RELEVANCE OF *IN VITRO* ANTIBACTERIAL ACTIVITIES OF CEFOTIAM AND CEFAZOLIN TO THEIR THERAPEUTIC EFFECTS ON EXPERIMENTAL PNEUMONIA CAUSED BY *KLEBSIELLA PNEUMONIAE* DT-S IN MICE

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The MICs of cefotiam and cefazolin against *K. pneumoniae* DT-S were unaffected by the inoculum size and were 0.1 and 1.56 μg/ml, respectively. Bactericidal and bacteriolytic activities of the cephalosporins were more potent in bacterial concentrations of 10^7 colony-forming units (CFU)/ml than in concentrations of 10^8 CFU/ml. Both activities of cefotiam were more markedly influenced by bacterial concentrations than those of cefazolin. Therapeutic activity of cefotiam was about 9~15 times as potent as that of cefazolin in experimental pneumonia caused by *K. pneumoniae* DT-S in mice, and this finding was in accordance with the ratio of *in vitro* antibacterial activities of the two cephalosporins as judged by the MICs or the bactericidal and bacteriolytic activities in bacterial suspension of 10^7 CFU/ml. The range of concentrations of cefotiam which induced cell filamentation *in vitro*, was wider than that of cefazolin. This difference, however, was not reflected on the therapeutic activities of the two cephalosporins in the model infection. In the pneumonic lungs, definite therapeutic doses of both cephalosporins (80 mg of cefotiam per kg and 640 mg of cefazolin per kg) produced mainly bacteriolysis of the challenge organisms.

In vitro antibacterial activity of β-lactam antibiotics is influenced by the bacterial concentrations of inoculum suspensions, and the antibiotics induce various morphological changes in Gram-negative rods, such as cell filamentation and lysis[1,2,3]. Filamentous cells are able to resume cell division and growth after removal of the antibiotics[2,4,5]. The range of concentrations which induce cell filamentation varies markedly among β-lactam antibiotics[1,2,5,6]. Cefotiam, a broad-spectrum cephalosporin, shows excellent *in vitro* and *in vivo* antibacterial activities, especially, against Gram-negative rods[7]. In some cases, however, the antibacterial activity of cefotiam is influenced markedly by the inoculum size, while that of cefazolin is only slightly affected[8]. Cefotiam induces filamentation of cells over a wide range of concentrations from less than the minimum inhibitory concentration (MIC) to one higher than the MIC, while the range of concentration of cefazolin which induces filamentation of cells is relatively narrow[8,9]. Some workers suggest that cephalosporins showing marked changes in *in vitro* antibacterial activities depending on the inoculum size and having a wide range of concentrations that induce filamentous cells, may be unfavorable for chemotherapy[5,8,10]. Practically, however, the chemotherapeutic activity of cefotiam is much more potent than that of cefazolin[11,12]. Therapeutic activity of cefotiam superior to that of cefazolin in experimental *Klebsiella* pneumonia in mice has also been confirmed with various dosage regimens[13]. The therapeutic significance of these *in vitro* effects of inoculum size and morphological change, however, has not been well established.

In the present paper, experiments were conducted to determine whether or not the differences in these *in vitro* effects of inoculum size and morphological change due to cefotiam and to cefazolin was accompanied by differences in their therapeutic efficacies on experimental pneumonia caused by

Klebsiella pneumoniae DT-S in mice.

Materials and Methods

Cephalosporins

Cefotiam was prepared at the Takeda Chemical Industries, Ltd., Osaka, Japan. Cefazolin (Cefamezin, Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan) was obtained from a commercial source.

Organism

K. pneumoniae DT-S, biotype *edwardsii*, capsular type I, is a well established derivative of strain DT, and the biological characteristics of the strain have been described previously[14].

In vitro Studies

The minimum inhibitory concentrations (MICs) were determined by the agar dilution method by using Trypticase soy agar (TSA; BBL) with a inoculum size of one loopful of bacterial suspension (10^6 and 10^8 colony-forming units (CFU)/ml)[7]. The MIC was defined as the lowest concentration of cephalosporin that prevented visible growth after overnight incubation at 37°C. Bactericidal and bacteriolytic activities were studied in Trypticase soy broth (TSB; BBL). An overnight culture of *K. pneumoniae* DT-S in TSB at 37°C was diluted in TSB to obtain a concentration of about 10^6 CFU/ml, and the cultures were incubated with shaking at 37°C in an automatic continuously recording photometer (Bio-Log II, Jasco Juan-Quentin SA) and 3-cm path length cells. Two-fold serial dilutions of cephalosporins were added at the point when bacterial counts reached about 10^7 CFU/ml (transmission, 90%) or about 10^8 CFU/ml (transmission, 50%). Opacity was recorded continuously. Aliquots were removed at various time intervals, and CFU were determined by the plate count method.

In vivo Studies

Experimental pneumonia was induced in mice according to the method described previously[14]. Four-week-old Slc: ICR male mice, weighing 18~22 g, placed in the exposure chamber, were infected with *K. pneumoniae* DT-S by the aerosol method resulting in a deposition of about 10^4 CFU of bacteria per lung. Therapy was started 30 hours after infection when mice had a well-developed pneumonia with bacterial growth in the lung reaching the late exponential phase[14]. The cephalosporins were dissolved in 0.85% NaCl and injected subcutaneously (0.1 ml/mouse) according to the schedules presented in each experiment (Table 1, Figs. 3, 4, 7 and 8). The numbers of dead mice were recorded daily, and surviving animals were sacrificed on the day following completion of medication for determination of gross pulmonary pathology and bacterial recovery from the lungs. The lungs were homogenized in 4 ml of distilled water in a Teflon tissue homogenizer. Tenfold serial dilutions of the homogenate in distilled water were made, and 1 ml of the sample was mixed with molten TSA. The plates were incubated at 37°C for 24 hours and the colonies were counted. The number of bacteria was expressed as the log number of CFU per lung. The amount of cephalosporin (mg/kg) required for 50% survival of the animals (50% survival dose, SD_{50}) and that required for an eradication of challenge organisms from the lung of 50% of the animals tested (50% clearance dose, CD_{50}) were calculated at the end of the observation period by the method of REED and MUENCH[15].

Cephalosporin Assay

Five-week-old Slc: ICR male mice, weighing 25~29 g were injected subcutaneously with the cephalosporins (0.1 ml/mouse) four times at 30-minute intervals. Groups of five mice were sacrificed by exsanguination at various intervals during the 8-hour period after the first medication. Blood specimens were collected from the axillary artery and vein, and plasma was separated by centrifugation from the heparinized blood specimens. After the animals were killed by bleeding, the lungs were removed and homogenized with 9 volumes of 10 mM phosphate-buffered saline, pH 7 (PBS)[14] in a Teflon tissue homogenizer. The homogenates were centrifuged and the supernatants were assayed. Specimens were stored at −80°C until assay. The concentrations of cefotiam and cefazolin in each specimen were assayed by the cylinder plate diffusion technique by using *Proteus mirabilis* ATCC 21100 and *Bacillus subtilis* PCI 219 as the test organisms, respectively[16,17].

Morphological Studies

K. pneumoniae DT-S was cultured in TSB with shaking at 37°C, and the antibiotics were added in the late exponential growth phase (about 10^8 CFU/ml). A loopful of each culture was smeared on a glass slide at various intervals during the 6-hour period after the addition of cephalosporins. Drying and fixation were performed according to the conventional method and cells were stained with methylene blue. About 100~200 cells were observed with a light microscope and morphological responses were distinguished as follows: Cells having a similar size and a degree of staining to those of the control cells were defined as normal, cells with more than four times the normal length were defined as filaments, and cell debris or the cells retaining a bacillary form with unstained cytoplasm were defined as lysed cells. Samples for scanning electron microscopy were prefixed in 1% glutaraldehyde (electron microscopic reagent) for 2~3 hours and after washing in a 1/15 M phosphate-buffered solution (pH 7) were post-fixed in 1% osmic acid (electron microscopic reagent) for 16~18 hours. Dehydration was performed with graded concentrations of ethanol and iso-amyl acetate. The specimens kept in gelatin capsules were rapidly frozen with liquid nitrogen and were cut by using a freezing-cut apparatus (TF-1, Eiko Engineering Co. Ltd.). They were then dried with a critical point dryer (HCP-2, Hitachi Koki Co. Ltd.); and, for the surface conductivity, were spatter-coated with gold by using an ion coater (1B-3, Eiko Engineering Co. Ltd.). Observation and photographings were performed in a scanning electron microscope (MSM 4C101, Akashi Seisakusho Ltd.), operated at an acceleration voltage of 15 KV.

Results

In Vitro Antibacterial Activity

The MICs of cefotiam and cefazolin against *K. pneumoniae* DT-S were 0.1 and 1.56 μg/ml both with inoculum sizes of 10^6 and 10^8 CFU/ml, respectively.

Comparable reduction in the number of surviving cells was observed at 0.1 μg of cefotiam per ml and at 1.56 μg of cefazolin per ml with bacterial concentrations of 10^7 CFU/ml (Fig. 1). Cefotiam at 0.2 μg/ml and cefazolin at 3.13 μg/ml showed almost the same and strong bactericidal effects. When the cephalosporins were added to bacterial suspensions reaching 10^8 CFU/ml, marked reduction in bactericidal activities of both cephalosporins was noted and the degree of the reduction was more marked with cefotiam. With 10^8 CFU/ml suspension, obvious bactericidal effects were seen at 0.78 μg of cefotiam per ml and at 3.13 μg of cefazolin per ml. Bacteriolytic activities of both cephalosporins, like bactericidal activities, were affected by the bacterial concentrations in suspension (Fig. 2). With 10^7 CFU/ml suspensions (transmission, 90%), cefotiam at concentrations higher than 0.1 μg/ml and cefazolin at higher than 1.56 μg/ml showed obvious bacteriolytic effects. With the 10^8 CFU/ml sus-

Fig. 1. Bactericidal effect of cefotiam and of cefazolin on *K. pneumoniae* DT-S.
Arrow indicates time of drug addition.
a: μg/ml.

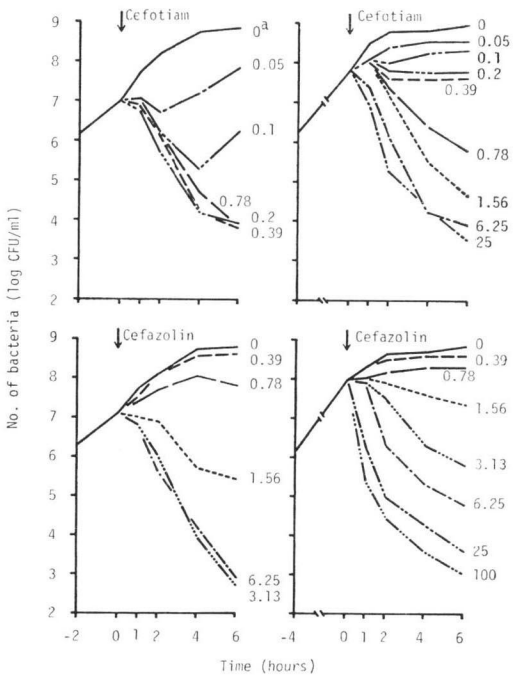


Fig. 2. Bacteriolytic effect of cefotiam and cefazolin on *K. pneumoniae* DT-S.
Arrow indicates time of drug addition. a: μg/ml.

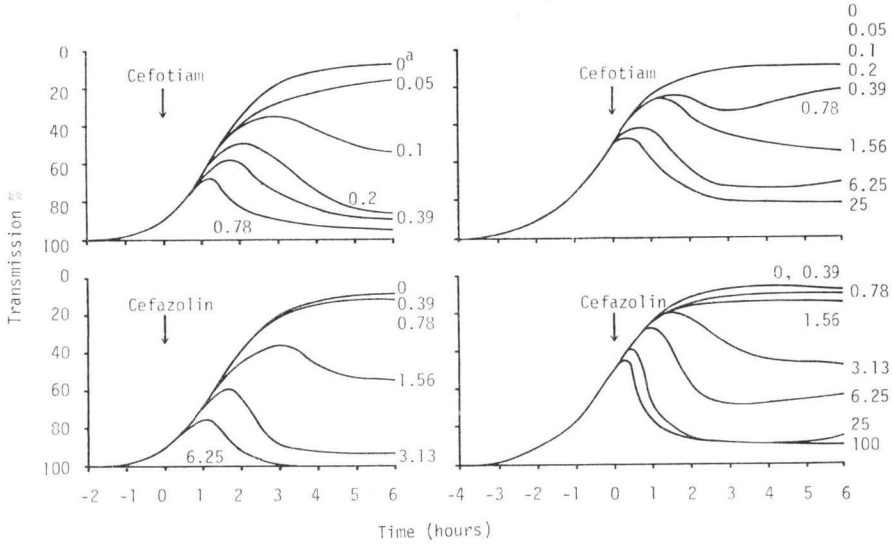


Table 1. Therapeutic effect of cefotiam and cefazolin on experimental pneumonia caused by *K. pneumoniae* DT-S in mice.[a]

Treatment[b] mg/kg			Survival[d] rate (survival/ total)	Time to[e] death (day; mean± S.D.)	Rate of positive mice for[f]		No. of bacteria[g] in lung (log CFU/ tissue; mean± S.D.)	50 % effective dose (mg/kg)[h] calculated by	
Cephalo-sporin	Dose[c] (mg/kg)	No. of dosing series			Gross lesion (positive/ survival)	Bacterial recovery (positive/ survival)		Survival rate (SD_{50})	Eradication rate (CD_{50})
Cefotiam	40(10×4)	20	0/10	3.3±1.3					
	80(20×4)	20	0/10	6.8±3.0					
	160(40×4)	20	10/10		10/10	9/10	4.96±1.22	117	265
	320(80×4)	20	9/10	5.0	9/ 9	2/ 9	5.05±0.35		
	640(160×4)	20	10/10		10/10	0/10			
Cefazolin	320(80×4)	20	0/10	3.7±1.1					
	640(160×4)	20	0/10	4.8±2.3				1,736	2,392
	1,280(320×4)	20	1/10	6.0±2.5	1/ 1	1/ 1	6.12		
	2,560(640×4)	20	10/10		10/10	4/10	5.03±2.67		
Infected control			0/10	2.9±0.3					

[a] Four-week-old Slc: ICR male mice weighing 18~22 g were used. Bacterial suspension ($10^{9.03}$ CFU/ml) was nebulized at a pressure of 1 kg/cm^2 for 40 minutes.

[b] Therapy was started at 30 hours after infection and continued for ten days. Two dosing series of cephalosporin, each consisting of four subcutaneous injections at 30-minute intervals, were made daily starting at 8 a.m. and 4 p.m., respectively.

[c] Dose was expressed as total amount of cephalosporin administered in a dosing series (individual dose × no. of injections).

[d] Survival rate at 11 days after infection.

[e] Time to death was calculated only for fatal cases.

[f] Surviving animals were sacrificed 11 days after infection and examined for gross pulmonary lesions and bacterial recovery from the lung.

[g] Number of bacteria in lung was calculated only for positive lungs.

[h] SD_{50} (50 % survival dose) and CD_{50} (50 % clearance dose) were expressed as total doses administered over a dosing series.

pension (transmission, 50%), 1.56 μg of cefotiam per ml and 3.13 μg of cefazolin per ml exhibited the same degree of bacteriolytic effects. These results indicate that the bactericidal and bacteriolytic activities of cefotiam are about 16 times as potent as those of cefazolin with the 10^7 CFU/ml suspension and about two to four times as potent as those of cefazolin with the 10^8 CFU/ml suspension.

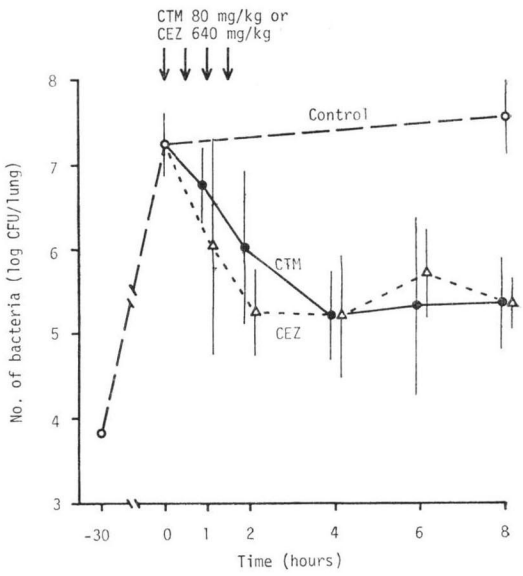
In Vivo Antibacterial Activity

The therapeutic effect of cefotiam on *Klebsiella* pneumonia in mice was compared with that of cefazolin. Therapy was started 30 hours after infection and continued for ten days. Two series of medication, each consisting of four subcutaneous injections at 30-minute intervals, were made daily. As judged by the SD_{50} and CD_{50} values, cefotiam proved 15 and 9 times as active as cefazolin, respectively (Table 1). Individual doses of cefotiam at 80 mg/kg and of cefazolin at 640 mg/kg provided almost the same therapeutic effects in the model infection. Eradication of the challenge organisms from the lungs was observed in 7 of 10 mice for cefotiam and 6 of 10 mice for cefazolin, and the number of bacteria in the lungs calculated for positive lungs was almost equal for both cephalosporin-treated groups.

Mice infected with *K. pneumoniae* DT-S by the aerosol method were treated with four subcutaneous doses of 80 mg of cefotiam per kg or 640 mg of cefazolin per kg at 30-minute intervals starting 30 hours after infection. Decrease in the number of bacteria in the lung was observed 1 hour after the onset of medication. The bacterial counts dropped to about one-hundredth the pretreatment level with cefotiam 4 hours after the first injection and with cefazolin 2 hours after the start of treatment, and this level was sustained at least as long as 8 hours after the start of medication (Fig. 3).

Fig. 3. Effect of cefotiam (CTM) or of cefazolin (CEZ) on the number of bacteria in the lungs of mice infected with *K. pneumoniae* DT-S by the aerosol method.

Cephalosporin administration, consisting of four subcutaneous injections at 30-minute intervals, was started 30 hours after infection. Each point represents the geometric mean of the values obtained from six to ten mice. Vertical bars indicate standard deviations.



Cephalosporin Levels in Plasma and Lung

The cephalosporin levels in the plasma and lungs of normal mice following administration of four subcutaneous doses of 80 mg of cefotiam per kg or of 640 mg of cefazolin per kg at 30-minute intervals are shown in Fig. 4. The high cephalosporin levels in the plasma were maintained during the period that injections were given followed by a rapid fall. The cephalosporin levels in the lungs nearly paralleled those in the plasma, and the cefazolin levels were about ten times as high as those of cefotiam during the first 2 hours after the start of medication. Elimination of cefazolin from the plasma and lung, however, were slower than that of cefotiam. Biological half-lives of plasma levels of cefotiam and cefazolin were 0.18 and 0.44 hour, respectively.

Morphological Changes

Morphological response of *K. pneumoniae* DT-S exposed to increasing concentrations of the

Fig. 4. Plasma and lung levels of cefotiam (CTM) and of cefazolin (CEZ) after four subcutaneous injections at 30-minute intervals in mice.

Arrows show times of medication. Each point represents the mean of the values obtained from five mice. Vertical bars indicate standard deviations.

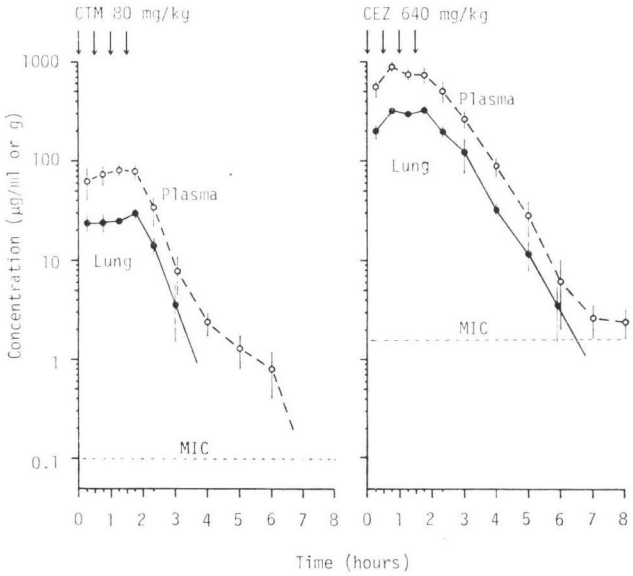
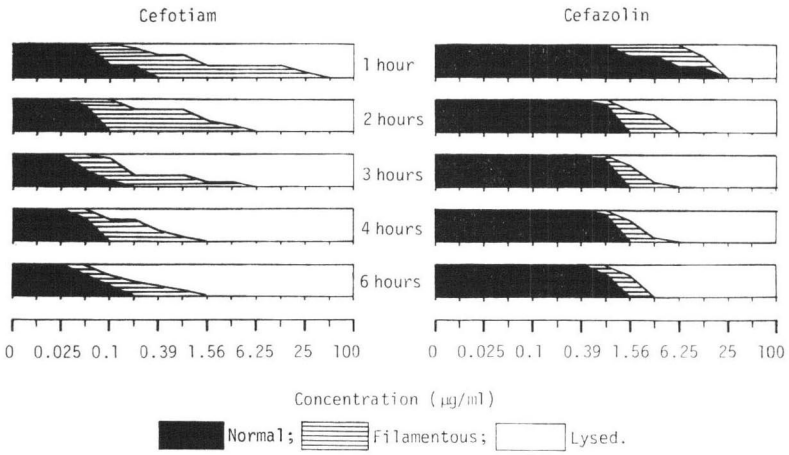


Fig. 5. Morphological changes of *K. pneumoniae* DT-S exposed to various concentrations of cefotiam or of cefazolin for 1 ~ 6 hours.



cephalosporins for 1 ~ 6 hours *in vitro* is illustrated in Fig. 5. After exposure to cefotiam for 1 hour, filamentous cells were induced at a concentration of 0.1 μg/ml, and lysed cell debris together with elongated cells were observed at 0.2 to 25 μg/ml. For a complete lysis of the cells within 1-hour exposure, a concentration of more than 50 μg of cefotiam per ml was required. With further incubation, however, the concentration of cefotiam required for complete cell lysis decreased, and after a 6-hour exposure, 1.56 μg of cefotiam per ml provided complete cell lysis. On the other hand, cefazolin

induced filamentous cells at 1.56 μg/ml and complete cell lysis at 25 μg/ml after 1-hour exposure. After 6 hours of exposure, 0.78 and 3.13 μg of cefazolin per ml provided filamentous cells and complete cell lysis of *K. pneumoniae* DT-S, respectively. At any time interval, the concentration range of cefotiam inducing filamentous cell was much wider than that of cefazolin. Filamentation of the cells became most marked after 3- to 4-hour exposure to the cephalosporins, and the cell length reached about 40 μm at 0.1 μg of cefotiam per ml and 1.56 μg of cefazolin per ml.

Morphological changes of *K. pneumoniae* DT-S in the lung of mice, given four subcutaneous doses of 80 mg of cefotiam per kg or of 640 mg of cefazolin per kg at 30-minute intervals beginning at 30 hours after infection, were examined by a scanning electron microscope. At 34 hours after infection, pulmonary alveoli in the center of pneumonic lesions of untreated infected animals harbored numerous bacilli about 3 μm in length. In the margin of the lesions a small number of the organisms was present (Fig. 6). Four hours after the onset of medication with cefotiam or cefazolin (34 hours after infection), most of the organisms in the lung had lysed (Fig. 7). Similar morphological changes of the organisms were seen up to 6 hours after the onset of medication. At 8 hours after the first dose, however, a small number of filamentous bacteria together with lysed cells were observed at some sites of pneumonic lesions of mice given either of the cephalosporin (Fig. 8).

Fig. 6. Scanning electron micrograph of the lung of mice infected with *K. pneumoniae* DT-S by the aerosol method.

Infected control, 34 hours after infection. (A) ×140. Bar=50 μm. (B) Higher power view of (A) ×1,400. Bar=5 μm.

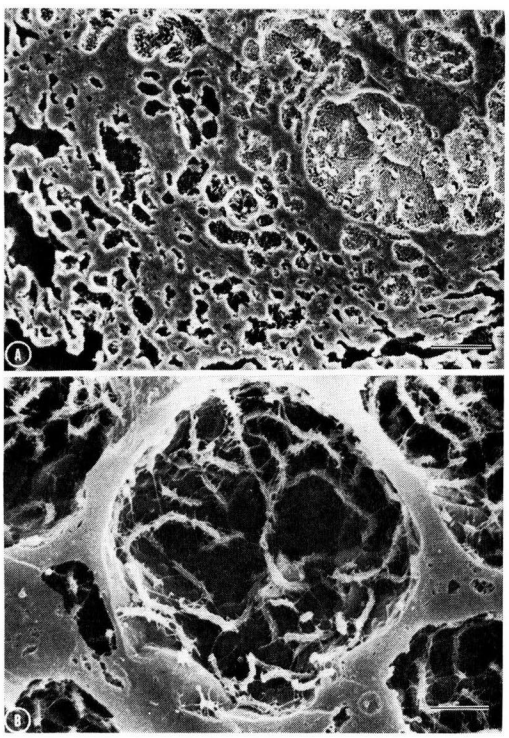
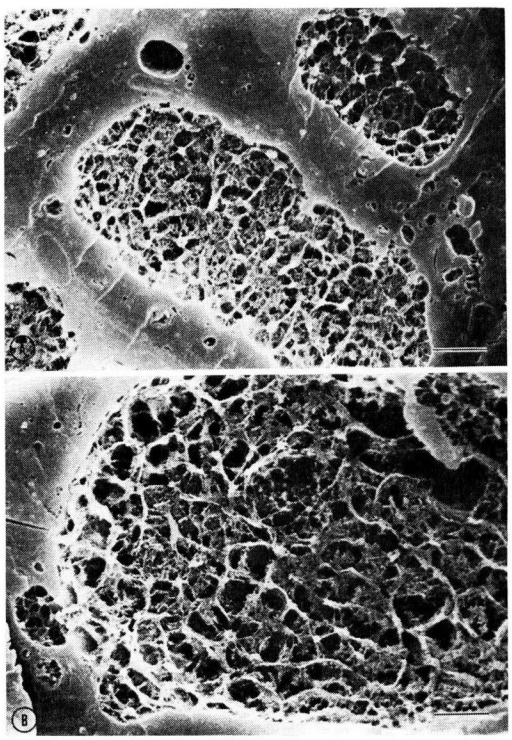


Fig. 7. Scanning electron micrograph of the lung of mice infected with *K. pneumoniae* DT-S by the aerosol method and treated with cephalosporin.

Four subcutaneous injections at 30-minute intervals of 80 mg of cefotiam per kg or 640 mg of cefazolin per kg given, starting at 30 hours after infection. Thirty-four hours after infection. Bar=5 μm. ×1,300. (A) Cefotiam. (B) Cefazolin.



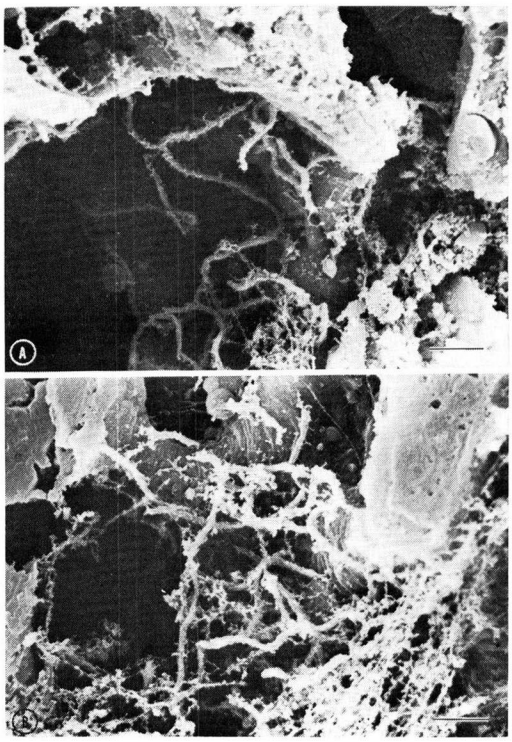
Discussion

At 30 hours after infection, the concentration of *Klebsiella* in the lung was about 10^8 CFU/g and that in the pneumonic lesions must have been much higher. The ratio of the SD_{50} or CD_{50} value of cefotiam to that of cefazolin in the model infection, however, accorded with that of the bactericidal or bacteriolytic activities of the cephalosporins in suspensions of 10^7 CFU/ml rather than that in 10^5 CFU/ml suspensions, and with that of the MICs obtained by the agar dilution method. It is inferred, therefore, that, for cefotiam and cefazolin the efficacy in comparable clinical conditions would be more relevant to the *in vitro* antibacterial activities measured with the moderate inoculum size rather than that obtained with the heavy inoculum size.

Fujii *et al.*[5] suggested a possible relationship between the appearance of filamentous forms and spheroplasts of *Escherichia coli* in the urine of a patient with chronic pyelonephritis, treated with cephalexin, and exacerbation of the disease. Comber *et al.*[4] reported that the protective activities of ampicillin and amoxicillin in mice infected intraperitoneally with *E. coli*, having the same susceptibility to both penicillins, correlated with *in vivo* bacteriolytic activities of these antibiotics. Cefotiam has a high affinity for penicillin-binding protein 3 of *E. coli* KN 126 and causes the development of filamentous cells over a wide range of relatively low concentrations[9]. The range of concentrations of cefotiam inducing filamentation of *K. pneumoniae* DT-S *in vitro* was much wider that of cefazolin. On the other hand, in the pneumonic lungs, therapeutic doses of both cephalosporins (80 mg of cefotiam per kg and 640 mg of cefazolin per kg) produced mainly bacteriolysis of the challenge organisms, but numerous filamentous cells were observed at subcurative doses (20 mg of cefotiam per kg and 160 mg of cefazolin per kg) (data not shown). These findings suggest that the difference between the concentration ranges for cefotiam and cefazolin which induce cell filamentation *in vitro* does not affect the therapeutic activity of the two cephalosporins in this model infection.

Fig. 8. Scanning electron micrograph of the lung of mice infected with *K. pneumoniae* DT-S by the aerosol method and treated with cephalosporin.

Four subcutaneous injections at 30-minute intervals of 80 mg of cefotiam per kg or 640 mg of cefazolin per kg given, starting at 30 hours after infection. Thirty-eight hours after infection. Bar = 5 μm. ×1,300. (A) Cefotiam. (B) Cefazolin.



Distribution of parenteral antimicrobial agents to the lung or to respiratory tract secretions is reported to differ markedly among antimicrobial agents[17~20]. In the present study, the cephalosporin levels in the normal lung tissue were roughly proportional to the doses administered. In pneumonic lesions due to *K. pneumoniae* DT-S, the challenge organisms are found mainly in the broncho-alveolar spaces[14]. Therefore, the therapeutic outcome may depend more on the concentration of the antimicrobial agents in respiratory tract secretions. It has been suggested, moreover, that antibiotics pass more readily through a "blood-bronchus barrier" when inflammation is present[21,22]. Accordingly, measurement of the antibiotic levels in the respiratory secretions in pneumonic lungs will be of much significance.

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