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Evidence of a trimolecular complex involving LPS, LPS binding protein and soluble CD14 as an effector of LPS response

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Abstract The kinetics of the interaction of lipopolysaccharide (LPS), lipopolysaccharide binding protein (LBP) and CD14 was studied using surface plasmon resonance. The association and dissociation rate constants for the binding of LPS and rsCD14 were 2.9×10^4 M^{-1} s^{-1} and 0.07 s^{-1} respectively, yielding a binding constant of 4.2×10^5 M^{-1}. Significantly, the presence of LBP increased not only the association rate but also the association constant for the interaction between LPS and CD14 by three orders of magnitude. Our experimental results suggest that LBP interacts with LPS and CD14 to form a stable trimolecular complex that has significant functional implications as it allows monocytes to detect the presence of LPS at a concentration as low as 10 pg/ml or 2 pM, and to respond by secreting interleukin-6. Thus, LBP is not merely transferring LPS to CD14 but it forms an integral part of the LPS–rLBP–rsCD14 complex.

Key words: Surface plasmon resonance; Lipopolysaccharide; Lipopolysaccharide binding protein; CD14

1. Introduction

The importance of innate immunity in recognizing microbial pathogens and mounting a response against them is now widely recognized. Bacterial endotoxin, lipopolysaccharide (LPS) of gram-negative bacteria, has been a model in the study of the innate immunity [1,2]. LPS plays a pivotal role in the septic shock syndrome in humans owing to its profound effects on the innate immune system. Septic shock is characterized by fever, hypoxia, hypoglycemia, acidosis, thrombocytopenia, hypotension, which often leads to multiple organ failure/dysfunction syndrome [3]. There have been various studies into the molecular basis of the septic shock syndrome that clearly demonstrate that LPS itself is not toxic. It is the ability of LPS to interact with and stimulate a variety of target cells that subsequently produce the potentially lethal mediators of septic shock [4]. The proinflammatory cytokines, including tumor necrosis factor (TNF)-α [5], interleukin (IL)-1β, IL-6 [6], IL-8 and IL-12 and interferon-γ, play a critical role in the inflammatory response [7].

CD14 and LPS binding protein (LBP) are known to play important roles in the pathway leading to the endotoxic shock caused by LPS. Blockage of LBP with antibodies or experiments using CD14 knockout mice resulted in prevention of LPS-induced shock [8,9]. CD14 is a 55-kDa glycoprotein that is present in a soluble form (sCD14) in blood and in a glycosylphosphatidyl-anchored, membrane-bound form (mCD14) on the surface of monocytes, macrophages and polymorphonuclear leukocytes (PMN). LBP, a 60-kDa serum acute phase protein, has been described as a lipid transfer molecule that catalyzes movement of phospholipids, in particular LPS monomers, from LPS aggregates to lipoproteins or to CD14 [10].

It has been shown earlier that LPS can directly bind to cells transfected with mCD14 [11], to monocytes [12] and to PMN [12] as well as to sCD14 [12–15]. LPS–sCD14 complexes activate endothelial or epithelial cells and U373 astrocytoma cells, which do not express mCD14 [12,14,16], and also enhance LPS stimulation of monocytes [14], macrophages [15], or PMN [13], even though these three cell types express mCD14. In the latter instances there seems to be a cooperative action of sCD14 and mCD14 whereby sCD14 delivers LPS to mCD14 [15]. LBP on its own is unable to trigger an LPS response [12]. However, LBP can catalytically transfer monomeric LPS from LPS aggregates onto mCD14 or sCD14 molecules and thus greatly enhance CD14-mediated responses to LPS [10,13,17,18]. Activation of cells via sCD14 occurs by binding of sCD14 to a yet undefined signal molecule, which is found on monocytes, endothelial cells and some epithelial cells [12]. Whether LPS is already bound to sCD14 or subsequently binds to mCD14, CD14 is required to bring LPS into close proximity to the toll-like receptor 4 (TLR4) [19] and to transfer it to the TLR4–MD-2 complexes present at the cell surface [20]. Although earlier studies give an insight into the interaction between the major players of endotoxic shock, there is still a need to quantitatively ascertain the parameters that govern these interactions. In particular how LBP increases the cellular responses of LPS has so far remained elusive.

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Abbreviations: LPS, lipopolysaccharide; LBP, LPS binding protein; sCD14, soluble CD14; mCD14, membrane-bound CD14; rsCD14, recombinant soluble CD14; SPR, surface plasmon resonance; RU, response units; k_1, association rate constant; k_{-1}, dissociation rate constant; K_a, association constant (k_1/k_{-1}); K_{eq}, equilibrium binding constant

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In this study, using surface plasmon resonance (SPR) we demonstrate that while the affinity of LPS for recombinant sCD14 (rsCD14) is intrinsically low, it increases by three orders of magnitude when present in complex with rLBP. The increase in affinity is largely related to an increase in the association rate constant indicating that LBP–LPS complex presents the determinants optimally for the recognition of CD14. The functional consequences of these results are complemented by analysis of IL-6 secretion by monocytes cultured under the influence of LPS in the presence or absence of rsCD14 and/or rLBP.

2. Materials and methods

2.1. Media and reagents

Monocyte cultures were established in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 20 mM L-glutamine (Life Technologies, Paisley, UK) and 1% human serum depleted of sCD14 and LBP (ELISA tests detected 10 ng/ml sCD14 and 2 ng/ml LBP after depletion) (Biometec, Greifswald, Germany). Recombinant human endotoxin-free sCD14 and rLBP were obtained from Biometec. LPS from *Escherichia coli* strain O55:B5 (Sigma) was repurified as described earlier [21].

2.2. SPR analysis

LPS samples were prepared and quantified using the endotoxin from Endosafe™, as a standard, by the *Limulus* amebocyte lysate assay in pyrogen-free water according to the method of Yin et al. [22], prior to all the assays. Biospecific interaction analysis was performed using a BIAcore 2000 biosensor system (Amersham Pharmacia Biotech, Uppsala, Sweden). For the analysis of rLBP–LPS interaction, LBP was covalently immobilized on a certified grade CM5 sensor chip (carboxymethylated) at a concentration of 40 μg/ml in 10 mM sodium acetate buffer, pH 4.8, using the amine coupling kit supplied by the manufacturer. Nearly 1500 resonance units (RU) of the protein were immobilized under these conditions, where 1 RU corresponds to immobilized protein concentration of ~1 pg/mm^2. The unreacted moieties on the surface were blocked with ethanolamine. All measurements were carried out in 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA. LPS was flowed over these surfaces at concentrations of 6, 8, 12, 20, 25, 35 nM at 25°C, in HEPES buffer at a flow rate of 10 μl/min in order to determine the association rate constant. For the determination of dissociation rate constant, the same buffer was passed at a flow rate of 10 μl/min. The surfaces were regenerated by a 10-s pulse of 10 mM glycine–HCl (pH 4.2) flowing at 50 μl/min. These surfaces have greater than 90% reproducibility if used in quick succession, i.e. within 24 h.

Recombinant sCD14 was also covalently immobilized on a certified grade CM5 sensor chip using the amine coupling kit at a concentration of 40 μg/ml. Nearly 1500 RUs of the protein were immobilized under these conditions. LPS (40, 55, 75, 100 nM) was passed over the immobilized rsCD14 to study the interaction of LPS and rsCD14. Also, LPS (200 nM) was mixed with increasing concentrations of rLBP (5, 10, 15, 25, 80, 140 nM) and passed over rsCD14 in order to study the interaction of LPS–rLBP complex with rsCD14. For the determination of association rate constant (k_1), LPS or the LPS–rLBP complex, as the case may be, was used at a flow rate of 10 μl/min, in HEPES buffer. Dissociation rate constant (k_{-1}) was evaluated subsequently by passing the same buffer at a flow rate of 10 μl/min. Also, as a control, rLBP alone was passed over immobilized rsCD14 and the data so obtained were subtracted from those obtained upon passing the complex of rLBP and LPS over immobilized rsCD14.

One of the surfaces was activated and blocked by ethanolamine. This was used as a control to negate the effect of any interaction between LPS, rLBP or rsCD14 with the ethanolamine used during the immobilization procedure. In all the cases there was no detectable increase in RUs upon passage of LPS, rLBP or rsCD14 over the control surface.

2.3. Data analysis

Rate constants k_1 and k_{-1} were obtained by fitting the primary sensorgram data using the BIA evaluation 3.0 software. The dissociation rate constant is derived using:

$$R_t = R_{t_0}\, e^{-k_{-1}(t-t_0)} \quad (1)$$

where R_{t_0} is the amplitude of the initial response, and k_{-1} is the dissociation rate constant. The association rate constant k_1 can be derived from the measured k_{-1} values, using:

$$R_t = R_{\max}\,[1-e^{-(k_1 C+k_{-1})\,(t-t_0)}] \quad (2)$$

where R_t is the response at time t, $R_{\max}$ is the maximum response, C is the concentration of the analyte in the solution, and k_1 and k_{-1} are the association and dissociation rate constants, respectively. The ratio of k_1 and k_{-1} yields the value of association constant K_a (k_1/k_{-1}). The parameters obtained from the binding interaction of the ligands with the protein on the surface of the chip were also plotted as per Scatchard analysis [23].

2.4. Monocyte culture and analysis of conditioned medium

Monocytes, obtained from normal healthy volunteers with their informed consent, were isolated by continuous flow centrifugation leukapheresis and counterflow centrifugation elutriation and cultured as in [24]. This technique yields monocytes with a 95–98% degree of purity, as judged by flow cytometry using the surface marker CD14. Monocytes were maintained in a humidified atmosphere, at 37°C, in the presence of 5% CO_2, in RPMI 1640 supplemented with 20 mM L-glutamine and 1% human serum, depleted of sCD14 and LBP and in the presence or absence of either LPS (5, 15, 30 or 60 pg/ml), and/or 2 μg/ml rsCD14, and/or 1 μg/ml rLBP. The final concentrations of CD14 and LBP as present in each assay system were less than 0.1 ng/ml and 0.05 ng/ml which was 0.005% of the extraneously added CD14 and LBP. Also, rsCD14 and LBP used in the assays were tested for endotoxin by Biometec and the endotoxin content was less than 0.1 ng/ml. In addition to this we also had the products tested by the endotoxin research laboratories of the Octapharma pharmaceutical company, which also could not detect endotoxin contamination. Monocytes (2×10^6 cells/ml/well) were cultured in 24-well plates (Nunc, Roskilde, Denmark). After incubation of the monocytes with rLBP, LPS or rsCD14 for 24 h, culture supernatants were harvested and IL-6 secretion was determined by sandwich ELISA according to the manufacturer's instructions (Immunotech, Marseille, France).

3. Results and discussion

SPR is a rapid method for evaluating the elementary steps of a reaction between a macromolecule and its complementary ligand as well as the affinities involved therein, as proven by the wealth of data in the literature [25–27]. Real-time biomolecular interaction analysis (BIA) relies exclusively on the mass change, without the need for labeling any of the interactants, which can sometimes alter the nature of the reaction. Also, this technique provides data for both the association and dissociation phases of a reaction in a single experimental run.

3.1. Analysis of the interaction of rLBP and LPS

The interaction of rLBP and LPS yielded k_1, k_{-1} and K_a values of 1.23×10^6 M^{-1} s^{-1}, 4.26×10^{-3} s^{-1} and 2.88×10^8 M^{-1}, respectively (Fig. 1A). The equilibrium binding constant (K_{eq}) calculated from the Scatchard plot was 1.4×10^8 M^{-1} (Fig. 1B). These values are in accordance with earlier studies utilizing indirect approaches such as ELISA and competitive binding reactions via fluorescently labeled polymyxin B, an extensively investigated ligand for LPS, reporting values of K_d for LPS–LBP interaction in the nanomolar range (i.e. $K_b = 1.25\times10^8$ M^{-1}) at 20°C [1]. Ulevitch et al. report a value of 2.85×10^8 M^{-1} for the same interaction [18].

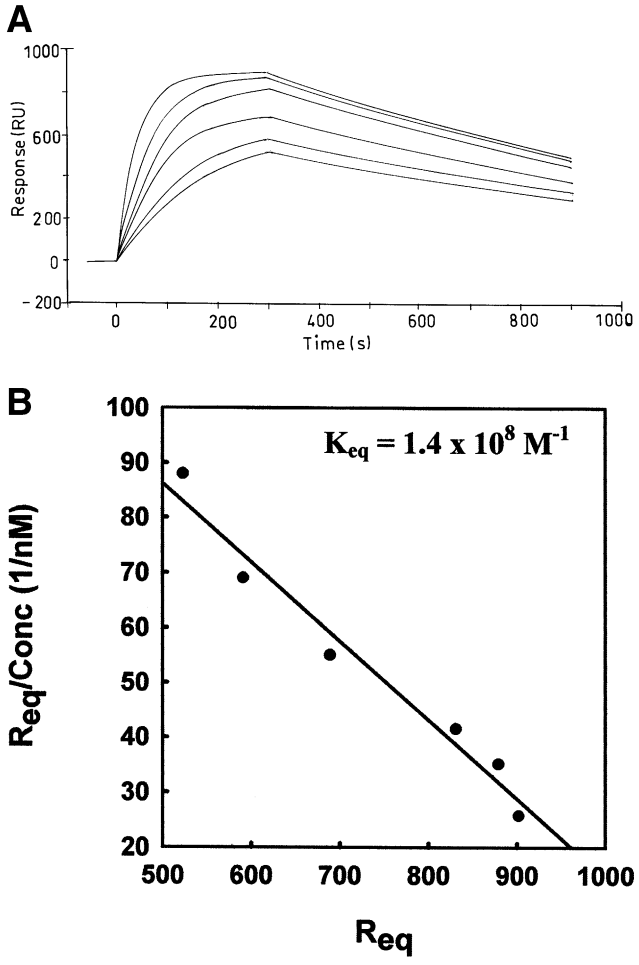

Fig. 1. A: Overlay plot of sensorgrams depicting the interaction of LPS with LBP. LBP was immobilized on CM5 sensor chip and LPS at concentrations of 6, 8, 12, 20, 25, 35 nM, from bottom to top, dissolved in 10 mM HEPES buffer, containing 150 mM NaCl, 3.4 mM EDTA, pH 7.4, was passed at a flow rate of 10 μl/min. The dissociation was studied subsequently by passing the same buffer at a flow rate of 10 μl/min. The surfaces were regenerated by a 10-s pulse of 10 mM glycine–HCl (pH 4.2) flowing at 50 μl/min. B: Scatchard plot analysis of the sensorgram data yielding a K_{eq} of 1.4×10^8 M^{-1}.

3.2. Analysis of the interaction of LPS and LPS–rLBP complex with immobilized rsCD14

The association and dissociation rate constants for the binding of LPS to immobilized rsCD14 were, respectively, 2.9×10^4 M^{-1} s^{-1} and 0.07 s^{-1} yielding a K_a value of 4.2×10^5 M^{-1} (Fig. 2A). The equilibrium binding constant was calculated as 9.8×10^5 M^{-1} (Fig. 2B). Next, a constant amount of LPS was incubated with increasing concentrations of rLBP and the complex so formed was passed over immobilized rsCD14. The amount of rLBP complexed under these conditions was calculated and used for the kinetic and Scatchard analysis of the data (Fig. 3). The data for the binding of LPS–rLBP complex were obtained after subtracting the response observed upon passing equivalent amounts of free LPS and free LBP over immobilized rsCD14. k_1 for the interaction of LPS–rLBP complex with immobilized rsCD14 was much more rapid than the one observed for the binding of LPS to rLBP and LPS to rsCD14. The association rate constant for the interaction of rLBP–LPS complex to rsCD14 was calculated as 1.3×10^7 M^{-1} s^{-1} whereas the dissociation rate constant was observed to be 0.06 s^{-1}, giving the value of overall binding constant of 2.16×10^8 M^{-1}. Scatchard analysis of the SPR data yielded a K_{eq} of 1×10^8 M^{-1}. The binding constants so obtained clearly demonstrate the role of LBP in the interaction of LPS to rsCD14.

LPS in complex with LBP binds mCD14, on the surface of responsive cells such as monocytes and macrophages. However, it is not yet resolved whether this interaction requires a trimolecular complex between LPS, LBP and CD14. Indeed, Hailman et al. [13] were unable to observe, by native polyacrylamide gel electrophoresis, a stable complex of rLBP with rsCD14 and LPS while Gegner et al. [28] have reported that LPS forms a ternary complex with LBP and mCD14 at the cell surface.

Our studies demonstrate that LBP perhaps orients LPS in a manner that favors its recognition by CD14 (Figs. 3 and 4). The agreement between kinetically determined values of association constants (k_1/k_{-1}) and those determined by Scatchard analysis of the SPR data suggests that the association and dissociation reactions are monoexponential in nature and describe faithfully the energetics of the system. The observation of a monoexponential phase for the interaction between LPS–

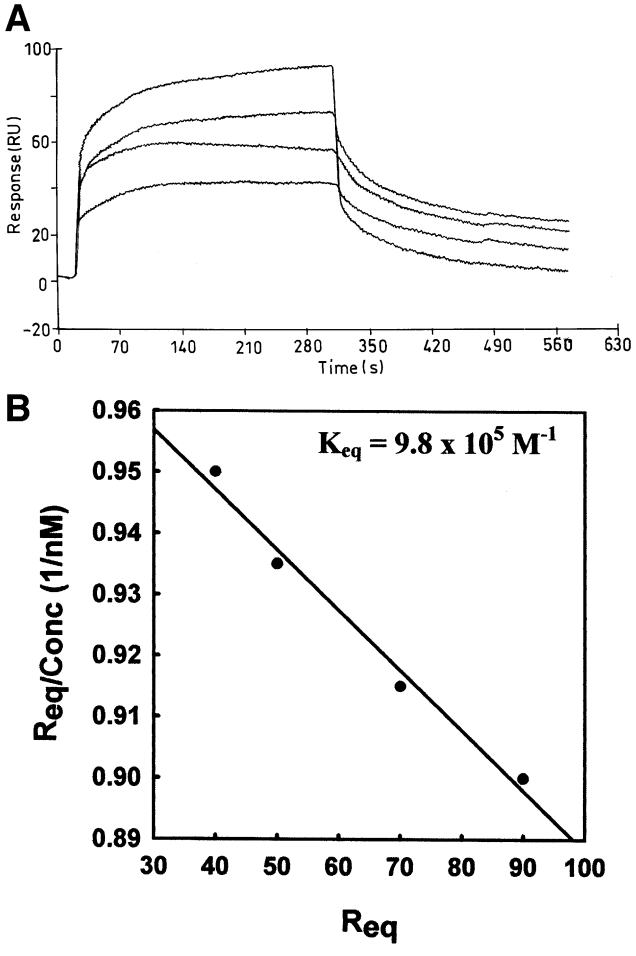

Fig. 2. A: Interaction of rCD14 with LPS. RsCD14 was immobilized on CM5 sensor chip using the amine coupling kit and the surface blocked by using ethanolamine. LPS was passed over this at concentrations of 40, 55, 75, 100 nM at a flow rate of 10 μl/min. B: Scatchard plot analysis of the binding data with a K_{eq} of 9.8×10^5 M^{-1}.

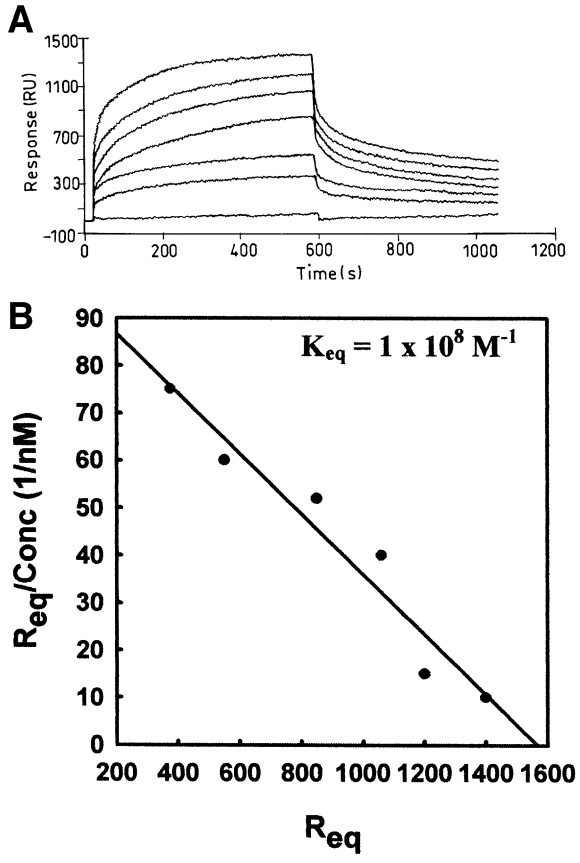

Fig. 3. A: Interaction of LBP–LPS complex with immobilized rCD14. LPS (200 nM) was mixed with increasing concentrations (5, 10, 15, 25, 80, 140 nM; second sensorgram from bottom to top) of rLBP and passed over immobilized rsCD14 at a flow rate of 10 μl/min. rLBP alone (first sensorgram from the bottom) when passed over immobilized rsCD14 showed no evidence of interaction even when a five-fold higher concentration was passed as compared to the experiments with the highest concentrations of the complex (viz. rLBP–LPS complex). B: Scatchard plot analysis of the same sensorgram with a K_{eq} of 1×10^8 M^{-1}.

rLBP complex and rsCD14, together with dramatically increased RUs for the reaction between the complex of LBP–LPS and rsCD14 as compared to the binding of LPS alone, suggests that LBP is not merely transferring LPS to the latter but associating itself, as well as forming an integral part of an LPS–LBP–sCD14 trimolecular complex. Also, the large increase in the value of K_a for the interaction of rsCD14 with LPS in the presence of LBP occurs mostly due to three orders of magnitude increase in the association rate constant.

3.3. *Monocytes secrete IL-6 when cultured in the presence of minute amounts of LPS in the presence of rsCD14 and/or rLBP*

TLR family members expressed on monocytes, B cells, adipocytes, intestinal epithelial and dermal endothelial cells [29] recognize LPS complexed to CD14 and transduce the signal for NF-κB to the nucleus. This in turn causes the release of cytokines and cellular mediators to elicit an inflammatory response intended to eliminate the infection. In this context, we studied the release of IL-6 from $CD14^+$ monocytes as an indicator of their activation by LPS in the presence or absence of LBP and/or sCD14.

Stimulation of monocytes from different donors gave results which were qualitatively similar, considering the magnitude of increase of IL-6 secretion as a function of stimulating conditions. However, quantitative differences were observed between donors. Two representative examples are shown in Fig. 4. Thus, incubation of monocytes in 1% human serum depleted of sCD14 and LBP with minute amounts of LPS (5–60 pg/ml) entails barely detectable IL-6 secretion. LBP has a concentration-dependent dual role in the pathogenesis of Gram-negative sepsis: low concentrations of LBP enhance the LPS-induced activation of mononuclear cells, whereas the acute phase rise in LBP concentrations inhibits LPS-induced cellular stimulation [30]. Thus, we used low concentrations of rLBP in the experiments. The addition of 1 μg/ml rLBP induced secretion of IL-6 only when LPS was also

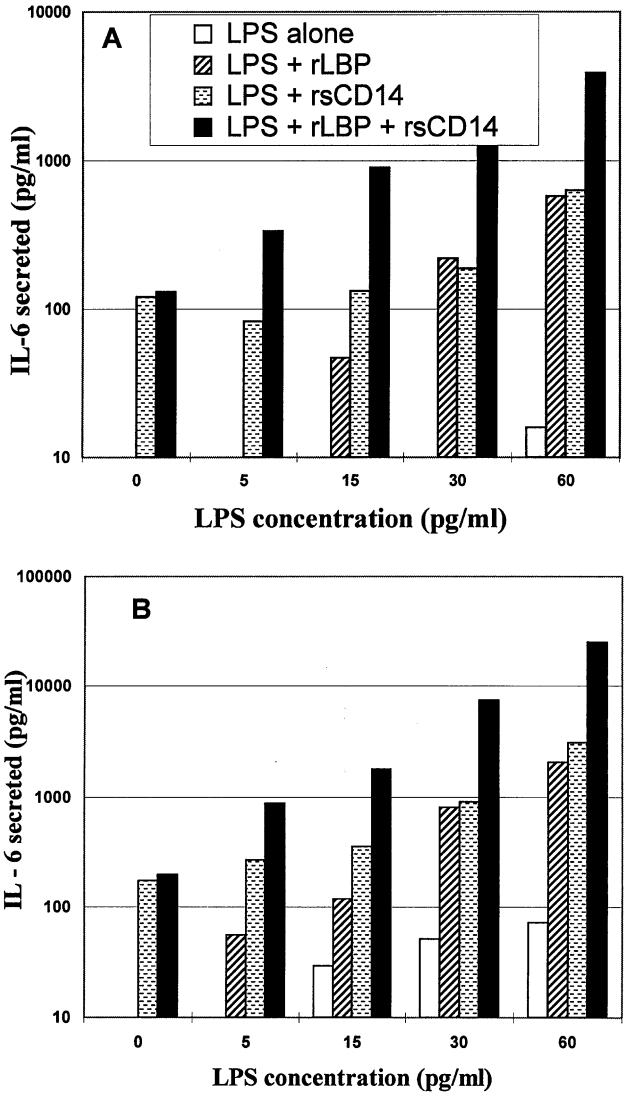

Fig. 4. Secretion of IL-6 by monocytes. Histograms A and B show the effect of various concentrations of LPS (0, 5, 15, 30, 60 pg/ml) on the secretion of IL-6 by freshly elutriated monocytes obtained from two different donors and cultured in the presence of 1% human serum depleted of sCD14 and LBP. The consequences of the presence of 1 μg/ml rLBP or 2 μg/ml rsCD14, or both of these molecules in the culture medium are shown. Note in B that 60 pg/ml of LPS induced secretion of 72 pg/ml of IL-6, while the same amount of LPS in the presence of rLBP and rsCD14 induced secretion of 25 052 pg/ml of IL-6.

present. Addition of 2 μg/ml rsCD14 to the monocytes led to the secretion of 174 pg/ml of IL-6, in the absence of any added LPS, an observation that can be correlated with the report by Landmann et al. [31] who showed that under serum-free conditions, endotoxin-free rsCD14 induced monocytes to secrete TNF-α. However, under physiological conditions this activation is precluded by a platelet-derived possibly lipidic inhibitor. The secretion of IL-6 was increased by more than five-fold upon incubation of the monocytes with 2 μg/ml rsCD14 and 60 pg/ml of LPS. Finally, the addition of both 1 μg/ml rLBP and 2 μg/ml rsCD14 in the presence of 60 pg/ml LPS led to secretion of 2836 pg/ml of IL-6 and an increase in the sensitivity of the monocytes to LPS. Secretion of IL-6 is observed for even very low concentrations of LPS (5 pg/ml). It has been shown earlier that addition of immunopurified sCD14, in the apparent absence of LBP, enabled responses to LPS by cells that do not express mCD14, like the astrocytoma line U373 or endothelial cells (for levels of LPS more than 1 μg/ml and less than 10 ng/ml, respectively) [12,13]. Likewise, it has been shown that rsCD14 substituted for serum to enable LPS stimulation of paroxysmal nocturnal hemoglobinuria mCD14-deficient peripheral blood mononuclear cells, but also LPS stimulation of mCD14-replete normal monocytes. Indeed, mCD14-bearing monocytes, incubated with low concentrations of LPS (10 ng/ml), in serum-free conditions, i.e. in the absence of both sCD14 and LBP, appear to be extremely sensitive to small concentrations of rsCD14 [14]. Likewise, the stimulation of $CD14^+$ PMN by LPS is dramatically enhanced by rsCD14 even in the absence of LBP [13]. If in our system the addition of rLBP alone failed to induce IL-6 secretion from monocytes, its addition in the presence of minute amounts of LPS clearly induced such secretion. The ability of rLBP to trigger a response in the presence of pg quantities of LPS was previously reported by Lee et al. [11], in a study of the conditions of IgM expression at the surface of a murine pre-B cell line transfected by mCD14. However, the monocyte response to LPS in the presence of rLBP is striking when the latter molecule is added concomitantly with rsCD14. Under these conditions, one observes IL-6 secretion at LPS levels as low as 5 pg/ml (corresponding to 0.045 EU/ml). Thus, our results show that the increase in the affinity of LPS for rsCD14, in the presence of rLBP, reduces the amount of LPS required to trigger IL-6 secretion by monocytes. They emphasize the high sensitivity of monocytes to tiny amounts of LPS, when incubated with sCD14 and LBP (Fig. 4).

Finally, it was recently shown that CD14 (in either its membrane or soluble form) is essential for LPS to be brought into close proximity and transferred to the TLR4–MD-2 complexes present at the cell surface [19,20]. Our functional studies underline the efficiency of LPS–LBP–sCD14 trimolecular complexes in this transfer and suggest that minute amounts of LPS are better delivered by sCD14 than by mCD14 to the TLR4–MD-2 complex.

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