Journal of Cellular Biochemistry 79:386–394 (2000)

Binding of *Escherichia coli* Lipopolysaccharide to Fasciculata-Reticularis and Glomerulosa Cells Evaluated by Flow Cytometry

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Abstract Binding of *Escherichia coli* lipopolysaccharide (LPS) to the two cell types of the adrenal cortex: fasciculata-reticularis and glomerulosa cells has been studied by flow cytometry and using fluorescein-labeled lipopolysaccharide (FITC-LPS). The binding characteristics were different in relation to time course and number of binding sites. Both fasciculata-reticularis and glomerulosa cells bound LPS in a specific and saturable process. Fasciculata-reticularis cells showed a higher affinity for LPS binding than glomerulosa cells as deduced from Hill plots. Unlabeled LPS decreased FITC-LPS binding in both fasciculata-reticularis and glomerulosa cells, suggesting competition of both ligands for a limited number of binding sites. Lipid A seemed not to be essential for binding of LPS to fasciculata-reticularis cells. However, serum constituents inhibited FITC-LPS binding to both cell types, possibly due to cell interaction with HDL. The exposure of cells to LPS during cell culture did not modify the number of binding sites, but revealed cell size and surfaces structure changes. J. Cell. Biochem. 79:386–394, 2000.

Key words: lipopolysaccharide; LPS; endotoxin; binding; fasciculata-reticularis cells; glomerulosa cells; adrenal cortex; flow cytometry

Lipopolysaccharide (LPS, endotoxin) is a major component of the outer membrane of Gram-negative bacteria and plays an important role in the pathogenesis of endotoxic shock and development of multiple organ system failure. It is generally assumed that endotoxin must interact with cellular surfaces before it can modulate cellular behaviour. The LPS molecule interacts with and stimulates a variety of host cells, some of which subsequently produce the potentially lethal mediators of endotoxic shock. Since lipopolysaccharides of different bacterial origins have similar endotoxic activities, it has been proposed that the biological activation of the target systems may occur via a receptor recognizing specific LPS components, such as lipid A and the inner core, which represent the most conserved parts of LPS molecule. Recent studies have confirmed the presence of membrane localized LPS binding proteins on a variety of mammalian cells using different techniques: radioligand assay [Shnyra and Lindberg, 1994; Xu et al, 1995], photoaffinity cross-linking [Lei and Morrison, 1988a; Sancho-Tello et al., 1992], immunogold labeling [Municio et al., 1990; Kriegsmann and Bräuer, 1993], and flow cytometry [Heine et al., 1994; Pedron et al., 1994]. Several LPS-binding cell surface components have been detected: CD14, a 55kDa cell-surface glycoprotein [Wright et al., 1990]; CD11c/CD18, a leukocyte integrin [Wright et al., 1989]; proteins with different molecular weights, 80 kDa in murine splenocytes and granulosa cells [Lei and Morrison, 1988b; Sancho-Tello et al., 1992], 95 kDa in macrophage-like cell lines and in human blood monocytes [Hampton et al., 1988], 73 kDa in human leukocytes [Halling et al., 1992], 11 kDa in hemocytes of *Bombix mori* [Xu et al., 1995]; lectin-like binding sites on human monocytes and on rat hepatocytes [Couturier et al., 1991; Parent, 1990]; scavenger receptor

Grant sponsor: DGICYT; Grant number: MEC PB94-0244; Grant sponsor: Multidisciplinary Grant from Universidad Complutense; Grant number: PR 218/94-5677.

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Received 23 September 1999; Accepted 17 March 2000

[Shnyra and Lindberg, 1994; Hampton et al., 1991]. In addition, there are soluble LPS binding proteins such as LBP [Tobias et al., 1988], BPI [Marra et al., 1992], septin [Wright et al., 1992], and sCD14 [Pugin et al., 1993].

In spite of advances in the understanding of LPS binding to blood and related cells, less is known about the binding sites in other types of mammalian cells. The adrenal gland plays an important role in the adaptive response to stressors, such as infection and sepsis. Adrenalectomy increases the organism's sensitivity to endotoxins whereas glucocorticoid treatment can often prevent endotoxin's lethal effects [Hinshaw et al., 1985]. Glucocorticoids are maximally elevated in experimental models of sepsis [Bosch et al., 1988; Givalois et al., 1994] but there is an impairment in the response to ACTH in adrenocortical cells isolated from endotoxemic rats [Garcia et al., 1990], so, endotoxin may modulate adrenocortical secretion. Furthermore, patients with sepsis and with a blunted cortisol response to ACTH stimulation show a poor prognosis [Aygen et al., 1997]. The adrenal cortex is composed of three zones: an outer zona glomerulosa producing mineralocorticoids and two inner zones, zona fasciculata and zona reticularis, which appear to function as a unit and synthesize mainly glucocorticoids. The aim of this study was to analyze the binding of LPS of *Escherichia coli* 0111:B4 to two cell preparations from adrenal cortex (fasciculata-reticularis and glomerulosa cells) by flow cytometry, using fluorescein isothiocyanate-conjugated LPS (FITC-LPS) as ligand.

MATERIALS AND METHODS

Reagents and Media

Fluorescein-isothiocyanate (FITC)-labeled LPS (7.8 mg FITC/mg LPS) and the unlabeled LPS were from *Escherichia coli* serotype 0111: B4, obtained by phenol-water extraction and were purchased from Sigma Chemical Co. (St. Louis, MO). Collagenase was from Seromed CLS I (Germany) and DNA-ase was obtained from Boehringer-Manheim Diagnostica (Germany). Dulbecco's modified Eagle's medium (DMEM) was from Sigma.

Animals

Adult male Wistar rats (Charles River, Spain) weighing 200–250 g were used in all the experiments. Animals were maintained on a standard diet. Food and water were available *ad libitum.* All handling and procedures were performed in adherence to the CEE (86/609) and Ministerio de Agricultura guidelines (Spain, BOE 223/1988, 265/1990) for care and use of laboratory animals.

Isolation of Glomerulosa and Fasciculata-Reticularis Cells

The adrenal glands were excised. After removing the surrounding fat, the capsule was carefully dissected away from the rest of the gland. The capsular and the inner tissues were used to isolate glomerulosa (Glom) and fasciculata-reticularis (Fas-ret) cells, respectively. The capsular and the inner tissues were minced and dissociated with collagenase (200 U/ml) and DNA-ase (0.1 mg/ml) for 30 min at 37°C on an orbital shaker. After incubation, the dispersion of cells was enhaced mechanically by gently flushing through a sterile syringe. The cells were then filtered through a nylon filter mesh and collected by centrifugation at 310 g 10 min. Red blood cells, which co-isolate with the desired cells (fasciculata-reticularis or glomerulosa), were removed by hypotonic treatment for 30 sec. Then, the cells were centrifuged in DMEM plus 2.5% BSA and washed once with DMEM. Cells were resuspended in DMEM and counted using a hemocytometer. Capsular and inner tissues from 10 rats were pooled to yield a single cell sample per experiment. The viability of isolated cells was assessed by trypan blue exclusion and found to be 80–90%. The purity of each cell type was assessed by optical and electronic microscopy; the inner cell contamination in capsular cell preparations was less than 7%. When cells were isolated to be cultured, all the procedures were performed under aseptic conditons.

Cell Culture

Fasciculata-reticularis cells were resuspended in DMEM suplemented with 25 mM HEPES, 3.7 g/l $NaHCO_3$,10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin and seeded in six-well plates at a density of 1×10^6 cells/well. Media were changed after 18–20 h to remove cell debris and unattached cells and cultures were performed in the absence or presence of LPS (10

and 100 μg/ml) for 5 days at 37°C in a humidified 5% CO_2/air atmosphere. Media were changed every 48 h and after 5 days in culture, cells were detached from culture wells with 0.02% EDTA in PBS. Cells were pooled, centrifuged at 310*g*, resuspended in fresh medium and then, their capacity to bind LPS was studied.

Binding Studies of FITC-LPS

Cells (10^6/ml) were incubated with FITC-LPS (1–100 μg/ml) at 25°C for different times (0–90 min) according to the experiment. Non-specific binding was obtained by incubating the cells in the presence of an excess of unlabeled LPS (1 mg/ml). Specific binding of FITC-LPS was evaluated as the difference between total and non-specific binding. Data were also analyzed by Hill plots and Hill binding constants (K_D) were calculated as the abscissa value where log [B/(B_{max}-B)] = 0.

Flow Cytometry Analysis

The binding of FITC-LPS was determined by flow cytometry analysis using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Propidium iodide (PI, Sigma) was added to all the samples at a final concentration of 0.005% before the analysis, in order to discriminate viable and non viable cells. Simultaneously, the autofluorescence of untreated cells was measured as a reference value. In order to analyze FITC-LPS binding to viable cells, the mean FITC fluorescence of the PI negative population in each sample was determined. At least 10,000 cells were analyzed for each sample and the data were analyzed with the LYSIS II Program (Becton Dickinson).

RESULTS

The interaction of endotoxin with cell plasma membranes is the first step for exerting its biological activity. Serum constituents have been shown to participate in LPS binding to host cells. In order to get optimal conditions for the binding of FITC-LPS to fasciculata-reticularis (Fas-ret) and glomerulosa (Glom) cells, we examined the effect of serum on the binding of FITC-LPS to both cell types. As shown in Figure 1, the amount of cell-associated LPS (expressed as arbitrary units of mean fluorescence) is less in the presence of 10% fetal bovine serum (FBS) in both cell types

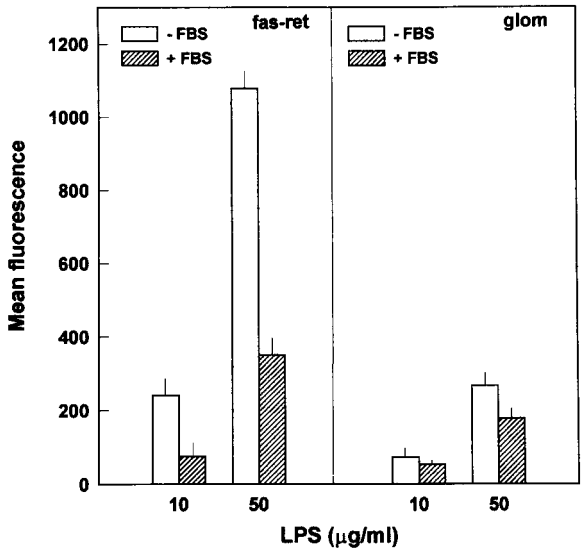

Fig. 1. Effect of serum on FITC-LPS binding to fasciculata-reticularis and glomerulosa cells. Cells were incubated at 25°C with 10 and 50 μg/ml FITC-LPS in the absence or presence of 10% fetal bovine serum (FBS) for 45 min. Results are expressed as mean fluorescence and represent the mean value ± SD of duplicates of one representative experiment.

when they were first incubated with 10 and 50 μg LPS/ml for 45 min, and this effect is more pronounced in Fas-ret cells. Based on these results, all the experiments were performed in the absence of serum.

Fas-ret and Glom cells were incubated with FITC-LPS (10 μg/ml) at 25°C either in the absence or in the presence of a large excess of unlabeled LPS (100-fold exces) and the capacity to bind FITC-LPS was analyzed by flow cytometry. In Figure 2 the fluorescence intensity vs. number of cells, at different times, is shown. In the absence of unlabeled LPS, FITC-LPS progresively bound to both cell types that gradually displayed up to higher fluorescence intensity (Fig. 2A), however, Fas-ret cells were more stained than Glom cells. In the presence of unlabeled LPS there is an inhibition of the binding of FITC-LPS to both cell types (Fig. 2B) and the displacement produced by unlabeled LPS was less marked in Glom cells.

To test the capacity of unlabeled LPS to compete with FITC-LPS binding, Fas-ret and Glom cells were preincubated at 25°C for 20 min with different concentrations of unlabeled LPS. After adding FITC-LPS (10 μg/ml) cells were incubated for 45 min and then analyzed by flow cytometry. The results show that FITC-LPS binding was inhibited by unlabeled LPS in a

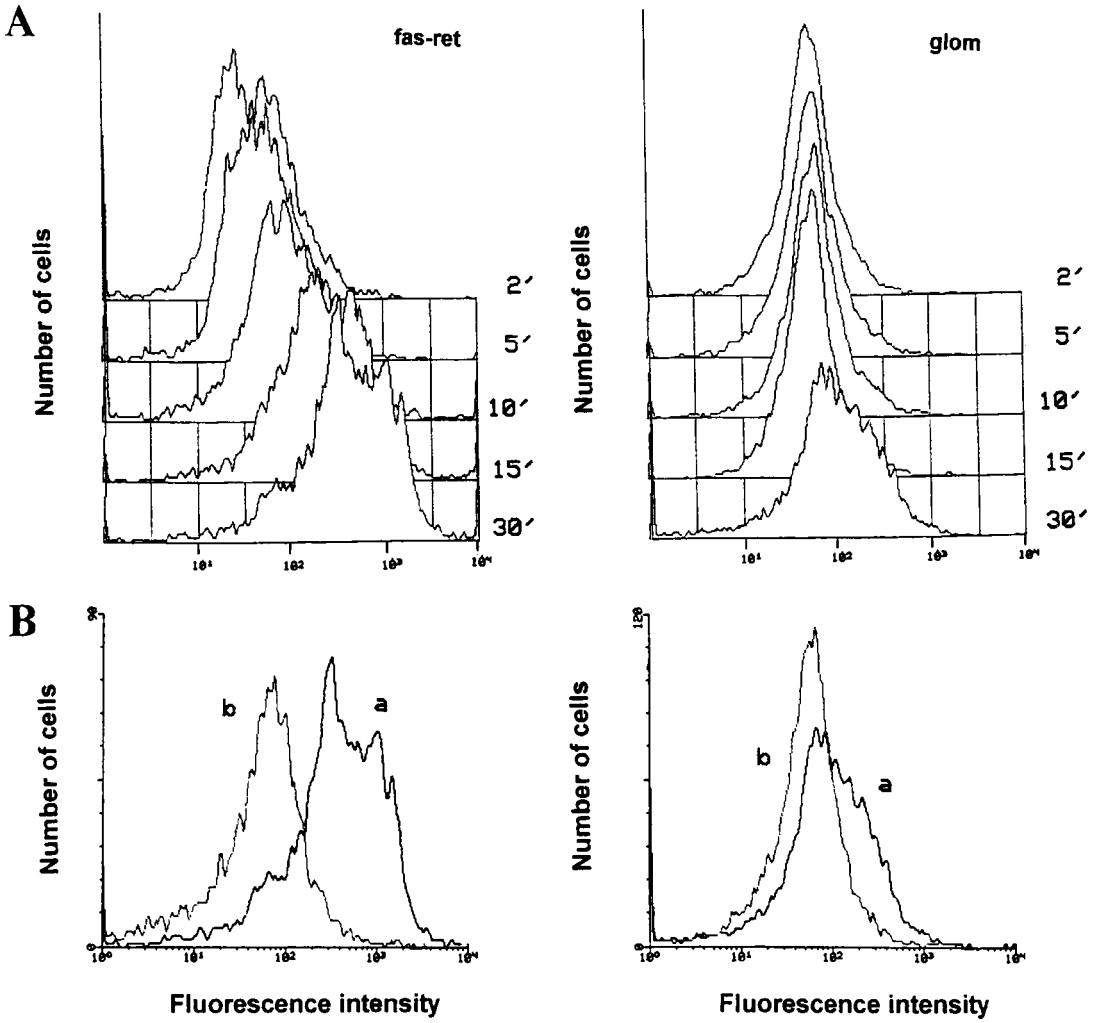

Fig. 2. Representative experiment of a FACS analysis of the kinetic of FITC-LPS binding to fasciculata-reticularis and glomerulosa cells. **A**: Cells were incubated at 25°C with 10 μg/ml FITC-LPS for different times. **B**: Cells were incubated at 25°C with 10 μg/ml FITC-LPS for 30 min in the absence (a) and the presence (b) of unlabeled LPS.

dose-dependent manner in both cell types (Fig. 3), although the inhibition was higher in Fas-ret cells. In some experiments, unlabeled LPS from other serotype (*E. coli* 0127) was used, obtaining analogous effects (data not shown).

In order to determine the kinetics of FITC-LPS binding, Fas-ret and Glom cells were incubated, separately, at 25°C for times ranging from 5 to 90 min with FITC-LPS (10 μg/ml). The non-specific binding was obtained by incubating the cells with a 100-fold excess of unlabeled LPS. Total, specific and non-specific binding are shown in Figure 4. The results are expressed as increase in fluorescence relative to that of untreated cells. FITC-LPS binding to Fas-ret cells (Fig. 4a) was time dependent, increasing over time and reaching a plateau after 60 min. Total, specific, and non-specific binding of FITC-LPS to Glom cells are shown in Figure 4b and behaved in a similar fashion. Binding of FITC-LPS (1–100 μg/ml) by Fas-ret and Glom cells was analized by flow cytometry. Figure 5a shows the specific binding of FITC-LPS (expressed as mean fluorescence) to Fas-ret and Glom cells versus FITC-LPS concentration. Specific LPS binding to both Fas-ret and Glom cells increased in a dose-dependent manner and behaved as a saturable process. Mean fluorescence associated to Fas-ret cells were higher than to Glom cells. The number of cells that bound LPS increased with the doses in both cell types (data not shown). The binding

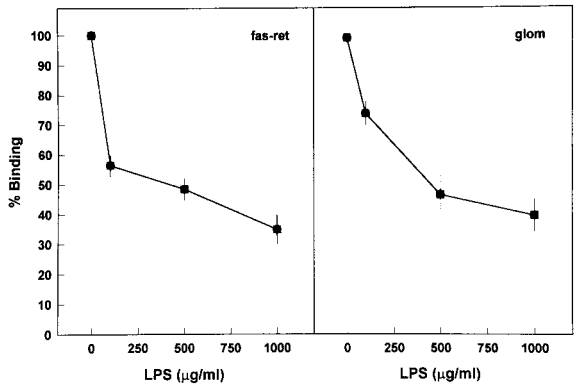

Fig. 3. Inhibition of FITC-LPS binding to fasciculata-reticularis and glomerulosa cells. Cells were incubated at 25°C with 10 μg/ml FITC-LPS for 45 min, in the presence of increasing amounts of unlabelled LPS. Mean fluorescence with FITC-LPS alone was set as 100%. Results are the mean values ± SD of three separate experiments.

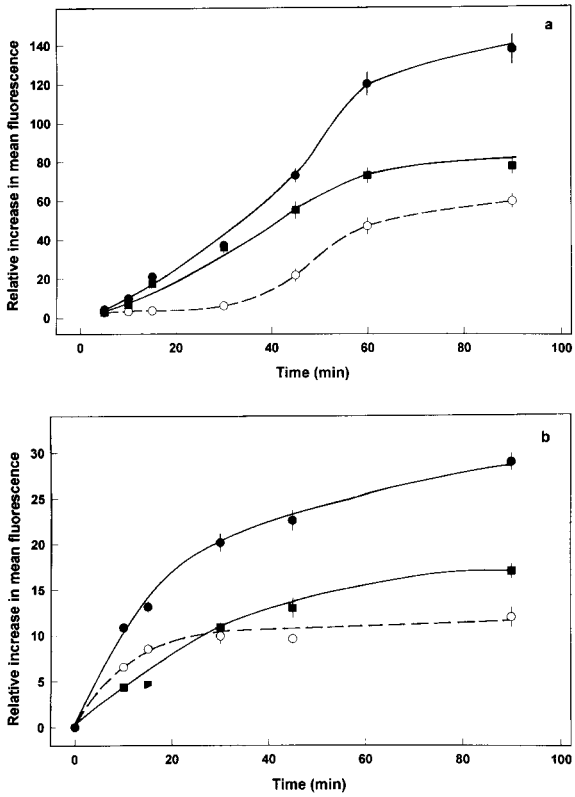

Fig. 4. Kinetics of FITC-LPS binding to fasciculata-reticularis (**a**) and glomerulosa (**b**) cells. Cells were incubated at 25°C with 10 μg/ml FITC-LPS in the presence (○) and absence (●) of an excess of unlabelled LPS (1 mg/ml); (■) specific binding. Binding of FITC-LPS is expressed as increase in fluorescence relative to untreated cells. Results represent the mean value ± SD of duplicate determinations in one representative experiments.

data were also analized by Hill plots (Figs. 5b,c). The Hill binding constants (K_D), calculated as the abscissa value where log [B/(B_{max}-B)] = 0, were 6.3 μg/ml and 8.5 μg/ml for Fas-ret and Glom cells, respectively.

The binding properties of FITC-LPS to both cell types were also studied at 4°C. In these experimental conditions very low levels of bound endotoxin were obtained and the non-specific binding was higher than at 25°C (data not shown), suggesting that metabolically active cells are needed to bind endotoxin.

Modification of LPS binding. Serum inhibited LPS binding to Fas-ret and Glom cells (Fig. 1) and this effect can be due to the interaction of LPS with serum proteins. In order to know the involvement of serum constituents in this inhibition, FITC-LPS binding to Fas-ret cells was carried out in the presence of albumin or HDL (high density lipoproteins). The cells were incubated with FITC-LPS (10 μg/ml) and different concentrations of albumin (0–1,000 μg/ml) or HDL (0–100 μg/ml). The results, expressed as percent of mean fluorescence of untreated cells (Table I), show that albumin inhibited slightly the binding but HDL had a strong inhibitory effect on the binding of LPS to Fas-ret cells. To test for the mechanism of interaction of LPS with the cellular membrane, we examined the effect of polymyxin B (PMB) which binds to LPS through lipid A [Morrison and Jacobs, 1976]. The cells were incubated with FITC-LPS (10 μg/ml) and PMB (0–100 μg/ml) for 45 min. The results (data not shown) of the analysis by flow cytometry indicate that PMB did not modify the FITC-LPS interaction with Fas-ret cells.

FITC-LPS binding to cultured cells. Fas-ret cells were cultured either in the absence or in the presence of LPS (10 and 100 μg/ml) for 5 days and after detaching, cells were incubated with FITC-LPS (10 μg/ml) and the binding was analized by flow cytometry as described. The percentage of cells that bind FITC-LPS and the mean fluorescence (a.u.) are shown in Table II. Data for freshly isolated cells are also included.The kinetics of interaction were quite different from that of freshly isolated cells.In cultured cells the number of cells that bind FITC-LPS increased with time, but mean fluorescence reached a plateau at 30 min, and the presence of LPS in the culture medium did not produce any significant modification in the pattern of binding. In addition,

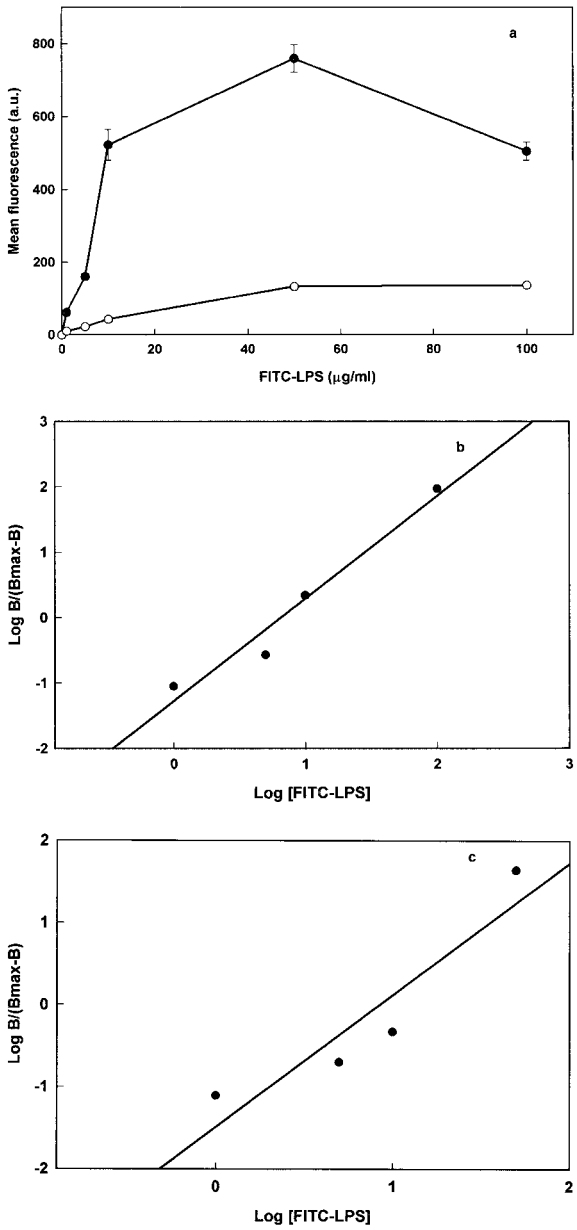

Fig. 5. Dose-response in FITC-LPS specific binding. Fasciculata-reticularis (fas-ret) and glomerulosa (glom) cells were incubated at 25°C with FITC-LPS (1–100 μg/ml) for 45 min in either the absence or presence of unlabelled LPS (1 mg/ml) to evaluate total and non-specific binding respectively. Specific binding of FITC-LPS was evaluated as the difference between total and non specific binding. Data were also analyzed by Hill plots and Hill binding constants (K_D) were calculated as the abscissa value where log [B/(B_{max}-B)] = 0. **a**: Specific saturation binding of FITC-LPS to fas-ret (●) and glom (○) cells. **b,c**: Hill plots of FITC-LPS binding to fas-ret and glom cells, respectively. Hill binding constants were 6.3 μg/ml and 8.5 μg/ml, respectively.

TABLE I. Effect of HDL and Albumin in the Binding of FITC-LPS to Fasciculata-Reticularis Cells*

HDL (μg/ml)	FITC-LPS binding (%)	ALB (μg/ml)	FITC-LPS binding (%)
0	100	0	100
25	94 ± 10	100	109 ± 9
50	75 ± 8	500	89 ± 8
100	60 ± 6	1000	90 ± 9

*Fas-ret cells were incubated with FITC-LPS in the presence of different concentrations of HDL and albumin. Results are expressed as percentage of the binding with FITC-LPS alone and represent the mean value ± SD of three independent experiments.

TABLE II. Binding of FITC-LPS to Cultured Fasciculata-Reticularis Cells*

	FITC-LPS binding cells (%)	Mean fluorescence (a.u.)
Fresh isolated cells		
+ 10 μg/ml FITC-LPS:		
30 min	43	100
45 min	70	240
60 min	85	475
Basal cultures		
+ 10 μg/ml FITC-LPS:		
30 min	76	304
45 min	79	280
60 min	84	281
Cultures with 10 μg/ml LPS		
+ 10 μg/ml FITC-LPS:		
30 min	78	272
45 min	79	259
60 min	82	289
Cultures with 100 μg/ml LPS		
+ 10 μg/ml FITC-LPS:		
30 min	77	257
45 min	79	260
60 min	80	305

*Cells were cultured in DMEM + 10% FBS alone (basal cultures) or in the presence of LPS (10 and 100 μg/ml) for 5 days, then the cells were detached and the subsequent binding of FITC-LPS (10 μg/ml) was analyzed by flow cytometry. The results are expressed as the percentage of cells that bind FITC-LPS and mean fluorescence (arbitrary units). Data represent the mean of duplicates.

the light scattering properties of culture Fas-ret cells were studied by flow cytometry evaluating forward angle light scatter (FSC) and 90° side angle light scatter (SSC). These parameters are related with cell size and cell surface

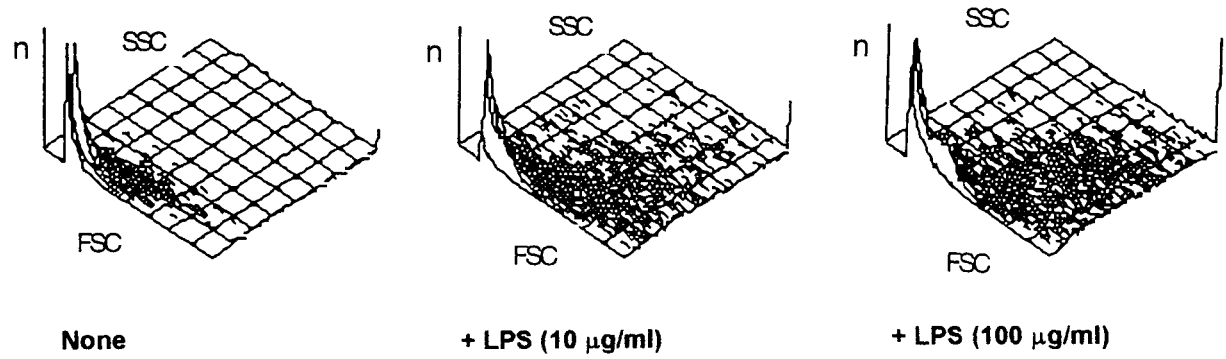

Fig. 6. Histograms of FACS analysis of SSC, FSC, and number of cells. Fasciculata-reticularis cells were cultured in DMEM + 10% FBS alone (none) and in the presence of 10 and 100 μg/ml LPS for 5 days, then the cells were detached, incubated with FITC-LPS (10 μg/ml) and analyzed by flow cytometry.

structures, respectively [Saltzman et al., 1979]. LPS induced an increase in both FSC and SSC and this effect was dose dependent (Fig. 6).

DISCUSSION

In previous studies, using the whole population of cells isolated from the adrenal cortex, we had shown that LPS binds to adrenocortical cells inducing a decrease in ACTH-induced steroidogenesis [García et al., 1985]. Since the adrenal cortex consists of two functionally distinct zones, the outer zona glomerulosa and the inner fasciculata-reticularis, the aim of this study was to characterize the binding of *E. coli* 0111:B4 LPS labeled with fluorescein to both cell types, Fas-ret and Glom cells, by flow cytometry.

The binding characteristics of LPS to both cell types were different in regards to kinetic and number of binding sites. In Fas-rat-cells the interaction was slow at short incubation times, but afterwards, increased quickly up to 70–80 relative mean fluorescence. The time required to get the half maximal incorporation was 40 min. On the other hand, in Glom cells the binding seems to be faster than in Fas-rat cells, half maximal incorporation was obtained after 25 min and the maximal amount of LPS bound was lesser than in Fas-ret cells (Fig. 4). The mean fluorescence (proportional to the number of binding sites/cell) was higher in Fas-ret than in Glom cells (Figs. 1 and 5), thus suggesting a higher number of binding sites in Fas-ret than in Glom cells. The LPS specific binding to both Fas-ret and Glom cells was a saturable process (Fig. 5a). The binding data obtained by flow cytometry were analyzed by Hill plots, as in other previous studies [Catalá et al., 1999], indicating a higher affinity in the LPS binding to Fas-ret cells than to Glom cells. In competition experiments the unlabeled LPS displaced FITC-LPS from its binding sites in a dose-dependent manner, in both cell types, suggesting competition of both ligands for a limited number of common binding sites. These results are in agreement with the data obtained previously with the two populations of adrenocortical cells and (^{14}C)-LPS [García et al., 1985]. However, we have now observed that in Fas-ret cells the displacement was slightly higher than in Glom cells. Analogous percentages of inhibition have been also observed with unlabelled LPS from other serotype (*E. coli* 0127), thus, indicating that lipid A or the inner core, the most conserved parts of the LPS molecule, may be implicated in the LPS binding to Fas-ret and Glom cells. However, the results of the experiments carried out in the presence of polymyxin B seem to suggest that lipid A is not essential in the interaction of LPS with Fas-ret cells, since polymyxin B, that binds to LPS through lipid A [Morrison and Jacobs, 1976], did not inhibit FITC-LPS binding.

The binding of LPS to both type cells was reduced when serum was included in the incubation medium. This inhibition can be due to albumin or HDL as it is shown in Table I, but HDL produced a higher decrease than albumin, possibly because the HDL interaction with its specific receptors in adrenocortical cells impairs LPS binding to plasma membrane. HDL can also bind LPS but longer times

are needed for HDL-LPS complexes can be formed [Van Lenten et al., 1986].

In serum-free conditions, proteins that bind LPS have been described in PMN [Weersink et al., 1994] and in monocytes [Corrales et al., 1993], as well as LPS receptors of low affinity in mouse bone marrow cells [Girard et al., 1997]. The binding to scavenger receptors on Küpffer and endothelial cells is also serum independent [Shnyra and Lindberg, 1994].

In the present study, cultured Fas-ret cells with 10% FBS for 5 days bound FITC-LPS and when cells were cultured for 5 days in the presence of LPS the subsequent binding of FITC-LPS was not modified (Table II). Other authors have described the induction of LPS receptors by treatment of cell cultures with LPS [Chaby et al., 1993; Pedron et al., 1994]. Our results indicate that there are no significant changes in the number of binding sites and mean fluorescence in Fas-ret cells cultured in the presence of LPS (10 and 100 μg/ml) for up to 5 days (Table II). However, when FSC and SSC were studied by flow cytometry, increases in cell size and cell surface structures were observed (Fig. 6). These results could be related to the mechanism of interaction of LPS with plasma membrane, further internalization and localization in subcellular organelles in agrement with our previous studies carried out with Fas-ret cells [Municio et al., 1990], hepatocytes [Diaz-Laviada et al., 1991] and Type II pneumocytes [Risco et al., 1991].

In conclusion, LPS binds to fasciculata-reticularis and glomerulosa cells showing different binding characteristics in both cell types in relation to time course and number of binding sites. Serum constituents (albumin, HDL) inhibit the FITC-LPS binding to both cell types. However, when cultured cells are exposed to LPS in the presence of serum, the pattern of FITC-LPS binding is not modified, indicating that the exposure of Fas-ret cells to LPS did not induce the expression of LPS binding sites.

ACKNOWLEDGMENTS

The authors are particularly grateful to A. Alvarez (Centro de Citometría de Flujo de la Universidad Complutense de Madrid) for his technical assistance. A.E.S. was supported by a predoctoral fellowship from UCM.

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