THE STUDY OF NF-κB TRANSCRIPTION FACTOR ACTIVATION BY *PSEUDOMONAS AERUGINOSA* RECOMBINANT PROTEINS IN EUKARYOTIC CELL CULTURE

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The transcription factor NF-κB is a key factor in the activation of immune responses; it is in turn activated by pattern recognition receptors, such as TLR and NLR receptors. The search for ligands activating innate immunity receptors is an important scientific problem due to the possibility of their use as adjuvants and immunomodulators. In this study the effect of recombinant *Pseudomonas aeruginosa* OprF proteins and a toxoid (a deletion atoxic form of exotoxin A) on the activation of TLR4, TLR9, NOD1, and NOD2 receptors has been investigated. The study was carried out using free and co-adsorbed on $Al(OH)_3$ *P. aeruginosa* proteins and eukaryotic cells encoding these receptors and having NF-κB-dependent reporter genes. The enzymes encoded by the reported genes are able to cleave the substrate with the formation of a colored product, the concentration of which indicates the degree of receptor activation. It was found that free and adsorbed forms of the toxoid were able to activate the TLR4 surface receptor for lipopolysaccharide. OprF and the toxoid activated the intracellular NOD1 receptor, but only in the free form. This may be due to the fact that the cell lines used were not able to phagocytize aluminum hydroxide particles with protein adsorbed on them.

Key words: *Pseudomonas aeruginosa*; TLR; NOD; NF-κB; recombinant proteins

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INTRODUCTION

The transcription factor NF-κB is one of the key components of intracellular signaling pathways responsible for the immune response in the human body; it is involved in the functioning of certain intracellular signaling pathways that regulate innate and adaptive immune responses [1–3]. The sphere of influence of NF-κB covers genes encoding of cytokines such as interleukins IL-1, IL-2, IL-6, IL-12, TNF-α, granulocyte-monocyte colony-stimulating factor GM-CSF, chemokines (such as IL-8, CCL3, CCL5, and CCL11), acute phase proteins, adhesion molecules, and inducible enzymes (e.g. iNOS, COX-2). In addition, NF-κB plays a critical role in regulating the survival, activation, and differentiation of innate immune cells and T cells involved in inflammation [1–3].

Various stimuli, such as cytokines and ligands of pattern recognition receptors and receptors of the TNF, TCR, and BCR superfamilies, cause NF-κB activation [1].

In this study we have investigated the effect of recombinant proteins of *Pseudomonas aeruginosa* OprF and a toxoid on pattern recognition receptors: (i) Toll-like receptors TLR4 (receptors for lipopolysaccharides) and TLR9 (ligand is CpG-rich DNA) [2, 3]; (ii) intracellular NOD1 and NOD2 receptors. Ligands for NOD1 and NOD2 are muropeptides, fragments of peptidoglycans containing structural motifs specific for bacteria, such as γ-D-glutamyl-meso-diaminopimelic and N-acetylmuramic acids [4, 5]. The results of recent studies have shown that other substances can also serve as ligands for these receptors [5].

The bacterium *P. aeruginosa* is one of the most important causative agents of opportunistic infections in modern clinical practice. The treatment of opportunistic infections is a serious problem due to the prevalence of antibiotic resistance among clinical isolates of this bacterium [6]. In this regard, studies of methods of vaccinal prevention of infections caused by *P. aeruginosa* are especially important [7].

Abbreviations used: ALR – AIM2-like receptors; AP-1 – activating protein-1; BCR – B-cell receptor; CLR – C-type lectin-like receptors (CLR); COX-2 – cyclooxygenase-2; EU – endotoxin units according to the International Standard for Endotoxin; EA – units of enzyme activity; MTT test – colorimetric test for assessing the metabolic activity of cells (from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide); iNOS – inducible nitric oxide synthase; NF-κB – nuclear factor kappa-beta; NLR – NOD-like receptor; OprF – outer membrane protein F of *Pseudomonas aeruginosa*; RLR – RIG-I-like receptors; SEAP – secreted embryonic alkaline phosphatase; TCR – T cell receptor; TLR – toll-like receptor; TNF – tumor necrosis factor.

Recently, in vaccinology, special attention has been paid to the mechanisms of activation of innate immunity by the action of vaccines [8]. The mechanisms of action of adjuvants including the adjuvant properties of bacterial proteins are currently studied by researchers [9].

The aim of this study was to investigate the activation of TLR4, TLR9, NOD1, and NOD2 receptors in eukaryotic cell culture by *P. aeruginosa* recombinant OprF and a toxoid (separately, together, and adsorbed on aluminum hydroxide). OprF is a protein with a mass of 38.9 kDa, and the toxoid is a deletion form of exotoxin A, which lacks the toxic activity, but causes the formation of neutralizing antibodies to exotoxin A, with a mass of 65.8 kDa [10–12].

MATERIALS AND METHODS

Cell Lines

The following cell lines purchased from InvivoGen (France) were used in the work:

1. Raw Blue cells are mouse macrophage leukemia cells induced by the Abelson virus. They contain a reporter gene encoding secretory embryonic alkaline phosphatase (SEAP) under the control of the NF-κB- and AP-1-dependent promoter; these cells express TLR-, NLR- and CLR-receptors;

2. HEK293-hTLR4-CD14/MD2 are human embryonic kidney cells containing the β-galactosidase gene under the control of the NF-κB/AP-1-dependent promoter and expressing the TLR4 receptor and CD14 and MD2 molecules, the components of the CD14/TLR4/MD2 receptor complex that recognizes lipopolysaccharide;

3. HEK293-TLR9 Blue cells are human embryonic kidney cells containing the SEAP reporter gene under the control of an NF-κB/AP-1-dependent promoter and expressing the TLR9 receptor;

4. HEK293-NOD1 cells are human embryonic kidney cells containing the β-galactosidase gene under the control of the NF-κB/AP-1-dependent promoter and expressing the NOD1 receptor;

5. HEK293-NOD2 are human embryonic kidney cells containing the SEAP gene under the control of the NF-κB/AP-1-dependent promoter and expressing the NOD2 receptor.

The recombinant forms of *P. aeruginosa* proteins were obtained in the Laboratory of Protective Antigens (I.I. Mechnikov Research Institute of Vaccines and Sera) and studied as vaccine candidates [10–12]. The complex of OprF and toxoid proteins adsorbed on an aluminum hydroxide gel showed protective properties exceeding those of individual proteins [13].

Protein concentrations in the studied preparations were similar to their content in the complex preparation. The content of aluminum hydroxide in the preparations of adsorbed proteins was also equivalent, 3 times higher than the protein concentration. The following compositions have been investigated:

1. OprF (50 μg/ml) + toxoid (100 μg/ml) in 0.9% NaCl saline solution;

2. Toxoid (100 μg/ml) in 0.9% NaCl saline solution;

3. OprF (50 μg/ml) in 0.9% NaCl saline solution;

4. OprF (50 μg/ml) + toxoid (100 g/ml) + + $Al(OH)_3$ (450 g/ml) in 0.9% NaCl saline solution;

5. Anatoxin (100 μg/ml) + $Al(OH)_3$ (300 μg/ml) in 0.9% NaCl saline solution;

6. OprF (50 μg/ml) + $Al(OH)_3$ (150 μg/ml) in 0.9% NaCl saline solution.

The corresponding ligands (all from InvivoGen) were used as a positive control confirming the functional competence of receptors and reporter genes:

1. Tumor necrosis factor TNF-α (10 ng/ml) for the control cell line HEK293-null and HEK293-null1-k;

2. The *Escherichia coli* serotype 0111:B4 lipopolysaccharide (1 μg/ml with an activity of 1×10^6 endotoxin units (EU) according to the International Endotoxin Standard/ml) for TLR4;

3. A synthetic oligonucleotide ODN 2006 (1 μg/ml) for TLR9;

4. A synthetic fragment of bacterial peptidoglycan C12-iE DAP (10 μg/ml) for NOD1;

5. L18 MDP (a muramyl dipeptide derivative 1 μg/ml) for NOD2; TDB (Trehalose-6,6-dibehenate) a synthetic analogue of trehalose-6,6-dimicolate from *Mycobacterium tuberculosis* (10 μg/ml) for Raw Blue cells.

The recombinant forms of the proteins were obtained in the Laboratory of Protective Antigens (I.I. Mechnikov Research Institute of Vaccines and Sera) using *E. coli* producer strains. The proteins were purified by metal-chelate affinity chromatography to a concentration of ~95% according to Laemmli polyacrylamide gel electrophoresis. The content of endotoxin, determined using the ENDOSAFE® ENDOCHROME™ LAL test (Charles River Laboratories, USA), did not exceed 35 EU/ml.

Thus, in all dilutions of the preparations used, the content of lipopolysaccharide was hundreds and thousands of times less than in the positive control (1000 EU/ml); this excludes the possibility of stimulation due to lipopolysaccharide contaminations.

Measurement of the Expression Level of Secretory Alkaline Phosphatase in Eukaryotic Cell Cultures

The cell lines were cultured on the DMEMx1 medium (Eagle's medium modified by Dulbecco) supplemented with 10% heat-inactivated fetal calf

serum, 4 mM L-glutamine, and the antibiotics streptomycin and penicillin (to a final concentration of 50 U/ml penicillin and 50 μg/ml streptomycin) .

Cells (5×10^4 for Raw Blue and 2.5×10^4 for HEK-NOD2 Blue and HEK-TLR9 Blue cells in 100 μl of the culture medium) were seeded in a 96-well culture plate. Test substances and controls were added to the cells 24 h after incubation in a CO_2 incubator (37°C, 5% CO_2). 24 h after these additions, the level of enzymatic activity of SEAP encoded by the NF-κB/AP-1-dependent gene was assessed by using a colorimetric method [14]. The method is based on measuring the rate of cleavage of a colorless substrate nitrophenyl phosphate disodium salt) with the formation of yellow *para*-nitrophenol. The color intensity of the solution, and, consequently, the optical density value, is directly proportional to the level of alkaline phosphatase enzymatic activity, which, in turn, is directly proportional to the level of activation of the transcription factor NF-κB in cells.

A freshly prepared solution of the substrate (4-nitrophenyl phosphate hexahydrate disodium salt; 2 mg/ml) in SEAP buffer (0.5 M $NaHCO_3$, 0.5 mM $MgCl_2$, pH 9.8) was added (150 μl) to each well of a new 96-well plate. Next, 50 μl of the medium was transferred from the cell culture plate to the assay buffer plate to measure SEAP activity. Immediately after the transfer of the medium, the optical density of the solution was measured in all samples at 405 nm (OD_0, T_0) using a BioTek Synergy H1 spectrophotometer (BioTek, USA). Next, the plate was incubated at 37°C for 10-60 min and the optical density was measured at a 405 nm until the positive control reached an optical density value of 1.8 and higher (OD_1, T_1).

Alkaline phosphatase activity was calculated by the formula:

$$EA = \frac{OD_1 - OD_0}{t_1 - t_0} \times \frac{K_{dil}}{K_{un}},$$

where EA — enzyme activity units, OD_0 — optical density at the first measurement, OD_1 — optical density at the last measurement, t_0 — time of the first measurement, t_1 — time of the last measurement, K_{dil} — dilution factor of the medium in the wells equal to 20, K_{un} – coefficient for 1 milliunit, defined as the amount of phosphatase that hydrolyzes 1 pmol of 4-nitrophosphate per minute, which corresponds to 0.04 mU per minute.

Measurement of the Expression Level of β-Galactosidase in Eukaryotic Cell Cultures

Cell lines HEK293-hTLR4-CD14-MD2 and HEK293-NOD1 were seeded (2.5×10^4 cells per well in 100 μl of culture medium) in a 96-well culture plate. After the 24 h of incubation (at 37°C and 5% CO_2), test samples and controls were added to the cells and 24 h after the additions, the activity of β-galactosidase encoded by the NF-κB/AP-1 responsive was measured. For β-galactosidase assay the culture medium was taken from the wells of the plate, and then a buffer with a β-galactosidase substrate (1 mM $MgCl_2$; 0.25 M Tris-HCl, pH 7.4; 0.02% NP40; 2 mg/ml *o*-nitrophenyl-β-D-galactopyranoside) was added. The level of β-galactosidase activity was measured on a BioTek Synergy H1 spectrophotometer at 405 nm by altered degree of the substrate color intensity in the wells, without calculating enzyme activity units.

Cells that do not express TLR and NOD receptors but contain the reporter SEAP gene (HEK293-null1-k) or the β-galactosidase gene (HEK293-null2) were used as controls confirming the specificity of receptor activation.

The MTT Test

The toxic effect of tested substances, which may be the cause of non-specific activation of NF-κB and, as a result, an increase in the expression level of β-galactosidase or SEAP, was determined using a quantitative colorimetric analysis determining cell survival (the MTT test using 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide). Cell viability analysis was carried out according to the standard procedure for the reduction reaction of pale yellow MTT to blue-violet formazan. The optical density was measured on a spectrophotometer using 540 nm and 620 nm filters. The results were calculated by the formula:

$$X = \frac{(OD_{540} - OD_{620})_{experimental}}{(OD_{540} - OD_{620})_{intact}} \times 100,$$

where X is the proportion of surviving cells, %.

The significance of differences between the compared values was determined by nonparametric basic statistics using the Mann-Whitney U-test. Differences were considered as statistically significant at $p \le 0.05$.

RESULTS AND DISCUSSION

The results of the study have shown that at the concentrations studied the tested substances had no effect on the HEK293-null1-k and HEK293-null2 control cell lines, which do not express TLR- and NOD-receptors, but contain the SEAP or β-galactosidase genes, respectively. At the same time, the reliability of the results is confirmed by the activation of the nuclear factor NF-κB during the interaction of the classical ligand, TNF-α, with its own TNF receptor on the surface of control cell lines (Tables 1, 2).

Table 1. Activation of NF-κB/AP-1-dependent SEAP in HEK293-null1-k cells by *P. aeruginosa* OprF and the toxoid

Additions	Dilutions		
	1:10	1:100	1:1000
	Enzyme activity (EA)		
OprF + toxoid	0.76±0.02 *p*=0.238	0.70±0.01 *p*=0.402	0.72±0.03 *p*=0.264
Toxoid	0.73±0.03 *p*=0.248	0.73±0.03 *p*=0.412	0.72±0.03 *p*=0.451
OprF	0.91±0.28 *p*=0.460	0.67±0.03 *p*=0.589	0.73±0.12 *p*=0.587
OprF + toxoid, adsorbed on $Al(OH)_3$	0.70±0.04 *p*=0.531	0.74±0.08 *p*=0.481	0.70±0.07 *p*=0.556
Toxoid, adsorbed on $Al(OH)_3$	0.72±0.001 *p*=0.360	0.68±0.01 *p*=0.539	0.66±0.01 *p*=0.565
OprF, adsorbed on $Al(OH)_3$	0.67±0.01 *p*=0.505	0.74±0.11 *p*=0.530	0.64±0.03 *p*=0.752
Positive control (TNF-α, 10 ng/ml)	9.11±0.10 *p*=0.007		
Negative control (without stimulation)	0.60±0.24		

Table 2. Activation of NF-κB/AP-1-dependent β-galactosidase in HEK293-null2 cells by *P. aeruginosa* OprF and the toxoid

Additions	Dilutions		
	1:10	1:100	1:1000
	Enzyme activity (OD_{405})		
OprF + toxoid	1.84±0.16 *p*=0.643	1.86±0.04 *p*=0.439	1.82±0.02 *p*=0.919
Toxoid	1.84±0.01 *p*=0.719	1.86±0.04 *p*=0.674	1.81±0.03 *p*=0.991
OprF	1.71±0.10 *p*=0.229	1.79±0.01 *p*=0.804	1.81±0.06 *p*=0.920
OprF + toxoid, adsorbed on $Al(OH)_3$	1.80±0.10 *p*=0.200	1.79±0.01 *p*=0.734	1.79±0.01 *p*=0.831
Toxoid, adsorbed on $Al(OH)_3$	1.70±0.05 *p*=0.482	1.82±0.03 *p*=0.911	1.78±0.02 *p*=0.648
OprF, adsorbed on $Al(OH)_3$	1.78±0.08 *p*=0.833	1.80±0.02 *p*=0.872	1.79±0.12 *p*=0.899
Positive control (TNF-α, 10 ng/ml)	10±0.20 *p*=0.006		
Negative control (without stimulation)	1.81±0.10		

The first stage of the work was to study the effect of the tested substances on NF-κB/AP-1 activation on Raw Blue mouse macrophage leukemia cells expressing many pattern recognition receptors and the SEAP reporter gene under the control of NF-κB and an AP-1-dependent promoter.

Results of our experiments have shown (Table 3) that all the tested substances at a concentration of 1:10 promote a pronounced expression of NF-κB/AP-1-dependent SEAP compared with the control (0.97±0.03 EA), but do not affect expression of NF-κB/AP-1 at a concentration of 1:1000. It should be noted that OprF+toxoid (1.34±0.11 EA; *p*=0.043), OprF (1.30±0.05 EA; *p*=0.000), OprF+toxoid+ aluminum hydroxide (1.84±0.07 EA; *p*=0.003), anatoxin+aluminum hydroxide (1.39±0.04 EA; *p*=0.005), and OprF+aluminum hydroxide (1.53±0.02 EA; *p*=0.001) were also active at a concentration 1:100. The complex of two proteins OprF+toxoid adsorbed on aluminum hydroxide exhibited the greatest effect (a 1.9-fold increase).

In order to confirm the specific activation of nuclear factors induced by the tested substances, their toxic effect on Raw Blue cells was studied. A dilution of 1:10 can be considered as toxic due to the pronounced toxic effect of all substances (Table 4), so the activating effect of drugs at this dose can be considered nonspecific.

Table 3. Activation of NF-κB/AP-1-dependent SEAP in Raw Blue cells by *P. aeruginosa* OprF and the toxoid

Additions	Dilutions		
	1:10	1:100	1:1000
	Enzyme activity, EA		
OprF + toxoid	5.6±0.37 *p*=0.003	1.34±0.11 (↑ 1.38-fold) *p*=0.043	0.85±0.01 *p*=0.007
Toxoid	4.69±0.22 *p*=0.002	1.06±0.07 *p*=0.270	0.82±0.04 *p*=0.001
OprF	4.84±0.02 *p*=0.000	1.3±0.05 (↑ 1.34-fold) *p*=0.000	0.89±0.03 *p*=0.078
OprF + toxoid, adsorbed on $Al(OH)_3$	3.62±0.15 *p*=0.002	1.84±0.07 (↑ 1.9-fold) *p*=0.003	1.15±0.05 *p*=0.045
Toxoid, adsorbed on $Al(OH)_3$	2.56±0.11 *p*=0.002	1.39±0.04 *p*=0.005	0.96±0.05 *p*=0.719
OprF, adsorbed on $Al(OH)_3$	1.90±0.03 *p*=0.000	1.53±0.02 (↑ 1.58-fold) *p*=0.001	0.94±0.19 *p*=0.822
Positive control (TDB, 10 μg/ml)	4.13±0.19 *p*=0.002		
Negative control (without stimulation)	0.97±0.03		

Table 4. Evaluation of the toxic action of *P. aeruginosa* OprF and the toxoid on survival of Raw Blue cells evaluated using the MTT test

Additions	Dilutions		
	1:10	1:100	1:1000
	Cell survival, %		
OprF + toxoid	33.1±0.71 *p*=0.005	56.8±0.63 *p*=0.014	109±12.8 *p*=0.250
Toxoid	44.4±0.7 *p*=0.008	92.4±0.07 *p*=0.926	135±3.46 *p*=0.013
OprF	48.4±2.69 *p*=0.011	118±6.36 *p*=0.050	131±1.06 *p*=0.012
OprF + toxoid + $Al(OH)_3$	45.2±2.33 *p*=0.009	110±7.35 *p*=0.123	112±11.38 *p*=0.171
Toxoid + $Al(OH)_3$	44.6±0.78 *p*=0.008	64±5.30 *p*=0.036	92±7.14 *p*=0.899
OprF + $Al(OH)_3$	77.6±9.62 *p*=0.197	103±2.80 *p*=0.404	125±2.83 *p*=0.020
Negative control (intact cells)	93±6.08		

During the next stage, the effect of the tested substances on cells carrying certain pattern-recognizing receptors was evaluated (Tables 5–8).

The tested substances promoted a pronounced activation through TLR4 at a toxic dose of 1:10. In a 1:100 dilution, the data were only significant for the toxoid and toxoid with aluminum hydroxide.

In the range of studied concentrations, no pronounced activation during interaction with TLR9 was detected, the data were not statistically significant.

Studying the interaction with the NOD1 receptor, statistically significant data were obtained for the OprF proteins, the toxoid, and their combination at a dilution of 1:100; the increase in activation was the most pronounced in the case of the toxoid. The action of OprF+anatoxin and OprF was also noted at a dilution of 1:1000. Proteins in the complex with aluminum hydroxide did not cause NOD1 activation.

Activation of the NOD2 receptor by the studied drugs was not detected (data were not statistically significant).

The innate immune system provides the first line of defense against microbial pathogens; it is mediated by phagocytes such as macrophages and dendritic cells. These cells recognize microbial infection, engulf pathogens, and trigger inflammatory responses. Activation of these cells by ligand stimulating pattern recognition receptors is the initial and very important stage of the immune response, which determines its further features and direction [3].

Table 5. Activation of NF-κB/AP-1-dependent β-galactosidase in HEK293-hTLR4-CD14/MD2 cells by *P. aeruginosa* OprF and the toxoid

Additions	Dilutions		
	1:10	1:100	1:1000
	Enzyme activity (OD_{405})		
OprF + toxoid	0.82±0.008 *p*=0.000	0.53±0.09 *p*=0.084	0.38±0.05 *p*=0.232
Toxoid	0.76±0.03 *p*=0.002	0.50±0.05 *p*=0.036	0.43±0.04 *p*=0.059
OprF	0.59±0.04 *p*=0.009	0.46±0.06 *p*=0.103	0.38±0.09 *p*=0.457
OprF + toxoid + $Al(OH)_3$	0.85±0.04 *p*=0.004	0.42±0.04 *p*=0.089	0.39±0.03 *p*=0.087
Toxoid + $Al(OH)_3$	0.68±0.007 *p*=0.000	0.47±0.03 *p*=0.019	0.38±0.01 *p*=0.038
OprF + $Al(OH)_3$	0.48±0.01 *p*=0.005	0.43±0.08 *p*=0.212	0.41±0.07 *p*=0.219
Positive control (*E. coli* lipopolysaccharide, 1,0 μg/ml)	0.83±0.004 *p*=0.002		
Negative control (without stimulation)	0.33±0.001		

Table 6. Activation of NF-κB/AP-1-dependent SEAP in HEK-TLR9 Blue cells by *P. aeruginosa* OprF and the toxoid

Additions	Dilutions		
	1:10	1:100	1:1000
	Enzyme activity, EA		
OprF + toxoid	0.99±0.01 *p*=0.074	0.83±0.01 *p*=0.497	0.72±0.16 *p*=0.759
Toxoid	0.78±0.003 *p*=1.000	0.81±0.04 *p*=0.718	0.87±0.02 *p*=0.291
OprF	0.96±0.02 *p*=0.104	0.80±0.02 *p*=0.782	0.76±0.005 *p*=0.771
OprF + toxoid + $Al(OH)_3$	0.77±0.008 *p*=0.883	0.79±0.008 *p*=0.884	0.87±0.009 *p*=0.276
Toxoid + $Al(OH)_3$	0.99±0.008 *p*=0.074	0.85±0.08 *p*=0.556	0.80±0.07 *p*=0.848
OprF + $Al(OH)_3$	0.81±0.05 *p*=0.738	0.83±0.06 *p*=0.597	0.93±0.07 *p*=0.245
Positive control (ODN 2006, 1,0 μg/ml)	2.05±0.32 *p*=0.050		
Negative control (without stimulation)	0.78±0.06		

Cells of the innate immune system, such as macrophages, dendritic cells, and neutrophils, express pattern recognition receptors (PRRs). These receptors detect conserved microbial components, known as pathogen-associated molecular structures (PAMPs), as well as damage-associated molecular structures (DAMPs). DAMPs are molecules secreted by necrotic cells and damaged tissues. Mammalian cells express various PRRs, which are currently divided into five families based on structural homology: TLR, RLR, NLR, CLR, and ALR [15]. Different families of PRRs have different structural properties and respond to different ligands, but share common high similarity in downstream signaling pathways. These include activation of the canonical NF-κB pathway, which is responsible for the transcriptional induction of pro-inflammatory cytokines, chemokines, and additional inflammatory mediators in various types of innate immune cells [4, 16–18].

One of the most important sets of pattern recognition receptors is the family of Toll-like receptors. In humans it consists of 11 proteins, which initiate intracellular signaling cascades during cell exposure to certain substances characteristic of pathogenic microorganisms. The other important family of pattern recognition receptors is the family of NOD-like receptors. In humans it consists

Table 7. Activation of NF-κB/AP-1-dependent β-galactosidase in HEK293-NOD1 cells by *P. aeruginosa* OprF and the toxoid

Additions	Dilutions		
	1:10	1:100	1:1000
	Enzyme sctivity (OD_{405})		
OprF + toxoid	0.67±0.08 *p*=0.305	0.72±0.01 *p*=0.007	0.77±0.07 *p*=0.097
Toxoid	0.86±0.13 *p*=0.148	0.82±0.09 *p*=0.002	0.58±0.07 *p*=0.243
OprF	0.74±0.04 *p*=0.048	0.72±0.001 *p*=0.004	0.73±0.008 *p*=0.005
OprF + toxoid + $Al(OH)_3$	0.72±0.06 *p*=0.119	0.72±0.16 *p*=0.423	0.52±0.13 *p*=0.788
Toxoid + $Al(OH)_3$	0.79±0.17 *p*=0.309	0.71±0.16 *p*=0.448	0.6±0.002 *p*=0.059
OprF + $Al(OH)_3$	0.61±0.02 *p*=0.039	0.58±0.30 *p*=0.591	0.55±0.10 *p*=0.899
Positive control (Cl12 DAP, 10.0 μg/ml)	0.98±0.10 *p*=0.001		
Negative control (without stimulation)	0.56±0.01		

Table 8. Activation of NF-κB/AP-1-dependent SEAP in HEK293-NOD2 cells by *P. aeruginosa* OprF and the toxoid

Additions	Dilutions		
	1:10	1:100	1:1000
	Enzyme activity, EA		
OprF + toxoid	0.65±0.05 *p*=0.685	0.66±0.08 *p*=0.843	0.66±0.08 *p*=0.843
Toxoid	0.71±0.02 *p*=0.571	0.81±0.002 *p*=0.083	0.68±0.002 *p*=1.000
OprF	0.79±0.006 *p*=0.112	0.67±0.01 *p*=0.831	0.66±0.008 *p*=0.672
OprF + toxoid + $Al(OH)_3$	0.95±0.02 *p*=0.026	0.83±0.09 *p*=0.267	0.78±0.02 *p*=0.155
Toxoid + $Al(OH)_3$	0.79±0.07 *p*=0.305	0.71±0.09 *p*=0.789	0.74±0.12 *p*=0.682
OprF + $Al(OH)_3$	0.71±0.07 *p*=0.774	0.72±0.09 *p*=0.724	0.68±0.02 *p*=1.000
Positive control (L18 MDP, 1.0 μg/ml)	1.84±0.15 *p*=0.017		
Negative control (without stimulation)	0.68±0.04		

of 23 proteins which are located in cytoplasm and recognize certain structural motifs of pathogens; NOD-like receptors play a role in the activation of immune cells [5, 17–20]. Different families of pattern recognition receptors act synergistically, functionally complementing each other [3].

The study of the processes of cell activation by means of pattern-recognizing receptors and the search for ligands stimulating them is an urgent scientific problem. Certain evidence exists that one receptor can be stimulated by various ligands differing in structure and origin: for example, in the case of TLR4 they can interact not only with lipopolysaccharides, but also with *P. aeruginosa* proteins including its outer membrane proteins, ExoS toxin, alginate [2, 18].

In the present work, using a mouse macrophage leukemia cell line as an example, it has shown that recombinant *P. aeruginosa* proteins are able to activate NF-κB signaling pathways in myeloid cells and the addition of an aluminum hydroxide adjuvant enhanced this effect.

Next, we have investigated their influence on some individual pattern-recognition receptors. At a (toxic) 1:10 dilution, all studied drugs activated TLR4. Since the toxoid dose was higher than OprF, it was impossible

to evaluate, which of the proteins had a stronger effect. At the same time, the effect of adsorption of these proteins on aluminum hydroxide was not revealed. At a non-toxic dilution of 1:100, the toxoid had similar properties, while the adjuvant $Al(OH)_3$ did not enhance these properties.

The stimulating effect of toxoid proteins, OprF and their combination on the intracellular NOD1 receptor was also noted; however, no additive effect was found. In the form adsorbed on aluminum hydroxide, these proteins did not stimulate NOD1. This may be due to the fact that the adsorption of proteins reduces their solubility in water, while HEK293 cells, being non-phagocytic due to their non-hematopoietic origin [21], do not absorb $Al(OH)_3$ particles with adsorbed proteins. Interestingly, OprF and its combination with the toxoid were able to activate NOD1 even at a 1:1000 dilution.

Stimulation of TLR9 and NOD2 receptors was not detected in the present study. Thus, the data obtained suggest that *P. aeruginosa* proteins are capable of activating the membrane receptor TLR4 and the intracellular NOD1 receptor.

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COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any research involving humans or using animals as experimental objects.

CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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ИССЛЕДОВАНИЕ АКТИВАЦИИ ТРАНСКРИПЦИОННОГО ФАКТОРА NF-κB РЕКОМБИНАНТНЫМИ БЕЛКАМИ *PSEUDOMONAS AERUGINOSA* В КУЛЬТУРЕ ЭУКАРИОТИЧЕСКИХ КЛЕТОК

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Транскрипционный фактор NF-κB — ключевой фактор активации иммунных реакций, который, в свою очередь, активируется через ряд паттерн-распознающих рецепторов, таких как TLR- и NLR-рецепторы. Поиск лигандов, активирующих рецепторы врождённого иммунитета, является актуальной научной задачей в связи с возможностью их использования в качестве адъювантов и иммуномодуляторов. Исследовали влияние рекомбинантных белков *Pseudomonas aeruginosa* OprF и анатоксина (делеционной атоксической формы экзотоксина A) на активацию рецепторов TLR4, TLR9, NOD1 и NOD2. Для этого использовали свободные и совместно адсорбированные на $Al(OH)_3$ белки и эукариотические клетки, кодирующие данные рецепторы и имеющие NF-κB-зависимые репортерные гены. Кодируемые ими ферменты способны расщеплять субстрат с образованием окрашенного продукта, концентрация которого указывает на степень активации рецептора. Было выявлено, что анатоксин в свободном и адсорбированном виде способен активировать поверхностный рецептор к липополисахариду TLR4. OprF и анатоксин активировали внутриклеточный рецептор NOD1, но только в свободном виде. Это может быть связано с тем, что использованные клеточные линии не были способны фагоцитировать частицы гидроксида алюминия с адсорбированным на них белком.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: *Pseudomonas aeruginosa*; TLR; NOD; NF-κB; рекомбинантные белки

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