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Isothermal titration calorimetric investigations of endotoxin binding to macrophages and the inhibition by polymyxin B

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Abstract

It is reported on the binding of lipopolysaccharide (LPS), a virulence factor of Gram-negative bacteria, with important cells of the human immune system, macrophages, applying isothermal titration calorimetry (ITC). Macrophages are responsible for the secretion of a variety of cytokines such as interleukins and tumor-necrosis-factor-α (TNFα) after stimulation with LPS, which may lead at sufficient LPS concentrations to the highly dangerous sepsis syndrom. The production of cytokines can be blocked by the addition of suitable antibiotics such as polymyxin B (PMB). The ITC measurements show that the interaction of LPS to macrophages is connected with a strong exothermic reaction probably in first line due to the binding of LPS to cell membrane proteins such as CD14 and membrane-bound mLBP, which are well-known LPS binding structures. This exothermic reaction can be considerably reduced by the addition of PMB to the cells, showing that the reduced cellular activation is connected with a strongly reduced interaction of LPS with the membrane surface. These first data prove the applicability of ITC for the LPS:cell interaction.

Keywords: Lipopolysaccharide; Titration calorimetry; Peptide antibiotics; Cytokine induction

1. Introduction

Lipopolysaccharides (LPS) are the main amphiphilic components of the outer leaflet of the outer membrane of Gram-negative bacteria. Due to their ability to induce a variety of biological effects in mammals, in particular the production of proinflammatory cytokines, they are called endotoxins [1]. However, at low endotoxin concentrations the biological effects induced by LPS may be even beneficial, since cytokines such as tumor-necrosis-factor-α (TNFα) have been shown to possess, among others, anti-tumor activity [2]. LPS consists of a sugar portion with varying length of oligo- or polysaccharide chains. The sugar moiety is covalently linked to the hydrophobic moiety of LPS, lipid A, which anchors the LPS molecule to the membrane. For enterobacterial LPS, lipid A is composed of a diglucosamine backbone which is phosphorylated in positions 1 and 4′ and acylated by six to seven hydrocarbon chains ester and amide linked in positions 2, 3 and 2′, 3′ [3]. Since lipid A is already able to elicit the biological responses, it is called the ‘endotoxic principle’ of LPS.

The biological activity of LPS is a result of its interaction with target cells, such as the highly immunocompetent mononuclear cells (monocytes and macrophages) of the white blood system (leucocytes). This interaction is not well undersood, it is assumed that LPS can bind to certain surface structures such as CD14 [4,5], membrane-bound lipopolysaccharide-binding protein (mLBP) [6,7], and receptor proteins such as the Toll-like receptors [8]. Also, the potassium channel MaxiK was found to to be stimulated by the interaction with LPS [9]. The biological activity of the LPS-induced cytokine induction can be inhibited to a large extent by the application of natural antibiotics such as the well-known polycationic decapeptide polymyxin B (PMB). This may be understood by a scavenger function of PMB preventing the LPS aggregates to react with or bind to the target cells. For an understanding of these processes, the knowledge of the binding energy of LPS with these target proteins would be important. However, up to now such data are not avaialble. Therefore, in the present paper an attempt is made to study for the first time the binding energy of LPS aggregates with target cell of the immune system, macrophages,

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and its reduction by the addition of PMB. The results clearly show a strong exothermic reaction due to binding of LPS with the macrophages and a drastcc decrease of the enthalpy change due to addition of PMB.

2. Materials and methods

2.1. Reagents

Lipopolysaccharide from deep rough mutant Re from *Salmonella minnesota* strain R595 and were extracted from bacteria grown at 37 °C by the phenol/chloroform/petrol ether method [10].

The known chemical structure of lipid A from LPS Re was checked by the analysis of the amount of glucosamine, total and organic phosphate, and the distribution of the fatty acid residues applying standard procedures. The amount of 2-keto-3-deoxyoctonate (Kdo) was also checked and in no case exceeded 5%.

Polymyxin B was purchased from Sigma–Chemie (Deisenhofen, Germany) and used without purification.

2.2. Macrophage preparation

Monocytes were isolated from peripheral blood from healthy donors by the Hypaque–Ficoll gradient method. To differentiate the monocytes to macrophages, cells were cultivated in teflon bags in the presence of 2 ng/ml M-CSF in RPMI 1640 medium (endotoxin 0.01 EU/ml; Biochrom, Berlin, Germany) containing 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, and 4% heat-inactivated human serum type AB at 37 °C and 6% CO2. On day 6 cells were used for calorimety at 10^7 cells/ml in buffer.

For stimulation with LPS in the absence and presence of PMB, macrophages were resuspended in serum-free medium, and their number was adjusted to 10^7/ml. For stimulation, 200 ml per well heparinized MNC (10^7/ml) were filled into 96-well culture plates and stimulated with endotoxins in serum-free medium. The stimuli were serially diluted in serum-free RPMI 1640 and added to the cultures at 20 ml per well. The cultures were incubated for 4 h at 37 °C and 5% CO_2. Supernatants were collected after centrifugation of the culture plates for 10 min at 400 × *g* and stored at −20 °C until determination of cytokine concentration.

The immunological determination of TNFα in the cell supernatants was determined in a sandwich-ELISA. Ninety-six-well plates (Greiner, Solingen, Germany) were coated with a monoclonal antibody against TNF (clone 6b from Intex, Germany). Cell culture supernatants and the standard (recombinant TNF, Intex) were diluted with buffer. The plates were shaken 16–24 h at 4 °C. For the removal of free antibodies, the plates were washed six times in destilled water. Subsequently, the color reaction was started by addition of tetramethylbenzidine in alcoholic solution and stopped after 5–15 min by addition of 0.5 M sulfuric acid. In the color reaction, the substrate is cleaved enzymatically, and the product can be measured photometrically. This was done on an ELISA reader (Rainbow, Tecan, Crailsham, Germany) at a wavelength of 450 nm, and the values were related to the standard.

2.3. Sample preparation

The lipid samples were usually prepared as aqueous 0.1 mM dispersions in 20 mM Hepes content for the ITC experiment. In all cases, the lipids were suspended directly in buffer and temperature-cycled several times betwen 5 and 70 °C and then stored at least 12 h before measurement.

2.4. Isothermal titration calorimetry (ITC)

Microcalorimetric experiments of endotoxin binding to cells were performed with a MCS isothermal titration calorimeter (Microcal Inc., Northampton, MA, USA) at 37 °C essentially as described in [11]. The endotoxin samples at a concentration of 0.1 mM—prepared as described above—were filled into the syringe compartment (volume 100 μl). Into the microcalorimetric cell (volume 1.3 ml).

Different reagents were filled (schematic see Fig. 1):

(1) A macrophage suspension in RPMI with $N = 10^7$ cells.
(2) The same macrophage suspension in the presence of 6.6 μM polymyxin B.
(3) Pure PMB (6.6 μM) in buffer.
(4) Pure buffer.

All suspensions were thouroughly degassed prior to use. After temperature equilibration, LPS was titrated in 3 μl

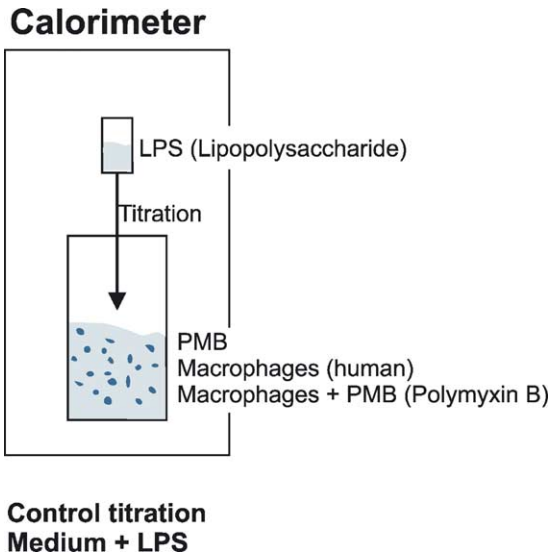

Fig. 1. Design of the ITC experiment. The calorimetric cell was filled with human macrophages (10^7 cells/ml), with macrophages + the antibiotic PMB (6.6 μM), or with PMB or medium alone (control experiments). In the titration cell, 0.1 mM lipopolysaccharide LPS Re was filled, and the titration was performed with 3 μl every 5 min.

portions every 5 min into the cell, which was stirred constantly, and the heat of interaction after each injection measured by the ITC instrument was plotted versus time. Afterwards, the total heat signal from each experiment was determined as the area under the respective single peaks and plotted versus the [peptide]:[lipid] molar ratio. Since the instrument works in temperature equlibrium at a constant 'current feedback' corresponding to a power of approximately 74 μW, the occurrence of an exothermic reaction leads to a lowering of this current and of an endothermic reaction to an increase.

All titration curves were repeated at least four times.

3. Results and discussion

A typical titration experiment shown in Fig. 2, in which 10^7 macrophages within the microcalorimetric cell are titrated every 5 min with 3 μl of a 0.1 mM suspension of deep rough mutant LPS Re from *Salomonella minnesota*. Clearly, a strong exothermic reaction takes place deduced from the reduction of the feedback power after each titration, which is nearly constant within the measuring period of 7000 s (nearly 2 h), this means under these conditions no saturation is observed. The strong exothermic reaction is superimposed by a slow exothermic process, a drift of the base-line from 73 μW at $T = 0$–67 μW at $t = 7000$ s. The strong exothermic reaction can be attributed to a binding of the LPS aggregates to cell-surface proteins such as CD14 amd mLBP, and the weak exotherm to a metabolic reaction of the macrophages, which are, e.g. known to change their adherence to the vessel walls. In Fig. 3 the enthalpy change of the exothermic reaction is plotted versus the LPS concentration, showing apparent enthalpy changes of more than 900 kJ/mol LPS. The enthalpy change is called 'apparent', since it can be assumed that a superposition of the binding processes with the metabolic processes of the macrophages takes place.

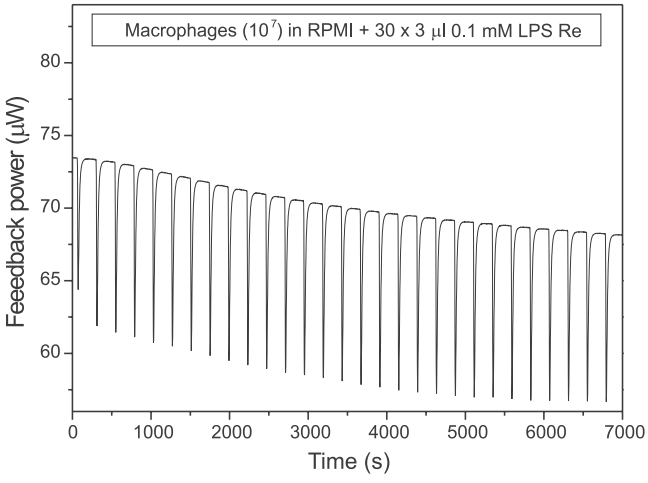

Fig. 2. Isothermal calorimetric titration of 10^7 macrophages in RPMI medium with 0.1 mM LPS Re. The 1.3 ml macrophage suspension was titrated every 5 min with 3 μl LPS.

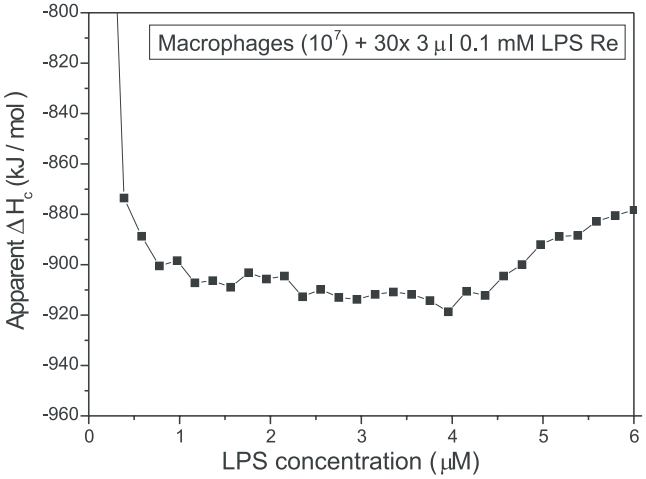

Fig. 3. Enthalpy change of the macrophage suspension (10^7 cells) titrated with LPS (0.1 mM) vs. LPS concentration according to Fig. 2.

In the next experiment, an amount of 6.6 μM PMB was added to the macrophages, the other experimental details remaining constant. There is a strong reduction of the apparent enthalpy changes over the entire time period, with again no saturation of binding to the macrophages and/or PMB (Fig. 4). The [PMB]:[LPS] molar ratio at the end of the measurements had a value of 1, which is beyond the saturation of LPS binding to PMB [11,12]. Thus, the presence of PMB drastically decreases the binding of LPS to macrophages.

Parallel to this, in the biological cell test the PMB in the medium causes a drastic decrease of the LPS-induced stimulation of the macrophages (Fig. 5). It can be seen that 10 ng/ml LPS Re induce more than 1800 pg/ml TNFα, which is reduced to less than 200 pg/ml when PMB is added at a concentration 1 μg/ml. A similar decrease in TNFα production is observed at a concentration of 1 ng/ml LPS.

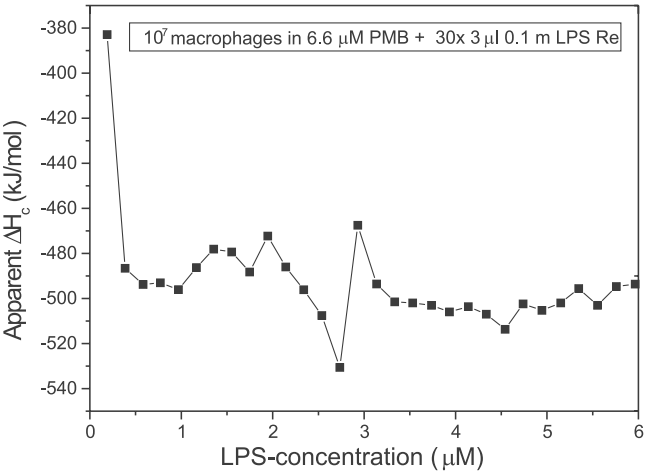

Fig. 4. Enthalpy change of the macrophage/PMB suspension (10^7 cells: 6.6. μM PMB) titrated with LPS vs. LPS concentration.

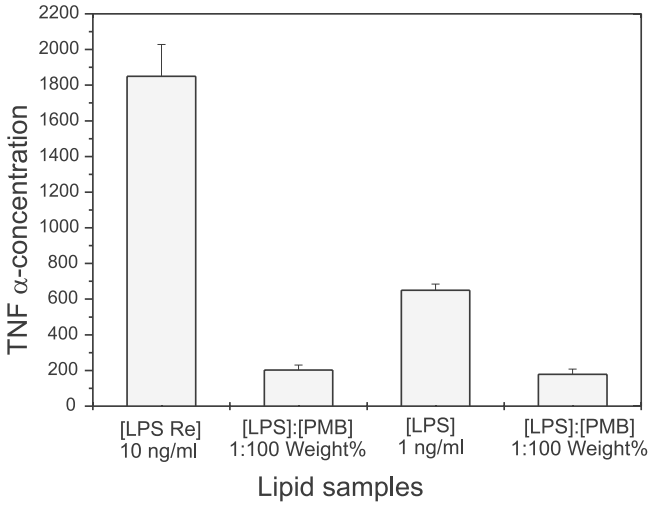

Fig. 5. Production of tumor-necrosis-factor-α of human mononuclear cells induced by LPS Re in the absence and presence of PMB.

For an assessment of the data it has to be taken into account that the pure dilution of the LPS suspension into the cell medium as well as the binding of LPS to the PMB in the medium may superimpose the binding process. Therefore, in control measurements these effects were investigated (Fig. 6). Clearly, at the beginning of the titration relatively strong exotherms due to LPS dilution (A) as well as to binding to PMB (B) can be seen. However, after 5–10 titrations the measured enthalpy changes are small as compared to those recorded in Figs. 3 and 6.

The presented data clearly indicate that ITC is well applicable for binding studies of bacterial virulence factors with interacting cells such as mononuclear cells. The binding can be assumed to take place essentially with membrane receptors CD14 [4] and mLBP [6,7], which have been shown to transport LPS to the sites of the signalling proteins probably by intercalation into the macrophage membrane. A quantification of the binding with respect to the cell number and LPS molecules is presently impossible. An estimate shows that under the conditions applied 10^{10} LPS molecules per cell are present. However, most of the LPS molecules are in large aggregates and therefore not available for binding. This is true in a smilar way for the cells, which can be assumed to adopt a multilayer. Furthermore, the exact number of copies of the LPS-receptor proteins is widely unknown. In further work it is intended to vary the LPS concentrations in a large concentration range which might give first estimates for the heat of binding with the target structures on a molecular level. This knowledge seems to be very important. Up to now there are no informations regarding the binding energy or binding constant of LPS to mCD14 or the Toll-like receptors such as TLR4, which have been reported to be responsible for the signalling into the cell interior [8,13]. This knowledge, however, would be important for a therapeutic approach with antagonistic lipid A part structures such as synthetic tetraacyl lipid A ‘406’ [14]. For an effective antagonistic action, these part structures should bind stronger than the agonistic LPS, which necessitates the knowledge of the respective binding energies.

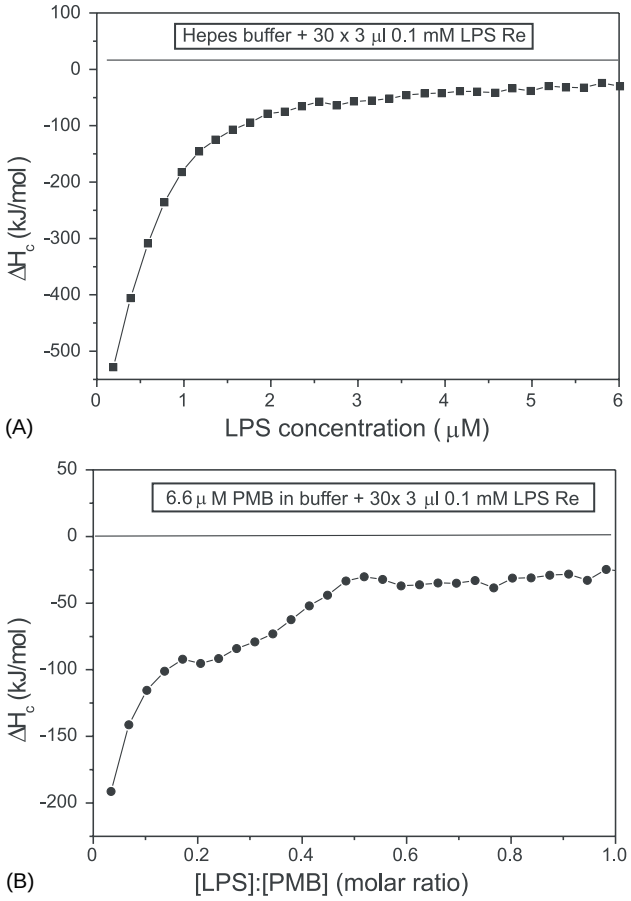

Fig. 6. Control experiments with 0.1 mM LPS Re titrated into pure buffer (A) and 6.6 μM PMB (B).

Acknowledgements

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