

Full Research Paper

Drastic Attenuation of *Pseudomonas aeruginosa* Pathogenicity in a Holoxenic Mouse Experimental Model Induced by Subinhibitory Concentrations of Phenyllactic acid (PLA)

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Abstract: The discovery of communication systems regulating bacterial virulence has afforded a novel opportunity to control infectious bacteria without interfering with growth. In this paper we describe the effect of subinhibitory concentrations of phenyllactic acid (PLA) on the pathogenicity of *Pseudomonas aeruginosa* in mice. The animals were inoculated by oral (p.o.), intranasal (i.n.), intravenous (i.v.) and intraperitoneal (i.p.) routes with *P. aeruginosa* wild and PLA-treated cultures. The mice were followed up during 16 days after infection and the body weight, mortality and morbidity rate were measured every day. The microbial charge was studied by viable cell counts in lungs, spleen, intestinal mucosa and blood. The mice batches infected with wild *P. aeruginosa* bacterial cultures exhibited high mortality rates (100 % after i.v. and i.p. route) and very high cell counts in blood, lungs, intestine and spleen. In contrast, the animal batches infected with PLA treated bacterial cultures exhibited good survival rates (0 % mortality)

and the viable cell counts in the internal organs revealed with one exception the complete abolition of the invasive capacity of the tested strains. In this study, using a mouse infection model we show that *D*-3-phenyllactic acid (PLA) can act as a potent antagonist of *Pseudomonas (P.) aeruginosa* pathogenicity, without interfering with the bacterial growth, as demonstrated by the improvement of the survival rates as well as the clearance of bacterial strains from the body.

Keywords: Phenyllactic acid, *Pseudomonas aeruginosa*, *in vivo* holoxenic mouse model, virulence attenuation

Introduction

Upon contact with the host cells many pathogens use intercellular communication mechanisms for the regulation of virulence factors expression. One of the newest strategies for the prevention and treatment of bacterial infections is the inhibition of cell-to-cell signalling by inhibitors which do not interfere with the microbial growth [1]. *Pseudomonas aeruginosa* is an increasingly prevalent opportunistic pathogen and is the most common Gram-negative bacterium found in nosocomial and life threatening infections of immunocompromised patients [2]. *P. aeruginosa*, besides its natural resistance to a large number of antibiotics effective against other Gram-negative bacilli such as the A group penicillins, 1st-3rd generation cephalosporins, tetracyclins and trimetoprim, has developed acquired resistance to the so-called “antipseudomonals”: carboxypenicillins (ticarcillin, carbenicillin, 25-30 % resistance), acyl-ureidopenicillins (ceftazidime, cephsulodin), carbapenems (imipenem, 20-25 %), gentamycin (47 %) and ciprofloxacin (35-40 %).

The intrinsic resistance of *Pseudomonas* cells towards antimicrobial agents is further increased when the bacteria is growing in biofilms. In fact, biofilms are known for their high resistance to antimicrobial agents, thus explaining their implication in the etiology of infectious diseases, with an incidence as high as 65 % [2]. Human *P. aeruginosa* infections involving bacterial adherence and biofilm development on medical devices are very difficult to treat and eradicate, due to their increased resistance to the anti-infective host defense [3]. The attempts made until present to attenuate bacterial pathogenesis due to *P. aeruginosa* by interfering with bacterial cell to cell communication, without interfering with growth have used natural antagonists produced by superior organisms (furanones) or chemically synthesized [6].

The present study was aimed at attenuating the pathogenicity in *P. aeruginosa* by using subinhibitory concentrations of phenyl lactic acid (PLA). Our approach is based on the fact that this compound was found in the cell-free cultures of *Lactobacillus* probiotic strains and its antibacterial activity was already demonstrated for Gram-positive microorganisms [7]. Our previous studies with cell-free probiotic cultures liquids have also demonstrated that the probiotic bacteria secrete soluble factors interfering with the pathogenic bacteria and host cell signaling mechanisms, in the first case by inducing changes in the expression of surface molecules and consequently, the shift of the adherence pattern from aggregative to the diffuse one, and the stimulation of endocytic processes of cultured cells, respectively [8].

Results and Discussion

The ability of pathogenic bacteria to survive in different environments illustrates the importance of understanding the regulation of bacterial virulence genes and those implicated in the detection of the environmental signals for the elaboration of adequate anti-infective strategies [9]. The success of any pathogen in the colonization of a sensitive host and the development of an infectious process is dependent on its ability to sense its environment and to modulate the expression of the gene encoding factors required for the establishment and adaptation to the new habitat [10].

Table 1. Mortality and body weight dynamics in animals infected with *P. aeruginosa* wild cultures.

No.	Bacterial strain/ Inoculation mode	Day 0			Day 1			Day 2		
		no.	g/b	g/m	no.	g/b	g/m	no.	g/b	g/m
1.	<i>P. aeruginosa</i> 247/1464 i.p.	5	107	26.75	2	20	10	1	23	23
2.	<i>P. aeruginosa</i> 247/1464 i.v.	5	102	25.5	–	–	–	–	–	–
3.	<i>P. aeruginosa</i> 247/1464 i.n.	5	92	23	4	90	22.5	4	90	22.5
4.	<i>P. aeruginosa</i> 492 i.p.	5	110	27.5	–	–	–	–	–	–
5.	<i>P. aeruginosa</i> 492 i.v.	5	99	24.75	3	55	18.53	–	–	–
6.	<i>P. aeruginosa</i> 492 i.n.	5	92	23	4	90	22.5	4	96	24
7.	<i>P. aeruginosa</i> 492 p.o.	5	94	23.5	4	90	22.5	4	94	23.5
8.	<i>P. aeruginosa</i> 37 i.p.	5	95	23.75	–	–	–	–	–	–
9.	<i>P. aeruginosa</i> 37 i.v.	5	92	23	–	–	–	–	–	–
10.	<i>P. aeruginosa</i> 37 i.n.	5	92	23	4	89	22.25	4	96	24
11.	<i>P. aeruginosa</i> 37 p.o.	5	97	24.25	4	95	23.75	4	106	26.5
12.	<i>Ps. aeruginosa</i> 1489 i.p.	5	88	22	–	–	–	–	–	–
13.	<i>P. aeruginosa</i> 1489 i.v.	5	77	19.25	–	–	–	–	–	–
14.	<i>P. aeruginosa</i> 1489 i.n.	5	107	26.75	4	78	19.5	4	86	21.5
15.	<i>P. aeruginosa</i> 1489 p.o.	5	82	20.5	4	87	21.75	4	95	23.75
16.	Control	5	74	18.5	4	76	19	4	76	19

Table 1. Cont.

No.	Bacterial strain/ Inoculation mode	Day 7			Day 9			Day 16		
		no.	g/b	g/m	no.	g/b	g/m	no.	g/b	g/m
1.	<i>P. aeruginosa</i> 247/1464 i.p.	1	24	24	1	21	21	1	28	28
2.	<i>P. aeruginosa</i> 247/1464 i.v.	-	-	-	-	-	-	-	-	-
3.	<i>P. aeruginosa</i> 247/1464 i.n.	4	95	23.75	4	95	23.75	4	107	26.75
4.	<i>P. aeruginosa</i> 247/1464 p.o.	4	93	23.25	4	100	25	4	112	28
5.	<i>P. aeruginosa</i> 492 i.p.	-	-	-	-	-	-	-	-	-
6.	<i>P. aeruginosa</i> 492 i.v.	-	-	-	-	-	-	-	-	-
7.	<i>P. aeruginosa</i> 492 i.n.	4	117	29.25	4	119	29.75	4	135	33.75
8.	<i>P. aeruginosa</i> 492 p.o.	4	105	26.25	4	105	26.25	4	117	29.25
9.	<i>P. aeruginosa</i> 37 i.p.	-	-	-	-	-	-	-	-	-
10.	<i>P. aeruginosa</i> 37 i.v.	-	-	-	-	-	-	-	-	-
11.	<i>Ps. aeruginosa</i> 37 i.n.	4	116	29	4	119	29.75	4	121	30.25
12.	<i>P. aeruginosa</i> 37 p.o.	4	118	29.5	4	123	30.75	4	122	30.5
13.	<i>P. aeruginosa</i> 1489 i.p.	-	-	-	-	-	-	-	-	-
14.	<i>P. aeruginosa</i> 1489 i.v.	-	-	-	-	-	-	-	-	-
15.	<i>P. aeruginosa</i> 1489 i.n.	4	118	27	4	112	28	4	130	32.5
16.	<i>P. aeruginosa</i> 1489 p.o.	4	100	25	4	120	30	4	138	34.5
17.	Control	4	77	19.25	4	79	19.75	4	100	25

no. = number of mice/batch; g/b = gram/batch; g/m= gram/mouse; - = animals died during the experiment

Table 2. Mortality and body weight dynamics in animals infected with PLA treated *P. aeruginosa* cultures.

No.	Bacterial strain/ Inoculation mode	Day 0			Day 1			Day 2		
		no.	g/b	g/m	no.	g/b	g/m	no.	g/b	g/m
1.	<i>P. aeruginosa</i> 247/1464 i.p.	5	122	30.5	4	118	29.5	4	120	30
2.	<i>P. aeruginosa</i> 247/1464 i.v.	5	120	30	4	127	31.75	4	132	33
3.	<i>P. aeruginosa</i> 247/1464 i.n.	5	130	32.5	4	135	33.75	4	122	30.5
4.	<i>P. aeruginosa</i> 247/1464 p.o.	5	130	32.5	4	119	29.75	4	140	35
5.	<i>P. aeruginosa</i> 492 i.p.	5	120	30	4	117	29.25	4	120	30
6.	<i>P. aeruginosa</i> 492 i.v.	5	115	28.75	4	119	29.75	4	120	30
7.	<i>P. aeruginosa</i> 492 i.n.	5	115	28.75	4	128	32	4	133	33,25
8.	<i>P. aeruginosa</i> 492 p.o.	5	120	30	4	123	30.75	4	124	31
9.	<i>P. aeruginosa</i> 37 i.p.	5	120	30	4	116	29	4	118	29.5
10.	<i>P. aeruginosa</i> 37 i.v.	5	120	30	4	124	31	4	122	30.5
11.	<i>Ps. aeruginosa</i> 37 i.n.	5	115	28.75	4	120	30	4	121	30.25
12.	<i>P. aeruginosa</i> 37 p.o.	5	120	30	4	122	30.5	4	122	30.5
13.	<i>P. aeruginosa</i> 1489 i.p.	5	110	27.5	4	106	26.5	3	70	23.33
14.	<i>P. aeruginosa</i> 1489 i.v.	5	130	32.5	4	120	30	4	130	32.5
15.	<i>P. aeruginosa</i> 1489 i.n.	5	120	30	4	117	29.25	4	119	29.75
16.	<i>P. aeruginosa</i> 1489 p.o.	5	120	30	4	110	27.25	4	120	30
17.	Control	5	130	26	5	132	26.4	5	122	30.5

no. = number of mice/batch; g/b = gram/batch; g/m = gram/mouse; – = animals died during the experiment

Table 2. Cont.

No.	Bacterial strain/ Inoculation mode	Day 7			Day 9			Day 16		
		no.	g/b	g/m	no.	g/b	g/m	no.	g/b	g/m
1.	<i>P. aeruginosa</i> 247/1464 i.p.	4	130	32.5	4	140	35	4	149	37.25
2.	<i>P. aeruginosa</i> 247/1464 i.v.	4	135	33.75	4	143	35.75	4	148	37
3.	<i>P. aeruginosa</i> 247/1464 i.n.	4	141	35.25	4	146	36.5	4	150	37.5
4.	<i>P. aeruginosa</i> 247/1464 p.o.	4	143	35.75	4	155	38.75	4	158	39.5
5.	<i>P. aeruginosa</i> 492 i.p.	4	131	32.75	4	145	36.25	4	147	36.75
6.	<i>P. aeruginosa</i> 492 i.v.	4	129	32.25	4	138	34.5	4	144	36
7.	<i>P. aeruginosa</i> 492 i.n.	4	136	34	4	142	35,5	4	147	36.75
8.	<i>P. aeruginosa</i> 492 p.o.	4	139	34.75	4	147	36.75	4	152	38
9.	<i>P. aeruginosa</i> 37 i.p.	4	120	30	4	124	31	4	146	36.5
10.	<i>P. aeruginosa</i> 37 i.v.	4	122	30.5	4	145	36.25	4	152	38
11.	<i>P. aeruginosa</i> 37 i.n.	4	122	30.5	4	137	34.25	4	142	35.5
12.	<i>Ps. aeruginosa</i> 37 p.o.	4	135	33.75	4	144	36	4	148	37
13.	<i>P. aeruginosa</i> 1489 i.p.	3	72	24	2	58	29	2	62	31
14.	<i>P. aeruginosa</i> 1489 i.v.	4	120	30	4	148	37	4	150	37.5
15.	<i>P. aeruginosa</i> 1489 i.n.	4	119	29.75	3	92	30.6	3	105	35
16.	<i>P. aeruginosa</i> 1489 p.o.	4	125	31.25	4	122	30.5	4	137	34.25
17.	Control	5	124	24.8	5	143	28.6	5	148	29.6

no. = number of mice/batch; g/b = gram/batch; g/m = gram/mouse; - = animals died during the experiment

Table 3. Cont.

No	Bacterial strain, inoculation route	CFU/ml dil. 10 ⁻⁶		CFU/ml dil. 10 ⁻⁷		CFU/ml dil. 10 ⁻⁸		CFU/ml dil. 10 ⁻⁹	
		PLA-	PLA+	PLA-	PLA+	PLA-	PLA+	PLA-	PLA+
7	<i>P. aeruginosa</i> 37 i.v.								
	Gall bladder	NC	–	NC	–	NC	–	NC	–
	Lung	NC	–	NC	–	NC	–	NC	–
	Intestine	10	–	–	–	–	–	–	–
	Blood	–	–	–	–	–	–	–	–
8	<i>P. aeruginosa</i> 1489 i.p.								
	Gall bladder	127	–	21	–	–	–	–	–
	Lung	NC	–	NC	–	260	–	96	–
	Intestine	NC	–	NC	–	–	–	–	–
	Blood	NC	–	NC	–	NN	–	NC	–
9	<i>P. aeruginosa</i> 1489 i.v.								
	Gall bladder	NC	356	186	110	62	23	6	–
	Lung	NC	–	NC	–	NC	–	NC	–
	Intestine	108	–	36	–	–	–	–	–
	Blood	–	–	–	–	–	–	–	–

NC- not countable at the chosen dilution

For opportunistic bacteria, such as *Pseudomonas aeruginosa*, the outcome of the complex bacterial cell-host interactions depend on cellular density [3]. Our *in vivo* experimental study using a holoxenic mice infection model showed that subinhibitory concentrations of PLA could act as a potent inhibitor of *P. aeruginosa* pathogenicity, without affecting the bacterial cells viability, this antagonistic effect depending on the infection route and the microbial tested strain.

Conclusions

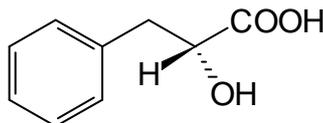
The present study has proved the role of subinhibitory concentrations of PLA in the attenuation of *P. aeruginosa* virulence and pathogenicity, using an *in vivo* holoxenic mouse infection experimental model. Our results are accounting for the hypothesis that subinhibitory concentrations of PLA, which are not acting by affecting the bacterial cell viability, are probably interfering with the intracellular communication and the sequential and coordinated expression of different virulence factors, altering the success of this pathogen in the colonization of a sensitive host and the development of an infectious process.

Experimental

General

The pathogenic *Ps. aeruginosa* strains used in our study were isolated in 2006 from invasive infections and identified comparatively by classical biochemical reactions and API 20NE microtests (BioMérieux). *D*-3-phenyl lactic acid, purity ≥ 97.0 % (PLA, Figure 1) supplied by Sigma-Aldrich Division (Milan, Italy) was used in the study.

Figure 1. *D*-3-phenyllactic acid.



Conventional (holoxenic) mice, purchased from Darvari Farm (Bucharest), were used in these experiments. In order to obtain comparative results the animals were grouped in different batches of five animals each as follows: one negative control (batch 1) and six infection batches and six batches for investigation of the influence of PLA on bacterial pathogenicity. Each batch contained 5 animals.

Study design

The bacterial strains were seeded on agar medium and incubated for 24 hours at 37 °C. Starting from isolated colonies developed on the agar medium, microbial suspensions of 0.5. MacFarland density ($1-3 \times 10^8$ CFU/ml) were prepared in sterile saline. 100 μ l of this inoculum were distributed in two culture tubes for each strain, one containing 10 ml nutrient broth and the other one 10 ml nutrient broth with *D*-3-phenyl lactic acid at a final concentration of 1 mg/ml. The concentration of PLA used in this study (1 mg/ml) proved to be subinhibitory for all tested strains, meaning that it did not affect the cultures viability, as demonstrated by the viable cell counts (CFU/ml) performed on the inoculum giving similar values in the presence as well as in the absence of *D*-3-phenyllactic acid. The initial solution of 100 mg/ml was prepared in vol:vol methanol:water solution. The solution was sterilised by 22 μ m filter membrane. After adding the *D*-3-phenyllactic acid in the nutrient broth, the pH medium was controlled (in order to exclude the medium acidification due to the addition of the PLA). The two tubes were incubated for 24 hours at 37 °C. The obtained overnight cultures of *P. aeruginosa* grown with and without addition of subinhibitory concentrations of PLA (1 mg/ml) were centrifuged and the sediment was washed three times in PBS (phosphate buffered saline). Starting from the cell sediment, bacterial suspensions of $\sim 10^7$ CFU/ml were prepared in PBS and used for mice inoculation in one single dose given by oral (p.o.), intranasal (i.n.), intravenous (i.v.), and intraperitoneal (i.p.) routes. The mice were followed up to 16 days after infection and the body weight, mortality and morbidity rate were measured at different time intervals. The course of infection was determined by daily examination of the animals in each batch, as well as by final viable cell count (Colony Forming Units, CFU number) performed on different organs and tissues, in order to appreciate the pathogen invasion (lungs, spleen, intestinal mucosa and blood). The tissue specimens (collected after the animals died from infection or were euthanized) were weighted and homogenised in sterile PBS. Serial ten-fold

dilutions from homogenate were plated (0.1. ml/plate) onto King A medium specific for the isolation and counting of *P. aeruginosa*. The seeded media were incubated at 37 °C for 24 hours and the specific *P. aeruginosa* colonies were counted and the results were expressed in CFU/ml.

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