Lactoferrin inhibits the binding of lipopolysaccharides to L-selectin and subsequent production of reactive oxygen species by neutrophils

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Abstract The activation of leukocytes by lipopolysaccharides (LPS), resulting in the oxidative burst, contributes to the pathogenesis of septic shock. The binding of LPS to L-selectin, which was reported as a serum-independent LPS receptor on neutrophils, induces the production of oxygen free radicals. Human lactoferrin (hLf), an anti-inflammatory glycoprotein released from neutrophil granules during infection, binds to LPS. In this study, we investigated the capacity of hLf to inhibit the L-selectin-mediated activation of neutrophils. Our experiments revealed that hLf prevents the binding of LPS to L-selectin in a concentration-dependent manner. Inhibition was maximum (87.7 ± 0.5%) at a concentration of 50 μg/ml of hLf. Furthermore, hLf inhibited up to 55.4 ± 0.5% of the intracellular hydrogen peroxide production induced by LPS in neutrophils. These findings suggest that the anti-inflammatory properties of hLf are due, at least in part, to their ability to prevent the binding of LPS to neutrophil L-selectin.

Key words: Lactoferrin; L-Selectin; Lipopolysaccharide; Neutrophil; Oxygen free radical

1. Introduction

Bacterial lipopolysaccharides (LPS), the major components of Gram-negative bacteria cell walls [1], are powerful mediators of inflammation. In particular, LPS activate polynuclear neutrophils which, in turn, produce abundant reactive oxygen species [2] important for the killing of ingested microorganisms and for cell-mediated cytotoxicity. Such a defense mechanism is triggered by the plasma LPS-binding protein (LBP) which catalyzes the transfer of LPS to CD14 [3], a glycosylphosphatidyl inositol-anchored molecule present on monocyte macrophages, and to a lesser extent on neutrophils [4,5]. However, at high LPS doses, other pathways participate to the activation of neutrophils, that leads to the overproduction of oxygen free radicals and subsequent damaging of host tissues [6]. The β2-integrin complex, CD11/CD18, has been described as an LPS receptor [7], but its role in the production of reactive oxygen species was not clearly elucidated. Indeed, leukocytes from CD18-deficient patients were able to produce normal levels of superoxide anion in response to LPS, suggesting that CD18 is not essential for mediating such a production [8]. Recently, L-selectin, a cell surface integral membrane glycoprotein involved in leukocytes trafficking, thrombosis and inflammation, was shown to mediate both LPS binding and signal transduction on neutrophils [9,10]. The interaction of LPS with L-selectin is serum- and calcium-independent and induces the production of superoxide and hydrogen peroxide [9–12]. Interestingly, mice deficient in cell surface L-selectin were resistant to lethal doses of LPS in a septic shock model [13].

Lactoferrin (Lf), one of the LPS-binding molecules released from neutrophils during infection [14] could rapidly inactivate LPS, thus limiting the damage of tissues caused by excess production of oxygen radicals. As a matter of fact, Lf was shown to protect mice [15,16] and germ free piglets [17] against lethal doses of LPS. The protective effect may be explained by the ability of Lf to interfere with the LBP/CD14 pathway in neutrophils [18] and monocytes [17,19]. Alternatively, Lf may interfere with one of the LBP/CD14-independent pathways. Up to now, the effect of Lf on the binding of LPS to neutrophil L-selectin has not been investigated.

In this paper, the binding of fluorescent-labelled *Escherichia coli* O55:B5 LPS to isolated human neutrophils under serum-free conditions was analyzed by flow cytometry. The preferential binding of LPS to L-selectin was checked using anti-L-selectin monoclonal antibodies. Then, the effect of human Lf (hLf) on the binding of LPS to L-selectin and subsequent production of oxygen free radicals in neutrophils was studied.

2. Materials and methods

2.1. Materials

Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}, *E. coli* O55:B5 LPS, *E. coli* O55:B5 LPS labelled with fluorescein isothiocyanate (FITC-labelled LPS) and 1,2,3-dihydrorhodamine were purchased from Sigma Chemical Co (St. Louis, MO, USA). Anti-L-selectin and anti-CD24 monoclonal antibodies (IgG1) were obtained from Immunotech (Marseille, France). Anti-CD11b/CD18 antibody (IgG1) was from Dako (Glostrup, Denmark). *Limulus* amoebocyte lysate assay was from BioWhittaker (Walkersville, MD, USA).

2.2. Preparation of hLf

Native hLf was purified from pooled human lactoserum by ion-exchange chromatography as previously reported [20]. Homogeneity of the protein was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Native hLf was 18% iron saturated. All buffers were prepared with pyrogen-free water. Since the interaction between LPS and Lf was abrogated by NaCl concentrations higher than 0.4 M [21], 50 mg of purified hLf were injected on a 7×1 cm SP-Sepharose fast flow column equilibrated in 0.1 M NaCl and then washed with 70 ml of 0.5 M NaCl. Human Lf was eluted with 2 M NaCl and desalted on PD-10 G-25 column equilibrated in 0.1 M NaCl. All buffers were prepared with pyrogen-free water. The LPS contamina-

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Abbreviations: Lf, lactoferrin; hLf, human lactoferrin; LPS, lipopolysaccharides; LBP, LPS-binding protein

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tion of these hLf fractions was less than 50 pg of endotoxin/mg of protein, as estimated by the *Limulus* amoebocyte lysate assay.

2.3. Preparation of neutrophils

Human neutrophils were isolated from human peripheral blood using dextran sedimentation [9]. After 30 min, the plasma layer was removed and centrifuged ($600\times g$, 45 min) in a solution of Ficoll. The supernatant was removed and the pellet treated with a hypotonic lysis buffer to remove red blood cells. The neutrophils were pelleted and resuspended in PBS without Ca^{2+} and Mg^{2+}. Viability was over 96% as determined by trypan blue dye exclusion.

2.4. Binding of LPS to neutrophils

Neutrophils at a concentration of 1.5 10^6 cells/ml were preincubated 30 min at 37°C in PBS containing 10 mM NaN_3, 2 mM NaF and 5 mM deoxyglucose (binding buffer) to prevent LPS internalization [22]. Then, FITC-labelled LPS at concentrations ranging from 0 to 10 μg/ml were added to the cells. Non-specific binding was measured in the presence of a 100-molar excess of unlabelled LPS. After 30 min at 37°C, cells were washed once with PBS and analyzed with a Becton Dickinson FACScalibur flow cytometer. Cells were gated for forward and side angle light scatters and 5000 particles of the gated population were analyzed. The fluorescence channels were set on a logarithmic scale and the mean of fluorescence intensity was determined. The fluorescence mean variations, corresponding to the specific binding of LPS to cells, were calculated after subtraction of the non-specific fluorescence intensity from the total fluorescence intensity.

Monoclonal anti-L-selectin and anti-CD11b/CD18 antibodies were used to assess the binding of LPS to neutrophils in the absence of serum. A monoclonal anti-CD24 antibody which bound to neutrophils without preventing LPS binding was used as a negative control. Cells were resuspended in the binding buffer and incubated for 30 min at 37°C with 0.75 μg/ml of anti-L-selectin, anti-CD11b/CD18 or anti-CD24 antibodies. After one wash with PBS, cells were incubated with FITC-labelled LPS (5 μg/ml) for 30 min at 37°C and analyzed by flow cytometry. Similar experiments were performed with the anti-L-selectin antibody at concentrations ranging from 0 to 1.5 μg/ml.

The binding of FITC-labelled LPS (5 μg/ml) to neutrophils in the presence of 1 to 50 μg/ml hLf was estimated as described above.

2.5. Effect of hLf on the production of oxygen free radicals induced by LPS

1,2,3-Dihydrorhodamine has been described as a probe for detecting changes in the production of reactive oxygen species inside various cells. Indeed, 1,2,3-dihydrorhodamine diffuses in cells and after oxidation by H_2O_2 and O_2^- to 1,2,3-rhodamine, emits a bright fluorescent signal upon excitation by 488 nm [23,24].

Isolated neutrophils were pretreated with 1,2,3-dihydrorhodamine (3 μg/ml) in PBS for 10 min at 37°C and then stimulated 15 min at 37°C by 100 μg/ml of *E. coli* O55:B5 LPS as previously described [9]. In some experiments, LPS was preincubated for 30 min at room temperature with native hLf at concentrations ranging from 1 to 50 μg/ml. The cells were stored on ice prior to flow cytometry analysis. A control was made in similar conditions but in the absence of both LPS and hLf. The fluorescence channels were set on a logarithmic scale and the means of fluorescence intensities were determined. The results were expressed as percentages of activation considering the fluorescence mean obtained with LPS alone as 100%.

2.6. Statistical analysis

Data are presented as the mean ± standard error of the mean (S.E.M.) for the indicated number of independent experiments. Statistical significance was analyzed with a Student's *t*-test for unpaired data. Values with $P < 0.05$ were considered to be significant.

3. Results

3.1. Binding of LPS to neutrophils

After incubation with 5 μg/ml of FITC-labelled *E. coli* LPS in the absence of serum, neutrophils exhibited a high fluorescence intensity (mean = 21.4 ± 0.5) which was significantly decreased by addition of a 100-molar excess of unlabelled LPS (mean = 3.8 ± 1) (Fig. 1A). This result demonstrates that

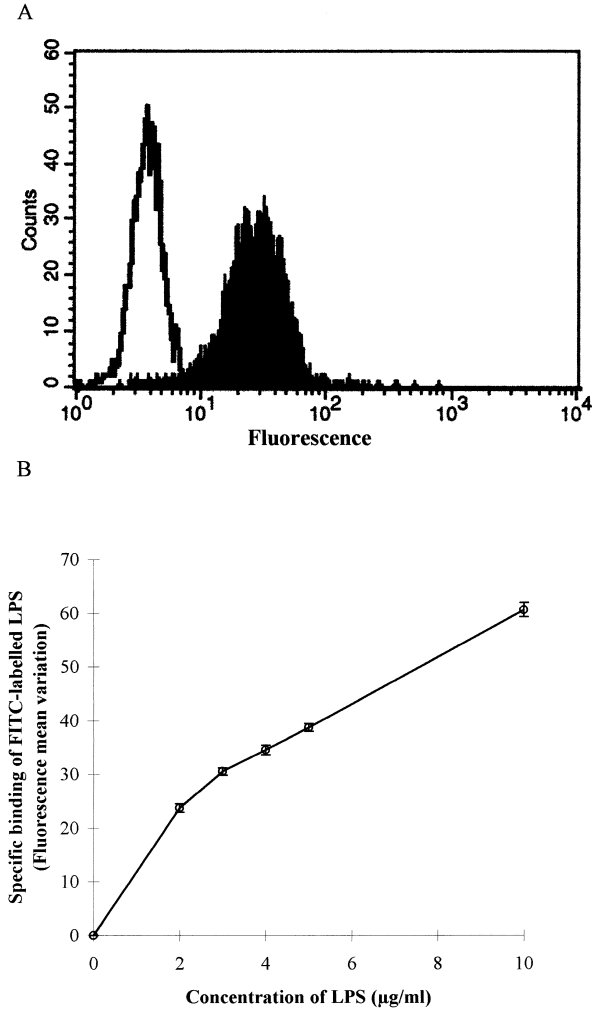

Fig. 1. Binding of LPS to neutrophils. A: Freshly isolated human neutrophils were incubated with 5 μg/ml of FITC-labelled LPS at 37°C for 30 min in the binding buffer (shaded curve). The non-specific binding was measured in the presence of a 100-molar excess of unlabelled LPS (unshaded curve). B: Human neutrophils were incubated with various concentrations of FITC-labelled LPS as described in Section 2. The intensity of fluorescence associated with the cells was measured. Data are expressed as fluorescence mean variations ± S.E.M. from triplicates and are representative from three separate experiments conducted with neutrophils from different donors.

E. coli O55:B5 LPS specifically bound to neutrophils in the absence of serum LBP. Furthermore, LPS binding was concentration-dependent and saturable from about 3 μg/ml LPS (Fig. 1B).

As shown in Fig. 2A, anti-L-selectin antibodies inhibited 67.3 ± 1.7% of the total LPS binding whereas CD11b/CD18 only suppressed 22.0 ± 3.7% of this binding (Fig. 2A). A control experiment performed with anti-CD24 antibodies which recognize cell surface of neutrophils did not interfere with the LPS binding. Such an inhibitory effect was concentration-dependent and up to 87 ± 1.2% inhibition of the total LPS binding was obtained in presence of 1.5 μg/ml of anti-L-selectin antibodies (Fig. 2B). These experiments clearly indicate that L-selectin is the major LBP-independent neutrophil site for LPS binding. Our results are consistent with previous published data [9].

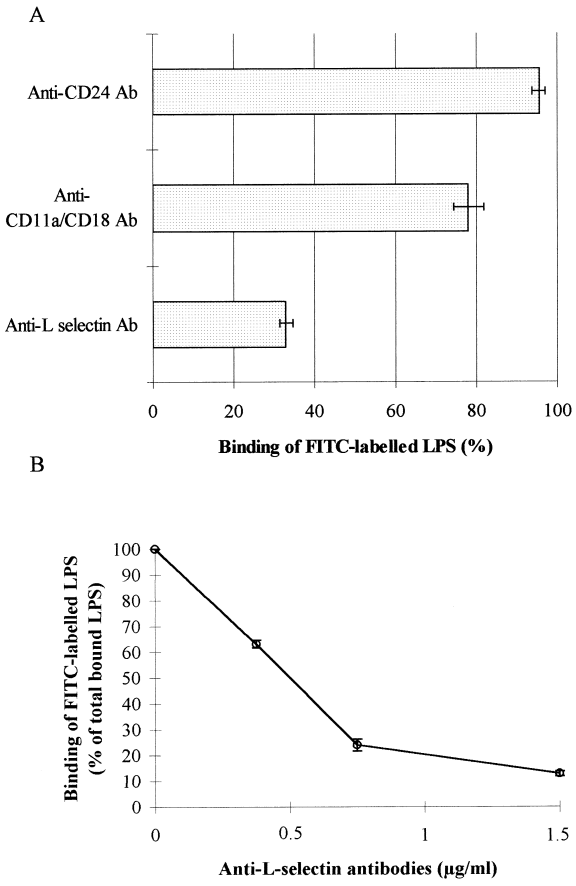

Fig. 2. Inhibition of LPS binding to neutrophils by various antibodies. As described in Section 2, neutrophils were preincubated with antibodies for 30 min before addition of 5 μg/ml of FITC-labelled LPS. A: Preincubations were performed with 0.75 μg/ml of anti-CD24, anti-CD11b/CD18 or anti-L-selectin antibodies. B: Various concentrations of anti-L-selectin antibodies were used. The results are expressed as percentages ± S.E.M. from triplicates of the total LPS bound to cells without antibodies. Data are representative from three separate experiments.

3.2. Effect of hLf on the LPS binding to neutrophils

The binding of LPS to neutrophils in the presence of hLf was studied. As shown in Fig. 3, hLf decreased the binding of FITC-labelled LPS to cells in a concentration-dependent manner. The inhibition of LPS binding reached 82.2 ± 1% and 87.7 ± 0.5% in the presence of 20 and 50 μg/ml hLf, respectively. Such an inhibition rate was previously observed with 1.5 μg/ml anti-L-selectin antibody (87.0 ± 1.2%) (Fig. 2B). This result suggests that hLf, in a similar way to anti-L-selectin antibody, impeded the binding of LPS to L-selectin.

3.3. Effect of hLf on the LPS-induced production of reactive oxygen species

As previously described [9], LPS stimulates the production of hydrogen peroxide in neutrophils. Fig. 4 shows that such a production was inhibited in a concentration-dependent manner by native hLf. More particularly, 20 and 50 μg/ml hLf caused 36.7 ± 7.2% and 55.4 ± 0.5% inhibitions of the intracellular hydrogen peroxide production, respectively. A control was performed with cells pretreated with 1,2,3-dihydrorhodamine but not stimulated with LPS (data not shown). This result shows that hLf was able to inhibit the LPS-induced production of reactive oxygen species in neutrophils.

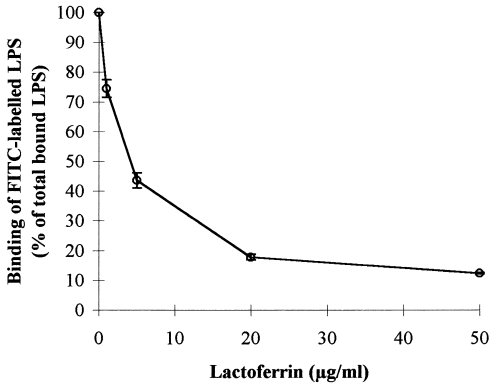

Fig. 3. Inhibition of LPS binding to neutrophils by hLf. Cells were incubated with 5 μg/ml of FITC-labelled LPS in the presence of increasing concentrations of hLf as described in Section 2. The results are expressed as percentages ± S.E.M. from triplicates of the total LPS bound to cells without hLf. Data are representative from three separate experiments.

4. Discussion

The oxidative burst of neutrophils contributes to the pathogenesis of the septic shock. Lactoferrin, an LPS-binding glycoprotein with anti-inflammatory properties, is released from neutrophils during infection. The protective effect of Lf against the septic shock was shown through in vivo experiments [15,16] but its mechanism(s) of action was (were) not clearly elucidated. Wang et al. [18] have shown that hLf inhibited the release of superoxide from neutrophils induced by low amounts of LPS using the LBP/CD14 pathway. More recently, Malhotra et al. [9] reported the importance of L-selectin in triggering the oxygen free radical production in neutrophils at high LPS concentrations, i.e. in septic shock conditions. We confirm here that L-selectin is the major LBP-independent LPS-binding site on neutrophils. Furthermore, we demonstrate that hLf, at serum concentrations encountered in pathological conditions (up to 20 μg/ml) [25], impedes the binding of LPS to L-selectin on neutrophils and

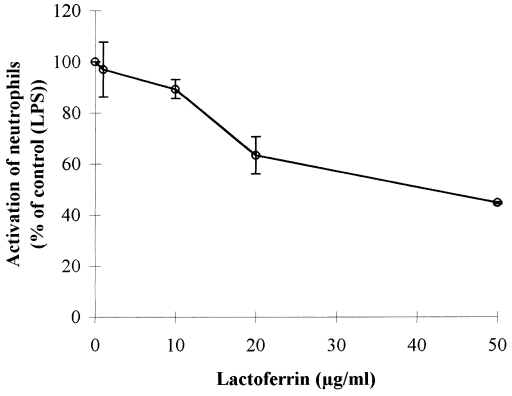

Fig. 4. Inhibition of LPS-induced reactive oxygen species production in neutrophils by hLf. Isolated neutrophils, loaded with 1,2,3-dihydrorhodamine, were incubated for 15 min at 37°C with LPS preincubated or not with various concentrations of hLf. The percentage of activation was determined from the activation with LPS without hLf. Data are expressed as percentages ± S.E.M. from triplicates and are representative from three separate experiments conducted with neutrophils from different donors.

hence decreases the intracellular production of oxygen radicals.

The LPS-binding properties of hLf are likely to explain such a biological effect. As a matter of fact, hLf binds to the lipid A with a high affinity (K_d = 2–5 nM) [26]. Two basic regions involving amino acid residues ^{2}RRRR^{5} and ^{28}RKVRGPP^{34} of hLf have been identified as a recognition site for anionic molecules such as LPS [21,27]. In the C-type lectin domain of L-selectin, the basic sequence ^{84}KKNKE^{88} was proposed as the binding site for negatively charged phospholipids [28]. This site is distinct from the carbohydrate-binding site [28]. Though the affinity of L-selectin for LPS was not reported [10], it is likely that the high affinity interactions of hLf with LPS impede further binding of LPS to L-selectin.

In conclusion, our results show that hLf competes with L-selectin for LPS binding and downregulates the production of oxygen radical species induced by high LPS concentration levels in neutrophils. They shed light on one of the negative feedback mechanisms of inflammation by which the organism is able to prevent tissue damage following infection.

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