RELATIONSHIP OF IRON AND EXTRACELLULAR VIRULENCE FACTORS TO *PSEUDOMONAS AERUGINOSA* LUNG INFECTIONS

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SUMMARY. The iron concentration in the culture medium used to prepare the inocula influenced the virulence of *Pseudomonas aeruginosa* in a chronic pulmonary infection model in rats. Groups of rats were given transtracheal inocula of agar beads in which were embedded *c.* 10^4 cfu of *P. aeruginosa* strain PAO and the mutants of strain PAO, Fe5 and Fe18. When strain PAO was grown in low-iron medium before infection, it caused severe parenchymal changes including a dense mononuclear cell infiltration in the alveolar spaces, as well as intra- and peribronchial inflammation. When strain PAO was grown in high-iron medium, the pathological changes in lungs were restricted to intra- and peribronchial inflammation. Strain Fe5, in which the effect of iron on yields of elastase is deregulated, produced similar pathological changes regardless of whether it was grown in low- or high-iron media. All rats infected with strain Fe18, in which the effect of iron on yields of toxin A is deregulated, died within 48 h after infection. These data indicate that the iron concentration of the culture medium can influence the pathogenesis of *P. aeruginosa* in a chronic respiratory infection. These studies also suggest that the regulation of extracellular virulence factors by iron is important in the determination of *P. aeruginosa* virulence.

INTRODUCTION

Some of the extracellular products of *Pseudomonas aeruginosa* such as toxin A, proteases, and lipopolysaccharide (Gray and Kreger, 1979; Pennington, 1979; Woods *et al.*, 1982a; Blackwood *et al.*, 1983; Cash, Straus and Bass, 1983) have been implicated as virulence factors in lung infections due to this organism. A model of *P. aeruginosa* pulmonary infection has been developed in rats to study the role of potential virulence factors in chronic pulmonary disease due to the organism (Cash *et al.*, 1983). In this model, it has been shown recently that toxin A and elastase are important virulence factors (Woods *et al.*, 1982a; Cash *et al.*, 1983). Tox⁻ mutants and a mutant producing a temperature-sensitive elastase were shown to be less virulent than their parental strain in this model (Woods *et al.*, 1982a), whereas rats given

intratracheal inocula of purified toxin A or proteases developed pulmonary histopathological changes similar to those induced by experimental infection (Cash *et al.*, 1983).

Yields of toxin A, elastase, and alkaline protease in culture supernates of *P. aeruginosa* are inversely proportional to the iron concentration of the medium (Bjorn, Sokol and Iglewski, 1979). Mutants have been isolated that are resistant to the regulation of either toxin A or elastase by iron, which indicates that iron regulates these exoproducts independently (Sokol, Cox and Iglewski, 1982).

Previously, we have shown that the iron concentration of the culture medium used to prepare the inocula influenced the pathogenesis of *P. aeruginosa* infections of the mouse cornea (Woods, Sokol and Iglewski, 1982*b*). When *P. aeruginosa* strain PAO was grown in low-iron medium it was more virulent than when it was grown in high-iron medium. However, the virulence of strain Fe18, a mutant resistant to the effect of iron on yields of toxin A, was unaffected. These studies suggested that the regulation of toxin A production by iron may be important in *P. aeruginosa* virulence in an animal model of acute infection.

In the present study we attempted to determine whether the iron concentration of the culture medium can influence the pathogenesis of *P. aeruginosa* in a chronic respiratory infection. Additionally, mutants in which the effect of iron on toxin A or elastase was deregulated were used to investigate the ability of iron to regulate extracellular virulence factors in chronic infections.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. aeruginosa* strain PAO (Holloway, Krishnapillai and Morgan, 1979) was the parental strain of the mutants used in this study. Strains Fe5 and Fe18 are independently isolated mutants which have been recently described (Sokol *et al.* 1982). Strain Fe5 is resistant to the effect of iron on yields of elastase in culture supernates. Strain Fe18 is resistant to the effect of iron on yields of toxin A; it is also a hyperproducer of toxin A in comparison to the parental strain PAO (Sokol *et al.*, 1982). A comparison of the parent and mutant strains with respect to the amount of toxin A and elastase produced in low- and high-iron media is shown in table I. These mutants are identical to the parental strain in all other characters that have been examined.

Cultures were grown in trypticase soy broth (Difco) which was deferrated with Chelex-100 (Bio-Rad Laboratories, Richmond, CA, USA), dialysed, and supplemented with monosodium

TABLE I
Comparison of mutant and parent strains

Strain	Yield of extracellular product ($\mu\text{g/ml}$)			
	Toxin A*		Elastase†	
	Low-iron medium	High-iron medium	Low-iron medium	High-iron medium
PAO (parent)	1.0	0.2	25.0	3.0
Fe5 (elaC)	1.0	0.2	28.5	12.0
Fe18 (tox C)	3.0	1.5	24.0	2.8

* Toxin A activity was determined by ADPR transferase activity as previously described (Iglewski and Sadoff, 1979; Sokol *et al.*, 1982; Woods *et al.*, 1982*b*).

† Elastase was measured by radioimmune assay as previously described (Ohman, Cryz and Iglewski, 1980; Sokol *et al.*, 1982).

glutamate 0.05 M and glycerol 1% (TSB-DC) (Ohman, Sadoff and Iglewski, 1980). The iron concentration of this low-iron medium was 0.05 $\mu\text{g/ml}$. FeCl_3 was added to TSB-DC to give a concentration of 5 μg of Fe/ml (high-iron medium; Woods *et al.*, 1982b). Organisms were embedded in agar beads as previously described (Cash *et al.*, 1979) after overnight growth in these media.

Experimental animals. Tracheostomies were performed under ether anaesthesia on six groups of ten adult male Sprague-Dawley rats weighing 200–250 g each, and 0.05 ml of an agar-bead suspension of the appropriate strain of *P. aeruginosa* was placed in a distal bronchus in the left lobe via a bead-tipped curved needle. Three and ten days after inoculation, five animals from each group were exsanguinated by cardiac puncture under anaesthesia. The lungs of three animals from each group were processed for quantitative bacterial culture as previously described (Cash *et al.*, 1979).

The lungs from two animals from each group were prepared for histological examination by removing the heart and lungs in a block and fixing it in 10% buffered formalin. Saggital slices of the entire left lobe of the fixed lungs, *c.* 3 mm thick, were dehydrated in graded alcohols, embedded in paraffin and cut into sections 6 μm thick.

Mounted sections were stained for light microscopy with haematoxylin and eosin. Infiltration of the lung with inflammatory cells and exudate was measured by the point counting method of Dunnill (1962). With a Zeiss integrating eyepiece (Zeiss, Oberkochen, West Germany), the number of points overlying the surface area of the infiltrate was divided by the total number of points counted over the entire surface area of the left lobe to obtain a measure of the percentage infiltration. This procedure was repeated with three left-lobe slices from two animals in each group.

RESULTS

Quantitative bacteriology of infected lungs

The mean numbers of cfu $\pm$ SEM of *P. aeruginosa* recovered from homogenised lung tissue 3 and 10 days after inoculation are shown in table II. Groups of animals were given transtracheal inocula of agar beads containing *c.* 10^4 cfu of *P. aeruginosa* strains PAO, Fe5, or Fe18 grown in either low-iron or high-iron medium. The number of organisms recovered from lungs infected with strain PAO grown in either low- or high-iron medium was approximately the same at 3 and 10 days after inoculation (*c.* 10^6 cfu). The number of organisms recovered from lungs infected with strain Fe5, however, fell considerably from *c.* 10^6 cfu at 3 days to *c.* 10^3 cfu at 10 days after challenge. The iron concentration of the culture medium had no effect on the ability of

TABLE II

Quantitative recovery of P. aeruginosa from rat lungs after inoculation of bacteria in agar beads

Strain	Medium	Mean cfu $\pm$ SEM ($\times 10^5$) recovered after	
		3 days	10 days
PAO	Low-iron	17.2 $\pm$ 1.0	13.3 $\pm$ 4.1
	High-iron	72.5 $\pm$ 6.3	16.0 $\pm$ 6.7
Fe5	Low-iron	83.4 $\pm$ 9.8	0.005 $\pm$ 0.002
	High-iron	50.1 $\pm$ 20.0	0.08 $\pm$ 0.06
Fe18	Low-iron	... *	... *
	High-iron	... *	... *

* No survivors after 48 h.

P. aeruginosa to survive in the lung, as the numbers of strains PAO and Fe5 recovered after each time interval were approximately the same with each iron concentration. Bacterial counts were not obtained from lungs of animals infected with strain Fe18 because all animals in this group died within 48 h of infection.

Pathological changes in lungs infected with P. aeruginosa grown in low-iron medium

Microscopic examination of lung tissue taken 3 days after infection with either strain PAO or strain Fe5 grown in low-iron medium revealed a pathological picture similar to that previously described for strain PAO (Woods *et al.*, 1982a); the pathological changes were limited mainly to the bronchi, although some parenchymal involvement was noted. The airways were surrounded by acute inflammatory cells which consisted primarily of polymorphonuclear leukocytes. Polymorphonuclear leukocytes were also present intrabronchially.

Ten days after infection, lungs from animals infected with strain PAO or strain Fe5 showed severe parenchymal as well as bronchial changes (figs. 1 and 2). In the lung parenchyma, dense mononuclear cell infiltration accumulated in the alveolar spaces and alveolar septal thickening was noted. There were no significant differences between the pathological changes in lungs with strains PAO and Fe5 when the organisms were grown in low-iron medium.

Pathological changes in lungs infected with organisms grown in high-iron medium

In contrast to the pathological findings in lungs infected with strain PAO grown in low-iron medium, the changes were much less dramatic when strain PAO was grown in

FIG. 1.—Photomicrograph of rat lung 10 days after inoculation with strain PAO grown in low-iron medium. Haematoxylin and eosin stain. Bar = 100 μ m.

FIG. 2.—Photomicrograph of rat lung 10 days after inoculation with strain Fe5 grown in low-iron medium. H and E stain. Bar = 100 μ m.

FIG. 3.—Photomicrograph of rat lung 10 days after inoculation with strain PAO grown in high-iron medium. H and E stain. Bar = 100 μ m.

FIG. 4.—Photomicrograph of rat lung 10 days after inoculation with strain Fe5 grown in high-iron medium. H and E stain. Bar = 100 μ m.

high-iron medium before inoculation. No parenchymal involvement was noted; pathological changes were restricted to the airways throughout the 10-day study period (fig. 3). Acute inflammatory cells (principally polymorphonuclear leukocytes) surrounded the airways. Inflammatory cells were also noted intrabronchially. Those pathological changes observed in the lungs of animals infected with strain Fe5 grown in high-iron medium (fig. 4) were similar to those seen in animals infected with strain Fe5 grown in low-iron medium (fig. 3). Those pathological changes were essentially identical to those seen in lungs of animals infected with strain PAO grown in low-iron medium (fig. 1).

Quantitative pathology

Pathological changes seen after 10 days in infected animals were measured as percentage infiltration by the point counting method of Dunnill (1962). In animals infected with strain PAO grown in low-iron medium, $31.2 \pm 0.7\%$ (mean $\pm$ SEM) of the left lobe of the lung was infiltrated. In contrast, in animals infected with strain PAO grown in high-iron medium, only $5.9 \pm 0.1\%$ of the left lobe was infiltrated with inflammatory cells and exudate. In animals infected with strain Fe5, cells grown in low-iron medium produced $33.1 \pm 0.2\%$ infiltration while cells grown in high-iron medium produced $17.9 \pm 0.8\%$ infiltration of the left lobe. Therefore, although the percentage infiltration of lungs infected with organisms grown in high-iron medium was much less than that of lungs infected with organism grown in low-iron medium, the infiltration observed with the mutant strain Fe5 was significantly greater than that with the parental strain grown in high-iron medium.

Animals infected with strain Fe18

None of the animals infected with strain Fe18 survived beyond 48 h. Thus, neither quantitative lung counts nor histopathological examinations were performed. At death, all animals had a frothy serous discharge from the nose, mouth and eyes.

DISCUSSION

Previous studies have indicated that exotoxin A and elastase play a role in chronic lung infections due to *P. aeruginosa* (Woods *et al.*, 1982a). Using a toxin-deficient mutant and a mutant producing non-toxic cross-reacting material (crm), Woods *et al.* demonstrated that the mutants were less virulent than their parent strain in the chronic lung infection model in rats. A mutant producing a temperature-sensitive elastase was also found to be less virulent in this model. Although neither of these factors was required for initiation of infection, both were involved in the production of the pathological changes observed in this infection.

Our data also suggest that toxin A and elastase may be important determinants of virulence in the chronic lung infection model. When *P. aeruginosa* is grown in low-iron medium it is actively synthesising toxin A and elastase at the time of infection. When the organisms are grown in high-iron medium they are not synthesising very high levels of toxin A or elastase at the time of infection and do not elicit the same pathological changes. The pathological effects observed in lungs from rats infected with the organisms grown on high-iron medium resembled the pathological changes observed with the toxin-deficient mutants and the temperature-sensitive elastase mutant (Woods *et al.*, 1982a). Pathological changes in these animals were restricted to intra- and peribronchial inflammation. The organisms which were grown in low-iron medium, however, which were actively producing toxin A and elastase, caused parenchymal changes as well as intra- and peribronchial inflammation.

The mutant strain Fe5, in which the effect of iron on elastase production is deregulated, caused similar pathological changes regardless of whether the inoculum was grown in low-iron or high-iron medium, although the degree of parenchymal involvement was greater in lungs of animals infected with organisms grown on the low-iron medium. Although strain Fe5 would not be actively synthesising toxin A when grown in high-iron medium, it would still be producing nearly normal amounts of elastase and, therefore, remained virulent. Production of either toxin A or elastase may be sufficient for a strain to be virulent in this model. Cash *et al.* (1983) previously demonstrated that purified toxin A or proteases, inoculated into rats intratracheally, either alone or in combination, produced pulmonary pathological changes similar to those induced by experimental infection. However, the studies of Cash *et al.* (1983) and Woods *et al.* (1982a) demonstrated that both toxin A and elastase are required for maximum virulence.

Strain Fe5 caused the same degree of pathological changes as strain PAO at 10 days even though the numbers of bacteria cultured from the infected lungs at this time were much lower with strain Fe5 than with strain PAO. This emphasises that elastase is an important virulence factor. It is not clear why the number of bacteria of strain Fe5 recovered from the lungs was so much lower than the number of strain PAO recovered.

All rats infected with the mutant strain Fe18, which is resistant to the effect of iron

on yields of toxin A and is a hyperproducer of toxin, died within 48 h after infection. The conclusion that these animals died due to the increased amounts of toxin A secreted by strain Fe18 is supported by a previous study which showed that rats given 15 µg of pure toxin A usually died within 48 h (Cash *et al.*, 1983). The manner of death of the rats infected with strain Fe18 was very similar to that described; they exhibited marked respiratory distress before death, and had a frothy discharge tinged with blood around the nose, mouth and eyes. Therefore, it appears that these rats died as a result of the large amount of toxin A produced by this strain.

In previous studies in a corneal-infection model we demonstrated that the iron concentration of the medium used to prepare the inocula influenced the pathological progression of *P. aeruginosa* acute infections. It was shown that organisms must be actively synthesising toxin A at the time of inoculation for maximum virulence. In this acute infection model, the maximum amount of damage is usually observed within 3 days of infection (Woods *et al.*, 1982b).

The present studies indicate that organisms must also be actively synthesising toxin A or elastase or both at the time of inoculation for maximum virulence in a chronic infection model. As in our studies of acute infection (Woods *et al.*, 1982b) the iron concentration of the culture medium also influences the pathological progression of *P. aeruginosa* infection in this chronic infection model. Thus, particular attention should be paid to the physiological state of bacteria before inoculation of animal models.

The use of the point-counting method (Dunnill, 1962) to measure pathological changes in chronically infected animals adds another dimension to the rat model originally developed by Cash *et al.* (1979). The remarkable consistency of the values obtained between lung slices and between animals infected with the same bacterial strain indicates that this technique may be a valuable addition to the measurable parameters associated with this animal model. Certainly, in the present study it has aided the quantitative differentiation of virulence based on effects of growth media.

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