Experimental Pneumonia Produced by Clinical Isolates of *Pseudomonas cepacia* in Mice

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Abstract

An experimental *Pseudomonas cepacia* lung infection was induced in ddY mice pretreated with cyclophosphamide. A single dose of 250 mg of cyclophosphamide per kg resulted in leukopenia which lasted for four days. At the lowest PMN levels, the mice were exposed to various doses of bacteria by either intratracheal inoculation or aerosol inhalation. Experimental pneumonia was established by intratracheal inoculation of $1 \times 10^7 - 2 \times 10^8$ colony-forming units of *P. cepacia*. The duration of survival time of the mice and the number of viable bacteria in their lungs were determined. A dramatic rise in the viable counts of *P. cepacia* was found within 24 hours after intratracheal inoculation of 1×10^8 colony-forming units of *P. cepacia*. It was impossible to establish *P. cepacia* pneumonia by aerosol inhalation, since the bacteria were immediately cleared from the lung. Mice which had not been treated with cyclophosphamide remained healthy and did not show any lung lesions. Thus, neutrophils appear to play an important role in the early defense mechanisms of the lung against *P. cepacia*. This animal model could be of use in evaluating additional therapies for the infection, and the pathologic determinants of infection caused by *P. cepacia*.

Introduction

Pseudomonas cepacia is an aerobic, glucose-nonfermenting gram-negative bacillus, that proliferates under conditions of minimal nutrition, and can survive even in the presence of certain disinfectants^{1,2)}. Over the past decade, this species has been increasingly recognized as a nosocomial pathogen²⁾⁻⁷⁾. The incidence of pulmonary colonization with *P. cepacia* has also increased in patients attending certain cystic fibrosis treatment centers and has been correlated with increased morbidity and mortality rates⁸⁾⁻¹¹⁾. We recently reported the clinical features of *P. cepacia* pneumonia in immunocompromised patients for whom

nebulizers were identified as the environmental reservoir for the epidemic strains¹²). However, little is known about the biological behavior and pathologic determinants of *P. cepacia* in experimental animal infections^{13,14}). Therefore, the present study was designed to develop an experimental model to determine whether direct exposure of the respiratory tract to *P. cepacia* could lead to infection of either normal or immunosuppressed mice.

Materials and Methods

Background

Between January 1990 and August 1991, the respiratory tracts of 37 patients admitted to our Department of Internal Medicine with hematological malignancies or solid tumors were colonized by *P. cepacia*. Extensive surveillance cultures of the hospital environmental surfaces and respiratory equipment revealed that all nebulizing devices were contaminated with *P. cepacia*. Sixteen of these 37 patients developed pneumonia, and in 14 the pneumonia was considered to be due to *P. cepacia*¹²).

Microorganism

In this study, we used a clinically isolated strain of *P. cepacia* (KF-1). This strain produced protease and lipase, but not lecithinase and hemolysin, which was determined by plate assays as described previously¹⁵⁻¹⁸). In the experiments described below, a stock culture was first transferred to a brain heart infusion agar slant (brain heart infusion [Difco], solidified by adding agar [Difco] to 1.5%), and the slant was incubated overnight at 37°C. Organisms from this culture were transferred to brain heart infusion broth, which was then incubated overnight at 37°C. The cells in this broth culture were sedimented by centrifugation at 12,000 ×g for 20 min and were then suspended in phosphate-buffered saline (PBS).

Mice

The mice were 4-weeks-old males of the ddY strain (Shizuoka Agricultural Cooperative Association for Laboratory Animals).

Induction of experimental *P. cepacia* pneumonia

The animals were bacterially challenged by intratracheal inoculation and aerosol inhalation.

Intratracheal inoculation

Mice were deeply anesthetized with ether and the ventral cervical region was cleaned with a 95% alcohol rub. A 0.2-inch medial longitudinal incision was then made to expose the trachea, and 0.05 ml of washed suspensions of various concentrations of *P. cepacia* was placed in it. The incisions were allowed to heal naturally, and rapid closure was noted without evidence of infection.

Aerosol inhalation

The aerosol device used for the infection is illustrated in Fig. 1¹⁹). Mice were placed in the exposure chamber (50 × 45 cm) without restriction. The bacterial suspension, charged in a nebulizer (Vaponefrin Pocket Nebulizer, USV Pharmaceutical Co., Tuckahoe, N.J.), was aerosolized with compressed air. This aerosol was drawn through the exposure chamber then passed through five air-washing bottles connected to an aspirator. The first four air-washing bottles contained 4 liters of 70% methanol, and the fifth contained 4 liters of water. The atmospheric pressure in the exposure chamber was maintained slightly lower (1.3-cm water column) than that of the ambient pressure. At the end of nebulization, fresh air was introduced into the exposure chamber. When a bacterial suspension of 10⁹ colony-forming units (CFU) per ml was nebulized at an operating pressure of 1 kg/cm² for 40 min, the maximal number of organisms in the chamber air, about 500 CFU/ml, was reached 10 min after the beginning of nebulization, and this level was maintained. After the nebulizer was turned off, the number of bacteria in the chamber air declined rapidly, and after 30 min, no bacteria were detected¹⁹).

Fig. 1 Diagram of the aerosol exposure apparatus

*Survival studies in leukopenia mice after challenge with *P. cepacia* aerosol*

To determine the correlation between the mortality rate and the intensity of infection, groups (10 mice per group) of control and immunosuppressed animals were challenged by either intratracheal inoculation or aerosol inhalation of different numbers of bacterial ($5 \times 10^6 - 2 \times 10^8$ CFU/mouse) on day 4 after cyclophosphamide treatment, and the survival rates were recorded.

*Determination of *P. cepacia* clearance from the lung*

The efficacy of the host's pulmonary defense mechanisms in clearing viable *P. cepacia* organisms from the lung was evaluated in mice treated with cyclophosphamide. These mice were challenged by an intratracheal inoculation of 1×10^8 CFU/mouse or exposed to aerosols of a bacterial suspension containing 2.5×10^9 CFU/ml. At various times after inoculation, groups of mice were exsanguinated by cardiac puncture under ether, the thoracic cavity was opened, and the lungs were aseptically removed. The lungs were weighed, then homogenized in 5 ml of sterile PBS at 4°C with a Brinkman polytron (Brinkman Inst.). Serial 10-fold dilutions were made from the homogenate, and 0.1-ml quantities of selected dilutions were plated on Trypticase Soy Agar and incubated overnight at 37°C. The following day colonies were counted, and the concentrations of *P. cepacia* in lung specimens were calculated as the total number of bacteria present in an entire lung and are described as the total bacterial count (TBC) per set of lungs. For convenience the TBC is expressed in logarithmic units to the base ten ($\log_{10}$ TBC).

Histopathological examination

Groups of animals treated with cyclophosphamide were sacrificed 24 and 48 h after inoculation. Lungs were fixed in 7.5% formalin, embedded in paraplast, cut into 3- to 5- μ m thick sections, stained with hematoxylin and eosin, and examined under a light microscope.

Results

Production of leukopenia in mice treated with cyclophosphamide

Leukopenia was found in mice treated with a single intraperitoneal dose of 250 mg of cyclophosphamide per kg. Three days after applying the alkylating agent, the WBC counts had fallen from about $8 \times 10^3/\mu$ l of blood to around 1×10^3 cells/ μ l and persisted at this level until Day 4. Recovery from the myelosuppressive effect started on Day 5, and a dramatic rise in WBC counts, indicating a strong rebound effect, was observed on Days 7 and 8. Peripheral PMN counts showed a similar profile of the WBC counts in their decline and recovery after cyclophosphamide treatment. They fell from around 9×10^2 cells/ μ l of blood to around 1×10^3 cells on Day 3 after treatment. PMN counts remained at this low level on Days 4

Fig. 2 Survival of mice after intratracheal inoculation of *P. cepacia* KF-1 (10 mice in each group). (A) Intratracheal inoculation of 2×10^8 ($\square$ — $\square$), 1×10^8 ($\blacktriangle$ — $\blacktriangle$), 5×10^7 ($\blacksquare$ — $\blacksquare$), 1×10^7 ($\circ$ — $\circ$), and 5×10^6 ($\bullet$ — $\bullet$) *P. cepacia*. (B) Aerosol inhalation of 2×10^8 ($\bullet$ — $\bullet$) *P. cepacia*.

Fig. 3 Recovery of vialbe *P. cepacia* KF-1 from the lung tissues of mice.

(A) Intratracheal inoculation of 1×10^8 CFU/mouse (mean $\pm$ SE in 3 independent experiments). (B) Aerosol inhalation of 2.5×10^9 CFU/ml (mean $\pm$ SE in 3 independent experiments)

Fig. 4 Pathology

(A) Segmental units of the lung revealed pronounced proliferation of bacteria in edema fluid in alveolar sacs without marked infiltration by leukocytes ($\times 82.5$). (B) Alveolar septa are infiltrated by small numbers of neutrophils and histiocytes, where proliferation of the bacilli is great ($\times 654$)

and 5. A dramatic increase of up to 1×10^4 cells/ μ l was observed on Day 7.

Establishment of *P. cepacia* pneumonia

Initially, it was impossible to establish *P. cepacia* pneumonia in normal mice by either intratracheal inoculation or aerosol inhalation. In addition, pneumonia could not be established even in WBC-depleted

mice by aerosol inhalation even at the highest concentration of *P. cepacia* (2.5×10^9 CFU/ml). *P. cepacia* pneumonia was established in only WBC-depleted mice by intratracheal inoculation.

Survival rate of mice after inoculation or inhalation with P. cepacia KF-1

All 10 immunosuppressed mice intratracheally inoculated with 2×10^8 CFU of *P. cepacia* per mouse died within 48 h. However, if the number of organisms was lower, the number of surviving animals and the survival time increased (Fig. 2A). In contrast, none of the non-immunosuppressed animals exposed to even 2×10^8 CFU of *P. cepacia* aerosol per mouse showed any clinical signs, and all survived (Fig. 2B).

Recover of viable P. cepacia KF-1 from the lung tissue of mice

The means $\pm$ SE of CFU of *P. cepacia* recovered from homogenized lung tissue at various times after inoculation are shown in Fig. 3. *P. cepacia* was usually isolated in pure culture. After an initial increase from about 6.0×10^7 CFU inoculated per lung on Day 0 to about 3.3×10^9 CFU per lung on Day 1, all the mice died (Fig. 3A). This was in contrast to the clearance of bacteria within a week when they were administered by aerosol inhalation (Fig. 3B).

Histology

Histological evaluation of hematoxylin and eosin-stained sections of mouse lung tissue is shown in Figure 4. The lungs are generally edematous. Large numbers of bacilli are present in the alveolar space in units of segments with bronchioles in their centers. Segments with less bacterial proliferation tended to have bacteria limited to alveolar spaces around the bronchioles. Infiltration of inflammatory cells is generally unremarkable despite the evident proliferation of bacilli in the alveolar space. However alveolar septa were infiltrated by small numbers of neutrophils and histiocytes, where proliferation of bacilli was greater.

Discussion

Many gram-negative bacteria, including *P. cepacia*, can multiply to very high levels in tap water, which is commonly used as a reservoir for inhalation therapy. The role of this organism in pulmonary disease remains controversial. Unlike *Pseudomonas aeruginosa*, *P. cepacia* is relatively nonvirulent, and it was not considered to be a primary human pathogen for many years because of its low degree of virulence and limited invasiveness. However, it has been implicated in a variety of respiratory tract infections, such as necrotizing and other forms of pneumonia²⁰⁾, and lung abscesses²¹⁾. We previously described the clinical features of epidemic nosocomial *P. cepacia* pneumonia in immunocompromised patients¹²⁾. In our survey, pneumonia occurred in 16 of 37 colonized patients, of whom 14 were considered to be infected solely by *P. cepacia*. We attempted to establish experimental pneumonia with clinical isolates of *P. cepacia* in mice.

However, it was impossible to establish *P. cepacia* pneumonia by aerosol inhalation even in WBC-depleted mice. It was possible to deliver only 1×10^6 CFU/lung even when 2.5×10^9 CFU/ml was inhaled, and these bacteria were immediately cleared from the lung. The determinants of lung clearance are multiple, including physical removal of bacteria, bacterial killing, and bacterial multiplication. It is generally believed that the decrease with time in the number of viable bacteria remaining in the lung after exposure to a bacterial aerosol is due both to physical removal of bacteria by the mucociliary system and to *in situ* bacterial killing. However, it should be considered that in humans, aerosols in the nebulizer are directly transported to the upper respiratory tract, and a relatively high concentration of bacteria could be delivered. In addition, in a clinical setting, when gram-negative bacilli are introduced by contaminated reservoir nebulizers through endotracheal tubes, or via tracheostomies, the mucociliary transport system is bypassed and large numbers of bacteria (10^7 or more) may enter the lower respiratory tract.

In contrast, experimental pneumonia was established by intratracheal inoculation of WBC-depleted mice with 1×10^8 CFU/mouse. Under these conditions, 6.0×10^7 CFU/lung was delivered. However, in

normal mice these bacteria were cleared immediately and pneumonia could not be established, suggesting that WBC are closely related to the clearance of *P. cepacia* from the upper respiratory tract.

Relatively little is known about the virulence of *P. cepacia*²³⁾. Because the strains obtained from these patients produced protease and lipase, *P. cepacia* may damage the pulmonary tissue directly by releasing toxins and enzymes. In this study, lung infection by *P. cepacia* was restricted to immunosuppressed animals. Cyclophosphamide treatment predisposed these mice to vigorous proliferation of *P. cepacia* and probably more concomitant toxin production. Histologically there were numerous bacilli in the pulmonary alveoli, but inflammatory cell infiltration was weak, probably because of cyclophosphamide predisposition.

In addition, treatment of *P. cepacia* infection is complicated by the almost universal resistance of this organism to commonly used anti-pseudomonal agents^{24,25)}. Therefore, this model may be useful in helping to establish effective prophylaxis against clinical isolates of *P. cepacia*.

In conclusion, we believe that this experimental mouse model will be useful for evaluating the pathogenesis and treatment of *P. cepacia* pneumonia.

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マウスにおける *Pseudomonas cepacia* 肺炎モデルの作成

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要 旨

Pseudomonas cepacia (*P. cepacia*) 臨床分離株の病原性を明らかにするためマウスにおける *P. cepacia* 肺炎モデルの作成を試みた。4—5週齢の ddy 系雄マウスに *P. cepacia* 臨床分離株を経気管投与、または噴霧吸入投与を行い、肺炎を惹起するか否かを検討した。Cyclophosphamide (250 mg/kg i.p.) 投与後の白血球減少マウスにおいては、*P. cepacia* 1×10^8 CFU/マウス以上を経気管投与することにより、マウスは全例死亡した。病理

組織像で細気管支周囲を中心とした肺泡腔内に菌や白血球の浸潤が認められ、肺炎の存在が確認された。一方、*P. cepacia* の噴霧吸入投与では 2.5×10^9 CFU/ml 濃度の菌液投与でもマウスは全例生存し、肺炎を惹起しえなかった。白血球正常マウスでは経気管投与、噴霧吸入投与とも肺炎を作成することはできなかった。この肺炎モデルは、*P. cepacia* の病原性因子の解析、治療の選択に関して有用な情報をもたらはしうと考えられた。