JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1975, p. 175-179

Vol. 1, No. 2
Printed in U.S.A.

Reduced Virulence of *Pseudomonas aeruginosa* Grown in the Presence of Benzalkonium Chloride

FRANK W. ADAIR,* HUI-LIAN LIAUW, SAM G. GEFTIC, AND JUSTUS GELZER[1]

Research Department, Pharmaceuticals Division, CIBA-GEIGY Corporation, Summit, New Jersey 07901

Received for publication 25 October 1974

Resistant cells of *Pseudomonas aeruginosa* ATCC 9027 which were grown in the presence of 1 mg of benzalkonium chloride (BC) per ml caused only a mild conjunctivitis when they were dropped onto the scratched corneas of rabbits. In contrast, cells of the BC-sensitive parent strain induced a severe keratoconjunctivitis. In addition, the BC-grown cells also had a reduced capacity to produce kidney infections in mice as compared to the parent strain. BC-grown cells acted as weak complex antigens which conferred slight protection against lethal doses of BC-grown cells. No cross-protection to cells of the parent strain occurred. The data indicate that growth in the presence of BC results in cells with reduced virulence.

In a previous study (1), we reported the ability of a resistant strain of *Pseudomonas aeruginosa* to survive and multiply in the presence of high levels (1 mg/ml) of benzalkonium chloride (BC), a mixed alkyl quaternary ammonium compound (quat). Growth under these conditions resulted in unusual cultural, metabolic, and ultrastructural changes in the cells (3, 8). BC and other quats are widely used as disinfectants and antimicrobial preservatives to prevent accidental bacterial contamination of multiple-dose parenteral and ophthalmic solutions. However, the contamination of solutions of quats by resistant species of the genus *Pseudomonas* has caused serious and fatal nosocomial infections (4). Therefore, it was of interest to determine the effect of growth in BC upon the virulence of *P. aeruginosa*. The traumatized rabbit eye was selected as a model. The capacity of the BC-resistant and BC-sensitive strains to induce kidney infections in mice was also compared.

MATERIALS AND METHODS

Organism and growth. The BC-sensitive parent strain was *P. aeruginosa* ATCC 9027. This is the same strain that is designated for testing the efficacy of preservatives in sterile, multi-dose parenteral and ophthalmic drugs (13). This strain will not grow in BC concentrations above 200 μg/ml. The BC-resistant strain was obtained by gradually increasing the BC concentrations as described previously (1). Both sensitive and resistant strains were cultured in a D-glucose, Casamino Acids (Difco), expanded salts medium (2). In the case of the resistant strain, BC (1 mg/ml) was always present in the growth medium, unless otherwise designated. Cultures of both strains were grown stationary at 30 C. Because of a long lag phase and a slower growth rate (3), the resistant strain was grown for 72 h (log phase), whereas the sensitive strain was grown for 24 h (log phase). After incubation, cells of both strains were then centrifuged, washed three times, and resuspended in sterile, 0.9% (wt/vol) NaCl solution (hereafter referred to as saline solution) and adjusted to various optical densities. Plate counts were made to determine the number of cells per milliliter in each suspension. These cell suspensions were used for all subsequent work.

Experimental infections. To compare the virulence of the BC-sensitive and BC-resistant strains for the traumatized eye, the corneas of albino, male rabbits, weighing approximately 3 kg, were anesthetized with 0.1 ml of a sterile, aqueous solution of 1% procaine HCl. After 15 min, the corneas were carefully scarified three times in parallel with a sterile 26-gauge needle. At this time, 0.1-ml volumes of each of the different cell suspensions were then carefully dropped onto the corneas. The concentrations of the BC-sensitive cell suspensions were 6×10^7 and 1.8×10^8 cells per 0.1 ml, whereas the cell suspensions of the BC-resistant strain had the following concentrations: 1.8×10^7, 1.7×10^8, and 1.7×10^9 cells per 0.1 ml. Only one eye per rabbit was infected. The eyes were examined grossly on a daily basis for a period of 3 weeks. The severity of the *P. aeruginosa*-induced corneal infection was scored according to the total area of corneal involvement as follows: 0, no reaction; 1+, up to 25% affected; 2+, 25 to 50% affected; 3+, 50 to 75% affected, and 4+, 75 to 100% affected.

The virulence of both the BC-resistant and BC-sensitive strains was measured by their capacity to cause kidney infections in mice systemically. The study was carried out by intravenously (i.v.) injecting 0.5-ml volumes of the washed cell suspensions into a lateral tail vein of groups of five male, CF1 mice, weighing

[1] Present address: Department of Biology, CIBA-GEIGY Ltd., CH 4002, Basel, Switzerland.

approximately 20 g. The BC-sensitive strain was injected in a concentration of 2×10^6 cells per mouse, whereas the BC-resistant strain was injected at a level of 1.6×10^6 cells per mouse. Groups of mice were sacrificed on a daily basis for the first 5 days; thereafter, groups were sacrificed every 2 days through day 11, then every 3 days through day 17. The last group was sacrificed on day 21. The kidneys were aseptically removed, pooled, and homogenized in sterile saline solution. The homogenate was then diluted in sterile saline solution and plated in Trypticase soy agar (BBL). After incubation at 30 C for 48 h, the colonies were counted, and the average number of organisms per kidney was calculated. The measurement of mean lethal dose (concentration of cells needed to cause 50% mortality in a group of animals) end points (12) was carried out for the BC-resistant and BC-sensitive strains by intraperitoneally (i.p.) injecting groups of 10 mice, each with 0.5-ml volumes of the various washed cell suspensions. A total of six groups of mice were injected with the following concentrations of the BC-sensitive strain: 2.6×10^7, 3.2×10^7, 4.0×10^7, 4.6×10^7, 5.2×10^7, and 6.0×10^7 cells. In the same manner, a total of six groups of mice were injected with the following concentrations of the BC-resistant strain: 8.6×10^7, 1.8×10^8, 2.5×10^8, 3.4×10^8, 4.3×10^8, and 5.7×10^8 cells. The mice were then observed for 5 days at which time the cumulative mortalities were recorded and LD_{50} end points were calculated.

Determination of resistance. The percentage of resistant cells in a population of BC-resistant cells, which were grown in the absence of BC for 24 h, was determined by first diluting and plating the cells in D-glucose, Casamino Acids, and expanded salts agar. The plates were incubated at 30 C for 48 h. A total of 100 individual surface colonies from growth on a plate containing a minimum of 100 surface colonies were then transferred into Kahn tubes containing 0.5-ml volumes of D-glucose, Casamino Acids, expanded salts broth, and 0.1% BC. After incubation at 30 C for 5 days, the tubes were observed for growth as indicated by turbidity.

Immunization. The immunization procedure consisted of injecting the first three immunizing doses by the subcutaneous route and the fourth by the i.v. route. Cell suspensions of the BC-resistant and BC-sensitive strains were injected subcutaneously into groups of male, CF1 mice, weighing between 17 to 20 g according to the following immunization schedule: day 1, 1.2×10^7 cells; day 2, 2.4×10^7 cells; day 3, 3.6×10^7 cells. After 7 days, 3.0×10^7 cells were injected by the i.v. route. Challenge doses equivalent to approximately 6 LD_{50} were injected i.p. after 21 and 35 days. The cumulative mortalities were recorded for 5 days after the challenge dose.

RESULTS

The inoculation of the scratched corneas with approximately 6×10^7 cells of a suspension of the BC-sensitive strain caused a range of reactions as shown in Table 1. A total of five out of six rabbits exhibited a moderate to severe infection within 48 h. This type of reaction was characterized by a blepharitis which was accompanied by a purulent exudate (Fig. 1a). As a result, the eyelids were sealed together. After cleansing of the eyelids with cotton swabs soaked in saline solution, the eyelids were gently separated to reveal a definite corneal haze and a whitish infiltrate (Fig. 1b). In the example shown, 25% (2+ reaction) of the corneal area was infiltrated when approximately 1.8×10^8 cells of the BC-sensitive strain were dropped onto the scratched cornea. A marked conjunctivitis was also apparent. The infection increased in intensity and, at 5 days postinfection, reached a stage characterized by a severe opaque, white infiltrate covering 100% (4+ reaction) of the corneal area (Fig. 1c). These infections started to regress after 8 days and were almost completely resolved after 21 days. Little or no scarring or clouding of the corneas could be detected. Conversely, cells of the BC-resistant strain caused only a mild conjunctivitis in all four rabbits at an infecting dose of approximately 1.7×10^9 cells per eye (Table 1 and Fig. 1d). The use of lower concentrations of cells of the BC-resistant strain caused no discernible eye infection.

TABLE 1. *Progression of ocular pathology induced by BC-sensitive and BC-resistant strains of P. aeruginosa*

Rabbit no.	Strain	Pathology score								
		Day (post-infection)								
		1	2	3	5	8	12	14	18	21
1	BC-S[a]	4	4	4	4	4	4	4	3	1
2	BC-S	4	4	4	4	3	3	3	3	1
3	BC-S	3	4	4	4	2	2	2	2	1
4	BC-S	3	3	4	4	2	2	2	1	0
5	BC-S	1	3	3	3	1	1	1	1	0
6	BC-S	0	1	1	1	0	0	0	0	0
7	BC-R[b]	0	1	1	1	1	0	0	0	0
8	BC-R	0	1	2	1	1	1	0	0	0
9	BC-R	0	0	1	1	1	0	0	0	0
10	BC-R	1	1	1	1	1	1	1	0	0

[a] Benzalkonium chloride-sensitive (6.0×10^7 cells per eye).

[b] Benzalkonium chloride-resistant (1.7×10^9 cells per eye).

In another experiment, when resistant cells were grown in the absence of BC for 24 h, they retained the same reduced level of pathogenicity for the rabbit eye as cells grown in the presence of BC. Upon examination of the resist-

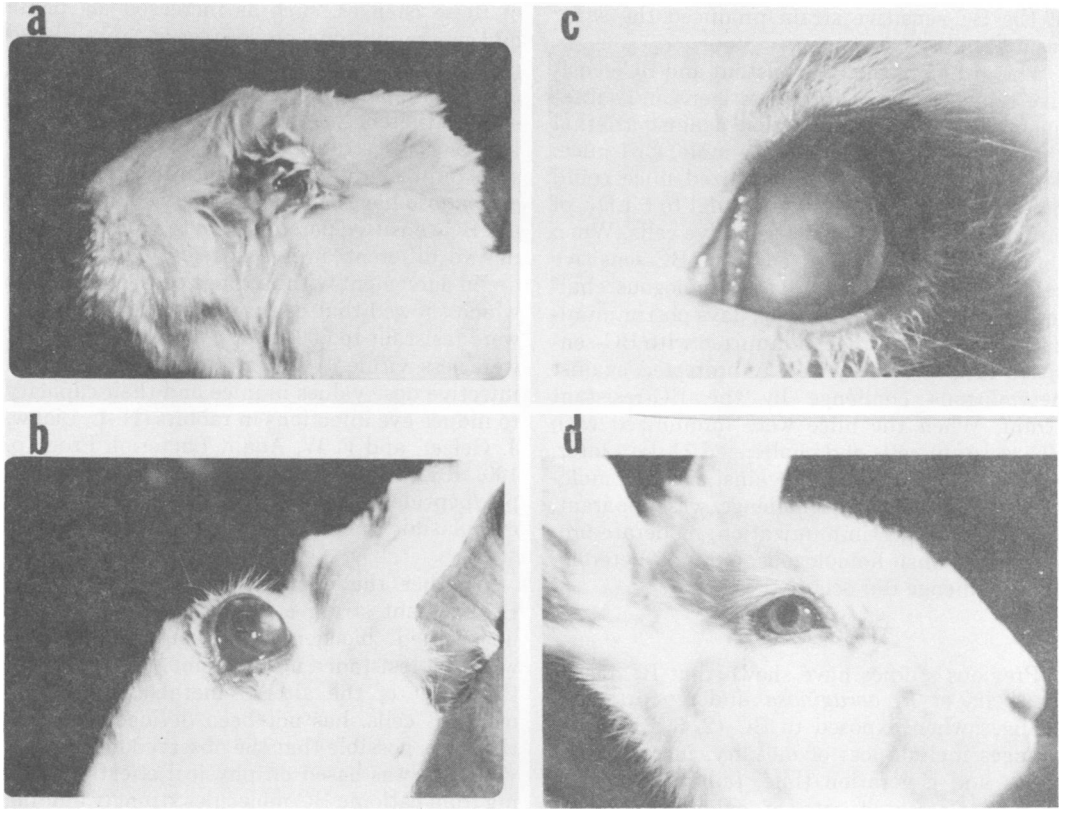

FIG. 1. *Ocular pathology of the BC-sensitive and BC-resistant strains. (a) Blepharitis and purulent exudate 72 h after inoculation with 6 × 10⁷ cells of the BC-sensitive strain; (b) corneal ulcer (3+ reaction) and conjunctivitis 72 h after inoculation with 1.8 × 10⁸ cells of the BC-sensitive strain; (c) corneal ulcer (4+ reaction) 8 days after exposure to 6 × 10⁷ cells of the BC-sensitive strain; (d) mild conjunctivitis (1+ reaction) 72 h after inoculation with 1.7 × 10⁹ cells of the BC-resistant strain; no corneal infiltration was apparent.*

ance level of these cells (see Materials and Methods), it was found that 95% of the colonies contained cells still capable of growing in the presence of 0.1% BC.

The virulence of the BC-sensitive and BC-resistant strains was further compared by their capacity to infect groups of mice after i.v. injection. The mouse kidney is very susceptible to infection by gram-negative bacteria with initial lesions occurring in the renal cortex (6). The results presented in Fig. 2 show that the number of sensitive cells in the kidneys progressively increased throughout the course of the study and reached a peak at 17 days postinfection. Between this time (day 17) and the end of day 21, at which time the experiment was terminated, all of the animals succumbed to the infection. In contrast, the average number of viable BC-resistant cells rapidly decreased in the kidneys during the first 3 days postinfection and appeared to establish a mild infection (Fig. 2). No deaths occurred in the animal groups

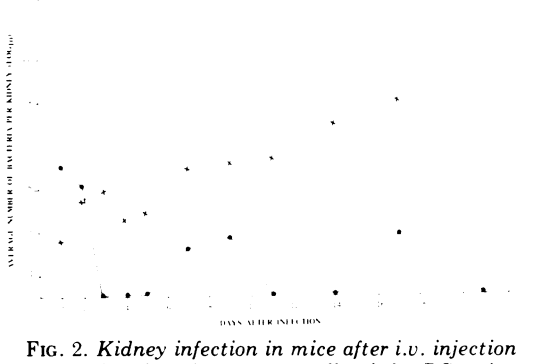

FIG. 2. *Kidney infection in mice after i.v. injection (lateral tail vein) of 1.6 × 10⁶ cells of the BC-resistant strain (●) and 2 × 10⁶ cells of the BC-sensitive strain (×). Each data point represents the average number of bacteria in the kidneys of five mice.*

that were infected with BC-resistant cells. The concentration of BC-resistant cells required to obtain an LD_{50} after i.p. inoculation was 3.8×10^8, whereas significantly fewer (4.8×10^7) cells

of the BC-sensitive strain produced the same results.

The ability of the BC-resistant and BC-sensitive cells to act as complex antigens and subsequently to provide protection against a lethal challenge was examined using male, CF1 mice. Table 2 shows that unimmunized mice could not survive a challenge dose equal to 6 LD_{50} of either BC-resistant or BC-sensitive cells. When the mice were immunized with BC-sensitive cells, they were resistant to homologous challenge administered 21 and 35 days postimmunization. However, mice immunized with BC-sensitive cells were only weakly protected against heterologous challenge by the BC-resistant strain. When the mice were immunized with BC-resistant cells and challenged 21 days later, essentially no protection against either homologous or heterologous challenge was apparent. After 35 days postimmunization, moderate protection against homologous but not heterologous challenge did occur.

DISCUSSION

Previous studies have shown that BC-resistant cells of *P. aeruginosa* undergo dramatic changes when exposed to BC (2, 3, 8). These changes include loss of motility, increased lag phase and generation time, failure to oxidize glucose, decreased rate of oxidation of substrates such as Casamino Acids, a negative cytochrome oxidase reaction, stabilization to lysis, and unique cytoplasmic reorganizations. Some of these changes, such as increased lag phase and generation time, could suggest a diminished capacity for competitive survival.

TABLE 2. *Infectious resistance in mice after immunization with BC-sensitive and BC-resistant strains of P. aeruginosa*

Immunizing strain[a]	Challenge strain[b]			
	Day 21		Day 35	
	BC-S	BC-R	BC-S	BC-R
None	0/10[c]	0/10	0/9	0/9
BC-S	13/14	1/14	13/15	4/15
BC-R	3/14	0/14	2/15	9/15

[a] Mice immunized with three consecutive subcutaneous injections 24 h apart, representing approximately 1.2×10^7, 2.4×10^7, and 3.6×10^7 bacteria, followed after 4 days by 1 i.v. injection of 3.0×10^7 bacteria.

[b] Mice challenged i.p. with approximately 6 mean lethal infective doses (BC-S, 2.9×10^8 cells; BC-R, 2.3×10^9 cells). Abbreviations: BC-S, benzalkonium chloride-sensitive; BC-R, benzalkonium chloride-resistant.

[c] Total number of survivors/total number challenged.

In this report, we have shown another interesting difference between the BC-resistant and BC-sensitive strains of *P. aeruginosa*. The results indicate that the BC-resistant strains of *P. aeruginosa* have significantly less capacity than the BC-sensitive parent strain to induce disease in two different animal species. These results are in agreement with findings presented earlier which showed that cells of *P. aeruginosa* which were resistant to 600 μg of polymyxin B per ml were less virulent with regard to mean lethal infective dose values in mice and their capacity to induce eye infections in rabbits (H.-L. Liauw, J. Gelzer, and F. W. Adair, Bacteriol. Proc., p. 100, 1971). Polymyxin B is a cyclic, cationic, polypeptide antibiotic which, like BC, has the cytoplasmic membrane as its primary active site.

Whether the decrease in virulence of the BC-resistant strain is the direct effect of still unspecified biochemical changes associated with the resistance mechanism, or is indirectly the effect of the altered metabolism of BC-resistant cells, has not been defined. In addition, it is possible that the observed decrease in virulence was based on physical effects, resulting from cationic BC molecules strongly binding with the negatively charged cell envelope (7, 10). It is important to note, however, that cells of the resistant strain, which were grown for 24 h in the absence of BC, also had a lowered disease-producing capacity for the rabbit eye, while also maintaining a high degree of resistance. This may be taken as an indication that attachment of high concentrations of BC molecules to the cell envelope does not play an important role in the reduced virulence of the organism, since the concentration of BC molecules should have been diluted during division. However, the possibility that low levels of cell envelope-attached BC molecules may affect the virulence of the organism cannot be excluded. We are currently comparing the BC-resistant and BC-sensitive strains with regard to their capacity for production of extracellular substances, such as vascular permeability factor (9) and collagenase (5), both of which are thought to be active in the pathogenesis of *P. aeruginosa* infections. The inability of cells of the BC-resistant strain to act as complex antigens and provide protection against lethal doses of the BC-sensitive strain suggests biochemical differences between the two strains. In this regard, the chemical compositions of the cell en-

velopes, especially the lipopolysaccharide components, of the two strains are being compared in our laboratories.

Maxcy et al. (11) reported that cells of *Escherichia coli*, which were adapted to grow in the presence of 28 μg of a quat similar to BC per ml, also acquired an increased tolerance to lysis. However, these organisms had a markedly diminished ability to survive storage as washed cell suspensions when compared with the quat-sensitive parent strain. Maxcy et al. (11) postulated that development of resistance to higher concentrations of the quat would probably result in an even greater weakening of the cells for competitive survival. These authors suggested that the ability of bacteria to become resistant to quats may be of little significance in the current commercial situations where quats are used as disinfectants. The acceptance of such a premise by the drug industry could lead to serious contamination problems. It is, therefore, important to emphasize that, even though our findings indicate that the BC-resistant strain has reduced virulence under some conditions, the results should not be construed to mean that such microorganisms are nonpathogenic and are not potentially dangerous contaminants of manufacturing equipment, clinical equipment, and drugs. Indeed, the literature cites several examples of quat-resistant microorganisms which have caused serious infections (4). Thus, the use of quats as disinfectants and as antimicrobial preservatives should continue to be accompanied by a keen awareness of the ability of *Pseudomonas* species to acquire resistance to these compounds.

LITERATURE CITED

1. Adair, F. W., S. G. Geftic, and J. Gelzer. 1969. Resistance of *Pseudomonas* to quaternary ammonium compounds. I. Growth in benzalkonium chloride solution. Appl. Microbiol. **18**:299-302.
2. Adair, F. W., S. G. Geftic, and J. Gelzer. 1971. Resistance of *Pseudomonas* to quaternary ammonium compounds. II. Cross-resistance characteristics of a mutant of *Pseudomonas aeruginosa*. Appl. Microbiol. **21**:1058-1063.
3. Adair, F. W., S. G. Geftic, J. Gelzer, and H.-P. Hoffmann. 1971. Effect of a hostile environment on *Pseudomonas aeruginosa*. Trans. N.Y. Acad. Sci. **33**:799-813.
4. Bassett, D. J. C. 1971. Causes and prevention of sepsis due to gram-negative bacteria. Common-source outbreaks. Proc. Roy. Soc. Med. **64**:980-986.
5. Diener, B., L. Carrick, Jr., and R. S. Berk. 1973. In vivo studies with collagenase from *Pseudomonas aeruginosa*. Infect. Immunity **7**:212-217.
6. Gorrill, R. H., and S. J. DeNavasquez. 1964. Experimental pyelonephritis in the mouse produced by *Escherichia coli*, *Pseudomonas aeruginosa*, and *Proteus mirabilis*. J. Pathol. Bacteriol. **87**:79-87.
7. Hamilton, W. A. 1968. The mechanism of the bacteriostatic action of tetra-chlorosalicylanilide: a membrane-active antibacterial compound. J. Gen. Microbiol. **50**:441-458.
8. Hoffman, H.-P., S. G. Geftic, J. Gelzer, H. Heymann, and F. W. Adair. 1973. Ultrastructural alterations associated with the growth of resistant *Pseudomonas aeruginosa* in the presence of benzalkonium chloride. J. Bacteriol. **113**:409-416.
9. Kusama, H., and R. H. Suss. 1972. Vascular permeability factor of *Pseudomonas aeruginosa*. Infect. Immunity **5**:363-364.
10. MacGregor, D. R., and P. R. Elliker. 1958. A comparison of some properties of strains of *Pseudomonas aeruginosa* sensitive and resistant to quaternary ammonium compounds. Can. J. Microbiol. **4**:499-503.
11. Maxcy, R. B., N. P. Tiwari, and P. R. Soprey. 1971. Changes in *Escherichia coli* associated with acquired tolerance for quaternary ammonium compounds. Appl. Microbiol. **22**:229-232.
12. Reed, L. J., and H. Muench. 1938. A simple method for estimating fifty percent end points. Am. J. Hyg. **27**:493-497.
13. United States Pharmacopeia, 18th ed. 1970. Antimicrobial agents—effectiveness test, p. 845-846. Mack Publishing Co., Easton, Pa.