Effect of Chemotype on *Escherichia coli* Interactions with Bactericidal Proteins

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We studied the effects of bactericidal proteins (lysozyme and lactoferrin) on endotoxin release from cell wall and inhibition of the growth of *Escherichia coli* colonies of different chemotypes. The structure of LPS core was found to be essential for the mechanisms of the interactions of the studied proteins with the cell wall. Cell viability after contact with cationic proteins is determined not by the amount of released LPS, but by the mechanism of damage to the cell wall.

Key Words: *lysozyme; lactoferrin; LPS release; Escherichia coli; chemotype*

Endotoxins or LPS are the main structural component of the cell wall in gram-negative bacteria. They exhibit various types of biological activity only in a free state and are involved in the pathogenesis of septic shock [8]. Located on the inner side of the gram-negative bacterial cell wall, LPS are easily accessible for contact with serum components, which release up to 30% endotoxins from *E. coli* cells [12]. Plasma proteins bind free and outer membrane endotoxins.

Lysozyme is the main cationic protein of neutrophils; it induces lysis of some bacteria, destroying the mureine layer located between the cell wall and cytoplasmatic membrane. Lysozyme binds released endotoxins, which leads to a decrease in its enzymatic activity and immunostimulating activity of LPS [9].

Lactoferrin is characterized by antibacterial activity towards gram-negative bacteria. Direct interaction between cationic lactoferrin and anionic LPS is followed by LPS release into the medium, osmotic destruction of the outer membrane, and bacterial death [4]. It was shown that lactoferrin binds LPS with high affinity [3]. Moreover, lactoferrin interacts with porins of *E. coli* cell wall, thus weakening bonds between porins and endotoxin molecules and inducing their release into the environment [5].

The interactions between plasma proteins and endotoxins of cell wall of gram-negative bacteria are little studied. It is obvious that endotoxin availability for proteins and type of their interactions depend on the structure of the cell wall LPS and the characteristics of the protein.

We studied the impact of LPS core for the interactions between plasma proteins and cell wall of *E. coli* of different chemotypes.

MATERIALS AND METHODS

Commercial lysozyme (Chicken Egg White) and lactoferrin (Bovine Milk; both preparations from Sigma Chemical Corp.) were used in the study.

Lipopolysaccharides were extracted from *E. coli* D21 (Ra-LPS) and *E. coli* JM 103 (Re-LPS) with mixture of phenol, chloroform, and petroleum ether [7].

Two *E. coli* K-12 strains were used: D 21 wild strain from the Russian Collection of Microorganisms, Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, and JM 103 from the German Collection of Microorganisms (DSMZ GmbH), differing by the LPS

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core structure. *E. coli* D21 cells were cultured in M 9 agarized medium, *E. coli* JM 103 in medium 382 (DSMZ GmbH) at 37°C during 24 h. The media contained the same concentrations of Ca^{2+} and Mg^{2+} ions, because these ions modulate the formation of cell wall during bacterial growth and LPS release from the cell wall [11].

For evaluation of the interactions between bacteria and plasma proteins, the cells were washed from agar surface with buffered saline (BS; pH 7.4), brought to a concentration 10^8 cell/ml, centrifuged, washed, and resuspended in BS containing the proteins in concentrations corresponding to those during acute phase of inflammation (150 μg/ml lysozyme and 100 μg/ml lactoferrin). Control cells were resuspended in BS. Incubation with proteins was carried out at 37°C and gentle shaking for 1 h.

The content of LPS was measured by spectroscopy with carbocyanin dye [1] after cell incubation with proteins and subsequent centrifugation. The spectra were recorded on a Hitachi spectrophotometer. The absolute count of released LPS in the sample was evaluated by the plots representing the dependence of absorption of LPS-carbocyanin complex on LPS concentration at λ=468 for Ra-LPS and at λ=464 for Re-LPS. The proteins present in the sample also form complexes with carbocyanin, but do not absorb in this wave range. Cell precipitate was suspended in BS and the numbers of CFU formed by control cells and cells incubated with proteins were determined after inoculation on agar medium.

RESULTS

The studied *E. coli* strains belong to the R-chemotype and differ by the core structure. *E. coli* D21 is characterized by complete Ra-structure of the core, while LPS from *E. coli* JM 103 contains Re-core with the minimum structure. The core structure determines the size and charge of the LPS molecule, which can be decisive during cell interactions with plasma proteins. In contrast to *E. coli* JM 103, the *E. coli* D21 LPS includes two additional phosphate groups of phosphorylated sugars in the inner core, which together with carboxyl groups of keto-deoxyoctulosonic acid and phosphate groups of lipid A participate in the formation of the integral negative charge on the cell surface [10].

Study of the effects of inflammatory acute phase proteins lysozyme and lactoferrin on LPS release from cell wall showed that the volume of released LPS in control cells incubated in BS at 37°C for 1 h depended on bacterial chemotype and increased with elongation of the LPS saccharide fragment (Table 1). The release of LPS from control cells into the medium is caused by cell wall instability in BS without addition of Ca^{2+} and Mg^{2+} ions providing membrane stability [11]. Since bacteria of different chemotypes released different amounts of LPS in control samples, for more correct comparison of the effects of protein on *E. coli* cells the results of experiments are presented as histograms. The volume of released LPS and CFU for control cells of different strains were taken for one unit.

The content of LPS released under the effect of lysozyme was comparable with the control for *E. coli* JM 103 cells and 1.4 times surpassed the control for *E. coli* D21 cells. Lactoferrin increased LPS release from *E. coli* D21 and *E. coli* JM 103 by 3 and 3.6 times, respectively, in comparison with the control.

For evaluation of the impact of LPS release under the effects of different proteins for cell viability, the number of CFU formed by cells incubated with plasma proteins and by control cells were compared. The number CFU decreased significantly under the effects of the studied proteins in comparison with control cells. Lysozyme exhibited the most pronounced suppression of *E. coli* D21 colony growth (6.6 times in comparison with the control). The growth of *E. coli* JM 103 colonies decreased 3-fold. Lactoferrin (cationic protein) decreased the number of CFU by 4 and 3.3 times in comparison with the control in cells with Re- and Ra-structures of LPS core, respectively.

Comparison of the effects of different proteins on LPS release from cell wall of both *E. coli* strains showed that these effects depended on bacterial chemotype.

Cells with Re-structure of LPS core treated with lysozyme released lesser amount of LPS than cells with Ra-structure, which can be attributed to different compactness of LPS packing in bacterial cell walls. In order to destroy the peptidoglycane layer, lysozyme has to penetrate through the outer layer of the external membrane, which is more difficult in cells with more compact LPS packing (hypothesized in bacteria with the LPS core Re-structure). Indeed, the least amount of LPS was released from

TABLE 1. Effect of Plasma Proteins on LPS Release from Cell Wall of *E. coli* of Different Chemotypes (n=5, μg/ml)

Incubation conditions	Ra-chemotype (*E. coli* D21)	Re-chemotype (*E. coli* JM 103)
Control	6.30	3.50
Lysozyme, 150 μg/ml	8.82	3.85
Lactoferrin, 100 μg/ml	11.90	18.90

these cells. In addition, less pronounced suppression of colony growth was observed in these cells in comparison with the cells with Ra-structure treated by lysozyme.

The main components of *E. coli* cell wall participating in lactoferrin binding are porin proteins. Lactoferrin tightly binds to the *E. coli* OmpF and OmpC and weakly to PhoE proteins weakening the bonds between these porins and endotoxin molecules. Presumably, LPS O-chains shield porins protecting them from interactions with lactoferrin [5]. It was shown that the degree of lactoferrin interaction with cell surface increases with transition from S- to R-structure of LPS in *E. coli* H10407 [6]. Our findings suggest that core sugars in cells with LPS Ra-structure impede lactoferrin access to porin, in contrast to the short core in cells with LPS Re-structure. As a result, more LPS molecules were released from *E. coli* JM 103 cells and colony growth was more strongly suppressed. Our results are in good agreement with the data on lactoferrin interactions with the S- and Ra-chemotype bacteria [2].

The results suggest that lactoferrin interactions with *E. coli* cells are realized by the mechanism involving reduction of cell viability proportional to LPS release. If *E. coli* cells are treated with lysozyme in physiological concentrations, the suppression of colony growth is not linked with damage to the outer layer of the membrane and LPS release from bacterial cell wall. Hence, bacterial chemotype is essential for the mechanisms of the interactions of the studied proteins with the cell wall. Cell viability after contact with cationic proteins is determined not by the amount of released LPS, but by the mechanisms of damage to the cell wall. Our data on the interactions of plasma proteins with gram-negative bacteria of different chemotypes are not only of theoretical, but also of practical importance, because can be used in the choice of antibacterial therapy.

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