Effects of Liposomes of Different Lipid Composition on *In Vitro* Growth of *Mycobacterium Tuberculosis* H37Rv

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The growth of *M. tuberculosis* H37RV in culture medium was studied after addition of liposomes from different lipids (phosphatidylcholine, cardiolipin, and glycosphyngolipids). Addition of phosphatidylcholine into culture medium did not modify the growth and multiplication of mycobacteria. Addition of glycosphyngolipids and their mixture with phosphatidylcholine partially inhibited the growth. Addition of cardiolipin inhibited the growth of mycobacteria and even suppressed it, depending on the dose. Presumably, high concentrations of cardiolipin added into the culture medium, can transfer the mycobacteria into an uncultivable state.

Key Words: *Mycobacterium tuberculosis H37Rv; in vitro culture; liposomes; phospholipids; cardiolipin*

M. tuberculosis, the agent of tuberculosis, is an intracellular pathogen penetrating into macrophages with phagosomes and multiplying in them due to its capacity to prevent phagosome fusion with liposomes [12]. It is assumed that mycobacteria utilize glycerol and sugars as the sources of nutrition in the extracellular space and fatty acids in the phagosome [6].

Some characteristics of mycobacteria (acid and thermal resistance) change after changes in environmental conditions [15]. According to the data of transcriptome analysis of *M. tuberculosis*, this process is paralleled by significant changes in the gene expression profiles [7].

Our previous experiments on *M. smegmatis* cultured in depleted Soton's medium with glycerol showed that addition of liposomes consisting of phosphatidylcholine (PC) and a mixture of PC with cardiolipin (CL) induced resistance of the bacteria to antituberculosis drugs in comparison with culturing in medium without liposomes [5]. It was also found for *M. avium* (a bacterium initially resistant to isoniazid) that isoniazid inhibited culture growth in the presence of PC liposomes [3]. The bacterial properties are modified in the presence of phospholipids, because the nature of the alimentary substrate determines metabolic routes of its transformation [13].

We evaluated the effects of various lipid classes in the form of liposomes: zwitterionic PC and anionic CL and a mixture of glycosphyngolipids (GSL) on the *in vitro* growth and viability of virulent *M. tuberculosis* H37Rv strain.

MATERIALS AND METHODS

Soybean PC, Lipoid S-100 (Lipoid), CL (Biolek), and porcine brain GSL (Techcon) were used in the study.

Large unilamellar vesicles (further called liposomes) were obtained by extrusion of multilamellar vesicles from PC or CL or GSL and from PC+GSL mixture (4:1 by weight) using a LiposoFast Basic extruder (Avestin) [5]. Liposomes were obtained using

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zwitterionic phospholipid PC (a component of macrophage membrane), negatively charged phospholipid CL (the main lipid of mycobacterial plasma membrane), and a complex mixture of GSL including cerebroside (45%), cerebroside sulfate (14%), cholesterol and triglycerides (11%), phosphatidylethanolamine (18%), and phosphatidylserine (12%).

The diameter of liposomes measured by turbidimetry [4] was 240-300 nm. Liposome suspension was sterilized by filtration through sterile Millipore filter (0.2 μ).

The dynamics of culture growth in liquid Dubos medium with liposomes was evaluated for a suspension of solitary mycobacterial cells in the same growth phase by a previously described method [1]. Liposomes from PC, CL, GSL, and PC+GSL mixture in different proportions were used in concentrations of 50 to 750 μg/ml. The effects of liposomes on the growth of *M. tuberculosis* H37Rv culture in Dubos broth were evaluated on days 4, 9, and 14. Culture without liposome suspension served as the control. The initial concentration of *M. tuberculosis* was 10^4 CFU. CFU were counted for each term of the study by two methods: inoculation of 10-fold dilutions of suspension of newly grown mycobacteria into Dubos agar (macrocolonies were counted on day 18 of culturing) and by quantitative real-time PCR (in triplicates). DNA was isolated from the entire *M. tuberculosis* precipitate using Proba-rapid kit (DNA Technology). PCR was carried out with the *M. tuberculosis complex* amplification kit for real-time mode (DNA Technology) with DNA reference samples corresponding to 10^3, 10^5, and 10^7 mycobacterial CFU/sample. The concentrations of mycobacterial cells in the experimental and control samples were evaluated by the calibration curve.

RESULTS

Liposomes from zwitterionic PC in concentrations 50-500 μg/ml did not modify the growth of *M. tuberculosis* H37Rv (Fig. 1). It is noteworthy that we previously obtained a similar result (no effect of PC liposomes) by another method, [^{3}H]-uracil incorporation into *M. tuberculosis* RNA [14]. A specific feature of this method is measurement of mycobacterial multiplication during the initial period of incubation (days 1-4), while in the present study PCR was used to evaluate the cell growth in the middle of the logarithmic phase (days 9 and 14 of growth).

Addition of PC liposomes with 25% GSL or liposomes from GSL alone led to a significant inhibition of mycobacterial growth on days 9 and 14. The inhibitory effect in these cases was determined by GSL concentration exclusively, while the presence of PC had no additional effect (Fig. 1).

The next step of the study was evaluation of the effect of liposomes from CL in concentrations of 50-750 μg/ml. Addition of 50-250 μg/ml CL liposomes to the incubation medium produced no appreciable changes in culture growth in comparison with the control, while in the presence of 500-750 μg/ml CL liposomes the growth of *M. tuberculosis* was completely suppressed (Fig. 2).

In the presence of these concentrations of CL, the viability of mycobacteria was suppressed, no CFU were found (Fig. 3).

These data indicate that the effects of liposomes on the growth and viability of *M. tuberculosis* H37Rv

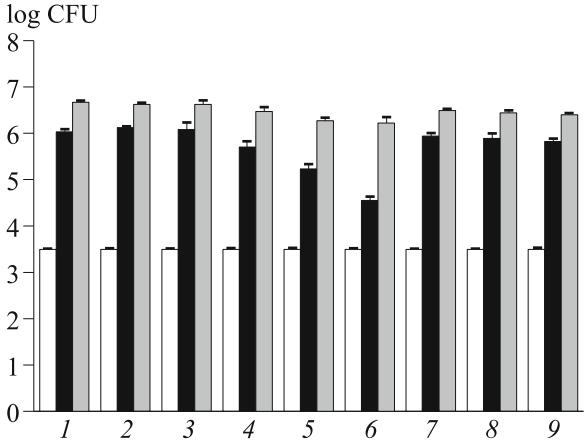

Fig. 1. Effects of liposomes of different lipid composition on the growth of *M. tuberculosis* H37Rv. *1*) H37Rv; *2*) H37Rv+50 μg/ml PC; *3*) H37Rv+500 μg/ml PC; *4*) H37Rv+50 μg/ml GSL; *5*) H37Rv+250 μg/ml GSL; *6*) H37Rv+500 μg/ml GSL; *7*) H37Rv+50 μg/ml PC+GSL mixture (4:1); *8*) H37Rv+250 μg/ml PC+GSL mixture (4:1); *9*) H37Rv+500 μg/ml PC+GSL mixture (4:1). Light bars: day 0; dark bars: day 9 of culturing; gray bars: day 14 of culturing.

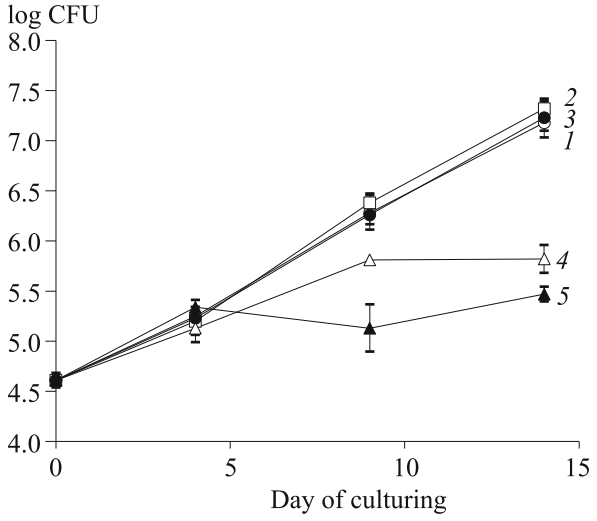

Fig. 2. *M. tuberculosis* growth in Dubos broth with CL liposomes (quantitative real time PCR). *1*) H37Rv; *2*) H37Rv+50 μg/ml CL; *3*) H37Rv+250 μg/ml CL; *4*) H37Rv+500 μg/ml CL; *5*) H37Rv+750 μg/ml CL.

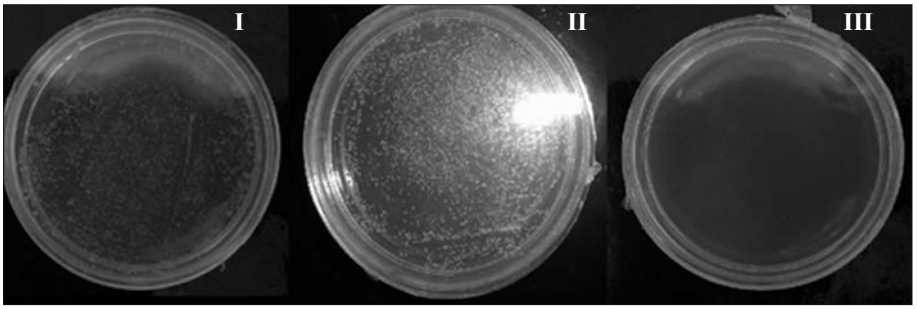

Fig. 3. *M. tuberculosis* H37Rv macrocolonies in Dubos agar on day 18 of culturing. *I*) H37Rv; *II*) H37Rv+50 μg/ml CL; *III*) H37Rv+750 μg/ml CL.

depend on their lipid composition. PC liposomes in a wide range of concentrations exhibited no effects of any kind; animal GSL inhibited the growth of *M. tuberculosis* H37Rv; CL (a component of cell membrane in this bacterium) used in a high concentration completely suppressed the growth and viability of the bacteria.

The inhibitory effect of CL on the growth of *M. tuberculosis* should be regarded with consideration for published data on the signal function of this phospholipid in bacteria. It was shown that, depending on its dose, CL forms domains in the bacterial membrane, no domains are formed at low CL concentrations [8]. It was found that CL is involved in *Bacillus subtilis* osmotic adaptation [10] and in secretion of *E. coli* enzymes [2]. Changes in the localization of fluorescent label-bound CL sporulation process were demonstrated for *E. coli* [11] and a significant increase in CL content in spores was shown for *Bacillus subtilis* [9]. These data indicate that changes in CL content can be an important factor modulating the process of the bacterium transition from one status to another (from active to unculturable) and from the logarithmic growth phase into stationary one.

Addition of CL liposomes to *M. tuberculosis* culture medium led to reduction or complete inhibition of bacterial growth. Our results do not answer the question whether this fact is explained by transition into an uncultivable state or addition of CL in high concentrations into medium is toxic for bacteria and leads to their death, associated with impairment of the cell integrity.

Presumably, if CL content in the plasmatic membrane is changing during *M. tuberculosis* multiplication, this process regulates the formation of domains, which can signal the transfer into an uncultivable state. Addition of exogenous CL during the initial period of multiplication can modify this process. Verification of this hypothesis is the task of further research. In addition, these results can be useful in the development of new antituberculosis drugs.

REFERENCES

1. S. N. Andreevskaya, L. N. Chernousova, T. G. Smirnova, *et al.*, *Probl. Tuber. Bol. Legkikh*, No. 12, 43-48 (2006).
2. E. V. Anisimova, A. O. Badyakina, N. V. Vasilyeva, and M. A. Nesmeyanova, *Mikrobiologiya*, **74**, No. 2, 179-184 (2005).
3. V. I. Golyshevskaya, A. A. Selishcheva, L. P. Martynova, *et al.*, *Probl. Tuber. Bol. Legkikh*, No. 8, 61-64 (2006).
4. A. S. Minina, G. M. Sorokoumova, A. A. Selishcheva, *et al.*, *Biofizika*, **49**, No. 4, 674-679 (2004).
5. Yu. N. Shakina, O. A. Troshkina, E. E. Petrova, *et al.*, *Zh. Mikrobiol. Epidemiol. Immunol.*, No. 6, 24-28 (2007).
6. H. I. Boshoff and C. E. Barry 3rd, *Nat. Rev. Mikrobiol.*, **3**, No. 1, 70-80 (2005).
7. H. I. Boshoff, T. G. Myers, B. R. Copp, *et al.*, **279**, No. 38, 40,174-40,184 (2004).
8. R. M. Epand and R. F. Epand, *Biochim. Biophys. Acta*, **1788**, No. 1, 289-294 (2009).
9. F. Kawai, M. Shoda, R. Harashima, *et al.*, *J. Bacteriol.*, **186**, No. 51, 1475-1483 (2004).
10. C. S. López, A. F. Alice, H. Heras, *et al.*, *Microbiology*, **152**, Pt. 3, 605-616 (2006).
11. R. Mukhopadhyay, K. C. Huang, and N. S. Wingreen, *Biophys. J.*, **95**, No. 3, 1034-1049 (2008).
12. P. Mueller and J. Pieters, *Immunobiology*, **211**, Nos. 6-8, 549-556 (2006).
13. E. J. Munoz-Elias and J. D. McKinney, *Nat. Med.*, **11**, No. 6, 638-644 (2005).
14. V. Sosunov, V. Mischenko, B. Eruslanov, *et al.*, *J. Antimicrob. Chemother.*, **59**, No. 5, 919-925 (2007).
15. N. Sung, K. Takayama, and M. T. Collins, *Ibid.*, **70**, No. 3, 1688-1697 (2004).