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Research Paper

Pathogenic effects of biofilm with chronic pseudomonas aeruginosa lung infection in rats ☆

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Abstract

Objective: To establish an animal model of *P.aeruginosa* biofilm associated with chronic pulmonary infection and investigate the pathogenic effects of biofilm. **Methods**: Experiments *in vitro*, measuring the MICS, MBCS of levofloxacin(LFX), ceftazidime(CAZ) in PAO579 in alginate beads and planktonic PAO579. Rats were challenged with 0.1 ml of PAO579(10^9CFU/ml) in alginate beads or 0.1 ml of planktonic PAO579(10^9 CFU/ml), 3,7,14 days after challenging, bacteriological, pathological features were observed. **Results**: The MICS, MBCS of LFX, CAZ in PAO579 in alginate beads were higher than those in planktonic PAO579 **in vitro**. CFU/lung in alginate beads group was significantly higher than that in planktonic bacteria group($P = 0.002$, $P = 0.004$, $P = 0.002$, respectively); macroscopic lung pathology and the inflammation in alginate beads group were significantly more severe compared to those in planktonic bacteria group *in vivo*. **Conclusion**: *P.aeruginosa* biofilm protected bacterium from killing of antibiotics and might mediate the host immune damage in the lung tissue and made bacterium evade the host immune defense.

Key words: *P. aeruginosa*; biofilm; pulmonary infection; pathogenic effect

INTRODUCTION

P.aeruginosa is an common opportunistic pathogen responsible for nosocomial infections and chronic lung infections in cystic fibrosis(CF) patients[1-5]. A report showed that 80%-90% CF patients have biofilm of *p. aeruginosa* in their lungs. *P.aeruginosa* can produce exopolysacharde glycocalyxpolyers:alginate which forms biofilm, alginate makes bacterium firmly adhere to lung epithelium[6]. Recent research indicates that biofilm information of *p.aeruginosa* is an important factor for difficult to cure lung infections[7-9]. In this study, we try to understand the pathogenic effects of biofilm through antibiotic experiment in vitro and have established an animal model of *p.aeruginosa* biofilm associated with chronic pulmonary infection in vivo and investigated bacteriological, pathological features. Through this we aim to provide theoretic evidence for the prevention and therapy of biofilm associated infections.

MATERIALS AND METHODS

Animals

Sixty healthy male SPF Wister rats (Guangxi medical university laboratory animals center, China) 8-9 weeks old with body weights of 150-180 g were used. The rats were randomly divided into two groups, 30 in alginate beads group and 30 in planktonic bacteria group.

Challenge strain

p.aeruginosa PAO579 (International Antigenic Typing System O:2/5) was used, which was provided by department of clinical microbiology, university hospital of Copenhagen, Denmark.

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Reagents

Tris-HCl buffer(Sigma Chemical Co, USA), 1.1% alginate solution(60% guluronic acid content, Norway).

Equipments

Homeothermia totter incubaton(Scientific instrument factory of WuHan, Chinese Acadamy of Science), SPX type biochemistry incubaton(Jiangnan instrument factory of NingBo, China), Hypothermia high-speed centrifuge (Callegra 64R, BEAKMAN, America), Alginate manufacture apparatus(Panum medical school, Univerisity of Copenhagen, Denmark), Magnet-stirrer(scientific instrument factory of jiangsu, China), Enzyme-mark apparatus(ELX808, America), Pathology image analysator(DMR+Q550, LEICA, Germany), Scanning electron microscope(S-800, Japan).

Preparation of p.aeruginosa in alginate beads

The methods can be found in the documents[10-12]. In brief, one *p.aeruginosa* colony from the culture dish was incubated in 100 ml LB broth for 20 h on a homeothermia totter incubator at 37℃. After the culture was centrifuged, we put moderate LB broth in it. Then 1ml of the *p.aeruginosa* bacterial culture was mixed with 9 ml of alginate beads, and the mixture was forced once with oppressed air through a cannula into a solution of 0.1 M $CaCl_2$ in 0.1 M Tris-HCl buffer(pH7.0). It was centrifuged after continuous stirring 1 h and washed twice in sterile 0.9% NaCl. The suspension(Fig 1) was incubated at 37℃ for 24 h and adjusted to yield 10^9 CFU/ml and the yield was confirmed by colony counts. The beads was stored in a refrigerator at 4℃ until used.

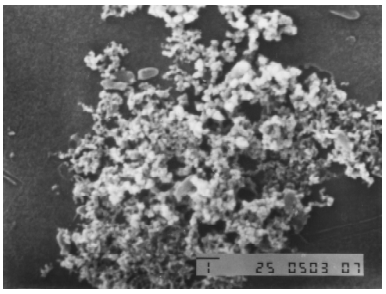


Fig 1 PAO579 in alginate beads(scanning electronic microscope, × 7 500)

Preparation of planktonic *p.aeruginosa*

One *p.aeruginosa* colony was incubated in 100 ml LB broth for 20 h on a homeothermia totter incubator at 37℃. We put moderate LB broth in it after the culture was centrifuged. The suspension(Fig 2) was incubated at 37℃ for 24 h and adjusted to yield 10^9CFU/ml and the yield was confirmed by colony counts. The suspension was stored in a refrigerator at 4℃ until used.

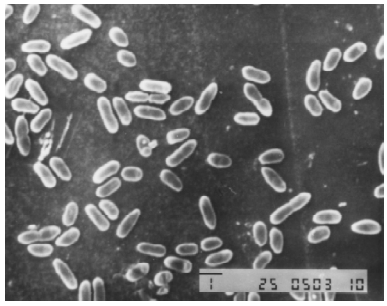


Fig 2 Planktonic PAO579 (scanning electronic microscope, × 7 500)

Antibiotic experiment in vitro

MICS, MBCS of LFX ,CAZ in PAO579 in alginate beads and planktonic PAO579 were measured by test tube doubling dilution[13-14].Sensitivity classification adopts the standards recommended by the National Committee for Clinical Laboratory Standards[NCCL (2002)].

Establishment of the animal model of *p. aeruginosa* biofilm and planktonic *p.aeruginosa* associated with pulmonary infection

According to the documents[10-12], we anesthetized the rat, incised the trachea, through this incision the curved bead-tripped needle with the syringe was entered. The tip of the needle was led through the left principal bronchus to the lower left bronchus and 0.1 ml of PAO579 (10^9 CFU/ml) in alginate beads or 0.1 ml of planktonic PAO579(10^9 CFU/ml) was installed. The wound was closed with a suture. 3, 7, 14 days after intratracheal challenge, the rats were sacrificed and lung samples were obtained.

Macroscopic lung pathology

The gross pathological changes on the lungs were assigned four different scores according to the severity of inflammation[15-16]. Ⅰ, normal lungs; Ⅱ, swollen, hyperemia, and small atelectasis lungs(<10 mm^2); Ⅲ, pleural adhesions and atelectasis(<40 mm^2); Ⅳ, abscesses, large atelectasis, and hemorrhages.

Histopathologic scoring

The lung pathology was assigned microscopically one to four scores according to the severity of inflammation[15-16]. ① normal histology; ② mild focal inflammation; ③moderate to severe focal inflammation with areas of normal lung tissue; and ④severe inflammation to necrosis or severe inflammation throughout the lung. In addition, according to the proportions of neutrophils(PMN) and mononuclear leukocytes(MN) in the inflammatory foci, acute inflammation was defined as an inflammatory infiltration in which PMNs were predominant(PMN≥ 90%, MN≤10%), whereas chronic inflammation was

defined as a preponderance of MNs(MN≥90%, PMN ≤10%), which included lymphocytes and plasma cells, and the presence of granulomas[15-16].

Lung bacteriology

Each lung tissue sample was homogenized. CFU/ml (CFU/lung) of each lung=practical colonies × diluted multiple × 10 × 5(× 10, for 0.1 ml lung homogenate was incubated in the culture dish; × 5, for lung tissue sample was homogenized in 5 ml 0.9% NaCl).

Statistcal analysis

Use SPSS10.0 statistical package to analyze. Unpaired differences in continuous data were analyzed by the Mann-Whitney U test and ANOVA test, and categorical data were compared using the χ^2-test.

RESULTS

Antibiotic experiment in vitro

PAO579 in alginate beads were less sensitive to LFX, CAZ than planktonic PAO579(Tab 1).

Tab 1 The MICS, MBCS of LFX, CAZ in PAO579

Bacterium	LFX(μg/ml)		CAZ(μg/ml)	
	MIC	MBC	MIC	MBC
PAO579 in Alginate beads	0.5	1.0	8	16
Planktonic PAO579	0.25	0.5	2	4

Lung bacteriology

3,7,14 days after intratracheal challenge with *p. aeruginosa*, CFU/lung in alginate beads group was significantly higher than that in planktonic bacteria group ($P = 0.002$, $P = 0.004$, $P = 0.002$, respectively). On the 14 th day, *p.aeruginosa* could be seen in alginate beads group and *p.aeruginosa* could not be seen in planktonic bacteria group when it was incubated ($P = 0.002$)(Tab 2).

Macroscopic lung pathology

Alginate beads group for rats had mainly a score of Ⅳ and planktonic bacteria group for rats had a score of Ⅰ and Ⅱ. On 3, 7, 14 days, the score of Ⅳ and the incidence of lung abscesses in alginate beads group were significantly higher than those in planktonic bacteria group($P < 0.001$, $P = 0.013$; Tab 3).

Tab 2 Median numbers of CFU of *p.aeruginosa* 3,7,14 days after intratracheal[Median (range)]

Group	Bacterial count(CFU/lung)		
	3 d	7 d	14 d
Alginate beads($n = 6$)	$3.63 \times 10^7(7.2 \times 10^5\text{-}1.4 \times 10^8)^*$	$5.1 \times 10^5(2.1 \times 10^4\text{-}2.5 \times 10^6)^*$	$8.8 \times 10^4(8.1 \times 10^4\text{-}1.5 \times 10^5)^*$
Plankton($n = 6$)	$100(0\text{-}6.6 \times 10^3)^{**}$	$100(0\text{-}6.4 \times 10^2)^{**}$	0^{**}
P value	0.002	0.004	0.002

Compared among Alginate beads group, $^*P = 0.002$; Compared among Planktonic group, $^{**}P > 0.05$.

Tab 3 Macroscopic lung pathology 3,7,14 days after intratracheal challenge with *p.aeruginosa* [Total no. of rats(%)]

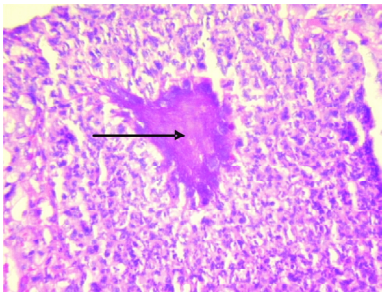
Time (day)	Score	Alginate beads($n = 9$) *n* (%)	Plankton($n = 9$) *n* (%)	*P* value
3	Ⅰ - Ⅱ	0	7(78)	
	Ⅲ	0	2(22)	
	Ⅳ	9(100)	0	< 0.0001
	Lung abscess	9(100)	0	< 0.0001
7	Ⅰ - Ⅱ	0	8(89)	
	Ⅲ	3(33)	1(11)	
	Ⅳ	6(67)	0	0.013
	Lung abscess	6(67)	0	0.013
14	Ⅰ - Ⅱ	5(56)	9(100)	
	Ⅲ	1(11)	0	
	Ⅳ	3(33)	0	0.087
	Lung abscess	3(33)	0	0.087

Histopathological changes in the lungs

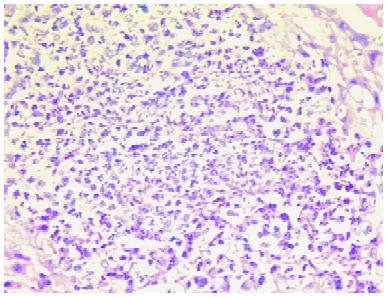
3, 7, 14 days after challenge with *p.aeruginosa*, 4 rats were selected randomly to make for histopathology from alginate beads group and planktonic bacteria group, respectively. Acute inflammation in lung tissues with an inflammatory infiltration in which PMNs were predominant was found in the two groups on the 3th day (Fig 3). Chronic inflammation was found in alginate beads group and normal lung was found in planktonic bacteria group(Fig 4) on the 14th day. Furthermore, the inflammation in alginate beads group was significantly more severe compared to that in the planktonic bacteria group. A score of 4 in alginate beads group and a score of 3 in planktonic bacteria group on the 3th day. 3 of 4 rats with a score of 4 in alginate beads group and 4 rats with a score of 1-2 in planktonic bacteria group on the 7th day. 3 of 4 rats with a score of 4 in alginate beads group and 4 rats with normal lungs in planktonic bacteria group on the 14th day.

DISCUSSION

Biofilm is a microbially derived sessile community characterized by cells that are irreversibly attached to a biomedical device or the surface of an organism tract or to each other, embedded in a matrix of extracellular polymeric substances such as polysaccharide, brinolase which they have produced[17-18]. Currently, studies show that biofilm is the main reason of many clinical chronic infectious diseases and infections related biomedical materials[10,19]. So far, there has not been a standard about establishing a model of biofilm in vivo worldwide. In this study, we introduced an animal model of the chronic

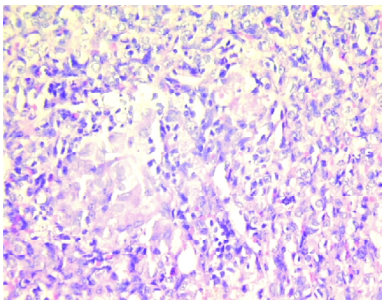


Algitate beads group, the arrow shows that the alginate beads were surrounded by numerous PMN.

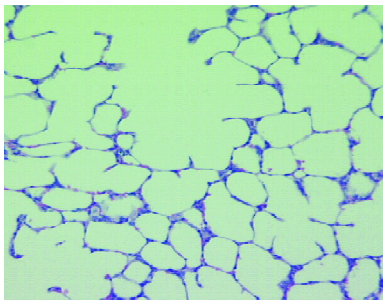


Planktonic group, the infilfration of numerous PMN.

Fig 3 Detection of lung tissue pathology after *p.aeruginosa* on the 3th day(HE, × 400)



Algitate beads group, the presence of numerous hyperplasia of lymphocytes, macrophages, multinucleated giant cells.



Planktonic group, nomal lung tissue.

Fig 4 Detection of lung tissue pathology after *p.aeruginosa* on the14th day(HE, × 400)

p.aeruginosa pulmonary infection which was established by Johansen HK and Hoiby N[10]. The major characteristic about this model in vivo is that the main component of biofilm-alginate produced by similarly live mucoid *p.aeruginosa* is used to embed the bacterium, which more closely resembled the infections caused by a naturally bacterial biofilm.

In our study, the antibiotic experiment in vitro and lung bacterology indicated that antibiotics and host immune system are sensitive to planktonic *p.aeruginosa*. The host immune defense system could rapidly eradicate the invading planktonic *p.aeruginosa*. However, things were completely opposite in the rats with chronic *p.aeruginosa* lung infection in alginate beads group, *p.aeruginosa* partly planted, multiplied, and existed for a long time in the lung. Clinical studies found that alginate production or mucoidy was an important characteristic in CF patients with chronic *p.aeruginosa* lung infection and a main reason of the progress of the disease as well. It was demonstrated that biofilm formation could make bacterium significantly resist antibiotics and host defense mechanism, it was also the reason of chronic infection [20-21].Song and colleagues[22] found that lung bacterial loads in nonmucoid *p.aeruginosa* group was significantly lower than that in mucoid *p.aeruginosa* group in a mouse model of lung infection. The positive correlation between lung bacteriology and lung macroscopic pathology in the mucoid Alginate $^{+}$PAOmuca22 group suggested alginate production not only impeded pulmonary clearance but also resulted in more severe lung damage. The results of macroscopic lung pathology and histopathological changes in the lungs in our study corresponded to the report above.

PMNs are the most important blood cells for the host defense against bacterial infections. PMNs are also the main inflammation cells early in the *p.aeruginosa* pulmonary infection for immune-perfected host. It is usually thought that the infiltration of PMNs clearing pathogens is the main reason resulting in lung damage after lung infection[23]. In our study, lung bacteriology showed that PMNs were predominant 3 days after challenge with *p.aeruginos*, and normal lung was found in planktonic bacteria group on the 14th day, which indicated planktonic *p.aeruginosa* invading the lungs caused an acute inflammatory reaction. In the inflammatory process, *p. aeruginosa* was cleared away, the focus of infection was absorbed and damaged lung recovered original structure and function. Currently, the studies of infection associated with biofilm suggest that in a certain condition, bacterium inside biofilm separate from biofilm fragments now and again, result in acute infection, and gather to form new colonies in new parts, which emerge repeatedly, thus cause infection repeatedly[24]. In this study, proliferation of mononuclear leukocytes and

lymphocytes, reaction of xanthoma cells, presence of multinucleated giant cells or/and red blood cells, fibroplasa were seen microscopically in alginate beads group, which indicated that it had perisistent reaction for host on damage, for bacterium embeded biofilm released intervally, the infiltrated inflammatory cells resulted in inflammation difficult to recover. Consequently, acute inflammation was changed to chronic inflammation. Furthermore, the inflammation in alginate beads group was significantly more severe compared to that in planktonic bacteria group, which suggested that biofilm could stimulate the production of PMNS. Currently, it is thought[8,25] that PMNS absorbed to biofilm are unable to kill the bacterium inside biofilm, but endocellular enzymes, such as lycoome, perocidate, elastase, release, then injure lung tissue around biofilm.

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