Macrophages (Mφ) are the most described and characterized target and host of mycobacteria. Like other cells of innate immunity Mφ have a wide range of receptor molecules which interact with different pathogen associated molecular patterns (PAMPs). Immunodominant proteins MPT63 and MPT83 that are synthesized in abundance by Mycobacterium bovis or Mycobacterium tuberculosis strains could be involved in development of tuberculosis infection. The aim of this study was to search for effects of these mycobacterial antigens on target cells. For this aim full-sized sequences of MPT83 (rMPT83full) and MPT63 antigens were cloned into plasmid pET24a(+). The increase of phagocytic activity of murine peritoneal macrophages was demonstrated, but not of macrophage-like cells from J774 cell line, which were treated by rMPT63 and rMPT83full proteins for 24 h. This effect of such antigens can be considered as a way to facilitate the consumption of mycobacterial cells by macrophages to avoid other effector mechanisms of innate and adaptive immunity.

Key words: Mycobacterium tuberculosis, Mycobacterium bovis, macrophages, phagocytosis, MPT63, MPT83.

The innate immune system, including Mφ, is the main chain of all immune networks, which is responsible for elimination and deactivation of numerous pathogens. However, inner environment of Mφ is a favorable place for reproduction and survival of M. tuberculosis [2]. Apparently, active phagocytosis of different bacilli by Mφ leads to pathogen lysis and future patient recovery. However, pathogenic mycobacterial strains survive inside macrophages by arresting the normal maturation of their phagosome and limiting fusion with pre-formed lysosomes [3]. Obviously, the components of bacteria cell wall and some proteins products are involved in these processes.

MPT63 is 16kDa secreted protein that had relatively high expression level in mycobacteria. Firstly this antigen was obtained from M. tuberculosis culture fluid in 1991 by Nagai and colleagues [4]. Analysis of the nucleotide sequence of mpt63 identified open reading frame, and it was shown that the protein consists of 159 amino acids and contains two components: a signal peptide that has 29 amino acids and entire MPT63, which contains 130 amino acids [5]. The orthologs MPB63 from M. bovis and...
MPT63 from *M. tuberculosis* have difference of one nucleotide in their gene sequences but are not distinguished in functional products. The recombinant MPT63 isolated from *Escherichia coli* and natural protein of *M. tuberculosis* MPT63 did not differ by the results of serological tests [6, 7].

Moreover, homologs of MPT63 have been found in different strains, but polyclonal antibodies against MPT63 from *M. tuberculosis* do not cross-react with them [8, 9]. Some mycobacterial species have mpt63 gene which are not translated into protein product [8]. Therefore, rMPT63 is a valuable target for immunological studies.

Though the function of MPT63 remains unknown, there have been few studies of MPT63 effect on *in vitro* systems. However there is little information available about targets of MPT63 on cells and activation cascades associated with them. As a rule, bacteria proteins have effect on cytokine production, such as tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6). Degranulation and release of histamine and hexaminidase from mast cells caused by MPT63 is demonstrated [10]. Surprisingly, that other *M. tuberculosis* antigens ESAT-6 and MTSA-10 showed a similar result.

Another important immunodominant antigen of *M. tuberculosis* is a membrane binding lipoprotein MPT83 [11]. This molecule is one of the ligands for toll-like receptor 2 (TLR-2) and could bind with human TLR-2 with affinity about 4.42×10⁻⁸ M [12]. Also *M. tuberculosis* has a number of molecules that bind and activate TLR-2 (lipoproteins LpqH, LprG, LprA and PhoS1, lipoarabinomannan, lipomannan and phosphatidylinositol mannoside (PIM)). Prolonged stimulation of macrophages with these ligands leads to the production of immunosuppressive cytokines (including IL-10 and IL-4) and reduction of major histocompatibility complex (MHC) class II expression and inhibition of antigen processing [13]. Probably, TLR-2 is not the only possible target for MPT83. Thus, MPT83 stimulation of Mφ from TLR-2 knockout mice resulted in a significant increase of TNF-α, IL-6 and IL-12 synthesis, although lower than for wild-type mice macrophages [14]. Similarly, the blocking of TLR-2 with antibodies reduces but not abolishes MPT83 effects [12].

Chambers and colleagues [12] offered that glycosylated and acetylated protein could take influence on matrix metalloproteinase (MMP) expression through interaction with TLR-2, TLR-1 or TLR-4. Nevertheless, it was shown that recombinant protein (not glycosylated and not acetylated) could activate the production of metalloproteinases too. The above evidence provides the value and adequacy for using the recombinant proteins MPT63 and MPT83 in investigation of their functions.

According to the literature macrophages and macrophage-like cells are the main target of mycobacteria, but for many *Mycobacterium* antigens the functions have not been found yet. Our goal was to show some effects of *M. tuberculosis/M. bovis* antigens MPT63 and MPT83 on selected cells models.

We have chosen two models to achieve the objective, primary culture of macrophages from peritoneal cavity of mice and macrophage-like cells of J774 cell line which is also murine. Effects could differ after treatment of primary culture and malignant cells by antigens. The absence of registered changes in phagocytosis assay in J774 cell line has focused our investigation on peritoneal macrophages only.

We previously cloned MPT63 and fragment of MPT83 that corresponded to sequence 115-220 aa of fasciclin-like domain (FLD) (94-217 aa) [6]. In the current study we focused on the effects of rMPT63 and rMPT83full on macrophages phagocytosis activity. Our results have shown that both rMPT63 and rMPT83 increase percentage of macrophages that actively consume EGFP expressed *E. coli*. Moreover, such influence of recombinant antigens did not depend on *E. coli* expression system, because control proteins, which were expressed and obtained from similar expression system, had no effect on phagocytosis.

**Materials and Methods**

*Mice and cells.* BALB/c female white mice (30-35 g of body mass) were used in the experiments. Mice were kept in cages of research vivarium, fed a standard diet and had free access to water. The animals (*n* = 12) were used for obtaining peritoneal macrophages.

The experiments are consistent with the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986) and with ethical norms as laid down in the laws of Ukraine.

Sterile saline was injected in the plot of peritoneal cavity. Belly was massaged gently for Mφ washed per 1 min. A small patch of skin was cut by scissors without damage of the peritoneal cavity in-
tegrity. Using a syringe carefully selected fluid with cells and transferred it to clean dishes. The liquid was centrifuged during 5 min at 300 g, the supernatant was collected and cells were washed twice in phosphate buffered saline (PBS). Cells’ precipitate was resuspended in RPMI-1640 medium with L-glutamine, 50 mg/l cefalotin and with addition of fetal bovine serum (FBS) to final concentration of 10% and transferred to a new dish. Peritoneal MΦ and macrophage-like cells of J774 cell line were cultivated in cell culture flasks and maintained at 37 °C in a humidified incubator in 5% CO₂ atmosphere. After MΦ adhesion to the bottom of the dish there are twice washed by PBS from on the medium, serum and red blood cells. Cells were removed by 30 mM EDTA solution in PBS for 15 min at 37 °C and active mixing.

Cloning of MPT83 full in Escherichia coli. Fragment of mpt83(full) gene was amplified by PCR from vaccine strain M. bovis BCG-Russia by 2 oligonucleotides: MPT83 L SP 5’-TGGATCCAGCACAAACCCGTGTCGCCA-3’ and MPT83 L ASP 5’-AACATCTGAGCCTGCAGCAAC-3’. PCR amplification was performed under the following conditions: incubation at 94 °C for 3 min; 28 cycles at 94 °C for 30 s, at 55 °C for 30 s and at 72 °C for 1 min; and extension at 72 °C for 7 min. PCR products were extracted and purified using GeneJet Gel Extraction Kit. Plasmid DNA was obtained by GeneJet Plasmid Miniprep Kit. All manipulations were performed according to the manufacturer's manual recommendations. The amplified mpt83(full) gene was ligated with the E. coli expression vector pET24a(+) (Novagen, Germany) using BamH1 and Xhol restriction sites. The obtained construct was used to transform E. coli Rosetta (DE3) host cells (Novagen, Germany) for protein expression. Several clones verified with PCR were frozen.

Immobilized-metal affinity on-column chromatography of His-tag proteins. MPT63 and MPT83full expressed E. coli cells were grown in LB medium with 50 μg/ml kanamycin and 1% glucose at 37 °C and active mixing (250 rpm) to optical density A₅₆₀ 0.3-0.5. The expression of mycobacterial proteins was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cell precipitates of MPT63 expressed E. coli were resuspended in wash buffer (50 mM Na₂HPO₄, (pH 8.0), 0.5 M NaCl), which contains 6 M urea and treated on ice with ultrasonic disintegrator Labsonic (Sartorius, Germany) and centrifuged during 20 min at 13,000 rpm. The supernatant was applied to a pre-equilibrated Ni-NTA agarose (Qiagen, Germany) column and washed with 6 M urea buffer. Elution of purified proteins was conducted in buffer with 250 mM imidazole and 6 M urea. After purification recombinant protein was dialyzed in PBS (0.8% NaCl, 0.02% KCl, 0.144% Na₂HPO₄, 0.024% KH₂PO₄, pH 7.4).

MPT83full expressed E. coli cells were resuspended in PBS and were treated and centrifuged as described above. MPT83full protein extraction was carried out in non-denaturating conditions using wash buffer without urea reagent. SDS-PAGE and Western blotting. Protein molecules were separated by 10% SDS-PAA electrophoresis under denaturing condition in Tris-tricine buffer system. We used a buffer sample that contained β-mercaptoethanol for prevention of dimers formation and buffer without disulfide reduction components. Staining was performed using Coomassie Brilliant Blue G-250. Protein concentration was determined by densitometry using special computer program TotalLab TL120.

Protein transfer to nitrocellulose membrane was performed using semi-dry Western blot during 2 h and current strength 38 mA in the buffer which contained 25 mM Tris-HCl, pH 8.3, 20% methanol, 192 mM glycerine.

After transferring the membrane was blocked by 5% non-fat milk dissolved in PBS during 1 h at 37 °C. Then the membrane was washed three times by T-PBS (0.8% NaCl, 0.02% KCl, 0.144% Na₂HPO₄, 0.024% KH₂PO₄, 0.04% Tween-20, pH 7.4) and incubated with anti-His tag antibodies (1:3000) conjugated with horseradish peroxidase. Bands have been detected by 3,3′-Diaminobenzidine tetrahydrochloride (DAB) chromogenic substrate.

Flow cytometry and Phagocytosis assay. Peritoneal MΦ and macrophage-like cells from J774 cell line incubated in cell culture flasks (1×10⁶ cells/dish) with rMPT63, rMPT83 and mCherry (0.25×10⁶ mM) as a control for 0.5, 1, 2 and 24 h. After cultivation the macrophages were incubated with EGFP expressed E. coli cells during 30 min at 37 °C in the ratio of 100 bacterial cells per one macrophage. Cells were washed from not phagocytized bacteria and in PBS-BSA (0.8% NaCl, 0.02% KCl, 0.144% Na₂HPO₄, 0.024% KH₂PO₄, pH 7.4, 2% BSA). Samples were analyzed by flow cytometry (Beckman Coulter Epics XL) using FL1 channel laser and calibrator with 50 000 total events/sample.
The temperature, lack of sodium azide meet conditions for active uptake of EGFP expressed *Escherichia* by macrophages. Bacteria with the accumulated protein fluoresces actively even after the takeover by macrophages. Green laser channel was identified increase in fluorescence, which means an increase of phagocytized bacteria by a permanent amount of macrophages.

**Results and Discussion**

*Vector construction for rMPT83full expression.* Full mpt83 gene was amplified by PCR (Fig. 1, A) and cloned into vector for protein expression pET24a(+) (Novagen, Germany) by restriction sites BamHI and XhoI (Fig. 1, B). The number of copies of pET24a-mpt83(full) vector were amplified in *E. coli* DH10B strain. The mpt63 gene was cloned into pET24 vector previously [6].

*E. coli* Rosetta DE3 strain cells were transformed by pET24a-mpt83. Cells were grown on solid culture medium LB at 37 °C with addition of selective antibiotic kanamycin. Selected clones were tested for the presence of the correct inserted gene fragment by PCR (Fig. 1, C). The expected amplified fragment length was approximately 900 b.p.

*Expressed recombinant proteins obtaining and purification.* Selected clones of MPT83full and MPT63 expressed *E. coli* were induced with 1 mM IPTG and incubated for 4 h at 30 °C with intensive aeration. His-tag labeled proteins were isolated by IMAC on Ni-NTA agarose (Qiagen, the Netherlands). Elution was performed by competitive displacement of protein molecules with 250 mM imidazole solution in wash buffer under denaturing (MPT63) and non-denaturing (MPT83full) conditions. Final fractions had been analyzed by 10% SDS-PAGE (Fig. 2, A). Also, the electrophoretic mobility of the rMPT83full protein depends on the presence or absence of disulfide reduction component in sample buffer (Fig. 2, B).

The cloning of the full length rMPT83 mycobacterial protein was needed to study the effect of all sequence of the antigen, but not only FLD rMPT83(115-220) [6], on Mφ. Despite the lower yield of protein from 1 liter of *E. coli* culture, full-size MPT83 was identified in the soluble cytoplasmic fraction that gives it in correctly folded conformation. In contrast, FLD rMPT83 (115-220) was identified in insoluble inclusion bodies (Fig. 2, A). Furthermore, full-size MPT83 does not form dimers,
Fig. 2. Electrophoresis assay of novel obtained protein MPT83full. **A** – lane 1 and 2 – insoluble and soluble fractions of E. coli cell lysates, which contain non-purified MPT83full. Lane 3, 4 – MPT83full purified on Ni-NTA agarose under denaturing conditions. Lane 5, 6 – FLD rMPT83 obtained and purified under denaturing conditions. Samples 7, 8, 9 – BSA for protein concentration calibration. **B** – electrophoretic mobility of MPT83full within the sample buffer without (lane 1) and with (lane 2) disulfide bonds reduction reagent DTT. Different mobility of MPT83full provides existence of the inner disulfide bond unlike its short analog (Fig. 2, **B**; Fig. 4). This also proves the value feasibility of using the product of a full gene sequence in which the N’-terminal amino acids play a potentially important role in protein folding. Therefore, the action of FLD rMPT83 (115-220) on phagocytosis assay was not studied.

**Primary culture but not malignant macrophages have higher phagocytic activity after 24 h rMPT63 and rMPT83full stimulation.** For phagocytosis test we used a transformed green fluorescent protein E. coli, according to the protocol described in Gille et al [15]. Protocol has been tested on J774 cells. Phagocytosis level was estimated by flow cytometry in order to obtain data for the large number of cells.

After rMPT63 and rMPT83full stimulation the number of phagocytic peritoneal MΦ was increased. However, any significant differences in phagocytosis level for incubation period of 30 min, 1 or 2 h was not found. However after 24 h rMPT63 and rMPT83full stimulation of peritoneal MΦ the number of phagocytic cells was increased twice. The control proteins did not lead to such effect and the number of phagocytic cells was at the level of unstimulated cells (Fig. 3, **A**, **B**).

Unlike peritoneal murine MΦ the malignant macrophage-like cells from J774 cell line were not characterized by any increase in phagocytosis, even after 24 h of stimulation.

The goal of our study was to investigate by flow cytometry the quantitative parameters of phagocytosis of EGFP expressed E. coli cells by mouse MΦ under application of mycobacterium proteins to them. Flow cytometry allows analyzing a larger sample of cells compared with the conventional microscopic analysis [16, 17]. Furthermore, the method allows an objective quantitation of the phagocytic activity to give two parameters, such as phagocytic rate and index. The present results show that the phagocytic activity of cultured mouse peritoneal MΦ increased after the exposure to rMPT63 or rMPT83full. While preincubation of the cells with mCherry or EGFP showed a negligible change in the amount of actively phagocytic MΦ, we concluded that the effect of rMPT63 and rMPT83full was specific.

There are two potential ways to increase activity of phagocyte link of innate immunity of the host. First of them is the increase of the number of phagocytic cells which intake the same number of particles (e.g. EGFP expressed E. coli). Another way implies intensification of phagocytosis activity of each individual cell, which is belonging by constant rate to cell population. However, intensification of
Recombinant MPT63 and MPT83full induce active intake of EGFP expressed E. coli by peritoneal macrophages after 24 h preincubation. The level of bright luminous macrophages after EGFP expressed E. coli capture was observed by flow cytometry after exposure to MPT63 and MPT83 compared to control antigen. The percentage of actively phagocytic cells was increased 2 times for both mycobacterial antigens (A, B) compared with unstimulated macrophages and stimulated by mCherry.

Using flow cytometry we have shown not only the increase of the intensity, but also of the number of phagocytic cells, which grew twofold in both cases under the action of studied mycobacterial proteins. The percentage of stained cells increased to 19.1% after MPT63 treatment in comparison with 10.5% of intact (without treatment) cells and 10.9% which were in preincubation with mCherry and to 19.8% after MPT83full stimulation in comparison with 10.5% of intact cells and 10.9% which were pre-incubated with mCherry (Fig. 3). Furthermore, this effect was caused by long-term stimulation of
cells. Treatment of mouse \( M\Phi \) for 0.5, 1 and 2 h had no effect on the increase of phagocytic activity compared with control samples. This effect was caused only after 24 h of incubation, which was probably related to changes in gene expression than to release from the inner cell store molecules which had been involved in phagocytosis.

The fact that malignant mouse macrophage-like cells from J774 cell line did not respond to the effect of mycobacterium proteins observed in primary cultures of \( M\Phi \) is to be discussed. Some malignant cell lines have excessive activation, including phagocytic activity rate [18]. Therefore, we cannot detect changes in phagocytic activity of these cells under stimulation with mycobacterial proteins. Also, we suppose that these immunodominant mycobacterial proteins facilitate bacteria cell phagocytosis for their survival inside \( M\Phi \). This can represent one of the ways how bacteria could escape from the environment outside the cell in the organism, which has many factors of innate and adaptive immunity that can eliminate pathogen.

As a consequence of phagocytosis mycobacteria are able to grow and reproduce within phagocyte. Perhaps, one of the many actions of MPT63 and MPT83 could be directed precisely to such “specific” protecting of the pathogen from the host immune system. Of course, this is only the first evidence, and further study of these antigens is required.

Thus, obtained previously MPT63 and for the first time cloned and obtained MPT83full strengthen phagocytic function of immune system cells. The value of full-size antigen in comparison with its short fragment FLD rMPT83(115-220) obtained previously in our lab shows its identification in the soluble fraction, which indicates the correct folding of molecules in spite of a lower yield of new protein. The ability to induce phagocytosis by products of contamination of prokaryotic expression system (e.g. lipopolysaccharide (LPS), teichoic acids) were offset by no increase of phagocytosis after control proteins treatment despite the same plasmid DNA, where their genes were inserted and obtained and purified by the same methods as for MPT63 and MPT83. The investigated effect caused by the chosen mycobacterial antigens was observed in the primary culture, but not in malignant cells, probably due to genetic determinism over activation of the latter. The enhancing of bacteria phagocytosis by macrophages after the exposure to mycobacterial antigens can be explained as the deception of immune system by mycobacteria, seeking to be captured by phagocytes for future survival and reproduction within the host.

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Fig. 4. Dimers of FLD rMPT83 identified by Western blot with anti-HisTag-HRP conjugate

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cauised by the bacterial wall components, like other features of products from recombinant origin.

For many infection diseases \( M\Phi \) provide the last niches for pathogen degradation and elimination, whereas mycobacteria can evade and coexist with these cells of host organism. Tuberculosis bacilli affect \( M\Phi \) and persist in these cells avoiding endosome-lysosome fusion [3]. As a result, vesicles do not become acidic and bacteria survive.

The authors proposed that \( M\Phi \) were the first target of MPT63 and MPT83 proteins of mycobacteria and theirs receptor molecules are localized at the surface of these cells. Also, we suggest that these immunodominant mycobacterial proteins facilitate bacteria cell phagocytosis for their survival inside \( M\Phi \). This can represent one of the ways how bacteria could escape from the environment outside the cell in the organism, which has many factors of innate and adaptive immunity that can eliminate pathogen.

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Acknowledgments
Authors thank Andrii Labyntsev, Mykhailo Guzyk and Kyrilo Pyrshev for technical support with flow cytometry. This work was supported by the State order for scientific and technical products.

АНТИГЕНИ Mycobacterium tuberculosis МРТ63 ТА МРТ83 ПІДВИЩУЮТь ФАГОЦИТАРНУ АКТИВНІСТЬ ПЕРИТОНАЛЬНИХ МАКОФАГІВ МИШІ

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Макрофаги – найбільш описана та охарактеризована мішень та клітина-хозяїн для мікобактерій. І, як і іншими клітинам природного імунітету, макрофагам притаманний широкий спектр рецепторних молекул, що взаємодіють із різноманітними патогенассоційованими молекулярними паттернами. Імунодомінантні антигени МРТ63 та МРТ83, що продукуються в значній кількості в штамах Mycobacterium bovis та Mycobacterium tuberculosis, можуть бути залучені до розвитку інфекції. Целью нашого дослідження був пошук деяких ефектів цих мікобактеріальних антигенів на клітини-мишени. Для цього було клоновано повноразмерні антигени МРТ63 та МРТ83 в плазмідній ДНК pET24a (+). Показано зростання фагоцитарної активності макрофагів в перитонеальній полості миші, але не макрофагоподібних клітин лінії J774, які були стимульовані rМРТ63 та rМРТ83full протягом 24 год. Ефект цих антигенів можна розглядати як спосіб сприяння захоплення мікобактерій макрофагами для уникнення інших механізмів дії природного та набутого імунітету.

Ключові слова: Mycobacterium tuberculosis, Mycobacterium bovis, макрофаги, фагоцитоз, МРТ63, МРТ83.
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Received 29.08.2016