The comparison of susceptibility to endogenous *Pseudomonas aeruginosa* septicemia between endotoxin responder and non-responder mice

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Pseudomonas free specific pathogen free (SPF) mice were orally inoculated with *Pseudomonas aeruginosa* D-4m strain via drinking a 0.45% NaCl solution containing *P. aeruginosa*. The animals also received ampicillin (ABPC) to promote indigenous colonization. The Pseudomonas colonized mice, rendered neutropenic by cyclophosphamide injection, developed fatal *P. aeruginosa* bacteremia. Using this model, a comparison of mortality due to *P. aeruginosa* endogenous septicemia was made between C3H/HeN (endotoxin-sensitive) and C3H/HeJ (endotoxin-resistant) mice. C3H/HeJ mice were hypersusceptible to *P. aeruginosa* endogenous septicemia, while C3H/HeN mice were resistant. *P. aeruginosa* levels in the livers were high enough to cause death in C3H/HeJ mice, while the bacteria were eliminated from the livers of C3H/HeN mice. When both strains of mice were also inoculated with *P. aeruginosa* intravenously, the 50% lethal doses (LD_{50}) of *P. aeruginosa* D-4m strain were $10^{2.4}$ and $10^{2.8}$ CFU/mouse in C3H/HeJ and C3H/HeN mice, respectively. The clearance rate of *P. aeruginosa* from blood was similar in the two groups of mice. These results suggest an important difference between endotoxin-sensitive and -resistant mice in endogenous infection.

Key words: Endogenous septicemia, *Pseudomonas aeruginosa*, neutropenia, endotoxin

Introduction

Bacteremia caused by Enterobacteriaceae or *Pseudomonas aeruginosa* is recognized as one of the leading causes of death in neutropenic cancer patients[1]. The indigenous microflora of the gastrointestinal (GI) tract often invade the bloodstream in patients treated with antibiotics or anti-neoplastic agents[2~4]. The passage of viable indigenous bacteria from the GI tract to other organs, known as bacterial translocation, has been studied in animal models[5,6]. Bacterial translocation occurs in mice with a depressed immune system but not in healthy mice free of specific pathogens[7~10].

To study the septicemia occurring in immunosuppressed patients, a neutropenic mouse model was developed that closely resembles that of Pseudomonas infection in humans[6]. Two kinds of neutropenic mice, in which *P. aeruginosa* had colonized the GI tract, were used for comparison of changes in bacteremia and mortality. C3H/HeJ mice are relatively refractory to the toxic and mitogenic effects of endotoxin due to a defect in the *Lps* allele on the fourth chromosome and other undefined genetic influences. On the other hand, syngenetic and fully histocompatible C3H/HeN mice are fully responsive to the effect of endotoxin[11~14].

This study provides evidence of an important role for responsiveness to endotoxin in neutropenic host defense to endogenous Pseudomonas infection.

Materials and Method

Mice: Male C3H/HeN and C3H/HeJ, 5 weeks

old, weighing 20~25 g body weight, specific pathogen-free (SPF) mice were purchased from Charles River Japan Inc. (Yokohama, Japan). All animals were housed in a pathogen-free environment and received sterilized food and water in the laboratory animal center for biomedical science at Nagasaki University. The mice were observed for a week in the laboratory animal center before use.

Bacterial strain and growth conditions: *P. aeruginosa* D-4m strain was a rough colony mutant of wild type murine bacteremia strain D-4 of serotype G. Bacteria were stored at −70℃ in a brain heart infusion broth (BBL Microbiology System, Cockeysville, MD) supplemented with 10% (volume/volume) glycerol and 5% (weight/volume) skim milk (Yukijirushi Co., Tokyo) until use. For infection, bacteria were grown on trypticase soy agar (BBL) plates at 37℃ for 24 hours. The cells were harvested and washed twice with sterile saline. Bacterial number was adjusted to the desired concentration using a McFarland barium sulfate standard set (DIFCO).

Reagents: Injectable cyclophosphamide (Endoxan; Shionogi Co., Ltd., Osaka) and ampicillin (ABPC: Viccillin; Meiji, Tokyo) were obtained commercially. Cyclophosphamide and ABPC were diluted in endotoxin free sterile water and saline, respectively, before use.

Infection model: A 200 mg/kg/day dose of ABPC was administrated intraperitoneally on days 0, 1, 2, and 4. From day 1 to day 3, *P. aeruginosa* D-4m were acquired orally by drinking water inoculated with approximately 10^7 CFU/ml of bacteria. These animals were given 150 mg/kg of cyclophosphamide intraperitoneally on days 7 and 10. Animals were examined twice a day to record their survival and blood was drawn from the heart for bacteriological examination.

Testing for translocation of *P. aeruginosa*: Testing for translocation was performed as described by Berg[15]. Mice were sacrificed by cervical dislocation. The skin of the abdomen and chest was soaked in 70% alcohol and an incision was made with sterile scissors. Cardiac blood, liver and spleen were taken aseptically for bacteriological examination. After the organs had been removed, mouse feces were removed aseptically with another set of scissors and pincettes, and placed in 1 ml of sterile saline. The blood from the heart was inoculated directly onto trypticase soy agar plates. The liver and spleen were weighed and homogenized with a teflon homogenizer (Iuch, Co., Osaka) in a double volume of sterile saline. To quantitate translocated *P. aeruginosa*, adequately diluted homogenized samples were inoculated on trypticase soy agar plates, and incubated for 48 hours at 37℃. The fecal samples were weighed, homogenized in sterile saline, and serially diluted. The samples were inoculated onto NAC agar plates (Eiken Chemical Co., Ltd., Tokyo) for selection of *P. aeruginosa*.

Clearance of bacteria from blood: Mice were given 150 mg/kg of cyclophosphamide twice 3 days apart. Forty eight hours after the second cyclophsohamide administration, the mice were injected via a tail vein with 0.2 ml of saline containing suspended bacteria (ca. 10^8 CFU/ml). Then, 20 μl blood samples were obtained from the retroorbital plexus with disposable micropipettes. The samples were serially diluted into sterile saline and plated onto trypticase soy agar.

The 50% lethal dose (LD_{50}) determination: *P. aeruginosa* D-4m was serially diluted in endotoxin free sterile saline, and the desired number was injected intravenously in a volume of 0.2 ml. Five mice were used for each dilution. LD_{50} was calculated based on cumulative mortality by the method of Behrens-Karber.

Statistic analysis: The Kay test was used to assess statistical differences among survival rates. Student's *t* test was used to compare mean numbers of bacteria snd neutrophils.

Result

Comparison of the susceptibilities of C3H/HeJ and C3H/HeN mice to endogenous *P. aeruginosa* infection: To establish infection equally in the two strains of mice, preliminary experiments assessing the number of leukocytes and fecal bacteria levels, were performed. In mice receiving 150 mg/kg of cyclophosphamide twice times with 3 days apart, peripheral blood neutrophils decreased significantly and the number of neutrophils significantly more decreased in C3H/HeJ than in C3H/HeN mice (Fig. 1). When cyclophosphamide was administered after oral *P. aeruginosa* inoculation, a significantly higher mor-

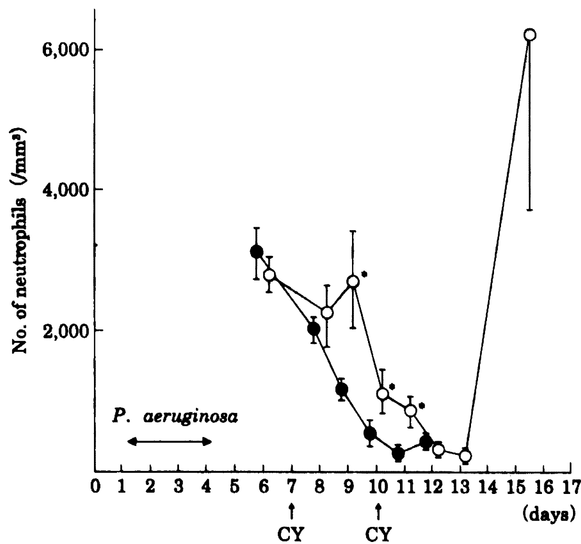

Fig. 1. The numbers of neutrophils in C3H/HeN (○) and C3H/HeJ (●) mice which received 150 mg/kg of cyclophosphamide twice times at a 3 day interval (each group n=5).

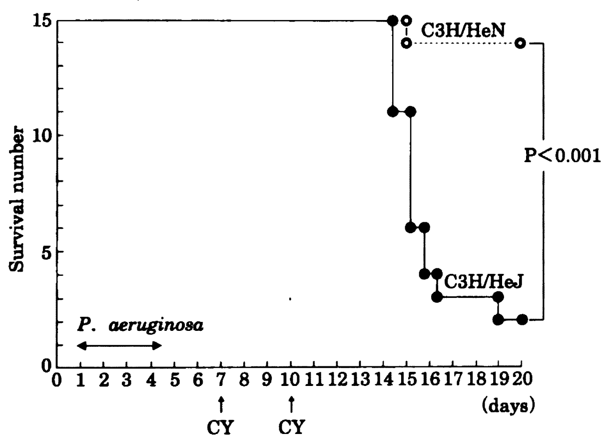

Fig. 2. Survival numbers (n=15) of C3H/HeN (○) and C3H/HeJ (●) mice given *Pseudomonas aeruginosa* orally prior to cyclophosphamide treatment.

tality rate was observed in C3H/HeJ mice, than in C3H/HeN mice ($p<0.001$; Fig. 2). A large number of *P. aeruginosa*, serotype G, were isolated from the cardiac blood of all dead mice.

The numbers of *P. aeruginosa* D-4m isolated from the liver and cardiac blood: The numbers of *P. aeruginosa* isolated from the livers of both strains of mice are shown in Fig. 3a. (The spleen recovery data were essentially the same as to those from the liver.) The number of *P. aeruginosa* isolated from the liver was similar in C3H/HeN and C3H/HeJ mice until 12 days after inoculation. However, the increase was much higher in C3H/HeJ mice than in C3H/HeN

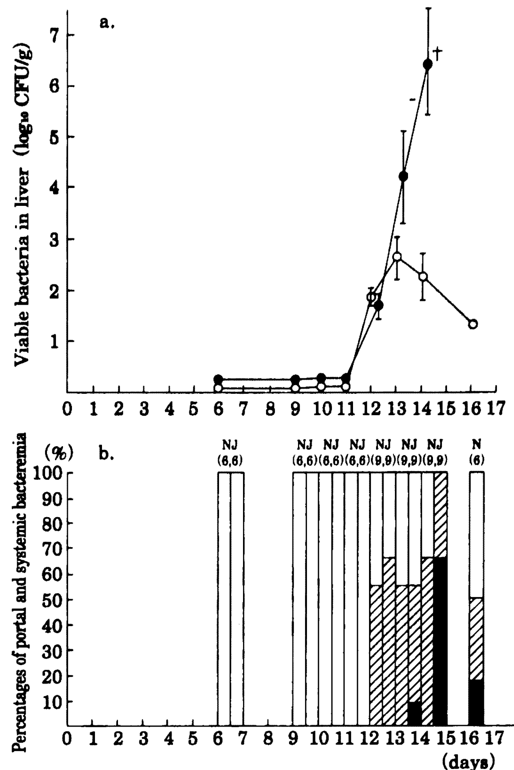

Fig. 3. a; The recoveries of *Pseudomonas aeruginosa* from the livers of C3H/HeN (○) and C3H/HeJ (●) mice (each group n=5).
b; The percentages of mice from which *P. aeruginosa* was isolated from cardiac blood (systemic bacteremia; (■) or only the liver (portal bacteremia; (▨) in C3H/HeN and C3H/HeJ mice at various times, and no isolation (□) (each group n=10).

13 days after inoculation. To assess host defense mechanisms against bacterial invasion through the mucosal surface of the GI tract, we examined changes in the isolation rates of *P. aeruginosa* from cardiac blood (systemic bacteremia) or only the liver (portal bacteremia or temporal bacteremia) in C3H/HeN and C3H/HeJ mice (Fig. 3b). There was no significant difference in bacterial isolation before day 13.

Clearance of *P. aeruginosa* D-4m from the circulation in C3H/HeJ and C3H/HeN mice: Fig. 4 shows the experiment demonstrating blood clearance of *P. aeruginosa* D-4m in C3H substrains. The two C3H substrains cleared *P. aeruginosa* from the blood stream at a similar rate.

LD_{50} of intravenously inoculated *P. aeruginosa* D-4m: The LD_{50} of the *P. aeruginosa* D-4m strain, which had been administered intravenously to neutropenic C3H/HeJ and C3H/HeN

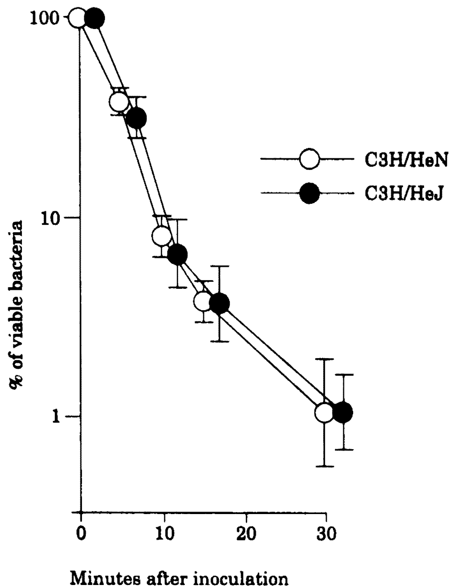

Fig. 4. The blood clearances of *Pseudomonas aeruginosa* D-4m in C3H/HeN (○) and C3H/HeJ (●) mice.

mice, were $10^{2.4}$ and $10^{2.8}$ CFU, respectively.

Discussion

Tancrède[4] demonstrated clinically that intestinal colonization was the predominant harbinger of gram-negative bacteremia and when the gut was colonized by *P. aeruginosa*, the organism was most likely to be isolated from the blood in neutropenic patients with hematologic malignancies. It was also suggested that the mechanism of bacteremia is similar to the mechanism of the endogenous bacteremia of indigenous organisms as described previously in our animal model[6]. This study provides evidence that endotoxin responsiveness determines host resistance to endogenous *P. aeruginosa* bacteremia in neutropenic hosts. Endotoxin resistant mice, C3H/HeJ, were significantly more susceptible to infection than their respective endotoxin sensitive counterparts, C3H/HeN mice. C3H/HeJ mice are hypersusceptible to *Salmonella typhimurium* septicemia[10] and to *Escherichia coli* urinary tract infections[12,13]. Using two routes of infection, UTI or intraperitoneal infection, Eden[12] demonstrated that the resistance to *E. coli* was correlated with the *Lps* genotype, but that *S. typhimurium* infection was influenced by at least two separate resistance factors, one of which might be controlled by the *Lps* gene. And their separate analysis of mucosal and systemic phases of infection revealed that the effect of *Lps* was especially pronounced after mucosal infection.

In the present study of endogenous infection, the number of *P. aeruginosa* recovered from the liver and the rate of positive liver culture 24 hours after the second cyclophosphamide treatment showed no difference between C3H/HeN and C3H/HeJ mice. Subsequently, in C3H/HeJ mice, *P. aeruginosa* multiplied to a level sufficient to cause death. In contrast, *P. aeruginosa* tended to be eliminated from the livers of C3H/HeN mice. This result emphasizes the importance of effector mechanisms in the clearance of *P. aeruginosa* from systemic sites, rather than the indigenous mucosal barrier. These results are assumed to contrast with the above results reported by Hageberg. This contrast can easily be explained by the use of different bacterial species, murine host defense mechanisms, and the mucosal site of infection.

The reticuloendothelial system (RES) is concerned with defense against micro-organisms invading the bloodstream[6,16~18]. We designed these experiments to compare the capacities of neutropenic C3H/HeJ and C3H/HeN mice to clear the circulation of bacteria injected into the tail vein, and to compare the LD_{50} for intravenously administered of *P. aeruginosa* D-4m in C3H/HeN and C3H/HeJ mice. Our data show that C3H/HeJ mice clear bacteria from he circulation as well as C3H/HeN mice, and that the two strains of mice are equally susceptible to intravenous *P. aeruginosa*. There was a close correlation between a defective endotoxin proliferative response and susceptibility to endogenous Pseudomonas infection, but little relation between endotoxin responsiveness and susceptibility to intravenous Pseudomonas infection.

In our previous study of a murine endogenous septicemia model[7], bacterial translocations were observed in mesenteric lymphnodes, the portal vein blood, or the liver before systemic Pseudomonas bacteremia developed. By translocation of the endotoxins of gram-negative bacteria, such as *E. coli* and *P. aeruginosa*, the reticuloendothelial system might have previously evoked host resistance and promoted bacterial clearance from the blood.

Our study demonstrates an important difference between genetic determinants of host resistance

to endogenous infection and intravenous infection. In the intravenous infection model, both sensitive and resistant strains of mice lack further effective resistance to Pseudomonas infection. With endogenous infection, in the endotoxin sensitive animals, previously translocated endotoxin might induce host defense mechanisms aimed at inhibition of bacterial growth, while endotoxin resistant animals are not capable of such resistance.

Koch and collagues[19] demonstrated that infusion of 40 μg/kg of endotoxin for 1 or 4 hours before *E. coli* injection in a rabbit model produced a significant delay in blood clearance of the inoculated bacteria. They concluded that the diminished systemic bacterial elimination reflected reticuloendothelial dysfunction induced by previously infused endotoxin. Their conclusion is inconsistent with our results, but the inoculum dose, the route of endotoxin inoculation, and the pathogen were different. In our experiment, the routes of pathogen and endotoxin inoculation were more natural and gradual than those used in their experiment.

Our investigation indicates that in endogenous infection, which is similar to clinical septicemia, previously translocated endotoxin induces host defense mechanisms aimed at inhibition of bacterial growth.

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内因性緑膿菌敗血症における endotoxin 感受性および非感受性マウスの比較

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緑膿菌を内因性に持たない specific pathogen free (SPF) マウスに緑膿菌 D-4m 株を経口的に接種し，ampicillin を同時に投与して腸管に定着させた。さらに，マウスに 150 mg/kg のサイクロフォスファマイドを2回投与して，顆粒球減少状態にすることで，内因性の緑膿菌による敗血症を惹起するモデルを作成した。このモデルを用いて，Endotoxin 感受性の異なる C3H/HeN マウス（感受性）と C3H/HeJ マウス（抵抗性）の緑膿菌による内因性感染症に対する致死性と臓器内菌数の比較を行った。その結果，C3H/HeJ マウスは C3H/HeN マウスに比べ，緑膿菌性内因性敗血症に対し感受性であった ($p<0.001$)。また，C3H/HeJ マウスの肝臓や脾臓，心臓の血液から分離される緑膿菌の菌数は経時的に増加したが，C3H/HeN マウスでは増加が抑制された。緑膿菌 D-4m 株の静注による LD_{50} は，両群のマウスで有意差をみなかった。また緑膿菌を静注した後の血液からの除菌率の検討でも，両群のマウスに差はみられなかった。これらのことから，endotoxin 感受性マウスでは，以前に我々が報告した全身性敗血症にさきだつ bacterial translocation による endotoxin の血流中への流入が網内系の活性化をもたらし，敗血症発症に対する抵抗性を獲得するものと考えられた。

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