IN VITRO AND *IN VIVO* CHANGES OF SEROTYPE IN *Pseudomonas aeruginosa* ISOLATES BY ANTI-PSEUDOMONAL DRUGS

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(Received for publication June 10, 1993)

The present study was designed to clarify whether anti-pseudomonal drugs affected *in vitro* and *in vivo* changes in serotypes of *Pseudomonas aeruginosa* isolates. Forty-two isolates of *P. aeruginosa* belonging to different serotype groups were each incubated in Mueller-Hinton broth including piperacillin, cefsulodin, ceftazidime, imipenem, gentamicin or norfloxacin at 1~4 MICs at 35°C for 20 hours. The bacterial cells in the media were serotyped after the incubation. In each experiment with these six drugs, serotypes of 4 (9.5%) to 8 (19.0%) of the 42 isolates changed to other different groups. There was no relationship between the serotypes of the variants formed with the anti-pseudomonal drugs and kinds of the drugs. In the case of *P. aeruginosa* TA-2 isolate, the different concentrations (1/2 MIC and 2×MIC) of cefsulodin induced the distinct changes in serotypes such as the poly-agglutinable (M, G) and non-typable groups, respectively. The time course for the formation of the variant cells was investigated during the incubation of *P. aeruginosa* TA-2 isolate in the presence of cefsulodin, respectively. In this case, the variant cells appeared 6 hours after incubation and continued to grow together with the intact cells. On the other hand, the variant cells with anti-pseudomonal drugs (cefsulodin, imipenem and gentamicin) were also formed in the model infections in mice. The present results indicated that the co-existence of colonies with different serotypes in some isolates of *P. aeruginosa* was partially due to the alternation of serotypes with anti-pseudomonal drugs given to patients with infection.

In our previous study, we found that a considerable number of *Pseudomonas aeruginosa* isolated from patients with various types of infection formed colonies different in serotypes and other biological characteristics[1], and partially in drug-susceptibility[2]. The co-existence of different types of colonies in individual isolates posed some problems for its biological and clinical significances, and the mechanisms of such co-existence of the different cells has not been well clarified. On the other hand, B. OJENIYI *et al.* showed that in cystic fibrosis patients infected with *P. aeruginosa*, changes in serotypes were caused by the infection with bacteriophages contained in the sputum of the patients[3]. Furthermore, it was demonstrated that changes in serotype were caused by cell to cell contact between the 2 distinct strains of *P. aeruginosa* isolated from cystic fibrosis patients[4]. Other studies indicated that changes in serotype and the *O*-antigen structure of *P. aeruginosa* were caused by bacteriophage infection[5~7].

In the present study, we investigated the *in vitro* and *in vivo* effects of anti-pseudomonal drugs on changes in serotype of the pathogens in patients infected with *P. aeruginosa*.

Materials and Methods

Specimens and Test Colonies

Sputum, urine, pus and otorrhea were obtained from patients with various infections from 19 hospitals throughout Japan in 1989[1]. These specimens were transported to our laboratory within two days. These

fresh specimens were each streaked on to CLED agar (OXOID, Hants, England), and then incubated aerobically at 35°C for 24~48 hours. From the agar plates 8 to 16 colonies were taken randomly and examined for gram-staining to screen for *P. aeruginosa*. Additionally, to identify *P. aeruginosa*, the colonies were examined using Bio-test No. 2 (a kit for *P. aeruginosa*-glucose-non-fermentative bacteria, Eiken Chemical Co., Ltd., Tokyo, Japan), and acylamidase production, pigment production and morphology (rough or smooth) were examined[8]. In this study, each colony was sub-cultured overnight in Mueller-Hinton broth (Difco, Detroit, U.S.A.) at 35°C as needed, to obtain the sub-strains. The biological characteristics of the colonies or sub-strains were tested, based on the methods described in Manual of Clinical Microbiology (5th edition)[8], unless otherwise mentioned.

Serotyping

Slide-agglutination tests with live antigens from an overnight blood agar sub-culture of the test colonies were carried out using a set of monoclonal antibodies towards HOMMA's serotyps (Meiji Seika Kaisha, Ltd., Tokyo, Japan)[9].

Anti-pseudomonal Drugs

The drugs used in this study included piperacillin (Toyama Chemical Co., Ltd., Tokyo, Japan), cefsulodin (Takeda Chemical Industries, Ltd., Osaka, Japan), ceftazidime (Glaxo Inc., Durham, N.C.), imipenem (Banyu Pharmaceutical Co., Tokyo, Japan), gentamicin (Schering-Plough Corp., Bloomfield, N.J.), and norfloxacin (Kyorin Pharmceutical Co., Ltd., Tokyo, Japan).

Treatment of *P. aeruginosa* with Drugs

Mueller-Hinton brothes each containing anti-pseudomonal drugs at concentrations of 1/4 MIC~ 4 × MIC were inoculated with *P. aeruginosa* isolates at an inoculum size of 10^5 colony forming units (CFU) per ml, and incubated at 35°C for 20 hours. Each 0.1 ml-aliquot was spread on CLED agar plates and incubated at 35°C for 20 hours. The grown colonies, different in morphology, were picked up for serotyping.

Growth Curve Serotype Variants

Mueller-Hinton brothes each containing cefsulodin (1/2 MIC~2 × MIC) were inoculated with *P. aeruginosa* TA-2 isolate at an inoculum size of 10^5 CFU/ml. The media were incubated at 35°C, samples at 0, 1, 2, 4, 6, 8 and 24 hours, and diluted serially with 0.9% saline. Each 0.1 ml-aliquot of the dilutions was spread on CLED agar plates using glass stick. The plates were incubated at 35°C for 20 hours, and colonies were counted. The geometric mean and standard derivations were calculated.

Drug-susceptibility

Minimum inhibitory concentrations (MICs) of the test drugs were determined by the agar dilution method[10]. Mueller-Hinton agar (Difco, Detroit, U.S.A.) was used for the test medium. A final inoculum of 10^4 CFU, prepared by dilution of a fresh overnight broth culture (Trypticase soy broth, BBL, Md., U.S.A.) was applied to Mueller-Hinton agar plates with a multipoint inoculator (Sakuma Seisakusho Co., Ltd., Tokyo, Japan). After incubation at 35°C for 20 hours, the lowest concentration that inhibited macroscopic colonial growth was defined as the MIC.

Model Infections

The test strains of *P. aeruginosa* were cultured overnight on Mueller-Hinton agar at 35°C and then suspended in saline, using the quantity necessary to yield the required cell counts per ml. Male ICR-strain mice aged 5 weeks were used in groups of 5. Mice were inoculated intraperitoneally with 0.5 ml of the suspension and were given a single intramuscular dose (ED_{50}) of the test drugs 1 hour after challenge. Eight hours after dosing, the blood was collected by puncture of the heart, and the kidneys removed and then homogenized with sterile saline. One ml of serial dilutions of the blood or homogenates were mixed with the melted Mueller-Hinton agar (9 ml) in a petridish. The agar media were incubated at 35°C for 20 hours before colony counting.

Results

Effect of Anti-pseudomonal Drugs on *In Vitro* Changes in Serotypes

Forty-two isolates of *P. aeruginosa* from patients with various types of infections were each incubated in Mueller-Hinton broth containing piperacillin, cefsulodin, ceftazidime, imipenem, gentamicin or norfloxacin (1 × MIC to 4 × MIC) at 35°C for 20 hours, and serotyped using monoclonal antibodies. As shown in Table 1, the changes in serotypes of test isolates were the most frequent in the presence of norfloxacin; 8 (19.0%) of the 42 isolates changed to different groups after incubation. Four (9.5%) to 6 (14.3%) of the 42 isolates also changed to different groups by other 5 drugs tested. In these experiments with 6 kinds of the drugs, serotypes of 14 (33.3%) of the test isolated changed to other groups by the contact with drugs used. Although the data were not shown, the serotypes of these variant cells did not differ after several transfers in Mueller-Hinton agar (35°C, 20 hours). Furthermore, *P. aeruginosa* isolates could be divided into two categories (stable and unstable isolates) according to their variability of serotype after *in vitro* exposure to anti-pseudomonal drugs. Table 2 shows the relationship between the isolates changed to other serotypes and the test drugs. These was no relationship between serotypes of the variants formed with the 6 drugs and kinds of the drugs. However, the 5 drugs except ceftazidime changed the serotype of *P. aeruginosa* No. 4S from G to C group; piperacillin, cefsulodin and ceftazidime changed the serotype of No. 5S from B to G group; the 5 drugs except cefsulodin changed the serotype of No. 57

Table 1. Incidence of changes in serotype of *Pseudomonas aeruginosa* isolates by anti-pseudomonal drugs.

Drug[a]	Piperacillin	Cefsulodin	Ceftazidime	Imipenem	Gentamicin	Norfloxacin	Total
Number of isolates changed in serotype / Number of isolates tested	5/42[b]	5/42	5/42	4/42	6/42	8/42	14/42
(%)	(11.9)	(11.9)	(11.9)	(9.5)	(14.3)	(19.0)	(33.3)

[a] 1 × ~4 × MIC, 35°C, 20-hour incubation.
[b] Test isolates.

Table 2. Changes in serotype of *Pseudomonas aeruginosa* isolates by anti-pseudomonal drugs.

Isolate (serotype)	Piperacillin	Cefsulodin	Ceftazidime	Imipenem	Gentamicin	Norfloxacin
1*R* (C)	—[a]	—	—	—	—	E
2*S* (B)	—	—	—	—	C	—
2*R* (C)	—	—	—	—	N.T.[b]	E
4*S* (G)	C	C	—	C	C	C
4*R* (G)	—	—	—	—	—	E
5*S* (B)	G	G	G	—	—	—
TA 2 (G)	M, G	N.T.	M, G	—	—	—
13 (A)	—	N.T.	N.T.	—	B	N.T.
50 (C)	—	N.T.	—	N.T.	—	—
57 (D)	B	—	B	B	B	B
174 (F)	N.T.	—	—	—	—	—
2 (G)	—	—	—	—	—	B
47 (I)	N.T.	—	—	—	—	—
111 (K)	—	—	N.T.	—	—	—
8 (M)	—	N.T.	N.T.	N.T.	G	N.T.

[a] No change.
[b] Non-typable.

from D to B group; the 5 drugs except piperacillin changed the serotype of No. 8 from M to G or non-typable group. The respective serotypes of other 11 isolates also changed to different one or two groups.

In the case of *P. aeruginosa* TA-2 isolate, although piperacillin and ceftazidime at 2 × MIC changed the serotype of TA-2 isolate from G group (a mono-agglutinative) to M and G groups (a poly-agglutinative), cefsulodin at different concentrations for the same isolate gave the different serotypes; the non-typable group of serotype was induced at 2 × MIC of the drug, and a poly-agglutinative group (M and G) at 1 × MIC (the data not shown). The low concentrations of cefsulodin, ceftazidime and gentamicin, respectively, at 1/4, 1/2 and 1/2 MIC, were also effective for changes in serotype of the TA-13 isolate.

Time Course for Growth of Serotype Variants

Mueller-Hinton broth containing cefsulodin at 1/2 MIC or 2 × MIC was each inculated with *P. aeruginosa* TA-2 isolate at an inoculum size of 10^5 CFU/ml, and incubated at 35°C for 24 hours. The viable parent and variant cell counts were determined. As shown in Fig. 1, in the absence of cefuslodin the parent cells only grew to the level of 10^9 CFU/ml, without any changes in their serotypes, at the end of incubation. However, in the presence of the drug at 1/2 MIC, the parent cells showing the serotype G group grew together with the poly-agglutinative cells for the M and G groups. In this case, the poly-agglutinative cells were not detectable by the first 4-hour incubation.

In the presence of the higher concentration of the drug (2 × MIC), the variant cells belonging to the non-typable in serotyping, apart from the parent cells (G group), started to grow from 6 hours after incubation and grew over the parent cells by 24 hours. A similar experiment was performed with *P. aeruginosa* TA-13 in the presence of gentamicin at 1/2 MIC (the data not shown). The parent cells only grew in the incubation without the drug, and any other cells were not detected throughout the incubation, but in the incubation with the drug at 1/2 MIC, the variant cells grew together with the parent cells from 6 hours after incubation. These results showed that both isolates tested did not originally contain the distinct cells different in serotype, and therefore indicated the formation of phenotypic variants by

Fig. 1. Growth curves of serotype variants of *Pseudomonas aeruginosa* TA-2 induced by cefsulodin.
a) Control (drug free), b) 1/2 MIC (0.78 μg/ml), c) 2 × MIC (3.13 μg/ml).

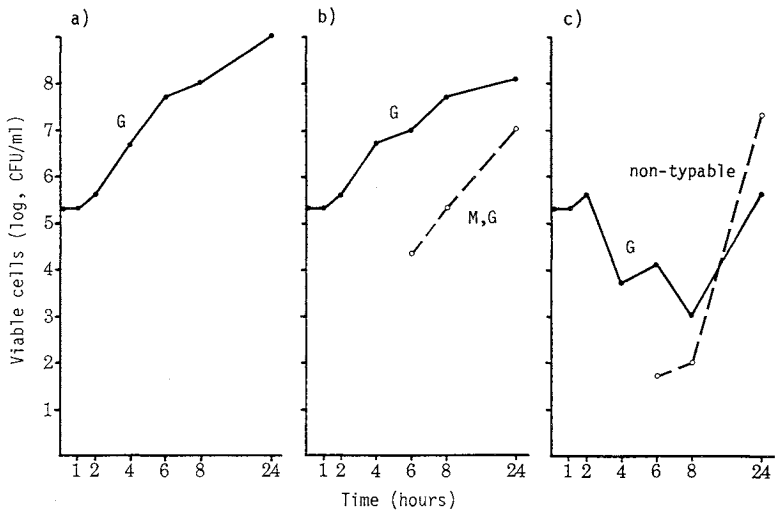
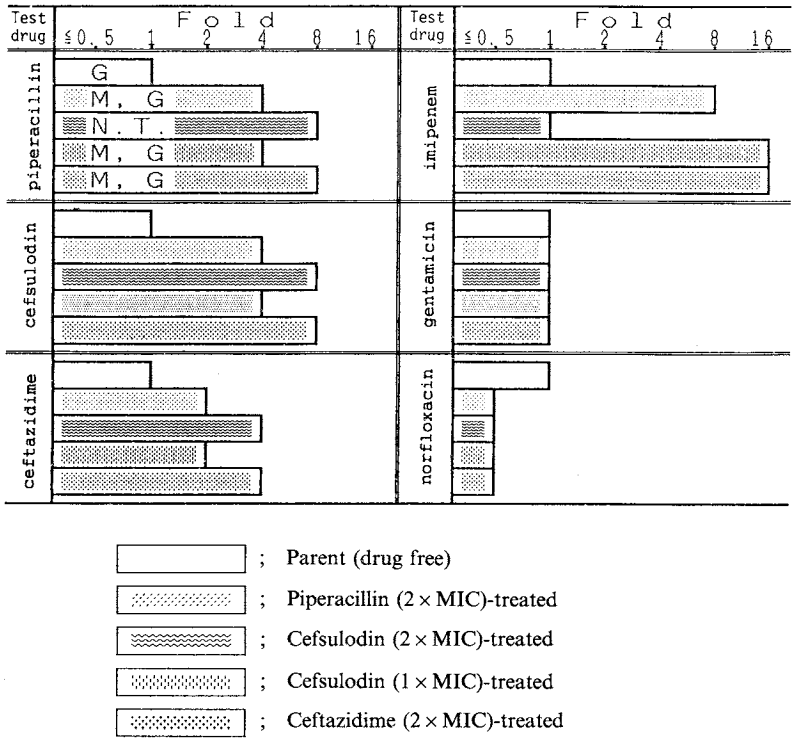


Fig. 2. Changes in drug-susceptibility of serotype variants of *Pseudomonas aeruginosa* TA-2 by anti-pseudomonal drugs.
(Variant MIC/parent MIC)



anti-pseudomonal drugs.

Drug-susceptibility of the Serotype Variants

Susceptibility of some variants to 3 anti-pseudomonal drugs was determined and compared with that of the respective parents. Variants of *P. aeruginosa* TA-2 isolate obtained after incubation in the presence of piperacillin (2 × MIC), cefsulodin (1 × or 2 × MIC), ceftazidime (2 × MIC) were used as test strains. The results were expressed as ratios of MICs for the variants to respective parents. In this experiment, the normal range of variation of MIC values was considered to be 0.5 ~ 2 × MIC. As shown in Fig. 2, the MIC ratios of gentamicin and norfloxacin for all the test strains were below 2; no changes in susceptibility of the variants to these 2 drugs were observed. However, MIC values of piperacillin and cefsulodin for the variants, obtained by the incubation were 4-fold or more higher than those of the parent, and those of ceftazidime for 2 of the 4 variants were 4-fold higher than those of the parent.

Susceptibilities of the variants obtained by the incubation with three drugs each were highly resistant to imipenem (the MIC ratios; 8 ~ 16 fold) except one variant.

Changes in Serotype of *P. aeruginosa* Isolates in Model Infections

P. aeruginosa TA-2, TA-13 and TA-21 isolates, which were confirmed to change their serotypes the *in vitro* experiments, were used as the challenge bacteria for the model infections in mice. *P. aeruginosa* ATCC-27853 was also used as a control strain with no changes in serotype. As described in Materials and Methods, mice were injected intramusculary with cefsulodin, imipenem or gentamicin in the respective

Table 3. Effect of anti-pseudomonal drugs on serotype of *Pseudomonas aeruginosa* isolates in model infections in mice.

Drug	Isolate (serotype)	Serotype (viable cells·log)	
		Blood	Kidney
Cefsulodin	TA-2 (G)	G (4.0)	G (4.9)+none (2.5)
	TA-13 (A)	A (4.7)	A (6.0)
	TA-21 (A)	A (6.4)	A (6.4)
	ATCC-27853 (G)	G (3.2)	G (4.8)
Imipenem	TA-2	G (4.1)+none (2.3)	G (5.7)
	TA-13	A (3.5)	A (4.6)
	TA-21	A (4.3)	A (4.2)
	ATCC-27853	G (3.5)	G (4.4)
Gentamicin	TA-2	G (3.1)	G (3.0)
	TA-13	A (6.6)	A (7.5)+none (7.7)+M (7.3)
	TA-21	A (5.3)	A (6.7)+none (5.0)
	ATCC-27853	G (3.0)	G (3.0)
Control	TA-2	G (8.0)	G (8.8)
	TA-13	A (4.6)	A (5.6)
	TA-21	A (6.2)	A (6.6)
	ATCC-27853	G (7.5)	G (8.0)

Mice: ICR strain, 5 weeks old, 5/group. Infection: Intraperitoneal challenge. Therapy: Intramuscular dose (ED_{50}), 1 hour after infection.

doses of ED_{50}s 1 hour after challenge. Eight hours after dosing, the blood was collected, and the kidneys were removed, and the bacterial cell counts in the samples and their serotypes were determined. As shown in Table 3, after dosing with cefsulodin, the non-typable cells were detected together with the intact cells of the G group in the kidneys of mice infected with *P. aeruginosa* TA-2 isolate. After dosing with imipenem, the same results were also obtained with the blood of mice infected with TA-2 isolate. Furthermore, after dosing with gentamicin, the non-typable and M group cells were detected with the intact cells of A type in the kidneys of mice infected with *P. aeruginosa* TA-13 isolate.

In infection with a standard strain, *P. aeruginosa* ATCC-27853 (G group), no changes in serotype were found in the bacterial cells isolated from the blood and tissue, after dosing with the three drugs. Control experiments without the drugs were performed, and no changes in serotype were confirmed in mice infected with four strains of *P. aeruginosa*.

Discussion

P. aeruginosa has emerged as a major opportunistic pathogen in recent year, causing a considerable part of nosocomial infections. Therefore, it is still now the most important pathogen for antibacterial chemotherapy. On the other hand, it is well known that the bacteria are heterogenous for biological characteristics such as serotype, phage type, pyocin type and biotype, and the clinical isoltes are classified into several types on the basis of these characteristics[1]. In the previous paper, we reported that some isolates formed colonies of more than one kind of serotype, when many isolates were cultured on Mueller-Hinton agar[1]. Each colony co-existing in the same isolates had, in part, different susceptibilities to some anti-pseudomonal drugs[2].

It is difficult to conclude whether the colonies with different serotypes in the individual isolates were due to mixed infections with distinct strains of the same species, or due to a heterotypic population of the colonies in single species. In cystic fibrosis patients infected with *P. aeruginosa*, mono-agglutinable cells are reported to be transformed to poly-agglutinable cells, owing to alternation of *O*-antigen structure on the bacterial surface by the infection with bacteriophages which were frequently contained in the sputum of

the patients[5~7,12]. However, in our studies, since various kinds of specimens included not only the sputum but also urine, pus and otorrhea, the sources were not limited to the patients with cystic fibrosis. Our results could not be explained by those obtained with patients with cystic fibrosis. In resent years, OJENIYI also reported an example of the alternation of the bacterial surface resulting in serotype changes during chemotherapy with tobramycin and ciprofloxacin for the patient infected with *P. aeruginosa*[7]. The results indicated the possibility that the serotypes of *P. aeruginosa* could be changed *in vitro* or *in vivo* with some of anti-pseudomonal drugs.

As a consequence, the co-existence of cells different in serotypes in individual isolates of *P. aeruginosa* from patients may be induced by anti-pseuomonal drugs given to the patients. In the present study, the effect of several anti-pseudomonal drugs on changes in serotypes of *P. aeruginosa* was investigated *in vitro*, and the changes of serotypes were observed in a considerable number of isolates tested. There were no cells different in serotypes in either experiment with *P. aeruginosa* TA-2 or TA-13 isolates in the absence of drugs. However, 6 hours after incubation in the presence of drugs, the variant cells different in the serotype appeared and grew together with the intact cells. These results *in vitro* confirmed OJENIYI's observation and speculation[7], and supported our findings that the co-existence of the cells different in serotypes of the individual isolates is due to alternation of serotypes with anti-pseudomonal drugs given. Furthermore, the *in vivo* experiments for evaluation of the effect of anti-pseudomonal drugs were also performed using the model infections with some isolates of *P. aeruginosa*. There were some changes in serotypes of the bacterial cells isolated from the heart blood and kidneys in mice infected with *P. aeruginosa* isolates and dosed with the anti-pseudomonal drugs. Furthermore, the patterns of *in vivo* serotype changes in *P. aeruginosa* TA-2 and TA-13 isolates by the drugs did not accord with those obtained in the *in vivo* models. We suggest that defense mechanisms in the animal body due to immune systems affect the bacterial surface, with changes in *O*-antigen structures.

The results in Fig. 2 showed that some serotype-variant cells had decreased susceptibilities to anti-pseduomonal drugs. The resistance to imipenem was especially high. A number of investigators have discussed the relationship between serotypes of *P. aeruginosa* strains and their drug-susceptibility[13~17], but a definite conclusion has not been made. It is well known that the resistance of *P. aeruginosa* to drugs is associated with different mechanisms such as: β-lactamase production, various modifications of penicillin-binding proteins, and different descreases in drug-permeability. Therefore, it may be necessary to determine the resistant mechanisms involved in the variants.

For this problem, attention should be paid to our data that most of the serotype variants were highly resistant to imipenem. Furthermore, it is well known that the drug exhibits antibacterial activity against wide ranges of bacteria, by permeating the porin structure on the cell surface[18]. Serotype changes in the variant cells may be expected to be associated with alternation in the *O*-antigen structure of the lipopolysaccharide of *P. aeruginosa*, the relationship between changes in serotypes and the *O*-antigen structure of the lipopolysaccharide and drug-susceptibility of variants obtained from this study are in progress.

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