ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1977, p. 736-744

Vol. 12, No. 6
Printed in U.S.A.

Comparative Effects of Amoxycillin and Ampicillin on the Morphology of *Escherichia coli* In Vivo and Correlation with Activity

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Received for publication 21 July 1977

An experimental mouse intraperitoneal infection due to *Escherichia coli* was treated with subcutaneous amoxycillin or ampicillin. Specimens of blood and peritoneal washings from the infected animals were assayed for antibiotic concentrations and examined microscopically for observation of the effects produced by the two penicillins on the morphology of bacteria growing in vivo. Amoxycillin was significantly more effective than ampicillin in protecting the animals from the lethal effects of the infection, although the antibiotic concentrations in the body fluids were very similar for both compounds. However, microscopic examination showed marked differences in the morphological effects produced at equivalent dose levels by the two compounds against the bacteria present in blood and peritoneal fluid. Treatment with amoxycillin at dose levels that produced peak antibiotic concentrations in the body fluids ranging from one-quarter to three times the minimum inhibitory concentration resulted in the formation of spheroplast forms, which lysed rapidly. In contrast, at the same concentrations, ampicillin produced relatively stable filaments or long cell forms, which lysed much more slowly, although at higher dose levels the effects produced were generally similar to those seen with amoxycillin. It is concluded that the superior therapeutic activity of amoxycillin compared with ampicillin is due to its greater bacteriolytic activity in vivo.

Amoxycillin [D-(−)-α-amino-*p*-hydroxybenzylpenicillin] and ampicillin [D-(−)-α-aminobenzylpenicillin] show similar activities in conventional growth inhibition tests, but amoxycillin has been reported to exert more rapid bactericidal effects in vitro than ampicillin (10, 14). In more recent studies by Rolinson and his colleagues (15; G. N. Rolinson, A. C. Macdonald, and D. A. Wilson, Abstr. 9th Int. Congr. Chemother, London, abstr. 302, 1975), microscopic examination of cultures of *Escherichia coli* exposed to the two penicillins revealed that at concentrations just above minimum inhibitory concentration (MIC) values amoxycillin caused the formation of spheroplasts, which lysed readily, whereas exposure to ampicillin resulted in the formation of filamentous cells, which were relatively stable. The more rapid rate of lysis demonstrated by amoxycillin as a result of its production of spheroplasts accounts for its greater bactericidal activity compared with ampicillin.

It is reasonably well established that amoxycillin is significantly more active than ampicillin against experimental infections, even when blood and tissue concentrations of the two compounds are similar (1, 3, 10). Also, there is evidence to show that in these infections amoxycillin produces greater bactericidal effects in the infected animal than does ampicillin, and it is suggested that this may largely explain the superior chemotherapeutic activity of amoxycillin compared with ampicillin (3, 10).

It seemed desirable to investigate the effects produced by amoxycillin and ampicillin on the morphology of bacteria during the course of experimental infections to determine if there was any correlation between activity and morphological effects in vivo, as has been demonstrated in vitro. The experiments reported describe results observed during the treatment of an intraperitoneal mouse infection due to a strain of *E. coli* with ampicillin and amoxycillin.

MATERIALS AND METHODS

Antibiotics. The penicillins tested were preparations of sodium amoxycillin and sodium ampicillin (Beecham Pharmaceuticals, Worthing, Sussex).

Mice. Male or female albino mice (18 to 22 g),

MFI strain (Olac 1976 Ltd., Bicester, Oxon), were used throughout.

Mouse protection tests. Mice were injected by the intraperitoneal route with 0.5 ml of a suspension in 3% hog gastric mucin (1701W, Wilson Laboratories, Chicago, Ill.) of a dilution of an overnight broth culture of *E. coli* 8 standardized to give an infective inoculum of 100 median lethal doses. The antibiotics were administered by the subcutaneous route immediately after infection, 0.2 ml/20-g mouse in phosphate-buffered saline. In a second series, mice were infected with 10,000 median lethal doses, the inoculum used for the microscopical studies, and were treated at the time of infection and 4 h later. Groups of 20 mice were treated at each dose level. The numbers of animals surviving 4 days after infection were recorded, and the dose of penicillin required to produce 50% survival of infected animals was calculated (11).

Antibiotic concentrations in mouse blood and peritoneal washings. Mice were infected intraperitoneally with *E. coli* 8 and dosed subcutaneously with amoxycillin or ampicillin immediately after infection. Groups of five mice were killed by dislocation of the neck at intervals during the 2-h period after infection. Samples of blood were collected from the axillary region, and 0.3-ml volumes were added to 0.3-ml volumes of heparin (100 U/ml; Weddal Pharmaceuticals Ltd., Wrexham, Clwyd). For determination of antibiotic concentrations in the peritoneum, the abdomen was swabbed with 75% alcohol and the skin was reflected, care being taken not to open the peritoneal cavity, and 2 ml of saline was injected forcefully. The abdomen was carefully massaged to ensure adequate mixing. A small incision was made in the peritoneum, and a sample of washings was collected with a sterile Pasteur pipette. The specimens of blood and peritoneal washings were assayed by large-plate agar diffusion assay with *Sarcina lutea* NCTC 8340 as the assay organism. Specimens of heparinized mouse blood were assayed against standard solutions of amoxycillin or ampicillin in whole horse blood, a diluent shown not to differ significantly from mouse blood in the assay procedures. Specimens of peritoneal washings were suitably diluted in phosphate-buffered saline and assayed against standard antibiotic solutions prepared in the same diluent. The assay plates were incubated overnight at 30°C, inhibition zone diameters were measured, and antibiotic concentrations were derived from standard lines prepared from standard solutions.

Microscopy and photography. Mice were infected intraperitoneally with approximately 10^7 cells of a suspension in 3% mucin of an overnight broth culture of *E. coli* 8 and were treated with a single subcutaneous dose of amoxycillin or ampicillin. The animals were killed at intervals after administration of the penicillins, and specimens of blood and peritoneal fluid were collected for microscopic examination. For the observations made on the peritoneal washings, the mice were dosed by the subcutaneous route immediately after infection, but for the studies on the blood the mice were not treated with penicillins until 6 h after intraperitoneal infection to ensure there was a sufficient bacteremia at the time of therapy. Specimens of peritoneal washings were suspended in an equal volume of 2% glutaraldehyde fixative in 0.05 M sodium cacodylate (osmolality, 305 mOsmol/kg); blood samples were suspended in 2% glutaraldehyde fixative containing 20 U of heparin (osmalality, 320 mOsmol/kg). The osmolalities of the fixatives were prepared to be the same as previously determined osmolalities of mouse peritoneal washings and blood. The glutaraldehyde fixative was shown in preliminary experiments to preserve the morphology of the bacteria and to prevent any further antibacterial activity of the penicillin. Specimens for microscopy were mounted on a thin film of agar prepared by spreading 0.6 ml of molten 0.8% brain heart infusion agar over the surface of a no. 1 cover slip (22 by 56 mm), which was trimmed to a 10-mm square after the agar had solidified. The sample was placed in the center of the agar and covered with a second cover slip for high-resolution microscopy, using a Ziess WL microscope fitted with differential interference contrast optics. Photography of the samples was carried out with a 120-format roll film camera and a 100-W tungsten-base lamp.

RESULTS

Activity against *E. coli* intraperitoneal infection. The relative activities of amoxycillin and ampicillin in protecting mice from the lethal effects of intraperitoneal infection with *E. coli* 8 are shown in Table 1. In the first study, in which the infective inoculum was approximately 10^5 cells (100 median lethal doses), the animals were treated with a single subcutaneous dose immediately after infection, as was the case in earlier studies to compare the in vivo activities of amoxycillin and ampicillin (1, 3). At all dose levels, amoxycillin was more effective than ampicillin, and the median protective dose of amoxycillin was significantly lower than that of ampicillin ($P < 0.05$). In the second study, the inoculum was much larger (10^7 cells) and was chosen for the microscopy studies. Against this larger inoculum, representing about 10,000 median lethal doses, treatment with a single dose of either compound was relatively ineffective, except at doses of 200 mg/kg or greater. However, administration of a second dose 4 h after infection resulted in effective therapy (Table 1), and amoxycillin was again signficantly more effective than ampicillin ($P < 0.05$).

Antibiotic levels in blood and peritoneal fluid. Data in Fig. 1 show the mean values obtained in three experiments to measure the antibiotic concentrations obtained in the blood and peritoneal cavities of mice infected with *E. coli* 8 after a single subcutaneous dose of amoxycillin or ampicillin. The concentrations of amoxycillin or ampicillin were the same in the

TABLE 1. *Relative activities of amoxycillin and ampicillin by the subcutaneous route against an intraperitoneal mouse infection due to E. coli 8*

Inoculum (no. of cells)	Dose (mg/kg)	Amoxycillin		Ampicillin	
		No. of survivors (20 mice/group)	PD_{50}[a] (mg/kg)	No. of survivors (20 mice/group)	PD_{50} (mg/kg)
10^5[b] (100 MLD[c])	200	19		19	
	100	19		17	
	50	17		12	
	25	16	4.0	11	23.5
	12.5	13	(2.4–8.6)[d]	8	(15.5–35.5)
	6.25	12		1	
	3.12	9		2	
	0	0		0	
10^7[e] (10,000 MLD)	200	20		20	
	50	19	7.2	19	22.0
	12.5	16	(4.6–11.2)	3	(16.3–29.7)
	3.12	3		0	
	0	0		0	

[a] PD_{50}, Median protective dose.
[b] Dosed immediately after infection.
[c] MLD, Median lethal dose.
[d] 95% confidence limits.
[e] Dosed immediately and 4 h after infection.

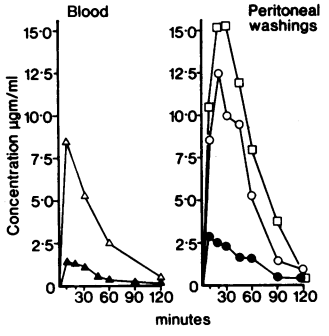

FIG. 1. *Concentrations of amoxycillin and ampicillin in blood and peritoneal washings after a single subcutaneous dose to mice infected with E. coli 8. Symbols: Blood–(△) 12.5 mg of amoxycillin and ampicillin per kg; (▲) 3.1 mg of amoxycillin and ampicillin per kg. Peritoneal washings–(□) 12.5 mg of amoxycillin per kg; (○) 12.5 mg of ampicillin per kg; (●) 3.1 mg of amoxycillin and ampicillin per kg.*

blood at both dose levels, 12.5 and 3.1 mg/kg, and in the peritoneal fluid at the 3.1-mg/kg dose level. In the blood, the dose of 12.5 mg/kg produced a peak level of 8.5 μg/ml at 10 min, which fell rapidly to undetectable levels (<0.01 μg/ml) by 120 min; at 3.1 mg/kg, the concentrations also fell rapidly from the peak value of 1.5 μg/ml measured at 10 min. The antibiotic concentrations measured in the peritoneal cavity were rather higher than those obtained in the blood. At 12.5 mg/kg, the peak level of ampicillin was 12 μg/ml at 20 min compared with 15 μg/ml for amoxycillin, and the levels of both penicillins fell rapidly to low values 2 h after treatment. In general, at this dose level the amoxycillin levels were about 20% higher than those of ampicillin. At the 3.1-mg/kg dose, peak concentrations of about 3.0 μg of amoxycillin and ampicillin per ml were measured in the peritoneal washings after 10 min.

Morphological effects in vivo. (i) Peritoneal fluid. The effects produced by a single subcutaneous dose of ampicillin (12.5 mg/kg) on the morphology of *E. coli* 8 in the peritoneal cavity of infected mice are illustrated in Fig. 2. Normal rod-shaped cells were observed up to 30 min after dosing, after which there was a progressive increase in cell length. Thus, at 1 h most bacteria observed were two to three cells in length, and these were replaced by cells of increasing length: at 2 h, with filaments 5 to 10 cells long, and from 3 h onwards with filaments 10 to 20 cells in length (Fig. 2a). Most of the filaments had normal motility, and many of them showed small bulges at one or more points along the cell. By 4 h many of the filaments had lysed, but some showed evidence of fragmentation, with regeneration of cells of normal morphology. Septation generally occurred at the tip of the filaments, though some cells showed multiple fragments (insert, Fig. 2a). At 5 h large numbers of filaments were still present, and increased macrophage activity was also evident so that many of the resid-

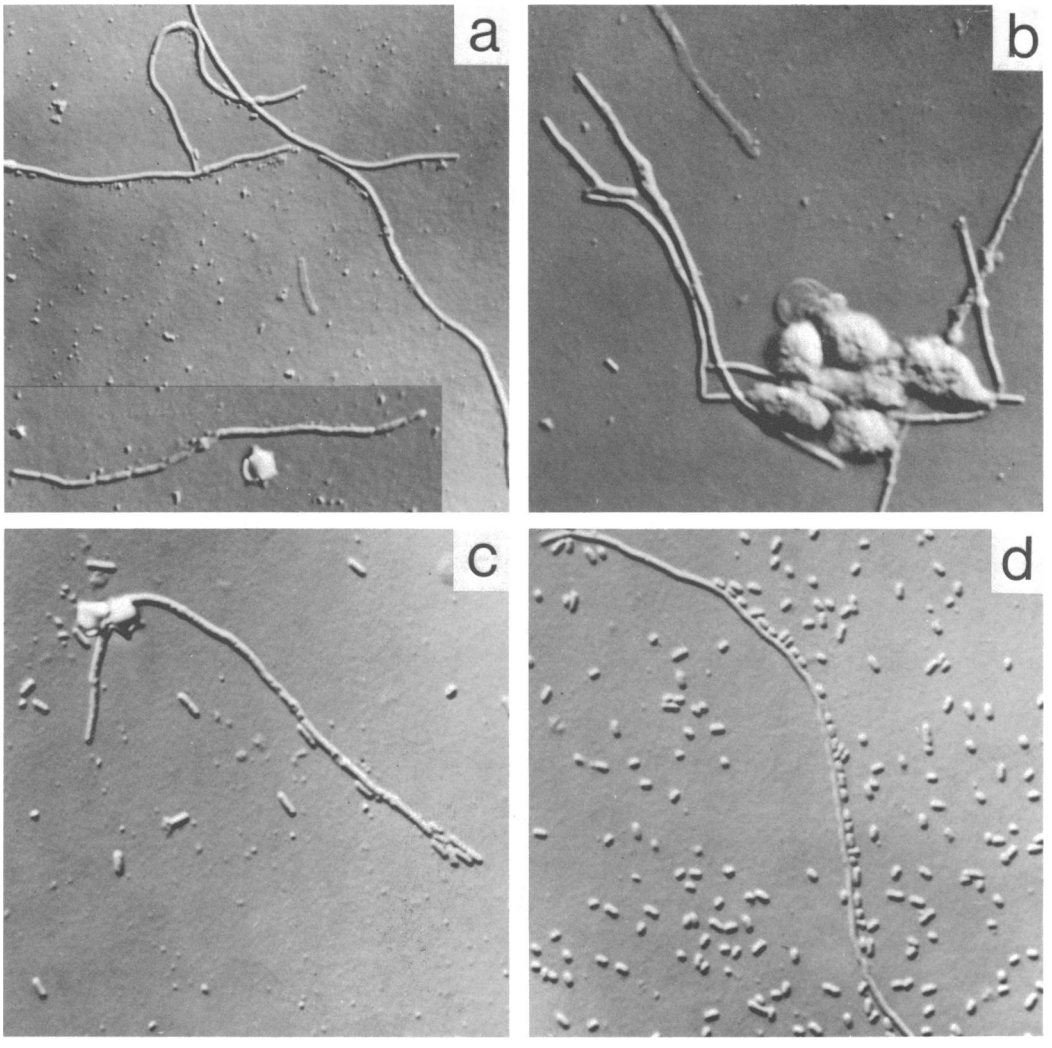

FIG. 2. *Effects of a single subcutaneous dose of ampicillin (12.5 mg/kg) on the morphology of E. coli 8 in the mouse peritoneum at (a) 3 h, (b) 5 h, (c) 9 h, and (d) 14 h. Differential interference phase contrast (×1,100).*

ual long forms and normal bacteria were in the process of being engulfed by the macrophages (Fig. 2b). At 9 h, large numbers of normal cells were being regenerated from the filaments, and by cell division (Fig. 2c), so that by 14 h very large numbers of normal cells were seen in each microscope field, together with the occasional greatly elongated cell (Fig. 2d).

At a higher dose level (50 mg/kg), treatment with ampicillin initially caused spheroplast formation and a rapid reduction in the numbers of bacteria, after which there was a progressive increase in cell length so that from 90 min to 2 h small numbers of elongated cells four to eight cells in length were seen in each microscope field. Between 3 and 9 h, when the ampicillin concentration in the peritoneal fluid had fallen to subinhibitory levels, filaments 10 to 20 cells long were present. Many of these gradually lysed over the next few hours, so that 6 h after dosing ghost forms of filaments were seen and surviving filaments were showing signs of fragmenting, and at 9 h normal cells were more numerous. There was no evidence of filament formation after treatment with a dose of 200 mg of ampicillin per kg, and only small numbers of normal cells were seen during the 14-h period after dosing.

In contrast to the findings with ampicillin, a

dose of 12.5 mg of amoxycillin per kg caused the rapid formation of spheroplasts, which lysed readily, and there was no evidence of filament formation. Instead, slightly elongated cells, about two cells in length, with spheroplasts, were seen 40 to 60 min after dosing (Fig. 3a), 10 to 30 min after the peak antibiotic concentrations (15 μg/ml) were measured in the peritoneal fluid. Soon thereafter, the majority of spheroplasts had lysed, and only small numbers of normal cells and an occasional spheroplast were seen 2 h after dosing. Similar results were obtained with higher doses of amoxycillin.

Treatment with a lower dose of amoxycillin (3.1 mg/kg) also resulted initially in the rapid formation of spheroplasts 60 to 80 min after dosing (Fig. 3b), i.e., 50 to 70 min after peak antibiotic levels (3.2 μg/ml) were measured in the peritoneum, and only small numbers of slightly elongated cells were seen up to 90 min after dosing. However, from 2 h onwards, by which time the antibiotic concentrations in the fluid had fallen to very low levels, the surviving cells elongated and filament formation became evident. In contrast to ampicillin-induced filaments, many of these long cells had bulbous swellings (Fig. 3c), and by 3 h there was a great variety of cell forms, including long forms with multiple bulbous swellings and bulges

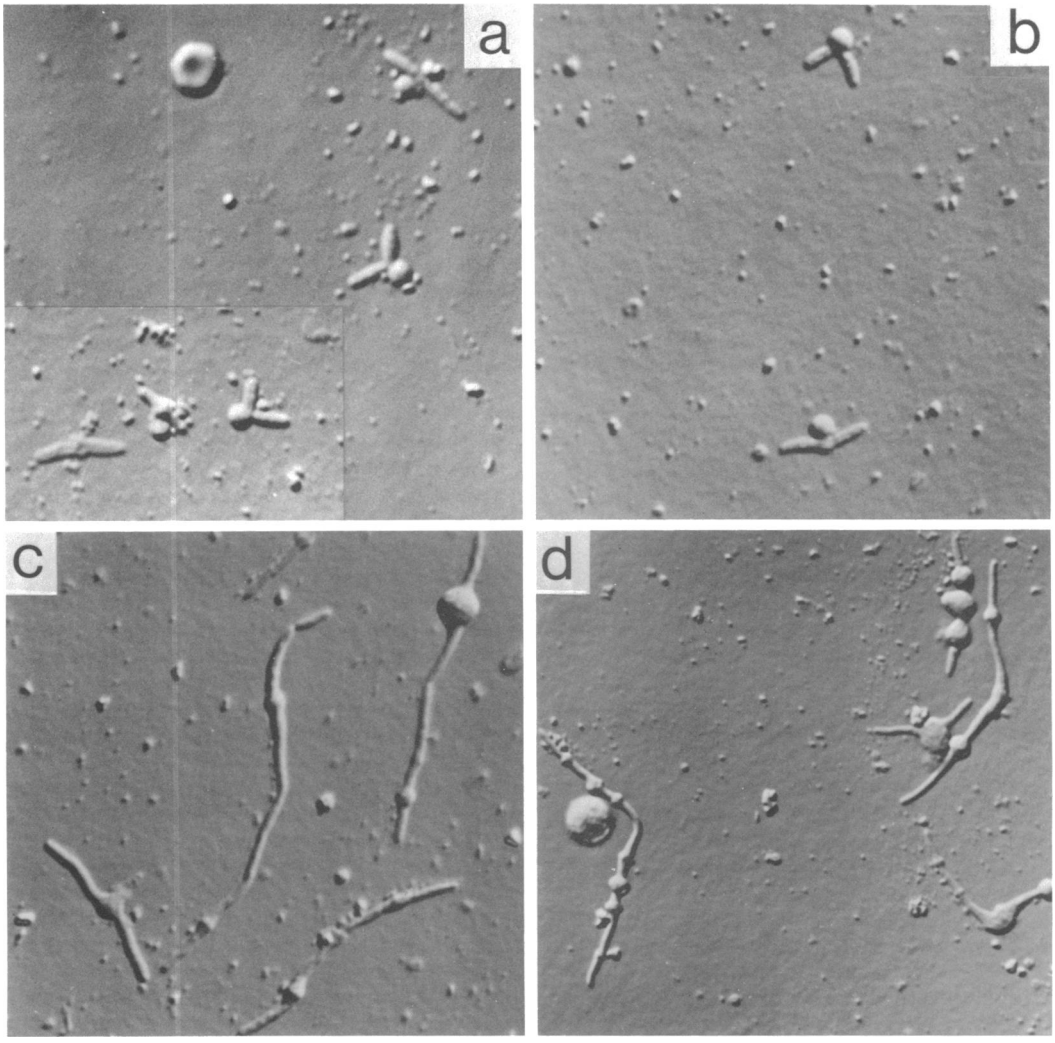

FIG. 3. *Effects of a single subcutaneous dose of amoxycillin on the morphology of E. coli 8 in the mouse peritoneum: (a) 12.5 mg/kg, 40 min; (b) 3.1 mg/kg, 80 min; (c) 3.1 mg/kg, 2 h; (d) 3.1 mg/kg, 3 h. Differential interference phase contrast (×1,500).*

along the filaments (Fig. 3d). At 4 h many of the long cells were still present, some showing evidence of lysis and others of fragmentation. By 9 h macrophage activity had increased, large numbers of normal cells were seen, and a number of filaments were still present.

(ii) Blood. The results of treatment with a single subcutaneous dose of 12.5 mg of ampicillin per kg on the morphology of *E. coli* 8 in the blood of infected mice are illustrated in Fig. 4. As was the case in the peritoneum, this dose of ampicillin caused characteristic cell elongation and filament formation. Relatively large numbers of normal cells were observed up to 30 min after dosing, but these were replaced after 60 to 90 min, when the antibiotic concentrations had fallen to subinhibitory levels, by filaments four to six cells in length, many with central bulges or collars (Fig. 4a). By 2 h, large numbers of elongated cell forms were seen, and filaments six to eight cells long were present (Fig. 4b). Fragmentation of filaments and the reappearance of normal cells were observed 3 to 5 h after dosing (Fig. 4c), and, by 9 h, normal cells were seen in large numbers of the blood of surviving animals. At a higher dose, 50 mg/kg, there was some evidence of cell elongation and cells of two to four cells in length were observed 1 to 2 h after treatment, but there was no sign of filament formation during this period, although it is likely this would have occurred later after the fall in ampicillin concentrations in the body.

The administration of amoxycillin at 12.5 and 50 mg/kg resulted in the formation of spheroplasts and rapid lysis of bacteria in the blood, as in the peritoneal cavity, and there was no evidence of filament formation. Typically, large numbers of spheroplasts were observed in the blood after 40 to 50 min (Fig. 4d), and by 60 min there was a significant reduction in the number of bacteria present, so that only the occasional spheroplast or normal cell was seen.

Again, rather different effects were observed at the lower dose of amoxycillin, namely, 3.1 mg/kg. Relatively large numbers of normal cells were observed after 30 min, but these evolved between 50 to 60 min to slightly elongated forms, about two cells in length, many with large swellings or spheroplasts (Fig. 4e). In addition, numerous ghost cells were seen. Between 1 and 2 h there was a marked decrease in the number of spheroplasts and a few normal cells were observed, together with slightly elongated cells with central collars. At 3 h many of the surviving bacteria had elongated, and filamentous cells were observed, many with large, single or multiple swellings (Fig. 4f). Between 3 and 5 h after dosing, a number of the bizarre forms lysed while fragmentation occurred in some of the filamentous forms, so that at 5 h a variety of cell forms was seen, including elongated cells with swellings as well as increased numbers of normal cells.

DISCUSSION

In the studies reported here amoxycillin was significantly more effective than ampicillin in protecting mice from the lethal effects of intraperitoneal infection with a strain of *E. coli*. Both compounds produced similar antibiotic concentrations in blood and peritoneal washings of the infected animals, in agreement with the results of earlier studies (3), so that the difference in the activities of the compounds cannot be explained in this fashion. In the earlier studies mentioned above, amoxycillin was shown to produce greater bactericidal effects than ampicillin in the infected animals, but this aspect was not investigated here.

In this study, microscopic examination of specimens of blood and peritoneal fluid from infected animals revealed striking differences in the effects produced by equivalent doses of the two penicillins on the morphology of the bacteria growing in vivo. In essence, amoxycillin typically produced spheroplast forms, which lysed rapidly, whereas ampicillin only did so at relatively high doses compared with amoxycillin. Characteristically, ampicillin produced filamentous or long cell forms at dose levels at which amoxycillin was producing spheroplasts, and these variants lysed relatively slowly. However, at high doses ampicillin caused spheroplast formation in the same way as amoxycillin, whereas at low dose levels treatment with amoxycillin resulted in the formation of filamentous cells after initial spheroplast formation, so that the differences between the compounds are essentially concentration dependent rather than reflecting differences in modes of action.

To a great extent, the morphological effects produced by the two penicillins during the course of treatment of this experimental infection resembled the effects described by Rolinson and his colleagues in their in vitro studies (15; Rolinson et al., Abstr. 9th Int. Congr. Chemother., abstr. 302, 1975). The similarities in the effects produced in vitro and in vivo are somewhat unexpected in view of the differences between the two experimental systems. For instance, in the infected animal the bacteria are exposed to the body fluids and to the host immune mechanism, factors that are not present in cultures incubated in vitro. In addition, in in vitro studies the bacteria are exposed to a

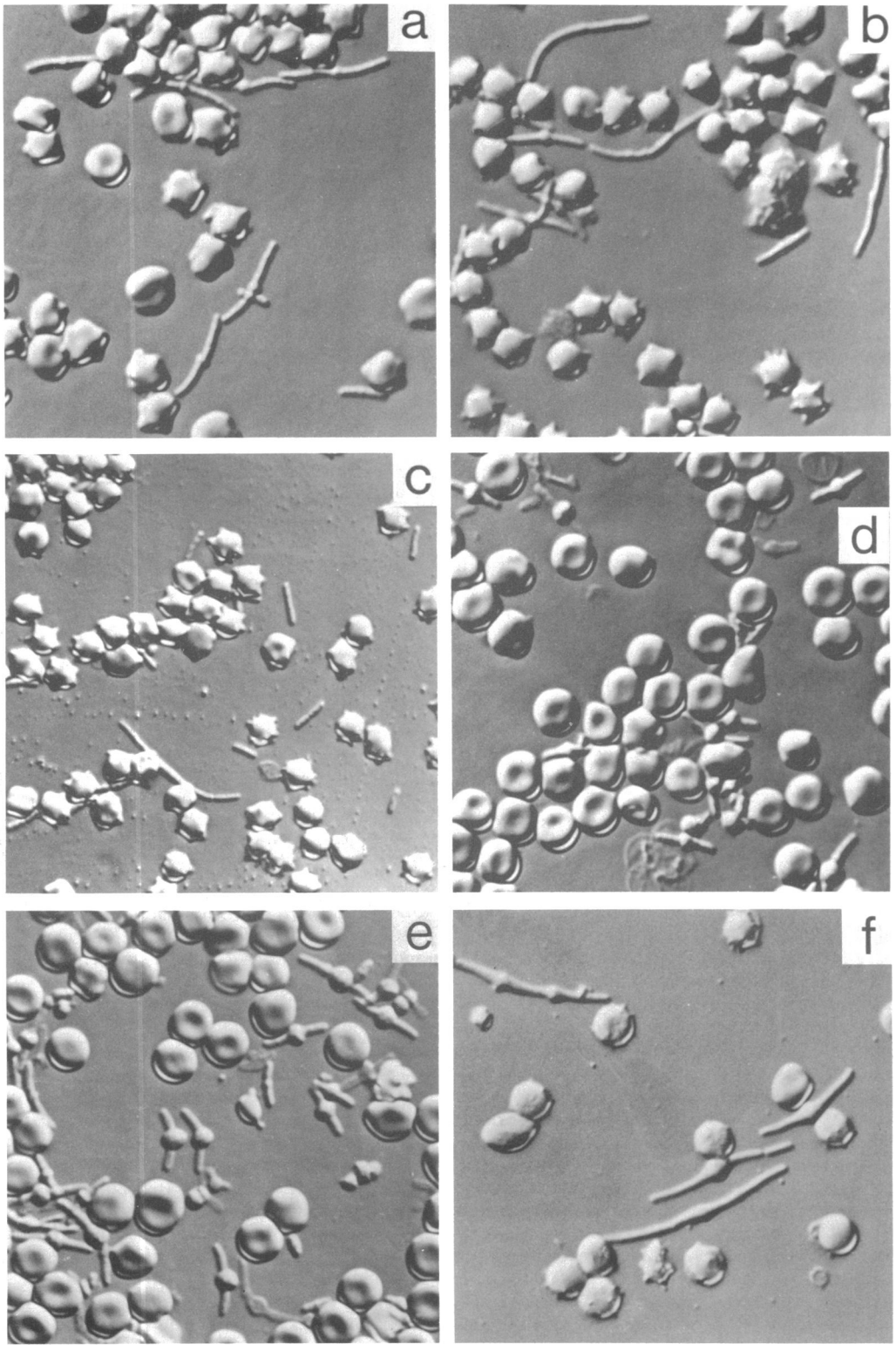

FIG. 4. *Effects of a single subcutaneous dose of ampicillin or amoxycillin on the morphology of E. coli 8 in the blood of infected mice. Ampicillin (12.5 mg/kg) at: (a) 60 min; (b) 2 h; (c) 4 h. Amoxycillin: (d) 12.5 mg/kg, 40 min; (e) 3.1 mg/kg, 1 h; (f) 3.1 mg/kg, 3 h. Differential interference phase contrast (×1,100).*

constant concentration of antibiotic for a relatively long period of time, whereas in the animal studies described here, peak antibiotic concentrations were attained rapidly (within 15 min) and the levels declined rapidly as the antibiotics were excreted from the host. The dynamic nature of the in vivo system is illustrated by the changing morphology of the bacteria in the infected animals with the passage of time. For example, at a low dose of amoxycillin (3.1 mg/kg), spheroplasts were produced soon after peak antibiotic levels were attained in blood and peritoneal fluid, but later, as the compound was being excreted from the animal, the low concentrations remaining evoked the production of filamentous forms. Similarly, with a dose of 50 mg of ampicillin per kg, spheroplast formation, seen soon after peak antibiotic levels were attained, was followed by cell elongation as the ampicillin concentrations in the body fell to subinhibitory levels.

Although filament formation was evident with low doses of amoxycillin as well as at higher doses of ampicillin, there were obvious differences in the appearances of the long cell forms. For instance, with amoxycillin, the filaments had characteristic multiple swellings, like inflated balloons, along the length of the cells, whereas these abnormal forms were not seen with ampicillin. These swellings became evident some length of time after the initial rapid formation of spheroplasts by amoxycillin had ceased and at a time when amoxycillin was no longer detectable in the body fluids.

Perhaps the most striking feature of this study is the very rapid bacteriolytic effect produced in vivo during treatment with amoxycillin at dose levels that resulted in the bacteria being exposed to subinhibitory concentrations of the compound for only short periods of time. For example, at a dose level of 3.1 mg/kg, amoxycillin produced peak blood antibiotic concentrations of about 1.5 μg/ml at 10 min, and these fell to about 0.25 μg/ml at 60 min and were undetectable at 120 min. The MIC of amoxycillin (and ampicillin) against the test organism, *E. coli* 8, was found in repeated tests to be 5.0 μg/ml, so that at best the bacteria in the blood of the infected animal were exposed to a concentration of one-fourth the MIC for a very short period of time. Nevertheless, treatment with this dose of amoxycillin resulted in the rapid formation of spheroplasts in the blood followed by rapid lysis, i.e., within 60 min after administration of the compound. Consequently, amoxycillin appeared to be notably more bacteriolytic in vivo than was observed in the in vitro studies of Rolinson and his colleagues (15), where filament formation occurred at subinhibitory concentrations and lysis of the filaments only took place after a period of hours. In contrast, the effects produced in vivo by ampicillin were more in keeping with those observed in vitro by Rolinson et al. (15) and Greenwood and O'Grady (6), in that spheroplast formation was observed in vitro and in vivo only when the bacteria were exposed to ampicillin concentrations well in excess of MIC levels. At concentrations not greatly in excess of MIC values (up to two to three times the MIC), and at subinhibitory concentrations, treatment with ampicillin resulted typically in the formation of filaments in vitro and in vivo.

The morphological effects produced by benzylpenicillin on susceptible bacteria were observed soon after the antibiotic was isolated (5), and the presence of abnormal varieties, in animals or patients, after penicillin therapy has been described (2, 4, 8, 9, 12). More recently, there has been a resurgence of interest in the various morphological effects produced by β-lactam antibiotics as a result of awareness of the variety of effects caused by compounds of differing structures (4, 6, 14, 17), and differences in effects on morphology have been correlated with differences in affinity for binding sites (7, 13, 16). However, to the best of our knowledge, the study reported here represents the first attempt to seek a correlation in vivo between antibiotic effect on morphology and the results of therapy. The apparent similarity between in vitro and in vivo observations is of obvious interest.

It is evident from the results of this study that the differences in the therapeutic activities of amoxycillin and ampicillin against experimental infections are associated with the differences produced on the morphology of the bacteria in vivo by the relatively low transient concentrations of the compounds present in the blood and tissues of the infected animals during therapy. The rapid production of spheroplasts and the rapid lysis observed with amoxycillin in vivo contrasts markedly with the filament formation and slower rate of lysis seen with ampicillin. The significantly greater bacteriolytic activity of amoxycillin in infected animals compared with ampicillin provides the explanation for its greater bactericidal activity in vivo and is the reason for the superior therapeutic activity of amoxycillin.

ACKNOWLEDGMENTS

We are grateful to David A. Wilson for helpful discussions and to Charles Winter for skilled assistance in printing the photographs.

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