Recombinant Plasmid Constructs Expressing Gene for Antimicrobial Peptide Melittin for the Therapy of Mycoplasma and Chlamydia Infections

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In view of growing number of pathogenic microbial strain resistant to routine antibiotics, antimicrobial peptides become promising agents for the therapy of infectious diseases. We studied *in vivo* effects of melittin, an antimicrobial peptide expressed in a recombinant plasmid vector, on infection with urogenital pathogens *Chlamydia trachomatis*, *Mycoplasma hominis,* and *Mycoplasma gallisepticum*. We obtained recombinant plasmid constructs, where melittin gene is under the control of tetracycline-dependent human cytomegalovirus promoter. Inhibition of experimental *C. trachomatis*, *M. hominis*, and *M. gallisepticum* infection after administration of recombinant plasmid vectors expressing melittin gene to BALB/c mice was demonstrated.

Key Words: *recombinant plasmid vectors; antimicrobial peptides; Mycoplasma hominis; Chlamydia trachomatis; Mycoplasma gallisepticum*

The resistance of various infectious agents to antibiotics continuously grows in the world during recent decades. The development of antimicrobial resistance is a natural biological response to wide use of antibiotics promoting selection, survival, and propagation of resistant strains of microorganisms [7].

The resistance to antibiotics is a great social and economic problem and is considered as a national security threat for each country and for the world community. Infections caused by resistant strains usually run a long-term course, more often require hospitalization, prolong the terms of hospital treatment, and worsen the prognosis. Inefficiency of preparations of choice necessitates the use of second and third line drugs, which are usually more expensive, less safe, and not always available. This increases the direct and indirect costs and the risk of spreading of the resistant strains in the world.

Recent attention of scientists and biotechnological companies was focused on antimicrobial peptides (AMP) as an alternative to antibiotics. AMP is a unique and very diverse group of compounds, the main component of innate immunity in all living organisms from fungi to vertebrates. AMP exhibit a wide range of activities against gram-positive and gram-negative bacteria, viruses, fungi; they are produced by various tissues as components of antimicrobial factors, which act synergistically against the infection *in vivo*. AMP also mediate signaling between the innate and adaptive immune systems: they actively modulate induction of proinflammatoiry cytokines and chemokines, activation and degranulation of mast cells, activation of macrophages, neutrophils, monocytes, and dendrite cells [3,12].

More than 1000 AMP from various sources are now identified. They are peptides produced by in-

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vertebrate, plant, and animal cells, main cytokines and chemokines, some neuropeptides and hormones, and fragments of high-molecular-weight proteins. AMP database is steadily grows, especially at the expense of fragments of high-molecular-weight proteins.

Leading pharmacological companies develop AMP-based drugs for the treatment of infectious skin diseases, trophic ulcers, and oral cavity infections. Some of these drugs have now entered stage III clinical trials [6]. However, despite evident advances of AMP, the use of artificially synthesized or isolated from various sources peptides in clinical practice is limited. The main obstacles are high cytotoxic activity for humans and animals and low selectivity of AMP [4].

This problem can be solved via using recombinant genetic constructs carrying AMP genes, which are characterized by pronounced bactericidal activity and can selectively eliminate pathogens from the host organism. The proposed approach is based on introduction of recombinant constructs expressing AMP genes (on the programmed basis) into the infected organism. Thus, we propose to replace the concentration influence of AMP on the target by their local targeted influence. The efficiency of this approach was demonstrated in experiments with recombinant plasmid vectors (pPV) expressing gene of melittin, one of AMP [1].

Here we studied *in vivo* inhibition of experimental *Chlamydia trachomatis, Mycoplasma hominis* and *Mycoplasma gallisepticum* infections in laboratory animals.

MATERIALS AND METHODS

Synthetic oligonucleotides (78 b.p.) corresponding to the sequence of melittin gene (GenBank X02007) were cloned by the restriction site PvuII in PV pBI-EGFP (BD Biosciences Clontech) containing 2 minimal inducible promoters of human cytomegalovirus and gene encoding green fluorescing protein (GFP). This PV was named pBI/mel2. Then the gene encoding rtTA transactivator protein was amplified using PV pTet-On (BD Biosciences Clontech) as the template and cloned by sites BglII in pV pBI/mel2. Thus, the PV named pBI/mel2/rtTA (Fig. 1) consisted of rtTA transactivator protein gene under the control of constitutive early promoter of human cytomegalovirus (CMV), genes of melittin and *gfp* under the control of CMV inducible minimal promoters, and tetracycline-responsive element TRE. Transactivator protein rtTA is a protein consisting of tetracycline repressor protein (TetR, a sequence from tetracycline-resistant operon of *E. coli* transposon Tn10) and activator domen of VP16 protein of herpes simplex virus. rtTA binds to the sequence of TRE element and activates transcription in the presence of tetracycline.

Mycoplasma hominis isolate 1862.3 (minimum inhibitory concentration of doxycycline 1 mg/liter) kindly provided by A. E. Taraskina (D. O. Ott Research Institute of Obstetrics and Gynecology, Russian Academy of Medical Sciences, St Petersburg) was cultured in 3 passages in PPLO medium containing arginine before using for infection of experimental mice.

Chlamydia trachomatis (strain D/UW-3/Cx, ATCC VR-885, minimum inhibitory concentration of doxycycline 0.08 mg/liter) kindly provided by Eva Hjelm (Uppsala University, Sweden) was cultured in McCoy cells. McCoy cells were grown in MEM containing 10% FCS and 2 g/liter glucose. *C. trachomatis* elementary bodies were purified by ultracentrifugation in urografin density gradient.

M. gallisepticum virulent strain 1226 (minimum inhibitory concentration of doxycycline 2 μg/ml) was grown in medium B containing 10% inactivated equine serum (Life Technologies) until the middle exponential phase determined by the change in indicator color in culture medium. The number of viable mycoplasma in the suspension was determined after seeding 10-fold dilutions to a culture medium containing 1% agar.

The animals were divided into 3 groups. Group 1 animals were infected with *M. hominis, C. trachomatis, M. gallisepticum* without treatment with PV pBI/mel2/rtTA and doxycycline. Group 2 animals received doxycycline in the corresponding dose followed by infection with *M. hominis, C. trachomatis* or *M. gallisepticum.* Group 3 animals received PV pBI/mel2/rtTA and doxycycline and then were infected with the corresponding infectious agent.

For infection with *M. hominis* and *C. trachomatis* we used 6-8-week-old female BALB/c mice weighing 18-22 g.

Estradiol (Intervet UK) in a dose of 0.5 mg/mouse was injected subcutaneously in a volume of 0.1 ml (4 injections one time per week). Progesterone (Depo-Provera, Upjohn) in a dose of 2.5 mg/mouse was injected subcutaneously in a volume of 0.1 ml (4 days before *C. trachomatis* injection).

Suspension of *M. hominis* (titer 10^9 cell/ml) was injected intravaginally in a volume of 50 μl after the 2nd injection of estradiol (each group comprised 6 mice; 2 independent experiments).

The fraction of *C. trachomatis* elementary bodies (titer 10^6 U/ml was injected intravaginally in a volume of 50 μl after injection of progesterone

TABLE 1. Effect of rPV pBI/mel2/rtTA on Dynamics of *M. hominis* Isolation from the Vagina of Infected Mice (lg/ml)

Group	Day of experiment			
	7	14	21	28
1	5.9	3.8	4.1	2.4
2	6.8	4.3	4.3	3.1
3	4.1	2.5	2.5	1.8

Note. *$p<0.01$ compared to groups 1 and 2.

(each group comprised 6 mice; 2 independent experiments).

For infection with *M. gallisepticum* we used 21-day-old Ross broiler chickens (Babolna Agriculture Company).

The chickens (each group consisted of 15 birds, 2 independent experiments) were one-by-one placed into a 0.0224 m^3 aerosol chamber and infected with 10 ml *M. gallisepticum* culture (titer 3×10^8 cells/ml). Each inoculate was sprayed over 2 min (particle size 7-10 μ)

rPV pBI/mel2/rtTA was administered to animals infected with *M. hominis* and *C. trachomatis* intravaginally using Effectene Transfection Reagent (Qiagen GmbH) twice: 24 h before infection *M. hominis* or *C. trachomatis* and 14 h after infection in a dose of 2 μg DNA per mouse in a volume of 25 μl with 25 μl cacao oil for increasing suspension viscosity.

To animals infected with *M. gallisepticum*, rPV pBI/mel2/rtTA was administered in aerosol using Effectene Transfection Reagent (Qiagen GmbH) in culture medium (50 μg DNA per 10 ml inoculate).

For evaluation of the titer of *M. hominis* after administration of rPV pBI/mel2/rtTA, 10-fold dilutions of urogenital tract washout fluid (to a concentration of 10^{-8} in 1 ml culture medium) were incubated at 37°C. The highest dilution when the color of the culture medium changed was taken as the titer of *M. hominis* culture.

For evaluation of the titer of *C. trachomatis* using the reaction of direct fluorescence, samples of vaginal washout fluid from infected mice were added to McCoy cell culture, centrifuged for 1 h at 3000*g* at room temperature, and incubated at 37°C for 48-72 h. The reaction of direct immunofluorescence was carried out with monoclonal antibodies to the major outer membrane protein conjugated with fluorescein (Orion Diagnostica). The number of chlamydial inclusions was counted under a Lyumam luminescent microscope (LOMO) at ×900 in at least 50 fields of view.

For evaluation of the titer of *M. gallisepticum*, the chickens were autospied and smears from the trachea, air sacs, liver, lungs, spleen, kidney, and heart were obtained. The material was incubated for 7 days on dishes with agarized medium B and the colonies were counted.

RESULTS

After administration of rPV pBI/mel2/rtTA followed by *M. hominis* infection we observed inhibition of the infection (Table 1). The titer of *M. hominis* in vaginal washout fluid from group 1 mice decreased from 5.9 to 2.4 lg cells/ml over 4 weeks. At the same time, in group 3 mice the titer of *M. hominis* varied from 4.1 to 1.8 lg cells/ml.

After administration of rPV pBI/mel2/rtTA followed by *C. trachomatis* infection, the percent of inhibition was 45-80% (Table 2).

Although the mice were not completely free from mycoplasma and clamydia within the observation period, the rate of elimination of the infectious agent in group 3 was higher than in the control groups. For instance, three mice of group 3 infected with *M. hominis* were free from the agent by day 21 after infection, while in groups 1 and 2 *M. hominis* were isolated from all mice. Four mice infected with *C. trachomatis* in group 3 were free from the infection by day 27.

It should be noted that we found no significant differences in the titers between groups 1 and 2 mice infected with *M. hominis* or *C. trachomatis,* which attests, first, to the absence of uncontrolled

TABLE 2. Effect of Treatment with rPV pBI/mel2/rtTA on Dynamics of *C. trachomatis* Isolation from the Vagina of Infected Mice (Number of Inclusions per 1 ml)

Group	Day of experiment						
	2	6	9	13	16	20	27
1	12 950	8490	4250	5220	2510	1070	1570
2	12 600	9750	3930	4850	2140	980	1470
3	6850	2710	1920	2090	1080	370	350

Note. *$p<0.05$ compared to groups 1 and 2.

expression of the melittin gene, and second, that the chosen concentration of the inductor (doxycycline) had no effect on the development of the infectious process.

Moreover, additional treatment with rPV pBI/mel2/rtTA 14 days after infection and after 28 days (data not shown) produced no significant inhibition of *M. hominis* and *C. trachomatis* infection. This was probably related to decreased efficiency of rPV de-livery into the infected cell [10].

We also observed inhibition of *M. gallisepticum* infection after aerosol administration of rPV. In group 1 chickens, *M. gallisepticum* was successfully isolated from 36 of 42 samples of the respiratory tract organs (trachea, lungs, and air sacs, Table 3) and from 18 of 56 samples from visceral organs (liver, spleen, kidney, heart). At the same time, in group 3 chickens *M. gallisepticum* was isolated from 30 of 42 samples of the respiratory organs and from only 6 of 56 samples of visceral organs. In none chicken of this group *M. gallisepticum* was isolated from the liver, spleen, and heart. The frequencies of *M. gallisepticum* isolation from respiratory and visceral organs of groups 1 and 2 chickens were similar.

We previously showed inhibition of *M. hominis* infection in HeLa/Tet-On and HeLa/Tet-Off cells transfected with rPV expressing melittin gene under the control of tetracycline CMV promoter [8]. However, the gene encoding transactivator protein rtTA in this case was constitutively expressed in HeLa/Tet-On and HeLa/Tet-Off cells. The use of these plasmid constructs *in vivo* requires simultaneous introduction of two rPV containing AMP gene and transactivator protein gene, respectively,

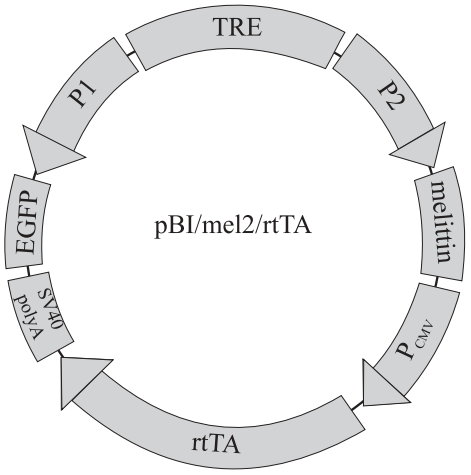

Fig. 1. Scheme of rPV expressing melittin gene. Melittin: melittin gene; P_{CMV}: constitutive human CMV promoter; P1 and P2: inducible human CMV promoters; SV40 polyA: polyadenylation signal; EGFP: GFP gene; TRE: tetracycline-responsive element; rtTA: transactivator protein.

which is associated with a decrease in the efficiency of vector delivery because of the necessity of the co-transfection stage. Here we constructed rPV pBI/mel2/rtTA containing melittin gene under the control of tetracycline CMV promoter and transactivator protein rtTA gene under constitutive early CMV promoter (Fig. 1). This vector consists of transactivator protein rtTA gene under the control of constitutive early CMV promoter, melittin and GFP genes under the control of inducible minimal CMV promoters, and tetracycline-responsive element. Transactivator protein rtTA is a protein consisting of tetracycline repressor protein (TetR, a sequence from tetracycline-resistant operon of *E. coli* transposon Tn10) and activator domen of

TABLE 3. Effect of rPV pBI/mel2/rtTA on Dynamics of *M. gallisepticum* Isolation from the Respiratory and Visceral Organs of Infected Chickens (n=14)

Organ	Number of animals from which *M. gallisepticum* was isolated		
	Group 1	Group 2	Group 3
Respiratory organs			
trachea	14	14	14
air sacs	12	14	10
lung	10	11	6
total number of re-isolates	36*	39	30
Visceral organs			
liver	4	3	0
spleen	3	4	0
kidney	8	7	6
heart	3	3	0
total number of re-isolates	18	17	6**

Note. *p<0.05 compared to: *groups 2 and 3, **groups 1 and 2.

VP16 protein of herpes simplex virus. rtTA binds to TRE-element sequence and activates transcription in the presence of tetracycline.

This construct allows precise regulation of the expression of AMP genes in the organism by varying the dose of the inductor, which is very important in cases when the products of expressed genes are toxic [5].

The effects of membrane-active AMP inhibiting mycoplasma and clamydia infections both in cell culture and *in vivo* are primarily mediated by their direct action on these bacteria [9]. Moreover, previous experiments showed [2] that *in vitro* treatment of various mycoplasma strains with amphipathic peptides (cecropin A, melittin, magainin 2) leads to depolarization of their plasma membranes, changes in cell morphology, and inhibition of their mobility. We previously showed that expression of melittin gene in HeLa cell culture reduced transmembrane potential of transfected cells [8], which, in turn, disturbs the process of mycoplasma adhesion to cells and normal cycle of their development.

We believe that this mechanism can be realized during introduction of rPV pBI/mel2/rtTA to laboratory animals.

Moreover, in these *in vivo* experiments elimination of the infectious agent from the organism after treatment with AMP can be effected through another mechanism. Recent *in vivo* studies on mice demonstrated selective lysis of macrophages infected with *B. abortus* by synthetic membrane-active peptides (including melittin), which, apart from the decrease in pathogen titer, led to activation of specific immune response 4-6 weeks after injection of peptides [11].

Thus, we showed inhibition of *M. hominis, C. trachomatis, M. gallisepticum* infection on laboratory animals receiving rPV expressing melittin gene under the control of tetracycline CMV promoter. These findings suggest that rPV expressing AMP genes can be considered as potential agents for prophylactics and treatment of mycoplasma and chlamydia infections.

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