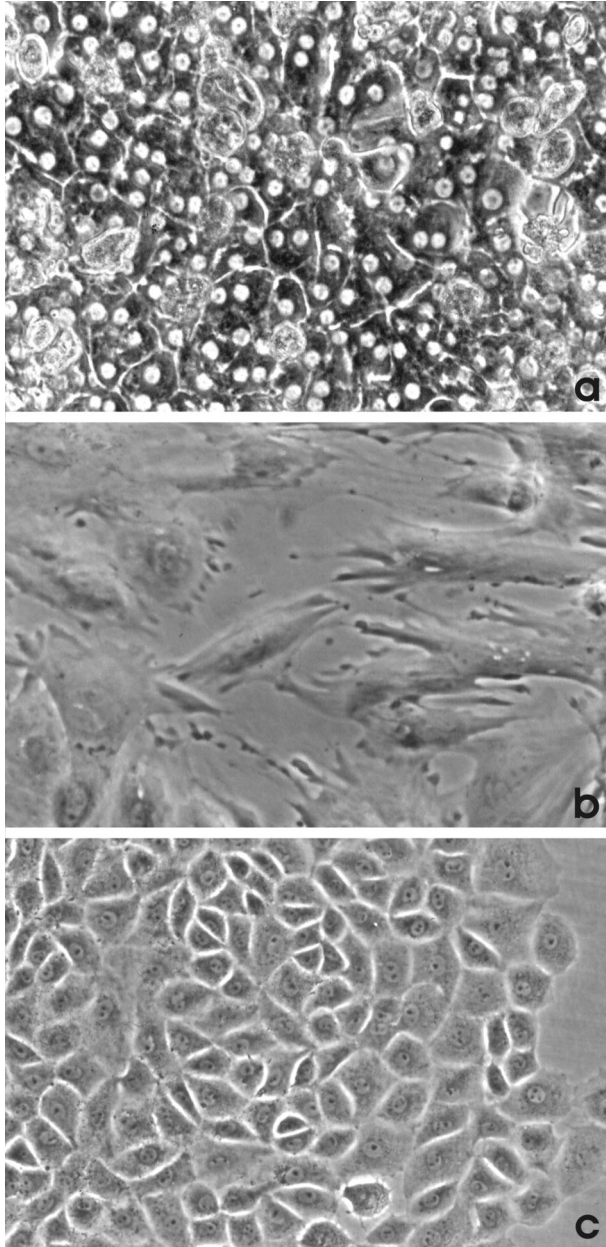


Fig. 1. Control cell cultures observed by optic phase contrast microscopy. **a.** Monolayer of 24h-cultured parenchymal liver cells, from adult Wistar rats. x 240. **b.** Monolayer of 48h-cultured sinusoidal cells, Kupffer cell-enriched, from adult Wistar rats. x1100. **c.** Monolayer of 15th-day cultured proliferating rat liver cells from 20-day adult male Wistar rats. x 240

and their high adhesion capacity allowed an optimal observation of their structure even after reaching confluence.

In order to study the action of LPS from *Escherichia coli* 0111:B4 on the cytoskeleton, a sonicated LPS suspension was added to cultures for treatments at different doses and incubation times. To perform cytoskeleton studies and in order to observe the LPS effect on actin microfilaments versus microtubules, the double immunolabelling technique (anti- α -tubulin FITC antibody for microtubules and phalloidin-rodamin for microfilaments) was performed in fixed cell monolayers. The double-stained cells were observed with different microscopical techniques, optic fluorescence microscopy and confocal laser scanning microscopy (CLSM), depending on cell type and technical resolution.

When cultured non-replicating parenchymal cells were examined by fluorescent microscopy, after staining with specific probes, cytoplasmic microfilaments and microtubules showed a normal distribution around the nucleus toward the cell periphery. As previously seen, these parenchymal cells need high doses of LPS (500 μ g/ml/15min) to respond and a cytoplasmic retraction is always induced by the endotoxin (Pagani et al., 1988). Cytoskeleton of control cells showed well-preserved microfilaments (Fig. 2a) and microtubules (Fig. 2b). The effect of LPS (500 μ g/ml/15min) on cytoskeleton was difficult to observe due to the cytoplasmic retraction. In some flattened areas cells showed a normal microfilament pattern (Fig. 2c) with a loss of architecture in peripheral microtubules (Fig. 2d).

The cytoskeleton changes observed in Kupffer cells included in the sinusoidal cultures could have a special interest due to their macrophagic activity that plays an important role in the LPS clearance. These cells are more sensitive to LPS and, when observed after the LPS treatment (100 μ g/ml/15 min), they showed a well-preserved microfilament pattern (Fig. 2g) and a peripheral loss of microtubules (Fig. 2h) when compared with non-treated cells (Fig. 2e,f). In order to improve the image resolution a confocal laser scanning microscopy (CLSM) with Normanski optic was used. Micrographs of control cells showed a well preserved nuclei and flattened cytoplasm (Fig. 3a) with an evident cytoskeleton fibres network (Fig. 3b). Immunofluorescence figures showed normal cytoskeleton components distributed within their cytoplasm (Fig. 3c,d).

The lowest LPS doses used (10 μ g/ml/15min) induced evident alterations with cytoplasm microvacuolisation, as is shown by Normanski micrographs (Fig. 4a,b). The immunofluorescence showed a peripheral reduction of microtubules (Fig. 4d,e) although, with higher LPS doses and short time (100 μ g/ml/5min), microtubules appeared better preserved (Fig. 4c, f).

Cells were then treated with LPS (10 μ g/ml/15 min) and we could observe the microvacuolisation induced by LPS (Fig. 5a,b), the loss of microtubules (Fig. 5c,d),

Cytoskeleton alterations induced by LPS

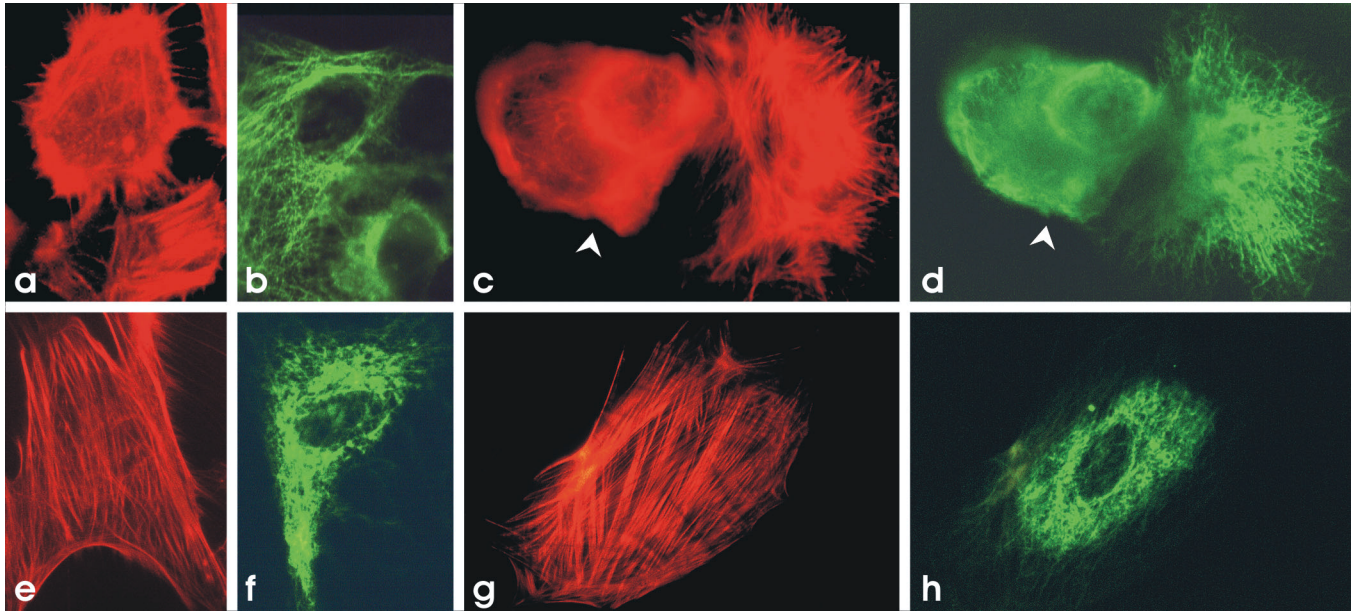


Fig. 2. Cytoskeleton immunocytochemical study by optic fluorescence microscopy of 24h-cultured parenchymal and 48h-cultured Kupffer cells. Parenchymal control cells: **a**, Microfilaments (x500); **b**, Microtubules (x 900). Parenchymal cells treated with *E.coli* 0111:B4 LPS (500 mg/ml/15min): **c**, Microfilaments (x 720); **d**, Microtubules (x 720). Cytoskeleton can be observed in adherent cells, cytoplasm retraction (arrowhead) induced by LPS, does not allow the observation of the cytoskeleton structure. Kupffer cells control cells: **e**, Microfilaments x 2000; **f**, Microtubules (x 2000). Kupffer cells treated with *E.coli* 0111:B4 LPS (100 μ g/ml/15min): **g**, Microfilaments (x 2000); **h**, Microtubules x 2000. Cytoskeleton can be observed in adherent cells with a loss of peripheral microtubules and a normal distribution of microfilaments.

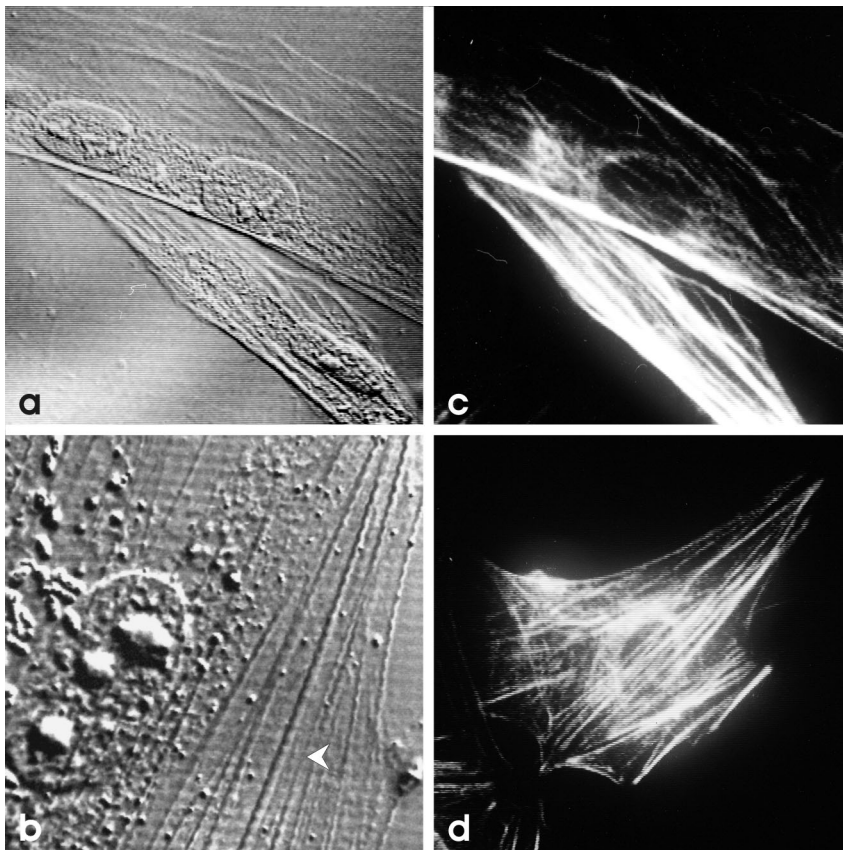


Fig. 3. Cytoskeleton immunocytochemical study by CLSM of 48h-control-cultured Kupffer cells. **a**, **b**. CLSM (Normanski optic) micrographs showing nuclei, flattened cytoplasm (**a**, x 2500) and cytoskeleton fibres (arrowhead) (**b**, x5000); Immunofluorescence micrographs showing a normal cytoskeleton pattern: **c** (x 2500); **d** (x 2000)

which remained at a perinuclear localisation and well preserved actin microfilaments, both at nuclear (Fig. 5e) or peripheral level (Fig. 5f).

In order to improve the cytoskeleton study we used proliferating rat liver cells in vitro, obtained from twenty-day-old Wistar rats (Vergani et al., 1991, 1994, 1999) for their high adhesion capacity that allows a better observation of the microtubules and microfilaments. The double labelling technique and the confocal laser scanning microscopy (CLSM) allowed us to observe the microtubules and actin microfilaments involved in their cytoskeleton structure in detail (Fig. 6a,c).

Mitotic figures were evident and appeared in a high number of cells. Using an image processing facility (changing the red colour for actin into blue) the images are more defined (Fig. 6b). These cells were more sensitive to endotoxin than normal non-replicating parenchymal cells from adult rats. When proliferating parenchymal cells were treated with LPS (10 $\mu\text{g}/\text{ml}/15\text{min}$) we did not observe an altered microfilament pattern but microtubules were only evident in mitotic spindles (Fig. 6d) when compared to

control cells (Fig. 6c). At shorter incubation times (10 min), proliferating parenchymal cells, treated with LPS (10 $\mu\text{g}/\text{ml}$) showed a normal microfilament pattern (Fig. 6f) with a loss of organisation and a disrupted microtubule pattern (Fig. 6h) when compared to control cells (Fig. 6e,g).

Discussion

LPS action on liver cells could be considered one of the most important pathways to understand the molecular mechanisms leading to endotoxic shock. In previous studies, a particular response to the LPS from *Escherichia coli* 0111:B4 has been observed on different liver cell types related to their function. Parenchymal and sinusoidal liver cells bind the LPS, uptake it and respond enhancing or depressing their metabolism. It is worth emphasizing that the endotoxin does bind to some constituent of the cell membrane bilayer either in parenchymal (Pagani et al., 1981; Díaz Laviada et al., 1991) or in sinusoidal cells (Kupffer and endothelial cells) (Catalá et al., 1999). In Kupffer cells, the LPS is mainly found in phagocytic vacuoles, and can also be

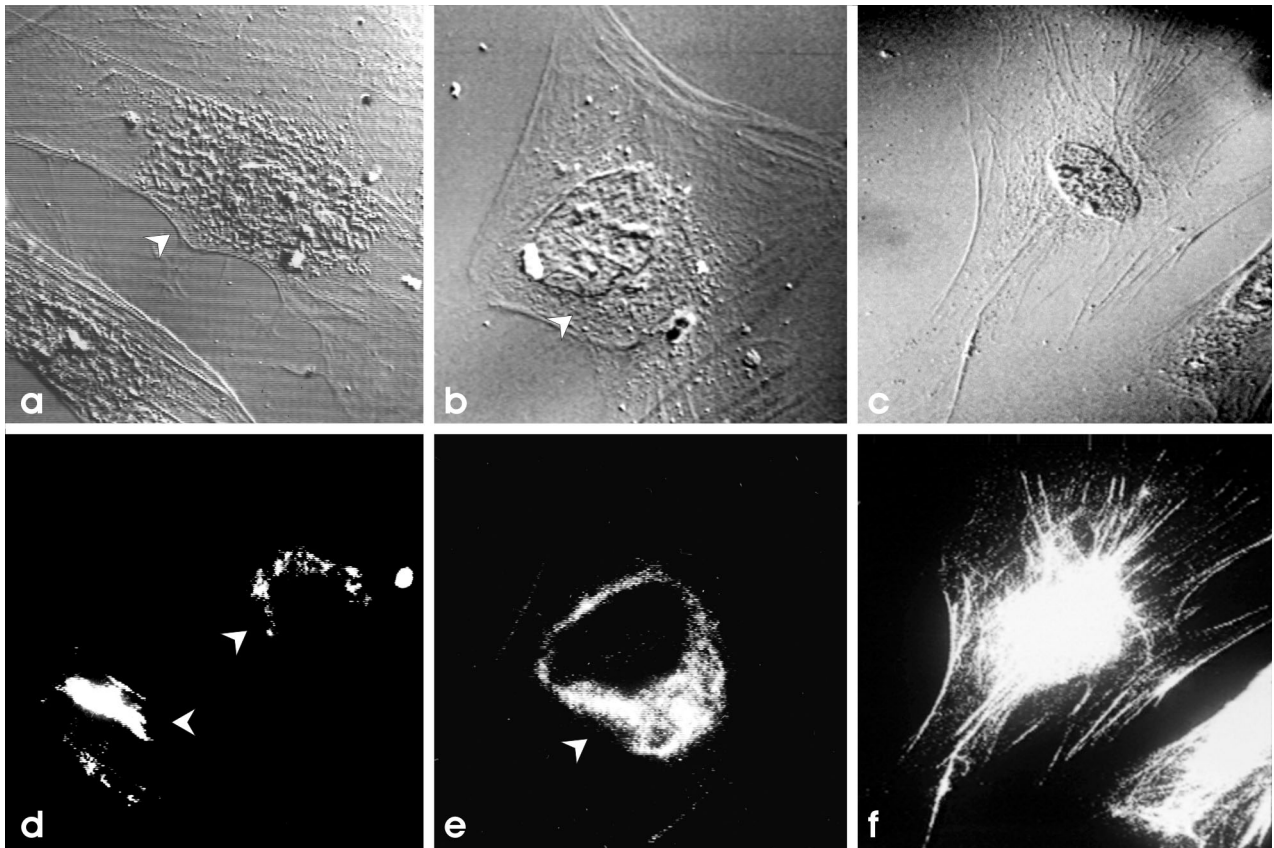


Fig. 4. Cytoskeleton immunocytochemical study by CLSM of 48h-cultured Kupffer cells treated with different doses of *E.coli* 0111:B4 LPS. **Fig. 4a, b.** CLSM (Normanski optic) micrographs of Kupffer cells treated with *E.coli* 0111:B4 LPS (10 $\mu\text{g}/\text{ml}/15\text{ min}$). Nuclei and vacuolated cytoplasm (arrowhead) can be observed. The immunofluorescence micrographs show a loss of microtubules which remain in a perinuclear localization (arrowhead) (**d, e**). The effect is less evident with higher LPS doses (100 $\mu\text{g}/\text{ml}/5\text{ min}$) (**c, f**). a, c-f, x 2700; b, x 3000

Cytoskeleton alterations induced by LPS

seen at the mitochondrial and nucleus level either *in vivo* or *in vitro* (Van Bossuyt et al., 1988; Pagani et al., 1996). LPS binding induces a membrane destabilisation, changing its fluidity (Portolés et al., 1987; Bursten et al., 1991; Pagani et al., 1996) and permeability with LDH leakage (Portolés et al., 1989a). The effect on the plasma membrane may represent a primary consequence of the modification of surface molecules such as glycoproteins, which are known to be relevant in plasma membrane organisation and cell adhesion to the substratum.

After LPS treatment, a severe cytoplasmic vacuolisation and retraction has also been shown in rat parenchymal cells (Pagani et al., 1988) as well as in sinusoidal, endothelial and Kupffer cells (Van Bossuyt et al., 1988; Pagani et al., 1996). The abnormalities of the cell surface and the cytoplasmic retraction must also reflect a damaged cytoskeleton that could explain the intracellular impaired metabolite transport and the metabolic dishomeostasis observed in LPS-treated cells. The importance of the cytoskeleton on cell function and metabolism is well established. It has been shown that the interaction of LPS with different cells, like mesangial cells (Bursten et al., 1991), type II pneumocytes (Risco

et al., 1991), microglia (ABD-el-Basset and Fedoroff, 1995), intestinal fibroblasts (Chakravorty and Kumar, 2000), and monocytes (Ruszwurm et al., 2000) alter their morphology inducing severe cytoskeleton alterations that could be considered one of main causes of cytotoxicity in particular tissues, as endothelium (Bannerman and Goldblum, 1997).

On that basis, by immunofluorescence and different microscopical techniques, we have studied the main cytoskeleton components, microtubule and actin using different liver cell types: parenchymal non-proliferating cells and Kupffer cells from adult rats and a proliferating liver cell population, previously used for morphological studies (Vergani et al., 1999) in order to observe their specific response to *Escherichia coli* 0111:B4 LPS.

Under our experimental conditions, we observed a loss of peripheral microtubule with a non-evident alteration of the microfilament distribution in all cell types. The microtubule change could be related with the cytoplasmic retraction observed in previous studies (Díaz-Laviada et al., 1991; Pagani et al., 1988; Vergani et al., 1999).

The LPS action was more evident on Kupffer cells at

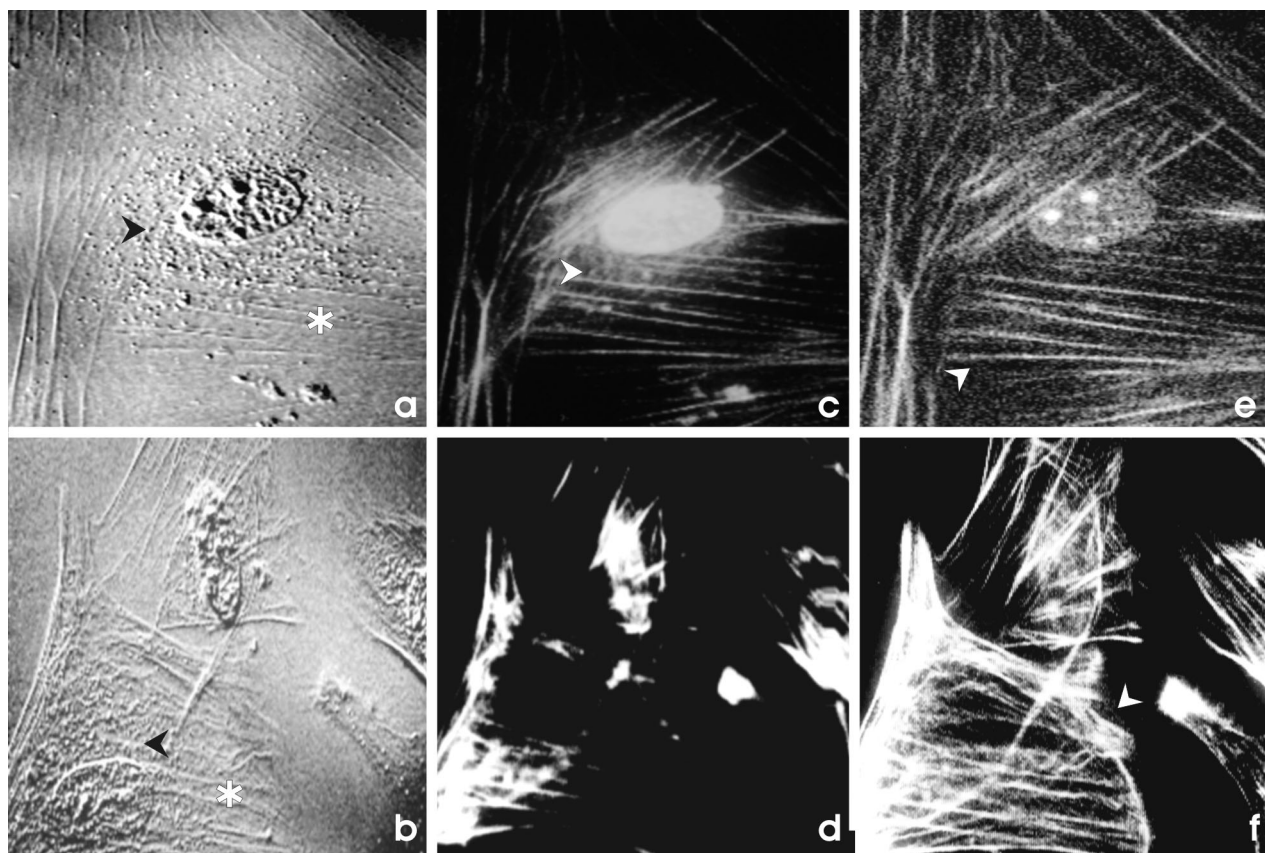


Fig. 5. Cytoskeleton immunocytochemical study by CLSM of 48h-cultured Kupffer cells treated with *E.coli* 0111:B4 LPS (10 μ g/ml/15 min). **a, b.** CLSM (Normanski optic) micrographs of Kupffer cells showing nuclei, vacuolated cytoplasm (arrowhead) and cytoskeleton fibres (*). Immunofluorescence micrographs of microtubules (**c, d**) and microfilaments (**e, f**). LPS action induces a cytoplasm microvacuolisation and a reduction in microtubules which remain in a perinuclear localisation (**c**) or disappear (**d**). Microfilaments (arrowhead) are well preserved (**e, f**). x 2700

low doses, but parenchymal non-replicating cells needed high LPS doses. Special attention has to be drawn to proliferating liver cells that were shown to be very sensitive to LPS at low dose ($10 \mu\text{g/ml}$) and short exposure time. In these cells, microtubules appeared highly disorganised after a 10 min LPS treatment and practically disappear, except in mitotic spindles, at 15 min.

The microtubule disturbance could be related to a direct action of the LPS on tubulin, as previously suggested by several authors (Valberg et al., 1988; Risco et al., 1993a, 1993b; Russwurm et al., 2000) or through an indirect damage due to cellular dishomostatic mechanisms (Ding et al., 2000).

The direct interaction of LPS with the microtubule has been shown by biochemical and electron microscopy analysis (Risco et al., 1991, 1993a) and can be related with the amphipatic and anionic nature of LPS, which acts as a polyanion at physiological pH, forming globular aggregates when sonicated which can be related with its activity (Rietschel et al., 1990; Risco et al., 1993b; Seydel et al., 2000). Physicochemical studies with LPS from *Azotobacter vinelandii*, showed that some polyanions, like nucleic acids, also inhibit microtubule assembly *in vitro*, supporting an interaction mainly related to the capacity of tubulin for interacting with membranes and liposomes (Olins and Warner, 1967; Corces et al., 1980). Different mechanisms have been proposed for the LPS-tubulin interactions. LPS

aggregates might act as membranous structures into which tubulin would insert, probably through hydrophobic forces (Hargreaves et al., 1986; Risco et al., 1991, 1993a). It has also been suggested that LPS inhibits, *in vitro*, microtubule formation on monocytes due to the exclusion of microtubule-associated proteins (MAPs) (Ding et al., 1992; Russwurm et al., 2000). Under our experimental conditions, a direct LPS action on microtubules could be responsible for the tubulin disturbances shown at the periphery of the cell cytoplasm when cells were treated with high LPS doses (more than $100 \mu\text{g/ml}$), as in non-proliferating parenchymal cells and Kupffer cells. This *in vitro* evidence could also be related to the *in vivo* ability of some cells to accumulate LPS (Raetz et al., 1991).

On the other hand, an indirect damage could be caused, primarily, by the intracellular calcium dishomoeostasis that could also alter the polymerisation-depolymerisation of cytoskeleton components (Bursten et al., 1991; Yamamoto et al., 1999) and then induce the TNF (Decker et al., 1987) or interleukines (Bauer et al., 1984, 1986) production. TNF- α promotes cell injury through several mechanisms including the mitochondrial over-production of reactive oxygen species involved in cytotoxicity (Fiers, 1991; Van Deuren et al., 1992; Goosens et al., 1995). Oxygen-derived radicals, generated mainly by phagocytic cells, can stimulate lipid peroxidation inducing an irreversible modification of membrane structure and functions, including alterations

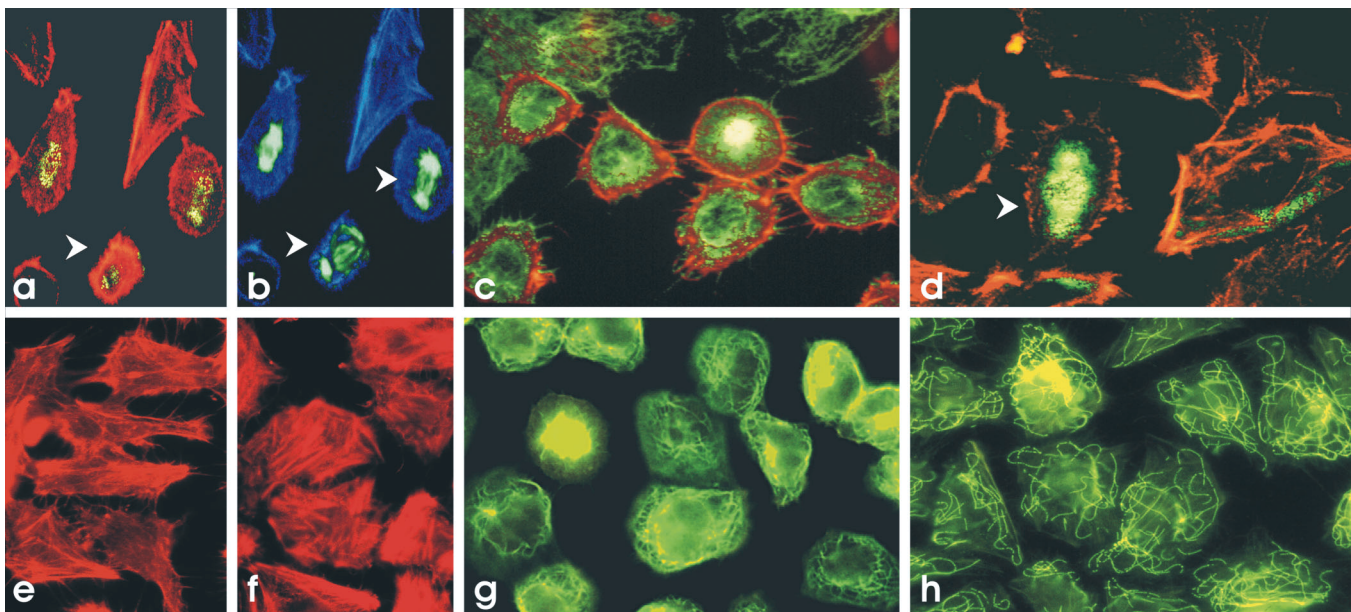


Fig. 6. Cytoskeleton immunocytochemical study observed by CLSM and optic fluorescence microscopy of proliferating rat liver cells. **a, b.** CLSM micrograph of proliferating rat control cells: microfilaments and microtubules observed in double stained preparations. **b.** shows microfilaments (blue) and microtubules (green), this image allows a better observation of the mitotic spindles (arrowhead). **c.** Cytoskeleton pattern of control cells by CLSM. **d.** CLSM micrograph of proliferating cells treated with *E.coli* 0111:B4 LPS ($10 \mu\text{g/ml/15min}$). Microtubules are evident only in mitotic spindles (arrowhead). Microfilaments are well preserved. **e, f.** Optic fluorescence micrograph of microfilaments of proliferating rat cells. **e.** Control cells. **f.** LPS-treated cells: microfilaments are well preserved. **g, h.** Optic fluorescence micrograph of proliferating rat cells. **g.** Control cells. **h.** LPS-treated cells: microtubules present a loss of organization and a disrupted pattern. **a-c,** x 600; **d-g,** x 750; **h,** x 800

in fluidity and permeability with LDH leakage and cellular calcium dishomeostasis (Portolés et al., 1989b; Bautista et al., 1990; Ding et al., 2000) causing metabolic disturbances and cell damage (Bors et al., 1990; Franceschi et al., 1990). It is well established that intracellular calcium levels and kinases control the organisation of microtubules and microfilaments (Li and Aderem, 1992; Bannerman and Goldblum, 1997) producing liver injury during endotoxic shock.

Previous studies have shown that LPS produces, on isolated parenchymal and sinusoidal cells, an increase in intracellular calcium (Portolés et al., 1991), lipid peroxidation (Portolés et al., 1993) and alteration of cell antioxidant status (Ding et al., 2001; Portolés et al., 1994, 1996; Catalá et al., 2002) which can induce damage to the lipid membrane, proteins and nucleic acids, resulting in a severe tissue injury.

This indirect mechanism of action could be responsible for the different sensitivity shown by different cell types, which could be explained considering their functionality. Low doses (10 µg/ml) could act by inducing rapid functional changes in cells with high responsiveness to xenobiotics, like in macrophagic Kupffer cells.

Microfilaments, as previously stated, appeared well preserved in all cases. A marked asymmetric distribution of actin mRNA was localised in the cellular periphery at filopodial and lamellopodila extensions, presumably sites of new actin protein synthesis (Bannerman et al., 1998; Bursten et al., 1991; Chakravorty and Kumar, 2000). This part of the cytoskeleton is also involved in cytosol transport and may be essential for endo and exocytosis processes.

Isowa et al. (1999) showed changes in actin filaments at the cell periphery to be attributed to a disturbed actin gene expression, these changes were reversible and microfilaments need longer times (24 h) than microtubules to be irreversibly damaged. The short times used in our experiments, 15 min maximum, could explain the normal appearance of microfilament patterns in all cases.

Considering the importance of the cytoskeleton disturbances on endotoxic shock, in order to improve the methodology, we used proliferating hepatocytes (Vergani et al., 1984; Thorgeirsson, 1993) and confocal laser scanning microscopy. These liver cells have almost all the functions of parenchymal cells (Vergani et al., 1994) and are particularly interesting for cytoskeletal studies due to their high adhesion to substrate and their sensitivity to LPS. Previous studies have shown that severe alterations were induced with low *E. coli* LPS doses (10 µg/ml) such as the increase in binucleated cells, abnormal mitosis, mitochondrial damage, cytoplasmic vacuolisation and retraction. An alteration in the $[Ca^{2+}]$ influx related to angiotensin was also observed (Vergani et al., 1999).

Their sensitivity to the LPS, similar to Kupffer cells, produces a dramatic microtubule disruption that can be observed by conventional fluorescence microscopy.

Their high LPS response could be due to their origin as epithelial cells (Thorgeirsson, 1993) which makes them more similar to sinusoidal cells. In fact, Isowa et al. (1999) showed that LPS altered microfilaments and microtubules in rat alveolar epithelial cells (macrophages and monocytes). Proliferating liver cells show a high mitotic rate after LPS treatment, well evidenced by immunofluorescence and imaging treatment. The mitosis induction can be related with an LPS-DNA interaction. It has been evidenced that LPS can also reach the nucleus and thus could interact with DNA inducing apoptosis (Lin et al., 2001; Ockner, 2001). A possible explanation for the nuclear damage found in the proliferating liver cells could be the irreversible binding of electrophilic LPS globular aggregates to macromolecules such as DNA and RNA. These phenomena suggest substantial changes in cytoskeletal rearrangement that could be related to the multiorgan dysfunction syndrome (DeMeester et al., 1998).

All these data can be correlated with the LPS interaction with microtubules and microfilaments as a first step of cellular response, especially in Kupffer cells. They could also contribute to the parenchymal cell response, although in these cells the importance of other mediator action to justify all metabolic changes is evident (Pagani et al., 1987).

Although further investigations on LPS action are required to determine primary cell alterations induced by endotoxin, these results prove the contribution of the cytoskeleton to the physiology and pathology of liver parenchymal and sinusoidal cells through a direct or indirect mechanism. Proliferating rat liver cell cultures could provide a specific model that can be used as a suitable tool for a deeper study on cytoskeleton.

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Cytoskeleton alterations induced by LPS

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